12th International Symposium on Reproductive Physiology of Fish

"Reproductive science for aquaculture production and conservation"

15 - 19 May 2023

Aldemar Knossos Royal Hotel | Crete, Greece

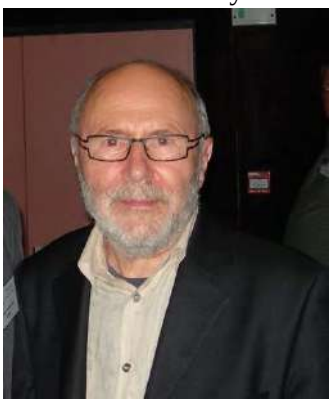
PROGRAM AND ABSTRACTS

This is the updated version of the printed Program and Book of Abstracts (only in pdf format), and contains additional Abstracts that were either submitted late or were excluded from the printed version by mistake. It also includes the Concluding – Summary Presentation given at the end of the symposium, as well as the Student Awards for best poster and oral presentations. Finally, some photos from the symposium are also included.

Thank you all for making this symposium a success. See you in 2026!

Constantinos C Mylonas

The **International Symposium on the Reproductive Physiology of Fish (ISRPF)** is an internationally highly recognized symposium, devoted to discuss and present the most advanced basic and applied knowledge in the field the reproductive physiology of fish and related areas. With its history of four decades, this worldwide scientifically well-established symposium takes place every 4 years, since its creation in 1977 by **Prof. Dr. Roland Billard**, in France. Professor R. Billard passed away in 2019.



This is a compact symposium with no parallel sessions enabling participants to attend all the invited plenary lectures and oral presentations. The ISRPFs status as the premier meeting in fish reproduction and its organisation is especially appropriate for students who are able to contact with experts in varied topics beyond their field of study. Posters are being displayed for the duration of the meeting to promote interactions and discussion.

Previous **Book of Abstracts** can be found in the website of the Institut National de Recherche pour l'Agriculture, l'alimentation et l'Environnement (National Research Institute for Agriculture, Food and the Environment), INRAE.

https://isrpf.hub.inrae.fr/different-editions

RÉPUBLIQUE FRANÇAISE
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INRAE

1st ISRPF - Paimpont, France, September 19-21, 1977

2nd ISRPF - Wageningen, The Netherland, August 2-6, 1982

3rd ISRPF - St John's Newfoundland, Canada, August 2-7, 1987

4th ISRPF - Norwich, United Kingdom, July 7-12, 1991

5th ISRPF - Austin, Texas, U.S.A., July 2-8, 1995

6th ISRPF - Bergen, Norway, July 4-9, 1999

7th ISRPF - Mie, Japan, May 18-23, 2003

8th ISRPF - Saint-Malo, France, June 3-8, 2007

9th ISRPF - Cochin, India, 9-14 August, 2011

10th ISRPF - Olhão, Portugal, 25-30 May, 2014

11th ISRPF - Manaus, Brazil, 3-8 June, 2018

12th ISRPF - Crete, Greece, 15-19 May 2023

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The fish drawings decorating the pages of the 12th ISRPF Program and Book of Abstracts are a kind contribution from ***Viotopos Publishers*** *(https://www.viotopos.com), and represent some of the major fish species used in aquaculture around the world. They were prepared specifically for the 12th ISRPF by* ***Mrs Thalia Zeimpeki****, whom we thank sincerely!*

Welcome to the 12th ISRPF!

Our Logo: The "**Phaistos Disk**", one of the oldest transportable text to be found to date, (still to be deciphered), has been excavated from the Phaistos Palace in Crete, and dates back to the Minoan Civilization (2nd millennium B.C.)

12th International Symposium on Reproductive Physiology of Fish
"Reproductive science for aquaculture production and conservation"

Dear colleagues,

It is with great pride and full understanding of the responsibility associated with the undertaking, that I welcome you to the **12th International Symposium on the Reproductive Physiology of Fish (12th ISRPF)** in Chersonissos, Crete, Greece, hosted by the Institute of Marine Biology & Biotechnology (IMBBC) of the Hellenic Center for Marine Research (HCMR). The symposium is **co-organized by the Municipality of Chersonissos** and obtained financial support from the **Region of Crete,** where HCMR and the symposium venue are located.

Hospitality is a personal attribute of which we are very proud of in Greece and, in a way, one can say that it is encoded in our genes! This is attested by the fact that from the Classical Greek Pantheon, "**Zeus**", the God of Gods, was the one responsible for protecting guests and strangers! In fact, in the Greek language the word for "guest", "stranger" and "foreigner" is the same: **«ξένος», pronounced "*xénos*"**. And this multiplicity of meanings is reflected in the importance that Greeks, since Classical times, give on welcoming and caring for people they do not know, members of other cultures or cities, or travelers from distant places. Therefore, we will all do our best to welcome you in this event and host you in the best possible way!

I first attended the 4th ISRPF (1991) in Norwich, UK as an MSc student, and have not missed any of the subsequent symposia ever since! More than thirty years afterwards, having gained immensely, both professionally and personally, from attending these unique –in many ways- symposia and from my association with a great number of remarkable scientists and people, I took on the challenge of organizing this symposium, together with many colleagues-friends from Europe who are also loyal participants of the ISRPF. I did this, as a way to express my gratitude to this organization and to offer my services to this community of outstanding professionals and students, hoping to contribute to the continuation of the ISRPF. As Greece does not have a critical mass of researchers working in the area of fish reproduction, instead of a local organizing committee, we have set up a **European Scientific Program Committee**, which was responsible for (a) the development of the program, (b) the evaluation of the submitted abstracts and selection of oral presentations, and (c) the editing of a Special Issue in the journal General and Comparative Endocrinology.

We have maintained the traditional format of the ISRPF with **no parallel sessions**, which is one of the characteristics that make this conference a very successful one, enabling all participants to attend all presentations and follow all ensuing discussions. As usual, posters will be displayed throughout the whole meeting in a nearby specialized area, and two dedicated **Poster sessions** are also organized to allow presenters to explain their work fully to the participants.

Through the generosity of a number of sponsors, we have managed to provide some benefits to our students, such **as free registration to the group excursion and the symposium dinner.** These sponsors include IRIDA SA (an aqua feed production company), the Hellenic Aquaculture Producers Organization (HAPO), StavRAS Aquatic Solutions Ltd (an aquaculture consulting and equipment provider), Galaxidi Marine Farm SA (a hatchery and grow-out company of Mediterranean marine fish), the Region of Crete and the Municipality of Chersonissos. This symposium was always a great way for young aspiring students to meet the most eminent researchers from around the world, and develop professional and personal relationships with them, by being together and interacting -professionally during the sessions and socially during the lunches, dinner and excursion- for a whole week, in a single-session scientific conference.

At the conclusion of the 12th ISRPF, you are invited to submit your work for publication in a Special Issue that will be published by the journal **General and Comparative Endocrinology**, edited by **Mylonas, CC, Bobe, J, Piferrer, F** and **Schulz, R**. These colleagues will coordinate the review process, each according to his expertise in the specific area of the submitted manuscripts. We welcome any of you that have completed a project and are ready to disseminate the results, to consider submitting your manuscript for evaluation, which will follow the regular procedures of the journal. Previous such Special Issues from the ISRPF have been very successful and very well citied, as Dr. Deborah Power, one of the Editors-in -Chief, confirmed. **Submission will open in 1 June 2023 and the deadline is 31 August 2023.**

The scientific host of the 12th ISRPF is **the Institute of Marine Biology, Biotechnology and Aquaculture** of the **Hellenic Centre for Marine Research**, of which I am currently the director. The HCMR was established in 2001 by merging the Institute of Marine Biology of Crete (IMBC, a private institute established in 1985 in Heraklion, Crete) with the National Centre of Marine Research (NCMR, a public institute established in 1985 in Athens). The HCMR consists of three institutes, the other two being the Institute of Marine Biological Resources & Internal Waters and the Institute of Oceanography.

The IMBBC has facilities in Crete and mainland Greece (Anavyssos). Its headquarters and its main facilities are in the premises of HCMR in Crete, named "Thalassokosmos" (see picture below). The "Thalassokosmos" complex is located 15 km east of the city of Heraklion (and 15 km from the ISRPF venue) and includes the aquaculture facilities "Aqualabs" and the public aquarium "Cretaquarium". The IMBBC is the only European Union marine institute that operates a **pilot scale commercial net pen facility** (30 mT of annual production). The IMBBC is also unique in Europe in operating an **Underwater Biotechnological Park** near its premises. This is an open-sea underwater research facility (50,000 m^2), about 1 km off the coast of "Thalassokosmos" supporting multi-disciplinary research and technology demonstration.

The IMBBC has two Research Sectors, the Marine Biology & Biotechnology (**MB&B**) and the Aquaculture Sector (**AQUA**).

The **MB&B** Research Sector carries out work in the fields of genetics/genomics, bioinformatics, bioanalysis and biotechnology, marine biodiversity, and marine ecology and ecosystem management. Modern approaches are employed to assess biodiversity and ecosystem functioning in the marine realm, integrating field observation, documentation and collection, taxonomy, experimental setups, genetics/genomics and modeling.

The **AQUA** Research Sector carries out research in the fields of fish biology, reproduction, ethology, nutrition, pathology of all developmental stages (larvae to harvestable size), and in final product quality improvement and production technologies. Beyond the widely farmed species in the region, emphasis is also given in species diversification, in order to develop a profitable and sustainable aquaculture industry. **AQUA** and **MB&B** collaborate in various research areas, the most important being genetics/genomics of cultured fishes, integrated multitrophic aquaculture (IMTA) and the use of genetics and epigenetics to develop improved strains and broodstock management methods.

Currently, **IMBBC has 25 permanent researchers**, 16 Post-docs, 34 PhD, MSc and Undergraduate students, and 70 technicians and other personnel. More than 65% of the technicians are on contract, paid by research grants and private funding. Annual funding during the last 5 years came from competitive Greek (52%) and European Union (28%) grants, and 18% from private funds in the form of contract research and the provision of goods and services.

On behalf of the Scientific Program Committee, I would like to welcome you to the **12th ISRPF 2023 in Chersonissos, Crete, Greece** and wish you a great time, scientifically and socially.

Constantinos (Dinos) C Mylonas
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Director, Institute of Marine Biology, Biotechnology & Aquaculture
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Scientific Program Committee

Julién Bobe, INRA, France
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Oliana Carnevali, Uni of the Marche, Italy
Neil Duncan, IRTA, Spain
Yoav Gothilf, Uni Tel Aviv, Israel
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José Antonio Muñoz-Cueto, Uni of Cadiz, Spain
Francesc Piferrer, ICM-CSIC, Spain
Hanna Rosenfeld, NCM-IOLR, Israel
Rüdiger Schulz, Uni Utrecht, Netherlands
Berta Levavi-Sivan, Uni Jerusalem, Israel
Yonathan Zohar, IMET-Uni Maryland, USA

Sponsors

IRIDA SA is a feed production company, providing top-quality nutrition for aquatic farmed organisms. The company also provides end-to-end solutions for its customers and has recently invested in **Aquatic Biologicals SA**, a spin-off company of the Hellenic Centre for Marine Research producing autogenous vaccines and providing disease diagnostic services for the Greek aquaculture sector.

The **Hellenic Aquaculture Producers Organization (HAPO)** has a membership of 23 companies, which represent 80% of the Greek aquaculture production. The objective of HAPO is to promote its products to selected domestic and foreign markets.

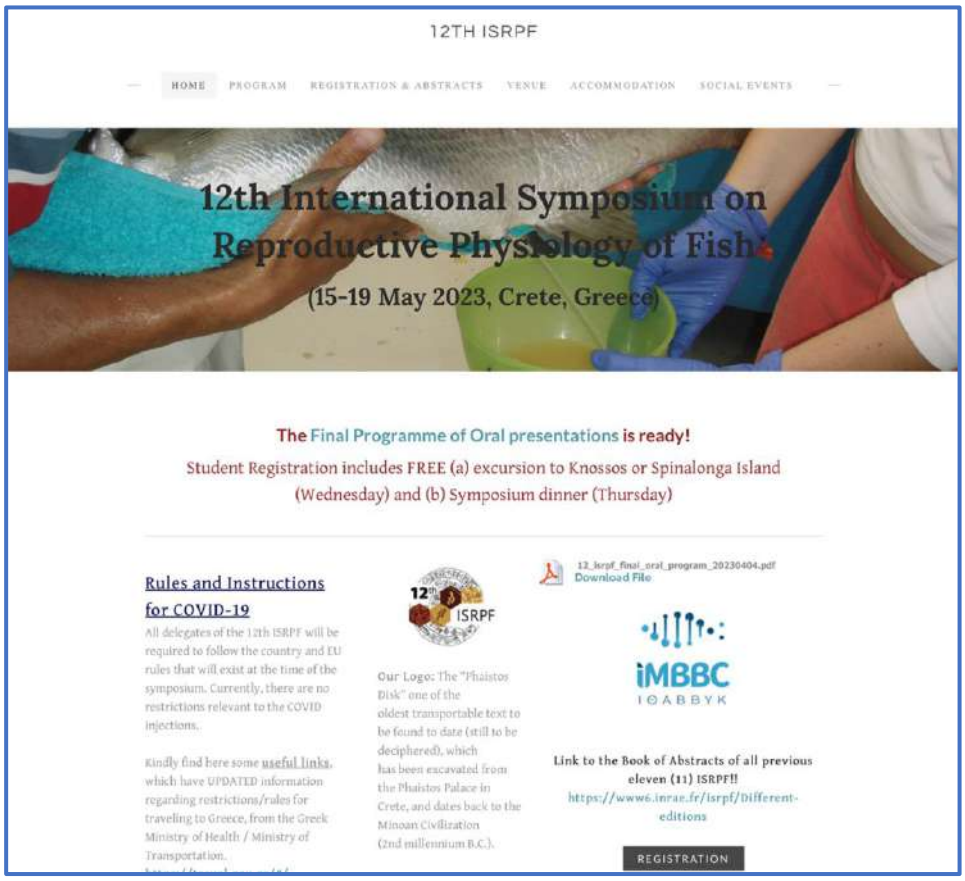
StavRAS Aquatic Solutions Ltd is a small aquaculture consulting and product provider based in Cyprus. The company specializes in the design, sourcing and construction of complete Recirculation Aquaculture Systems (RAS). In addition, **StavRAS** represents important aquaculture suppliers in the Eastern Mediterranean region, such as Fish Farm Feeder, Pentair, BernAqua, UltrAqua and others.

Galaxidi Marine Farm SA is a hatchery and grow-out company of Mediterranean marine fish operating for more than 30 years in the Corinthian Gulf. The company is the main producer of organic marine fish in Europe, and its products follow the most prestigious standards, such as NATURLAND, GLOBAL G.A.P and others.

The **Region of Crete** is one of the 13 regions of Greece. The island of Crete is the 5th largest in the Mediterranean Sea and the largest one in Greece. Crete is an important part of the economy and cultural heritage of Greece, preserving its own cultural elements. Between the years 3000-1400 BC, the Minoan civilization, one of the first civilizations of Europe, flourished on the island, with its main centers being Knossos and Phaistos. The island is very mountainous with three main mountain ranges, Dikti (Lasithi, 2148 m), Ida (Psiloritis, 2456 m) and the White Mountains (2454 m), which cross the island from east to west. About 3,5 million tourists visit the island every year, to enjoy what has been called “A continent in an island”, due to its wide diversity of landscapes and ecosystems.

The 12th ISRPF is **co-organized with the Municipality of Chersonissos**. The Municipality of Chersonissos and its wider area has an important cultural heritage that is part of the place and its history, and reflects the culture and the physiognomy of the society. With the history of the area dating back to the pre-Minoan era, the area has a significant number of monuments complemented by modern cultural infrastructure, making up an integrated cultural network. The importance of the area's cultural resources is a dynamic element in the development of the area. The Municipality of Chersonissos has a rich natural environment, combining large mountain ranges, plains, rivers and gorges, creating a unique landscape. The coastal character of the area in combination with the constant changes of the landscape and the ideal climate of the region contributes to the existence and development of many species of flora and fauna.

The website of the 12th ISRPF (12isrpf.weeebly.com**)**



A screen-shot from the website of the 12th ISRPF during the preparation of the symposium. It will remain active for the next year, to inform everyone of issues related to the Special Issue in the journal General and Comparative Endocrinology, as well as the final Book of Abstracts.

Acknowledgements

As the president of the Scientific Program Committee of the 12th ISRPF, I would like to acknowledge the presence and contribution to this symposium, of the **only person who attended all twelve (12) ISRPF**, beginning with the first one in Paimpont, France (1977).

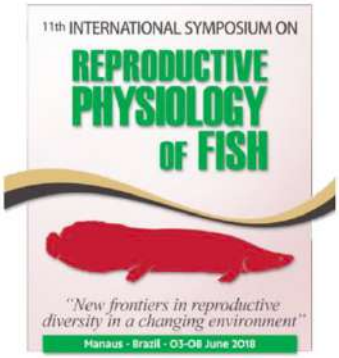
Professor Yonathan (Yoni) Zohar (Institute of Marine and Environmental Technology and University of Maryland Baltimore County, USA)

I take extreme pride of being his first registered PhD Student (1991), and I am sure that so does every scientist -be it student, researcher of professor- who ever associated with this remarkable and wonderful person and scientist! He is still as active as ever in research, teaching and publishing, and I look forward to seeing him in yet another ISRPF. Congratulations Yoni!

Constantinos (Dinos) C Mylonas

Memories of ISRPF Past

11th ISRPF – Manaus, Brazil (2018)



10th ISRPF – Olhao, Portugal (2014)

9th ISRPF – Cochin, India (2011)

8th ISRPF – St Malo, France (2007)

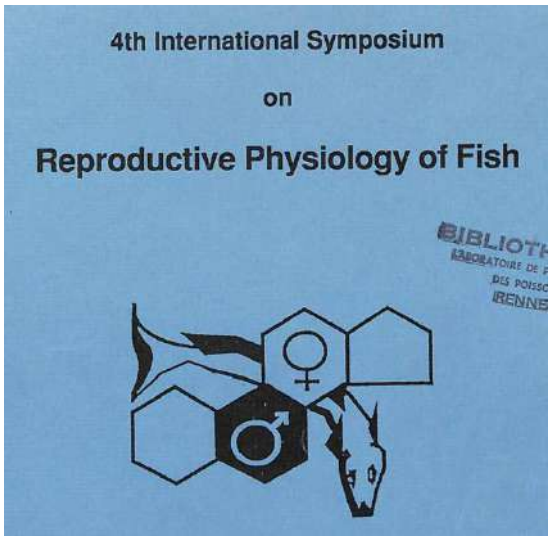
7th ISRPF – Mie, Japan (2003)

6th ISRPF – Bergen, Norway (1999)

6TH INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH

5th ISRPF – Austin, Texas (1995)

4th ISRPF – Norwich, United Kigdom (1991)



General Information

Traveling to Crete and the 12th ISRPF

Since Crete is an island, you can arrive by airplane from Athens or directly from several European cities. The national airlines are **Aegean Airways and Olympic Airways** (affiliated with **Star Alliance**). Crete is also accessible daily by ferry from Athens (Piraeus Harbor) and the trip takes 9 hrs (21:30 - 06:30). The venue is 25 km from the **international airport of Heraklion** (also spelled Iraklio), **"Nikos Kazantzakis" (HER).**

From the airport in Heraklion, you can get to the venue in **Chersonissos** (Aldemar Knossos Royal Beach Resort,) by **bus** or **taxi**. You can also rent a car, from a large number of different companies (both International and local). **Be careful if you will drive yourself**. Greeks are notorious of being "adventurous" drivers, and the road system in Crete is not the best!

The **bus station** is located at the entrance of the airport site, at the right side of the traffic lights as you are looking away from the airport. The bus will get you from Heraklion Airport to Chersonissos in 45 minutes, and the ticket will cost €3-5. Tickets can be bought from the bus driver when boarding or in a ticket kiosk at the bus stop.

Taxis are available right at the exit from the airport building (arrivals). A taxi trip will last 25 minutes, and its cost is €40-50.

Crete

Crete is the 5th largest island in the Mediterranean, and one of the most popular vacation destinations in Greece, with more than 3 million tourists spending their summer holidays every year. Crete was home to one of the oldest civilizations in Europe (2700-1420 BC), the Minoan Civilization who gave the name to the continent (Europa).

The island's tourism infrastructure caters to all tastes, including a very wide range of accommodation. The island offers large luxury hotels with complete facilities, swimming pools, sports and recreation, as well as smaller family-owned apartments, camping facilities and others. Visitors reach the island via two international airports in Heraklion and Chania (international charter and domestic flights starting May) or by boat to the main ports of Heraklion and Chania.

Popular tourist attractions include the archaeological sites of the Minoan civilization, the Venetian old city and port of Chania, the Venetian castle at Rethymno, the Gorge of Samaria, the small nearby islands of Chrysi, Elafonisi, Gramvousa, Spinalonga and the Palm Beach of Vai, which is the largest natural palm forest in Europe. The island has a number of gorges, such as the Samariá Gorge and Imbros Gorge. Many islands, islets, and rocks hug the coast of Crete. Some of the islands that can be visited are:

- Gramvousa (Kissamos, Chania) the pirate island opposite the Balos lagoon,
- Elafonisi (Chania), which commemorates a shipwreck and an Ottoman massacre,
- Chrysi island (Ierapetra, Lasithi), which hosts the largest natural cedar forest in Europe.

Chersonissos

Chersonissos (also spelled Hersonissos) is one of the oldest towns of Crete. It was first inhabited in the distant Minoan period, and in a former settlement, archeologists found a lot of valuable discoveries. The city has many sights, the main of which are the Roman port, Roman theater, Early Christian Basilica, and the Roman Fountain. At the same time, Chersonissos is the typical holiday seaside resort, with a nice series of beaches to discover, good restaurants and tavernas, bars, plenty of shops, souvenir stores and video game arcades for children. And of course, a very lively nightlife.

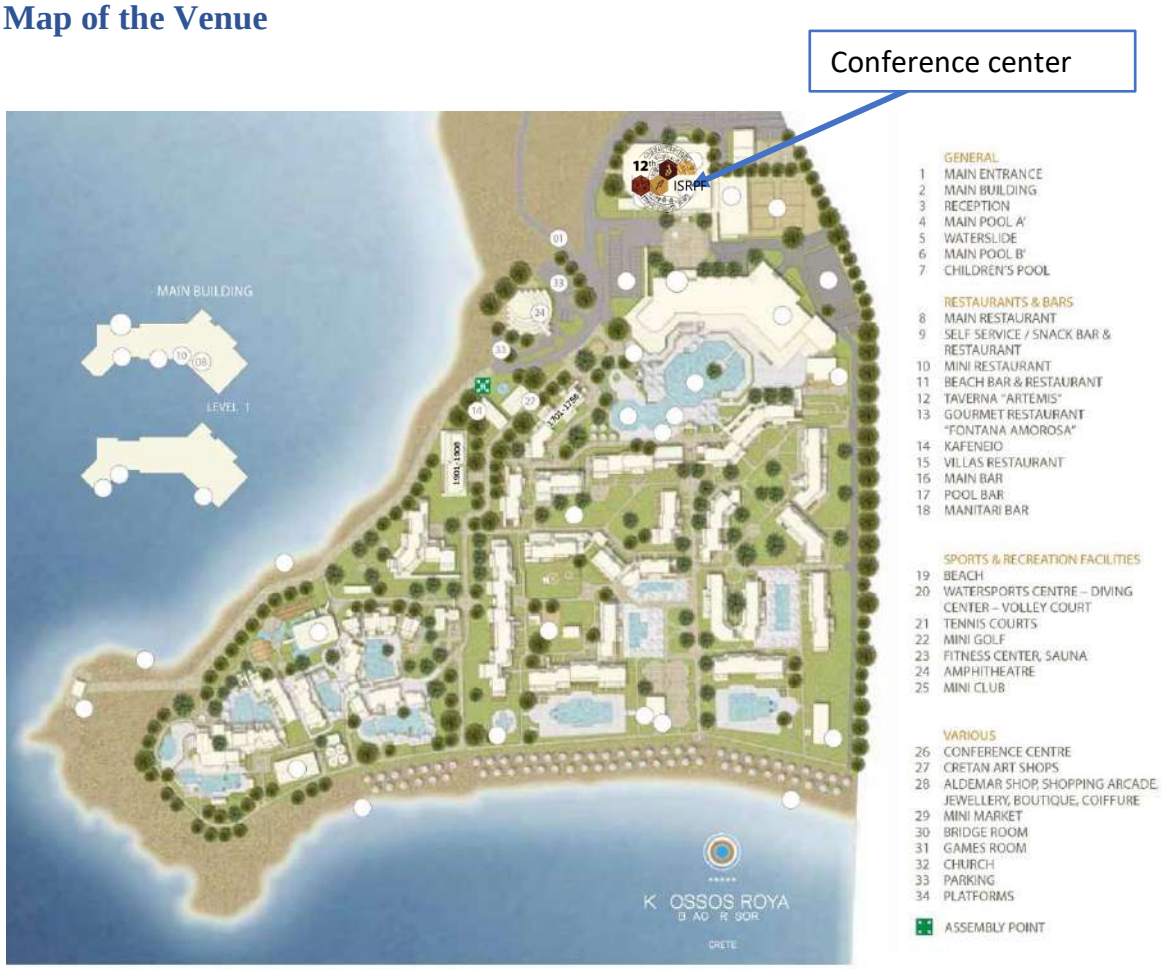
The Venue

Aldemar Knossos Royal Beach Resort is the Venue of the 12th International Symposium on Reproductive Physiology of Fish. This is a 5-star family resort, located in one of the most popular tourist areas of Crete.

It is located at the tip of the Annisaras Peninsula in the community of Chersonissos, with a beautiful beach right in front of it. Bathed in sunlight, calmed by the gentle waves of the Aegean Sea rests this idyllic and stylish resort. It is 25 km east of the Nikos Kazantzakis International Airport and the capital of the island Heraklion. Minoan-style architecture combined with recently refurbished rooms, gleaming swimming pools and lush gardens put the visitor in the perfect relaxing mood. Please visit www.aldemarknossosroyal.gr for more information.

Aldemar Knossos Royal Beach Resort's conference center has two large halls, tastefully decorated, and equipped with state of the art audiovisual and simultaneous interpreting systems. The conference hall can take 300 participants with appropriate projection and sound systems. The area for the poster session is separate and located 50 meters from the conference hall. The coffee breaks will take place around the Posters to facilitate their viewing.

Map of the Venue



The CRETAquarium at Thalassokosmos

The **CRETAquarium** belongs to the **Hellenic Center for Marine Research (HCMR)** and is located in the Thalassokosmos complex 15 km from the 12th ISRPF venue towards the city of Heraklion. Thalassokosmos is the largest complex for marine research, technology and entertainment in the Mediterranean area, and the **CRETAquarium** offers visitors a unique opportunity to explore the magnificent Mediterranean seaworld. From large predator sharks to small sea horses and spectacular jellyfish, the diversity of marine life is showcased against a backdrop of Cretan underwater seascapes, such as the rocks at Matala (South Crete) and the seabed at Vai (Southeast Crete).

The Mediterranean seascape comes to life in 62 different tanks varying in size from 125 to 900,000 L of sea water, totaling 1.8 million L in all. There are one hundred observation points for visitors to admire approximately 2,500 Mediterranean and tropical organisms. A full tour taking in all exhibits and species lasts about 2 hours.


After your Tour, you can visit the CRETAquarium gift-shop for gifts and souvenirs that will complete your visiting experience. It's name is «Octopus» and it addresses visitors of all ages. On the aquarium premises you will also find a snack bar / restaurant, and there is a long sandy beach in front of the aquarium, and several other beaches further east.

CRETAquarium is open to the public daily from 09:30am to 19:00pm

General entrance fee	**12 €**	Personal Audio Guide	**3€**
Children 5-17 and seniors over 65 years old	**6 €**	Virtual Reality experience	**5€**
Students (with ID**)**	**6 €**		

Social activities

Welcome reception – Sunday 14 May 2023 (19:00)

The ISRPF 2023 Welcome Reception will take place at the Aldemar Knossos Royal Beach Resort, which offers the ideal scenery for welcoming the participants to Crete and to see old friends and colleagues, and meet new students and young researchers! The Welcome Reception is **free for all participants**, courtesy of the Hellenic Aquaculture Producers Organization. We wil get to sample fish from the Greek aquaculture industry, courtesy of Galaxidi Marine Farm SA.

Group Excursion - Wednesday 17 May 2023 after lunch (13:30)

Wednesday afternoon is devoted to socializing and networking. Participants may visit some of the most important historical sites of Crete, at an extra cost for regular registrants (45-47€), but **free for students**, courtesy of IRIDA SA. There are two options available: the Knossos Palace (Heraklion Region) or Spinalonga Island (Lassithi Region).

Guided tour to the Knossos Palace (the cradle of the Minoan Civilization)

Amongst all the ancient monuments in Crete, Knossos is the archaeological site that is a must-see in order to understand the greatness of the Minoan Civilization (2000 BC). The tour takes you to the island's capital, Iraklio (Heraklion), and from there onto the village of Knossos where you will be led through the ruins of the ancient palace complex: the labyrinth, galleries and rooms of the Palace, and the Royal Palace of King Minos, the son Zeus (the God of Gods, in Greek Mythology) and Europa, who gave her name to our continent! Return to the hotel is after approximately 5 hours. The excursion includes transfers to/from Knossos Palace with luxury a/c coaches, a professional-official English-speaking guide per coach, and entrance fee and full guidance in Knossos Palace.

Guided tour to Spinalonga Island (a Venetian fortress, an Ottoman village and a Leper colony)

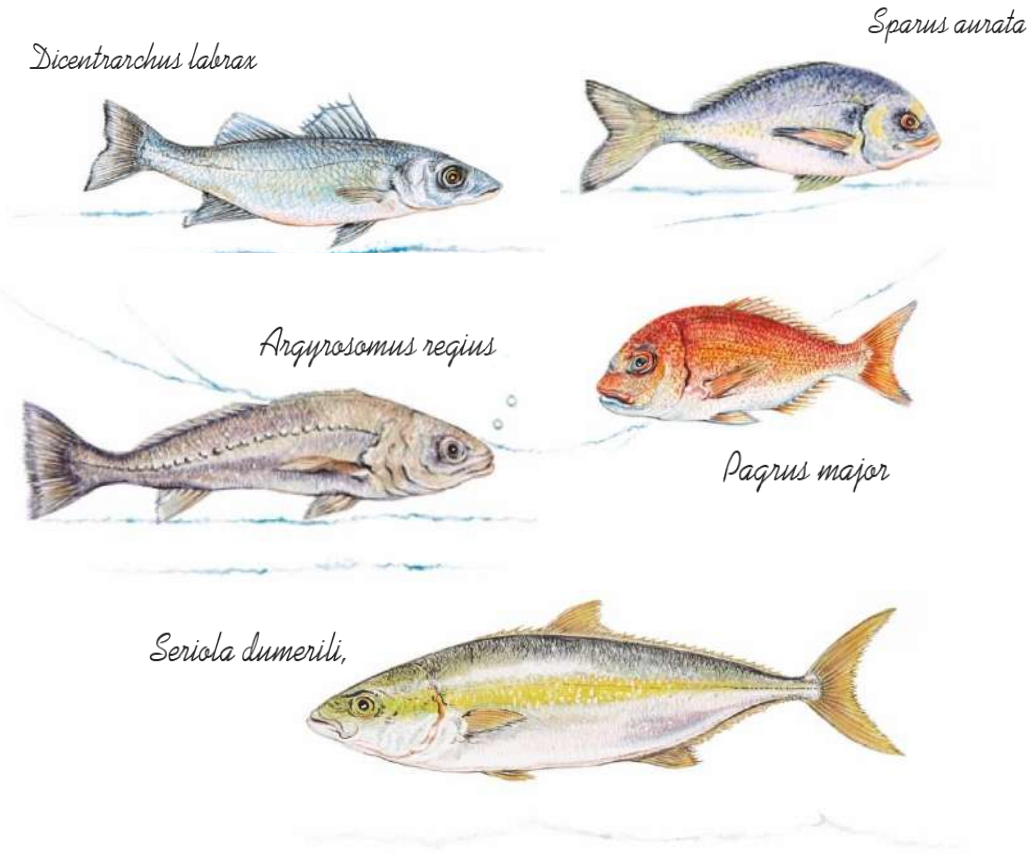
Participants will be picked up from the hotel and transferred to the town of Elounda and then with a 10 min boat ride across to the island of Spinalonga, which has an exceptionally interesting history. Once a Venetian Fortress built in 1579 and later an Ottoman village, in the 20th century it has become synonymous with human pain. In 1903, Spinalonga developed into a gathering place for people infected with Leprosy (Hansen's disease) from all over Crete, where they spent the rest of their life! Today, with the painful story forgotten, the colony is being restored and it offers a pleasant vist with spectacular scenery! After this nice visit the boat will bring participants back at Elounda or Ag. Nikolaos. The excursion includes transfers to/from Elounda with luxury a/c coach, a professional-official English-speaking guide per coach, boat ride to/from Spinalonga Island, and entrance fee and full guidance to the Island.

Symposium dinner – Thursday 18 May 2023, evening (19:00)

The Symposium dinner will take place on Thursday 18th May at a traditional restaurant. It is located at the picturesque Cretan village "Ano Karouzana" and it is 17.5 km from the Symposium venue. The bus transfers between the venue and the restaurant will be done by coach, leaving the entrance of the venue (Aldemar Knossos Royal Beach Resort) at 19:00. One bus will depart from the restaurant to bring participants back to the hotel at 23:00. The remaining buses will depart from the restaurant between 24:00-01:00, depending on how the party goes!

The Symposium dinner is optional and at an extra cost for regular registrants (50€), but **free for students (**courtesy of IRIDA SA). We are sure that all will enjoy the hospitality and festive atmosphere in a local "γλέντι" (translated as "party", pronounced "glendi") with Cretan food and folk dances, so get your ticket too!

Fish cultured in Grece and the Mediterranean Sea



Instructions for Participants

Oral Presentations

A presenter's desk will be available near the registration desk. All presenters should submit their Oral presentation to the organizers, **the day before the presentation.** Bring your presentation (in Microsoft Powerpoint for **Windows not macOS**) saved in a USB memory stick. If you are using an Apple Mac computer to prepare your presentation at home, make sure that it runs well on a Windows-based computer, especially if you have videos or soundtracks.

The file should be labeled as "Oral **X_Lastname**_Session **Z**", where:
X = sequence number of the oral presentation, according to the Symposium Program,
Lastname = the last name of the presenter, and **Z** = Special session number

You should check your presentation in the presenter's desk computer, and make sure that all runs well. All presentations will be loaded in the projection computer the day before each Special Session, and no modifications can be made during the day.

See later, for suggestions/advice on preparing your oral presentation.

Poster presentations

The accepted size for the Posters is A0 upright (119 cm height x 84 cm width). The poster must be printed on gloss or matt paperor light fabric, but **not heavy canvas or rigid plasticize material**, which is too heavy to stay on the board! We will provide pins, double sided tape, and blue tag for putting up the posters. Posters should be placed on their corresponding board on Sunday 14 or at the latest Monday 15 May 2023 in the morning, and will be organized according to Special Session. Each Poster will be given a sequence number, and the boards will be numbered accordingly. Presenters having an odd number of poster (P1, P3 etc) should stand by and present their posters during the Tuesday 16 May 2023 Poster Session (I). Presenters having an even number of poster (P2, P4, etc) should stand by and present their posters during the Thursday 18 May 2023 Poster Session (II). Abstracts submitted late and after the conclusion of the Program, will be given a sequence number from 101 onwards, and will be displayed in a different location than the Special Session to which they are allocated, at the end of Special Session 9.

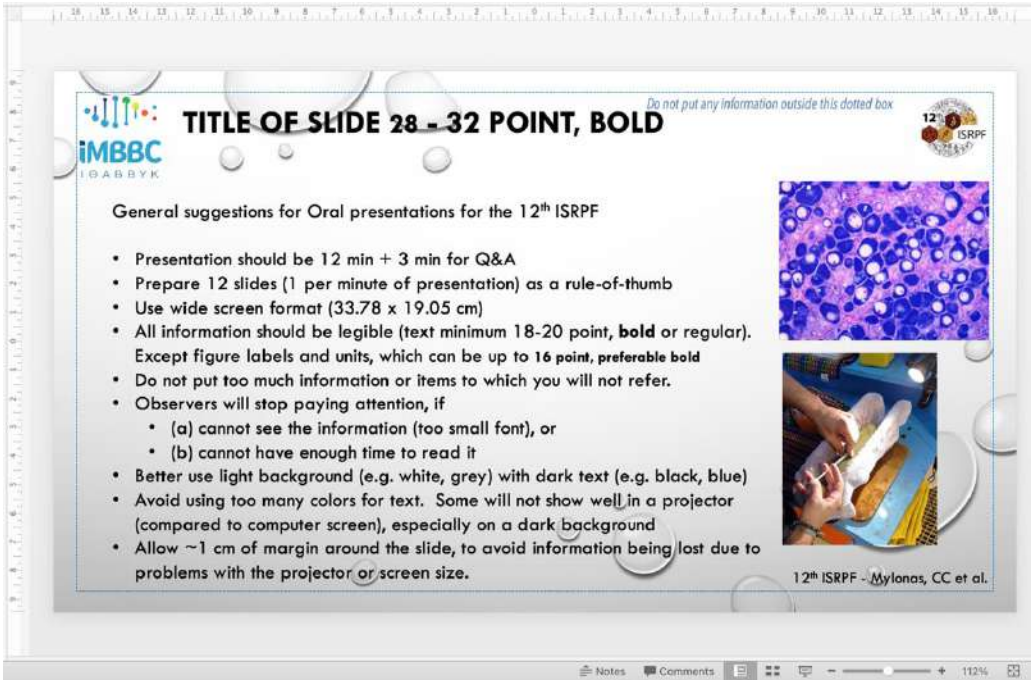
See later, for suggestions/advice on preparing your poster presentation.

Best Student Oral and Poster presentation

There will be two (2) awards for best **Student Oral Presentation** and two (2) awards for best **Student Poster Presentation**. The awards will also include a financial reward. The evaluation of the presentations will be made by the members of the Scientific Program Committee, and the announcement and award ceremony will be held on Friday 19 May 2023, during the closing session (18:00). To be easier for the evaluation committee members to identify student posters and oral presentations, these have been identified in the Program and Book of Abstracts (student). It is suggested that an identification symbol is added to the poster itself (next to the photo of the presenter), as well as to the opening slide of the oral presentation (next to the title of the presentation), such as the **one provided here** (graduation cap and diploma).

General suggestions for Oral presentations for the 12th ISRPF

- Presentation should be **12 min + 3 min** for Q&A
- Prepare 12 slides (1 per minute of presentation) as a rule-of-thumb
- For the **Title of slide use 28 or 32-point text, bold**
- Use wide screen format (33.78 x 19.05 cm)
- All information should be legible (**text minimum 18-20 point**, **bold** or regular). Figure labels and units, may be up to **16 point, preferably bold**
- Do not put too much information or items to which you will not refer.
- Observers will stop paying attention, if
    - (a) they cannot see the information (too small font), or
    - (b) cannot have enough time to read it
- Better use light background (*e.g.* white, grey) with dark text (*e.g.* black, blue)
- Avoid using too many colors for text. Some colours will not show well in a projector (compared to computer screen), especially on a dark background
- Allow ~1 cm of margin around the slide, to avoid information being lost due to problems with the projector or screen size. **See sample slide below:**

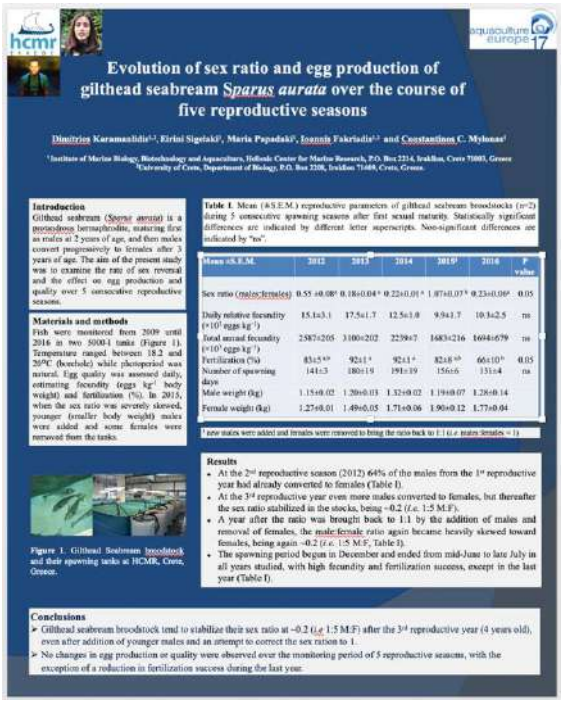


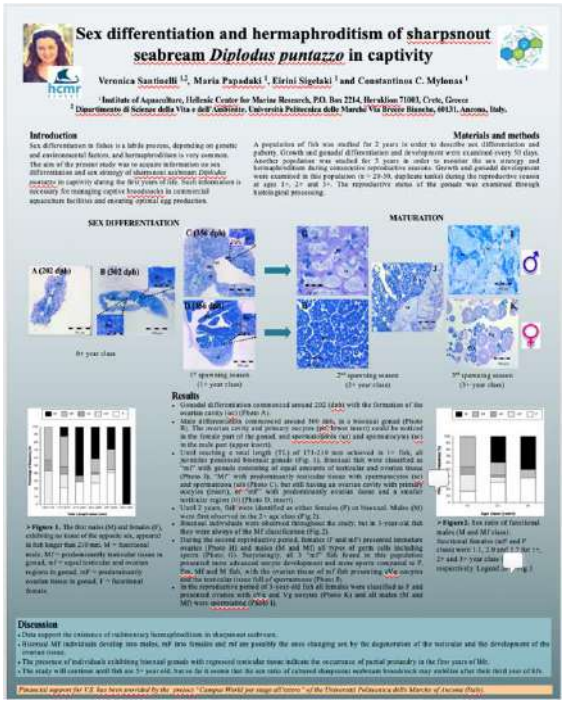
General suggestions for Poster presentations for the 12th ISRPF

The accepted size for the Posters is A0 upright (119 cm height x 84 cm width). The poster must be printed on gloss or matt paper (not canvas or rigid plasticize material, which is too heavy to stay on the board!) Posters should be placed on their corresponding board on Sunday 14 or at the latest Monday 15 May 2023 in the morning, and will be organized according to Special Session. Each Poster will be given a sequence number, and the boards will be numbered accordingly. We will provide pins, double sided tape, and blue tag for putting up the posters.

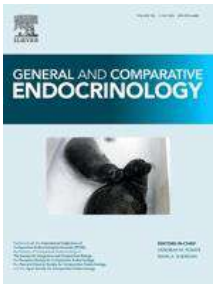
- Place a photo of the person **presenting the poster** next to the title. This will help participants find the presenter, if they want to discuss about the poster.
- For the **Title of the poster use >60-point text, bold**
- All information should be legible (text minimum 36 point, **bold** or regular). Except figure labels and units, which can be up to **28 point, preferably bold**
- Observers will often not stand at a Poster, if
    - (a) they cannot read the information easily (too small font), or
    - (b) there is too much information and too many details
- Use eye-cathing photos and graphics
- Focus more on the Objectives and less on the methods
- Results are often easier to be read, if in a bullet format
- Conclusions should be clear and well-supported by the data and statistical analysis
- Display the logos of the affiliated University/Research organization and the project funding the research
- Always acknowledge the funding agency and project contract number

See sample poster below:





Special Issue in the journal General and Comparative Endocrinology

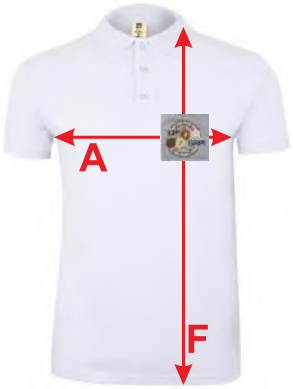


As in previous ISRPFs, we will have a Special Issue (SI) in the journal General and Comparative Endocrinology (GCE) with manuscripts from the Oral and Poster presentations of the 12th ISRPFs. The SI will be edited by Mylonas, CC., Bobe, J., Piferrer, F. and Schulz, R. Manuscript submission will be through the regular platform of GCE, **between 1 June 2023 and 31 August 2023**, with expected publication time the beginning of 2024. Manuscripts considered for publication should not necessarily include "endocrine" work or data, since the ISRPF includes research in all aspects of Fish Reproductive Physiology and not just endocrinology. However, they must come from work presented in the 12th ISRPF or by an author registered in the symposium presenting other work in the area of Fish Reproductive Physiology.

T-shirts

We have produced a Polo t-shirt with the logo of the symposium. The t-shirts will be available at the registration desk, beginning on Tuesday 16 May 2023 at a cost of 15€ each. There is only one color and a unisex-style, in sizes S, M, L, XL, 2XL, 3XL and 4XL, according to the chart below (in cm).

MK215	1/2 Pecho 1/2 Chest	Largo delantero Front length
SIZE	A	F
S	50	69
M	53	71
L	56	73
XL	59	75
2XL	63	77
3XL	66	80
4XL	69	83
5XL	72	86



A certificate of attendance will be sent by email to all participants, **after** the conclusion of the symposium.


CERTIFICATE OF ATTENDANCE

THIS IS TO CERTIFY THAT

AMALIA MAVROEIDI

has participated at the

12th International Symposium on Reproductive Physiology of Fish

15 to 19 May 2023 at Chersonissos, Crete, Greece

Constantinos C. Mylonas
President of the Scientific Program Committee
Institute of Marine Biology, Biotechnology & Aquaculture
Hellenic Centre for Marine Research
Heraklion, Crete, Greece

Program at a glance

Sunday 14 May	Monday 15 May	Tuesday 16 May	Wednesday 17 May	Thursday 18 May	Friday 19 May
14:30 – 18:00 Registration	**08:30 – 09:00** Welcoming **Dr. Constantinos (Dinos) C. Mylonas**, HCMR, Greece	**09:00 – 10:00** **Plenary** by **Pr. Daniel Pauly**, UBC, Canada	**09:00 – 12:15** SS5. Climate change & anthropogenic impacts (10:30 -11:00 Coffee break)	**09:00 – 12:00** SS6. Reproduction in aquaculture (10:30 -11:00 Coffee break)	**10:00 – 13:00** SS8. Behaviour & pheromones (11:30 -12:00 Coffee break)
19:30 Welcome reception (dinner and drinks)	**09:00 – 10:00** **Plenary** by **Dr. Sylvie Dufour**, CNRS, France	**10:00 – 13:00** SS3. Oogenesis/ vitellogenesis & ovulation (11:00 – 11:30 Coffee break)	**12:15 - 13:30** Lunch	**12:00 - 12:15** Group Photo	**13:00 - 14:30** Lunch
	10:00 – 13:00 SS1. Sex determination & Differentiation (11:00 – 11:30 Coffee break)	**13:00 - 14:30** Lunch	**13:30 – 19:30** *Excursion to Knossos Palace (Heraklion) or Spinalonga Island (Lasithi)	**12:15 - 13:30** Lunch	**14:30 -17:30** SS9. Reproductive biotechnologies (16:00 -16:30 Coffee break)
	13:00 - 14:30 Lunch	**14:30-17:00** SS4. Spermatogenesis & spermiation		**13:30 - 16:00** SS7. Gamete & egg quality	**17:30 – 18:00** Summary **Birgitta Norberg**, IMR, Norway
	14:30-17:30 SS2. Brain- pituitary – gonad axis (16:00- 16:30 Coffee Break)	**17:00- 19:00** Poster 1 (Odd numbers) & Coffee break		**16:00 -18:00** Poster 2 (Even numbers) & Coffee break	**18:00 – 18:30** Closing **Dr. Constantinos (Dinos) C. Mylonas**, HCMR, Greece
				19:00 *Symposium Dinner	

*Optional and at additional charge but **free of charge** for students with valid University ID.

Session chairs and co-chairs, and State-of-the-art speakers

Special Session	Chair	Co-chair	State-of-the-art speaker
1. Sex determination and differentiation	Piferrer, Francesc	Chang, Ching-Fong	Shao, Changwei
2. Brain-pituitary-gonad axis	Levavi-Sivan, Berta	Golan, Matan	Golan, Matan
3. Oogenesis/vitellogenesis and ovulation	Rosenfeld, Hanna	Yilmaz, Ozlem	Yilmaz, Ozlem
4. Spermatogenesis and spermiation	Schulz, Rüdiger	Chauvigné, François	Crespo, Diego
5. Climate change and anthropogenic impacts	Norberg, Birgitta	Carnevali, Oliana	Servili, Ariana Moreira, Renata
6. Reproduction in aquaculture	Migaud, Hervé	Horvath, Akos	Akos Horvath
7. Gamete and egg quality	Bobe, Julién	Żarski, Daniel	Żarski, Daniel
8. Behaviour and pheromones	Duncan, Neil	Li, Weiming	Li, Weiming
9. Reproductive biotechnologies	Zohar, Yonathan	Yoshizaki, Goro	Yoshizaki, Goro

Program

Sunday 14th May 2023	
14:30 – 18:00	Registration
19:30	Welcome Reception

Monday 15th May 2023	
08:30 – 09:00	Welcoming **Mylonas, Constantinos (Dinos) C** President of Scientific Program Committtee
09:00 – 10:00	Plenary Talk 1 **Dr Dufour, Sylvie** *"A historical perspective of Fish Reproductiuve Physiology Research"*
10:00 – 11:00 **SS1.** **Sex determination and differentiation** Chair: Piferrer, Francesc Co-chair: Chang, Ching-Fong	Invited State-of-the-Art Presentation 1 **Shao, Changwei** *"Epigenetic regulation of sex determination and differentiation in fish: The interaction between genes and the environment"* Oral Presentation 1 **Srikulnath, Kornsorn** *"Exploring the genomic basis of sex determination in African catfish and bighead catfish"* Oral Presentation 2 **Patil, Jawahar G** *"Sex and sexability: A curious case of Gambusia holbrooki"*
11:00 – 11:30	Coffee Break
11:30 – 13:00 **SS1.** **Sex determination and differentiation** Chair: Piferrer, Francesc Co-chair: Chang, Ching-Fong	Oral Presentation 3 **Chakraborty, Tapas** *"Oct4 and sexuality in fish gonad; an understanding using medaka, Oryzias latipes"* Oral Presentation 4 **Murata, Ryosuke** *"Sex change evolution and signaling involving TIS cells in groupers"* Oral Presentation 5 **Sánchez-Baizán, Núria** *"Effects of sex and temperature in the European sea bass epigenome"*

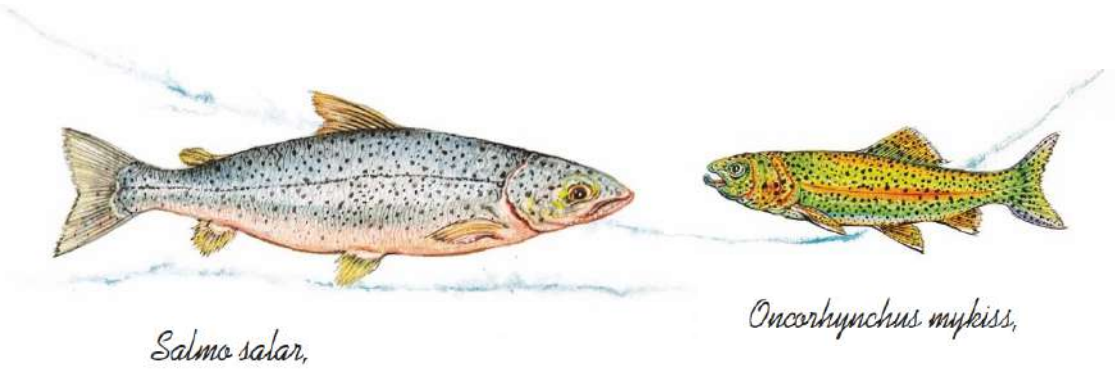
Monday 15th May 2023	
11:30 – 13:00 **SS1.** **Sex determination and differentiation** Chair: Piferrer, Francesc Co-chair: Chang, Ching-Fong	Oral Presentation 6 **Panthum, Thitipong (**student) *"Polygenic sex determination or recent emergence of a new sex determining region in the Siamese fighting fish (Betta splendens)"* Oral Presentation 7 **Bhandari, Ramji K** *"Methylome of medaka sperm and eggs and their reprogramming in post-fertilization stage embryos and primordial germ cells"* Oral Presentation 8 **Tseng, Peng-Wei** (student) *"The potential mechanism of sex change (secondary sex determination) in the protandrous black porgy, Acanthopagrus schlegelii"*
13:00 - 14:30	Lunch
14:30 – 16:00 **SS2.** **Brain-pituitary-gonad axis** Chair: Levavi-Sivan, Berta Co-chair: Golan, Matan	Invited State-of-the-Art Presentation 2 **Golan, Matan** *"Networking for reproduction: how direct cell-cell communication in the teleost HPG axis shapes its development and its output"*
	Oral Presentation 9 **Zmora, Nilli** *"Possible roles for GnRH3 in regulating pituitary organization in female zebrafish as revealed by neuronal ablation and single cell RNA sequencing"* Oral Presentation 10 **Wang, Bin** *"Gonadotropin-inhibitory hormone (GnIH) and its receptors in the European sea bass (Dicentrarchus labrax): intracellular signaling pathways and interaction with other neuroendocrine factors"* Oral Presentation 11 **Chen, Jie** (student) *"Somatostatin signaling is a key regulator in the allocation of metabolism to reproduction."* Oral Presentation 12 **Mennigen, Jan** *"Reproductive consequences of CRISPR/Cas9-Based avp knock-out in zebrafish (Danio rerio)"*
16:00 – 16:30	Coffee Break

Monday 15th May 2023	
16:30 – 17:30 **SS2.** **Brain-pituitary-gonad axis** Chair: Levavi-Sivan, Berta Co-chair: Golan, Matan	Oral Presentation 13 **Cohen Rothschild, Noam** (student) *"Characterization of a novel fast-growing zebrafish: a new approach to GH transgenesis"* Oral Presentation 14 **Andersson, Eva** *"Loss of fshr inhibits maturation in male Atlantic salmon"* Oral Presentation 15 **Galotta, Mariel** (student) *"Multi-tissue targeted DNA methylation analysis of gonadotropins in chub mackerel (Scomber japonicus) using a cost-effective sequencing method"* Oral Presentation 16 **Ferrão, Leonor** (student) *"Superoxidase dismutases in the European eel: characterization and expression in vivo under different temperature conditions"*

Tuesday 16th May 2023	
09:00 – 10:00	Plenary Talk 2 **Professor Pauly, Daniel** *"On the Direction of the Causal Arrow Linking Growth and Reproduction in Fishes"*
10:00 – 11:00 **SS 3. Oogenesis/vitellogenesis and ovulation** Chair: Rosenfeld, Hanna Co-chair: Yilmaz, Ozlem	Invited State-of-the-Art Presentation 3 **Yilmaz, Ozlem** *"Role of multiple vitellogenins in early development of fishes"* Oral Presentation 17 **Lewis, Blake** (student) *"Unpacking the egg's earliest life support system – identification of putative cortical alveoli proteins in zebrafish, Danio rerio"* Oral Presentation 18 **Tokumoto, Toshinobu** *"Phenotypic analysis of gene knock-out strains of highly upregulated genes during ovulation in zebrafish"*
11:00 – 11:30	Coffee Break
11:30-13:00 **SS 3. Oogenesis/vitellogenesis and ovulation** Chair: Rosenfeld, Hanna Co-chair: Yilmaz, Ozlem	Oral Presentation 19 **Bobe, Julién (replacing Janatti-Idrissi, Sarah)** *"Hippo pathway-mediated regulation of micropyle formation by microRNA 202 (miR-202) in the fish oocyte"* Oral Presentation 20 **Papadaki, Maria** (student) *"MicroRNAs are involved in ovarian maturation of greater amberjack (Seriola dumerili) under captivity*" Oral Presentation 21 **Thermes, Violette** *"Quantitative analysis of asynchronous oogenesis dynamics in fish: Application of a modern 3D analysis approach to an old problem"* Oral Presentation 22 **Sehgal, Neeta** *"Genes encoding for non-phosphorylated vitellogenin and choriogenin express during the early stages of oogenesis in the Indian freshwater murrel, Channa punctatus"*

11:30-13:00 **SS 3. Oogenesis/vitellogenesis and ovulation** Chair: Rosenfeld, Hanna Co-chair: Yilmaz, Ozlem	Oral Presentation 23 **Sempere, Laura** (student) *"Seeking for the relationship between body size and maturity in female European sea bass (Dicentrarchus labrax L.)"* Oral Presentation 24 **Gioacchini, Giorgia** *"New insights by Fourier Transform InfraRed (FTIR) Microspectroscopy on yolk composition, distribution and role in Mustelus mustelus a placentotrophic viviparous shark"*
13:00 - 14:30	Lunch
14:30 – 17:00 **SS 4. Spermatogenesis and spermiation** Chair: Schulz, Rüdiger Co-chair: Chauvigné, François	Invited State-of-the-Art Presentation 4 **Crespo, Diego** *"Follicle-stimulating hormone effects and the regulation of early spermatogenesis: from model to aquacultured fish species"* Oral Presentation 25 **Nóbrega, Rafael** *"Fsh regulates the proliferation of embryonic-like germ stem cells in adult zebrafish testes"* Oral Presentation 26 **Kjærner-Semb, Erik** *"Lack of vgll3a delays onset of sexual maturation in Atlantic salmon (Salmo salar) males"* Oral Presentation 27 **Diaz, Noelia** *"European sea bass gene expression dynamics during spermatogenesis"* Oral Presentation 28 **Prat, Francisco** *"Expression of Gdnf-Gfra1-Ret system genes and nanos2 in the European seabass testis during the reproductive cycle and under unilateral orchiectomy (ULO) conditions"* Oral Presentation 29 **Zapater, Cinta** *"Involvement of the transcriptional coactivator Ncoa7 during initial stages of spermatogenesis in European sea bass (Dicentrarchus labrax)"* Oral Presentation 30 **Chauvigné, François** *"Differential regulation and function of two luteinizing hormone receptors during flatfish spermiogenesis"*

14:30 – 17:00 **SS 4.** **Spermatogenesis and spermiation** Chair: Schulz, Rüdiger Co-chair: Chauvigné, François	Oral Presentation 31 **Palaiokostas, Christos** *"A multi-omics study on male fertility in farmed Arctic charr (Salvelinus alpinus)"* Oral Presentation 32 **Bondarenko, Olga** *"The role of Ca2+ and pH in the regulation of trout spermatozoa motility"*
17:00 – 19:00	Poster 1 (Odd numbers) & Coffee break



Wednesday 17th May 2023	
09:00 -10:30 **SS5.** **Climate change and anthropogenic impacts** Chair: Norberg, Birgitta Co-chair: Carnevali, Oliana	Invited State-of-the-Art Presentation 5 **Servili, Arianna** *"Climate change impacts the reproductive neuroendocrine axis of fish"*
	Invited State-of-the-Art Presentation 6 **Moreira, Renata** *"The effects of aluminum, and water quality parameters, on the reproduction of Astyanax altiparanae (Characiformes: Characidae), a neotropical teleost"*
	Oral Presentation 33 **Sarih, Samira** *"Influence of temperature on early puberty of juvenile male European sea bass (Dicentrarchus labrax)"* Oral Presentation 34 **Yamamoto, Yoji** *"Multi-year field survey on the effects of environmental factors on the sex determination in the cobaltcap silverside Hypoatherina tsurugae"*
10:30 – 11:00	Coffee Break
11:00 -12:15 **SS5.** **Climate change and anthropogenic impacts** Chair: Norberg, Birgitta Co-chair: Carnevali, Oliana	Oral Presentation 35 **Piferrer, Francesc** *"Sex reversal in natural populations: types, causes and consequences"* Oral Presentation 36 **Devergne Jimmy** (student) *"Effect of climatic and estrogenic stress on the life cycle of an estuarine fish, Gasterosteus aculeatus"* Oral Presentation 37 **Lombó, Marta** *"Glyphosate exposure disrupts zebrafish spermatogenesis"* Oral Presentation 38 **Kestemont, Patrick** *"Reproductive physiology of zebrafish affected by contraceptive pill hormones: estetrol as an ecological alternative to ethinylestradiol?"* Oral Presentation 39 **Alix, Maud** *"Is teleost fecundity style a species-characteristic trait? – A re-evaluation of the ovulatory cycle of Atlantic cod (Gadus morhua) in a changing environment"*
12:15 - 13:30	Lunch
13:30 – 19:30	Excursion **Knossos Palace (Heraklion) or Spinalonga Island (Lasithi)**

Thursday 18th May 2023	
09:00 -10:30 **SS6.** **Reproduction in aquaculture** Chair: Migaud, Hervé Co-chair: Horváth, Ákos	Invited State-of-the-Art Presentation 7 **Horváth, Ákos** *"Cryopreservation research in aquaculture: what the industry needs and what it doesn't"* Oral Presentation 40 **Geffroy, Benjamin** *"Can we sex fish using circulating miRNAs? a comparative study"* Oral Presentation 41 **Felip, Alicia** *"The relationship between the IGF system and the early onset of puberty in male and female European sea bass (Dicentrarchus labrax)"* Oral Presentation 42 **Kleppe, Lene** *"Effects of bmp15 mutation on gonad development and fertility in Atlantic salmon"* Oral Presentation 43 **Rosenfeld, Hanna** *"Aquaculture improvement toolkit for grey mullet (Mugil cephalus): broodstock management & production of all-female genetic lines"*
10:30 – 11:00	Coffee Break
11:00 – 12:00 **SS6.** **Reproduction in aquaculture** Chair: Migaud, Hervé Co-chair: Horváth, Ákos	Oral Presentation 44 **Beato, Silvia** (student) *"DNA methylation during early development in diploid and triploid European sea bass"* Oral Presentation 45 **Jéhannet, Pauline** (student) *"|Improving the artificial reproduction of the European eel to enhance larval quality"* Oral Presentation 46 **Vallainc, Dario** *"Flat-head grey mullet (Mugil cephalus) farming conditions for producing bottarga, histological, physiological and biochemical processes during gametogenesis"* Oral Presentation 47 **Fatsini, Elvira** *"The use of sand substrate modulates stress response and enhances maturation in Senegales sole females"*
12:00 – 12:15	**Group Photo**
12:15 - 13:30	Lunch

Thursday 18th May 2023	
13:30 – 16:00 **SS7.** **Gamete and egg quality** Chair: Bobe, Julién Co-chair: Żarski, Daniel	Invited State-of-the-Art Presentation 8 **Żarski, Daniel** *"Do non-genetic inheritance factors blur mechanisms responsible for egg quality?"* Oral Presentation 48 **Jeuthe, Henrik** *"Exploring the occurrence of DNA-fragmentation in sperm of different Swedish Arctic charr (Salvelinus alpinus) broodstocks and its impact on offspring viability"* Oral Presentation 49 **Niepagen, Nils** (student) *"Abnormal development after gastrulation: a novel egg quality parameter for Atlantic halibut (Hippoglossus hippoglossus) in aquaculture"* Oral Presentation 50 **Anderson, Kelli** *"A multi-omics approach to studying egg quality in flow-through and RAS-conditioned Tasmanian Atlantic salmon broodstock"* Oral Presentation 51 **Pšenička, Martin** (cancelled) *"Post-ovulatory oocyte aging leads to a significant PGC decline, which affects sexual differentiation."* Oral Presentation 52 **Félix, Francisca** (student) *"Supplemented melatonin did not confer extra protection to Senegalese sole spermatozoa during cryopreservation"* Oral Presentation 53 **Schaerlinger, Berenice** *"Feeding during the resting period and oogenesis is critical for successful reproduction in Eurasian perch (Perca fluviaitilis)"* Oral Presentation 54 **Samarin, Azin Mohagheghi** *"Molecular mechanisms of aging in fish oocytes"* Oral Presentation 55 **Gayo, Patricia** (student) *"Evaluation of antioxidants on sperm quality in Senegalese sole"*
16:00 – 18:00	Poster 2 (Even numbers) & Coffee break
19:00	**Symposium Dinner**

Friday 19th May 2023	
10:00 -11:30 **SS8.** **Behaviour and pheromones** Chair: Duncan, Neil Co-chair: Weiming, Li	Invited State-of-the-Art Presentation 9 **Li, Weiming** *"Structure, function, and potential application of sea lamprey reproductive pheromones"* Oral Presentation 56 **Amano, Yuichi** (student) *"Using surrogate fish for eradicating invasive fish: Can surrogate triploid rainbow trout mate with their wild-type counterparts and produce lethal hybrids?"* Oral Presentation 57 **Oliveira, Rui** *"Coevolution of the oxytocin signaling pathway and reproductive behavior in African cichlids"* Oral Presentation 58 **Huertas, Mar** *"Olfactory sensitivity to conspecific odors released by striped bass (Morone saxatilis) during reproduction"* Oral Presentation 59 **Scaia, María Florencia** *"Two to tango: the importance of reproductive and hormonal variables in intrasexual aggression in Cichlasoma dimerus"*
11:30 – 12:00	Coffee Break
12:00 – 13:00 **SS8.** **Behaviour and pheromones** Chair: Duncan, Neil Co-chair: Weiming, Li	Oral Presentation 60 **Ashoori, Samyar** (student) *"Possible role of faeces in chemical communication in the Mozambique tilapia (Oreochromis mossambicus)"* Oral Presentation 61 **Sorensen, Peter** *"Acute olfactory sensitivity of bighead and silver carp to 9 sex steroids strongly suggests that novel mixtures of 21-carbon steroids function as species-specific priming pheromones in bigheaded carps"* Oral Presentation 62 **Siapazis, Christos** (student) *"Reproductive behavior and parental contribution of meagre (Argyrosomus regius) in aquaculture conditions"* Oral Presentation 63 **Fakriadis, Ioannis** *"Sound production in relation with breeding behaviour in meagre (Argyrosomus regius) in aquaculture conditions"*
13:00 – 14:30	Lunch

Friday 19th May 2023	
14:30 -16:00 **SS9. Reproductive biotechnologies** Chair: Zohar, Yonathan Co-chair: Yoshizaki, Goro	State-of-the-Art presentation 10 **Yoshizaki, Goro** *"Improved methods for long-term culture of germ cells capable of differentiating into eggs and sperm when transplanted into recipients"* Oral Presentation 64 **Moriya, Natsuko** (student) *"Luteinizing hormone gene over-expression in pre-pubertal rainbow trout can induce sperm production within a short period."* Oral Presentation 65 **Lancerotto, Stefano** (student) *"Gonadal maturation and spawning of hatchery-produced greater amberjack (Seriola dumerili) following administration of single-chain recombinant greater amberjack gonadotropins"* Oral Presentation 66 **Duncan, Neil** *"Sex-specific advance in pubertal maturation in response to in vivo application of recombinant Fsh and Lh to prepubertal meagre (Argyrosomus regius)"* Oral Presentation 67 **Wong, Ten-Tsao** *"Sterile salmonids produced by transient gene silencing and their applications in aquaculture and studying fish reproductive endocrinology."*
16:00 -16:30	Coffee Break
16:30 - 17:30 **SS9. Reproductive biotechnologies** Chair: Zohar, Yonathan Co-chair: Yoshizaki, Goro	Oral Presentation 68 **Luckenbach, J Adam** *"Investigation of approaches for sterility induction in sablefish Anoplopoma fimbria"* Oral Presentation 69 **Nayak, Rigolin** (student) *"Genome-wide comparative methylation analysis in zebrafish produced via surrogacy"* Oral Presentation 70 **Gao, Linan** (student) *"Replacement of mitochondria in sturgeon germline"* Oral Presentation 71 **Ichida, Kensuke** *Production of offspring derived from cryopreserved spermatogonia by surrogate broodstock in ayu (Plecoglossus altivelis)*
17:30 – 18:00	Summary **Norberg, Birgitta**
18:00 – 18:30	Closing **Mylonas, Constantinos (Dinos) C.**

Abstracts of Plenary Talks

Plenary Talk 1

An historical overview of fish reproductive physiology, from comparative physiology to ecological and evolutionary physiology

Dufour, Sylvie

National Museum of Natural History (MNHN), Sorbonne University, CNRS, IRD, Paris, France

E-mail: sylvie.dufour@mnhn.fr

Physiology is defined as the study of the functioning of living organisms and of their constituent organs, tissues or cells. If the invention of the term "physiology" is attributed to Jean Fernel in the 16th century, the roots of "physiology" in Europe can be traced back to ancient Greece, namely with Hippocrates and the foundation of medicine. Physiology has been and is still largely focusing on human, but the development by Claude Bernard of "experimental medicine" on animal models, opened the field of comparative physiology. By investigating various animal species, comparative physiology allows to decipher some conserved basic physiological processes, thanks to suitable model organisms, but also to reveal the potential variety of physiological functions and regulations through the animal kingdom. The raise of aquaculture in the past decades has largely promoted fish reproductive physiology, in order to solve reproduction-related bottlenecks. An increasing diversity of fish species is now being investigated worldwide for their relevance to basic and applied reproductive physiology. The diversity of fish models for physiological studies is facilitated by the generalization of broad cutting-edge approaches from genomics to field investigation. Fish reproductive physiology may pursue various objectives such as aquaculture diversification and sustainability, fisheries impact on life traits and population sustainability, biodiversity conservation, physiological mechanisms involved in species adaptation and evolution, as well as vertebrate models for medical research. Fish as aquatic organisms are specially threatened by anthropogenic pollutants which converge and accumulate into fresh and marine waters. Reproductive endocrine disruption has been first revealed in a freshwater fish and various fresh and marine fish models are now studied as sentinels, experimental models and biodiversity targets of pollutants. Reproductive physiology of fish, as poikilotherm organisms, is also threatened by global warming, from sex determination to regulation of reproductive cycles. In the current context of global changes and alarming loss of biodiversity, the field of fish ecological reproductive physiology thus attracts increasing attention. Even if "fish" species, share common features as aquatic coldblooded animals with fins and gills, phylogenetical studies highlight that they do not constitute a monophyletic group. "Fish" indeed encompass representative species of major vertebrate groups, including cyclostomes, chondrychthyans, actinopterygians and even basal sarcopterygians. This phylogenetic diversity further supports the relevance of "fish" studies for evolutionary biology, making "fish" major models for the rising field of vertebrate evolutionary reproductive physiology. In the frame of today's scientific challenges and global threats, fish reproductive physiology is contributing to the expansion of comparative physiology into ecological and evolutionary physiology.

Plenary Talk 2

On the direction of the causal arrow linking growth and reproduction in fishes

Pauly, Daniel

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The conventional view of the relationship, in fish, between growth and reproduction is the 'reproductive load' hypothesis, which states that, following the rapid growth of juveniles, the achievement of maturity cause 'energy' previously channeled to growth to be redirected to the elaboration of gonads, which then causes somatic growth to decline. This notion, which is mainly derived for the appearance of growth curve in length, becomes untenable when growth in weight is examined, in which first maturity (in virtually all fishes capable a attaining length of more than 10 cm) is reached well before growth rate is maximized (i.e., their growth accelerates after first maturity and spawning). This and multiple other features of fishes contradicting the conventional view of their relationship between growth and reproduction, led to a radical new view of fish physiology, also applicable to other water-breathing ectotherm (WBE; crustaceans, cephalopods, etc.), with emphasizes the role of respiration and oxygen in the life-history of WBE, i.e., the Gill-Oxygen Limitation Theory.

Abstracts per session - Oral Presentations

SS1. Sex determination and differentiation

Invited State-of-the-Art presentation 1

Epigenetic regulation of sex determination and differentiation in fish: The interaction between genes and the environment

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Environmental sex determination (ESD) occurs in divergent, phylogenetically unrelated taxa, and in some species, co-occurs with genetic sex determination (GSD) mechanisms. Although epigenetic regulation in response to environmental effects has long been proposed to be associated with ESD, a systemic analysis on epigenetic regulation of ESD is still lacking. Using the Chinese tongue sole (*Cynoglossus semilaevis*) as a model—a marine fish that has both ZW chromosomal GSD and temperature-dependent ESD—we investigated the role of epigenetic mechanisms including DNA methylation, ncRNAs and histone modifications in transition from GSD to ESD. Comparative analysis of the gonadal DNA methylomes of pseudomale, female, and normal male fish revealed that genes in the sex determination pathways are the major targets of substantial methylation modification during sexual reversal. Methylation modification in pseudomales is globally inherited in their ZW offspring, which can naturally develop into pseudomales without temperature incubation.We found that the stability of tongue sole methylome during embryonic reprogramming enables multigenerational environmental plasticity on sex determination.We also detected decreased levels of Ca2+ signaling pathway-related genes in spermatogonia, insufficient meiotic initiation in spermatocytes, and a malfunction of somatic niche cells in pseudomales using the single-cell sequencing. Transcript expression patterns shift during the sex differentiation phase, and ceRNA modulation occurs through crosstalk of differentially expressed long ncRNAs (lncRNAs), circular RNAs (circRNAs), microRNAs (miRNAs), and sex-related genes in fish. The circRNA from the sex-determining gene *dmrt1* (circular RNA *dmrt1*) and a lncRNA, called AMSDT, share the same miRNA response elements with *gsdf*, which has an up-regulated expression when they bind to miRNA cse-miR-196 and concurrent down-regulated female sex-related genes to facilitate testis differentiation. We also found that the higher expression of G9A/GLP increase the level of H3K9me2, which in turn recruit the DNA methylation, leading to the testis development. In conclusion, our results suggest a complex epigenetic regulation involving the DNA methylation, ncRNAs and histone modifications in the sex determination and differentiation in fish species.

The project received funding from the National Nature Science Foundation of China.

Oral Presentation 1

Exploring the genomic basis of sex determination in African catfish and bighead catfish

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INTRODUCTION

Sex determination (SD) mechanisms in many teleost fishes, including the aquaculturally important catfish, differ from vertebrates such as mammals and birds. The sex chromosomes studied in different species of catfish are mostly homomorphic or less differentiated, posing major challenges for the identification of specific sex determination genes. An emerging hypothesis posits that sex-determination system complexity between two parental species mightaffect sterility, and consequently hinder breeding and productivity. Here, we present our recentfindings from the investigation of two important catfish species, African catfish (*Clarias gariepinus*) and bighead catfish (*Clarias macrocephalus*) with previously unknown SD.

METHODS

We integrated cytogenetic/karyotyping, histological examination and genotyping by sequencing methods coupled with bioinformatic analysis and PCR-based marker validation to determine the mechanisms underlying the SD systems in both species. Meiotic chromosome preparations for light microscopy were made by the air-dry method for histological analysis oftestis sections. Genotyping of multiple SNP loci was accomplished using Diversity Arrays Technology Pty Ltd. (DArTseq™). Hybrid sex-linked loci were searched to find the genome-wide SNP of parents of all sex-linked loci that met our criteria and had a statistically significantassociation with phenotypic sex.

RESULTS & DISCUSSION

Results from African catfish revealed the existence of both male-linked and female-linked lociacross all the examined hybrid specimens. Most of these loci were not sex-linked in the parental species, suggesting that the hybrid exhibited a combination of different alleles, indicating a polygenic sex determination (PSG) system in African catfish. Annotation of the identified sex- linked loci revealed the presence of one female-linked locus homologous with the *B4GALNT1* gene, which is involved in the spermatogenesis pathway and hatchability. Findings from bighead catfish showed a male heterogametic XX/XY sex-determination system with one of the sex-linked loci annotated with the *GTSF1L* gene, showing a testis-enriched expression pattern. We further investigated the spermatogenic phenotype linked with male sterility in North African catfish and found a number of degenerated spermatocytes. The observed inter- species and interpopulation diversity in SD of both African and bighead catfish species reflectsthe possibility of sex chromosome turnover and confirms PSG in African catfish. This requires further testing by other methods such as genome assembly. Spermatogenic disruptions in the pachytene stage of the F1 hybrid resulted from the failure of homologous chromosome pairing due to chromosomal incompatibility between parental genomes and the two different SDs. Results provide novel insights into the sex-determination mechanisms in clariid catfish and willcontribute to genetic improvements in breeding programs. This project received funding from National Research Council of Thailand (N42A650233)

Oral Presentation 2

Sex and sexability in Gambusia holbrooki: An evolutionary nexus and applied consequences

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INTRODUCTION

The eastern gambusia *Gambusia holbrooki* along with its sister species, *G. affinis* are by far the most widely spread pest fish species in the world. To develop genetic control options for the former, we have been systematically characterizing its reproductive traits. Synthesised here are multiple developmental, cellular, molecular and genetic aspects highlighting its distinct and shared reproductive traits that are critical for managing their pest populations as well as for decoding evolutionary mechanisms of reproduction in vertebrates.

METHODS

All investigations were conducted on wild and/or captive-reared individuals that were obtained throughout Australia and elsewhere. Gravid spot size/intensity, gonopodium morphology, developmental staging, histology karyomorphology and data analyses were conducted using standard procedures. These were complemented by hormonal sex reversal and selective breeding techniques to verify the gamity. Sex-specific molecular markers were developed and validated on multiple populations and used to identify phenotypic trait/s for early (embryonic) detection of sex. The development of germ cells and their fates in respective sexes were investigated using *in situ* hybridisation techniques, while genomic comparisons between males and females were investigated using NGS technology.

RESULTS & DISCUSSION

The size and intensity of the gravid spot served as a reliable external indicator to predict the clutch size as well as the developmental stage, providing a reference for subsequent investigations in this and other live-bearing fish. Most interestingly, our work provides evidence for female heterogamety (ZZ/WZ), which contrasts with the XX/XY sex-determining system reported in this species previously. Taken together both male (XX/XY) and female (ZZ/WZ) sex-determining mechanisms occur in this species, an aspect shared with some members of poecilids. Evolutionarily, this provides a common platform to investigate how and when the transition between the two mechanisms occurred and occurs. From the management of pest population, this has a significant consequence as all the wild populations we tested, comply with female heterogamety. Specifically, this forms the basis for our Trojan W genetic strategy for controlling pest populations of this species. Our discovery of female-specific sex markers and its subsequent deployment to identify phenotypic correlates provides an opportunity for early, automated, and rapid sexing of neonates in this species. These sexually dimorphic traits were also evident at a cellular level e.g., in the proliferation rates of germ cells. Remarkably, germ cell development shared aspects specific to both preformation (as in zebrafish) as well as induction (as in mice) modes, which provide a direct evolutionary link between the two modes. Collectively, our work highlights the importance of eastern gambusia as a model to understand the evolutionary connectedness of reproductive adaptations and the functional diversity of genes involved in vertebrate reproduction, whilst laying a foundation for managing their pest populations globally.

Oral Presentation 3

Oct4 and sexuality in fish gonad; an understanding using Medaka, *Oryzias latipes*

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INTRODUCTION

Germ cells hold the genetic information and are the building blocks for future generation. Pluripotent stem cells (PSC), expressing various conserved stem cell markers (e.g., Yamanaka factors) and mainly found as an inner cell mass during embryogenesis, can be artificially reprogrammed from various adult cells, differentiated, and turned into all types of cells, including eggs and sperm. In our recent investigation, our group has found that any alteration in the initial germ cell population could ultimately change the phenotypic sex of medaka. Using adult medaka, we also found germ line stem cells (GSC) control the functional sex reversal during adulthood which further indicates towards pluripotency.

METHODS

QurtE and Cab strains of medaka (*Oryzias latipes*) were mainly used in this study. Adult mature/ immature male and female medaka were treated with estrogen or aromatase inhibitor to induce sex reversal and periodic samples were conducted for histological, transcriptional, epigenetic and GSC analysis. Fish specific Oct4 antibody (kindly donated by Dr. Yokinori Kazeto, National Fisheries Research Institute, Japan) was used for IHC. Further, Oct4-mcherry-olvas-eGFP double transgenic reporter line was developed and used for antagonist experiments and targeted *knock in* production. Later, GSC from Japanese anchovy (*Engraulis japonicus*) and chub mackerel (*Scomber japonicus*) were collected and Oct4 physiology were confirmed.

RESULTS & DISCUSSION

We found that, irrespective of fish species, Oct4, unlike mammals, expresses in both GSC and germ cells of fish gonad, however, only GSC shows nuclear localization of Oct4. It was observed that, during early stages of sex reversal (from 3-7 days) *Oct4* transcriptions were elevated in GSC, which later subsided (from 15-30 days; when GSC differentiation occurred). Surprisingly, we found that mere addition of Oct4 antagonist blocks the functional sex change. This suggests that Oct4 is essential for GSC maintenance. Later, we collected the GSC at various points of sex reversal and performed targeted epigenetic analysis. We found a strong age, sex and steroid dependent Oct4 methylation pattern. Further it was also found that, intronic regions before the nuclear localization signal (NLS) were more epimethylated from 7-30 days period, which in combination with our western blotting data suggests that Oct4 during GSC differentiation somehow loses its NLS and thus becomes unavailable for GSC maintenance. To prove this, we performed series of targeted *knock in* and conditional *knock out* experiment and preliminary found that deletion of fish specific region (after the NLS) does not affect GSC maintenance but hampers germ cell sex change. Whereas any deletion before NLS inadvertently results in GSC death. Cumulatively, our data suggests that Oct4 is an important regulator of gonadal sexual plasticity. However, further analyses are pertinent to understand the intricate details of Oct4 associated GSC to germ cell differentiation.

The project received funding from JSPS KAKENHI (Grant Numbers 16H04981, 19H03049, 22H00386, 22K05832, 22K19211) and BRAIN, Japan.

Oral Presentation 4

Sex change evolution and signaling involving TIS cells in groupers

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INTRODUCTION

Fishes belonging to the tribe Epinephelini in the subfamily Epinephelinae, family Serranidae, order Perciformes are commonly called groupers. They are found in tropical to temperate coastal areas worldwide. Groupers are protogynous hermaphrodites, that is, they change sex from female to male when they reach a specific size or depend on the social conditions. Our recent study indicated that the endogenous androgen (11-ketotestosterone; 11KT) might trigger the onset of their sex change. Subsequent studies demonstrated that pituitary follicular stimulating-hormone (FSH) plays an important role on the 11KT synthesis at the initiation of sex change. Additionally, we revealed that androgen-producing cells, termed testicular-inducing steroidogenic (TIS) cells, distributed in the tunica of even in the ovary, show positive immunoreactivity against Cytochrome-P450-11βhydroxylase (Cyp11b) which is a steroidogenic enzyme involved in 11KT synthesis, and that TIS cells are the main sites of 11KT production in the ovary of groupers. We describe here the commonalities and expected function of TIS cells on sex change in groupers.

METHODS

In this study, we collected wild 7 genera and 18 species of groupers. Additionally, we obtained 1 species belonging to the tribe Diplonini in the subfamily Epinephelinae, 2 species belonging to the subfamily Anthiinae, family Serranidae, and 1 species of the other family than Serranidae. The existence of TIS cells in gonads were confirmed by immunohistochemistry against Cyp11b using common antibody. Additionally, to clarify the endocrine mechanism of sex reversal signaling involving TIS cells in groupers, we investigated the gonadal gene expression profiles during sex change in the wild caught blacktip grouper, *Epinephelus fasciatus*.

RESULTS & DISCUSSION

The immunohistochemical analysis revealed anti-Cyp11b-immunoreactive TIS cells in the tunica of the gonads of all grouper individuals belonging to tribe Epinephelini investigated in this study, regardless of the gonadal status. Additionally, *D. bifasciatum* belonging to tribe Diploprionini, subfamily Epinephelinae, which is the nearest group of groupers, also showed TIS cells in the tunica of the ovary. However, the immunohistochemical analysis in the outgroups of Perciformes belonging to Sebastinae (gonochorism) and Anthiinae (protogynous hermaphroditism) revealed that there were no TIS cells in the tunica of the gonads. These findings strongly suggest that TIS cells are common structures in the species belonging to subfamily Epinephelinae. From these facts, we conclude that groupers may have acquired their autonomous transsexual ability via the TIS cells during their evolution. Subsequent studies using wild blacktip grouper showed that endogenous 11KT tends to increase with the progression of sex change. On the other hand, there was no change in E2 levels between immature female and male. Additionally, FSH receptor and Cyp11b genes expression levels in gonads were significantly increased during natural sex change from female to male. These results suggest that pituitary FSH stimulates their gonads to produce and secret 11KT at the initiation of sex change.

The project received funding from the JSPS KAKENHI Grant Number 19H03034.

Oral Presentation 5

Effects of sex and temperature in the European sea bass epigenome

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INTRODUCTION

The European sea bass is a fish with mixed genetic and environmental sex determination. Elevated temperature during early development affects sex ratios, causing masculinization. This makes this species an interesting model to study how epigenetics integrates information form the environment to result into different gene expression programs and ultimately phenotypes. The effect of sex and temperature is known to significantly affect two sex-related genes: *cyp19a1* and *dmrt1*. Also, from previous transcriptomic studies performed during sex development, we know that numerous genes are involved in the acquisition and maintenance of the sexual phenotype. Thus, the first objective is to know what other genes and regions are affected by sex and temperature. Besides, although most epigenetic marks are erased after fertilization and re-established after embryonic development, some environmentally-induced marks can escape reprogramming and can be potentially transmitted to the subsequent generations. The second objective is then to investigate what epigenetic marks are transmitted from sires that were exposed to low and high temperatures during their early development to their offspring which in turn were also exposed to exactly the same temperature treatment. Finally, we investigate whether there is an additive effect of epigenetic marks by comparison of the methylome from fish exposed zero, one (sire or offspring) or two times (sires and offspring) to elevated temperature.

METHODS

We created five families by crossing one female with five males: two reared low (16ºC, LT) and three reared at high (21ºC, HT) temperature during early development (12-60 days post fertilization). Then, offspring were also exposed to LT and HT temperatures during the same early period. Fish were sacrificed at one year old and gonadal tissue was obtained for DNA extraction. We produced 64 libraries by Reduced Representation Bisulfite sequencing technique to analyze the DNA methylome of five sires and one dam gametes (F0, n=6) and offspring gonad (F1, n=58). We obtained an average of ~22,8 million raw reads per sample. Alignment to the reference genome was 90.7% and resulted in >700,000 CpGs per sample, covered >10x.

RESULTS & DISCUSSION

Between males and females reared at LT, we found a total of 4,961 differentially methylated regions (DMRs, with methylation difference >10% and *q*-value < 0.05), of which up to 96.75% were hypomethylated regions in females. The distribution of those DMRs across chromosomes, genomic features, and coding genes with emphasis on those related to sex differentiation will be discussed. Regarding the effect of temperature, on the F1, when comparing HT males vs LT males from unexposed sires we found a total of 161 DMRs, while the same comparison in offspring of exposed sires resulted in a total of 2,580 DMRs, of which 78.14% were hypermethylated in the HT males, indicating a cumulative effect of temperature on the epigenome. Finally, we will examine which proportion of those epigenetic marks are transmitted from sire sperm to offspring gonads. Together, these results will help to explain the role of epigenetic regulatory systems and how the environment affects the epigenome during crucial early developmental stages. Also, these data will help to understand how epigenetic mechanisms may contribute to the next generation adapting to rapidly changing environments.

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Oral Presentation 6 (student)

Polygenic sex determination or recent emergence of a new sex determining region in the Siamese fighting fish (*Betta splendens*, Regan, 1910)

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INTRODUCTION

Siamese fighting fish (*Betta splendens*) show sexual dimorphism; males have long ventral and tail fins with vivid and uniform body coloration, whereas females have shorter fins and dull, patterned bodies, with biased offspring sex ratios observed in many previous studies probably resulting from environmental factors. Different populations showed various sex determining aspects, suggesting complex sex determination traits in this species. Further studies on Siamese fighting fish populations are required to elucidate the sex determination mechanisms in this lineage. Here, we investigated the mode of sex determination in several populations of Siamese fighting fish using genome-wide SNP and genome analysis.

METHODS

Multiple loci genotyping were sequenced with DArTseq™ to determine candidate sex-specific/sexlinked loci within 75 individuals (40 males and 35 females) in five populations. Gene ontology and *in silico* chromosome mapping with SNP loci were also used to search for homologies in Siamese fighting fish (accession no. GCF_900634795.3) and other vertebrates using comparative genomic analyses. Genome analysis and the distribution of repetitive sequences were also carried out using RepeatMasker (RM; version 1.332) using Krait version 1.3.3 to identify candidate sex-specific regions.

RESULTS & DISCUSSION

In total, 73 male-specific loci were identified and mapped to a 5.6 kb region on chromosome 9, suggesting a putative male-determining region (pMDR) containing localized *dmrt1* and *znrf3* functional sex developmental genes. Repeat annotations of the pMDR revealed an abundance of transposable elements, mainly the Ty3/Gypsy, with novel types of repetitive tandem arrays and high microsatellite distribution identified around this and the neighboring regions. These elements were often found on sex chromosomes in vertebrates, suggesting that they may contribute to the process of sex chromosome differentiation. Remarkably, two out of the 73 male-specific loci were located on chromosomes 7 and 19, implying the existence of polygenic sex determination, while five female-specific loci on chromosome 9 were also observed in certain populations, indicating the possibility of a femaledetermining region. Perhaps, female-specific loci in Siamese fighting fish recently emerged as new sexdetermining loci during domestication and repeated hybridization leading to sex chromosome turnover.

The project received funding from the High-Quality Research Graduate Development Cooperation Project between Kasetsart University and the National Science and Technology Development Agency (NSTDA) (6417400247).

Oral Presentation 7

Methylome of medaka sperm and eggs and their reprogramming in post-fertilization stage embryos and primordial germ cells

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INTRODUCTION

Eggs and sperm are responsible for the continuation of generations. Following the epigenetic reprogramming of the embryo, core epigenetic information present in the sperm and eggs is transmitted to offspring somatic cells before the blastula stage, which specifically influences gene expression in the cells. This information is established in mammals and poorly defined in fish. It is important to understand the epigenetic profiles of gametes, embryos, and developing germline cells to understand the epigenetic inheritance of health and disease. The present study profiled the methylome of eggs and sperm of medaka (*Oryzias latipes*, Hd-rR) DNA and examined their dynamics during cleavage stages of post-fertilization embryo and in primordial germ cells (PGCs).

METHODS

Genomic DNA obtained from unfertilized eggs, sperm, embryos at various stages of embryogenesis through the gastrula stage, and PGCs were subjected to whole genome bisulfite sequencing to determine the DNA methylation levels in each sample set. Using the curated bioinformatic pipelines, the data was analyzed by a professional bioinformatics analyst.

RESULTS AND DISCUSSION

The egg genome was hypomethylated compared to sperm. We identified gamete-specific differentially methylated regions (DMRs) in the medaka genome. A comparison of sperm methylome with that of eggs revealed that 10 DMRs are hypermethylated and 237 DMRs are hypomethylated in the eggs, suggesting that medaka maintain an allele-specific methylation pattern in gametes. Three regions showed a distinct pattern of maternal imprinted-like regions, which needs further confirmation. After fertilization, the methylation marks of the sperm genome are erased within the first cell cycle, and the embryonic genome remains hypomethylated from the zygote until the 16-cell stage. The DNA methylation (5-mC) level gradually increases from the 16-cell stage through the gastrulation stage. The 5-hydroxymethylation (5hmC) levels show an opposite pattern to DNA methylation. The pattern of genome methylation in medaka embryos is similar to that of mammals but not to zebrafish. We also generated a series of PGC methylomes across key stages from 8 days post fertilization (dpf) to 25 dpf coinciding with germ cell sex determination and gonadal sex differentiation. The results suggest that the PGCs undergo global demethylation in a two-step strategy. The first step occurs between the blastula and 8-dpf stages, and the second step occurs between the 10-dpf and 12-dpf stages. Both demethylation processes are global, except for CGI promoters, which remain hypomethylated throughout the stage of PGC specification. The ground state of methylome reprogramming occurs in PGCs between 15 dpf and 25 dpf. The pattern of genome methylation in the PGCs is similar to that of mammals but not to zebrafish. The present results suggest that medaka likely processes epigenome in gametes, embryos, and germ cells using similar mechanisms as mammals and open the avenue for research on the epigenetic inheritance of phenotypes caused by nutrition, lifestyle, and environmental stressors.

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Oral Presentation 8 (student)

The potential mechanism of sex change (secondary sex determination) in the protandrous black porgy, *Acanthopagrus schlegelii*

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INTRODUCTION

Protandrous black porgy with digonic gonads has a stable primary male phase during the first two reproductive cycles and fish occur sex change to female later. Plasma estradiol-17β (E2) levels and *cyp19a1a* transcripts were significantly increased during the natural sex change process. However, functional femaleness is rarely observed in the E2-induced fish, and a reversible sex change is found after E2 is withdrawn in < 2-yrs-old fish. Precocious femaleness is found in testis-removed fish in <2yrs-old fish which implies the importance of the regressed testicular tissue for further ovarian development. The Gnrh-Gths signaling and testicular *dmrt1* play a critical role in maintaining male fate; however, the communication between testicular tissue and ovarian tissue is still unclear. Therefore, we compare the ovarian expression profiles at early stage of secondary sex determination by transcriptomic studies.

METHODS

To investigate the potential mechanism in secondary sex determination, we surgically removed the testicular part of the digonic gonad at nonspawning season to advance functional femaleness in < 2-yrsold fish. BrdU was used to analyze the cell proliferative activity in gonads between control and testisremoved fish. We then analyzed gene expression profiles in known sex-related genes with qPCR. To identify the potential mRNA and miRNA involved in the early stage of secondary sex determination, the ovarian transcriptome between the control and testis-removed fish was further compared.

RESULTS & DISCUSSION

Histological results showed that oogonia proliferation activity is the first sign of secondary sex determination. qPCR analysis showed that most known sex-related genes are not yet different between the control and early stage of testis-removed fish. In mRNA-seq, we found 289 genes without known sex-related genes that reveal sexual fate dimorphic expression in the ovary between intact and testisremoved fish. Moreover, we found several DEGs annotate as oogenesis, reproduction, and meiosis I cell cycle process in GO term, and oocyte meiosis in the KEGG pathway. These data support the above results. In miRNA-seq, 123 miRNAs revealed differentially expressed in the ovarian tissues between the control and testis-removed fish. Target prediction results showed that increased miR-200a-3p in the control maleness suppresses *cyp19a1a*, and increased miR-223 in the testis-removed femaleness suppresses *dmrt1*. Our work demonstrated that the first characteristic of femaleness was the proliferation of germline cells, and testicular tissue could suppress ovary maturation. The transcriptomic analysis provides several candidate genes in the ovarian tissue that may involve in the early period of secondary sex determination. Moreover, our data provide some sex-bias miRNA which favor the secondary sex determination. These data are consistent with our previous findings about the regulation in plasma E2 levels, the expression of *cyp19a1a* and *dmrt1*, aromatase activity, *cyp19a1a* methylation, and gonadotropins levels during the natural sex change. The further information on the interaction between these potential mRNA and miRNA could provide new insights into secondary sex determination.

SS2. Brain-pituitary-gonad axis

Invited State-of-the-Art presentation 2

Networking for reproduction: how direct cell-cell communication in the teleost HPG axis shapes its development and its output

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INTRODUCTION

The generation of meaningful hormone pulses requires the orchestrated function of large populations of endocrine cells. Understanding the function of the endocrine cell networks that drive this orchestration at the level of the hypothalamus and pituitary gland is at the focus of an intense research effort. Although the pioneering discoveries in the field were made in mammalian models, development of transgenic technologies and live imaging techniques in several fish species in recent years have allowed us to extend our insights of endocrine cell networks to teleosts.

METHODS

At the level of the hypothalamus, neuroendocrine cell networks have been studied mainly in GnRH neurons, which migrate during embryonic development from the nasal placode to the preoptic area. Using calcium imaging of GnRH neurons in live zebrafish larvae, we recently discovered that these cells pause during their migration at the entrance to the brain to form an isolated, synaptically wired circuit. This cell network exhibits synchronized activity that induces a phenotypic switch in the cells that allows them to enter the brain and continue their migration towards their hypothalamic target. Therefore, larval neuroendocrine networks affect the fate of GnRH cells and control the assembly of the adult circuit. Interestingly, the strong coupling between fish GnRH neurons is not limited to early developmental stages, as adult hypothalamic GnRH neurons were shown to be coupled via gap junctions. This coupling allows the coordinated release of GnRH from multiple neurons simultaneously thereby providing a strong stimulus to the pituitary gland to secrete an LH surge.

RESULTS & DISCUSSION

The generation of transgenic fish and specific antibodies have also allowed us to revisit the hypothalamic regulation of anterior pituitary cells. Studies in multiple fish species reveal that upon entering the gland, hypophysiotropic axons make strong interactions with blood vessels, which can then deliver the neuropeptides to their target cells in the adenohypophysis. Such a dominant neurovascular mode of regulation is functionally related to the neuro-vascular coupling in the median eminence of tetrapods. Moreover, we and others described gap junction-coupled gonadotroph networks that are critical for the generation of large-scale long-lasting hormone pulses in response to short hypothalamic stimuli. Additional cell types are now being studied to understand how the fish pituitary gland can adapt its output to match the changing physiological needs of the organism.

Taken together, these insights suggest that the fish HPG axis does not function as a collection of individual cells but rather as a large scale tightly wired endocrine networks in which endocrine cells communicate with each other directly. This additional functional layer serves to shape the development of the HPG axis and to optimize the output of the fish reproductive neuroendocrine system.

Oral Presentation 9

Possible roles for GnRH3 in regulating pituitary organization in female zebrafish as revealed by neuronal ablation and single cell RNA sequencing

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INTRODUCTION

The brain neuropeptide, gonadotropin releasing-hormone (GnRH), is a pivotal regulator of reproduction in vertebrates through controlling LH and FSH expression and secretion from the pituitary. Most vertebrates possess 2-3 isoforms (i.e. GnRH1-3), in which GnRH1, expressed in neurons located in the hypothalamic preoptic area that innervate the pituitary, is the reproductive relevant form. GnRH2 is found in the midbrain tegmentum and plays roles in relaying metabolic cues. GnRH3, is a teleost specific form expressed in the olfactory bulbs and telencephalon, with roles mostly attributed to signal perception and neuro-modulation. Zebrafish possesses only two isoforms of GnRH, GnRH2 and GnRH3, and GnRH3 neurons populate both the forebrain and the preoptic area, hence for years GnRH3 was believed to function as GnRH1. Recent studies, including GnRH3 gene knockout and functional studies however, failed to conclusively establish this role in zebrafish. These findings have prompted studies aimed at understanding how reproduction is regulated in zebrafish and what is the exact role of GnRH3 at the level of the pituitary, which are now leaning toward a multi-factorial redundant control mode.

METHODS AND RESULTS

Results from single cell RNA sequencing of adult female pituitaries revealed that GnRH receptor is expressed only in LH and not in FSH gonadotropes or other pituitary cells. Further, a population of gonadotropes expressing both LH and FSH (termed bi-GtH) was identified, which intriguingly was more prominent in GnRH3 knockout female pituitaries. In order to answer the question whether GnRH3 regulates this population, we chemically ablated GnRH3 neurons by exposing Tg (gnrh3:nfsb) zebrafish mature females to metronidazole for 2 weeks and observed the effect on LH and FSH gonadotropes in the pituitaries. In our studies, this method ablates 80-100% of GnRH3 neurons. To our surprise, depletion of GnRH3 neurons in females has yielded a dramatic increase of the cells expressing both LH and FSH (from 16% to about 60% of total cell population), rendering most gonadotropes “bi-GtH”). The intensity of the LH and FSH signal following immunostaining was profoundly stronger in the ablated females compared the nonablated control females.

DISCUSSION

These results indicate that GnRH3 has a suppressing effect on LH and FSH expression thereof may be involved in the organization of the pituitary cell populations in zebrafish. This proposed function still needs to be further established in light of the fact that only LH cells express GnRH receptor. Furthermore, GnRH3 neuronal projections were described in the pituitaries of most teleosts, including those that express GnRH1. The fact that separate LH and FSH populations are unique to teleosts, raises the possibility that the teleostean specific GnRH3 may have a role in promoting this separation. The mechanism by which GnRH3 exerts this function still needs to be investigated.

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Oral Presentation 10

Gonadotropin-inhibitory hormone (GnIH) and its receptors in the European sea bass (*Dicentrarchus labrax*): intracellular signaling pathways and interaction with other neuroendocrine factors.

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INTRODUCTION

Results of previous studies provided evidence for the existence of a functional gonadotropin- inhibitory hormone (GnIH) system in sea bass, with GnIH exerting an inhibitory action on the brainpituitary-gonadal axis in this species. Herein, we further elucidated the molecular mechanisms of sea bass GnIH actions and the potential interactions with sea bass kisspeptin (Kiss) and gonadotropin-releasing hormone (GnRH) signaling pathways.

METHODS

Luciferase reporter assay system was used for deciphering the signaling pathways of three putative sea bass GnIH receptors (GnIHR, NPFFR2-1, and NPFFR2-2), such as cAMP response element (CRE-luc), serum response element (SRE-luc), and nuclear factor of activated T-cells response element (NFAT-RE-luc) for adenylate cyclase (AC)/cAMP/protein kinase A (PKA), extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) (principally considered protein kinase C [PKC]-mediated activation), and intracellular Ca^{2+} mobilization, respectively. In addition, whether the ERK pathway is activated by GnIH peptides was investigated by Western blot analysis.

RESULTS & DISCUSSION

Although GnIH1 and GnIH2 had no effect on basal CRE-luc activity, they significantly decreased forskolin-induced CRE-luc activity in COS-7 cells expressing their three receptors, respectively. Moreover, an evident increase in SRE-luc activity was observed when COS-7 cells expressing GnIHR were challenged with GnIH peptides, and this stimulatory effect was markedly reduced by two inhibitors of the PKC pathway. However, GnIH peptides did not alter NFAT-RE-luc activity and ERK phosphorylation levels through the three receptors. Notably, GnIH2 antagonized Kiss2-evoked CRE-luc activity in COS-7 cells co-transfected GnIHR and Kiss2 receptor (Kiss2R). Activation of sea bass GnIHR had no effect on GnRH activation of the CRE-luc pathway. These data indicate that sea bass GnIHR signals can be transduced via the PKA and PKC pathways, and GnIH can interfere with kisspeptin actions by reducing its signaling. Generally, our results provide more evidence for differential activation of signaling pathways by GnIH peptides in teleosts, and are a starting point for the study of interactions with other neuroendocrine factors on cell signaling.

This work was funded by a Grant from PAIDI2020 (Junta de Andalucía, Grant no P18-RT-5152) to JAM-C. Bin Wang was awarded a scholarship sponsored by the China Scholarship Council (CSC, File No. 201903260004).

Oral Presentation 11 (student)

Somatostatin signaling is a key regulator in the allocation of metabolism to reproduction

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INTRODUCTION

Energy allocation between growth and reproduction determines puberty onset and fertility are well defined in mammals. Peripheral hormones, such as leptin, insulin and ghrelin, convey metabolic signals to the central nervous system where gonadotrophin-releasing hormone neurons are activated to trigger puberty and maintain fertility. However, the regulatory signals and mechanisms integrating metabolism and reproduction are still largely unknown, especially in fish.

METHODS

We have used CRISPR/Cas9 system to target a candidate metabolism and reproduction integrated sensor – somatostatin – by establishing two zebrafish mutant lines, *ss1* and *ss3*. We compared their phenotypes, including fecundity and metabolic physiological parameters, to their wildtype siblings.

RESULTS & DISCUSSION

Ss1 mutants showed no significant differences in breeding efficiency (% of successful spawning attempts) and fecundity (number of eggs released) compared to wild type siblings. However, *ad libitum* feeding reduced their breeding efficiency and fecundity of *ss1* mutants. *Ss3* mutants showed both enhanced breeding efficiency and fecundity, regardless of normal or *ad libitum* feeding. Both mutant lines showed over-proliferation of primordial germ cells (PGC) via their receptors *sstr2a* and *sstr5*.

There were significant changes in metabolic physiology in both mutants. The *ss1* mutant line showed a lower energic expenditure phenotype, being hyperglycemic, with low glucose tolerance and higher triglyceride levels compared to wildtype due to an increase in pancreatic α-cell proliferation. In contrast, *ss3* mutants showed a higher energic expenditure phenotype, were hypoglycemic, with high glucose intolerance, lower triglyceride and total cholesterol compared to the wildtype due to increased pancreatic β-cell proliferation.

Our results demonstrated for the first time that somatostatin signalling tightly controls energy and fecundity through its inhibitory role on pancreatic cells and primordial germ cell proliferation. Considering that many somatostatin-like peptides and functions have been shown to be highly conserved, we suggest this regulatory role of somatostatin on metabolism and reproduction may be extensive to other vertebrates.

Oral Presentation 12

Reproductive consequences of CRISPR/Cas9-Based *avp* knock-out in zebrafish (*Danio rerio*)

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INTRODUCTION

The nonapeptide vasopressin (Avp) is an evolutionarily conserved nonapeptide which acts as neuromodulator and endocrine/paracrine signaling molecule. Circumstantial and mechanistic evidence from pharmacological manipulations of the Avp system in several teleost fishes suggest sex- and species- specific reproductive roles. While effects on reproductive physiology have been documented to involve both courtship behaviours or effects on the hypothalamic-pituitary-gonadal (HPG) axes, comprehensive studies investigating physiological and behavioural reproductive consequences of genetic ablation of Avp in genetically tractable fish model, such as the zebrafish are currently lacking.

METHODS

Here, we report the generation of a CRISPR/Cas9-based *avp* -/- zebrafish mutant, which we used to investigate reproductive roles of Avp.

RESULTS

Breeding pairs of *avp* -/- fish produce significantly fewer fertilized eggs per clutch compared to wildtypes (WT), an effect coincident with reduced female quivering courtship behaviour. Crossbreeding experiments showed the reproductive phenotype to be female-dependent, as *avp* -/- males reproduce normally when paired with female WT fish. Sectioning of female gonads revealed a reduction in overall oocytes, as well as fewer early-stage I oocytes but more stage V oocytes in the ovarian oocyte pool in *avp* -/- fish. Ovarian gene expression analysis revealed significant decreases in the germ cell marker *nanos2*, suggesting a potential role for Avp in germ-cell maintenance. Ovaries of Avp mutants also exhibited a significant reduction in concentrations of the prostaglandin, PGF2alpha, a known regulator of ovulation and female courtship behaviour in some female teleosts. This reduction coincided with significantly decreased transcript abundance of *pla2g4ab*, a phospholipase involved in mobilization of arachidonic acid, a precursor of PGF2alpha. Together, these findings provide further support for the emerging roles of Avp in female (teleost) reproduction and open translational research avenues in the domains of captive breeding in the context of aquaculture and species conservation. The findings are also anticipated to contribute to an improved understanding of possible modes of action of endocrine disrupting chemicals in the field of ecotoxicology.

The project received funding from the Natural Sciences and Engineering Research Council of Canada (NSERC) under the Discovery grant # RGPIN-2017-05290.

Oral Presentation 13 (student)

Characterization of a novel fast-growing zebrafish (*Danio rerio*): a new approach to GH transgenesis

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INTRODUCTION

Growth and reproduction are among the most critical processes in an animal's life and are intimately correlated. The somatotropic axis, which regulates growth, has been the subject of many transgenic manipulations to create fast-growing fish that could be used for research and aquaculture. Due to the extremely high GH expression and release, these transgenic lines often exhibit various defects and irregularities, affecting fish health and lowering consumer acceptance. In the current study, we took a different approach to GH transgenesis. We created a novel transgenic construct driven by the LH promoter to couple the exogenous GH expression to reproductive processes and ensure localized and lower GH secretion than the previously mentioned transgenic lines. This research aims to characterize the growth of the new line of transgenic zebrafish (LHp-GH fish) using hormonal and physiological parameters of growth and reproduction.

METHODS

We generated the GH transgenic zebrafish line using Invitrogen Multisite Gateway Manual and Tol2kit. After establishing the transgenic line, siblings larvae were spawned and separated to LHp-GH fish and wild-type. Fish weight and length were recorded weekly for three months to calculate Feed Conversion Ratio (FCR) and Specific Growth Rate (SGR). Following tissue collection, we measured gene expression, gonadotropin levels, somatic indexes, follicle diameter, and spermatogenesis stage frequencies.

RESULTS & DISCUSSION

Our experiment reveals that the LHp-GH fish exhibit accelerated growth in length and weight, lower FCR values, unchanged body proportion, and higher expression levels of LH and GH in the pituitary as well as IGF-1 in the liver. Furthermore, LH and FSH pituitary contents were lower in the transgenic fish, and the average diameter of the ten most prominent follicles was smaller in LHp-GH female's gonads. We believe our transgenic construct is a good candidate for genetic engineering of commercial aquaculture fish species due to its accelerated growth along with the preservation of body proportions. Our results also suggest that the coupling of exogenous GH expression with endogenous LH expression causes the redirection of metabolic resources toward somatic growth at the expense of reproductive processes. We present a potential model in which a positive feedback loop is created. Liver IGF-1 secretion is induced by GH. IGF-1 also upregulates LH expression and increases both LH and GH expression in the case of the novel transgenic construct.

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Oral Presentation 14

Loss of *fshr* inhibits maturation in male Atlantic salmon

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INTRODUCTION

Early puberty is a major problem in farmed Atlantic salmon males as it stunts growth and entails welfare problems due to the maturation-associated loss of osmoregulation capacity in seawater. A better understanding of the regulation of puberty is the basis for developing improved rearing protocols to avoid these problems. As puberty onset is controlled by activation of the brain-pituitary-gonad (BPG) axis, our aim here was to study if puberty is initiated when the gene encoding the follicle-stimulating hormone receptor (*fshr*) is rendered non-functional. It is known that medaka and zebrafish *fshr* KO males show no clear phenotype regarding fertility, which may be related to Lh receptor-activation and the ensuing androgen production stimulating testis maturation. In salmon, this may be different since Fsh cannot activate the Lh receptor and since Lh is usually not secreted until the spawning period. We therefore expected a phenotype different from *fshr* KO zebrafish and medaka males.

METHODS

We made *fshr* mutants using CRISPR-Cas9 technology. We first studied highly mutated F0 generation *fshr* crispants. *fshr* crispants and control males were reared in a common garden, and precocious maturation was induced by exposing the one-year old postsmolts to continuous light and 16º C water temperature for a period of three months. Samplings (testis, pituitary and plasma) took place 1, 2, 5 and 9 months post induction. For subsequent studies on the F1 generation, the sampling times were 7 and 11 months post induction.

RESULTS

At the first sampling (1 month), all males displayed low GSI values and no effect of the *fshr* KO could be detected on plasma androgen levels or stage of spermatogenesis. However, in the two last samplings, we observed slightly (5 months) and clearly (9 months) lower GSI values in *fshr* F0 crispants; histological analysis showed, despite a certain variability among individual F0 crispants, that testis maturation in the crispants started later but was completed sooner than in control males. Later analysis suggested that the variability among F0 crispants may be related to presence of in-frame mutations, which may leave some of the mutated protein at least partially functional. Most F0 crispants produced sperm (or eggs) and we crossed four highly mutated fish (2 of each sex), to create an F1 generation. At one-year of age, we identified sibling males having either wild-type, $fshr^{-/-}$ or $fshr^{+/-}$ genotypes with known mutations. When stimulating pubertal maturation in F1 fish, clear effects were observed. None of the $fshr^{-/-}$ (N=9) males entered puberty while 42% of the $fshr^{+/-}$ males (N=24) and 94% of the wild-type males (N=17) entered maturation.

CONCLUSION

We find that Atlantic salmon males do not enter puberty in response to stimulatory photoperiod/temperature conditions when lacking *fshr*, suggesting a stronger dependency on Fshr-mediated signaling for entering puberty in salmon compared to the model species studied so far. The absence of significant concentrations of circulating Lh in salmonids during the period of testis growth may be relevant in this context.

This project was funded by the Norwegian Research Council (projects 221648/SALMOSTERILE and 254870/POSTSMOLTMAT).

Oral Presentation 15 (student)

Multi-tissue targeted DNA methylation analysis of gonadotropins in chub mackerel (*Scomber Japonicus*) using a cost-effective sequencing method

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INTRODUCTION

In fish, the gonadotropins follicle-stimulating hormone (fsh) and luteinizing hormone (lh) are involved in seasonal gonadal growth and maturation. Epigenetic mechanisms, such as DNA methylation, could be controlling phenotypic responses of these genes due to the environment. Next Generation Sequencing (NGS) techniques make it possible to study in-depth epigenetic variations. Combining the high throughput power of NGS with target PCR sequencing, multiple samples can be processed simultaneously in a cost and time-effective way, which potentially could be a better alternative over conventional targeted bisulfite sequencing methods. Thus, the objective of this project is to study DNA methylation patterns of *fshb* and *lhb* genes regulatory regions in chub mackerel (*Scomber japonicus*), while optimizing a cost-effective target bisulfite sequencing workflow.

METHODS

Fish provided by two local fish farms from Karatsu, Japan, were sampled on three different occasions (October 2020, January, and April 2021) and several tissues collected (n=96). DNA was isolated and bisulfite-converted, and part of the regulatory region of *lhb* and *fshb* genes were amplified with target specific primers containing a synthetic 4bp 5`-adapter (barcode) for sample identification, as explained in the Vial-Pradel et. al. 2019 article. PCR products were pulled, purified, and processed for library preparation. Library quality was check with Agilent 2100 Bioanalyzer and then sequenced on a Novaseq platform (paired end, 2x250bp). In total, 8 unique barcodes and 12 Illumina single indexes were used, to make 96 unique combinations, one corresponding to each fish. Data processing was carried out with QIAGEN CLC Genomics Workbench (version 20.0.4) and R software.

RESULTS & DISCUSSION

Processing of all tissues was done in a similar way. For pituitary, a total of 5923530 (94.02%) reads were filtered after two rounds of demultiplexing. The remaining reads were adapter trimmed and low quality filtered. Of the initial reads, 51.85% could be mapped, but only CpG sites with read coverage higher than 393 were further analyzed, after a Fisher power analysis test (80% power, 0.2 size effect). A higher DNA methylation level trend was observed in 4 CpG sites located 1691~1178bp upstream of the start codon of *fshb* gene for samples taken in January, followed by October, with the least methylated group being pituitary of fish sampled in April, indistinct of sex. Fish sampled in April were older (~11month-old), in reproductive season and previous gene expression measurement showed higher expression of *fshb* in this group. Moreover, two of the four sites were 3~4% differentially higher methylated in April caught males than females (Fisher exact test $p < 0.05$). Whereas this difference is biologically relevant should be further studied. On the other hand, for samples taken on October 6 CpG located in the promoter region of *lhb* were also significantly more methylated in males (Fisher exact test $p < 0.01$), which also matches higher expression of *lhb* in females for this group. In both cases, higher gonadotropin gene expression correlates with lower DNA methylation level. Cumulatively, the present workflow is proven to be both time and cost-effective, compared to the conventional Sanger-seq method, while allowing the detection of small yet significantly differentially methylated CpG positions.

Oral Presentation 16 (student)

Superoxidase dismutases in the European eel: characterization and expression *in vivo* under different temperature conditions

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INTRODUCTION

The superoxide dismutases (SODs) are the most important antioxidant enzymes defenses against oxidative damage. In mammals, three SODs have been described: SOD1-3. Studies have shown that SODs are modulated by different conditions, having a crucial role in homeostatic balance. Recent transcriptomic analysis of European eel (*Anguilla anguilla*) males kept in low temperature showed that SODs are highly expressed in immature testis, indicating that SODs play a physiological function in eel maturation. However, SOD knowledge in fish reproduction is limited and does not consider the three SODs separately. Thus, the objective of this study was to characterize SODs in the European eel and to study their expression profiles using distinct maturation protocols.

METHODS

SODs sequences were characterized by carrying BLAST analyses in the European eel genome, followed by phylogenetic and synteny analyses. Then, SOD expression levels were measured in different tissues collected from both male and female eels. Lastly, male eels were transferred from freshwater to seawater (both at 20°C) and later submitted to standard hormonal treatment (weekly rechCG injections at 20°C), serving as control group. In parallel, other male eels were pretreated with cold seawater (10°C during 2 or 4 weeks) and then submitted to the standard hormonal treatment. Testis samples were collected from control eels under freshwater and seawater conditions and after 2 and 4 weeks of hormonal treatment. Testes were sampled from pretreated eels, after pretreatments and after 4 weeks of rechCG treatment. RT-qPCR expression analyses and a histological study were carried out.

RESULTS & DISCUSSION

SODs sequences analysis showed that the European eel presents one SOD1, two SOD2 (a and b) and two SOD3 (a and b), indicating a duplication of SOD2 and SOD3 at least in elopomorph lineage. In both genders, SOD1 was highly expressed in the gonads and in the brain parts. Similar pattern was observed in both SOD2 subtypes, suggesting that SOD1 and SOD2 subtypes play a role in eels BPG axis. SOD3a expression was mainly found in the ovary in females, and the males liver. SOD3b was detected in the brain parts and in peripheral tissues in both genders. Our results show that SODs expression is tissuespecific and gender-dependent. In control eels, differentiated SPGA were predominant in testis from both freshwater and seawater males. Salinity increase up-regulated SOD1 and SOD2 subtypes, but not SOD3 ones, indicating that SOD1-2 are involved in the onset of spermatogenesis. After 2 and 4 weeks of rechCG injections, SPGB and SPC, respectively, were abundant in the testis. SOD1 showed a declining pattern (not significant) while SOD2a decreased throughout hormonal treatment, contrarily to SOD2b. SOD3a expression decreased after 4 weeks of rechCG injections while SOD3b showed an increasing trend (not significant). These results indicate that SODs have differential functions during eel testis maturation. Low temperature pretreatment before rechCG injections downregulated SOD1 but increased SOD3a expression. Moreover, standard treatment at 20°C decreased SOD2a but not SOD2b. Concerning SOD3b, no significant variations on mRNA levels were found after low temperature pretreatment or standard hormonal treatment.

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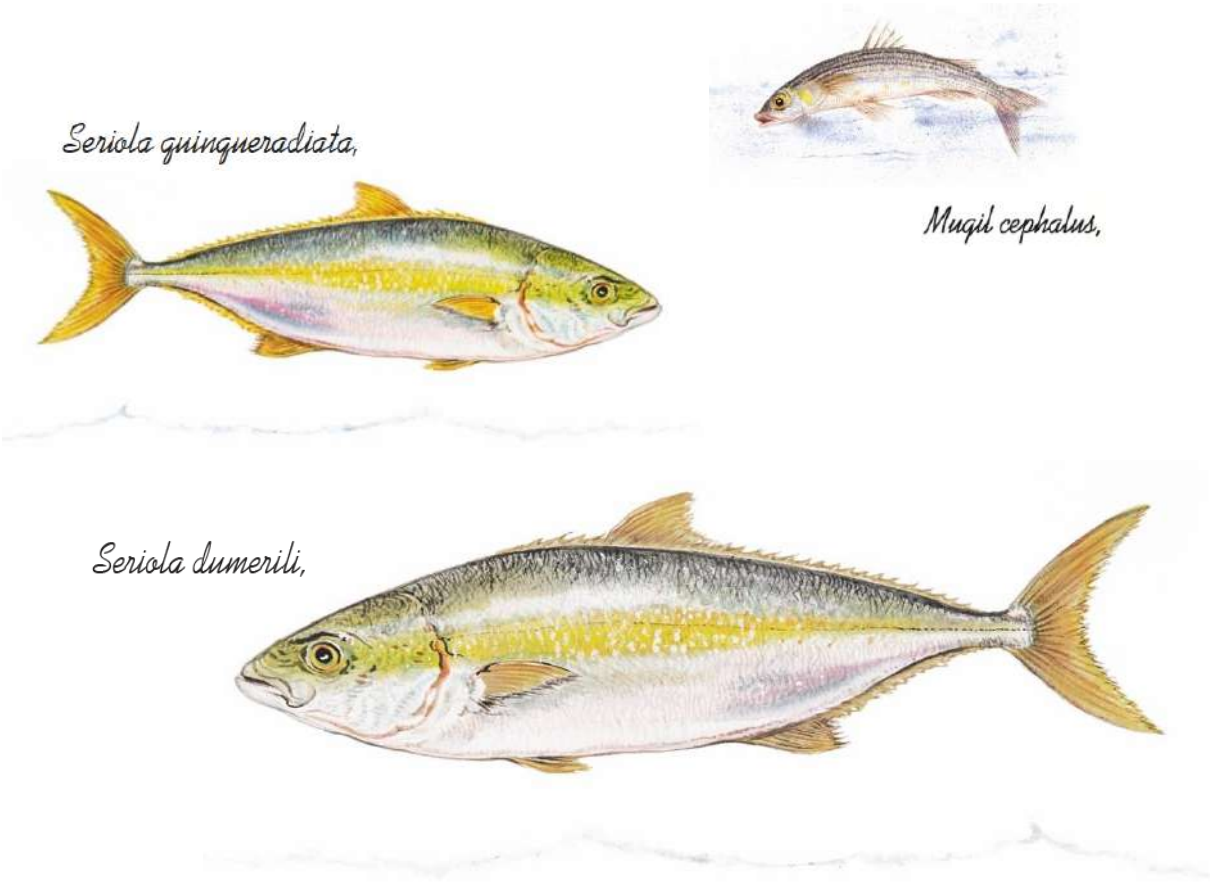
SS3. Oogenesis/vitellogenesis and ovulation



Invited State-of-the-Art presentation 3

Role of multiple vitellogenins in early development of fishes

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Functions of vitellogenins (Vtgs) have been in the limelight of fish reproductive physiology research for decades. The Vtg system of Acanthomorph teleosts consists of two paralogous complete forms of VtgA (VtgAa and VtgAb) as well as an incomplete form of Vtg, VtgC. Insufficient uptake and processing of Vtgs by cathepsins (CATs) into yolk proteins (YP) during oocyte growth, and into free amino acids (FAA) during oocyte maturation (OM), impairs oocyte hydration and acquisition of egg buoyancy. Differential proteolysis of YPs is key to neofunctionalization of different types of Vtgs. Early studies of Atlantic halibut with neutrally buoyant eggs indicated that the YPs derived from VtgAa are selectively proteolyzed to provide FAA for oocyte hydration and early embryo development; the relatively intact YPs derived from other Vtgs are used as nutrients by late embryos and larvae. In contrast, our studies of moronids, pelagic egg spawners, revealed that maturational yolk proteolysis involves limited degradation of the major YP product, lipovitellin heavy chain (LvH), of all three forms of Vtg. In our later studies of zebrafish, a freshwater benthic egg spawner, knock-out (KO) of type I and type III *vtgs*, orthologs of Acanthamorph *vtgAa* and *vtgC*, revealed type I Vtgs to have essential developmental and nutritional functions in both late embryos and larvae. Despite being a minor form of Vtg, type III Vtg was found to contribute importantly to the developmental potential of zebrafish zygotes and early embryos. Loss of three other forms of type I Vtgs and of type III Vtg resulted in increased Vtg7 levels, suggesting the presence of compensatory mechanisms, but these did not rescue the serious developmental impairments and high mortalities. Knock-down (KD) of type II zebrafish *vtg*, orthologous to Acanthamorph *vtgAb*, led to high mortalities during the first 24 hours of embryonic development. These studies also revealed that Vtgs have previously unreported regulatory functions in maternal reproductive physiology, including fecundity (type I Vtgs) and fertility maintenance (Vtg2 and Vtg3), in addition to embryo hatching rate and timing, and development of proper morphology in *vtg*-mutant offspring. Mutant larvae had pericardial and yolk sac/abdominal edema and spinal lordosis, with feeding and motor activities also being absent in *vtg1* KO offspring. By late larval stages, *vtg* mutations were either completely lethal (*vtg1* KO) or nearly so (*vtg3* KO, *vtg2* KD). Our most recent studies in halibut and in the European plaice, another pelagic egg spawner, showed that only LvH from VtgAa (LvHAa) and light chain lipovitellin from VtgAb (LvLAb) are utilized between the postvitellogenic oocyte and unfertilized (UF) egg stages, indicating their utilization during oocyte maturation in halibut, and between the UF egg and 10 days post fertilization (dpf) embryo stages, indicating their utilization during early embryogenesis in plaice. All the remaining YPs (LvLAa, LvHAb, LvHC, and LvLC) decreased on after 10 dpf, indicating their utilization from late embryo up to late larval development stages in both species. Observed decreases in FAAs around 12 dpf (hatching) followed by increases at 15 dpf, fortified the significance of the YP utilization findings and the potential for a tertiary YP proteolysis in halibut. The FAA profiles in plaice were different from halibut, with an increase between UF egg and 5 dpf embryo stages followed by a decrease until first feeding, indicating diversity in Vtg utilization even between closely related species. Additional analyses showed no differences in CAT gene expression during early and late larval developmental stages, while increases in CAT B, D and L activities were obvious between UF eggs and hatching embryos. The overall findings from this collection of studies suggest: 1) apart from their essential roles in early development, different types of Vtgs appear to have regulatory functions in maintenance of other reproductive functions such as fecundity, fertility, and embryonic hatching rates, 2) utilization of multiple Vtgs may involve compensatory mechanisms, 3) the functions and the mode of utilization of different types of Vtg are highly species-specific, and 4) signs of a tertiary YP proteolysis near hatching exist in halibut. Despite the advances in our understanding the multiple Vtg systems over the past 2 decades, a higher complexity of these systems is now evident, with much greater diversity between species in modes of Vtg utilization at different reproductive and developmental stages.

Oral Presentation 17 (student)

Unpacking the egg's earliest life support system – Identification of putative cortical alveoli proteins in zebrafish (*Danio rerio*)

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INTRODUCTION

The egg must be pre-equipped with the cellular machinery necessary to facilitate successful fertilization and, in oviparous species, to sustain and protect the embryo throughout development. An integral component of this early life support system is the thousands of maternally derived cortical alveoli (CA) which initiate the expansion of the perivitelline space upon egg activation/fertilization, facilitate prevention of polyspermy, and provide the developing embryo with a system of innate immunity. While the importance of CAs for successful fertilization is often recited, little is known regarding their composition. For this reason, we sought to identify candidate CA proteins from the perivitelline space of zebrafish eggs.

METHODS

Ovulated eggs were collected from zebrafish and activated via water exposure. Perivitelline fluid (CA rich), yolk, and chorions were collected from three fish (~100 eggs each). These samples were processed for LC-MS-based protein profiling and the resulting peptide sequences were subjected to database-dependent protein identification, utilizing UniProt's zebrafish protein database. Perivitelline fluid, yolk, and chorion protein datasets were compared in order to identify unique proteins (putatively CA in origin) associated with the perivitelline space.

RESULTS & DISCUSSION

Our preliminary analysis detected 78 proteins, each represented by at least two unique peptides, in the perivitelline fluid of zebrafish eggs, of which 41 proteins were identified as candidate CA proteins. Seven proteins have been selected for further interrogation by immunohistochemistry, qPCR, and/or antibody-mediated knock-down to further characterize zebrafish CA and shed further light on their functional significance during fertilization and early embryogenesis.

Oral Presentation 18

Phenotypic analysis of gene knock-out strains of highly upregulated genes during ovulation in zebrafish

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INTRODUCTION

Eleven genes that were selected as highly upregulated genes during the induction of ovulation in zebrafish by using an in vivo ovulation assay. The functions of these genes are evaluated by establishing gene knock-out strains by CRISPR/Cas9 system.

METHODS

F0 founder of crispants of each gene were produced by microinjection of mixture of crRNA, tracrRNA and Cas9 protein. Female and male F1 progeny that possessed same non-sense mutation were selected and paired. Then F2 homozygous mutant progenies were selected and paired. Phenotypic analysis, ovulation, fecundity, morphology of embryos etc., was conducted in F3 progenies or in more later generations.

RESULTS & DISCUSSION

From the phenotypic analysis of earliest established knock-out strains of three genes were conducted at present. A member of transcription factor of paired domain, *pax2a* (paired box2a) has been previously reported to be involved in eye, ear, kidney, thyroid, and central nervous system (CNS) development by anti-sense knock-down techniques. By establishing gene knockout strain, we found that *pax2a* is expressed as a maternal gene in oocytes and is necessary for oogenesis and early development. The starmaker gene (*stm*) has been previously reported to be involved in otolith formation during the early development of zebrafish. Our crispant also showed defect of otolith formation. However, they do not cause defects in swimming behavior. The Stm protein is expressed in the chorion and is responsible for the formation of fiber-supported knob-like structures (KS) on chorion. It was suggested that a lack of Stm caused a lower fertilization rate due to inadequate formation of the chorion. Furthermore, we found reduced fecundity; however, ovulation could be induced in the *prss59.1*, a trypsinlike proteolytic enzyme, mutant fish, which suggests that *prss59.1* is not essential for ovulation. Interestingly, we observed abnormal chorion formation. The morphology of fiber-supported knob-like structures (KS) was also altered in *prss59.1* mutants. The results suggested that Prss59.1 in the chorion was responsible for the elevation of the chorion, which is essential for the proper development of zebrafish. Phenotypes of knockout fishes of three genes showed that these genes are necessary for chorion elevation or early development. We showed that Pax2a is expressed during oogenesis and is necessary for early development. We also showed protease and calcium incorporation to KS structure is involved in chorion elevation. Thus, we provide the new insight into understanding the mechanism of chorion elevation. Although, new insights were identified, we are trying to identify genes for ovulationinduction. Therefore, we continue to establish strains of other candidate genes and to conduct its phenotypic analysis.

The project received funding from the JSPS KAKENHI (20K06719 to TT).

Oral Presentation 19

Hippo pathway-mediated regulation of micropyle formation by microRNA 202 (miR-202) in the fish oocyte.

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INTRODUCTION

Micro RNAs (miRNAs) are small non-coding RNA that post-transcriptionally regulate gene expression by targeting messenger RNA (mRNA) 3'UTR to trigger mRNA decay or repress translation. In medaka, invalidation of *miR-202*, a miRNA known to be mainly expressed in gonads in vertebrates, induced a strong organism level phenotype. Mutant females exhibit a reduced fecundity and fertility with a decrease of eggs production and early developmental success. In this context we hypothesized that miR202 controls oogenesis dynamics and the ability of the egg to be fertilized. The aim of the present work was to decipher the miR-202-controlled mechanisms leading to a reduced reproductive success, with a focus on early developmental success.

METHODS

In order to identify the origin of the reduced developmental success, eggs originating from *mir-202* knock-out (KO) females were assessed for their capacity to be fertilized and their ability to allow the entry of spermatozoa. Next, the functionality of the micropyle, a funnel shape canal in the chorion through which the spermatozoa enter the egg, was assessed using scanning electron microscopy (SEM). The formation of the micropyle, from a single precursor cell in the granulosa layer (i.e., the micropylar precursor cell, MPC) was thoroughly characterized in *mir202* KO females using immunohistological staining. Because MPC differentiation is known to be under the control of the Hippo signaling pathway, quantitative PCR on Hippo pathway and RNA-seq analyses were conducted on whole ovaries and isolated follicles, respectively.

RESULTS & DISCUSSION

In non-developing eggs originating from *mir-202* ko females crossed with WT males, no signal corresponding to the male *mir-202* locus could be detected in genomic DNA, indicating that the observed phenotype could be explained by a lack of sperm entry and therefore a lack of fertilization. Light microscopy search for the presence of the micropyle on the surface of *mir-202* ko eggs revealed the absence of the micropyle in 7.6% of the cases, which is statistically different from control WT eggs but most likely insufficient to explain the overall phenotype. Using SEM, we observed that in 95% of cases, the micropyle was non-functional and sometimes "closed". Using immunostaining we were able to identify different phenotypes of abnormal micropylar cell in mutant ovaries including discontinuous cytoplasm and abnormal shape. Using qPCR, we monitored the expression of the different members of the Hippo pathway in *mir-202* ko and WT ovaries. We observed a significant dysregulation of gene expression in *mir-202* ko fish for several Hippo genes (*mob1b, tead3b, mst1, taz, sav*). Interestingly, several members of the Hippo pathway appear to be possible targets of miR-202-5p (i.e., the active form of miR-202) in the medaka ovary, suggesting that miR-202 could regulate micropyle formation through a direct action on the Hippo pathway. RNA-seq analysis conducted on follicles at different stage of oogenesis shed light on underlying mechanisms and confirmed the dysregulation of Hippo pathway genes.

Oral Presentation 20 (student)

MicroRNAs are involved in ovarian maturation of greater amberjack (*Seriola dumerili)* under captivity

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INTRODUCTION

In fish, captivity exerts a significant negative effect in their reproductive performance, often leading to maturation incompetence and spawning failure. Greater amberjack (*Seriola dumerili*) is one of the fish species exhibiting reproductive dysfunctions when reared in captivity. MicroRNAs (miRNAs) are noncoding RNAs (ncRNA), known to influence the expression of genes at the post-transcriptional level, usually by inhibiting the expression of targeted mRNAs. They have been shown to be involved in different physiological processes, including fish reproduction. The aim of the present study was to explore the role of miRNAs in ovarian development of captive greater amberjack. With this aim, immature, maturing (late vitellogenesis) and spent ovaries of captive greater amberjack were collected, and the differential expression of miRNAs in the three different ovarian maturation stages was studied.

METHODS

Four female fish from each ovarian maturation stage were killed in ice, the gonads were excised and from the same gonad one piece was preserved in a formaldehyde solution for histology and another piece in RNAlater for RNA extraction. After RNA extraction and quality check, ncRNA libraries were generated, sequenced and differential expression analysis was conducted. More specifically, adapter sequences, low quality reads, reads < 10 nucleotides (nt) and read counts < 5 were removed. Sequences were annotated searching the miRbase (release 22.1). Conserved miRNAs were identified, and their expression was analyzed. Moreover, potential targets of highly differentially expressed miRNAs were identified.

RESULTS & DISCUSSION

The average number of reads for each sample after quality and adaptor trimming was 12 million reads, which is considered as a reliable sequencing depth for differential expression analysis. The read length distribution showed two main peaks in the three different ovarian maturation stages, one at 19-24 nt, corresponding to miRNAs and one at 25-30 nt, corresponding to piwi-interacting RNAs (piRNAs), another type of nc RNAs with a germ-cell specific role. The Principal Component Analysis of the differentially expressed miRNAs distinguished the three different ovarian maturation stages in three groups. The number of common and stage-specific differentially expressed miRNAs were shown in a Venn diagram. The differential expression analysis of miRNAs, the role of piRNAs, as well as the target mRNAs were related with the different ovarian maturation stages of captive greater amberjack.

Oral Presentation 21

Quantitative analysis of asynchronous oogenesis dynamics in fish: Application of a modern 3D analysis approach to an old problem

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INTRODUCTION

In multiple spawning fish, oogenesis involves anatomical structures in permanent turnover, the ovarian follicles, which support the development and growth of oocytes until spawning. Although many regulatory actors have already been identified, we still have an incomplete view of the dynamics of the follicular growth during life and reproductive cycles. This gap is mainly due to the lack of quantitative data describing the follicular population and its evolution. Traditionally, studies use manual methods of counting from dissociated follicles, or semi-automatic methods from two-dimensional (2D) ovarian sections. Such approaches are extremely time-consuming and limit the accuracy of the data. More recently, the development of modern 3D imaging methods and automatic image data analysis tools has raised new opportunities to overcome this limitation and reduce methodological bias. The objective of the present work was to exploit these novel approaches to visualize and precisely enumerate the whole population of ovarian follicles of medaka females of different ages, in normal and disrupted conditions.

METHODS

Ovaries were collected from medaka of different stages (larvae, juvenile and adults). Samples were processed for staining of follicular contours (MG nuclear dye) and subjected to clearing to allow confocal 3D imaging. To this aim, we established a clearing protocol (C-ECi) that combines both the CUBIC hyperhydration and the organic solvent ECi methods. For larvae samples, an iDISCO-inspired permeabilization protocol followed by pH3 immunostaining and ECi clearing were used for full 3D imaging. In order to accurately quantify and measure intra- ovarian follicles, we exploited recent Deep Learning algorithms (Noise2Void, Cellpose) that we integrated into an end-to-end processing pipeline allowing reliable measurement of diameters of almost all follicles, regardless of the female stage.

RESULTS & DISCUSSION

The follicular diameter distributions revealed different patterns of follicular density over the fish life. At the larval and juvenile stages, the density profile exhibits a single peak corresponding to the early follicle reserve. This profile then converges, in adults, to a more complex profile including 2 peaks of early follicles, intermediate follicles in a stationary phase and large follicles in a fast, daily maturation cycle. The detection of two early follicle peaks strongly suggests the existence of two distinct recruitments in adults (and two oocyte reserves). The first recruitment (between stages II and III) supplies previtellogenesis and the second one (between stages IV and V) marks the transition from pre-vitellogenesis to vitellogenesis. Analysis of follicular density in ovaries from females lacking miR-202 (KO *mir-202*-/-), a key regulator of fish fecundity, revealed a decrease in the number of follicles at stages I and IV and an increase at stages II/III, thus suggesting lower recruitment rates in absence of miR-202. Overall, the quantitative and temporal analysis of the ovary in 3D enabled to accurately describe the dynamics of the asynchronous oogenesis in Medaka and revealed the existence of two follicular reserves at the adult stage. This approach also allowed to highlight the role of *mir-202* in the regulation of these reserves. In the future, such exhaustive data will be of great value to build mathematical models to better understand the dynamics of Medaka oogenesis in relation to fecundity and its regulation by internal or secreted factors.

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Oral Presentation 22

Genes encoding for non-phosphorylated vitellogenin and choriogenin express during the early stages of oogenesis in the Indian freshwater murrel, *Channa punctatus*

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INTRODUCTION

The materials packaged within and around the oocytes during oogenesis are vital for successful embryogenesis in teleosts. Vitellogenin (Vg), the precursor of egg-yolk proteins and Choriogenin (Chg), the precursor of egg-envelope proteins, are synthesized in the liver under the influence of estrogen. These proteins are secreted into the blood and transported to the ovary where Chg gets deposited around the oolemma as chorion, but Vg is stored within the oocytes. We have earlier shown existence of phosphorylated Vg, and non-phosphorylated Chg in murrel, *Channa punctatus*. Here we describe the regulation of expression of genes encoding for nonphosphorylated Vg and Chgs, in addition to genes coding for phosphorylated Vgs and attempt to assess the roles played by these proteins during oogenesis and embryogenesis.

METHODS

Fish were injected with E_2, plasma was collected and subjected to gel filtration chromatography to isolate Vg and Chg. The proteins were characterized by SDS-PAGE, 2D and mass spectrometry. Total RNA was extracted from liver and processed for amplification and sequencing of *vgc* and *choriogenin* (*chgH, chgL*) genes. *In-silico* analysis was conducted using Bioinformatics tools. Regulation of expression of *vgc* and *chg* genes was studied under natural and experimental conditions.

RESULTS & DISCUSSION

The proteins eluting at Ve/Vo (1.85) when subjected to SDS-PAGE, separated into two peptide bands (33 kDa and 100 kDa). Both the peptides stained negatively for phosphorus. The smaller peptide (33 kDa) showed reactivity with antibodies raised against the egg envelope of murrel. The larger peptide was characterized as Vitellogenin C and the smaller peptide as choriogenin or zona pellucida related protein. The full-length transcript of *vgc* gene and partial transcripts of *chgH, chgL* genes were sequenced and their respective amino acid sequences were derived. Although the exact physiological function of VgC has not been established, its expression in the early stages of oogenesis suggests a specialized role. Gene expression analysis reveals that *vgc, chgH, chgL* genes are expressed in the liver of male murrel after E_2 administration. A correlation between seasonal changes in gonadosomatic index with E_2 levels and hepatic expression of *vgc* and *chgH* and *chgL* genes during the ovarian cycle of murrel was observed. Low titer of E_2 during preparatory phase induces expression of *chgH* gene followed by *chgL* and *vgc* in females. In early pre-spawning phase *vgb* and *vga* genes are expressed, thereafter, a decline in the expression of all the above-mentioned genes was observed during late pre-spawning and spawning phases. All the genes (*vga, vgb, vgc* and *chgH, chgL)* do not express in the resting phase. Expression of *chg* genes prior to *vg* genes indicates that Chg protein is synthesized at the onset of oogenesis, and it prepares the oocyte to incorporate a large amount of Vg during vitellogenesis. Expression of *vgc* gene at low levels of E_2 suggests that VgC (Incomplete Vg), is synthesized earlier than VgA and VgB (Complete Vgs) during oogenesis. Concomitant with Vg uptake, high expression of *vgr* subtype *lr8* during the mid-vitellogenic phase was noticed. The deduced amino acid sequences of multiple Vgs showed the presence of conserved domains (DUF1943, DUF1944 and VWD) which have been suggested to play immunity related functions in fishes.

Oral Presentation 23 (student)

Seeking for the relationship between body size and maturity in female European sea bass (*Dicentrarchus labrax*)

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INTRODUCTION

European sea bass is one the main teleost species produced in the European aquaculture industry. Females grow faster than males and thus a monoculture of female fish would be profitable for aquaculture producers. However, there is evidence that a percentage of females at two years old reach an advanced gonadal development, thus evoking divergent growth that leads to size dispersion and a lower biomass of the population. Therefore, the relationship between maturation and growth merits to be investigated as it might contribute to the genotype-assisted selection of farmed fish species.

METHODS

In this work, four female phenotypes were identified at 23 months of age including small-size females (S) and large-size females (L) which could have either immature gonads (I) or advanced gonadal growth (A). According to performance indicators such as body weight and gonadosomatic index, female sea bass phenotypes were determined as follow: SI, LA (i.e., considered as expected phenotypes) and SA, LI (i.e., considered as unexpected phenotypes). Growth history of these four female phenotypes was evaluated over their second year of life. Changes in the plasma levels of follicle-stimulating hormone (Fsh), 17β-estradiol (E_2), vitellogenin (Vtg) and insulin-like growth factor-1 (Igf-1) were also determined. In addition, liver and muscle RNA-seq analysis of each of the female phenotypes were done in order to identify key genes modulating differences in maturation and growth in 2-year-old sea bass.

RESULTS & DISCUSSION

Our results demonstrated that LI and LA female sea bass were 68.4% heavier than SI and SA fish over their second year of age, while SA and LA females had significantly higher GSI values (3.1 ± 0.6 %) than those of SI and LI fish (0.6 ± 0.1 %). On the other hand, SA and LA females showed higher plasma levels of Fsh, E_2 and Vtg in comparison to those of the other female phenotypes. Circulating levels of Igf-1 were similar in all female groups. After analyzing the liver and muscle transcriptome profiles of these four female phenotypes at 23 months old, a total of 1031.2 ± 283.3 and 977.8 ± 305.9 differentially expressed genes were obtained for the total of comparisons among phenotypes in each collected tissue, respectively. Important genes involved in the vitellogenesis process, *vtg6* and *vtg3*, showed the highest fold change >10 in the liver between those female phenotypes displaying immature and advanced mature gonads. In addition, pathways involved in signal transduction, lipid metabolism and the endocrine system were highlighted in both tissues among female phenotypes. Of note, genes belonging to the Igf system were also differentially expressed. These findings may provide important information for a better understanding of the relationship between maturation and growth traits in fish.

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Oral Presentation 24

New insights by Fourier Transform InfraRed (FTIR) Microspectroscopy on yolk composition, distribution and role in *Mustelus mustelus* a placentotrophic viviparous shark

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INTRODUCTION

Nowadays, the worldwide elasmobranchs overexploitation is a well-known problem; hence, the vulnerability condition of several Chondrichthyans species. Up to the present day, very little has been done for the conservation of these species, especially in the Mediterranean, where only a limited number of species benefit from protective measures. The baseline for sustainable fisheries programmes requires information about the targeted organism's biology, thus, reproductive biology. The aim of this study is to improve the knowledge and identify macromolecular changes underlying oocyte growth and maturation during oogenesis in the common smooth-hound *Mustelus mustelus*, a shark species classified as Endangered (EN) by IUCN Red List.

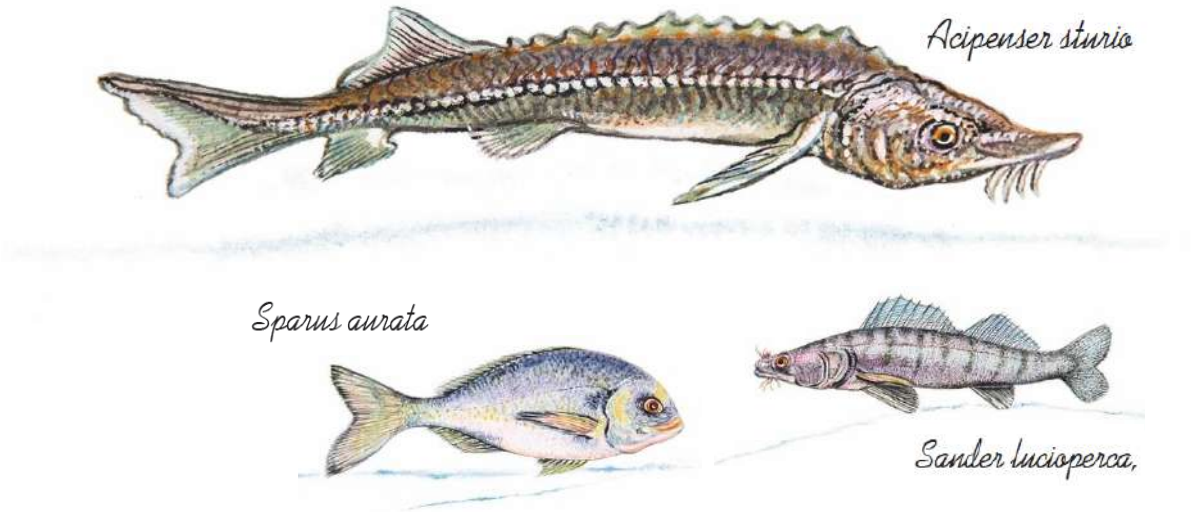
METHODS

The sampling area was the Central Adriatic Sea (FAO-Geographical Sub-Area 17, GFCM). Ovarian tissue containing oocytes at different stages of maturation were sampled from both the right and left ovary and processed for the further histological and FTIR analyses. Histological analysis was performed on ovaries sections stained with both Mayer's haematoxylin –eosin and the Periodic Acid-Schiff (PAS); sections were observed under a Zeiss Aixio Imager M2 microscope and microphotographed with a high-resolution camera Zeiss Axiocam 105 color. A Bruker VERTEX 70 interferometer coupled with a Hyperion 3000 Vis-IR microscope equipped with bidimensional Focal Plane Array (FPA) detector was used to obtain ovarian chemicals maps corresponding to oocytes at different development stages. By integrating specific spectral regions, false colour images representing the topographical distribution inside the oocytes and relative amount of the biochemical features such as lipids, proteins, carbohydrates and nucleic acids were obtained.

RESULTS & DISCUSSION

The knowledge on several aspects of the reproductive biology of elasmobranchs is still little understood. The common smooth-hound, is a viviparous placental organism, characterized by widespread distribution worldwide. Epigonal organ, oogonia, previtellogenic follicles and vitellogenic follicles were identified and described histologically and subjected for the very first time to spectral analysis by FT-IR in order to characterize the macromolecular changes during oogenesis of both somatic cells (Granulosa cells and theca cells) and germinal cells. Structural analysis of *Mustelus mustelus* oocytes at different stages of development obtained by histology, was coupled with IR spectral characterization highlighting for the first time the macromolecular changes of zona pellucida, ooplasm, germinal vesicle and yolk globules underlying follicle growth by both structural and macromolecular point of view. Results evidenced -proteins, sugars and lipids concentration and distribution within the oocytes are continuously changing in a highly regulated way during oocyte development; a peculiar uptake of vitellogenin-like macromolecule and yolk component accumulation within the oocyte. All these results are of great importance to better understand the significance of yolk role on a viviparous placental organism.

SS4. Spermatogenesis and spermiation



Invited State-of-the-Art presentation 4

Follicle-stimulating hormone effects and the regulation of early spermatogenesis: from model to aquacultured fish species

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Spermatogenesis in vertebrates is a developmental process in which a spermatogonial stem cell (SSC) population produces germ cells that go through a series of mitotic and then two meiotic divisi ons, eventually providing highly differentiated spermatozoa. Early stages of this cellular development are orchestrated by the pituitary follicle-stimulating hormone (FSH), through the production of Sertoli cellderived factors and by Leydig cell-released androgens. Extrinsic mechanisms, in particular those regulating "strategic" decisions of the SSC population, such as the level (quiescence vs. cell cycling) and quality (self-renewal vs. differentiation) of proliferation, are the basis for a precocious or delayed/limited onset of spermatogenesis that compromise sustainability, animal welfare, and efficiency aspects of finfish aquaculture. To approach these questions, we used zebrafish (Danio rerio) and Atlantic salmon (Salmo salar) as experimental models. First, we identified Fsh-regulated candidate growth factors relevant for spermatogenesis via gene expression profiling (microarrays, RNA sequencing). Among those, prominently regulated factors by Fsh such as insulin-related peptides Insl3 and Igf3, retinoic adic (RA), Wnt and Tgfb signaling pathways were selected. Next, we characterized the biological activity of identified candidate factors by gain-of-function and loss-of-function approaches, often using a primary testis tissue culture system (e.g. pharmacological approaches, production of recombinant proteins) or targeted gene knock-out by CRISPR/Cas9. Additionally, we studied the endocrine regulation of expression and/or release of identified candidate factors. We found that Fsh stimulated testicular RA production in zebrafish, a species lacking stra8 (key gene mediating RA effects in mammalian spermatogenesis), thus linking for the first time in vertebrates the endocrine system to local RA signaling. Genetic ablation of RA signaling in germ cells compromised spermatogenesis, but activated steroidogenesis, leading to an over-compensation of spermatogenesis and testicular hypertrophy. Editing of stra8 gene in Atlantic salmon resulted in increased germ cell apoptosis, but mutants compensated this cell loss by an elevated production of spermatogenic cysts, and produced functional sperm. Surprisingly, the highly Fsh-responsive growth factor Insl3 only moderately promoted germ cell differentiation in zebrafish. The genetic loss of insl3 is compensated initially, until germ cell apoptosis increased progressively starting at 9 months of age. Fsh also uses canonical (via Sertoli cellderived Igf3) and non-canonical (via Leydig cell-derived Wnt5a) signaling to achieve a balanced regulation of SSC self-renewal and differentiation. Hence, Fsh makes use of several, locally produced signaling molecules in zebrafish, operating in parallel to regulate spermatogenesis, such that – different from the situation in mammals – failure of one pathway is (partially) compensated by one or more alternative, parallel pathways. Even the loss of Fsh signaling is compensated in zebrafish, however, this is different in salmon, where puberty is blocked in male Fsh receptor mutants.

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Oral Presentation 25

Fsh regulates the proliferation of embryonic-like germ stem cells in adult zebrafish testes

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INTRODUCTION

A novel subpopulation of pluripotent stem cells, named embryonic-like stem cells (ELs), has been recently reported among spermatogonial stem cells in humans and mice. Furthermore, it has been shownthat ELs in testes and ovaries express FSHR, and FSH has a direct effect on these cells. In this study, we sought to investigate whether ELs were present in zebrafish testes.

METHODS

To address our aims, expression analyses (RT-qPCR) of genes involved in pluripotency were carried out during zebrafish embryonic stages as well as cultures of testicular explants incubated or not with recombinant zebrafish Fsh (rzfFsh). To further identify the pluripotent markers and Fshr, immunofluorescence, western blot or flow cytometry were employed on wild-type or Fshr::eGFP zebrafish reporter lines. Finally, RNAseq libraries were produced from total RNA extracted from zebrafish testicular explants cultivated with trilostane (an inhibitor of sexual steroid production) in presence or absence of rzfFsh (100 ng/mL).

RESULTS & DISCUSSION

We first demonstrated that the selected pluripotent genes, *pou5f3*, *nanog* and *nanos3*, showed higher expression levels at the blastula stage, and later, mRNA levels were significantly down-regulated over the gastrulation. Furthermore, we showed that Pou5f3, Nanog and Nanos3 were found among the different generations of spermatogonia although their staining pattern varied depending on the spermatogonial development in adult testes. The pluripotent markers were expressed at higher levels in early spermatogonia (type A undifferentiated (A_{und}) and differentiated (A_{diff}) spermatogonia) compared to type B spermatogonia, and no longer detected in meiotic and postmeiotic germ cells. Using a specificantibody, we observed that Fshr was expressed in somatic cells and in A_{und} and A_{diff}. We further evaluated whether the selected pluripotent genes were regulated by Fsh. Similar to mammals, we foundthat Fsh increased *pou5f3* mRNA levels, while *nanog* and *nanos3* were down- regulated after 7 days of Fsh exposure. RNAseq libraries also showed a deregulation for many mediators of the stem cell signaling pathway in the testes cultivated with Fsh. Finally, we observed that a transgenic zebrafish line carrying the GFP reporter gene under the control of a proximal promoter fragment of the *fshr* gene showed high GFP expression levels not only in somatic cells, but also in A_{und} and A_{diff}. Altogether, ourdata indicate the existence of Fshdependent proliferating ELs in adult zebrafish testes.

The project received funding from FAPESP (20/03569-8 and 21/06742-5).

Oral Presentation 26

Lack of *vgll3a* delays onset of sexual maturation in Atlantic salmon (*Salmo salar*) males

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INTRODUCTION

As one of the major problems in farming of Atlantic salmon (*Salmo salar*), early male maturation is causing increased disease susceptibility and osmoregulatory problems, leading to higher mortalities, production losses and reduced animal welfare. The *vgll3a* locus on chromosome 25 is strongly associated with time of maturation in Atlantic salmon, with alleles responsible for early and late maturation. However, it is not clear how *vgll3a* controls the onset of puberty. We therefore decided to generate a *vgll3a* loss-of-function mutant.

METHODS

Using CRISPR/Cas9 we have produced a *vgll3a* knockout salmon line which we have followed in both F0 and F1 generations until the fish were 2 years old. In both generations, males were subjected to environmental conditions known to stimulate the onset of puberty in salmon (16 degrees, constant light, freshwater, for 6 weeks) about one-year post hatching. Individual growth was recorded every second month after start feeding and the maturation status was determined either by dissection (F0) or by ultrasound (F1).

RESULTS & DISCUSSION

Most mutated males in the F0 generation showed high mutation rates (76% average mutation frequencies). Upon dissection 8 months after the maturation inducing conditions, we observed a larger proportion with mature gonads among the control fish (93%) compared to the mutated F0 crispants (22%). The F1 fish were divided into *vgll3a*$^{-/-}$, *vgll3a*$^{-/+}$ and *vgll3a*$^{+/+}$ genotype groups (homozygous knockout, heterozygous, and homozygous wild type, respectively). During early development, the *vgll3a*$^{-/-}$ males were smaller than the males in the two other genotype groups. Also, during the maturation triggering conditions, the homozygous knockout males showed reduced growth rates compared to males in the other groups. However, half a year after the maturation-inducing treatment, body mass differences were no longer recorded between genotype groups. To determine if the fish had matured, the males were scanned with an ultrasound reader three months after the maturation inducing treatment. This showed that 61-62% of the *vgll3a*$^{-/+}$ and *vgll3a*$^{+/+}$ fish had mature gonads, respectively, while no mature gonads were found in any of the 9 *vgll3a*$^{-/-}$ males.

CONCLUSIONS

Atlantic salmon males lacking *vgll3a* show a delayed entry into maturation. This provides a model for studying the molecular and physiological mechanisms underlying the timing for onset of puberty in Atlantic salmon. Further studies will reveal if the use of fish lacking *vgll3a* could be a potential solution to reducing the problem with early male maturation in Atlantic salmon farming.

The project received funding from the Norwegian Research Council (projects 324890 Maturewel and 254783 Matgen).

Oral Presentation 27

European sea bass (*Dicentrarchus labrax*) gene expression dynamics during spermatogenesis

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INTRODUCTION

The European Sea bass, Dicentrarchus labrax, is a highly valued species in Mediterranean aquaculture. The expansion of its culture requires basic studies to improve the understanding at the molecular level of the players involved in the first sexual maturation and, therefore, the onset of puberty. The findings from these studies will contribute to elaborate protocols to delay the onset of puberty, generally linked to growth retardation, and thus obtain larger fish with a higher economic value by marketing time.

METHODS

Fish were maintained under standard rearing conditions of annual natural photoperiod and water temperature fluctuations. Males were sampled over the first yearly maturation cycle. Histology was used to assess the maturation stage of the testis, classify them into the six canonical stages (Stages I-VI), and quantify the different cell population abundancies per stage. Moreover, directional bulk RNA-seq libraries for the six different maturation stages were generated to analyse the dynamics of gene expression during pubertal maturation in sea bass testis.

RESULTS & DISCUSSION

Spermatogenesis is characterised by a tightly spatio-temporal regulation of gene expression. In European sea bass males, puberty starts with the onset of the first spermatogenesis when primary spermatogonia start dividing and enter meiosis. In this study, we characterised gene expression dynamics during spermatogenesis, contributing to the knowledge of the molecular mechanisms that regulate the onset and progression of male pubertal development. In addition, the presence and expression of possible molecular markers involved in this process are also assessed.

The histological analysis provided a quality control tool for testicular maturation from a morphological and cellular population abundance perspective. Likewise, it was used to select the samples for further RNA-seq analysis of the six maturation stages. Directional RNA-seq was used to first de novo re assemble the European sea bass transcriptome and then to evaluate the differentially expressed genes between the testis maturation stages. The most similar stages from a transcriptomic point of view were SII (early recrudescence) and SIII (mid recrudescence) with 3,143 differentially expressed genes (DEG), while the most dissimilar transition was from SIV (late recrudescence) to SV (full spermiating testis; 25,708 DEG). The onset of puberty coincided with the up-regulation of genes involved in cell proliferation, cell cycle, meiosis progression, reproduction, and growth. In contrast, in the most advanced maturation stages, chromosome organisation and segregation, cell motility, DNA binding and ATPase/GTPase processes were dominant.

Altogether, these results represent an atlas of spermatogenesis gene expression during puberty. Furthermore, its evolutionary comparison to zebrafish single-cell testicular populations allowed for a cell-specific extrapolation of gene expression.

The project was funded by the Spanish Ministry of Science and Technology (SPERMATOGEST, RTI2018-094667-B-C21,) and (SPERMSTART, PID2021-122929OB-C31). ND was supported by the ‘Severo Ochoa Centre of Excellence’ accreditation (CEX2019-000928-S), and NS by a Severo Ochoa FPI scholarship (CEX2019-000928-S-21-4).

Oral Presentation 28

Expression of Gdnf-Gfra1-Ret system genes and *nanos2* in the European seabass (*Dicentrarchus labrax*) testis during the reproductive cycle and under unilateral orchiectomy (ULO) conditions

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INTRODUCTION

Spermatogenesis starts when undifferentiated type A spermatogonia (und-SpgA) become differentiated SpgA (diff-SpgA). Expression of *nanos2* in und-SpgA has been observed in several species and it is recognized as a spermatogonial stem cell marker. In the mammalian testis, it is well established that Sertoli cells produce GDNF (glial cell line-derived neurotrophic factor) that binds the GFRA1 (GDNF family receptor alpha-1), which is expressed by und-SpgA promoting its self-renewal and maintenance. The Gdnf-Gfra1 complex interacts with the Ret receptor tyrosine-kinase activating their intracellular signaling pathway. A few recent studies have addressed the potential involvement of the Gdnf-Gfra1 system in the regulation of gametogenesis in fish. As a first step to determine Nanos2, Gdnf, Gfra1and Ret involvement in the self-renewal and maintenance of the und-SpgA in the European seabass, we have studied their gene expression during testicular development.

METHODS

Adult testis samples from immature to fully matured stages were collected at different times of the reproductive cycle. In mid-September when testes contained mainly SpgA, some males were submitted to ULO. The right testis was removed in the ULO fish through an incision made into the abdominal cavity, while SHAM fish received only the incision without testis removal. Testis samples were collected at 0, 2, 7 and 12 weeks after the operation. Levels of expression of *nanos2*, *gdnfa*, *gdnfb*, *gfra1a*, *gfra1b* and *ret* were analyzed in all testis samples by quantitative real time PCR.

RESULTS & DISCUSSION

High levels of *nanos2*, *gdnfa*, *gdnfb*, *gfra1a*, *gfra1b* and *ret* mRNA expression were seen from June until early-September when testes contained only SpgA. The expression of all these genes decreased sharply to low levels coinciding with the appearance of type B-spermatogonia (October), except for *gfra1a* that still showed rather high levels of expression throughout the following months. These results suggest that Gdnfa/b-Gfra1a/b-Ret regulatory pathway, including Nanos2, can have key roles during the early stages of testicular development.

After the operation, both ULO and SHAM fish showed decreasing expression levels of all the genes studied, but with higher values in ULO fish. At 12 weeks after surgery a sharp increase of expression levels of *gdnfa*, *gdnfb*, *gfra1b* and *ret*, but not *nanos2*, was observed when fish were spermiating and testis were occupied by cysts at all developmental stages. The expression levels of *gfra1a* in ULO fish, however, showed constant high levels of expression from 2 -12 weeks after operation. Compensatory growth of the remaining testis occurred from week 7 post-ULO which, in turn, resulted in an advancement of spermiation at week 12. Then, it is likely that *gdnfa* and *gdnfb* at this time could rapidly induce production of und-SpgA and/or diff-SpgA to provide the remaining testis with new spermatogenic cysts and, therefore, fully maintain production of spermatozoa until the end of the spawning season.

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Oral Presentation 29

Involvement of the transcriptional coactivator Ncoa7 during initial stages of spermatogenesis in European sea bass (*Dicentrarchus labrax*)

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INTRODUCTION

Recent studies in European sea bass using a hemigonadectomy approach and transcriptomic analyses have revealed several genes potentially involved in early gametogenesis in gonads. One of these genes is *ncoa7* that encodes a nuclear receptor co-activator that, in mammals, increases the transcriptional activity of the estrogen receptor (Esr). In sea bass, the role of the estrogen receptors during early oogenesis has been investigated, but little is known about their contribution to the regulation of early events in spermatogenesis. In this scenario, this study is aimed at the molecular and functional characterization of the *ncoa7* gene, its relation to the Esrs, and their involvement in early spermatogenesis in European seabass.

METHODS

The complete cDNA sequence of European seabass *ncoa7* was cloned and mutants of the possible receptor binding sites were made. Transient co-transfection of HEK293 cells with expression plasmids for sea bass Ncoa7 (or their mutants) and nuclear estrogen receptors (Esr1, Esr2a, Esr2b) was used to assess functionality. The expression of sea bass *ncoa7, esr1*, *esr2a* and *esr2b* in different male tissues and also in adult testis at different stages of development was determined by RT-qPCR. Levels of E2 in plasma samples during all the male reproductive cycle were measured using a specific ELISA. *In vitro* tissue culture of immature testis stimulated with E2 or gonadotropins where performed to assess regulation and functionality. Localization and interaction of Ncoa7 and Esr1 in testis was investigated by co-immunofluorescence and co-immunoprecipitation, respectively. Finally, EMSA assays were done to demonstrate if the Ncoa7-Esr1 complex can interact with estrogen receptor responsive elements in immature testis.

RESULTS & DISCUSSION

Functional analyses of Ncoa7 cotransfected with nuclear Esrs and exposed to E2 showed that Ncoa7 was able to increase Esrs transcriptional activity. Single mutations were done in the predicted binding sites and mutated Ncoa7 lost its functionality. Regarding tissue expression, the highest expression of *ncoa7, esr1*, *esr2a* and *esr2b* was observed in brain and testis. Testicular expression of *ncoa7* during a reproductive cycle was highest in immature testis overlapping with the highest expression of *esr1* and high levels of E2 in plasma. E2 treatment of testis explants increased the proliferation of type A spermatogonia and rFsh treatment significantly decreased the expression of both *ncoa7* and *esr1* in immature testis. Immunofluorescence showed localization of Ncoa7 and Esr1 in type A spermatogonia. Moreover, co-immunoprecipitation and targeted mutation analysis showed that Ncoa7 interacts directly with Esr1 and identified the domains that are involved in this direct binding. Finally, EMSA assays supershifted with Ncoa7 antibody showed that in immature testis from July, Esr1 interacts with Ncoa7 for DNA binding. All together our data indicate that in immature testis of European sea bass, both Esr1 and Ncoa7 may be involved in type A spermatogonia proliferation and rFsh would regulate their expression to control the onset of spermatogenesis.

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Oral Presentation 30

Differential regulation and function of two luteinizing hormone receptors during flatfish spermiogenesis

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INTRODUCTION

In previous studies on the flatfish Senegalese sole, we reported that the ligand-activation of the luteinizing hormone receptor ba (Lhcgrba) in mature spermatids promotes spermatozoa differentiation. To better understand the regulation of this pathway in germ cells, we investigated the transcriptomic changes between immature and mature spermatids using RNA-seq. Amongst the upregulated mRNAs detected in mature spermatids we found a novel tandemly duplicated paralog of the Lhcgr, which we termed Lhcgrbb. The objective of the present study was to characterize the function of this novel Lhcgrbb, as well as to investigate its localization and regulation during the maturation of haploid cells and their differentiation into spermatozoa.

METHODS

The full-length sequence of Lhcgrbb was cloned from the testis and subcloned into the pCDNA3 expression vector for luciferase-based reporter assays in HEK293T cells, using homologous singlechain recombinant gonadotropins (rFsh and rLh). To study the differential localization of both Lhcgrba and -bb, we performed in situ hybridization (ISH) using specific RNA probes, as well as immunofluorescence microscopy employing antibodies specific for each receptor. The genomic upstream promoters of each receptor and mRNA 3´UTR were also isolated to gain insight into the transcriptional and translational regulation of each receptor in haploid germ cells.

RESULTS & DISCUSSION

Phylogenetic analysis confirms that the coexistence of two Lhcgrs is conserved in many teleosts. Functional assays in HEK293T cells showed that while the Lhcgrba is specifically activated by rLh, the Lhcgrbb is also able to respond to high doses of rFsh. ISH confirmed redundant mRNA expression of the lhcgrba and -bb in Leydig cells, as well as in immature and mature spermatids. The upregulation of lhcgrbb transcripts in mature sole spermatids detected by RNA-seq was confirmed by real-time quantitative qRT-PCR, while that of lhcgrba remains unchanged during spermatid maturation. Immunostaining experiments revealed a sequential protein expression of each receptor in spermatids, with the Lhcgrba being expressed in maturing spermatids and the Lhcgrbb expressed later on in elongating spermatids and spermatozoa. Interestingly, bioinformatic analyses of the gene promoters of lhcgrba and -bb predicted the presence of some common and specific potential binding sites for transcription factors, some of which are found to be upregulated in mature spermatids, which may explain the differential expression of the receptors in germ cells. In addition, amongst the regulated genes during spermatid maturation, we found several eukaryotic translation initiation factors, as well as hormonal pathways that could be involved in the divergent regulation of Lh receptors during spermatid maturation. Altogether, our results suggest the presence of specific Lhcgrba and -bb specific pathways regulating their expression in spermatids and controlling spermatozoa differentiation and maturation.

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Oral Presentation 31

A multi-omics study on male fertility in farmed Arctic charr (*Salvelinus alpinus*)

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INTRODUCTION

Arctic charr (*Salvelinus alpinus*) is a niche market species with a high commercial value for the Nordic aquaculture industry. Its superior ability to grow at low water temperatures compared to other salmonids constitutes Arctic charr particularly suitable for farming across the Holarctic region. Nevertheless, a highly variable reproductive success in captivity hampers the industry's expansion. Empirical data from the Swedish Arctic charr breeding program suggest that underlying genetic parameters affect fertility.

METHODS

Motility-related traits from over 500 Arctic charr males of known pedigree (Swedish Arctic charr breeding program) were recorded using a computer-assisted semen analysis (CASA) system. Furthermore, the motility of approximately 150 of those males was further recorded multiple times during the same reproductive season and in subsequent years (2020 – 2022). DNA from semen was extracted and used as a template for i) genotyping by sequencing aiming to detect genomic regions associated with male fertility and assess the potential of genomic selection to improve male fertility, ii) 16S and 18S sequencing (n = 84) for deciphering the core microbiome structure of semen and exploring for potential associations between the residing seminal microbiota and the recorded motility and iii) whole methylome sequencing (n = 48) screening for regions where the methylation levels affect motility.

RESULTS & DISCUSSION

Moderate heritability estimates were obtained, suggesting that genetics can explain approximately 20 – 30% of the observed phenotypic variance of the recorded motility traits. The conducted association study indicated that several genomic regions could affect motility. The most prominent one was found in chromosome seven (LG7), where a genome-wide significant genetic marker was in close proximity to *PTPN11*, a gene previously associated with sperm quality in mammals. Simultaneously, groups of males were identified as having either consistently high or variable motility-related parameters across the reproductive season. The conducted microbiome study showed that the core microbiome of the sampled population was comprised of seven operational taxonomic units (OTU). A strong negative correlation was detected between the relative abundance of a fungal OTU potentially belonging to the Leotiomycetes class and sperm motility ($r = -0.78$, $P = 0.004$). Finally, the whole methylome study indicated the existence of more than 20 regions where methylation levels were statistically significantly associated ($P < 0.05$) with motility parameters.

Oral Presentation 32

The role of Ca^{2+} and pH in the regulation of trout spermatozoa motility

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INTRODUCTION

As in the majority of fish species, trout spermatozoa are immotile in the seminal fluid. Motility occurs by contact of sperm cells with the aquatic environment under an abrupt change in extracellular ion composition, osmolality and pH. The dilution of seminal fluid K^+ is the crucial trigger for spermatozoa activation in trouts. The increase of extracellular K^+ leads to complete inhibition of motility, but Ca^{2+} can work as an antagonist of K^+ under some conditions. Nevertheless, under low K^+ concentration, certain pH and Ca^{2+} concentrations play an essential role in regulating spermatozoa motility. In the present study, we examined the effect of extracellular pH and Ca^{2+} on trout spermatozoa activation.

METHODS

Spermatozoa motility from 8 rainbow trout (*Oncorhynchus mykiss*) males was recorded in three replicates using ISAS digital camera (PROISER, Spain). Obtained records were analyzed with ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) using the CASA plugin (Wilson-Leedy and Ingermann 2007; Purchase and Earle 2012). Sperm samples with a motility percentage less than 5% and spermatozoa with VCL less than 20 μm/s were considered immotile. Statistica v. 13 (TIBCO Software Inc., USA) was used for statistical analyses of data. Sperm was activated in AM (10mM Tris-HCl) under different pH (7.5, 8.0, 8.5, 9.0, 9.5) in the presence or absence of 1 mM Ca^{2+} to plot a dependency curve of spermatozoa activation from extracellular pH. To understand the role of environmental Ca^{2+}, Ca^{2+} in pH 8.0 and 9.0 were titrated. The effect of various Ca^{2+} concentrations on trout spermatozoa motility under different pH was detected in the presence of 10mM NH_4, which was used to induce intracellular alkalization of spermatozoa.

RESULTS & DISCUSSION

We detected that the plot of spermatozoa motility in different pH is significantly shifted to an alkaline area when activation media does not contain Ca^{2+}, suggesting lower cell sensitivity to pH in the presence of extracellular Ca^{2+}. High motility (around 90%) was detected in AM pH 8.0 in the presence of 1mM Ca^{2+}, whereas no motility was obtained in Ca^{2+}-free AM pH 8.0. However, over 80% of motile spermatozoa were detected in Ca^{2+}-free media with pH 9.0. Thus, AM with pH 8.0 and 9.0 were chosen to study the role of extracellular Ca^{2+}. Our results show that a significantly higher Ca^{2+} concentration (around 2 mM) is needed for 50% activation of trout spermatozoa in AM pH 8.0 compared to low (around 0.25 mM) in pH 9.0. Alkalization of spermatozoa intracellular environment led to a slight increase of minimal Ca^{2+} concentration required for motility initiation (0.75mM of Ca^{2+} in the presence of NH_4) under pH 9.0 and a decrease of minimal Ca^{2+} concentration in pH 8.0 (1.2 mM of Ca^{2+} in the presence of NH_4). The relation between the Ca^{2+} concentration and the level of pH required for spermatozoa activation allows us to suggest that the Ca^{2+} and pH-dependent channels are involved in motility regulation passways. Since K^+ efflux is a primary trigger of spermatozoa activation cascade, we suppose the involvement of Slo-type K^+ channels, specifically Slo1 (Ca^{2+}-dependent K^+ channel) and Slo3 (pH-dependent K^+ channel) in regulation of motility. In our following study, we will study the presence and the functional role of these specific channels in mechanisms of sperm motility in trouts.

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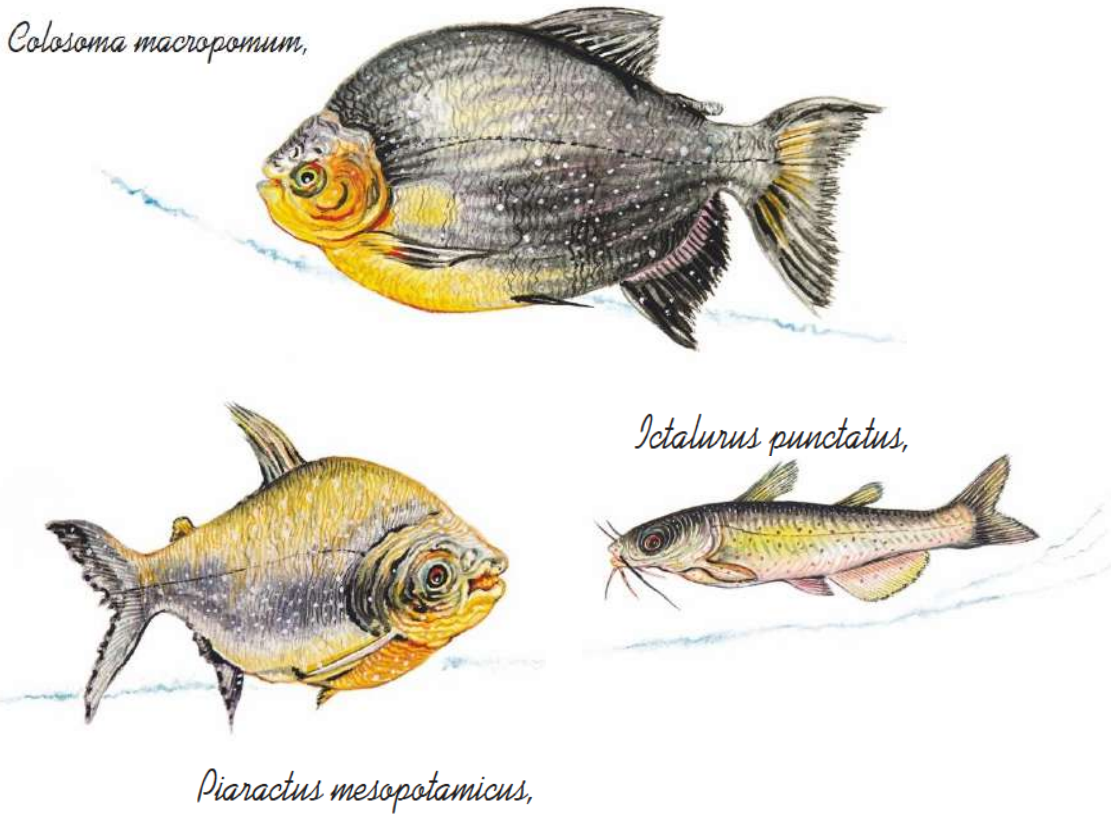
SS5. Climate change and anthropogenic impacts



Invited State-of-the-Art Presentation 5

Climate change impacts the reproductive neuroendocrine axis of fish

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Sexual reproduction is a key and energetically expensive process for species survival and evolution. In fish, this process is largely dependent on specific environmental cues that trigger or modulate sexual maturation, breeding and offspring development.

Anthropogenic emissions of carbon dioxide in the atmosphere have generated rapid changes in physico-chemical proprieties of sea and freshwater ecosystems, including variations in water temperature and salinity, increases in duration and frequency of hypoxia events and water acidification. Such ongoing rapid environmental changes can interfere with reproductive processes and may compromise breeding success and survival by acting on the neuroendocrine axis (brain-pituitary-gonad axis).

Temperature and photoperiod regimes variations are known to strongly affect sex differentiation and the timing and phenology of spawning in several fish species. Temperature mainly acts at gonad level by interfering with steroidogenesis. Salinity changes and water acidification are notably associated to disruption of sperm quality and reproductive output. Hypoxia events can impact gonad steroidogenesis with a result on the quality of gametes and reproductive success.

Although the precise mechanisms underlying the regulation of these effects are not always identified, different hypothesis will be discussed based on experimental data mostly focusing on a single factor variation (acidification) and multistress effect (acidification and warming) on sea bass and three-spined stickleback.

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Invited State-of-the-Art Presentation 6

The effects of aluminum, and water quality parameters, on the reproduction of *Astyanax altiparanae* (Characiformes: Characidae), a neotropical teleost

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Anthropogenic actions, such as the introduction of metals into water, can affect physiological processes, including reproduction. The metals can act as endocrine disruptors compounds (EDC), interfering with the hypothalamus-pituitary-gonadal (HPG) axis and affect gamete quality. The toxicity of metals, including aluminum (Al), can be enhanced by water quality factors, as temperature, pH, ionic composition, among other water quality variables, threatening the reproductive success.

To evaluate the effects of acute exposure to Al in fish reproduction, we have been performing experiments with *Astyanax altiparanae* males and females in the last decade, also varying some water quality parameters, as pH and temperature. *A. altiparanae* is a neotropical teleost model in different areas in the recent years, including ecotoxicology. In sexually mature females, exposure to Al in acidic pH alters the plasma level of thyroid hormone and the pattern of deposition and mobilization of energetic substrates, mainly ovarian proteins, hepatic lipids and muscle glycogen. The role of Al as EDC is also evident in the HPG axis, as it affects steroidogenesis, decreasing 17α- hydroxyprogesterone and cortisol plasma levels in mature females. These alterations in the endocrine system are followed by an impairment in reproduction and ovarian function, as Al exposure triggers a decrease in relative fecundity, an effect that is also caused by acidic pH.

Al also affects the reproduction of *A. altiparanae* males, acting as EDC in testicular steroidogenic, increasing testosterone plasma levels, but different from females, cortisol levels are not altered after acute exposure to this metal. We observed that the bioaccumulation of Al is temperature dependent, as the temperature rise increases the bioaccumulation of Al in the testes after 96 h, while after 24 h it already triggers the bioaccumulation of Al in the semen. Additionally, Al is potentially cytotoxic and genotoxic to erythrocytes and spermatozoa, effects that are potentiated by temperature and pH.

The effects of Al in the gametes were also investigated in *A. altiparanae*, considering the main sperm quality parameters. Al and increased water temperature reduce seminal osmolality and sperm concentration. When analyzing sperm kinetics, Al triggers a reduction in motility, and when this exposure time is longer, both acidic pH and Al reduce sperm motility. Al also affects the sperm ultrastructure, fertility, besides the morphology of the larvae. If Al is added to the activation/incubation medium at acidic pH, a condition that is observed in many rivers, fertilization, hatching, and embryonic development is impaired. When analyzed under *in vitro* conditions, Al negatively influences the sperm quality, impairing membrane vitality, sperm kinetics, and mitochondrial activity.

Therefore, the data obtained so far allowed to conclude that Al can be considered an EDC in *A. altiparanae* males and females, and water quality parameters, as pH and temperature modulate these effects. Additionally, due or not this EDC action, sperm quality is also damaged, in a temperaturedependent way, interfering with fertilization, hatching, and embryonic development.

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Oral Presentation 33

Influence of temperature on early puberty of juvenile male European sea bass (*Dicentrarchus labrax*)

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INTRODUCTION

Climate change is a serious concern for the aquatic environment which alters the physical and chemical properties of the seawater causing negative impacts on fish. Among many effects of climate change, the elevation of atmospheric greenhouse gases has led to an increase in average sea surface temperatures. Fish are poikilothermic and therefore extremely sensitive to changes in environmental temperature. Given the consequent changes in global temperature, it is essential to understand its effects on fish reproduction to cope with ocean warming. Therefore, our objective was to study the impact of high seawater temperature on the onset of early puberty in male European seabass (*Dicentrarchus labrax*).

METHODS

Eight-month-old juvenile sea bass were sized and weighed, and the smallest 85% individuals were considered potentially male fish (n = 510 fish). Two experimental groups were set in triplicate: i) control group (CG) kept under natural seawater temperature at the IATS facilities (40°08′15″ N; 0°10′12″ E) and ii) high-temperature group (HTG) kept 3-4 ºC above CG. The survival rate, body weight (BW) and fork length (FL) of fish were periodically measured. Collected plasma samples were stored at -20ºC until analysis of reproductive hormones (Fsh, Lh, T, 11-KT, E2). Five-eight fish per group were sacrificed from July to February during their first year of life. Testes were removed and weighed to determine the gonadosomatic index (GSI) according to the formula: GW(g)/BW(g)×100, where GW=gonad weight. Staging of testicular development was performed by histological analysis. The percentage of early maturing males was evaluated from November onwards by applying gentle hand pressure to the abdomen of all fish or also assessed by histological examination.

RESULTS & DISCUSSION

After five months, male sea bass reared at a high temperature showed a lower survival rate than that of the CG (66% and 96%, respectively). Differences in growth performance were observed between HTG and CG groups, where the mean weight of males in HTG was 35.8% lower than that of CG. Meanwhile, males in HTG were approximately 12.7% smaller in length than that in CG. The condition factor varied between 1.14 and 1.56 in fish at HTG, while it was around 1.171.91 in the CG. The mean condition factor of fish in GC was approximately 3.6% higher than that of HTG. First significant increase of the GSI was observed after 4 months of experiment in fish of the CG (GSI= 0.47 ± 0.42%) compared to individuals in HTG (GSI= 0.05 ± 0.03%). Accordingly, the histological analysis of testis revealed clear differences between CG and HTG. So far, non-early spermiating fish has been observed although the percentage of gonadal stage III (mid-recrudescence) in the CG reached 29%, whereas the HTG group displayed 0%. At the present time, the analysis in the circulating levels of sex steroids is in progress. In conclusion, rising water temperature negatively affects the growth performance and delays gonadal development of the European sea bass males during the first year of life.

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Oral Presentation 34

Multi-year field survey on the effects of environmental factors on the sex determination in the cobaltcap silverside *Hypoatherina tsurugae*

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INTRODUCTION

The cobaltcap silverside, an atheriniform species that inhabits the inshore waters of southern Japan and the Korean Peninsula, is known to have the Y-chromosome linked *amhy* as a genotypic sex determinant (hence XX and XY system) as well as temperature-dependent sex determination (TSD). It has been shown in laboratory experiments that sexual differentiation of XX genotype fish as males and of XY genotype fish as females occurs in response to high and low water temperatures, respectively. Although the effects of such environmental factors on sex determination have been reported in many fish species, few have demonstrated its existence in wild populations. In this study we report the results of a multiyear field survey on the genotypic and phenotypic sex ratios and on the causal relationship between water temperature and photoperiod conditions on male-to-female and female-to-male sex reversals in a wild population of cobaltcap silverside.

METHODS

The field survey was carried out between 2014 and 2021 in Tokyo Bay. Each sampling was conducted after the end of the spawning season of each year at the same sampling point. The occurrence of genotypic/phenotypic sex mismatches was assessed by gonadal histology and sex genotyping based on amplification of the *amhy* gene. To estimate the birth dates and approximate thermal/photoperiod history during the presumptive period of sex determination, otolith analysis of each individual was conducted. Associations between the thermal/photoperiod experience of each fish and phenotypic sex of fish from each year-class were assessed by generalized linear modelling (GLM).

RESULTS & DISCUSSION

A total of 1897 fish of each year-class were collected in during the survey (n = 88-338 per year). The percentages of genotypic female/phenotypic male (XX-males) varied from 7.3% in 2014 to 52.0% in 2016. Those of genotypic male/phenotypic female (XY/YY-females) were comparatively lower, ranging between 0% in 2016 and 2019 and 14.5% in 2014. The GLM analysis on the effects of the water temperature experienced by the fish between hatching and the critical period of sex determination on the possibility of sex reversal indicated an increase in the frequency of female-to-male transitions with increasing temperature for the XX model and male-to-female transitions with decreasing temperature for the XY model. This study also uncovered compelling evidence of the influence of photoperiod during early development on sex determination in cobaltcap silverside, with short and long photoperiod inducing masculinization and feminization, respectively. These results suggest that the sex determination of wild cobaltcap silverside is affected by a combination of environmental factors which need to be considered when examining the possible impacts of climate change and genotypic/phenotypic sex imbalances on natural resources.

Oral Presentation 35

Sex reversal in natural populations: types, causes and consequences

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INTRODUCTION

The resilience and viability of natural populations to stressors, either of natural or anthropogenic origin, is influenced by the sex ratio, a parameter important in demography that in turn is influenced by sex-determining mechanisms (SDMs) and the environment. Vertebrate SDMs range from genetic (GSD) to environmental sex determination (ESD). Sex reversals produce individuals that show a mismatch between genotypic and phenotypic sex, and include different types depending on the underlying SDM and which sex undergoes sex reversal. Aside genetic factors, the most frequent causes of sex reversal are exposure to abnormal temperature, exogenous chemicals (e.g., endocrine disrupting chemicals, EDCs), and sex steroids, a phenomenon that can occur even in some strict GSD species. An important issue for conservation biology is to understand the underlying causes responsible for the appearance of neomales, individuals with a female genotype but a male phenotype. It is not clear, however, whether among GSD species, those with polygenic sex determination (PSD) are more sensitive to the effects of masculinizing environments than those in which sex determination is chromosomal (CSD).

METHODS

To address this question, we used two approaches: literature review and experimental manipulation. In the first approach, we looked for cases of sex reversal in fish, amphibians, reptiles, birds and mammals. We focused on the offspring of sex-reversed parents vs. the offspring of non-sex reversed parents considering different environmental factors (mostly temperature, hormones and EDCs) and different SDMs. In the second approach, we used two wild zebrafish strains, Ekkwill and Nadia, which have CSD with a ZZ/ZW system, and the AB laboratory strain, which has PSD, and compared the effects of elevated temperature.

RESULTS & DISCUSSION

The literature survey showed that the offspring of sex-reversed individuals were more sensitive to environmental cues than the offspring of non sex-reversed parents. This was irrespective of whether sex reversal was found in the wild or generated in the lab. The inheritance of this increased sensitivity is likely to be underpinned by epigenetic modifications such as DNA methylation, which can be influenced by the environment and passed to subsequent generations if they escape reprogramming. Laboratory manipulations showed that even at the control temperature, some fish of the two wild strains with ZW and WW genotypes developed as neomales. At elevated temperatures, to our surprise, we observed that WW fish became neomales in about the same frequency as ZW fish and that the wild strains with CSD were equally susceptible to masculinization as the lab strain with PSD. Furthermore, at the control temperature, the testes of neomales contained less sperm than the testes of regular males but at elevated temperature, this relationship was reversed. These results indicate that in a context of global warming, CSD species may not benefit from a better protection against elevated temperatureinduced masculinization when compared to PSD species, suggesting a role for epigenetics in controlling the heritability of sensitivity, and uncovering GxE interactions in sex determination and spermatogenesis.

Oral Presentation 36 (student)

Effect of climatic and estrogenic stress on the life cycle of an estuarine fish (*Gasterosteus aculeatus*)

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INTRODUCTION

Through the climate change the anthropic activities lead to numerous alterations of the aquatic environments (ocean warming, acidification, etc). The physiology and resilience of the aquatic organisms, that depends of the evolution physico-chemical parameters of their environment, could be affected under such variations. Furthermore, aquatic organisms are exposed to an increasing number of chemical contaminants, including endocrine disruptors, which are likely to have an impact on their fitness. In this multi-stress context, by an experimental approach, this study aims to: 1/ characterize the effects of warming (+3°C) and acidification (-0.4 pH units) on the physiological functions of the marine stickleback (*Gasterosteus aculeatus*) and 2/ assess whether the presence of a contaminant with a known biological activity (oestrogenic stress with exposure to ethynylestradiol (EE2)) modulates these effects during its entire life cycle.

METHODS

This experiment was carried out on juvenile sticklebacks (F0) acclimated at 2 climatic scenarios (Current; RCP8.5) and on their offspring (F1) submitted, during the embryo-larval stage, to an additional stress: an environmentally realistic chemical contamination (15ng EE2.L^{-1}). The effect of climatic stress alone and in combination to EE2 contamination were evaluated in F0 and F1 respectively on growth, maturation and reproductive endpoints.

RESULTS & DISCUSSION

Growth rates were examined on the two generations of sticklebacks maintained in the laboratory (F0, F1) based on regular biometric measurements and food intake and demonstrated a significant influence of climatic conditions. The growth rates in F0 and F1 fish were lower in RCP8.5 condition, being the lowest when F1 where additionally contaminated with EE2. The reproductive success and output of F0 generation was assessed through the quality and the fertilisation rate of eggs and also the hatching and survival rates of the larvae. Sexual maturation was evaluated by the histological analysis of gonads and the gene expression in brains for both generations. Preliminary results show early effects of climatic and chemical conditions that might delay the maturation of sticklebacks. Altogether, the experimental data could suggest a higher maintenance costs and accelerated energy metabolism in warmer and acidified conditions, possibly modulated by an estrogenic stress.

Data analyses need to be completed and further research may be needed to better understand the effects of the combination of climatic and chemical stress on stickleback physiology and, ultimately, to anticipate the impact of multistress on the population dynamic.

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Oral Presentation 37

Glyphosate exposure disrupts zebrafish spermatogenesis

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INTRODUCTION

Glyphosate is an active component of most herbicides largely used in conventional agricultural practices worldwide. Growing evidence has reported that glyphosate disrupts the homeostasis of the endocrine system mainly due to its estrogen-like properties, thus having deleterious consequences for the reproductive health. In addition, this herbicide has been reported to promote epimutations in mammals, pointing to potential inheritance of its effects to the following generations. Since the environmental levels of glyphosate are alarmingly increased and, considering that the safety of this compound remains a controversial issue, more information is still needed. In this study, we used zebrafish as model species to have a deeper insight into the molecular changes affecting spermatogenesis and male breeding capacity.

METHODS

Zebrafish adult males were dietary exposed to three different doses of glyphosate: 0.5 mg/kg bw day (defined as acute reference dose by the EFSA), 5 mg/kg bw day and 50 mg/kg bw day (defined as the no observable adverse effect level dose) for three weeks. Fishes were fed twice a day using dry commercial food with or without glyphosate (control group). After the treatment, males were euthanized and testicles were immediately excised to assess the potential changes in their structure at histological level, whereas in testicular cells apoptosis was evaluated by TUNEL assay, the alterations at protein level by western blot, and the histone modifications by immunohistochemistry.

RESULTS & DISCUSSION

The results revealed that, although the gonadosomatic index of males was similar between control and glyphosate-exposed ones, testicles of males exposed to the highest dose displayed abnormal cystic structures, whereas those exposed to the lowest dose of glyphosate showed a higher frequency of spermatogonia. The TUNEL assay allowed the detection of an increased number of apoptotic cells in the testicles from males treated with 50 mg/kg bw day, but no changes were observed in the Casp3 levels in the whole gonads. Bearing in mind the crucial role of the endocannabinoid system in reproduction, the levels of the cannabinoid receptor CB1 were evaluated. In this case, exposure to both 0.5 and 5 mg/kg bw day decreased the levels of CB1, thus likely affecting different processes occurring during spermatogenesis, such as cell differentiation, chromatin remodeling, and sperm maturation. For this reason, immunohistochemistry was performed to study proteins involved in cell proliferation and differentiation as well as two epigenetic marks: the acetylation of K9 in H3 (H3K9ac) and the acetylation of K12 in H4 (H4K12ac). Altogether, these results suggest that male exposure to glyphosate promotes genotoxic and epigenotoxic effects in the testicular cells in a dose-dependent manner. Considering the lack of consensus about the toxic effects of glyphosate, these data could be of the utmost interest to agencies, such as the EFSA and EPA, and lawmakers to better regulate the use of this pesticide in areas devoted to crop production.

The project received funding from the Fondo Ateneo 2022 to FM and OC.

Oral Presentation 38

Reproductive physiology of zebrafish affected by contraceptive pill hormones: estetrol as an ecological alternative to ethinylestradiol?

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INTRODUCTION

The pill accounts for 50% of the contraceptive methods in industrialised countries, and ethinylestradiol (EE2) is the most widely used estrogen in combined oral contraceptives. This synthetic estrogen is well known to be a potent endocrine disruptor. Recently, the natural estrogen estetrol (E4) was approved as the estrogenic component of a new oral contraceptive in combination with the progestin drospirenone (DRSP).

The objective of the present study was to characterise and compare the endocrine disrupting potential of E4 and the mixture E4/DRSP with that of EE2 and EE2/DRSP in zebrafish *Danio rerio*. The effects of these compounds were analyzed after short-term exposure and after exposure over several generations. A particular attention was given to the mechanisms involved in steroidogenesis, oocyte maturation, fertilization and spawning.

METHODS

In a first short-term experiment, zebrafish breeders were exposed during 3 weeks to different concentrations of E4 or EE2 (10, 100 and 1000 X the predicted or detected environmental concentrations respectively). In a second experiment, fish were exposed over 3 generations (F0, F1 and F2) to environmentally relevant concentrations of E4/DRSP or EE2/DRSP (1 to 32 X the predicted/detected environmental concentrations). Aside descriptive endpoints such as fecundity, fertilization and hatching rates, gonad histopathology, vitellogenin and other endocrine-related parameters, the mechanisms of action of these two hormonal mixtures were investigated through a large set of gene expression.

RESULTS & DISCUSSION

While no impact of E4 was detected on fecundity and gonad histopathology during the first experiment (short-term exposure), the number of eggs spawned was significantly reduced in fish exposed to EE2 (100 and 1000 X the environmental concentration). This was accompanied with a severe alteration of female gonads including oocyte atresia, perifollicular cell atrophy, granulomatous inflammation and interstitial fibrosis. Moreover, several genes related to steroidogenesis (*hsd3b1, lhr, esr2b, cyp17a1* and *cyp19a1a*) were down-regulated in ovaries after exposure of fish to EE2 while only minor changes were detected at the highest concentration of E4.

In the second experiment (F0 to F2 generations), gonad and steroidogenesis disruption was observed in F0 generation after exposure to EE2/DRSP in comparison with E4/DRSP. In F1 generation, gonad development was impaired in fish exposed to EE2/DRSP at concentration as low as 3X the environmental concentration, with a significantly decreased fecundity, gonad histopathological damages (*e.g.* oocyte atresia and decrease of post-ovulatory follicles) and increased vitellogenin synthesis in both sexes. In contrast, E4/DRSP did not induce significant changes in the different reproductive physiology and histopathology endpoints, when compared to control. Mechanisms of action of E4/DRSP as compared with EE2/DRSP are still under investigation. Overall, the data obtained in these studies suggest that E4 might be an ecological alternative to EE2 in hormonal contraception.

Oral Presentation 39

Is teleost fecundity style a species-characteristic trait? – A re-evaluation of the ovulatory cycle of Atlantic cod (*Gadus morhua*) in a changing environment

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INTRODUCTION

Fecundity style in teleost is a fundamental reproductive trait, highly relevant for basic and applied research (e.g., monitoring of spawning stock biomass of harvested fish stock). The actual expression of fecundity, either indeterminate (with de novo oocyte recruitment) or determinate during the event of spawning respectively, may relate to several factors, such as temperature, body condition or stock demography. Northeast Arctic (NEA) cod (*Gadus morhua*), a classical determinate teleost, is an important fisheries stock undertaking migration over long distances, from feeding areas in the Barents Sea to spawning grounds in the Lofoten archipelago. Due to strict management regulations and a favorable climate, this stock – previously overharvested – has recovered age and size diversity over the last couple of decades. Therefore, we hypothesized that cod may have shifted towards a more indeterminate style under the present productive oceanic regime.

METHODS

Wild NEA cod spawners were captured at the main spawning ground in Lofoten, Northern Norway, in a relatively cold (2003) and warm year (2018) for vitellogenesis. In addition to demographic structure of female NEA cod, we analyzed oocyte recruitment and fecundity down regulation (atresia) by validating the recently developed ultrametric method and the use of histology, respectively. This reevaluation of the ovulatory cycle of NEA cod was placed in a longer perspective by investigating the fecundity dynamics and ovulatory cycle in wild NEA cod prespawners collected between 1986 and 2022 in Vesterålen (Norway). Hence, this study covers a broad span in temperature regimes and stock status.

RESULTS & DISCUSSION

At the spawning ground, demography structure of NEA cod has recovered in recent years with an increase in proportion of age-9+ females (from 5% in 2003 to 82% in 2018). Additionally, today's high fraction of repeat spawners presenting less atresia suggest that reproductive performances and hence fecundity, may have improved. We found that NEA cod may show a flexible fecundity style, i.e., de novo oocyte recruitment (to secondary growth) within the spawning season suggesting a semiindeterminacy. This flexibility was lower recently (4% in 2018) compared to the beginning of the century (15% in 2003). As there is a trend towards determinacy in cold-water and indeterminacy in warm-water species, temperature at feeding and spawning areas was consulted. Although environmental temperature for vitellogenesis was higher in 2018 compared to 2003, spawning temperature in Lofoten was similar between these two years. However, flexible fecundity may be attributed to the recent lengthier poleward migration (as the suitable feeding area is expanding) resulting in lower ambient temperatures for NEA cod. Our work challenges the dichotomous fecundity classification and emphasize the complexity of reproductive mechanisms in changing environment bringing up new interrogations for future studies.

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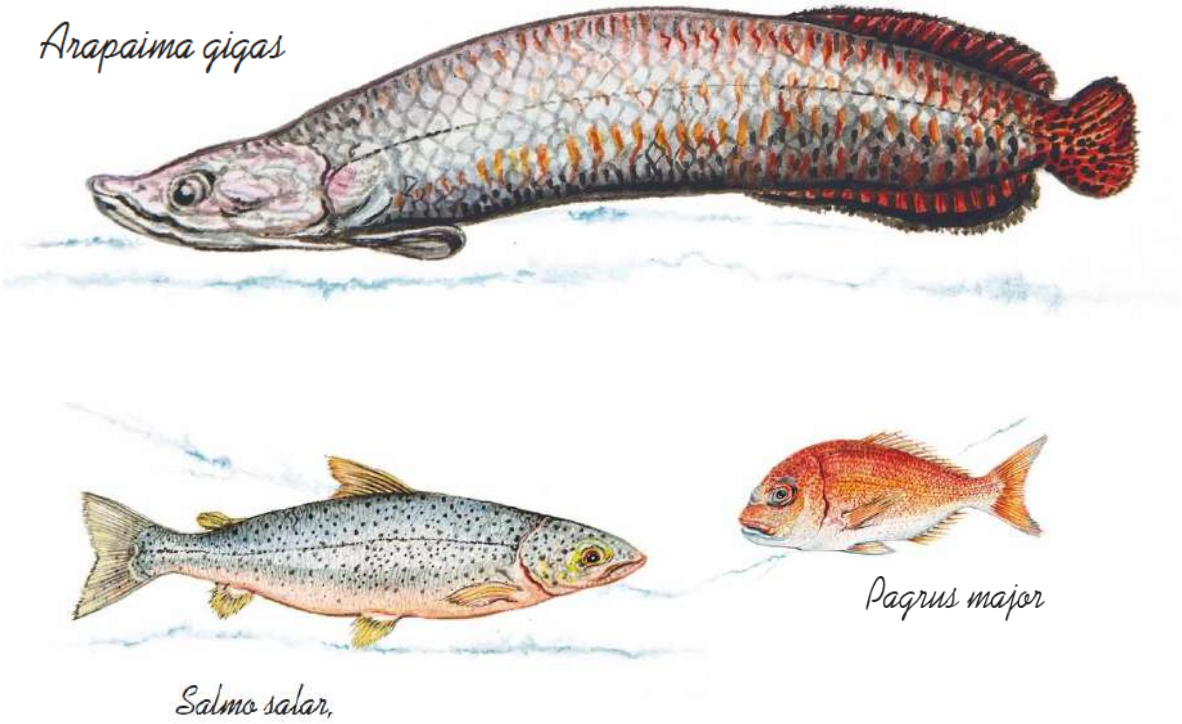
SS6. Reproduction in aquaculture



Invited State-of-the-Art Presentation 7

Cryopreservation research in aquaculture: what the industry needs and what it doesn't

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INTRODUCTION

Cryopreservation of live cells and tissues has long been recognized as an efficient tool for the conservation of genetic resources and is employed for commercial (farming) and conservation purposes, as well. In aquatic species, cryopreservation protocols have been developed for decades, however, they saw only limited use by the aquaculture sector. This review intends to investigate the areas of aquaculture where cryopreservation is actually applied to improve production.

METHODS

Spermatozoa are by far the most common cell types cryopreserved for reproduction purposes. Over the last 70 years, protocols have been developed for several hundred species. Sperm cryopreservation has been applied to aquaculture activities primarily in salmonids. National gene banks in collaboration with hatcheries have been established in a number of countries including Norway, Poland and France. These gene banks serve as repositories for genetic resources and are for the most part interconnected with conservation activities of the genetics resources of wild and farmed fish communities. The main competing interests among various actors are mass selection vs. individual selection, large quantities of sperm produced by an individual and needed for fertilization vs. small quantities that can be cryopreserved and differences in priorities between the research community and the aquaculture sector. However, increasing interest by aquaculture enterprises in the use of cryopreserved sperm resulted in a shift in priorities by the research community. Instead of the development of methods and protocols for cryopreservation (such as development of media and cooling rates) more attention is paid to standardization issues ensuring security and replicability of results, including quality control of sperm as well as standardization of sperm concentrations rather than dilution ratios. Improved quality control along with interest by representatives of the aquaculture sector has also raised the attention of companies serving the cryopreservation industry, thus, many of these now either aquaculture-oriented or have an aquatic species branch. Thus, there clearly seems to be an interest by all parties (aquaculture, research and cryopreservation industry) in further collaboration and development. On the other hand, creation and maintenance of these gene banks still seem to be initiated by public institutions and they largely depend on taxpayer funding rather than independent business investment.

RESULTS & DISCUSSION

Cryopreservation of fish eggs and embryos is currently largely considered a dead end due to the lack of reliable and affordable protocols stemming from the particular structure of eggs and embryos. More attention is paid to the cryopreservation of bivalvian (e.g. oyster) larvae and there is a genuine interest by the industry in these activities. Germ cell research has recently concentrated on the cryopreservation and transplantation of gemline stem cells (GSCs) such as spermatogonial and oogonial stem cells. Although these methods offer a good alternative to the cryopreservation of sperm and eggs/embryos in fish, their value is primarily conservational and they are less attractive for the aquaculture sector.

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Oral Presentation 40

Can we sex fish using circulating miRNAs? a comparative study

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INTRODUCTION

Identifying fish sex is crucial for understanding population dynamics. In many fish species, no sexual size dimorphism has been detected and it is most often impossible to identify fish sex based on external characteristics. In aquaculture, the identification of sex at the earliest stage is a predominant preoccupation for fish farmers. The development of new non-invasive technics to sex fish would be a clear asset in those domains. We propose to use circulating microRNAs (miRNAs) detected in the blood, as sex markers. MiRNAs are short and conserved sequences of nucleotides (20-22 nucleotides) involved in the regulation of multiple biological processes. Some circulating miRNAs were already shown to be markers of several functions, including stress, metabolism and immunity. Here we aimed at studying to what extent they can serve as makers of sexual development.

METHODS

Blood samples and gonads of five gonochoristic fish species (*Dicentrarchus labrax, Scophthalmus maximus, Caranx ignobilis, Acipenser baerii* and *Sciaenops ocellatus*) were sampled on immatures (n= 10 Males and 10 Females) and matures individuals (n= 10 Males and 10 Females). We also sampled blood and gonads of one protandrous hermaphrodite species (the gilthead sea bream, *Sparus aurata*) at five different stage (n= 10/ stage) of the transition from male to female. Total RNA of plasma samples was extracted and simultaneously, the sex of immature fish was determined by observation of the histological sections of gonads. After smallRNA sequencing, miRNAs were aligned and annotated using the software Prost!. We used the phylogenetically closest species, i.e. *Gasterosteus aculeatus* for the alignment. The package DEQSeq2 was used to identify miRNA differentially expressed between sex of gonochoristic species or intersexual stages (gilthead sea bream). We then validated some of these miRNAs by qPCR, with a focus on *Dicentrarchus labrax*.

RESULTS & DISCUSSION

The sequencing allowed identifying a list of miRNAs differentially expressed between males and females, although this was highly stage and species dependent. In juveniles European Seabass we detected that both miR-1388-3p and miR-7132a-5p were up-regulated in females, while miR-499a-5p was more abundant in the plasma of males. This was confirmed by qPCR. In the Seabream, we detected some miRNAs specific of each intersexual stage. We discuss their interests as non-lethal tools to monitor sex differentiation and sex-change in fish species and expand on their potential roles, thanks to *in silico* analysis.

The project received funding from the European Maritime and Fisheries Fund – miSS (miRNAs, Sex and Stress) n°498769.

Oral Presentation 41

The relationship between the IGF system and the early onset of puberty in male and female European sea bass (Dicentrarchus labrax)

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INTRODUCTION

Precocious puberty occurs in captivity when individuals reach sexual maturity earlier than expected. In the European sea bass, precocity affects both sexes thus altering final growth at harvest. Precocious males are smaller than their non-precocious counterparts at 23 months old, whereas precocious females are usually heavier than immature ones. This situation leads to size dispersion and a lower biomass of the population. Thus, although body size and maturity traits are interrelated, their relationship is still not entirely understood in fish. In the present work we study the association among the elements of the Igf system with key reproductive indicators in precocious sea bass influencing early gonad growth.

METHODS

European sea bass were maintained under natural conditions at the IATS facilities (Castellón, Spain, 40°N 0°E). Fish were individually tagged and growth and gonadal development were evaluated during the first and second year of age. The gonadosomatic index (GSI), the visceral somatic index (VSI) and the hepatosomatic index (HSI) were also evaluated. Gonads were fixed for histological analyses. Blood was collected and plasma was stored at -20°C until analyzed. Hormonal profiles, including Fsh, estradiol (E_2), vitellogenin (Vtg) and Igf-1 plasma levels were measured. The changes in gene expression of igf-1, igf-2 and igf-3 were analyzed by qRT-PCR as well as the relative mRNA expression levels of cell division and proliferation-related genes and steroidogenic genes were also considered. Male and female fish were sacrificed at the end of the first and second year of age, respectively, and reproductive performance indicators were restored to specific point-in time based on the gonadal development.

RESULTS & DISCUSSION

Precocious 1-yr-old male and 2-yr-old female sea bass are usually heavier than their immature counterparts with higher GSI and HIS values and lower VSI. Precocious males displayed higher plasma levels of Igf-1, while immature males showed higher plasma levels of E_2. Advanced mature females had higher circulating plasma levels of Fsh, E_2 and Vtg than the immature ones, whereas Ifg-1 plasma levels in precocious fish increased during late spring-early summer, one year before spawning. Interestingly, the relative mRNA expression gonadal levels of igf-1 were low in late stages of spermatogenesis, whereas igf-1 expression was high during early and late vitellogenesis in females. The igf-2 mRNA levels increased in males coinciding with increasing mitotic proliferation of spermatogonia, whereas igf3 expression peaked during meiotic phase (Stage III). In females, igf-2 and igf-3 mRNA levels were similar among ovarian stages. Based on these results, tissue distribution and temporal mRNA expression of the elements of the Igf system during early development are also planned to be analyzed. Examination of the expression profile of Igf system components in mature and immature prepubertal fish will contribute to assess the involvement of the Igf system in male and female sea bass reproduction. In addition, cell division and proliferation-related genes and genes related to the biosynthesis of steroids that accompany maturation will be analysed as key factors influencing early gonad growth in fish.

Project funded from MCIN (AGL2016-75400, PID2019-109548RB-I00) and supported by the ThinkInAzul programme (MCIN with funding from European Union NextGenerationEU, PRTRC17.I1, and GV, THINKINAZUL/2021/024). L.S. supported by an FPI fellowship from MCIN (Spain).

Oral Presentation 42

Effects of *bmp15* mutation on gonad development and fertility in Atlantic salmon

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INTRODUCTION

Inducing sterility in farmed Atlantic salmon (*Salmo salar*) prevents 1) genetic introgression from farmed escapees into wild populations and 2) unwanted precocious maturation in males, which both would contribute to a more sustainable production. One strategy to induce sterility is to inhibit the function of proteins that are essential for germ cell development and/or survival, such as the previously identified Dnd (Dead end). Bone morphogenetic protein 15 (BMP15/Bmp15) is a growth factor important for female reproduction. BMP15 is involved in ovarian development and maturation, and mutations in *BMP15/Bmp15* cause sterility in different mammalian species. However, the function of BMP15/Bmp15 in males is largely unknown. To investigate the role of Bmp15 in salmon gonad development and fertility, we produced *bmp15* mutated salmon using CRISPR/Cas9.

METHODS

F0 *bmp15* mutated salmon were generated and reared throughout a life cycle. While no apparent effect on gametogenesis was observed, surprisingly few offspring survived when crossing highly mutated (92100% mutated sequences by MiSeq) F0 broodfish (n=6 individuals). The F1 survivors (n=176), originating from 4 egg batches (1 mother and 4 fathers), were combined and reared in common garden with wild types (WTs, n=106)) throughout their life, and body growth, gonad development and fertility were compared between WT and mutant females and males.

RESULTS & DISCUSSION

Low survival of offspring from the F0 generation suggests that Bmp15 may be important for the development of functional gametes and/or embryo development in salmon. When the F1s were 18 months old, 21% of the mutant females displayed only 1 ovarian bulb, and 83% of the mutant males had germ cell-poor testes, also reflected by lower *vasa* (*ddx4*) expression compared to WTs. Furthermore, genes involved in estrogen synthesis and signaling (*cyp19a1a* and *esr1*) were upregulated in mutant females. At 22 and 26 months, no differences were detected between mutant and WT females. However, spermatogenesis in mutant males seemed more advanced than in WTs, showing higher gonado-somatic index values and cumulative frequency of spermiating testes (50% vs. 22%), and lower expression of genes involved in steroidogenesis (*cyp17a1* and *star*). From 36 months up until spawning, mutant females showed smaller body mass and different types of abnormal ovarian morphology, such as none or only 1 ovarian bulb, and mature ovaries with a high incidence of atresia. Analyses of oocyte morphology, sperm motility and fertility in correlation with the different mutation types are ongoing.

So far, our results suggest a role for Bmp15 in the regulation of germ cell development during early stages of both oogenesis and spermatogenesis in Atlantic salmon, and that loss of Bmp15 function advances spermatogenesis. Ongoing analyses will reveal if mutation in *bmp15* affects fertility.

The project was funded by the Norwegian Research Council (projects 221648 and 302532).

Oral Presentation 43

Aquaculture improvement toolkit for grey mullet (*Mugil cephalus*): broodstock management & production of all-female genetic lines

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INTRODUCTION

The grey mullet, *Mugil cephalus*, is fished and farmed world-wide. Following the success in closing the species' life cycle in captivity, we are now focused on culturing all-female mullet populations, that are faster growing than males and are well known for their highly prized roe called "bottarga". To achieve this goal, the current study has adopted the indirect feminization strategy: i.e, the masculinisation of genotypic females, and crossing the resultant neomales with normal females to produce a mullet all-female population. Accordingly, the following objectives were addressed: (i) optimize broodstock management and improve spawning success, (ii) establish functional neomales (iii) develop sex-specific molecular markers for identification of the neomales, (iv) produce an all-female grey mullet genetic line.

METHODS

Induced gonadal development and spawning- In females, the effects of combinations of Metoclopramide

(MET) with either recombinant FSH (rFSH) or implants for sustained release of the GnRH analogue (GnRHa-EVAc) were tested. Studies with males evaluated the effect of rFSH as a sole therapeutic agent vs. rFSH, which was used to prime the fish prior to the administration of methyltestosterone implants (MT-EVAc). To induce spawning, fully mature grey mullet females and males were treated with two consecutive injections consisting of GnRHa +MET given 22.5-h apart.

Induced masculinization- A masculinized phenotype was obtained by exposing for 4 months hatchery produced mullet fry at 3 age categories to food supplemented with MT (10 or 15 mg MT/Kg of food). The sex phenotype was monitored by histological secession of the gonads and/or gonadal biopsies.

Sex-specific molecular markers- Analysis of genomic alleles was based on whole- genome sequencing of a male and female. Variants that fit the XY system model were tested for association with sex by Sanger sequencing of sample of individuals from two families. The sex specific markers were validated and used to identify mullet neomales.

RESULTS & DISCUSSION

The optimal treatment consisting of MET+rFSH, resulted with 91% post-vitellogenic females within the treatment-group. All males that were primed with rFSH and then subjected to MT-EVAc implantation produced sperm. The spawning induction trials gave rise to high quality eggs and larvae resulting later in large numbers of robust juveniles during natural and shifted reproductive seasons. The results of the masculinizing studies highlight the period of 6 to 9 month of age, as a labile phase when the differentiating gonads are most susceptible to androgens. Regardless of the dose, administration of MT to the 6-month-old fish, produced 100 % males. After sexual maturity was achieved, spermiating mullet neomales were crossed with normal females giving rise to the first reported all-female mullet stock. Furthermore, body weight measurements revealed enhanced growth of the all-female mullet juveniles relative to mixed population of the same age class.

Altogether, our results, reinforce the notion that the culturing of all-female mullet populations would improve production significantly due to the faster growth of females in comparison to sibling males.

Oral Presentation 44 (student)

DNA methylation during early development in diploid and triploid European sea bass

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INTRODUCTION

Triploidy can be induced by retention of the 2nd polar body by temperature or pressure shocks. Induced triploidy is used in the aquaculture of some fish and mollusks to increase growth and avoid the problems associated with sexual maturation, since induced triploids are sterile. Most research on the consequences of induced triploidy has been focused on survival, growth, and reproductive physiology. However, less is known on the epigenetic regulation of gene expression and the limited literature reveals contrasting results. Thus, no differences were detected between diploid and triploid brown trout (*Salmo trutta*). In triploid oysters (*Crassostrea gigas*), different levels of DNA methylation were observed related to their fertility status. When compared to diploids, and regardless of sex, triploid oysters had more hypomethylated regions if they were infertile and more hypermethylated if they were fertile. In allotriploid cyprinids, DNA methylation was involved in dosage compensation, resulting in similar gene expression levels than diploids. Finally, DNA methylation was suggested to contribute to autopoliploidy–mediated speciation in marbled crayfish (*Procambarus virginalis*). However, no information is available on the effects of induced triploidy on DNA methylation patterns during early development in fish, which was the goal of this study.

METHODS

The European Sea bass (*Dicentrarchus labrax*) was used as a model. Triploidization was induced by a cold shock of eggs, at 5 minutes post fertilization (mpf), at 0°C for 10 minutes. Three stages of development (90% epiboly, 30 mpf; hatching, 92.5 mpf; mouth opening, 5 days post hatching, dph) and two distinct families were analyzed to account for biological variation. Triploidy was verified to be near 100% by erythrocyte size measurements. DNA was obtained by Phenol-Chloroform extraction.

Methylation was measured by Reduced representation bisulfite sequencing (RRBS) analysis. A total of 12 libraries were prepared using the Premium RRBS kit (C02030033, Diagenode) and multiplexed in 2 pools. Sequencing was done at single-end 50 bp and 16x coverage, using the Illumina HiSeq2500. Bioinformatics analysis was carried out using the trimmomatic and BSMAP software, and, R packages *methylKit* and *GenomicRanges*, respectively.

RESULTS & DISCUSSION

A total of 18.63 Gb of data were produced with an average of 40 million reads per sample. Two different types of comparisons will be reported. First, at each one of the three developmental stages DNA methylation at single nucleotide resolution will be reported for reach ploidy level. Differentially methylated CpGs (DMCs) are defined as having more than 15% methylation differences (False Discovery Rate, FDR <0.05) to ensure biologically meaningful results. Second, DMC will also be calculated between two adjacent developmental stages within each ploidy level in order to study possible alterations in DNA methylation patterns along embryonic and larval development. The localization of the DMC in different genomic regions and genomic features (CpG islands and shores) will also be reported. Finally, Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Pathways (KEGG) will be carried out focusing on genes containing DMCs. Funded by Ministry of Science and Innovation grant ‘Epipure’ (PID2019-108888RB-I00) to FP.

Oral Presentation 45 (student)

Improving the artificial reproduction of the European eel to enhance larval quality

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INTRODUCTION

The routine protocol to induce sexual maturation in European eels consists of long-term weekly injections with pituitary extracts (PE) from salmon or carp to induce oocyte growth, followed by a single 17α,20β-dihydroxy-4-pregnen-3-one (DHP) injection to induce oocyte maturation and ovulation. With this protocol, we produce larvae three times per week but only a minor fraction survives until exogenous feeding since high mortality is often experienced during the first week after hatching. Although much effort has been devoted to understanding the reasons behind larval mortality, the causes of abnormal development remain largely unclear. Over the past four years, we have conducted experiments to optimize the artificial reproduction protocol to increase egg and larval quality.

METHODS

PE is a mixture of hormones that might vary between batches and donor species. Contrary to using PE, recombinant gonadotropins (rGths) are species specific and the dosages of the gonadotropins are controllable. In a first experiment, we tested the effects of eel rGths on inducing oocyte development *in vitro* and *in vivo*. DHP is hypothesized to decrease egg fertility. In other fish species, progesterone (P) has been found to induce oocyte maturation and ovulation. In a second experiment, we compared DHP and P effectivity *in vitro* and *in vivo* in inducing oocyte maturation and ovulation. In a third experiment, we compared the transcriptomics of larvae of batches that survived less than 3 days and larvae of batches that survived for a least a week. In a fourth experiment, we tested the effects of antibiotics and disinfection treatment, alone or in combination, on hatching success and larvae survival.

RESULTS & DISCUSSION

Several females treated with rGths had very high gonadosomatic index values (77-80) in comparison with females injected with PE (40-60) suggesting that rGths induced strong vitellogenic growth. Larvae were produced for the first time with rGths, but dose and timing still need optimization to produce high quality eggs. When comparing DHP and P, we found that both steroids have the same potency to induce oocyte maturation and ovulation *in vitro* and *in vivo* but P is more attractive to use since it is 3,000 times cheaper than DHP. Larvae that died shortly after hatching initiated an immune response toward pathogens and tried to maintain homeostasis. The use of antibiotics increased larvae survival and decreased the amount of deformity. Finally, larvae deformities were classified for the first time in European eels.

The experiment using the rGths was performed in collaboration with I. Giménez Nebot (Rara Avis Biotec S.L., Valencia, Spain). The project has received funding from the DUPAN foundation; The Dutch Ministry of Economic Affairs and the European Union and European Maritime and Fisheries Fund, and as public-privat partnership from the Dutch Ministry of Agriculture, Nature and Food Quality and the DUPAN foundation.

Oral Presentation 46

Flathead grey mullet (*Mugil cephalus*) farming conditions for producing *bottarga*, histological, physiological and biochemical processes during gametogenesis

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INTRODUCTION

The Mugilidae flathead grey mullet (*Mugil cephalus*) has been identified as one of the most promising for the development of a profitable but more sustainable aquaculture of the future. The main commodity resulting from mullet added value practices results in a luxury product, the *bottarga*. Despite this growing interest, commercial aquaculture of the species is struggling to take off. The main cause could reside in the absence of a commercially applicable protocol for producing *bottarga* from farmed animals. Indeed, grey mullet fail to complete gametogenesis in captivity. The present study investigates the most suitable farming conditions to obtain sufficient gonadal development to produce *bottarga* (from females in advanced vitellogenesis).

METHODS

On July 2022 a population of 3 years old fish produced at IMC laboratories and farmed in fresh water ponds was subjected to an initial sampling for i) gamete maturation assessment (histology of the gonads and in vivo-oocyte measurements), ii) physiological reproductive status (sex steroids and gene expression of the fsh receptor in gonads) and biochemical composition (total lipids and fatty acid profiles), during the reproductive season (July-August). A sub-population of the farmed fish was transferred into a lagoon enclosure (> 37 ppt) and monitored for 6 weeks for the above reported analysis. At the same time the original population kept in freshwater and wild breeders undergoing reproductive migration were also studied.

RESULTS & DISCUSSION

The sex ratio of the farmed population resulted to be composed by 91.4% of females (36±4 cm TL and 483±163 g BW) and 8.6% of males (36±4 cm TL and 510±171 g BW). Wild breeders sex ratio was composed by 53% of females (35±8 cm TL and 425±302 g BW) and 47% of males (34±9 cm TL and 407±369 g BW). At the end of the experimental activity only 11% of the females held in freshwater presented vitellogenic oocytes while the vast majority of females (89%) presented previtellogenic early perinucleolar oocytes, whereas the fish moved to the lagoon enclosure developed advanced vitellogenic (25%) and fully mature oocytes (12.5%), with oocyte diameters averaging 426±40 µm. In the wild population 40% of the females were in full vitellogenesis while 20% resulted to have already spawned. None of the farmed males presented ripe testis, while 64% of wild males were fluent. Present results demonstrated that fresh water farmed flathead grey mullet female can mature similarly to wild individuals in 6 weeks if moved during the natural reproductive season to marine conditions such as those present in the employed lagoon enclosure. The results of the ongoing analysis will add useful information for shedding light on the physiological and biochemical processes underpinning grey mullet gametogenesis. Such data will help to define protocols that may favour the development of a flathead grey mullet roe industry no longer reliant on depleted wild populations. Funding for this trial was provided by the iLAB Project “Cluster 2” managed by the Municipality of Oristano (Sardinia, Italy).

Oral Presentation 47

The use of sand substrate modulates stress response and enhances maturation in Senegalese sole females

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INTRODUCTION

The use of enriched environment has been demonstrated to enhance animal welfare, reduce stress response, increase cognitive perception, and decrease anxiety-like behaviours. These benefits have been observed in several fish species at different levels like growth parameters and stress markers. Senegalese sole (*Solea senegalensis*) is a flatfish species with high importance in European aquaculture due to its high growth and survival rates. However, this species faces reproductive disorders which does not allow to close the life cycle in captivity. Our hypothesis was that conditioning the sole to natural environmental factors in early life stages may help to develop healthier fish and future potential breeders. The aim of this study was to assess the effect of using sand as environmental enrichment in stress response and maturation in Senegalese sole females from juveniles to pre-pubertal stages.

MATERIAL AND METHODS

A total of 1500 sole (~10g; 8 months) were established in 6 outdoor tanks in the experimental station of IPMA (Olhão, Portugal) maintaining natural temperature and photoperiod according to each season. The bottom of 3 tanks was covered by 2 cm of sand and the other 3 remained without it (fiber-glass). These fish were maintained in the same conditions for two years and 4 samplings (6 months each) were conducted to collect several biological materials and to take biometric parameters. One part of the ovary was fixed in 4% PFA for histological procedures (H&E staining). RNA was extracted from brain and ovary to run qPCR to observe differences among enriched conditions and time points. The genes *hsp70*, *hsp90* were used as stress markers in brain and quality in gonads; *nrd2*, *nr4a2*, *bdnf* and *c-fos* as cognitive markers in brain; *fshra* and *pgr* were used as maturation markers in brain and ovaries. Levels of testosterone (T) and estradiol (E_2) were determined in blood plasma (n=20 females/environment/sampling) using ELISA.

RESULTS & DISCUSSION

Females maintained in sand showed significant lower expression levels of *hsp90* in brain demonstrating that fiber-glass group might exhibit higher chronic stress response than females maintained in sand. The histological results showed that sand group was one stage more mature than the fiber-glass females from the second sampling, where the in the last sampling, the sand group reached previtellogenic oocytes. The levels of sex steroids were significantly higher in the sand group after two years under an enriched rearing environment. These results in maturation were in concordance with the expression levels of *fhra* and *pgr* in brain and gonads. These preliminary results indicate that enriched environment during the on-growing phase promotes gonadal development and enhance welfare, reducing chronic stress response. Environmental enrichment could be used in future breeders' selection in Senegalese sole.

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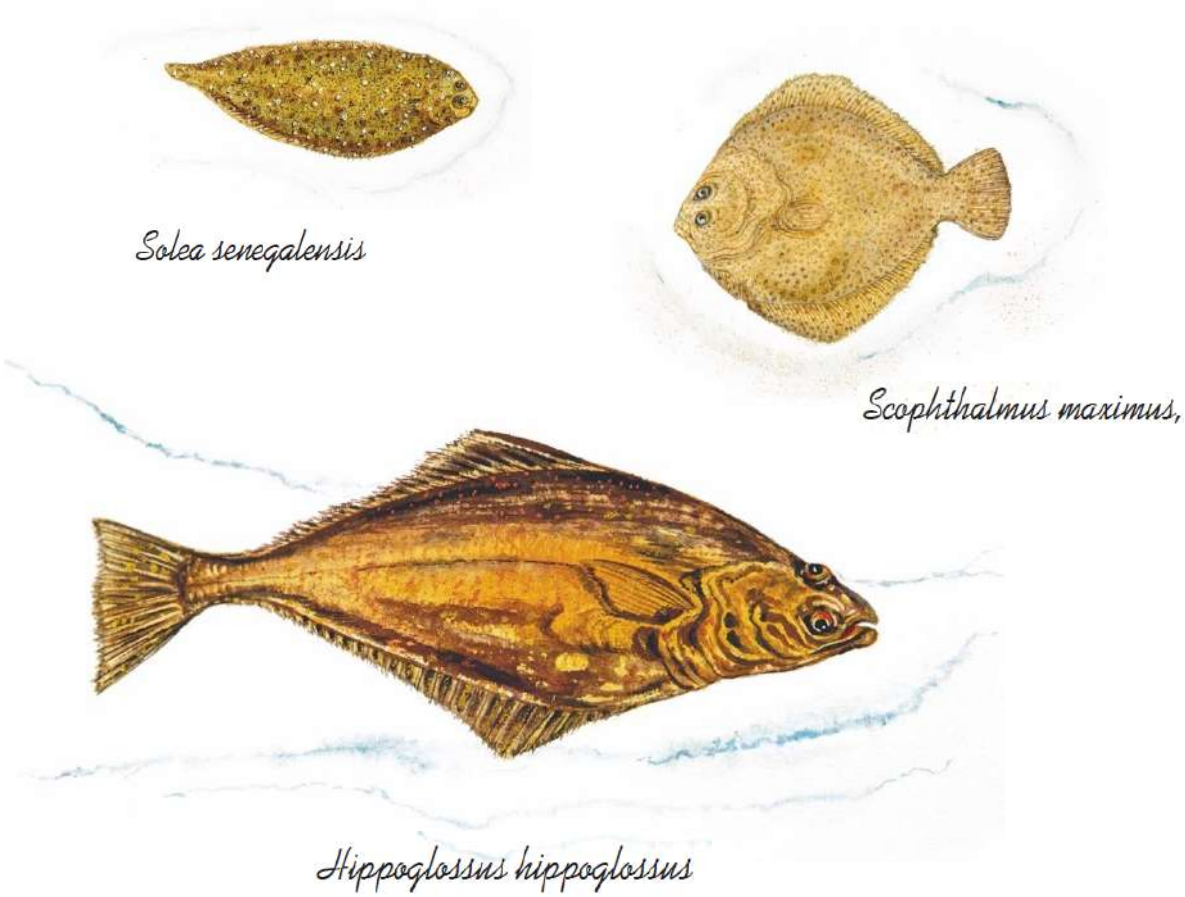
SS7. Gamete and egg quality



Invited State-of-the-Art Presentation 8

Do non-genetic inheritance factors blur mechanisms responsible for egg quality?

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INTRODUCTION

Egg quality is an important feature characterizing reproductive fitness in wild and cultured fishes. The growing pile of literature brings us closer to unraveling the actual role the maternally-derived molecules are playing. Here we discuss that egg-derived molecules are complex mixture of phenotypic determinants hindering understanding mechanism conditioning egg quality.

MOLECULAR PROFILING OF THE EGGS IN FISHES

Over the last two decades increasing attention in molecular profiling of pre-ovulatory oocytes and eggs has been observed. Most of the studies concentrated on finding egg quality markers, understanding molecular kinetics during oogenesis and processes determining egg quality. These pointed out involvement of several important genes (such as *npm2*) in the process of embryogenesis and allowed to identify, through the CRISPR/Cas9 knock-out technology, new actors conditioning gonadal development and early embryogenesis. However, the results were often hard to be confirmed in other species and even in other populations of the same species. This sheds light on huge inter- and even intraspecies variability when comes to molecular profile of the eggs, though causes and effects remains vague. This stems from the fact that molecular cargo is responsible not only for developmental competence, but is also phenotypic determinant of the progeny.

NON-GENETIC INHERITANCE

Non-genetic inheritance (NGI) includes diverse heritable factors affecting gene expression in progeny.

From this perspective, NGI is crucial mechanism in “communication” between female and progeny in fishes. Recent studies provide evidence that maternally inherited gene transcripts are playing important role not only during the early embryonic development (util maternal-to-zygotic transition), but may also shape the final phenotype of the progeny. This suggest variety of potential applications of “transgenerational programing” (TP), which has already been reported to be feasible through nutrition. The most common hypothesis is that TP is possible via epigenetic modifications. However, recent studies suggest that also other molecules (i.e. mRNA and proteins) may constitute important NGI factors suggesting TP to be more complex and flexible process. This indicates, that female’s experience may considerably modify overall molecular profile what, in consequence, may blur molecular mechanisms conditioning egg quality in fishes.

CONCLUSIONS

Considering NGI-related factors are important determinants of molecular cargo in fishes, it may be suggested that understanding the mechanisms responsible for developmental competence in eggs will require understanding the NGI factors first. Possibly, by “understanding the noise” coming from typical NGI-caused molecular variations we will be able to focus on the factors having direct effect on egg quality (i.e. key features of embryonic development). However, it should be emphasized that “removing the noise” may become highly exciting, but also very challenging scientific journey.

This study was funded by National Science Center, Poland (UMO-2020/38/E/NZ9/00394).

Oral Presentation 48

Exploring the occurrence of DNA-fragmentation in sperm of different Swedish Arctic charr (*Salvelinus alpinus*) broodstocks and its impact on offspring viability

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INTRODUCTION

Arctic charr often perform poorly during reproduction in an aquaculture environment. Previous studies on Arctic charr broodstock in Sweden have shown that i) the majority of non-viable eggs are lost due to embryonic mortality rather than failed fertilization, ii) part of this mortality can be connected to paternal factors, and iii) high levels of DNA-fragmentation can be found in sperm from Arctic charr sires. Hence, DNA-fragmentation is likely to be one of the limiting factors for offspring viability following artificial reproduction of this species. However, no direct connection between the two parameters have been found under routine hatchery conditions, thus far. The aim of the study was to validate previous results showing high fragmentation levels, map its extent throughout the industry in Sweden, and test for connections between DNA fragmentation levels in semen samples and subsequent embryo viability.

METHODS

DNA-fragmentation levels were measured using the SCD (Sperm Chromatin Dispersion) method (Halomax HT-TT40, Halotech DNA) in 93 semen samples collected from the five major Arctic charr hatcheries in Sweden. Sperm concentrations and sperm cell viabilities were also included in the mapping, measured using a NucleoCounter SP-100 (Chemometec). Nine of the semen samples from one location were chosen at random and used to fertilize eggs of one female. Subsequent embryo viability was assessed by measuring survival to the eyed stage and hatching.

RESULTS & DISCUSSION

Overall, the median value of sperm concentration was 3.6 x 10^9 cells/ml (range 0.3-11.5 x 10^9), cell viability was 90.3% (67.9-95.7), and DNA fragmentation (proportion of cells with severe fragmentation) was 31.5% (5.0-49.5%) among the studied Arctic charr sires. Significant differences in sperm quality (sperm concentration, cell viability, and DNA fragmentation) were found between the five different hatcheries (Kruskal-Wallis $p<0.05$). Weak positive correlations were found between sperm concentration and both sperm cell viability (Spearman $\rho=0.24$, $p<0.05$) and DNA integrity (cells with intact DNA; Spearman $\rho=0.31$, $p<0.05$), respectively. No correlation was found between sperm cell viability and DNA fragmentation level (Spearman $\rho=-0.001$, $p>0.05$).

Results from the fertilization trial revealed a strong positive correlation between DNA fragmentation levels in sperm from individual sires and subsequent abortion rates before the eyed stage (Spearman $\rho=0.68$, $p<0.05$), and a strong negative correlation with abortion rates from eyeing to hatching (Spearman $\rho=-0.70$, $p<0.05$). Still, hatching rates showed moderate correlation with sperm DNA integrity, although not statistically significant (Spearman $\rho=0.56$, $p\approx0.12$). Our interpretation is that eggs fertilized with sperm cells of higher fragmentation levels are terminated at an earlier development stage, while the overall hatching success is, to large extent, determined by maternal factors. Yet, sperm DNA fragmentation levels in individual sires are likely to play a significant role in explaining some of the variation in egg survival seen in Arctic charr hatcheries.

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Oral Presentation 49 (student)

Abnormal development after gastrulation: a novel egg quality parameter for Atlantic halibut (*Hippoglossus hippoglossus*) in aquaculture

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INTRODUCTION

Aquaculture of Atlantic halibut peaked in the early 2000s, but production has not kept up with expectations. Today, Norway is the only country producing significant amounts of halibut in aquaculture. One of the largest obstacles in production is the production of good quality eggs and juveniles. Several egg quality markers have been used for Atlantic halibut, but thus far they have not been effective in predicting the quality of an egg group in a large-scale commercial hatchery. We have followed a series of egg batches in a commercial hatchery over several spawning seasons and evaluated the egg quality markers in relation to the embryonic development and survival up to hatching.

METHODS

Seventeen egg batches were stripped and fertilized from broodstock over three spawning periods, and fertilization rates, 8 cell stage symmetry, survival until hatching and hatching rates were registered. The development of the egg groups was followed by visual inspection at 10 different stages until hatching. Several egg groups were incubated at small scale (800 ml flasks) to obtain hatching rates. The percentage of normally developing embryos was recorded at 8 cell stage and 50 °d post fertilization (°dpf) and correlated to hatching success and survival until hatching.

RESULTS & DISCUSSION

Fertilization success was variable (65 ± 17%) and did not correlate with hatching success. Both survival up to hatching (48 ± 23%) and hatching success (61 ± 25%) were highly variable and similar to values reported previously by several authors. The often-used early blastula symmetry at 8 cell stage did not correlate with hatching success or survival up to hatching. The percentage of normally developing embryos at 50 °dpf was significantly and positively correlated to both hatching success and survival until hatching. The first part of Atlantic halibut development is characterized by a series of meroblastic cleavages, followed by gastrulation, the onset of body axis formation and tissue differentiation. It appeared that eggs which showed severe deviations from normal at 8 cell stage would not undergo gastrulation. Malformations during gastrulation were visible approximately at 50% epiboly (ca. 30 °dpf), when absence of the embryonic shield became distinct. However, when gastrulation is complete (at 50 °dpf), deformities were more clearly pronounced and were easier to identify. We observed a range of deformities, such as lack of eye predecessors and reduced head size, headless embryos with thin and/or deformed vertebral axis, lack of lateral somite symmetry, and embryo undergoes gastrulation without further development of body axis or tissue differentiation. Therefore, we suggest to use the percentage of abnormal development after completion of gastrulation as a reliable egg quality marker in commercial Atlantic halibut aquaculture. Together with fertilization success this will help producers to allocate limited recourses to good quality egg batches.

Oral Presentation 50

A multi-omics approach to studying egg quality in flow-through and RAS-conditioned Tasmanian Atlantic salmon broodstock

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INTRODUCTION

Broodstock rearing conditions have a profound impact on the molecular cargo of eggs, which has downstream implications for the survival and fitness of offspring. While progress has been made regarding the molecular basis for altered offspring development and performance, multi-omics studies characterising the dynamics of fatty acid (FA), protein, and mRNA throughout development and/or at ovulation, for fish reared in different environments, are lacking. Tasmanian Atlantic salmon offers a unique model for studying the impacts of rearing condition on egg content and subsequent quality in a commercial setting, given that some hatcheries concurrently use flow-through (FT) and recirculating aquaculture systems (RAS) to condition broodstock.

METHODS

A single population of female Atlantic salmon were split into RAS (controlled) and FT (ambient) systems and were subsequently sampled to characterise: 1) the transcriptome, proteome, and FA profile of unfertilised eggs at stripping, 2) the FA profile of oocytes and muscle tissue (Norwegian quality cut, NQC) throughout reproductive development, and 3) traditional egg quality and embryo survival metrics.

RESULTS & DISCUSSION

Conditions in FT were warmer during vitellogenesis in summer, cooler during late vitellogenesis, and warmer during ovulation. Broodstock in the FT system were generally smaller, had a stable (as opposed to declining) condition factor, higher GSI, lower total fecundity, and larger oocyte diameter. Despite some apparently more favorable gross broodstock metrics, embryos originating from FT broodstock had a lower incidence of neural streak development and a lower proportion reached the eyed stage.

The temporal profiles of most FAs (with some exceptions) in NQC and oocytes were relatively stable over time, until significant changes were observed at stripping relative to the previous time point. At stripping, the larger eggs from FT broodstock had a higher abundance of FAs (mg/egg), especially monounsaturated FAs which were more abundant in both absolute amount (mg/egg) and proportion (%) compared to eggs from RAS. Eggs from RAS broodstock had a significantly higher proportion of essential FAs, e.g. eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA), and other polyunsaturated FAs. Eggs from FT broodstock were enriched for proteins associated with energy metabolism, while RAS eggs were enriched for proteins involved in proteolysis and lipid transport. Transcriptomic analysis of eggs revealed differential accumulation of mRNAs involved in metabolism, immune function, lipid usage, brain and nervous tissue development, blood vessel development, and stress response. The collective dataset provides a good basis for understanding the impaired development of embryos from FT broodstock, though more research is required to understand the commercial performance implications for the offspring produced, including their resilience in challenging grow-out environments.

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Oral Presentation 51

Post-ovulatory oocyte aging leads to a significant PGC decline, which affects sexual differentiation

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INTRODUCTION

Post-ovulatory oocyte aging causes lower fertilization and survival rate, and various offspring abnormalities during artificial reproduction in fish. The problems associated with oocyte aging are attributed primarily to oxidative stress. One of the most sensitive embryonic cells to oxidative stress are primordial germ cells (PGCs). Yolk streams in teleost fish embryos likely mediate the transfer of some important PGC determinants from the yolk to the blastomeres, which may also be affected by aging. In some fish species, including zebrafish, sexual differentiation is dependent on the number of PGCs. In this study, we evaluated the number of PGCs in embryos from slightly aged oocytes, tried to find the reasons for PGCs decline, and the effect on sex differentiation.

METHODS

We used vas::gfp transgenic zebrafish for visualization of PGCs. The eggs from each female separately were split roughly into two halves. The first half was fertilized with pooled sperm immediately, and the second half was fertilized 2 hours post striping. Fertilization and survival rate and the number of PGCs on the second day were evaluated. For confirmation, the embryos were subjected to qPCR for PGC specific vasa gene. The fish were further reared to adulthood to assess lifelong impact of oocyte aging on sex differentiation and possible transgenerational effects. A portion of the eggs was collected before fertilization for reactive oxygen species (ROS) assessment. To trace yolk stream dynamics influenced by oocyte aging, a portion of fertilized eggs was injected with rhodamine-dextran into the vegetal pole. Yolk stream function evaluated as distribution of dye in the embryo was assessed at the 1K-cell stage. Also transfer of PGC specific dnd1 mRNA was assessed by qPCR in yolk and blastodisc at the 1K-cell stage. The same experiments were conducted on common carp (other representative of meroblastic cleavage) and sterlet sturgeon (representative of holoblastic cleavage).

RESULTS & DISCUSSION

The embryos from fresh eggs compared to 2 h aged eggs had higher fertilization (70.8±5.4 vs 57.8±12.6%) and hatching rate (55.6±11.0 vs 34.4±17.6%). Number of PGCs significantly declined from 27.6±6.9 to 12.9±7.2 PGCs, which was confirmed by relative decrease of vasa transcript abundance from 1 to 0.6. Occasionally, we have observed an embryo completely devoid of PGCs. The female/male ratio was significantly shifted from 49.0% to 35.1%, which is consistent with the previously reported effect of PGC number on sexual differentiation. The disturbed function of yolk streams was confirmed, when rhodamine-dextran injected to vegetal pole migrated differently into blastodisc in embryos from fresh and aged eggs (69.4±21.3% vs 42.8±13.6%), and dnd1 transcript migrated 70% less. The concentration of ROS, which probably disturbed the function of yolk streams and PGC migration was increased by 37.7% in aged embryo. In this study, we showed the impact of oocyte aging on PGC number and sex differentiation in fish. The putative reason for reduced PGC numbers is increased ROS concentration and impaired yolk stream functionality. Similar results were observed in carp and sturgeon, however in sturgeon the decline in PGC number was not so dramatic, which was probably due to holoblastic cleavage without presence of yolk streams.

Oral Presentation 52 (student)

Supplemented melatonin did not confer extra protection to Senegalese sole spermatozoa during cryopreservation

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INTRODUCTION

Senegalese sole (*Solea senegalensis*) is a species mainly cultured in the Iberic peninsula, however, F1 males do not spawn naturally and present lower sperm quality and quantity, being cryopreservation and artificial fertilization a common practice to solve this problem. Melatonin is not only a clock-hormone that regulates circadian and seasonal processes, like reproduction but also a powerful antioxidant that our group previously demonstrated to be present in fish seminal plasma. This study explored the protective effect of melatonin against the cryodamage inflicted on spermatozoa during the freezing and thawing processes.

METHODS

In this study, a broodstock of F1 males established at Ramalhete station (University of Algarve, Faro, Portugal), was used. Fish were distributed within 6 fiberglass tanks, each with 18 animals, kept on a semi-closed system with a 2:1 sex ratio (male:female). Males were anesthetized with 300 ppm phenoxyethanol and sampled for sperm collection during the reproductive season. First, a melatonin toxicity test was performed (n=11) using different concentrations (0.01, 0.1, 1, and 10 mM) and exposure times (3, 5, 15 and 30 min), after which different sperm motility parameters were registered (TM, PM, VCL, VSL, LIN) using CASA system. The best conditions from the toxicity test (0.1 and 10 mM) were applied in a cryopreservation assay (n=11) and, to evaluate the protective effect of supplemented melatonin, a set of quality analyses were performed [motility, viability and Reactive Oxygen Species (ROS) (flow cytometry), lipid peroxidation (MDA), and DNA fragmentation (Comet assay)]. Melatonin-FITC was used to visualize if melatonin enters fish spermatozoa using confocal microscopy. Statistical analysis was performed on SPSS software, and significant differences were considered when $p<0.05$.

RESULTS & DISCUSSION

The melatonin toxicity test revealed that only concentration influenced sperm motility. Afterwards, the best treatments (0.1 and 10 mM) were used in the cryopreservation assay and showed different results in the post-thaw quality analysis: in motility, no significant differences were found, although 10 mM melatonin was the treatment that had the lower percentage of viable cells both in viability and ROS analysis. No significant differences were found in MDA content and DNA fragmentation. Overall, the obtained results suggest that supplemented melatonin did not confer extra protection to spermatozoa during cryopreservation, as hypothesized. Even though, at the confocal microscopy, it was demonstrated that melatonin enters the cell by passive diffusion. In addition, preliminary results from a complementary study, already indicated that melatonin receptors (*mel1*, *mel2*, *mel1c*) do not express in the spermatozoa, explaining the inefficacious of melatonin as an antioxidant during cryopreservation.

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Oral Presentation 53

Feeding during the resting period and oogenesis is critical for successful reproduction in Eurasian perch (*Perca fluviaitilis*)

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INTRODUCTION

Nutrient accumulation during egg formation is a major key step to ensure reproduction. They can be accumulated before oogenesis in adipose tissues to be later transferred to ovaries (capital breeders). Or, yolk reserves depend upon the feeding regime during oogenesis (income breeders). Between these two categories, there exists potential mixed breeders that depend in both adipose reserve and food during oogenesis. Mixed breeder species may have a greater ability to adapt, to guarantee their reproduction in captive conditions. Percid fishes considered as capital breeders. However, several studies showed that food ingested during oogenesis may lead to low egg quality. This study aimed at understanding the nutritional needs of Eurasian perch (*Perca fluviatilis*) females to obtain a good quality eggs.

METHODS

Two independent experiments were performed. Firstly, 3 groups of fish were fed during the oogenesis with various triacylglycerol/phospholipid ratios (from 16 to 2.5). Secondly, fish with various adipose reserve quantities due to either *ad libitum* (NOR) or restricted feeding regimes 2 months before the oogenesis were compared. Restricted females were fed again *ad libitum* either from the reproduction cycle induction (RI) or later during the cortical alveoli period (RV). Oogenesis progression and reproduction success parameters (embryonic survival and deformities rates) were studied.

RESULTS AND DISCUSSION

In the first experiment, neutral and polar lipids in addition to a large number of fatty acids present specific incorporation kinetics in eggs. However, lipid contents in ovaries didn't differ with feeding regime. In the second experiment, lipid reserves were lower in RI (HSI=0.7%±0.2 and VSI=4.7%±1.7) and RV (HSI=0.8%±0.2 and VSI=6.0%±1.0) females compared to NOR (HSI=1.6%±0.2 and VSI=11.4%±1.8) before the onset of reproduction. Gonad morphologies and GSI were not different between the 3 groups. Fertilization was not different, but after 24 h, RV spawning presented lower survival rates than the other groups (68.5%±1.0; 70.4%±0.4 and 49.1%±1.6 for NOR, RI and RV, respectively). This difference was even more drastic for hatching (55.9%±1.0, 47.4%±0.3 and 29.7%±0.2 for NOR, RI and RV groups, respectively). However, the deformities rates were significantly higher in RI (24.1%±0.1) and RV (64.9%±0.04) in comparison to NOR spawn (8.9%±0.01).

CONCLUSION

Our data indicate that even if females ate *ad libitum* from the oogenesis onset, they were not able to fully compensate previous adipose tissue losses. Nonetheless, a partial compensation of nutritional defects is possible and the food ingested during early oogenesis probably participate to the yolk egg formation. The present work shows that the Eurasian perch may be a mixed breeder. In addition, we showed that various lipid families may use distinct pathways to reach the developing oocytes. Consequently, females may probably have specific nutritional needs depending on the oogenesis period including the resting period between two oogenesis cycles to constitute yolk and guarantee reproduction success.

Oral Presentation 54

Molecular mechanisms of aging in fish oocytes

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INTRODUCTION

The time between ovulation and fertilization, known as post-ovulatory oocyte aging, can lead to changes in egg maternally incorporated components, thereby dropping egg quality. The underlying molecular mechanisms of post-ovulatory oocyte aging and its subsequent deleterious effects on the embryos and the later life of the offspring are still insufficiently understood. The involvement of oxidative stress, epigenetic regulators, and apoptosis pathways have been/are investigated in our research group as the probable mechanisms behind the progress of oocyte aging. Discovering the basic knowledge about the mechanisms involved in the fish oocyte aging process might also benefit other vertebrates.

METHODS

The oocyte aging process, either *in vivo* or *in vitro*, was evaluated in fifteen fish species up to the complete loss of egg fertilizing ability. We examined the role of oxidative stress in the progress of oocyte aging in five fish species. The oxidation products, antioxidant enzyme activities, and the mRNA abundance of selected transcripts associated with oxidative injury and stress response were investigated. In addition, we studied whether post-ovulatory oocyte aging leads to modifications in histone acetylation. Genome-wide DNA (hydroxy) methylation was investigated in fresh and different aged oocytes and the emerging embryos. Furthermore, whole genome bisulfite sequencing was applied to detect possible differentially methylated regions.

RESULTS & DISCUSSION

The time during which oocytes retain their fertilizing ability after ovulation depends on the fish species and the storage temperature ranging from a few minutes to a few weeks. With elapsing time postovulation, the amount of oxidation products and the activity of the antioxidant enzymes showed no significant difference. In addition, no significant changes in the relative mRNA levels of oxidative stress-related transcripts were observed. Oxidative stress is, therefore, unlikely to be the initiator but most likely appears in the advanced stages of the oocyte aging process. The activity of histone acetyltransferase increased during oocyte aging. The assessment of histone acetylation dynamics revealed significant modifications in specific histones during the progress of oocyte aging. The genomewide DNA methylation in embryos was not altered during post-ovulatory aging. However, the epigenetic modifications of global DNA hydroxymethylation significantly decreased in embryos produced from the aged oocyte. Currently, we are studying the apoptotic signaling pathways and their relation to the progress of fish oocyte aging. A detailed study of the association between oocyte aging and the incidence of anomalies in the progeny at the molecular level is of our interest for future works.

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Oral Presentation 55 (student)

Evaluation of antioxidants on sperm quality in Senegalese sole

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INTRODUCTION

Antioxidants are key modulator of sperm quality by protecting cells from the ROS excess in fish. They are routinely used to preserve sperm characteristics although the effects are species-specific and dose dependent. In this study, we evaluate the effect of six antioxidants on chilled and cryopreserved sperm using a CASA system in the flatfish Senegalese sole. Results are useful to improve the artificial fertilization protocols and support genetic breeding programs.

METHODS

Two trials were carried out to evaluate the effects of antioxidants in Mounib solution and Marine Freeze® (MF). Firstly, pure sperm from six soles (CCMAR, Faro, Portugal) with total motilities (TM) higher than 50% were selected and chilled separately in Mounib solution. Sperms were added different doses of vitamin C (0.02, 0.6 mM), Trolox (0.06, 0.12 mM), glucose and trehalose (100, 200, 300 mM), ZnSe (0.1, 0.5 mM), cysteine (1, 5, 20 mM), glycine (15, 30, 130 mM) and taurine (1, 50, 100 mM) and incubated for two minutes. A non-supplemented sample in Mounib solution was kept as control. Sperm quality was evaluated in an ISAS CASA system from 15s to 60s. In a second trial, sperm samples from sixteen F1 soles (CUPIMAR San Fernando, Spain) were collected directly in MF selecting 12 samples with TM higher than 40%. As samples were stabilized in MF, a sample pool was created and added different doses vitamin C (0.2, 0.5, 0.8 mM), Trolox (0.5, 1, 5 mM), trehalose (100, 200, 300 mM), ZnSe (0.1, 0.5, 1 mM) and cysteamine (2, 7.5, 15 mM). Three samples of each treatment were chilled and incubated for two hours. Three samples were cryopreserved after incubating for two minutes using nitrogen vapors in straws. A non-added MF sample was used as control in both conditions. Sperm quality was evaluated in a CASA AiStation at 15s. Data were processed using repeated-measures ANOVA.

RESULTS & DISCUSSION

Mounib supplemented with vitamin C (0.02 and 0.6 mM) improved 45 % TM at 15s with respect to non-supplemented samples. This positive effect was still evident at 60s using a longitudinal approach. In contrast, high concentrations of glucose and aminoacids, cysteine and glycine, significantly decreased sperm motility up to 55-73%. VCL remained unchanged except for three doses of glycine (up to 40% with respect to non-supplemented samples). In chilled MF-stabilized sperms, vitamin C also had a positive dose-dependent effect on TM (up to 19%) with respect to non-supplemented samples. In contrast, high doses of cysteamine significantly decreased ($p < 0.05$) velocity parameters. Trehalose, Trolox and ZnSe did not show statistically significant differences although a slightly increased TM and velocities (VCL and VSL). No changes in TM and velocities were observed in post-thawed samples with respect to control group ($p>0.05$).

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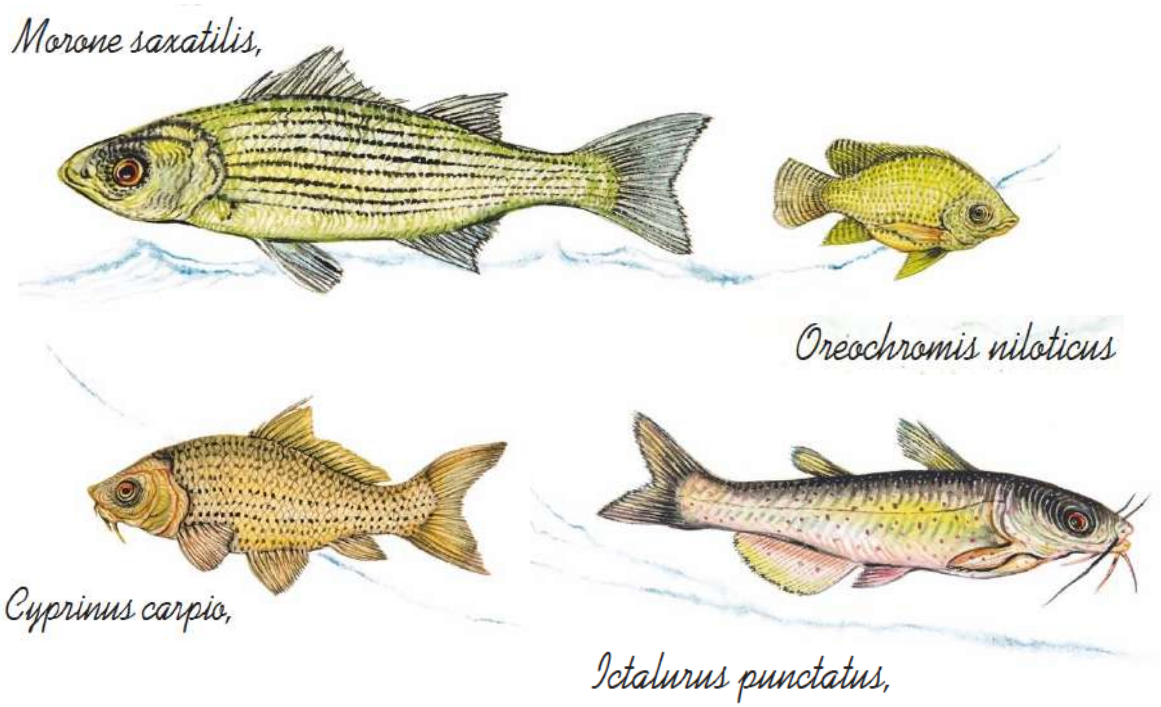
SS8. Behaviour and pheromones



Invited State-of-the-Art presentation 9

Structure, function, and potential application of sea lamprey reproductive pheromones

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Chemical cues and pheromones guide decisions in fish. We study how these olfactory cues regulate development and reproductive behaviors of sea lamprey (Petromyzon marinus), a jawless vertebrate. In this anadromous species, migratory adults rely on larval odorants to search for suitable spawning streams. After arriving on spawning grounds, males release pheromones that guide female mate search. This set of behaviors provides useful models for examining how olfactory cues enable long-distance communication among individuals of the same species. Our research is also driven by interest in means to misguide or disrupt olfactory communication in sea lamprey, a destructive invader of the Laurentian Great Lakes of North America. In this presentation, I will briefly review the known olfactory cues used by sea lamprey during migration and reproduction, and then describe our effort to define the structures and functions of male pheromones through integration of approaches from biology and chemistry. Sexually mature males have evolved efficient mechanisms that produce, transport, and release mixtures of diverse pheromone components. These compounds are potent olfactory stimuli recognized by specific receptors of the odorant receptor neurons in conspecifics. Upon detecting these odorants, adult females show life-stage specific behavioral responses that lead them to nests built by the signaling males and spawn. Structural analogs to main pheromone components alter or abate these female behaviors. Our results establish that the sea lamprey is a broadly useful model with which to study fish behavior and pheromone communication, and to develop strategies for invasive species management.

Oral Presentation 56 (student)

Using surrogate fish for eradicating invasive fish: Can surrogate triploid rainbow trout mate with their wild-type counterparts and produce lethal hybrids?

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INTRODUCTION

To date, the most common strategy to remove alien fish is capturing them using fishing gear. However, even if capturing can reduce the population to a low density, it is challenging to eradicate the entire population. Therefore, developing methods for eradicating low-density populations is key to controlling alien fish populations. To achieve this goal, we attempted to develop a novel method to inhibit the reproduction of alien fish by releasing surrogate males that produce sperm of different species. In this study, rainbow trout, a globally introduced and representative alien fish, was used as a model. By introducing male surrogate rainbow trout that produces brown trout sperm and releasing large numbers of the surrogates into rivers inhabited by rainbow trout, female rainbow trout are expected to mate with surrogate males in a stochastic manner and produce lethal fertilized eggs. To test the feasibility of this strategy, as a first step, we produced surrogate triploid male rainbow trout and tested whether they could mate with rainbow trout females in the river.

METHODS

The germ cells isolated from the brown trout were transplanted into the triploid rainbow trout larvae. Moreover, it is known that the rainbow and brown trout hybrids are lethal. The resulting surrogate recipients were reared until two years of age in running water (10 cm/s) to acclimate them to the river conditions. Furthermore, artificial redds were created in a 3.2 m wide river and optimized for rainbow trout spawning (water depth 20 cm, bottom velocity 10–20 cm/s, surface velocity 20–35 cm/s, and gravel size 1–10 cm). The spermiated surrogate males were released into the river together with freshly ovulated wild-type females. Their behavior was recorded using an underwater camera fixed around the redds for detailed observation of their mating behavior. When spawning was confirmed, the fertilized eggs were collected from the redds and used for species identification by polymerase chain reaction-amplified fragment length polymorphism.

RESULTS AND DISCUSSION

Two of the six released surrogate males mated with five wild-type females, and nine ovipositions were observed. Approximately 100–700 eggs were retrieved from the spawning redds. Their fertilization rates ranged from 66.7% to 91.7%. When they reached the hatching stage, they were subjected to DNA extraction for species identification. As a result, all fertilized eggs were identified as hybrids between rainbow and brown trout. All hatchlings died before the swimming-up stage. The results showed that triploid surrogate males possess the potency to mate naturally with females of the same species as surrogates and produce lethal hybrid offspring in the river. Future studies will focus on optimizing surrogate males' body size and age and the number of surrogates to be released for more efficient mating and utilizing this technology for controlling the alien fish population.

Oral Presentation 57

Coevolution of the oxytocin signaling pathway and reproductive behavior in African cichlids

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INTRODUCTION

The explosive speciation of cichlid fish in Lake Tanganyika generated an array of species that differ in their reproductive behavior, including a range of mating systems (monogamy, polygyny, polyandry, polygynandry) and parental care types (female or biparental substrate or mouthbrooding). Thus, Tanganyika cichlids offer a unique opportunity to study the evolution of mechanisms underlying reproductive behavior. On the other hand, the nonapeptides of the vasotocin/oxytocin family have been implicated in the regulation of social and reproductive behaviors across vertebrates. Therefore, we have hypothesized that the rapid diversification of reproductive behaviors in cichlids may be correlated with the molecular evolution of the nonapeptide signaling pathways. Here we have studied the correlated evolution between the oxytocin signaling pathway and reproductive behavior in Tanganyika cichlids.

METHODS

Based on whole genome sequencing of Tanganyika cichlids (N=243 species), we have mapped the occurrence of different mating and parental care types on a complete phylogeny of the cichlid radiation in Lake Tanganyika. In parallel, we have identified the genes involved in oxytocin signaling using the KEGG database (N|=154 genes), and inferred their orthologs in the cichlid genomes. Then, we studied their molecular evolution in the phylogenetic tree of Tanganyika cichlids and estimated the occurrence of different regimes of selection (i.e. neutral, purifying or positive) based on the the d_N/d_S ratio which assesses the balance between synonymous and non-synonymous mutations. Finally, we have used Pagel's MCMC method to detect correlated evolution of discrete binary traits, to assess the precedency and contingency between identified mutations under positive selection and the emergence of reproductive types (e.g. monogamy) in the phylogeny of Tanganyika cichlids.

RESULTS & DISCUSSION

Most genes of the oxytocin signaling pathway are under strong negative selection in Tanganyika cichlids. Nevertheless, we have identified a set of highly variable genes, with positively selected sites. Preliminary results suggest the occurrence of correlated evolution between some of these genes and pair-bonding, such that genetic variants precede and are contingent with gain and presence of pair bonding, respectively. Future work aims to validate these findings with more in depth bioinformatic analysis and to functionally validate it by developing specific KO mutants in zebrafish to assess their impact in mating behavior.

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Oral Presentation 58

Olfactory sensitivity to conspecific odors released by striped bass (*Morone saxatilis*) during reproduction

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INTRODUCTION

Striped bass (*Morone saxatilis*) is an important aquaculture species in US and a popular species for recreative fishing. Natural reproduction of striped bass in intensive farming set ups has been achieved without any hormonal induction. However, there is still a high percentage of mortality in the first steps of larvae development. Therefore, there is a need to understand additional factors that allows successful reproduction and production of healthy spawn. Sex pheromones are chemical signals used to coordinate reproduction between vertebrates and are often critical for successful spawn and larvae development in fish. To date, little is known about chemical communication in striped bass. Our aim is to identify odors produced during striped bass reproduction that can pinpoint the use of putative sex pheromones in this species.

METHODS

We collected urine, bile fluid, and rearing tank water samples of brood male and female striped bass during the reproductive event in April. Water samples were concentrated by solid-phase extraction. Olfactory sensitivity to each odorant candidate was assessed by electro-olfactogram (EOG) analysis in juvenile striped bass. In each experiment, juvenile striped bass was anesthetized in water containing 100 mg l^{-1} MS222 and immobilized by intramuscular injection of gallamine triethiodide (3 mg kg−1 in 0.9% NaCl) before recording the EOG response. The amplitude of olfactory response to the diluted urine, bile, and water samples was recorded as direct current potential by placing the recording electrode in the olfactory lamellae. The EOG amplitude (mV) was blank-subtracted and normalized using the responses to 10^{-3} mol l^{-1} L-serine.

RESULTS & DISCUSSION

EOG responses from striped bass showed high sensitivity to urine and bile samples. The EOG responses were larger for broodfish females than the brood males. Moreover, brood female urine had a higher dilution threshold (1: 10^5) compared to the brood males (1: 10^4). Preliminary data also showed that the most potent odors originated from females in the latest stages of ovary maturation. There was no difference in amplitude and sensitivity for bile fluid from either male or female, but responses where higher compared to samples of bile from other fish (catfish and trout). Water samples elicited parallel urine pattern (i.e., water extract from females elicit larger responses than water from males). The high sensitivity and specificity of striped bass urine odors points to the use of urinary chemical signals as putative sex pheromones bass during the reproductive cycle.

This work was supported by North Carolina Sea Grant 2023.

Oral Presentation 59

Two to tango: the importance of reproductive and hormonal variables in intrasexual aggression in (*Cichlasoma dimerus*)

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INTRODUCTION

Individuals living in social hierarchies engage in agonistic encounters throughout aggressive behavior, which induces experience-dependent shifts in social status. Aggression has been historically linked to males and androgen levels while female aggression is still widely understudied, even if females from different species also display aggressive behavior. While cichlid fish constitute ideal models to assess the physiological basis of aggression, most studies refer to African species. The aim of the present work is to disentangle how sex differences in social plasticity can be explained by sex steroid hormone levels, gonadal state and/or morphometric characteristics by using a Neotropical cichlid species.

METHODS

Intrasexual dyadic encounters were performed between male or female *Cichlasoma dimerus* of a wild stock from North Argentina. Aggressive and submissive displays from the winner and loser fish were quantified during all phases of one-hour contest: pre-conflict, conflict and post resolution. Morphometric variables, gonadal histology and hormonal levels were determined in both winners and losers.

RESULTS & DISCUSSION

This integral multivariate analysis suggests that the reproductive and hormonal variables analyzed explain the behavioral variation among winner and loser males and females, and that there are significant differences between sexes and contest outcome when individual morphometric variables are excluded from the analysis. Regarding gonadal histology in females, since both opponents were prespawning fish they show majority of mature follicles and vitellogenic oocytes, with a very low incidence of previtellogenic oocytes. Interestingly, there are no sex differences in aggressive and submissive behaviors, and clustering into winners and losers is mainly explained by specific behavioral displays, such as bites, chases, approaches, passive copings, and escapes. Correlation heatmaps show a positive correlation between estradiol with aggression and a negative correlation with submission, suggesting estrogens may have a dual role regulating agonistic behavior. Finally, these results suggest that size difference can help to understand aggression in females but not in males, and that assessment of the opponent´s body size is important to understand aggression also before the initiation of the contest in both sexes. Overall, this study constitutes an integral approach adding insights into the importance of reproductive and hormonal variables to understand social plasticity in males and females.

Oral Presentation 60 (student)

Possible role of faeces in chemical communication in the Mozambique tilapia (*Oreochromis mossambicus*)

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INTRODUCTION

In several species, including the Mozambique tilapia (*Oreochromis mossambicus*), accessibility to mates for reproduction depends on position of individuals in a hierarchy, which itself is modulated by different sensory inputs such as chemical cues. These chemicals cues or "pheromones" are released by an individual and received by the olfactory system of conspecifics in which they cause a variety of physiological and behavioral responses. Excretory products such as urine and faeces are potential vehicles for releasing pheromones. Recently, two pregnanetriol-3-glucuronates (P3Gs) have been identified in the urine of male tilapia that act as sex pheromones and prime ovulation in females. Here we hypothesize that, in a dominance hierarchy, female tilapia signal their ovulation status and attract conspecifics to mate through the use of faeces. During defecation, fecal odorants are released into the water where they can transfer particular information about the ovulation status. Therefore, the present study was designed to identify chemical signals (putative pheromones) in the faeces.

METHODS

After formation of male dominance hierarchies in family groups (two males, four females), faeces were taken from pre- and post-spawning females. Females were considered 'pre-ovulatory' on the day prior to their predicted ovulation and 'post-spawning' one day after they spawned. Individual fecal samples were extracted with C18 solid-phase cartridges and methanol eluates were fractionated by reverse-phase HPLC. Fractions were assessed in males for olfactory potency using the electro-olfactogram (EOG). The fraction(s) giving larger EOG responses were analyzed by liquid chromatography coupled to mass spectrometry (LC- MS).

RESULTS & DISCUSSION

Most olfactory sensitivity was contained in the C18 eluate, and three HPLC fractions (fraction 2, 14 and 15) from pre-ovulatory females evoked significantly larger amplitude EOG responses than those from post- ovulatory females. Also, the eluate fraction evoked significantly larger amplitude EOG response than that from post-ovulatory. LC-MS analysis identified amino acids (arginine, threonine, proline, valine, methionine, serine, asparagine, phenylalanine, tryptophan and leucine) in fraction 2 (2-4 min) from pre- ovulatory females, to all of which tilapia males had high olfactory sensitivity. Cholic acid and taurocholic acid were detected in fractions 14 and 15 in much higher concentration in pre-ovulatory than post-spawning females. Olfactory sensitivity to these bile acids was also high. During mouthbrooding, females do not eat which could explain the lower amount of bile acids in post-spawning female faeces. Before spawning and during the final ovulation, releasing bile acids through the faeces could signal the ovulation status to male conspecifics and mediate the mate-choice behaviors. This needs to be tested experimentally.

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Oral Presentation 61

Acute olfactory sensitivity of bighead and silver carp to 9 sex steroids strongly suggests that novel mixtures of 21-carbon steroids function as species-specific priming pheromones in bigheaded carps

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INTRODUCTION

The Order Cypriniformes (minnows) contains a dozen families including the Cyprinidae which contains the goldfish, *Carassius auratus*, as well as several related carps from Eurasia. These carps are all scramble spawners and have all evolved to use mixtures of 5 relatively common hormonal products including 21-carbon maturation inducing steroids (MIS) derivatives as potent sex pheromones to mediate their gonadal maturation and spawning synchrony. However, whether other families of fish in this order Cypriniformes might use similar suites of hormonal products as sex pheromones is not known. In this study, we asked whether bighead (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix)*, two other scramble spawners in the Family Xenocyprididae from Asia employ the same MIS-derived hormonal pheromones as the goldfish.

METHODS

Juvenile bighead and silver carp were obtained from hatcheries, raised in the laboratory at 18°C and fed planktonic food. The olfactory sensitivity of these fishes to several hundred common sex steroids and derivatives was then determined using electro-olfactogram (EOG) recording, a multi-unit electrophysiological technique that measures peripheral olfactory sensitivity. We tested responses to water control, 10^{-5} Molar (M) L-serine control (a food odor), and 21 mixtures of 10 to 14 sex steroids at a concentration of 10^{-9} M (N=223 steroids) thought to have the potential to function as sex pheromones. After testing all mixtures at least 6 times, those steroid mixtures that elicited notable and significant responses, were then broken up and their individual components tested on their own at least 6 times. Dose-response relationships were determined for the most stimulatory components, while the effects of androgen treatment were measured to determine if responses were sexually dimorphic.

RESULTS & DISCUSSION

The olfactory systems of bighead and silver carp were highly sensitive to 9 of the 223 steroids we tested, with small differences in sensitivity being noted between species. While bigheaded carp responded to 5 steroidal mixtures, silver carp responded to 7. Like goldfish, both Asian carps were extremely responsive to water-borne 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) as well as its glucuronidated and sulfated conjugates. Mixture experiments showed both Bigheaded carp species to detect these steroids in highly specific manners down to 10^{-12}M, also like goldfish. However, both species also detected cortisone sulfate with high sensitivity, a novel finding. In addition, the silver (but not bighead) carp was highly sensitive to 5α-pregnan-3α-17,20β-triol as well as its epimer, and 4-androstene-17-ol3-one. Goldfish are not known to detect any of the later 4 steroids, suggesting species and family level differences in pheromone function. Very likely, the MIS derivatives detected by the bighead and silver carp function as priming pheromones to synchronize reproduction in large flowing rivers. Other reduced sex steroids may impart species-specificity with the cortisone sulfate possibly functioning as a stress pheromone. Tests of these possibilities are now needed. (Funded by Legislative Citizen Commission on Minnesota's Natural Resources).

Oral Presentation 62 (student)

Reproductive behavior and parental contribution of meagre (*Argyrosomus regius*) in aquaculture conditions

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INTRODUCTION

The meagre (*Argyrosomus regius*) is an emerging species for the Mediterranean aquaculture industry and its reproductive dysfunctions in rearing conditions have been resolved using GnRHa-based spawning induction and spermiation enhancement protocols. However, to eventually be able to obtain fertilized eggs without the use of exogenous hormones, more information about the environmental requirement of the species and its behavior during reproduction is needed. The aim of this study was to describe the reproductive behavior and parental contribution of meagre in aquaculture conditions, after spawning induction with gonadotropin releasing hormone agonist (GnRHa).

METHODS

Two meagre broodstocks (Group 1 and 2, 2 females and 3 males, each) were utilized for two consecutive years (Year 1 and 2). At the expected spawning period, reproductive stage was evaluated, through ovarian biopsies and sperm quality evaluation, and fish were induced to spawn with GnRHa. Each breeder was externally tagged, and their behavior was monitored using underwater equipment. Egg production and quality was evaluated in terms of fecundity, fertilization success, 24-h embryo survival, hatching and 5-d larval survival. A sub-sample of the eggs from each spawn was collected for parental contribution analysis using a multiplex of 12 microsatellite loci and an exclusion-based method.

RESULTS & DISCUSSION

Spawning induction led to three consecutive daily spawns per broodstock per year, 2 days after GnRHa administration. No statistically significant differences were observed between years or groups (2-way ANOVA, $P>0.05$) in mean daily relative fecundity, hatching success and 5-d larval survival. However, fertilization success ($P=0.036$) and 24-h embryo survival ($P=0.016$) were higher in Year 2. The mean daily relative fecundity (n=2) was 159,743±23,315 eggs kg^{-1}, while mean hatching success was 98±1%, and 5-d larval survival was 96±1%. Mean fertilization success and 24-h embryo survival were 83±0 % and 93±0%, respectively for Year 1, and 92±4% and 100±0% for Year 2, respectively. Parental contribution analysis showed that 77±3% of the eggs were produced by the same female, while 77±9% of the eggs were fertilized by the same male in each group for both years. These spawning kinetics and productive results were similar to group and paired spawnings of meagre reported in earlier studies. The most aggressive male from Group 1 fertilized only 14±6%, while the larger one fertilized 68±9% of the eggs during the two years of the study. In Group 2, the most aggressive male fertilized 86±8 % of the eggs. The study demonstrated that hierarchies developed within meagre broodstocks, and even after exogenous GnRHa stimulation, spawning success was not equal among breeders, and a single individual of each sex dominated the produced progeny. It is not clear at this stage what factor contributes to dominance of the two sexes, but male size or aggressiveness did not correspond to enhanced parental contribution. Further analysis is underway to investigate other behavioral traits.

The project received funding from the EU Horizon 2020 Program (No 8626588, NewTechAqua).

Oral Presentation 63

Sound production in relation with breeding behaviour in meagre (*Argyrosomus regius*) in aquaculture conditions

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INTRODUCTION

Although sound production has been described so far in little more than 1100 fish species, recent studies demonstrate that this communication modality is shared by nearly two-thirds of actinopterygian species. Meagre (*Argyrosomus regius*) has a wide vocal repertoire and can emit sounds made of a single pulse up to more than 100 pulses. The calling rate and sound temporal features have been suggested as indicative of reproductive events. The aim of this study was to document sound production during spawning activities and to investigate how sounds relate with specific behaviors when fish are reared in aquaculture.

METHODS

Two broodstocks (Group 1 and 2, each with 2 females and 3 males) were used. At the expected spawning period, reproductive stage was evaluated through ovarian biopsies and sperm quality evaluation, and selected fish were injected with GnRHa. Each breeder was tagged externally, and their behavior was monitored using underwater cameras. Two HTI- 96-Min hydrophones were connected to the audio input of the camera. Using Adobe Premiere, audio files were extracted from the videos (May 5th to May 13th, 2022). All audio files were analyzed by audio and visual assessment (Raven Pro 64 1.4). Meagre sounds were manually labelled on the basis of the number of pulses as knocks, short, intermediate and long grunts (total of 243 h of recordings analyzed, 33,351 sounds selected). The occurrences in which a sound could be associated with distinct behaviors (*i.e*. trailing, darting and male-to-male agonistic behavior) or spawning, were annotated from the video observations. Sounds emitted during these events were measured for fine temporal features (number of pulses, pulse period and sound duration).

RESULTS & DISCUSSION

Fish spawned for three consecutive nights, beginning 2 days after GnRHa administration. The number of sounds produced increased significantly during spawning days, compared to the ones immediately before GnRHa treatment. Long grunts, in particular, were emitted only during spawning days, when knocks were emitted in long, dense series. Knocks were associated with all behaviors considered and were the only sound type associated with same-sex trailing and male-to-male agonistic interactions. Grunts, on the other hand, appeared associated with different-sex trailing during spawning. These results agree with previous studies that identified long grunts as potential indicators of spawning, as well as with studies conducted in the wild, which reported long choruses of knocks. Knocks and long grunts are, therefore, suggested as “carrier of information” during spawning in the meagre.

The project received funding from the EU Horizon 2020 Program (No 8626588, NewTechAqua).

SS9. Reproductive biotechnologies

Invited State-of-the-Art presentation 10

Improved methods for long-term culture of germ cells capable of differentiating into eggs and sperm when transplanted into recipients

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Our research group has developed the "surrogate broodstock technology," in which undifferentiated germ cells are transplanted into the recipients of different species or strains, allowing these recipients to produce eggs and sperm derived from the donor cells. Because the donor germ cells used for this transplantation can be cryopreserved in liquid nitrogen, this technique can be applied for long-term preservation of the genetic resources of elite strains used for aquaculture and endangered species. However, if the donor species is small in size or only small gonads are available, it may be difficult to create a recipient to produce sufficient quantities of eggs and sperm. In particular, when sampling germ cells from populations on the verge of extinction, the problem of inbreeding becomes apparent, and we have had actually encountered cases wherein only extremely small gonads were available. To overcome this obstacle, we have attempted increasing the number of germ cells by culturing them *in vitro* as the undifferentiated state.

In the early days of culture experiments, it was difficult to establish the survival of undifferentiated germ cells *in vitro*, and even when they did survive, their efficiency of migration to recipient gonads was low when transplanted into the recipients, probably due to their loss of germness and stemness. The key factors that solved these problems were the use of Sertoli cells, as feeder cells, and the addition of plasma derived from the same species to the culture medium. We performed our experiments on the rainbow trout, a species that retains only undifferentiated germ cells, the so-called type A spermatogonia, in its testes until approximately 1 year of age. Although the function of Sertoli cells changes according to the differentiation stage of adjacent germ cells, all the Sertoli cells of an immature rainbow trout are nursing type A spermatogonia. Therefore, we labeled these cells with *DsRed* transgene, isolated them by flow cytometry, and cultured them *in vitro* for amplification. When these cells were used as feeder cells, it was confirmed that type A spermatogonia proliferated exponentially for an approximately 2-month culture period. It is important to emphasize that these germ cells, when transplanted into recipients, migrated to their gonads, where they eventually differentiated into functional eggs and sperm. However, these germ cells also exhibited a gradual decrease in their ability to migrate to the recipient gonads (transplantability) with a further extended culture period. A gradual loss of stemness was also observed in mouse ES and iPS cells, which was considered as a common phenomenon during stem cell culture.

Therefore, to enable the culture of cells that can maintain their transplantability for an extended period of time, we used three inhibitors for intracellular signaling pathways that have been known to maintain the undifferentiated status of mouse ES cells. Specifically, we added three kinases, MAPK/ERK kinase, glycogen synthase kinase 3β, and TGF-β type I receptor to the culture medium and verified their effects. Consequently, we succeeded in improving the transplantability of the germ cells at 45 days after the start of culture by approximately threefold. Moreover, we purified germ cells with high transplantation ability immediately after the start of culture and those with reduced transplantation ability after long-term culture using a flow cytometer and subjected them to RNAsequencing. The obtained information was subjected to *in silico* analysis to explore the factors that restore the pathways whose activity is altered by long-term culture, and by adding these factors to the culture medium, we confirmed an improvement in the transplantation ability of germ cells after longterm culture. Currently, the application of this system to other species is ongoing.

Oral Presentation 64 (student)

Luteinizing hormone gene over-expression in pre-pubertal rainbow trout can induce sperm production within a short period

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INTRODUCTION

Surrogate broodstock technology was previously established, in which donor-derived gametes are produced in recipients by germ cell transplantation. This technique is expected to be very useful in shortening the generation time for breeding. Chinook salmon (*Oncorhynchus tshawytscha*) gametes, which take 3–6 years to make, were produced in 1–2 years by having rainbow trout (*Oncorhynchus mykiss*) produce them. However, rainbow trout recipients required at least 1 year to mature. To further shorten their maturation time, herein, super-precocious recipients were generated by overexpressing the luteinizing hormone (*lh*) gene in rainbow trout and evaluated for their suitability as recipients for germ cell transplantation.

METHODS

An Lh expression vector (*hsc:lh*) was constructed, containing the rainbow trout *heat-shock cognate 71* (*hsc71*) gene promoter, which is ubiquitously active in all tissues, and the *lh* gene encoding a fusion protein of the Gthα and Lhβ subunits. The gene construct was microinjected into the rainbow trout's fertilized eggs. F1 *hsc:lh* heterozygous transgenic offspring (*lh-tg*), generated by crossing the founder individuals with wild-type (WT), were used for phenotypic analyses. First, transgenic *lh*-, *lhcgr*-, and *fshr* gene expression analysis was performed by RT-PCR using the testes of 4-, 5-, and 6-month-old *lh-tg* individuals and WT individuals. Next, plasma 11-ketotestosterone (11-KT) levels were measured by ELISA, and their testes' histological analysis was performed to compare their maturational stages. In addition, *lh-tg* spermiation was examined by applying gentle pressure on their abdomen, periodically. As soon as their spermiation was observed, their sperm was used for inseminating eggs obtained from WT females. Finally, to evaluate the *lh-tg* individuals' suitability as recipients for germ cell transplantation, germ cells prepared from the rainbow trout's testes carrying the *vasa:Gfp* transgene whose germ cells specifically expressed green fluorescent protein (Gfp) were transplanted into the abdominal cavity of F2 *lh-tg* hatchlings sterilized by triploidization. The frequency of individuals retaining Gfp-positive cells in their genital ridges after 20 days of transplantation was quantified.

RESULTS & DISCUSSION

The *lh* transcripts from the transgene, *lhcgr*, and *fshr* mRNAs were detected in the *lh-tg* individuals' testes at 4–6 months of age. Plasma 11-KT levels in *lh-tg* individuals were remarkably higher than those in WT individuals at all ages. Moreover, *lh-tg* individuals had spermatozoa at 4 months of age and spermiated at 5.5 months of age, whereas WT individuals retained only spermatogonia at all ages examined. The resulting sperm showed normal morphology and produced offspring, which developed and grew normally. Therefore, this study succeeded in bringing juvenile rainbow trout, which are usually spemiated at 2 years of age, to early maturity at only 6 cm of their body length by introducing transgenic *lh* genes. The percentage of recipient larvae carrying donor-derived Gfp-positive germ cells in their genital ridges after 20 days of transplantation was 78.6% ± 7.1% in *lh-tg* individuals, indicating that *lh-tg* individuals retained the ability to attract and incorporate the transplanted donor-derived germ cells into their genital ridges. In the future, we will attempt to produce precocious female rainbow trout and aim to reduce the generation time, which is the greatest impediment to genetic breeding, by using surrogate broodstock technology with super-precocious recipients.

Oral Presentation 65 (student)

Gonadal maturation and spawning of hatchery-produced greater amberjack (*Seriola dumerili*) following administration of single-chain recombinant greater amberjack gonadotropins

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INTRODUCTION

Reports describing reproductively dysfunctional breeders are recurrent in greater amberjack (*Seriola dumerili*) held under farming conditions. The resulting discontinuity in the supply of gametes of good quality, highlights the priority of alleviating reproductive dysfunctions and augmenting spawning performance to secure egg production. Two experiments were conducted in order to examine the potential of recombinant single-chain *Seriola dumerili* gonadotropins (rsdGths) in inducing gametogenesis, in pre-pubertal or adult fish. We report here the efficacy of rsd Follicle stimulating hormone (Fsh) and Luteinizing hormone (Lh) in promoting gonadal recrudescence and enhancing reproductive success of greater amberjack.

METHODS

In a first experiment, twenty-month-old hatchery-produced F1 immature male and female greater amberjack were treated with rsdFsh (8 - 12 μg kg^{-1}), and rsdLh (10 - 12 μg kg^{-1}) every 7-days for 12 weeks. In the secondexperiment, rsdGths (starting at 6 μg and increasing to 12 μg kg^{-1} of rsdFSH and rsdLH) were administered weekly for six weeks to enhance maturation in 5-year-old adult hatchery-produced F1 greater amberjack reared in tanks. At the expected spawning season, fish were examined for maturation and gonadotropin-releasing hormone agonist (GnRHa, 50 μg kg^{1}) was administered to induce spawning. Plasma levels of sex steroids were measured using LCMS/MS and reproductive performance was evaluated through estimation of relative fecundity, fertilization success, larval survival and of sperm quality parameters.

RESULTS & DISCUSSION

Administration of rsdGths for 12 weeks increased significantly plasma androgen levels in both sexes. All treated males were in spermiation, whereas in Control males the testes were dominated by spermatogonia, withspermatocysts with spermatocytes at various stages representing only a small fraction of the gonadal content. On the contrary, no induction of vitellogenesis was observed in rsdGth-treated females, and ovaries were occupied with oocytes at primary growth. In the second experiment, 5y-old untreated females did not mature, and no spawns were obtained. On the other side, all fish treated with rsdGths underwent complete gametogenesis. Ovarian biopsies of treated females indicated a steady increase of oocyte diameter over time, with the most advanced ones exceeding 750 μm at week 6 after treatment, while spawning initiated earlier thanexpected, at week 4 with four spawns in the month of May. Nine additional spawns were obtained after GnRHaimplant administration, and its usage allowed to increase both mean (SD) relative fecundity and total fertilization success from 2,283 ± 508 before GnRHa induction to 5,961 ± 1,568 eggs kg^{-1} and from 7 to 35 %, respectively. The effect of rsdGTHs in plasma steroid levels and sperm quality in 5-year old fish is still underanalysis, however the current results prove that the combined usage of weekly rsdGths and a final administration of GnRHa might be beneficial in promoting gametogenesis, maturation, and spawning, and in enhancing the reproductive output in F1 greater amberjack maintained in tanks.

The project was funded by the project NewTechAqua (European Union ś Programme H2020, GA862658).

Oral Presentation 66

Sex-specific advance in pubertal maturation in response to *in vivo* application of recombinant Fsh and Lh to prepubertal meagre (*Argyrosomus regius*)

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INTRODUCTION

Biotechnologies to advance pubertal maturation and spawning would enable the aquaculture industry to accelerate genetic improvement in late maturing fish, such as meagre (*Argyrosomus regius*). Follicle stimulating hormone (Fsh) initiates pubertal maturation and combined with luteinizing hormone (Lh) controls gametogenesis through to spawning. The aim of the present study was to determine the *in vivo* effect of recombinant gonadotropins (rGths), rFsh and rLh, administered to prepubertal meagre.

METHODS

Gonadotropin sequences were used to produce meagre single-chain recombinant gonadotropins, rFsh and rLh using the CHO expression system (Rara Avis Biotec, S. L.). Prepubertal meagre, (≈18 months old, 1.07 ± 0.18 kg mean ± SD) were randomly distributed into rGth treatment (n=36) and control (n=36) groups and housed in a 10 m^3 tank under natural photoperiod (Oct. – March) and constant temperature (18.1 ± 0.3°C). A 21-week experiment was initiated on Oct. 27 in which the treatmed fish received weekly injections of a combination of rFsh (6 to 12 µg kg^{-1}) and rLh (3 to 10 µg kg^{-1}), and control fish saline injections. Gonadal development was assessed by GSI and histology on weeks 0 (n=12) and in both groups (n=12 $group^{-1}$) on weeks 6, 12 and 21. Plasma 17β-estradiol (E2) and 11ketotestosterone (11-KT) were determined for groups (n=12 $group^{-1}$) on weeks 0, 4, 6, 8, 12, 16 and 21.

RESULTS & DISCUSSION

The rGth treatment increased male maturation from 12.5% (Control) to 100% (rFsh + rLh). Males in the rGth group had a GSI of 1.9±0.2% (week 12), with testes full of luminal spermatozoa and free flowing spermiation of motile spermatozoa compared to non-maturing control males that had no presence of sperm, a GSI of 0.2±0.1% and testes containing mainly spermatogonia and spermatocytes. Testes development was driven by significantly elevated 11-KT plasma levels in the rGth group (weeks 6 to 21). In comparison females were not induced to mature and oocytes remained almost exclusively at primary growth, perinucleolar stage. Plasma levels of E2 remained low (50.8±12.3 to 104.4±12.4 pg mL^{-1}) across all groups and dates. The female GSIs were low between 0.1 ± 0.0 and 0.3 ± 0.1 %, but the GSIs of rGth treated females were significantly higher than control females on weeks 12 and 21. Four females (from 10 females, i.e. 40% - week 12 and 21 combined) had a low frequency (0.1 %) of oocytes in the early stages of vitellogenesis. The response to the rGth treatment to 18-month-old / 1 kg meagre was sex specific, as males matured and females did not mature. These findings suggest that ovaries had not fully developed the follicle structure with Fsh receptors and were not able to receive the rFsh signal to produce E2 and initiate puberty. Normally, female meagre enter puberty a year after males and appear to require more time to develop before puberty can be induced using rGths.

The study was funded by the European Union´s Programme H2020, project NewTechAqua, GA 862658.

Oral Presentation 67

Sterile salmonids produced by transient gene silencing and their applications in aquaculture and studying fish reproductive endocrinology

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INTRODUCTION

Reproductive sterility carries environmental significance along with economic benefits. It provides a proficient genetic containment strategy and minimizes energy input toward gonadal growth while enhancing muscle development, promoting health, and protecting valuable strains from unauthorized propagation. We developed a bath-immersion method to execute a transient gene-silencing technology to produce sterile fish. This non-GMO approach can maximize food production and minimize ecological impact, thereby achieving long-term environmentally, economically, and socially sustainable aquaculture development. In addition, sterile fishes are a valuable addition for studying fish reproductive endocrinology, specifically gonadal regulation of the reproductive endocrine network.

METHODS

Salmonid unfertilized eggs were immersed with Vivo conjugated Morpholino Oligomer (MO) against *deadend* (*dnd*-MO-Vivo) in modified L-15 or DMEM medium for 24 to 48 hours at 4 or 8 ºC. After immersion, eggs were washed with medium and fertilized with fresh milt. Germ cell marker gene *vasa* was analyzed by qPCR to predict sterility induction. Gonadal histology was used to confirm sterility. Pituitaries from 3 matured (3-year-old) fertile and 3 sterile rainbow trout females were isolated for single-cell cDNA library preparation and sequencing. The Cell Ranger Single Cell Software Suite 6.1.1. from 10x Genomics was used for RNA-seq data processing.

RESULTS & DISCUSSION

Using *dnd*-MO-Vivo bath-immersion, sterile salmonids were produced with success rates reaching 84% in rainbow trout, 75% in Atlantic salmon, and 20% in coho salmon. The levels of *vasa* mRNA in treated groups decreased to 24.6% of the control. Forty times GSI reduction was found in adult sterile female rainbow trout during spawning compared to the fertile females. In single-cell RNA-seq, the overall size of pituitary cells isolated from sterile female rainbow trout is 95% larger (25% larger in diameter) than the fertile female rainbow trout. For the pituitary single-cell cDNA libraries generated from fertile and sterile females, 324,703,406 and 326,026,680 reads, respectively, were obtained, of which 74.2% and 78.0% of the total reads were confidently mapped to the reference genome. Nine hormone-secreting endocrine cell types were classified, including lactotropes, somatolactotropes, somatotropes, thyrotropes, Fsh-gonadotropes, Lh-gonadotropes, gonadotrope-like cells, melanotrope, and corticotropes. The most remarkable changes at the pituitary cell population levels in sterile fish are the increase of Fsh-gonadotropes and decrease of Lh-gonadotropes percentages compared to the fertile fish, which suggest that gonadotropes likely have the most remarkable plasticity within the pituitary endocrine cell populations. The expression of *lhβ* dramatically decreased in both Lh-gonadotropes (77.4 folds) and gonadotrope-like (90.1 folds) of sterile fish. Both changes detected at cellular and population levels in Fsh- and Lh-gonadotropes demonstrated the responses of the pituitary to the absence of gonadal factors in sterile fish.

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Oral Presentation 68

Investigation of approaches for sterility induction in sablefish (*Anoplopoma fimbria*)

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INTRODUCTION

Escape of farmed fish and potential genetic pollution of wild populations is a global ecological concern. To mitigate this risk, we are investigating several non-GMO approaches for reproductive sterility induction, including bath immersion-based gene silencing and triploidization. Sablefish (*Anoploma fimbria*) is a marine aquaculture model species based on our understanding of its sex determination and early reproductive development, and availability of a complete genome and other molecular tools.

METHODS

Trials were conducted to test the efficacy of gene silencing (morpholino oligomer, MO) targeting the germ cell-specific gene, dead end (dnd). Sablefish eggs, either pre- or post- fertilization, were immersed in a solution containing a dnd-MO conjugated to a molecular transporter, Vivo, for 48 h and then stocked into standard hatchery rearing vessels. Triploid sablefish were produced for the first time for comparison to sterile sablefish generated by dnd gene silencing. Protocols for induction of triploidy using hydrostatic pressure or cold shock were developed, as well as methods for ploidy determination. In brief, pressure shock of 7,000-9,000 psi applied 10 min post-fertilization for 5 min, or cold shock at -1.5 ºC for 60–120 min induced triploidy in a high percentage of fish while minimizing adverse effects on early survival. Fish from both methods were reared until phenotypic sex and early gonadal development could be assessed by histology and ploidy and genotypic sex determined from blood samples. A subset of the fish was maintained through a grow-out cycle to assess subsequent gonadal development and preliminarily evaluate their performance, including survival and growth.

RESULTS & DISCUSSION

dnd-MO-Vivo treatments produced fish with three gonadal phenotypes: normal gonads, gonads with reduced germ cell counts, or gonads devoid of germ cells. The highest rates of sterility induction were obtained in the pre-fertilization treatment; ~10% of the fish had germ cell-free gonads, 15% had significantly reduced germ cell counts relative to controls, and 75% had normal gonads. During growout, significant differences in gonadosomatic index (GSI) were observed among females, with sterile individuals exhibiting a 25-fold lower GSI than control females. Phenotypic sex matched genotypic sex in 100% of sampled individuals, indicating that no sex reversal occurred in response to dnd-MO-Vivo treatment or sterility. Triploid female sablefish exhibited suppressed ovarian development, indicated by reduced numbers of underdeveloped oocytes compared to diploid females. Testes of triploid and diploid males were similar in appearance and composed of type-A spermatogonia. During grow-out, the GSI of triploid females was reduced ~10 fold relative to diploid females, while there was no difference in GSI between triploid and diploid males. Sex ratios for triploid fish were significantly female skewed.

This study provides proof-of-concept for sterility induction in sablefish using immersion-based *dnd* gene silencing or triploidy. Triploidy was especially disruptive to ovarian development as has been shown in other fishes. Critical next steps will be to optimize these methods for higher rates of sterility and to further assess fish performance in aquaculture. This project was supported by a National Aquaculture Research Program/ Maryland Sea Grant project (R/AQ-7) and NOAA Internal Competitive Aquaculture Funds (ICAF) grant.

Oral Presentation 69 (student)

Genome-wide comparative methylation analysis in zebrafish produced via surrogacy

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INTRODUCTION

Spermatogonial stem cell (SSC) transplantation is the tool to produce donor-derived gametes in fish via a surrogate parent. The isolated SSCs introduced to a sterile host, where only a few cells survive to build the complete gonad again. The gametogenesis of the donor SSCs occurs in a foreign environment. As known, epigenetics control cell differentiation and gene expression in living organisms, which may change due to environmental intervention. DNA methylation is one of the best-studied epigenetic modifications. In this study, we have explored the methylation changes in donor-derived sperm and their transgenerational effect on the progeny.

METHODS

We have performed inter- and intraspecific transplantation by taking vasa: EGFP line zebrafish as donor and AB line as host. For interspecific transplantation, the recipient was prepared by hybridization by crossing zebrafish and pearl danio, and for intraspecific dead-end knockdown recipients were used. Sperm and progeny from the donor and the germline chimera were collected for whole-genome bisulfite sequencing to study the DNA methylation at the single-base resolution (four biological replicates for each group). Data from three groups (MO, Hybrid, and donor) were compared to investigate genomewide and differentially methylated promoter (DMPs) regions, followed by Gene Ontology (GO) and KEGG pathway analysis. The significant DMPs were further validated with quantitative PCR to know gene expression status.

RESULTS & DISCUSSION

We observed homogeneity in the genome-wide methylation pattern between the donor sperm and the donor-derived sperm from the MO and hybrid germline chimeras and in the progeny. When looking at the different genomic features such as promoters, exons, and repeats (transposable elements), Hybrid progeny showed slight hypermethylation, while MO progeny showed more closeness to the donor. Next, we studied the Differentially methylated region (DMR) between all the groups. We found 3788 DMR genes between the Hybrid and donor progeny, 3814 between the MO and donor progeny, and 3766 between the MO and Hybrid progeny. Similarly, the sperm samples were compared between the groups, where we observed 3639 DMR between Hybrid and donor, 3527 between MO and donor, and 3708 for MO and Hybrid. Most of the DMRs resulted because of the hypermethylation in both the germline chimera. Since our utmost priority was understanding the potential disruption of cellular or molecular function, we only focused on promoter methylation (DMPs). Surprisingly we found significant hypermethylation in several promoter regions of protocadherin 1 gamma (PCDH1g) in MO progeny. The identified promoter regions are coding for *pcdh1gc6, pcdh1g22, pcdh1g30, pcdh1g33, pcdh1g2,* and *pcdh1g31*. The clustered PCDH codes for the group of homophilic cell adhesion proteins known as protocadherin, which requires cell-cell adhesion during vertebrate embryogenesis and neural development during zebrafish embryonic development. We checked the relative gene expression of the genes mentioned above by qPCR and did not find any significant difference, neither did we observe any malformation in the hatched larvae. The MO progeny inherits the PCDH1g hypermethylation from the donor-derived sperm, apparent by their methylation pattern in Integrative Genome Viewer (IGV). In addition, we also found the MAPK/P53 signal pathway enrichment in the Hybrid sperm due to the hypermethylation in the Tp53 gene and other genes responsible for apoptosis, which leads to the production of abnormal spermatozoa with low motility due to the lack of apoptosis in Hybrid recipient testis.

Oral Presentation 70 (student)

Replacement of mitochondria in sturgeon germline

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INTRODUCTION

Sturgeon is one of the most critically endangered species in the world mainly due to overfishing for caviar, it is urgent and necessary to preserve the remaining diversity and aid in the recovery of this endangered species. Cryopreservation is a method for storage of living cells for a virtually infinite period of time, and the cryopreservation of sperm has been successful in several aquatic species, but we still lack the technology to cryopreserve eggs and embryos, which leads to the fact that the maternal genetics cannot be successfully preserved. That means we urgently need to establish a new technology to conserve sturgeon maternal genetics – mitochondrial DNA. The aim of the present research is to implement the replacement of mitochondria in sturgeon embryo germline. We will take advantageous of specific primordial germ cell (PGCs) development in vegetal pole of sturgeon embryo, which is isolated from other cell line precursors.

METHODS

Mitochondria were isolated from eggs of donor sturgeon, then the host embryos underwent irradiation of vegetal pole with UV to eliminate the endogenous mitochondria of germplasm. The previously isolated mitochondria were injected into the vegetal pole of the embryo at the 1-4 cell stage allowing the mitochondria to colonize PGCs. A control group was injected with FITC-dextran, which labels PGCs. To test whether PGCs development was disturb after UV-irradiation, we injected FITC-dextran into UV- irradiated embryos. To test whether mitochondria can rescue the PGCs, we injected FITCdextran with PKH26 labelled or unlabeled mitochondria into UV-irradiated fertilized embryos. Finally, the numbers of PGCs in each experimental group were counted. The transplanted PGCs were identified by molecular and histological methods.

RESULTS & DISCUSSION

We found that UV irradiation can specifically and efficiently destroy PGCs and that injection of mitochondria from donor eggs (of the same or different sturgeon species) into UV-treated embryos rescued PGCs and restored them to the original numbers, the higher number of mitochondria injected, the higher number of PGCs rescued. We also found that mitochondria labeled with PKH26 rescued PGCs in the same manner as unlabeled mitochondria and can be successfully tracked in vivo. In addition, the mitochondria transplanted between different sturgeon species were detected using mtDNA specific primers in larvae. The preliminary results clearly demonstrate that the eliminated mitochondria can be replaced by interspecific transplantation.

Oral Presentation 71

Production of offspring derived from cryopreserved spermatogonia by surrogate broodstock in ayu (*Plecoglossus altivelis*)

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INTRODUCTION

The ayu (*Plecoglossus altivelis*) is an amphidromous migratory species and an important target for recreational fishing in Japan. Therefore, since a long time, juveniles from land-locked populations collected in a lake have been released as seedlings into rivers in various regions. It has long been believed that these released fish do not impact the genetic composition of native fish because of their low survival rates after migration to the ocean. However, recent detailed DNA analyses have revealed that genetic introgression by the land-locked populations has been occurring in several areas. Therefore, the establishment of cryobanking of their germ cells would provide an effective backup measure for conserving the genetic resources of the native populations of this valuable species. However, no methodology of germ cell transplantation and cryopreservation has yet been established. Here, we attempted to produce functional eggs and sperm derived from cryopreserved testicular cells through the combination of cryopreservation and transplantation of germ cells in the ayu.

METHODS

To identify the suitable stages as donor, 16- to 32-week-old juveniles were analyzed for the occurrence frequency of type A spermatogonia possessing translatability in germ cell transplantation. Next, to identify the suitable stages as recipients, we examined the developmental stages of the genital ridges in 6- to 12-day-old posthatching (dph) larvae. Immature testes collected from Gunma ayu strains that had been maintained by intrastock breeding for 52 generations were cryopreserved by slow freezing as donor testes. After thawing, the cells dissociated from the cryopreserved testes were labeled with PKH26 and intraperitoneally transplanted into sterile triploid recipients of Edogawa ayu strains. To determine whether donor-derived spermatogonia could differentiate into gametes in the recipient gonads, gametes collected from mature recipients were fertilized. Finally, microsatellite DNA analysis was performed to confirm whether the resulting F1 are donor-derived offspring.

RESULTS & DISCUSSION

Histological analysis revealed that 20-week-old juveniles contained a higher number of type A spermatogonia, and their occurrence frequency was also higher in their gonads. Further, in 12 dph larvae, endogenous primordial germ cells were incorporated into the genital ridges and completely covered by somatic cell layers, suggesting that larvae younger than 12 dph are optimal to be used as recipients. After the transplantation, PKH26-labeled cells were incorporated into the genital ridges of recipients when the 8–10 dph recipients were used. Their transplantation success rate was 86.6% ± 0.017%. At 1-year post-transplantation, 7 of 35 males and 4 of 33 females produced sperm and eggs. Through their artificial insemination, we successfully produced donor-derived offspring exhibiting identical microsatellite DNA patterns as those of the donor strains. The methodology developed in this study will contribute to the preservation of genetic resources of various native ayu populations.

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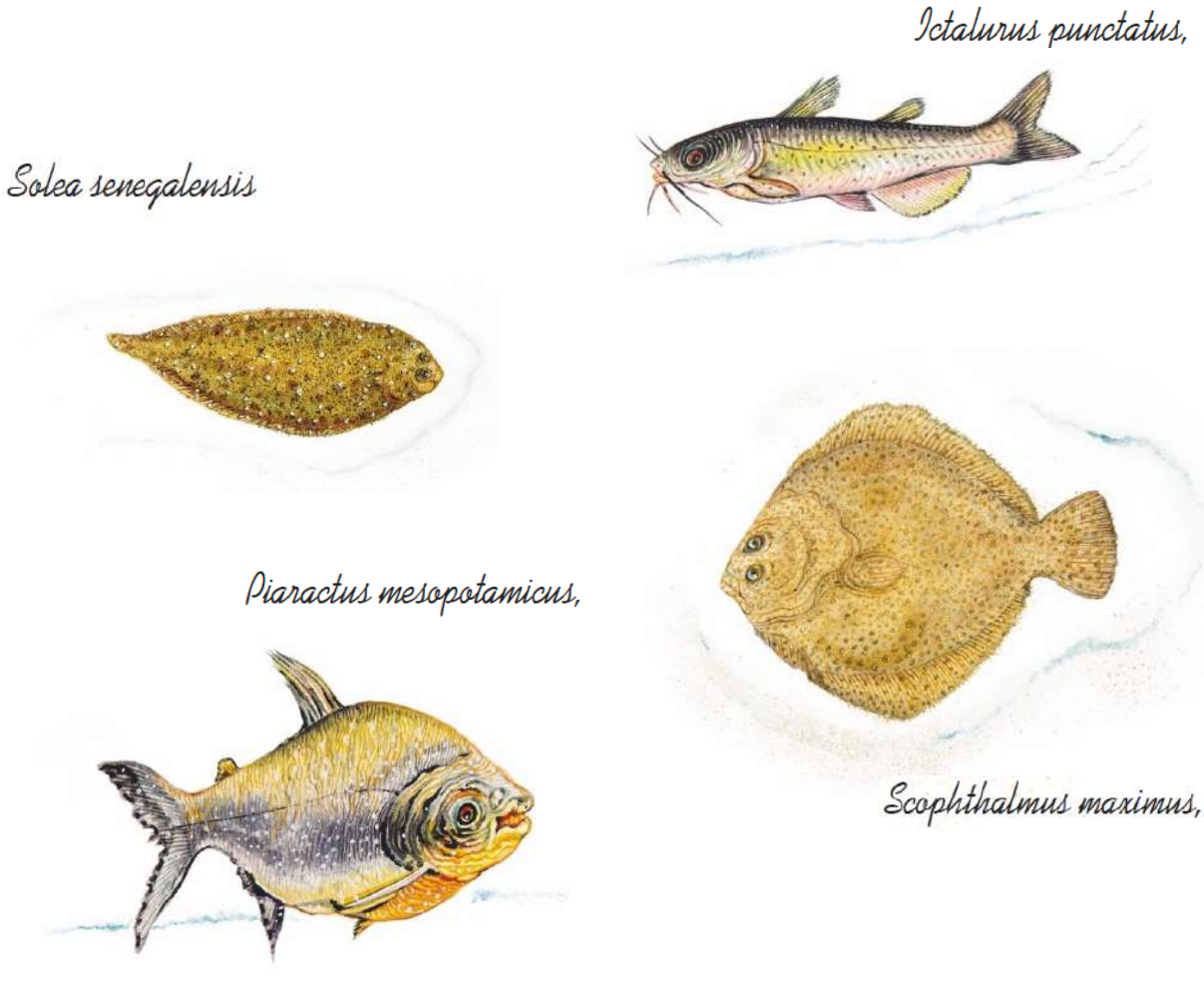

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P104	DEDUKH DMITRIJ	Gametogenic pathways in diploid and triploid asexual hybrids from *C. hankugensis-longicorpa* fish complex	S3. Oogenesis/vitellogenesis and ovulation
P105	RODRIGUEZ FORERO ADRIANA	Aspects of the reproductive biology of the tropical mullet, Mugil incilis.	S6. Reproduction in aquaculture
P106	RODRIGUEZ FORERO ADRIANA	Semen characterization and sperm structure of the tropical lisa *(Mugil incilis).*	S4. Spermatogenesis and spermiation

SS1. Sex determination and differentiation

Poster Presentation 1

Genotype by environment interaction explain sex determination in the European seabass

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INTRODUCTION

Traditionally, fish sex determination was considered to be governed by genetic or environmental factors. However, many teleost species defy this dichotomy. We combined genomic and transcriptomic approaches to characterize the temperature-dependent polygenic sex determination of European sea bass.

MATERIAL AND METHODS

We produced 8 families by mating 8 males with the same female. The progenies were reared in common garden under two thermal treatments in triplicate: a LT temperature protocol (16°C) and HT treatment (21°C). Fish were evenly sampled in the triplicates of each temperature treatment, at four different key developmental stages encompassing the temperature sensitive period. A total of 2030 offspring were genotyped using a 57k SNPs (DLabCHIP array) to estimate their genetic sex tendency (eGST), defined as the estimated genetic probability to become a given sex under a liability threshold model for sex determination. A subsample of these fishes was also taken for transcriptomic approaches, whole-body energy measurements and gonads' histological analyses. In addition, full sibling of sexed individuals at 1 year were also used for genome-wide profiling of DNA methylation levels.

RESULTS & DISCUSSION

The proportion of females obtained was 53.4% at LT and 25.3% at HT. As expected, the high temperature induced a marked masculinization, dividing by two the proportion of female relative to the LT treatment. We found that the eGST accurately predicted the future phenotypic sex. We also provided evidence that energetic pathways, concerning the regulation of lipids and glucose, are involved in sex determination and could explain why females tend to exhibit higher energy levels and improved growth compared to males. Besides, early exposure to high temperature upregulated *sox3*, followed by *sox9a* in individuals with intermediate eGST, but not in individuals showing highly female-biased eGST, providing the most parsimonious explanation for temperature-induced masculinization. This gonadal state was maintained likely by DNA methylation (hypomethylation in the promoter region of sox3) and the upregulation of several genes involved in histone modifications, including jmjd1c. Overall, we describe for the first time a sex determination system resulting from continuous genetic and environmental influences in an animal. Our results provide significant progress in our understanding of the mechanisms underlying temperature-induced masculinization in fish.

ACKNOWLEDGEMENT

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Poster Presentation 2

Gonadal sex differentiation in the ovoviviparous red stingray (*Hemitrygon akajei*)

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INTRODUCTION

The cartilaginous red stingray (*Hemitrygon akajei*) is commonly distributed in Japan. Red stingrays exhibit ovoviviparous reproduction. The embryos rely on nourishment from each yolk and lipid-rich fluid (uterine milk) secreted from the uterine wall. The morphological features of the developmental process in the embryo have been well described. The physiological features of embryos remain largely unexplored. This study investigated the gonadal sex differentiation of red stingray embryos in the uterus.

METHODS

Pregnant red stingrays were purchased from a local fishing market. The developmental stages of the embryos were in accordance with a previous study. Embryos were fixed in Bouin's solution, and the differentiation process of the gonads and reproductive ducts was observed histologically. Additionally, the concentration of estrogen in the maternal serum and uterine milk was measured using ELISA.

RESULTS & DISCUSSION

The developmental stages of embryos in each uterus were the same, suggesting a single timing of ovulation and fertilization in the red stingrays. The uterus of each stingray contained 9.5 ± 3.5 embryos. Embryos at stages 3, 4, 8, and 9 were obtained. In stage 4 (pectoral fins extend anteriorly in front of the eyes but remain unfused), sex identification was possible based on external observations (claspers; modified pelvic fins). The male-to-female sex ratio of the embryo in each uterus was 0.97: 1.03. The concentration of estrogen in the uterine milk did not differ significantly from the concentration in maternal serum (144.9 ± 20.8 vs. 148.0 ± 25.7 pg/ml). This suggests that sex determination/differentiation of red stingray embryos occurred under exposure to maternal estrogens in the uterus.

Histological observations revealed that few primordial germ cells (PGCs) were located in the gonads of the stage 3 embryos. In stage 4 of the female embryo, PGCs are localized in the dorsal region of the gonads. In contrast, PGC localization in the gonads of the male embryos was random. These differences in the localization of PGCs in the gonads might be the first morphological sexual differences in cartilaginous fishes. Müller and Wolff ducts also differentiated ventrally in the kidneys of the embryos. In females, the left Müllerian duct develops differentially into the uterus. Female Wolff's duct did not disappear and persisted. In contrast, Wolffian ducts developed in the male embryos. These results indicate that the sexual differentiation of the stingrays has many points in common with that of mammals. Further analysis is needed to elucidate the molecular mechanisms of sex differentiation in cartilaginous fishes.

This work was supported by JSPS KAKENHI (grant number 19K06229).

Poster Presentation 3

In vivo effect of recombinant Fsh and Lh administered to meagre (_Argyrosomus regius_) at the initial stages of sex differentiation

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INTRODUCTION

Recombinant gonadotropins, follicle stimulating (rFsh) and luteinizing hormone (rLh), offer the potential to induce gametogenesis in prepubertal fish. This study aimed to determine the *in vivo* effect of rFsh and rLh administered to prepubertal meagre juveniles at the initial stages of sex differentiation.

METHODS

Meagre single-chain recombinant gonadotropins, rFsh and rLh were produced with the CHO expression system by Rara Avis Biotec, S. L. Juvenile meagre, approximately 10 months old with mean weight of 222 ± 36 g (mean ± SD) were randomly distributed into seven experimental groups (n = 8 per group) that were treated weekly for three weeks with an acute injection of 6, 12 or 18 µg kg^{-1} of rFsh (groups, 6-rFsh, 12-rFsh and 18-rFsh) or 6, 12 or 18 µg kg^{-1} of rLh (groups, 6-rLh, 12-rlh and 18-rLh) or saline solution (control group). Two more groups (n=8) were set up and treated for 6 weeks, with 12 µg kg^{-1} of rFsh or saline control. The fish were held in a 10 m^3 tank with natural photoperiod (Feb. – March) and temperature 16.1 ± 0.4°C. At the start of the experiment (n = 8) and end of the 3-week experiment all fish were sacrificed and gonads dissected. Gonads were weighed, fixed in Bouin´s solution and processed for histological analysis. Blood was sampled from all fish at the start and end of the experiment (week 3 and 6) for 17β-estradiol (E2) and 11-ketotestosterone (11-KT) analysis.

RESULTS & DISCUSSION

Juvenile meagre at the start of the experiment were in the initial stages of sexual differentiation, indicated by the presence of the ovarian or sperm duct lumen that was surrounded by undifferentiated embryonic germ stem cells, which were in turn surrounded by somatic cells. At the end of the experiment, there was no significant difference in gonadosomatic index (GSI) amongst control (initial and saline treated) and the experimental groups. After three weeks of application of rFsh, rLh or saline all fish presented a similar gonadal structure as at the start of the experiment. However, the incidence of isolated developing germ cells (principally spermatogonia, spermatocytes, spermatids, but also chromatin nucleolar stage and perinucleolar stage oocytes) generally increased in rGth treated meagre. A mean of 44 % of meagre treated with rFsh or rLh presented isolate developing germ cells, mainly male cells. Seven of eight fish treated with 6 µg kg^{-1} rFsh had isolated male germ cells compared to saline treated fish that presented only undifferentiated germ cells. One fish in groups 6-rFsh, 12-rFsh and 18-rLh presented both male and female germ cells, intersex. Plasma steroid levels of E2 decreased significantly from the start of the experiments to the end. At the end of the experiment there were no differences between control fish and rGth treated fish. Plasma 11-KT showed no change from the start of the experiment to week 3 (six rGth groups and control group). However, a significant increase was observed in the rFsh group after six weeks of treatment compared to the start of the experiment and the control group on week 6. The application of rFsh or rLh to meagre at the initial stages of sex differentiation did not stimulate steroid production until week six and had a limited, but evident effect on the development of isolated germ cells. The rGths, rFsh or rLh did not stimulate large developmental changes in undifferentiated gonads.

The study was funded by the European Union´s Programme H2020, project NewTechAqua, GA 862658.

Poster Presentation 4

Gonadal sex differentiation in Pacific bluefin tuna (*Thunnus orientalis*)

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INTRODUCTION

The Pacific bluefin tuna (*Thunnus orientalis*; PBT) is among the most important species in the aquaculture industry worldwide. The aim of this study was to reveal the process of gonadal sex differentiation and its molecular mechanisms in PBT.

METHODS

We sampled PBTs reared from fertilized eggs at 41, 57, 83, and 100 days post hatching (dph). The dissected gonads were subjected to histological observation. The total RNA extracted from sampled gonads was also subjected to RNA-sequencing and quantitative RT-PCR (qRT-PCR). The genotypic sex of each sampled fish was identified using a male-specific DNA marker. Aromatase inhibitor (AI) treatment experiments (oral administration of letrozole between 31 and 70 dph) were conducted in terrestrial tanks using juveniles reared from fertilized eggs.

RESULTS & DISCUSSION

Gonadal sex differentiation was first histologically characterized by the formation of the ovarian cavity in females and of the efferent duct in males at 57 dph; the gonads then directly differentiated into ovaries or testes according to the genotypic sex until 83 dph.

Comparative transcriptomic analysis based on RNA-sequencing (100 dph; n = 3 fish for each sex) identified 522 and 281 genes that were upregulated in female and male gonads, respectively. Among them, *cyp19a1a* (coding for gonadal aromatase) was the most upregulated gene in females. Furthermore, expression of *foxl2*, a well-known transcription factor controlling *cyp19a1a* expression, was upregulated in females. qRT-PCR analysis showed that expression of both *cyp19a1a* and *foxl2* was upregulated in female gonads from morphologically sex-undifferentiated (41 dph) to differentiated (83 dph) stages. Additionally, we demonstrated that AI treatment completely induces sex-reversal of genotypic females into phenotypic males. Our results suggest that active estrogen synthesis by aromatase, which includes positive regulation of *cyp19a1a* expression by Foxl2, is essential for ovarian differentiation in PBT. In males, comparative transcriptomic analysis identified upregulated expression of *dmrt1* and *gsdf*, which are well-known key factors regulating testicular differentiation in fish. Furthermore, qRT-PCR analysis showed that the expression of *dmrt1* and *gsdf* was upregulated in male gonads from morphologically sex-differentiating (57 dph) to differentiated (83 dph) stages. Moreover, through qRT-PCR analysis, we confirmed that *dmrt1* and *gsdf* expression was upregulated in the masculinized gonads of genotypic females obtained from the AI experiment. Our results suggest that *dmrt1* and *gsdf* play an important role in testicular differentiation in PBT. Using Gene Ontology analysis and qRT-PCR, we also confirmed that androgen synthesis is upregulated in male gonad during sex differentiation, suggesting that androgens may also contribute to testicular differentiation in PBT.

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Poster Presentation 5 (student)

Development of a molecular method to identify YY supermales of cobaltcap silverside, a fish with both genetic- and temperature-dependent sex determination

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INTRODUCTION

The cobaltcap silverside, an atheriniform from the Northwest Pacific, is known to have genotypic sex determination driven by the Y-chromosome linked *amhy* and at the same time temperature-dependent sex determination. Previous field surveys revealed that sex-reversed XY females, probably caused by exposure to low water temperature during the critical time of sex determination, are present in the wild population at frequencies between 0 and 14.5% of the XY genotype. Although their proportion is low, if they are fertile, they might mate with normal XY males and cause a skew in the genetic sex ratio. More importantly, this cross may yield YY offspring which in turn could yield all XY progenies when mating with normal XX females. In order to estimate the possible impacts of climatic and anthropogenic factors on sex determination and of genotypic/phenotypic sex imbalances on natural cobaltcap silverside resources, it is necessary to develop methods to assess the genotypic/phenotypic sex structure of wild populations including the identification of the YY genotype. Given this background, we developed a method to distinguish the YY from the XY and XX genotypes through DNA quantification (qPCR) of *amhy* gene, assessed the existence of YY fish and their phenotypic sex in presumably the same yearclass of cobaltcap silverside from Tokyo Bay, and progeny-tested a YY individual in a laboratory experiment.

METHODS

The 5′ flanking region of *amhy* gene was used for designing the qPCR primers. This region was selected in order to avoid an eventual cross-amplification with the *amha* locus, which shares high identity with the coding region of *amhy* gene. A tentative genotypic screening using the primers was conducted on phenotypic males and females captured from the wild in 2021 (juveniles; n=77) and 2022 (adults; n=128). For distinguishing XX, XY, and YY genotypes, we performed a cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA) using the average values of *amhy* for two replicates from each individual after correction by the respective β-*actin* values. One sexually mature YY male and XY males for comparison were selected for progeny tests in crosses with XXfemales.

RESULTS & DISCUSSION

DNA quantification by qPCR with the *amhy* primers revealed 53.2% (n=41) XX, 45.5% (n=35) XY, and 1.3% (n=1) YY fish in the 2021 juveniles and 79.7% (n=102) XX, 19.5% (n=25) XY, and 0.8% (n=1) YY fish in the 2022 adults. The two presumptive YY fish were phenotypic male and the individual captured in 2022 was sexually mature and spermiating. Mating of this male and presumptive XY males to XX females yielded all *amhy*-positive progeny only in the case of the presumptive YY male whereas crosses with the XY males yielded balanced ratios of *amhy*-negative and *amhy*-positive genotypes in the progenies. These results indicated that the YY genotype was successfully distinguished from XY by qPCR and that YY individuals are both viable and fertile. Although only two individuals were found in this study, the results are suggestive of the strong tendency of YY fish to develop as males. Ongoing studies will analyze the samples collected over the past 8 years to confirm these results and to assess the trends in genotypic/phenotypic sex structure of the wild cobaltcap silverside population in Tokyo Bay.

Poster Presentation 6

Sturgeon embryo switched from holoblastic to meroblastic cleavage is viable but lacks primordial germ cells

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INTRODUCTION

A vertebrate embryo's cleavage pattern is either holoblastic (complete) or meroblastic (partial). Holoblastic cleavage is thought to be ancestral to vertebrates and is most likely to occur in amphibians, mammals, and chondrosteans. Meroblastic cleavage has evolved five times in vertebrate lineages, including hagfish, elasmobranchs, coelacanths, teleosts, and amniotes. Sturgeons are called living fossils and belong to the actinopterygian lineage, which evolved about 200 million years ago. The embryo of the sturgeon retains nearly the same characteristics as that of the *Xenopus laevis* (amphibian), such as localization of germplasm in vegetal cortex of egg, embryo's cleavage pattern, blastulation, gastrulation, and neurulation. It was speculated that vegetal blastomeres of sturgeon (holoblastic) are extraembryonic as in yolk of teleost (meroblastic). Hence, this study aims to switch the cleavage pattern of sturgeon eggs from holoblastic to meroblastic to know whether the VP-inhibited embryo can develop and what further consequences can occur (e.g., embryo sterility).

METHODS

The fate mapping of vegetal blastomeres was conducted using the microinjection of fluorescein isothiocyanate (FITC)-dextran, BrdU pules and chasing, and histology. To inhibit the vegetal blastomere, embryos were injected with optimal 2-4 decadienal (DD) percentage (0.01 v/v)—a model aldehyde for experimental studies—adversely affects the developing embryos of several aquatic species and subsequently irradiating them by visible light (91.15 – 44.86 W m2). Moreover, to clarify whether the vegetal pole-inhibited embryos are fertile or sterile, we utilized GFP-nos3 3′ UTR mRNA (300 ng/µl) coupled with said treatment. For embryo analysis and imaging, we utilized a Leica fluorescence stereomicroscope.

RESULTS & DISCUSSION

Normal cleave embryo of sturgeon revealed that vegetal blastomeres gave rise to primordial germ cells (PGCs), and the rest of the descendants were vegetal YCs. These YCs become transcriptionally inactive after mid-blastula transition and serve only nutrition. Interestingly, when the cleavage of the vegetal pole was inhibited (switched from holoblastic to meroblastic), embryogenesis continued, without producing the PGCs. Our results revealed that holoblastic cleavage and production of YCs in sturgeon embryo is due to localization of the germplasm in the vegetal cortex and YCs only facilitate the migration of PGCs towards the genital ridge during embryonic development. The present study also makes the way toward the sterile surrogate production of critically endangered fish species (sturgeon). Moreover, further research can be conducted to determine "which one is the best surrogate production technique" in the case of sturgeon using 1) Vegetal blastomere inhibition (present study), 2) depleted PGCs through UV irradiation, 3) morpholino oligonucleotide (MO) against dnd1 and 4) CRISPR/Cas9 dnd1 knockout.

Poster Presentation 7

New insights in zebrafish primordial germ cell survival and sex determination: the role of the autophagic protein Ambra1b

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INTRODUCTION

AMBRA1 is an intrinsically disordered protein in which the presence of specific domains and stretches of intrinsically disordered structure allows interaction with several proteins, thus regulating, by protein-protein interaction, many cellular processes, including autophagy, mitophagy, apoptosis, and cell cycle progression. The zebrafish genome contains two *ambra1* paralogous genes (*a* and *b*), both involved in the development, as demonstrated by morpholino knockdown, and expressed at high levels in the gonads. Moreover, the characterization of the zebrafish *ambra1* mutant lines, generated by CRISPR/Cas9 approach, showed that *ambra1a*$^{-/-}$ and *ambra1b*$^{-/-}$ mutants do not display overt developmental defects due to the activation of genetic compensation mechanisms. In contrast, double mutants cannot survive after larval stages. Interestingly, *ambra1b* knockout (KO) leads to an all-male population.

METHODS

In this work, we analyzed the effect of *ambra1a and ambra1b* KO on sex determination, gonads morphology, and reproductive performance, adopting many different experimental approaches ranging from histology, primordial germ cells (PGCs) analysis by Vasa-immunohistochemistry and *GFP-nos13'UTR* mRNA microinjection, morpholinos knockdown and rescue experiments with human *AMBRA1* mutated in the PP2A, LC3, TRAF6, and CULLIN4 binding sites. Moreover, follicles at different developmental stages were counted in the ovaries of *AMBRA1*$^{+/-}$ and *AMBRA1*$^{+/+}$ mice and expression analyses were used to validate the results in zebrafish and mice.

RESULTS & DISCUSSION

We demonstrated that the silencing of the *ambra1b* gene determines a reduction of PGCs, a condition that, in the zebrafish, leads to the development of all-male progeny. PGCs reduction was confirmed by knockdown experiments and rescued by injection of *ambra1b* and human *AMBRA1* mRNAs, but not *ambra1a* mRNA. Moreover, PGCs loss was not rescued by injection with *hAMBRA1* mRNA mutated in the CUL4-DDB1 binding region, thus suggesting that the interaction with this complex, but not with PP2A, LC3 and TRAF6 proteins, is involved in PGCs protection from loss. Results from zebrafish embryos injected with murine *Stat3* mRNA and *stat3* morpholino suggest that Ambra1b could indirectly regulate this protein through CRL4-DDB1 interaction. According to this, *Ambra1*$^{+/-}$ mice showed a reduced *Stat3* expression in the ovary together with a low number of antral follicles and an increase of atretic follicles, indicating a function of Ambra1 in the ovary of mammals as well. Moreover, in agreement with the high expression of *ambra1a* and *ambra1b* genes in the zebrafish testis and ovary, in these mutant lines we found a significant impairment of the reproductive process and pathological alterations, including tumors, mainly limited to the gonads.

Poster Presentation 8

Differential expression of sex reveal in brain and gonads of mullet (*Mugil cephalus*) at NGS transcriptome analysis

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INTRODUCTION

Next generation sequencing (NGS) techniques are having several applications. NGS assists to construct transcript and genomic library *de novo* without a reference animal sequence. The brain transcriptome analysis during the different stages of maturity of male and female is a novel approach to know sex differentiation in flathead mullet (*Mugil cephalus*). Present study has been carried out to know the differential expression of genes between male and female brain at the ovary and testis during the reproductively active fishes.

METHODS

Matured mullet has been captured from Pulicat Lake which is the second largest estuarine lake of India in the month of November 2021. Gonads of testis and ovary, and brain of male and female fishes sampled in RNAlater and proceeded towards separation, purification and qualitive check for RNA, and reverse transcripts to form cDNA library. Illumina NGS transcriptome library was prepared for the analysis.

RESULTS & DISCUSSION

The transcriptome library was prepared for the yolk laden vitellogenic ovary and spermiating testis of flathead mullet. The ovary and testis are showing the total of 16257 reads in which many are found to be non-annotated genes showing the non-significant expression. The ovary differentially expressed 7619 transcripts and the rest of the transcripts are specific to testis. In coherent with the gonadal expression, brain expressed 31579 transcripts together with male and female brains. The differential expression noticed with 748 genes between male and female brain. The differential expression of the sex specific transcripts will help to bring out the sex specific markers. Earlier study reveal that the sexual differentiation of the gonad is due to systematic and accurate coordination of the brain and gonad interactions through various signal mechanism. Although the inherent factors imprinted in genetic level, the governing control mechanism operated through brain is yet to be understood. The sexual/genetic expressional differentiation of brain controls the maturation of gonads at various level.

In addition, as per earlier investigators reported that the amh, dmrt, cyp19a, gsdf and sox9; FTZ-F1 and WT-1 are playing a crucial role for forming the testis and ovary. Hence, the present study inter-related to the specific gene expression at brain and gonadal level. The results form the basis for the exhibition of functional role of sex genes at brain and gonad at different maturity periods.

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SS2. Brain-pituitary-gonad axis

Poster Presentation 9

Sexual behavior and hormone gene expression in the Labyrinthici fish blue gourami (*Trichogaster trichopterus*) during reproduction

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INTRODUCTION

The blue gourami (*Trichogaster trichopterus*) belong to the Anabantidae family, which are ray-finned fish in the order Anabantiformes; they are commonly called labyrinth fish. Hormone control in males and female blue gourami along the gonadotropic brain-pituitary-gonad (BPG) axis are studied. Gene transcription is affected by environmental, biological, and behavioral factors.

METHODS

A proposed quality model is presented describing sexual behavior and hormone gene expression in male and female blue gourami labyrinth fish, for which relatively little information has been published.

RESULTS & DISCUSSION

In male based on gene transcription, gonadotropin-releasing hormone 1 (GnRH1) is involved in controlling spermatogenesis (spermatogonia to spermatids) via the BPG axis in non-reproductive and reproductive stages by controlling follicle-stimulating hormone (FSH), 11-ketotestosterone (11KT) and 17β-estradiol (E2). However, GnRH3 has a larger effect during the reproductive stage via the BPG axis (spermatids to sperm) on luteinizing hormone (LH), 11KT, and 17α-hydroxyprogesterone (17P). In the whole-brain transcriptome of female blue gourami, transcription of 17 genes changes during vitellogenesis in the brain. The hormones involved in reproduction in female blue gourami include: Kiss peptides (Kiss2 and its receptors 1 and 2 (KissR1 and 2)), GnRH1, 2 and 3, GnRH receptor, Pituitary adenylyl cyclase-activating polypeptide (PACAP) and PACAP-related protein (PRP), somatolactin, FSH, LH, growth hormone (GH), prolactin (PRL), E2, testosterone, vitellogenesis and 17α,20β-dihydroxy-4-pregnen-3-one (17,20P). A proposed quality model is presented regarding the sexual behavior and hormone gene expression in blue gourami males and females that has a Labyrinth organ about which relatively little information has been published.

Poster Presentation 10

Endocrine patterns during sexual maturation in female Pacific halibut (*Hippoglossus stenolepis*)

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INTRODUCTION

The Pacific halibut (*Hippoglossus stenolepis*) is a large migratory demersal flatfish species that occupies a top trophic role in the North Pacific Ocean and Bering Sea ecosystems, where it also supports various fisheries. Here we present results from recent studies conducted to investigate the endocrine signals that drive sexual maturation in female Pacific halibut.

METHODS

Female Pacific halibut (≥ 90 cm fork length) were captured in the Gulf of Alaska every month throughout an entire calendar year. Biological samples were collected for histology (ovary), gene expression (ovary and pituitary) and steroid hormone and vitellogenin determinations (plasma). Gene expression was quantified by quantitative real time PCR and steroid hormones and vitellogenin were quantified by enzyme-linked immunosorbent assay (ELISA). Biological samples from Pacific halibut females were classified into eight different female developmental stages based on histological characterization of oocyte developmental stages, as previously described (Fish et al. 2020. J. Fish Biol. 97: 1880-1885): primary growth perinucleolar (PGpn), cortical alveoli (CA), vitellogenic 1 (Vtg1), vitellogenic 2 (Vtg2), vitellogenic 3 (Vtg3), germinal vesicle migration (GVM), periovulatory (PO) and post-spawning (PS) stages.

RESULTS & DISCUSSION

First, we investigated the mRNA expression levels of gonadotropin beta subunits (*fshb* and *lhb*) in the pituitary and the mRNA expression levels of gonadotropin receptors (*fshr* and *lhr*) and key steroidogenic enzymes (*cyp19a1* and *hsd20b2*) in the ovary of Pacific halibut females at different stages during sexual maturation. Secondly, we measured the plasma levels of testosterone (T) and 17β-estradiol (E_2) in the same females. The mRNA expression levels of *fshb* in the pituitary increased gradually from the PGpn state until the Vtg3 stage, remained elevated until the PO stage and declined at the PS stage. The mRNA expression levels of pituitary *lhb* were low during the primary growth phase, increased slightly during the Vtg 1, Vtg 2 and Vtg3 stages, significantly increased at the GVM stage and remained elevated until the PS stage. The ovarian mRNA expression levels of *fshr* and *lhr* reached maximum values at the GVM and PO stages, respectively, immediately following the stage at which pituitary *fshb* and *lhb* mRNA expression first reached maximum values. The pattern of mRNA expression of *cyp19a1* in the ovary and of T and E_2 in plasma across the different female developmental stages followed quite closely that of *fshr* mRNA expression. Vitellogenin levels in plasma are currently being analyzed. The ovarian mRNA expression levels of *hsd20b2* gradually increased although at low levels until the PO stage, when a significant increase of *hsd20b2* expression was observed (>50-fold over the CA stage), decreasing subsequently at the PS stage. Our results indicate that increases in the mRNA expression levels of pituitary *fshb* during sexual maturation are shortly followed by increases in *fshr* and *cyp19a1* mRNA expression levels as well as concomitant increases in T and E_2 plasma levels, suggesting that FSH is likely acting on the ovary to stimulate the production of E_2. Furthermore, increases in the mRNA expression levels of pituitary *lhb* at later stages during sexual maturation are followed by increases in those of *hsd20b2*, that likely lead to the production of the maturation-inducing steroid. Overall, our data suggest that the vitellogenic phase and the oocyte maturation and ovulation phase are likely under the control of pituitary FSH and LH, respectively, during sexual maturation in female Pacific halibut.

Poster Presentation 11

In vivo effects of estradiol on the pituitary and testis of male European sea bass at different stages of spermatogenesis

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INTRODUCTION

The pituitary gonadotropins, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) are central endocrine regulators of gametogenesis in vertebrates. Their synthesis and secretion are regulated by brain and local factors, such as gonadotropin-releasing hormone (Gnrh). In addition, gonadal sex steroids have a feedback effect modulating the availability of gonadotropins. All these actions at the level of the pituitary have a direct impact on gametogenesis progression. Previous studies in sea bass showed that estradiol could reduce the stimulatory effect of Gnrh on gonadotropin production in primary cultures of pituitary cells. In this study, we have focused on the differential *in vivo* action of estradiol in males in pre-gametogenesis, early gametogenesis before meiosis, and initial stages of gametogenesis including phases after meiosis entry.

METHODS

Adult male European sea bass were obtained from a stock raised at the facilities of the Aquaculture Institute Torre de la Sal (IATS, 40º NL). For the *in vivo* experiments, E_2 implants were prepared with silastic medical grade elastomer (Dow Corning Corporation) and placed intraperitoneally in male sea bass to give a concentration of 25 □g of E_2/ g of fish. Control animals had empty implants. Treatments lasted from 10 to 30 days depending on the experiment and were done i) in July when testes are composed of proliferating type A spermatogonia (SgA), ii) in October, in testes with differentiated spermatogonia before entering meiosis, iii) in November, in testes that had just entered meiosis. At the end of the experiments, animals were sacrificed, blood was taken for hormone assessment, pituitaries were removed and stored at -80ºC for gene expression and hormone analysis. Gonads were taken also for GSI calculation and histological processing. Levels of Lh and Fsh were measured by homologous competitive ELISA. Plasma levels of E_2 were measured by EIA. The pituitary expression patterns of *esr1*, *esr2a* and *esr2b* during spermatogenesis as well as *fshb* and *lhb* in implanted fish by RT-qPCR.

RESULTS & DISCUSSION

Annual expression of the estrogen receptor genes (*esr1*, *esr2a* and *esr2b*) in male sea bass pituitary showed different profiles, and has been analyzed in relation with the stages of spermatogenesis, the pituitary gonadotropin content and the circulating levels of sex steroids. In the implanted animals we have seen different effects of E_2 depending on the gonadal stage of development. In pre-gametogenic animals, in summer, E_2 promoted a GSI increase while in animals in spermatogenesis E_2 treatment led to a decrease in GSI, indicating that E_2 blocks SgA entry in spermatogenesis but stimulates proliferation of undifferentiated SgA. E_2 inhibited Fsh at all levels: *fshb* expression, Fsh content in the pituitary and Fsh release, while the impact of E_2 on Lh, both pituitary expression of *lhb* or Lh content, varied depending on the gonadal stage. In all cases E_2 inhibited androgen production probably by inhibition of gonadotropins.

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Poster Presentation 12

Zebrafish NKBa modulated the brain-pituitary-gonad axis in female stinging catfish (*Heteropneustes fossilis*): An in vivo study

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INTRODUCTION

Tachykinins (TKs) are a family of peptides that are distributed along the central and peripheral nervous system. The most known tachykinin peptides are neurokinin A (NKA), substance P (SP), and neurokinin B (NKB). In mammals, neurokinin B (NKB) is a short peptide encoded by the gene tac3. It is involved in the brain control of reproduction by stimulating gonadotropin-releasing hormone (GnRH) neurons, mainly via kisspeptin. Recently, two tac3 genes have been identified in some teleosts, likely resulting from the teleost-specific whole genome duplication (3R).

METHODS

Acclimatized sexually mature catfish of pre-vitellogenic phase (March-April; Fish weight =50-60g; GSI=2.6g%) were divided into 5 groups (N=10 fish per group), and the fish were i.p injectioed with of 1, 10 and 100ng/g BW zfNKBa peptide. Vehicle control group fish were injected with fish saline. After 24 hr, fish were anesthetized with 0.01%MS222 solution and were decapitated to dissect their brains and ovaries. Tissues (brain, ovaries (100mg) were snap frozen in ice, stored in RNAlater at 4ºC and then stored at -20ºC till processed for gene expression. A similar experiment was conducted with another group of fish to collect pituitaries (~15 pituitaries per sample, n = 5 per group) group-wisefor estimation of gonadotropins.

RESULTS & DISCUSSION

In the present study, injection of zebrafish NKBa stimulated dose-dependently catfish brain *vt, it, cyp19a1b, gnrh2, kiss2,* and pituitary *lhβ, fshβ* and *gpα* expression suggesting a role of zfNKBa in the regulation of the reproductive axis of the catfish *Heteropneustes fossilis.* A dose-dependent biphasic effect of NKBa on *vt* gene expression was observed in the ovary compared with control, whereas *it, cyp19a1a, sgIIb, gnrh2* and *kiss2* expression were significantly increased. In the catfish *Heteropneustes fossilis,* the addition of zfNKBa showed differential expression patterns of the genes *vt, it, cyp19a1b, cyp19a1a, gnrh2, kiss2* in both brain and ovary, and *lhβ, fshβ* and *gpα* in the pituitary in a concentration dependent manner under in vivo condition. The present study suggests the involvement of this peptide in regulating reproductive phase along the Brain-Pituitary-Gonadal axis.

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Poster Presentation 13

Reproductive physiology of *Epinephelus marginatus* in captivity: hormonal therapies applied to the induction of sexual maturation in pre-spawning phase

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INTRODUCTION

The Dusky Grouper, *Epinephelus marginatus*, is an endangered species with great potential for aquaculture. However, when kept in captivity, this hermaphrodite species presents reproductive dysfunctions, limiting the success of its large-scale rearing. The following study aims to understand the physiological mechanisms involved in the hypothalamic-pituitary-gonad axis during the sexual maturation of this species, testing the use and efficacy of long-term sustained hormone release systems to induce gonadal maturation in captivity.

METHODS

Twenty-eight adult females were induced with long-term sustained hormone release systems with gonadotropin releasing-hormone analogs (GnRHa). These animals were divided into four experimental groups: control (CTR), 25ug GnRHa, 50ug GnRHa, and 100ug GnRHa. The doses of hormone were injected intramuscularly in the upper portion of the dorsal fin. The injections were repeated every two weeks during the experimental period (70 days), with a total of five injections. After the experimental period, the animals were anesthetized and euthanized. Blood, gonads, brain, and pituitary were sampled for molecular (brain, pituitary, and gonads), histology (gonads), and blood (sexual hormone profile) analyzes. Additionally, seven adult females were sampled before the start of induction with long-term sustained hormone release systems (zero).

RESULTS & DISCUSSION

Initial analyses suggest that the sex steroid profile has changed, particularly the 17□-estradiol concentration. The histological analyses of the gonads did not indicate morphological changes after induction. These data are under analysis. The samples were processed to conventional PCR for DNA amplification of their respective target genes. The quantification of these genes was performed in Real Time analysis using *primers* developed and patterned for *E. marginatus*. In the present, only gonadotropins, *fsh* and *lh*, and *ef1*□ have already been performed and were specific for *E. marginatus*. We are designing and testing new specific primers for *gnrh* and *cyp19a1b* (brain tissue), and *sox9, dmrt1, amh, foxl2, 20*□*-hsd,* and *cyp19a1a* (gonads tissue) for *E. marginatus*. These last primers will be specific for this species, and it is the first time that they will be described for *E. marginatus*. It is expected that the results of this study will show whether the endocrine disruption observed in animals kept in captivity is due to changes in the gene expression of neurohormones, gonadotropins, and gametogenesis regulators, which coordinate this reproductive axis. All this information is important for improving the artificial reproduction of *E. marginatus* through controlled breeding programmers, and these results may support future studies on Brazilian aquaculture of marine teleost species.

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Poster Presentation 14

Molecular mechanism of sexual plasticity in a natural sex changing fish

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INTRODUCTION

Teleostean sex change is a dramatic example of sexual plasticity, with the phenotypic and transcriptional ontogenies of hormone driven intersex developments being quite similar among vertebrates. Hermaphroditism has been documented in about 2% of all extant fish species. The bamboo leaf wrasse *Pseudolabrus silboldi* is a diandric protogynous labrid fish. The female spawns almost every day during the 2-month spawning period, and has a diurnal rhythm in oocyte growth, maturation, ovulation, and spawning. However, during absence of male, the largest female in the group can transform into functional male. These features make this fish a good model for studying the endocrine control of sexual plasticity. Gonadal sex change requires simultaneous changes in germ cells (e.g. sperm and their associated processes) and their surrounding somatic cells. Due to extreme histological and structural similarity between sex reversed gonochoristic fish and hermaphrodite fish, it is suggested that some common progenitor contributes to the sexual transformation. In this regard, it was recently found that the gonad houses a special population of stem cell which are increasingly considered as the precursor of germ cell in the adult gonad. So, in the present study, we investigated the potential of gonadal stem cells in sexual plasticity maintenance in fish.

METHODS

Adult wrasse fished out from sea (5-15 m depth) and transported to the laboratory. Males (brown color) and females (orange red color) were placed in 80L tanks @ 1:6. Special care was taken to keep 1 large and 5 relatively small females in each tank. After the acclimatization period, sampling was conducted at different time points (based on previous female to male sex reversal data), and pituitary, gonad and serum was collected for various analysis. Cell sorting was performed using gonadal single cell suspension to obtain various types of gonadal stem cell. Further single cell library was constructed, and transcriptomic analysis was performed. Simultaneously, pituitary and whole gonadal transcriptome were analyzed and validated.

RESULTS & DISCUSSION

For the first time, we have found two different types of stem cells, hereafter named as germline stem cell (GSC) and undifferentiated somatic cells (SSC) from ovary, testis and intersex fish. Notably, we have found similar cell population of GSCs in medaka (gonochoristic model species), and upon transplantation, these GSCs differentiated into both testicular or ovarian germ cell depending on the surrogate host. Interestingly, our experimental wrasse histology, cell sorting, and -omics analysis suggests that the cellular and molecular characteristics of both cell types are different from germ and somatic cells, which highlights that, both these cell populations are the starting point and prime candidate for sexual plasticity. Owing to the similarity among vertebrate sexual development, in-depth analysis of sexual re-differentiation associated molecular cues and dynamics of these cells are pertinent to unravel the sexual and plasticity process, thereby ensuring higher reproductive security. Further, we are also examining the molecular connections between pituitary and gonad during sex change.

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Poster Presentation 15

Acute stress affects differentially the expression and daily variations of gonadotropin-inhibitory hormone (GnIH) and its receptor in the brain of the European sea bass (*Dicentrarchus labrax*)

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INTRODUCTION

After the discovery of gonadotropin-inhibitory hormone (GnIH) in the Japanese quail in 2000, GnIH orthologs have been identified in other vertebrate classes, including fish. This neuropeptide seems to exert pleiotropic actions and has been involved not only in reproduction but also in other functions such as feeding, sexual/social behaviour and the stress response. Although the influence of stress on the GnIH system has been reported in birds and mammals, scarce information is available up to date in fish. To fill this gap in, we examined whether acute stress could affect the GnIH system in European sea bass.

METHODS

This study explored the potential role of GnIH as a mediator of stress-induced reproductive dysfunction after an acute stressor challenge (air exposure for 60 s) in male European sea bass, *Dicentrarchus labrax*. One hour after air exposure, brain was collected and frozen at -80ºC until analysed. Seven fish per experimental group (control and stressed), fed in the middle of the light phase (ML), were sampled at approximately every 4 h, during a 24 h cycle. The daily expression of GnIH and GnIH receptor was analysed using real time quantitative PCR with specific primers already tested in our laboratory.

RESULTS & DISCUSSION

Our results showed marked effects of acute stress on the GnIH system of sea bass, which were dependent of the brain area and the time of the day. Thus, in the telencephalic regions, GnIH expression exhibited marked daily variations, with higher nocturnal transcript levels, which were significantly increased in stressed animals. This stress-induced increase in GnIH expression was much more evident in the preoptic/hypothalamic regions, rising both nocturnal and diurnal GnIH transcripts after acute stress. In the midbrain/hindbrain regions, acute stress altered the daily pattern of GnIH expression, which peaked at daytime, when GnIH mRNA levels were also higher compared to control animals. Our results suggest that brain GnIH populations could respond in a regional-dependent manner to an acute stress challenge in sea bass and could have a differential contribution to the effects of the stress on the reproductive axis and other functions (e.g., feeding, behaviour) in this species.

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Poster Presentation 16

Gonadotropin expression, pituitary and plasma levels during gametogenesis of wild and captivereared greater amberjack (*Seriola dumerili*)

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INTRODUCTION

The greater amberjack (*Seriola dumerili*) is a species with a great potential for the Mediterranean aquaculture industry, due to its excellent flesh quality and worldwide consumer acceptability. A comparative study of wild-caught and captive greater amberjack was carried out to compare the endocrinological status of the pituitary-gonad axis related with reproductive developmental stage of this species, which usually exhibits reproductive dysfunctions in captivity.

METHODS

A total of 33 (14 males and 19 females) individuals from the wild and 24 (12 males and 12 females) wild-caught as juveniles and reared in captivity were sampled at three different times of the reproductive cycle. The fish were sampled for biometric data (fork length - FL, body mass - BM, gonad mass - GM), and blood, gonads and pituitaries were collected. Pituitary and plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were measured using ELISAs. Pituitary gonadotropin gene expression levels were evaluated using qPCR. After histological examination of the gonads, data were grouped according to reproductive stage of development as “developing”, “spawning capable”, “regressed” and “regenerating”.

RESULTS & DISCUSSION

Significant differences in pituitary FSH and LH content between spawning and developing wild females was observed, as well as between captive and wild females at the spawning capable stage. However pituitary *fshβ* and *lhβ* expression levels and plasma FSH and LH remained mostly unchanged both for the captive and wild females among the different reproductive stages examined. The only significant difference was in plasma FSH levels between the captive and wild females at the spawning capable stage. Captive females appeared to be particularly sensitive to repeated handling, as they failed to successfully complete vitellogenesis, even though they were at the same initial stage of development during the first sampling time. In males, no differences were observed in pituitary FSH content, pituitary *fshβ* and *lhβ* expression or plasma FSH and LH levels among the reproductive stages of either captive or wild males. However, significantly higher levels of plasma FSH and LH were recorded between captive and wild developing and spawning capable males, as well as pituitary LH content in spawning capable males. The latter increased significantly in wild males from developing to spawning capable, but not for the captive males, where an increase was observed between developing and regressed stages. On the other hand, significantly lower *fshβ* expression levels were recorded in wild males at the developing stage compared to captive males. In addition, they seemed to face reproductive dysfunctions in captivity even without handling, since differences from wild ones existed already at the first sampling.

The project received funding from the European Union 7FP (GA 603121, DIVERSIFY).

Poster Presentation 17 (student)

Interaction of Gonadotropin Inhibitory Hormone with FSHβ and LHβ: *in-vivo* and *in-silico* analysis in Indian freshwater murrel (*Channa punctatus*)

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INTRODUCTION

Gonadotropin Inhibitory Hormone (GnIH), hypothalamic neuropeptide, belongs to the RFamide family. Its inhibitory actions on synthesis and release of gonadotropins are mediated directly via its receptors (NPFF1 and NPFF2) located on GnRH neurons and on gonadotropes. Dopamine is a well-documented functional antagonist of GnRH in many teleosts that inhibits synthesis as well as release of gonadotropins. But in many species of the order perciformes this dopaminergic inhibition has not been demonstrated. The present study has been conducted on murrel, *Channa punctatus* that belongs to the order perciformes, to investigate the interaction of GnIH with gonadotropic hormones (FSH and LH).

METHODS

Adult female specimens of murrel were collected from the river Yamuna (Delhi) and acclimatized to laboratory conditions. Blood was collected every month and processed for estimation of gonadotropins in plasma. Brain and pituitary were excised over a period of one year and immediately processed for extraction of RNA. Degenerate primers were designed using the sequences retrieved from Genbank database to amplify *gnih, fsh-β* and *lh-β* genes. The annual gene expression profile of these genes was assessed using quantitative Real time PCR. In addition, retrieved sequence of GnIH peptide was synthesized and various doses were administered to study effect of GnIH peptide on transcript levels of genes encoding for gonadotropins in pituitary as well as gonadotropin levels in plasma. Various bioinformatics tools and molecular dynamic (MD) simulations were employed for characterization, homology modelling, interaction, and dynamic behavior of these neuropeptides.

RESULTS & DISCUSSION

The GnIH nucleotide sequence encodes a 199 aa precursor peptide containing three conserved LPXRFamide peptides. Both FSHβ (118aa) and LHβ (154aa) belong to the glycoprotein hormone beta chain superfamily. The expression of *gnih* gene was maximum during the resting phase of the reproductive cycle and mRNA levels started reducing with gonadal recrudescence. FSH and LH levels in plasma were inversely related with *gnih* transcripts. Minimum expression of *gnih* gene corresponded with surge of LH in plasma which culminates into ovulation. GnIH peptide administration to a gravid female during prespawning phase significantly reduces the mRNA levels of *fsh-β* and *lh-β* in pituitary after 6 hours of treatment. Even the titre of FSH and LH also decreases in plasma. Exogenous administration of synthesized GnIH suggests that probably it inhibits the synthesis and secretion of gonadotropins. The 3D homology structures for all the three genes were generated and validated. Protein-protein docking study was performed and the docked complexes of GnIH with FSHβ and LHβ were subjected to MD simulation which shows stability of docked complex and hydrogen bonding during a 50 ns simulation run.

The protein-protein interaction of GnIH with FSHβ and LHβ, indicates that the inhibitory action of GnIH may be mediated directly through gonadotropins. The variable expression of these proteins during different phases of the annual reproductive cycle suggests a potential role of GnIH as a functional antagonist in the HPG axis of murrel.

Poster Presentation 18 (student)

Insulin like growth factor 1 (IGF1) in the Hypothalamus-Pituitary-Gonad-liver (HPG-L) axis: Gene expression and *in silico* analysis in the Indian freshwater catfish (*Heteropneustes fossilis*)

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INTRODUCTION

Insulin like growth factors (IGF1 and IGF2) are involved in various physiological functions and are regulated by the somatotropic (GH- IGF) axis. Liver is the main source of IGF1 which is produced after the stimulation of GH from the anterior pituitary. In addition, liver is also the main site for synthesis of vitellogenin and choriogenin under the influence of estradiol-17β (E2). Hence, liver plays a vital role in the reproductive process influencing the HPG axis. The present study has been conducted on *H. fossilis* to correlate expression of *igf1* in brain, liver and ovary with GSI during annual reproductive cycle. Further interaction of IGF1 with GH and LH has been interpreted using *in silico* analysis.

METHODS

Fishes were collected from nature and acclimatized to the laboratory conditions. During pre-spawning phase, fish were administered with various doses of GnRH and GH. Blood was collected at different time points and processed for estimation of IGF1 and LH. Brain and ovary were excised and processed for RNA extraction. In addition, brain, liver and ovarian samples were also collected every month which were processed immediately for RNA isolation. cDNA was synthesized and degenerated primers were designed for amplification of *igf1* and *lhβ*. Relative expression of genes was analyzed by real-time PCR. Bioinformatics tools and software were used to interpret the interactions between IGF1, GH and LH.

RESULTS & DISCUSSION

Phylogenetic analysis of IGF1 deduced amino acid sequence suggests that it is a highly conserved protein and belongs to the Insulin/IGF superfamily. Although, no significant difference in the titre of LH as well as IGF1 in the plasma was observed on administering either GH or GnRH but, the expression of *lhβ* and *igf1* was significantly high in brain with GH. But, expression profile of *igf1* in brain, liver and ovary throughout the annual gonadal cycle elucidates that IGF1 plays a pivotal role in HPG-L axis. In brain and liver transcripts of *igf1* were significantly high during post-spawning phase (October to January) but downregulated in ovary. Post-spawning phase is a resting period for fish wherein food energy is mainly used for somatic growth. Further, at the beginning of gonadal recrudescence, mRNA of *igf1* was high in brain and liver but declined sharply and remained low during oocyte development. After attainment of maximum GSI, *igf1* started increasing during late prespawning and spawning phase in both the tissues. Whereas, in ovary, *igf1* expression exhibited direct correlation with gonadal growth. At the onset of oogenesis transcript levels of *igf1* increased and significant increase was observed during uptake of vitellogenin into the oocytes. Upregulation in ovary during oogenesis indicates energy shift from somatic to gonadal growth as synthesis of egg-yolk precursors is high energy consuming process. Even in fish with fully grown oocytes, high transcript levels were maintained till the oocytes spawned, suggesting its role in maintenance of postvitellogenic oocytes. Spawning of the oocytes coincides with downregulation of *igf1* in ovary. The docked 3D complexes of IGF1-LH and IGF1-GH indicate formation of hydrogen bonds and salt bridges at the interface area. Taking together *in silico* analysis and annual profile of *igf1* it can be speculated that IGF1 plays a pivotal role in somatic as well as gonadal growth.

Poster Presentation 19 (student)

FSH secretion and fish feeding are directly regulated by a novel regulator

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INTRODUCTION

In order to meet the increasing demand for fish production in aquaculture, it is necessary to optimize fish reproduction and growth. In all vertebrates, reproduction is controlled by two gonadotropin hormones secreted from the pituitary; Follicle stimulating hormone (FSH) and Luteinizing hormone (LH), which controls gonadal growth and final maturation, respectively. While Gonadotropin-releasing hormone (GnRH) from the hypothalamus regulates both LH and FSH secretion in fish, other factors that regulate FSH secretion remain unclear. In this study, we identified a long-sought FSH regulator in fish - namely, Cholecystokinin (CCK), a neuropeptide and gut hormone that plays a central role in the regulation of digestion and satiety. Thereby it has a key role in identifying a direct link between feeding and reproduction.

METHODS

Our model fish is the Nile tilapia (*Oreochromis niloticus*). In a cell-specific transcriptome, a novel CCK receptor was identified as the highest-expressed receptor in FSH cells. The specific localization of the novel CCK-R was verified by in situ hybridization. Using reporter assays on COS-7 cells expressing the receptor, we validated its activation by tilapia CCK peptide. Through intraperitoneal injections of tiCCK and measurements of FSH levels in the blood, the direct relationship between CCK and FSH secretion was examined in-vivo.

RESULTS AND DISCUSSION

The expression of the novel CCKR in the pituitary of Nile tilapia had been further validated using in situ hybridization, revealing high and specific expression of the receptor, mainly on FSH cells. FSH secretion was significantly increased in Nile tilapia after tiCCK injections, supporting the hypothesis that CCK is a trophic hormone that directly stimulates FSH release. There was also a direct effect on tilapia feeding, as the injected group consumed less food. Compared to non-fed fish, we found a direct effect on FSH levels in the blood, supporting the hypothesis that CCK increases after feeding lead to FSH secretion. These novel findings may lead to the development of CCK as an effective modulator of FSH and contribute to our understanding of the control of fish reproduction in aquaculture.

The project received funding from the U.S. National Science Foundation and the U.S.-Israel Binational Science Foundation Joint Funding Research Grants (# 947541) and Israel Science Foundation (# 1540/17).

Poster Presentation 20

Central and peripheral control of reproduction in zebrafish Adenomatous Polyposis Coli ($APC^{+/}$) mutant line

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INTRODUCTION

Several Colorectal cancers result from mutations in adenomatous polyposis coli (APC) which activates the Wnt/β-catenin signaling pathway and represents the primary transforming event in cancer. Moreover, zebrafish that are heterozygous for a truncating APC mutation, spontaneously develop intestinal, hepatic and pancreatic neoplasias. Thus, while the occurrence of gastrointestinal cancers in APC mutants has been largely described, the effects of the Wnt/β-catenin signaling pathway activation on reproduction has not been investigated.

METHODS

Apc $^{(+/+)}$ and Apc $^{(+/-)}$ adult were paired to obtain embryos. Embryo genotyping was performed and larvae were grown till 6 months, while monitored for the appearance of morphological shifts, color changes or alteration of the reproductive behavior. At sampling, part of the brains and ovaries were dissected out and fixed in Bouin's fixative for histology or stored at -80°C for molecular analysis. The remaining ovaries were teased into separate follicles using transfer pipettes without trypsinization; thereafter, follicles were separated into different maturation stages according to their diameters and class IIIb and IV follicles were collected and stored at −80 °C for molecular analysis.

RESULTS & DISCUSSION

In this study, the comparison of results obtained from apc $^{+/+}$ and apc $^{+/-}$ sibling female, allowed the investigation of the role of APC protein mutation in female zebrafish reproduction. Follicular stage count was performed on ovarian hematoxylin eosin stained sections and a slight increase of the number of previtellogenic follicles associated to a reduction of vitellogenic and maturing ones, was observed in mutant ovaries. At central level, a deregulation of the *kiss-gnrh3* system was observed in mutants, with an increase of *gnrh3* and a downregulation of *kiss* mRNA isoforms. Notheworthy is the upregulation of two cannabinoid receptors, *cnr1 and gpr55* and the dowregulation of *cnr2*, which could affect the levels of gonadotropin levels as suggested by *fshr and lhcgr* mRNA changes in class IIIb mutant follicles. In this regard, a deregulation of FSH level could be hypothesiszed as responsible of the accumulation of previtellogenic oocytes documented by histology. The lack of changes in progesterone receptor mRNA levels suggests that oocyte maturation is not affected by APC mutation, which condition, on the contrary seems to favor meiosis resumption, as suggested by *ccnb1* mRNA increase in class IV $^{+/-}$ follicles. Altogether the results suggest that although the quality of the different follicle classes is not affected by the mutation, it causes an impairment of ovarian growth as suggested by the increase of previtellogenic follicle and the downregulation of *inhbaa*. In this contest, the role of the endocannabinoid system, also at peripheral levels, and the modulation of the Wnt/β-catenin signaling pathway will be further investigated.

The project received funding from the National Research Council of Thailand within the Research project "Manipulation of the endocannabinoid system for cancer treatment: zebrafish as a model".

Poster Presentation 21

Sex-specific responses within the gonadotropic axis of Mozambique tilapia to growth hormone, prolactin, and luteinizing hormone

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INTRODUCTION

The Mozambique tilapia (*Oreochromis mossambicus*) is an established teleost model for aquaculture- and endocrine-related research that exhibits sexually dimorphic growth. Here, we describe sex differences in how growth hormone (GH), prolactin (PRL), and luteinizing hormone (LH) regulate the gonadal expression of factors underlying growth and reproduction.

METHODS

Adult male and female tilapia of the same size were hypophysectomized (Hx) and subsequently administered oGH, oPRL, and/or oLH (5 µg/g body weight) over the course of 5 days via intraperitoneal injections. At the conclusion of the hormone treatments, we determined gonadosomatic index (GSI) prior to assessing gonadal morphology through histological techniques. Next, we quantified the gene expression of the GH receptor (*ghr*), insulin-like growth factors (*igf1*, *-2*, and *-3*), estrogen receptors (*erα* and *-β*), and androgen receptors (*arα* and *-β*).

RESULTS & DISCUSSION

Hypophysectomized females exhibited ovarian follicular atresia together with a diminished GSI. The combination of oLH and oGH restored morphological conditions and GSI to patterns observed in shamoperated controls. In males, GSI followed a similar pattern to that observed in females whereas gonadal *ghr* expression responded differently to hormonal treatments between sexes. In testes, *ghr* remained unchanged following Hx but was inhibited by all hormone treatments containing oLH. Ovarian *ghr* expression was reduced following Hx and recovered by oGH and oLH. Both sexes exhibited reduced *igf1* expression following Hx. In testes, *igf1* was most strongly restored by oGH while ovarian *igf1* was restored by oPRL. In females, but not males, *igf2* was reduced following Hx and recovered by oGH. The *igf3* was dramatically reduced following Hx in both sexes with a partial recovery elicited by oGH and oLH in males or the combination of oLH with oPRL or oGH in females. oGH increased the gonadal expression of both *era* and *-β* in males, but not in females. Lastly, *arα* expression was not impacted by Hx; nonetheless, *arα* was stimulated by oLH in males. Following hypophysectomy, the expression of *arβ* increased in males but decreased in females. In females, the combination of oLH with either oPRL or oGH recovered *arβ* levels to those of sham-operated controls.

Our findings indicate that GH and LH act synergistically to regulate ovarian functions in tilapia. The higher *ghr* expression in ovaries supports gonadal development in females and the regulation of *igf3*. Sex-dependent and sub-type specific responses of *ers* and *ars* to oGH, oPRL, and oLH reflect differences in how steroid hormones regulate germ cells in males and females. In conclusion, gene transcripts associated with growth and reproduction exhibit sex-dependent regulation by GH, PRL, and LH, and therefore, provide a basis for sex-specific roles of the pituitary gland in tilapia and likely other species that exhibit sexually dimorphic growth.

The project received funding from NIFA Hatch (#HAW02051-H), NOAA (#NA18OAR4170347), NIH (1R21DK111775-01), and NSF (IOS-1755016 and -1755131).

Poster Presentation 22 (student)

The influence of specific recombinant gonadotropins on the initial stages of gametogenesis in male and female European eels (*Anguilla anguilla*)

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INTRODUCTION

The European eel is a species that, despite being critically endangered, has a high consumer demand. Reproduction in captivity is the only alternative to reduce the pressure on wild populations, but its complex reproductive physiology makes it difficult. Recombinant human chorionic gonadotropin (rhCG) has proven effective in inducing male eel maturation, but the same treatment does not work with females, and the administration of carp or salmon pituitary extracts is needed. This research includes new experiments using eel-specific recombinant gonadotropins (rGths: rFsh and rLh) and the study of their effects on the early gametogenesis of both male and female European eels.

METHODS

Homologous single-chain rGths were produced in a Chinese hamster ovarian cell line by Rara Avis Biotec S.L. Immature eels were acclimatized from freshwater to seawater for 7 days before the 4-weeks hormonal treatment. Males were weekly administered 6 µg/fish of rFsh or rLh, and weekly samplings were carried out. Females were weekly administered 8 µg/fish of rFsh or rLh, or 8 µg rFSH + 2 µg rLh per fish, and samplings were done after 1 and 4 weeks of treatment. Control groups received weekly physiological solution. For both sexes, blood samples were extracted for steroid analyses (E2, T and 11KT), biometric parameters (gonadosomatic and eye indexes; GSI, EI) were registered, and the stage of gonadal development was determined by histology. Some supposed females resulted being males due to the difficulty distinguishing sexes while they are immature and similar in size.

RESULTS & DISCUSSION

In males, rFsh treatment increased biometric parameters, and caused testis maturation and synthesis of androgens, while rLh slightly affected the biometric parameters and synthesis of androgens. When both rGths were administered, higher values of GSI and steroids, as well as an advanced stage of gonadal development, were found. Our results indicate that the administration of rFsh, and specially rFsh+rLh, shows better results in male maturation. In females, rGths did not induce changes in biometric parameters or oocyte maturation, but higher levels of E2 and 11-KT were found in the treatment with a combination of both gonadotropins. This result supports the role of androgens during the early phases of eel oocyte development. According to other studies, higher doses, longer treatments of rFsh+rLh and the use of larger females could be crucial to achieve ovarian development.

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Poster Presentation 23

Single-cell RNA sequencing reveals the changes of gene expression in the pituitary of sterile female rainbow trout

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INTRODUCTION

Reproduction is driven and controlled through the physiological network of the hypothalamic-pituitarygonadal (HPG) axis. Reproductively sterile fish are not only of production interest to the aquaculture industry, but also excellent resources to study the response of the HPG axis to the absence of gonadal development. Here, we describe the changes in gene expression in the pituitary of mature sterile female rainbow trout (*Oncorhynchus mykiss*) using scRNA-seq.

METHODS

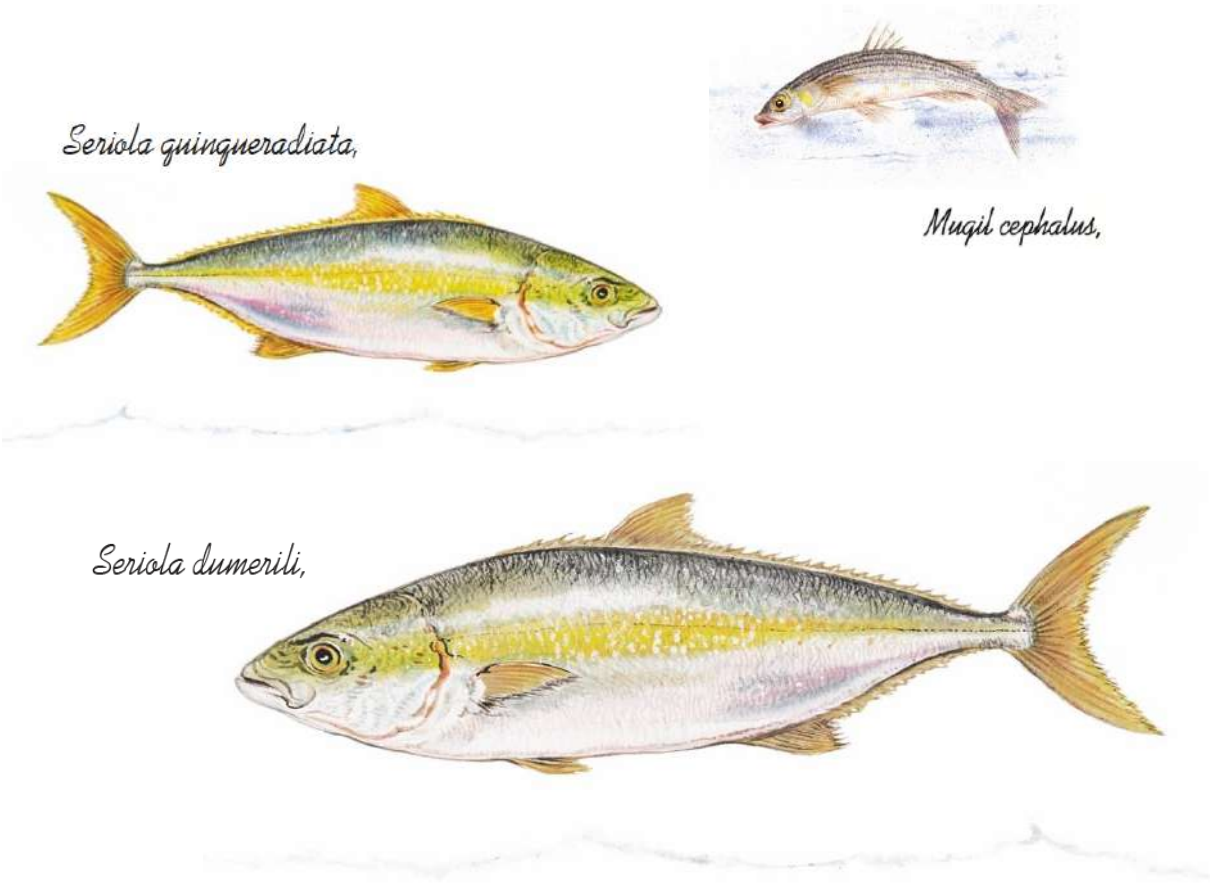
Sterile rainbow trout were achieved by immerse-treating eggs with morpholino-Vivo conjugates targeting at *dead end* gene. Pituitaries from sterile along with fertile females were dissected, dissociated and subjected to scRNA-seq (10X genomics) during their spawning season at 36 months old. Cell quality control, clustering, data integration and differentially expressed genes (DEGs) identification were performed using Seurat pipeline in R.

RESULTS & DISCUSSION

Ultimately, 4,325 and 5,975 cells for fertile and sterile samples, respectively, were filtered out and used for downstream analysis. In total, 14 cell clusters were identified in both fertile and sterile samples according to conserved marker genes of different pituitary cell populations. These include nine hormone-secreting endocrine cell types, namely lactotropes, somatolactotropes, somatotropes, thyrotropes, Fsh-gonadotropes, Lh-gonadotropes, gonadotrope-like cells, melanotrope, and corticotropes. Additionally, two potential progenitor clusters, *isl1+* group and *prop1+* group, red blood cells, a cluster with characteristics of immune cells, and an uncharacterized cluster were also identified. Among major hormone-producing cells, Fsh-gonadotropes represented a substantially higher proportion (23.1%) of pituitary cells in sterile fish compared to that in fertile fish (5.5%). On the other hand, Lhgonadotropes account for only about 2.5% of the pituitary cells in sterile fish, but approximately 8.3% in fertile fish. In consistent with cell population changes, *fshb* expression was significantly higher in both Fsh-gonadotropes (1.5-fold) and gonadotrope-like cells (5.5-fold) in the pituitaries of sterile fish, compared to fertile fish, while *lhb* expression was dramatically decreased in both Lh-gonadotropes (77.4-fold) and gonadotrope-like (90.1-fold) pituitary cells of sterile fish, to a deficient level. These changes in pituitary suggested that sex steroids, as probably the most prominent change in sterile fish, have inhibitory effects on Fsh production and stimulatory effects on Lh production. Overall, gonadotropes, including Fsh- and Lhgonadotropes and gonadotrope-like cells, had more DEGs than other cell types. In Fsh-gonadotropes, strikingly, most of the DEGs detected were downregulated in sterile fish pituitary in response to the absence of developed gonad, including nuclear receptors (*nr1d4b*, *nr1d1*, *nr1d2a*, *nr2f2*, *hr38*, *nr4a1*, *nr4a3*, *nr5a1b*, *erb2*), and other transcription regulators/factors (*egr1*, *egr3*, *egr4* and *egr4l*).

The project received funding from the University of Maryland, Baltimore County (STRT7TEN-1113).

SS3. Oogenesis/vitellogenesis and ovulation



Poster Presentation 24

Recombinant Fsh and Lh for the reproductive control of *Mugil cephalus* from previtellogenesis to fertilized tank spawning: hormonal inductions and transcriptomic analysis

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INTRODUCTION

Growth in flathead grey mullet (*Mugil cephalus*) aquaculture is limited as in intensive hatchery conditions; females remain arrested at previtellogenesis or early-vitellogenesis and males do not produce fluent sperm. The aim of the present study was to use long-term treatment with recombinant follicle stimulating hormone (rFsh) and luteinizing hormone (rLh) to induce gonadal development.

METHODS

In the first study, females ♀ (n = 9) and males ♂ (n = 3) received rFsh (15 µg kg^{-1}) injections on a weekly basis. In two additional studies, one in 2018 (n = 9 ♀, n = 4 ♂) and another in 2020 (n = 21 ♀, n = 9 ♂) combined rFsh (6 to 12 µg kg^{-1}) and rLh (2.5 to 24 µg kg^{-1}) doses were administered. Recombinant Fsh was applied during the first stages followed by a subsequent decrease with an increase of rLh to regulate late gametogenesis. To induce oocyte maturation and ovulation, priming rLh (15 or 30 µg kg^{-1}) injection and resolving Progesterone (P_4) (40 mg kg^{-1}) injection (rLh + P_4), or priming and resolving rLh (30 µg kg^{-1}) (rLh + rLh) were given. Spawning success, embryo presence, and hatching were registered. In 2018, oocytes were collected from five females at four stages of gonadal development for bulk RNA sequencing. Controls (total n = 27 ♀, n = 13 ♂) received saline injections or medium without hormone.

RESULTS & DISCUSSION

Males spermiated with fluent milt when treated with rFsh and rLh (n = 13 from 2018 and 2020). The rFsh application induced vitellogenesis to a maximum 425 ± 19 µm oocyte diameter, but did not complete vitellogenic growth. The co-application of rLh with rFsh at mid-vitellogenesis completed vitellogenesis (~600 µm) in all the females (29 fish) that received the complete treatment to demonstrate rGths induced the process of oogenesis. In 2020, 30 µg kg^{-1} rLh + P_4 (n = 9) and rLh + rLh (n = 6) induced 89 % and 100 % spawning (including reproductive behaviour), respectively, with 63 ± 21% eggs with embryo, and 58 ± 23 % hatching. Transcriptome analysis showed that the differentially expressed genes observed were typical of natural oogenesis in other fish species. During vitellogenesis, upregulated genes were enriched in pathways related to steroidogenesis, lipids uptake, and production of energy. The upregulation of pathways related to behaviour and the synthesis of C-21 steroid hormone suggests that while steroidogenic activity for the completion of vitellogenesis was still taking place, the oocyte was preparing for further processes regulated by Lh and the expression of its receptors. Control fish did not show further gonadal development. These results indicate the possibility of controlling reproduction of *M. cephalus* from early gonadal stages to fertilized tank spawning using rFsh and rLh.

The work was funded by the Spanish Government, MINECO (RTI2018-094710-R-I00 and PID2021126070OR-100) and EU (GA 603121, DIVERSIFY), AQUAEXCEL2020 (GA652831), supported by IMBBC-HCMR computational resources. S.R. had a PhD grant (FI-AGAUR) co-financed by the ESF.

Poster Presentation 25

Six years of experimentation to optimize the artificial reproduction protocol for European eel: What worked and what didn't?

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INTRODUCTION

In 2016, the Eel Reproduction Innovation Centre was launched, a collaborative initiative of Wageningen Livestock Research with the Dutch eel sector, united in the sustainable eel foundation DUPAN. The major aim of EELRIC is to close the production cycle of the European eel in order to supply eel aquaculture with glass eels and to alleviate fishery pressure on the natural population. Now, we are able to produce larvae batches three times per week but larval mortality and deformity rates can be high. Over the past years we have executed many experiments aiming to condition glass eels into high quality broodstock females and to optimize the artificial reproduction protocol.

METHODS

Feminization was applied as developed for Japanese eel by Chai et al. (2010) and involved feeding with 17β estradiol (E2) coated pellets for 5-7 months during the early elver stage. Feminized eels (> 300 g) were subjected to a ~3,000 km simulated migration as originally described by Mes et al. (2016). Simulated migration was also combined with a single CPE injection (20 $mg.kg^{-1}$), or with a dopamine antagonist implant (eticlopride 5 mg; Jolly et al., 2016). Males were matured by just a single hCG injection (1,000 IU; Kahn et al., 1987) followed up by 2nd injection 24 h before stripping. Females were matured by weekly CPE injections (20 $mg.kg^{-1}$) followed up by a CPE booster at ~10% body weight increase and 17α,20β-dihydroxy-4-pregnen-3-one (DHP; 2 $mg.kg^{-1}$) injection at ~20% body weight increase. Tested were implants with 17 methyltestosterone (17MT; 5 mg), E2 (2 mg) and 17MT+E2 combined (Palstra et al., 2022) as pretreatment of the weekly CPE injections, as well as a single hCG injection of 3,000 $IU.kg^{-1}$ (based on Tuan Nguyen et al., 2020). Three experiments have been executed testing weekly injections with recombinant FSH (recFSH: 6 or 12 µg) and LH (recLH: 10 or 20 µg) replacing CPE treatment.

RESULTS & DISCUSSION

Feminization provides 99% females of which 90% reaches 300 g in 12-30 months. Simulated migration makes these feminized eels silver (larger eyes, increased GSI). In combination with a single CPE injection the GSI increased further but not up to values indicating the onset of vitellogenesis, eticlopride did not give any additional effect to simulated migration. Both 17MT as E2 implants gave significant increase of GSI and oocyte diameter, the combined implant worked synergistically in advancing vitellogenesis (GSI of 6). Also, pretreatment with a single hCG injection showed yolk deposition in the oocytes. Both methods decreased the number of weekly CPE injections that were necessary to mature the eels. For the first time, European eel larvae were produced with recombinant gonadotropins but dose and durations still need optimization (see abstract Jéhannet et al.).

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Poster Presentation 26 (student)

Glyphosate chronic exposure impairs vitellogenesis and affects female zebrafish reproduction

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INTRODUCTION

Glyphosate, the active compound of several herbicide formulations, is commonly used for weed control in crops. Despite it was predicted to possess no action against organisms other than plants and bacteria, some evidence demonstrated its detrimental effects especially on aquatic animal species. Since its application widely increased over the years, its accumulation in the environment represents a concrete and severe risk for both wildlife and human health.

METHODS

Fish were exposed through the diet to 0.5 mg/kg/body weight/day (G1), defined by the EFSA as acute reference dose, 5 (G2) and 50 mg/Kg/body weight/day (G3), displaying no observable adverse effect (NOAEL), for three weeks, and results were compared to those of an untreated control group (C). During sampling, liver, ovaries and body weight were measured and the hepatosomatic and gonadosomatic indexes were computed. In order to analyze ovary follicle classes number, Hematoxylin and Eosin staining was performed on histological sections of paraffin embedded samples. Class III and IV follicles were also separately collected during sampling to perform expression analysis of master genes involved in reproduction, while in livers the expression of the seven vitellogenin isoforms and the estrogen receptors mRNA levels were evaluated.

RESULTS & DISCUSSION

Hepatosomatic index (HSI) ad gonadosomatic index (GSI) did not show differences among female exposed to different doses of glyphosate and C. Similar to HSI and GSI, also the frequency of follicles at different maturation stages was not affected by glyphosate exposure. On the contrary, the molecular analysis evidenced significant changes regarding genes involved in oogenesis. Gene expression analysis of class IIIb follicles revealed that the highest dose of glyphosate led to an increase of the gonadotropin receptor (*fshr* and *lhcgr*) and estrogen receptor transcripts (*esr1* and *esr2a*), suggesting the estrogenic effect of this xenobiotic. Progesterone receptor (*pgrmc1* and *pgrmc2*) levels resulted instead unaffected. No changes were observed in female exposed to G2 and G3 doses. Considering class IV follicles, no alteration of genes expression was observed, even if a similar trend to class III was evident for all genes analyzed. Moving to the hepatic expression of all the seven vitellogenin isoforms, the highest glyphosate dose decreased the expression of *vtg1*, *vtg2*, *vtg3* and *vtg4*, while the lowest dose caused a decrease of *vtg3*, *vtg4* and *vtg7*. Being hepatic vitellogenin levels under the control of estrogen receptors (ERs), the expression of *esr1* was evaluated and significant reduction was observed in all the treated groups compared to C, in agreement with the vitellogenin expression reduction. Additional investigations are in progress to better elucidate the effects of glyphosate on zebrafish female reproduction.

The project received funding from Fondo Ateneo 2022 to OC and FM.

Poster Presentation 27

Structural modelling of vitellogenin C protein in the Indian freshwater murrel, *Channa punctatus*

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INTRODUCTION

Three-dimensional (3D) structures of proteins are important to understand the molecular basis of their function and interaction with ligands. Prediction of 3D model by *in silico* methods is preferred as compared to time consuming techniques viz. X-ray crystallography and Nuclear magnetic resonance spectroscopy. Therefore, generation of three-dimensional structure of protein from its amino acid sequence by comparative or homology modelling is the most reliable method. Multiple vitellogenins (Vgs), female-specific egg-yolk precursor proteins, are synthesized in the liver of fish in response to estrogens. Complete vitellogenins (VgA, VgB) consist of all the yolk protein domains- Lv, Pv and β′c. On the other hand, incomplete vitellogenin or phosvitinless (VgC) consists of only Lv molecule lacking Pv and other yolk protein domains at C-terminal end. We have reported all the three types of Vg proteins (VgA, VgB, VgC) in murrel on the basis of their structure and presence of domains. In the present study, full-length cDNA of *vgc* from the Indian freshwater murrel was sequenced, 3D structure of derived protein sequence was predicted and validated by web-based softwares.

METHODS

Multiple amplicons of *vgc* were amplified using degenerated primers and assembled to a large single sequence. Rapid amplification of cDNA ends (RACE) technique was employed for sequencing the 5' and 3' ends of *vgc* sequence. The primary, secondary and 3D structure of VgC protein were modeled using bioinformatics tools like Expert Protein Analysis System (http://www.expasy.org/), SOPMA and I-TASSER. The best model for VgC was selected on the basis of threading sequence identity and confidence score (C-score). The reliability of model was validated by Procheck, WHATIF server and Errat analysis.

RESULTS & DISCUSSION

The complete nucleotide sequence of *vgc* was 4126 bp, contained initiation codon (ATG), open reading frame (ORF) of 3816 bp encoding CDs of 1272 amino acids, stop codon (TGA) with a 294 bp 3′untranslated region (UTR) and polyA tail. The predicted pI and molecular weight of VgC were 8.40 and 141 kDa respectively. The 15 aa long signal peptide sequence (MQELLLCGLVALATC) was predicted at N-terminal end, which helps in translocation of the protein to the endomembrane system and its secretion outside the cell. Alanine was major amino acid present in deduced protein sequence of VgC in murrel which is also a main constituent of lipovitellin. The secondary structure of complete protein sequence of VgC showed the presence of α helix (47.48 %), β turns (3.3 %), random coils (35.85 %) and extended strands (13.36 %). The best 3D model for VgC generated by I-TASSER web server was selected as the best structure which had a confidence score (C-score) of -0.43 with RMSD value and TM score of 10.5 ± 4.6Å and 0.66 ± 0.13, respectively. Then, the structure was submitted to YASARA server for energy minimization and the minimum levels of energy showed the model stability. Ramachandran plot analysis for the predicted VgC structure showed that 96.4% residues were present in the allowed regions, suggesting that the predicted model was reliable in terms of their backbone conformation. ProSA calculated the quality score for protein structures, wherein predicted Z-scores values was -9.28, evidencing highly reliable structure. The deduced primary sequence of VgC showed homology with VgC protein, purified and characterized from plasma (Pipil *et al.*, 2015), suggesting that the VgC in the blood of murrel is the translated product of the *vgc* cDNA in the liver.

Poster Presentation 28

The ovarian maturation changes during the main spawning season of largehead hairtail (*Trichiurus japonicus*) in Jeju Island from the Republic of Korea

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INTRODUCTION

Largehead hairtail (*Trichiurus* spp.) is one of the most important commercial fish species in Korea. They have synchronous maturation in all stages of oocytes at the same time and spawn multiple times in the main spawning season. Also, they react sensitive to changes in the marine environment and migrate accordingly throughout their life cycle. They migrate between the growth area and the spawning area according to changes in the environment. For these reasons, it is not easy to understand their reproduction. The Jeju Island region is a major fishing area for *T. japonicus* in Korea. However, their resources are decreasing, and the population is getting smaller due to overfishing and climate change. Nevertheless, the annual ovarian maturation of *T. japonicus* lacks detailed data on migration in the Jeju Island area. In this study, the final goal is to predict the spawning season of mature and immature females. As the first step, we investigated mature and immature female ovarian maturity during the main spawning period of largehead hairtail migrating in the Jeju Island area in 2022.

METHODS

Mature females (pre-anal length 45.2 cm ± 4.41) and immature females (pre-anal length 22.5 ± 1.3 cm) were investigated in the main spawning period from April to September. The monthly maturity level was determined based on the gonadosomatic index (GSI), hepatosomatic index (HSI), condition factor (K) and absolute fecundity. Ovary tissue was fixated with formalin and staining was performed with H&E. Ovarian maturity levels were confirmed by oocyte diameter, using microscopy and computer imaging systems. In addition, changes in blood estrogen concentrations have been identified from June to September. All data were significance differentiation verified at the $P < 0.05$ level using One-way ANOVA of statistics using SPSS 24.

RESULTS & DISCUSSION

As a result, mature females maintained a GSI level more than twice as high as immature females throughout the main spawning season. Mature females showed high ovarian maturity in May and August. The result pattern is similar to the proportion of mature oocytes in ovarian tissue. Immature females confirmed that the GSI level gradually increased from June to September. The percentage of mature oocytes increased in August and September. Blood estrogen concentration also showed similar pattern to GSI and histological results. These results suggest that mature females may spawn multiple times around May and August, during the main spawning season. Immature females were not all individuals mature enough to participate in spawning in August. However, some rapidly mature individuals suggest the possibility of participating in spawning throughout August and September. In the future, to identify more detailed maturation patterns, we will need more diverse sex hormone measurements such as LH and FSH. These results are expected to be important basic data on ovarian changes during the main spawning season of largehead hairtail which migrated around the Jeju area.

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Poster Presentation 29

Clonal gametogenesis is triggered by intrinsic stimuli in the hybrid's germ cells, but is dependent on sex differentiation

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INTRODUCTION

Interspecific hybridization can trigger the transition from sexual reproduction to asexual reproduction, but the specific reasons behind this change in reproduction mechanisms for hybrids are not yet well-understood. In many asexual hybrids, gametogenesis involves a stage of premeiotic endoreplication (PMER), where each chromosome duplicates before undergoing canonical meiosis. This process helps prevent issues related to improper chromosome pairing and ensures the clonality of offspring. Our study focused on fish hybrids from the *Cobitis taenia* complex, which have previously been reported to either be sterile (in the case of hybrid males) or reproduce clonally (in the case of hybrid females).

METHODS

We conducted a study where we transplanted triploid male gonial cells from interspecific hybrids into diploid parental species, and also transplanted diploid male gonial cells from parental species into hybrid fish. After a two-year period, we spawned potential chimeras and subjected them to multiple diagnostic methods, including microsatellite analysis, observation of pachytene/diplotene chromosomes, histology, and ploidy measurement.

RESULTS & DISCUSSION

When transplanted into clonal females, non-hybrid SSCs underwent regular meiosis and produced normally reduced gametes. However, when the hybrid's SSCs were transplanted into sexual males, it led to sterility. On the other hand, when the same hybrid SSCs were transplanted into sexual females, they maintained their ability to undergo asexual development and produce clonal eggs. This finding suggests that asexual gametogenesis is under complex control, where somatic gonadal tissue indirectly affects the execution of asexual development by determining the sexual differentiation of stem cells. Once such cells develop into female phenotypes, hybrid germ cells trigger PMER from their intrinsic signals.

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Poster Presentation 30 (student)

Potential role of Samd4 binding to *cyp19a1a* promoter involve in regulation of ovarian granulosa cells in the protogynous hermaphroditic ricefield eel (*Monopterus albus*)

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INTRODUCTION

The TGF-β and steroidogenesis signaling pathways co-regulate the development of ovarian follicles and the maintenance of fertility. Smad4 (mothers against decapentaplegic homolog 4) is a downstream effector of the TGF-β signaling pathway in the ovary of ricefield eel (*Monopterus albus*). Cyp19a1a is a key enzyme in the process of converting androgens into estrogen, and it interacts with the TGF-β and steroidogenesis signaling pathways. To date, potential role of Samd4 binding to *cyp19a1a* promoter involving in regulation of ovarian granulosa cells in the protogynous hermaphroditic ricefield eel have not been reported.

METHODS

In this study, we cloned the sequence of Smad4, and analyzed its expression patterns in different tissues by qPCR (quantitative polymerase chain reaction) and immunohistochemistry. We also identified the binding sites between Smad4 and the promoter of *cyp19a1a* using dual luciferase reporter system.

RESULTS & DISCUSSION

Our results show that Smad4 encodes 449 amino acids with the three typical conserved domains of SMAD protein families. Then, *smad4* was differentially expressed in different developmental stages of ovaries, and regulated by gonadotropin--FSH and hCG. Smad4 presented in oocytes, granulosa cells, and thecal cells, and co-localized with *cyp19a1a* in granulosa cells of middle and late vitellogenic follicles. Additionally, two Smad4 binding sites were identified in the proximal promoter region of *cyp19a1a*, namely SBE1 and SBE2. Mutation of SBE1 or SBE2 attenuated the activity of the transcription of *cyp19a1a* promoter in Chinese hamster ovary cells, and it appeared that the SBE1 site had higher binding activity by the dual-luciferase assay. Taken together, these findings suggest that Smad4 is involved in ovarian development, possibly by regulating both the oocyte and follicular cells, and may activate *cyp19a1a* in the ovarian granulosa cells during vitellogenesis in ricefield eel.

The project received funding from the National Natural Science Foundation of China (grant numbers 31972777, 2019; 31402286, 2015) and China Scholarship Council (202106915017).

Poster Presentation 31 (student)

The impact of genetic and karyotype divergence on gametogesis in laboratory obtained F1 hybrids of spined loach (Cobitis)

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INTRODUCTION

Hybridization has a great impact on reproductive outcomes and gametogenesis. The fate of the progeny, its fertility, and the type of reproduction are conditioned mainly by the divergence between hybridizing species. Hybridization may lead to the emergence of fully fertile progeny, sterile or inviable progeny. Less frequently, hybridization may lead to the formation of fertile hybrids with clonal reproduction. Spined loaches (*Cobitis*) are freshwater fishes that are known for frequent hybridization events in nature that gave rise to the so-called all-female asexual lineages. To evaluate the role of chromosomal and genetic divergence on reproductive outcome, we crossed eight species of different genetic divergence and analysed the gametogenesis in obtained F1 hybrid males and females.

METHODS

Eight parental species were crossed to obtain xx families of F1 hybrids. Samples for analysis of gametogenesis (larvae, juveniles, and adult female gonads) were collected during different stages of gonadal development. Using antibodies against *vasa* protein, we visualized germ cells in fish gonads using confocal microscopy. We applied immunofluorescent staining to investigate the mechanisms ensuring chromosomal pairing and recombination in meiotic cells. Additionally, we evaluated the ploidy of diplotene oocytes. Male gonads were used for flow cytometry to identify the presence of sperm cells. The exome-capture method and morphological analysis of chromosomes were used to evaluate the genomic and karyotype divergence used. Statistical analysis was performed to sex differences in chromosomal pairing, and to estimate the range of reproductive outcomes in F1 hybrids.

RESULTS & DISCUSSION

The majority of analyzed hybrid females from 7 out of 11 cross combinations produced clonal diploid gametes due to premeiotic genome endoreplication. Genome duplication was not observed in all gonial cells but in a small population of gonial cells. In one cross-combination we observed the normal pairing of chromosomes in meiosis and the formation of haploid gametes in both males and females. In another cross type, only females were able to produce haploid gametes, while males were sterile. In the majority of F1 hybrid males, we identified only non-duplicated pachytene cells with aberrant pairing and no further progression to the formation of spermatids. Similar chromosomal mispairing we observed in non-duplicated pachytene cells in hybrid females. In two cross types, sterility was rather caused by the lack of transition of gonial cells from mitosis to meiosis. Based on genomic and karyotype data, we defined the range of divergence required for the emergence of different reproductive outcomes. Specifically, we presume sequence divergence of gametogenic regulatory genes facilitates premeiotic endoreplication in clonal females. In addition, karyotype divergence between species leads to hybrid sterility caused by chromosomal mispairing in meiosis in non-duplicated cells. Interspecific hybridization may thus commonly affect gametogenesis in a specific way, allowing the formation of unreduced oocytes. At least among loaches, the emergence of asexual gametogenesis is tightly linked to hybrid sterility and constitutes an inherent part of the speciation process.

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Poster Presentation 32

The true life-history of a Neotropical invasive Characiform fish: to be or not to be an intersex species

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INTRODUCTION

Acestrorhynchus pantaneiro is a Neotropical medium-sized, short-distance-migratory and an ichthyophagous fish with an elongated pike-like body that fits the opportunistic description according to Winemiller (1989) life strategies classification. This species displays features that make it successful in colonizing new habitats, being considered, in many cases, as an invasive fish. The aim of this study was to describe some aspects of the life-history traits of this species.

METHODS

The reproductive strategy, sexual system and growth of *A. pantaneiro* was evaluated at a floodplain lake located in the Lower Paraná River (Argentina) after two-year monitoring (April 2015 – April 2017) in a monthly basis, by analyzing data of male and female morphological parameters, gonadal histology and development, spawning, fecundity, and growth rate, together with the retro calculated birthdate of larvae and juveniles derived from counting otoliths increments.

RESULTS & DISCUSSION

Acestrorhynchus pantaneiro is an opportunistic species that performs short displacements improving their body condition due to its continuous feeding habits, showing plasticity in terms of habitat/distribution requirements. During the sampled period, the study of the gonadosomatic index, gonadal maturation rates and larvae/juvenile birthdate showed that this characin was a partial spawner, reproductively active from October to January, with most spawning activity observed in November (spring at Southern Hemisphere). It was evidenced that water temperature modulated gonadal maturation, but it was the river water level the synchronizing stimulus that triggered spawning. *A. pantaneiro* presented size bimodality and, consistently with the obtained growth curves, fish would reach the first autumn to winter months with approximately 120 mm standard length, already as mature males. However, we found some fish, over a broad size range, that showed a conspicuous cavity at the within the testes, where primary oocytes were found adjacent to the cavity. In more advanced intersex gonads, the female tissue gradually replaced male tissue, possibly corresponding to a hermaphrodite gonadal remodelling process. The first females with mature oocytes were found at 210 mm standard length, being sexually mature between the second and third breeding seasons. According to growth models, the biggest fish caught in this study, a female of 320 mm standard length, was around three years old. By the time of spawning, sex ratio was biased towards males, with few large females that would achieve better fitness by becoming highly fecund. This is the first integrative study that includes body-length frequency distribution, sex differential size at first maturity and growth; and reports the presence of intersex gonads questioning its sexual system, and describing diagnostic features that allow the evaluation of a new hypothesis in which this characiform species could be a protandrous sequential hermaphrodite.

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Poster Presentation 33

Characterization of miRNAs in full grown follicles from the zebrafish ovary

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INTRODUCTION

Numerous studies have shown that microRNAs (miRNAs) are important regulators of ovarian development in mammals but by comparison there is limited information on their role in ovarian physiology of teleost fishes. This study set out to characterize miRNAs in full grown (FG) zebrafish follicles when treated with the steroid hormone 17α, 20β-dihydroxy-4-pregnen-3-one (17α,20βP) which promotes oocyte maturation and ovulation.

METHODS

Zebrafish full grown (FG) ovarian follicles were manually separated from other follicular stages and incubated *in vitro* for 1 hr in 500 µl of 60% L-15 culture medium with either 10 nM 17α,20βP or ethanol. miRNAs were extracted using the mirVana miRNA isolation kit (Thermofisher) and miRNA expressions were measured using the Advanced Taqman kit (Thermofisher). In follow up studies, FG follicles were incubated *in vitro* with 17α,20βP for 1, 3 or 5 hours or zebrafish were treated *in vivo* to waterborne 17α,20βP for 2 hr prior to the collection of FG follicles. Quantitative real-time PCR was used to identify the changes in miRNA (Qiagen miScript SYBR Green PCR kit) and mRNA (Sso Advanced SYBR Green PCR kit) between samples. Statistical analysis was done using R.

RESULTS & DISCUSSION

The microarray analysis identified 256 miRNAs with 18 upregulated and 6 downregulated (threshold of 1.8-fold change) following 17α,20βP treatment. Several of these differentially regulated miRNAs including dre-miR-181c, dre-miR181a-5p, dre-miR-430C, dre-miR 17a-5p and dre-miR731 were shown to have binding sites in genes involved in steroidogenesis (Cyp 19a1a, Cyp19a1b, StAR) and prostaglandin synthesis (ptsg2b, cPla2). The novel miRNA cgr-miR-1973 was also detected in FG follicles treated with 17α,20βP and this miRNA was only previously reported in the Chinese hamster ovary. Primers were developed and miRNAs that were successfully amplified and detected from a 1-hour 17α,20βP treatment in vitro included: dre-miR-181c, dremiR-181a-5p, dre-miR-430c, dre-miR-17a-5p, cgr-miR-1973, dre-miR-22a-3p and dre-miR-125a. When exposed to 17α,20βP for two hours, dre-miRNA-181a-5 showed a 2-fold increase (p=0.019) in average expression in while dre-miR-181c showed a 3-fold increase (p=0.010) in average miRNA expression. However, dre-miR-22a-3p and cgr-miR-1973 showed no significant change in expression after being exposure to 17α,20βP. Collectively, the findings of the current study support the overall hypothesis that miRNAs are involved in ovarian physiology in zebrafish ovary and that multiple miRNAs and targets exist.

The project received funding from NSERC.

Poster Presentation 34 (student)

Induction of *ptgs2a* and *ptger3* expression in ovarian follicles is essential for ovulation in Amur sturgeon (*Acipenser schrenckii*)

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INTRODUCTION

In general, final oocyte maturation in teleosts is regulated by luteinizing hormone (LH) and maturation-inducing steroids (MIS). Oocyte maturational and ovulatory competence are defined by the sensitivity of the MIS to reach oocyte maturation and ovulation, respectively. However, the molecular mechanisms underlying the acquisition of ovulatory competence remain unknown. We have previously shown that transcripts having high homology with *prostaglandin G/H synthase 2* (*ptgs2*) were upregulated in a limited manner in sturgeon that reached ovulation (ovulated sturgeon), which consequently induced ovulatory competence. In this study, we focused on two types of *ptgs2* and prostaglandin (PG) receptor genes and investigated their expression patterns in Amur sturgeon (*Acipenser schrenckii*) ovarian follicles during the ovulation process.

METHODS

Oocyte maturation and ovulation in the sturgeons used in this study were induced by LH-releasing hormone analog (LHRHa) priming injection (2 μg/kg body weight) and LHRHa high-dose injection (50 μg/kg body weight), 24 h after the priming injection. Two types of *ptgs2* cDNA (type A and B) were isolated from the ovarian follicles of Amur sturgeon. *Ptgs2a/b* mRNA levels in the ovarian follicles that were biopsied before and after priming/high-dose injections were measured by quantitative polymerase chain reaction (qPCR). Ovarian follicles that were sampled immediately before priming injection were incubated at 12℃ for 3, 6, 9, 12, 18, and 24 h in L-15 culture medium with or without 1000 ng/mL Amur sturgeon recombinant LH, and then mRNA levels of *ptgs2a/b* were measured in cultured ovarian follicles for each time point. Next, we screened the PG receptor genes upregulated by LHRHa injection only in the ovulated sturgeons with RNA-sequencing analysis using NGS reads and re-examined their changes in expression during induced ovulation by qPCR.

RESULTS & DISCUSSION

The amino acid sequences of Ptgs2a and Ptgs2b were similar, with 96% homology. Both Ptgs2a and Ptgs2b had amino acids essential for arachidonic acid binding, peroxidase activity, and cyclooxygenase activity, although multiple mutations between these sequences were found, especially in the signal peptide sequence, membrane-binding and catalytic domain. *In vivo*, *ptgs2a* mRNA levels in the ovarian follicles were upregulated with priming injection only in the ovulated sturgeons. *In vitro*, mRNA expressions were induced by recombinant LH. Meanwhile, *ptgs2b* mRNA levels were lower than *ptgs2a* both *in vivo* and *in vitro*. RNA-sequencing analysis showed that *prostaglandin E2 receptor EP3 subtype* (*ptger3*) was the only PG receptor gene induced by priming injection in ovulated sturgeons. qPCR analyses indicated that *ptger3* mRNA levels were elevated by priming injection and reached the highest level 8 h after the high-dose injection only in the ovulated sturgeons. These results suggest that *ptgs2a* transcriptions are important for ovulatory competence induction, and that ovarian follicles with ovulatory competence acquire the ability to induce *ptger3* during the late ovulatory process. This study suggests that the expression of PG/PG receptor systems is the most important condition influencing whether the sturgeon could reach successful ovulation or not.

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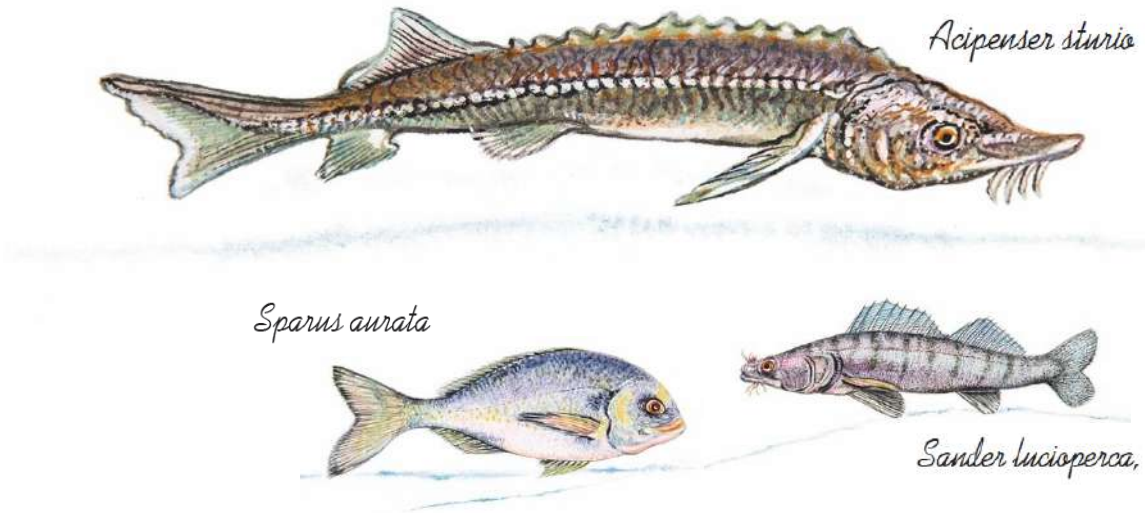
SS4. Spermatogenesis and spermiation



Poster Presentation 35 (student)

Steroidogenic activity of anti-Müllerian hormone in European male sea bass (*Dicentrarchus labrax*)

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INTRODUCTION

The Anti-Müllerian hormone (AMH) promotes the regression of Müllerian ducts during male sex differentiation in higher vertebrates, and signals through a heterodimeric receptor complex. Despite the absence of Müllerian ducts, teleosts present *amh* orthologous genes, whose encoded hormone participates in sex and gonad differentiation in males and shows a role in adult male and female gonads. In the gonads of European sea bass (*Dicentrarchus labrax*), *amhr2* expression shows little change during the annual cycle, while *amh* shows the highest level in early and final stages of spermatogenesis, and in post-vitellogenesis. Recombinant European sea bass Amh (sbAmh) was produced and its functionality was confirmed through its capability to activate the Amh signalization pathway (Smad pathway) in transfected COS-7 cells containing sea bass Amhr2.

METHODS

Explants of pre-meiotic testis of adult fish were cultured *in vitro* and treated with recombinant Amh. *In vivo* experiments were carried out by intramuscular injection of sbAmh expression plasmid to males in pre-meiotic phases. Species-specific antibodies for sbAmh and Amhr2 allowed their detection throughout the male reproductive cycle by Western Blot and their localization in testis of adult males at different stages of development by immunohistochemistry. Intracellular signaling of European sea bass Amh/Amhr2 and potential crosstalk between pathways was analyzed in COS-7 cells cotransfected with an expression vector containing sea bass Amhr2 and the reporter plasmids pBRE-Luc (Smad pathway) or pCRE-Luc (cAMP pathway/ steroidogenic pathway) and then treated with different concentrations of sbAmh, combined with the selective BMP-signaling inhibitor LDN-193189 or the cAMP pathway inhibitor Rp-cAMPS. The ability of sbAmh to interact and activate other receptors of the Bone Morphogenetic Protein (BMP) family was also tested by using the COS-7 cell system.

RESULTS & DISCUSSION

Contrary to the known inhibitory activity of Amh in teleost steroidogenesis, the addition of Amh in testis explants from European sea bass increased steroid production, which was blocked by adding the Rp-cAMPS inhibitor. In the same line, muscle injection of an *amh* expression plasmid resulted in an increase of circulating 11-KT in the injected animals. These results were confirmed in the COS-7 cell system. In addition to the canonical Smad pathway, the European sea bass Amh/Amhr2 pair is able to signal through the main steroidogenic pathway (cAMP pathway), as seen by activation of the reporter plasmid pCRE-Luc. This activation was inhibited by the cAMP antagonist RpcAMPs. Inhibitors for Smad (LDN-193189) and cAMP (Rp-cAMPS) signaling were used to investigate intracellular cross-talk between these pathways. Amh and Amhr2 were detected by Western blot throughout the entire annual cycle, but showing different proteolytic forms and isoforms and suggesting a translational rather than transcriptional regulation for their actions. Amh was immunodetected in Sertoli cells surrounding early germ-cell generations while Amhr2 was found in undifferentiated SgA in post-spawning and premeiotic testis and in postmeiotic germ cells in more advanced stages, showing the paracrine role of this system communicating germ and somatic cells. Finally, alternative roles for Amh by signalling through other BMP receptors have been explored. Funded by Spanish MCIN/AEI/10.13039/501100011033/ and by ERDF a way of making Europe (RTI2018094667-B-C22 and PID2021-122929OB-C32). PhD contract GRISOLIAP/2020/129 (GV) supported A.M.

Poster Presentation 36

Testis mRNA expression in wild and hatchery-produced greater amberjack (*Seriola dumerili*)

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INTRODUCTION

The greater amberjack (*Seriola dumerili*) is a promising emergent aquaculture species. Males caught from the wild and reared in captivity exhibited small seminiferous tubules, a precocious arrest of spermatogenesis and high levels of apoptosis. In the present study, we report a comparative analysis of testis transcriptome of wild *versus* hatchery-produced fish, as part of a research aiming at describing the effects of rearing in captivity on gene expression throughout the reproductive axis.

METHODS

Three wild and 6 hatchery-produced greater amberjack males were sampled on 31 May - 01 June 2021. Wild fish were caught around Lampedusa (Sicily, Italy) and sampled immediately. Hatchery-produced fish belonged to a broodstock produced from fertilized eggs obtained through hormonal induction of spawning and reared in a sea cage at Argosaronikos Fishfarming S.A. (Salamina, Greece). Fish reproductive state was evaluated by gonado-somatic index (GSI) and gonad histological analysis. Total RNA was extracted from testis samples, checked for quantity and quality and then used to prepare the mRNA libraries. A pooled sample was then submitted to sequencing through the Illumina NextSeq platform (Illumina Inc., U.S.A.) using paired-end 2x75 strategy. Sequencing raw data were qualitychecked, cleaned and aligned onto the greater amberjack reference genome using state of the art bioinformatics tools as well as in house scripts. Read counts per gene and differential gene expression analysis was carried out and pathways involving differentially expressed genes (DEGs) were investigated using KEGG (http://www.genome.jp/kegg/pathway.html).

RESULTS & DISCUSSION

Testes from wild greater amberjack (WIDL) showed normal spermatogenic activity. Among the six hatchery-produced fish, four showed normal spermatogenesis (non-dysfunctional farmed fish, NF) and two showed evident reproductive dysfunction (dysfunctional farmed fish, DF), characterized by low GSI, smaller seminiferous tubules and reduced spermatogenic activity. The three groups underwent a comparative transcriptome analysis using the RNA-seq technology. Nine libraries were produced, each of them resulting in the production of 25 million paired-end reads. About 90% of reads were uniquely mapped to the reference genome. A high number (2157) of DEGs was found between WILD and DF groups and between DF and NF groups (1986). The analysis of KEGG pathways evidenced that the observed reproductive dysfunction was associated to cell death, cell cycle and proliferation pathways. The present study improved our understanding of the molecular mechanisms underlying spermatogenesis impairment of greater amberjack reared in captivity. Ongoing analyses of pituitary and hypothalamus transcriptome will provide a comprehensive view of the effects of confinement in captivity on the reproductive axis of this species.

The study was funded by the European Union´s Programme H2020, project NewTechAqua, GA 862658.

Poster Presentation 37 (student)

Thyroid hormones deficiency impairs male germ cell development: a cross talk between hypothalamus-pituitary-thyroid, and gonad axes in zebrafish

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INTRODUCTION

In vertebrates, thyroid hormones are critical players in controlling different physiological processes such as development, growth, metabolism among others. There is evidence in mammals that thyroid hormones are also an important component of the hormonal system that controls reproduction, although studies in fish remain poorly investigated. Here, we tested this hypothesis by investigating the effects of methimazole-induced hypothyroidism on the testicular function in adult zebrafish.

METHODS

To induce hypothyroidism, thirty adult males were chemically exposed to methimazole (1mM), an antithyroid agent, for 21 days. As controls, fish were kept in water without treatment (negative control), or some were exposed to methimazole + T4 (T4 was added in the water from the second week until the end of exposure) (recovery group) for 21 days. After treatment, we evaluated the effects of thyroid hormones deficiency by histomorphometrical analysis of germ cells, testicular gene expression (*thrα*, *thrβ*, *fshr*, *cyp17a1*, *insl3*, *igf3*, *amh*, *gsdf*, *nanos2*, *dazl*, *sycp3l*, *odf3*), as well as 11- Ketotestosterone (11-KT) plasma levels. 11-KT release capacity of zebrafish testicular explants were evaluated after 18h *ex vivo* culture in the presence or absence of Fsh (100 ng/mL) from methimazole, negative control and recovery group. Subsequently, we investigated the methimazole-induced hypothyroidism on zebrafish brain and pituitary by evaluating expression of selected genes (*gnrh2*, *gnrh3*, *gnih*, *crf*, *lhβ*, *fshβ*, *tshβ*). Finally, zebrafish testes from methimazole exposure and negative control were incubated with Fsh (100 ng/mL), T3 (100 nM), or Fsh+T3 for 7 days, and histomorphometrical analysis of germ cells were carried out.

RESULTS & DISCUSSION

Treatment with methimazole increased proportion area occupied by type A_{und*}, type A_{diff} and type B spermatogonia and decreased spermatozoa number, while the proportion of meiotic and postmeiotic cells were not affected. Further, plasma 11-KT levels decreased significantly following treatment with methimazole, as well as the expression levels of several testicular and hypothalamicpituitary genes, suggesting that thyroid hormones deficiency impaired gonadal function. On the other hand, co-treatment with T4 restored zebrafish spermatogenesis and spermatozoa production, but increased the proportion of meiotic and post-meiotic cells, suggesting that THs are involved directly or indirectly with meiosis entry in the zebrafish testis. With respect to the steroidogenic capacity, our results showed that Fsh did not stimulate androgen release by testes of methimazole exposed fish, as compared to negative group. Also, in long-term culture, methimazole nullified the action of Fsh, T3 or Fsh+T3 on germ cell development. The impairment of spermatogenesis induced by methimazole can be correlated with significant changes in transcript levels for genes implicated in the control of reproduction. Altogether, these results reinforce the hypothesis that thyroid hormones are an essential element of multifactorial control of reproduction and testicular function in zebrafish and possibly other vertebrate species.

This project received funding from FAPESP (19/22997-3, 20/03569-8 and 22/13097-1).

Poster Presentation 38

Two genes coding for gonadal soma-derived factors act in early gametogenesis in European sea bass (*Dicentrarchus labrax*)

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INTRODUCTION

Puberty is the developmental period during which an individual becomes sexually mature for the first time and its regulation is not completely known in teleosts. The gonadal soma-derived factor (Gsdf) gene was found to be downregulated in precocious testis of male European sea bass (*Dicentrarchus labrax*) and proposed as an early gonadal marker of puberty. The genome of sea bass contains two *gsdf* duplicates, *gsdf1* and *gsdf2*, whose encoded proteins share 87% identity and have gonad-specific expression. Gsdf belongs to the transforming growth factor beta superfamily, is exclusively found in teleosts and it is apparently involved in the proliferation of type A spermatogonia.

METHODS

All the animals were kept under natural photoperiod and temperature conditions at the Instituto de Acuicultura Torre de la Sal (IATS) facilities. Adult specimens of sea bass (5-years-old) were sampled monthly during an entire annual reproductive cycle. One-year-old male European sea bass were sampled every two weeks from August to November. In each sampling only the smallest 15% (Small group) and the largest 25% (Large group) fish were selected for analysis. Gonads from all fish were staged according to gonadal histology. Gene expression was assessed by RT-qPCR. Sea bass Gsdf were detected in Western blot or immunohistochemistry with a species-specific antibody.

RESULTS & DISCUSSION

One-year-old males from the Small group did not arrive to full spermiation and showed higher *gsdf1* expression than the ones of the Large group during all the experiment time, with significant differences in August and October. Most of the animals of the Large group spermiated the next winter. In the present study, we have confirmed that expression of *gsdf1* and *gsdf2* decreases in testis of 1-year old sea bass males that enter precocious puberty compared with their siblings that remained immature.

In adult males, *gsdf1* showed maximum expression levels in premeiotic (immature) testis, that decreased as spermatogenesis progressed. The expression of *gsdf2* followed the same trend but was 5-fold lower. This expression profile matched with the presence of Gsdf protein in testis extracts, and with its location in Sertoli cells surrounding type A spermatogonia.

In adult females, mRNAs from *gsdf1* and *gsdf2* are present to a much lower level than in testis, and the highest levels correspond to *gsdf2* in post-ovulatory ovaries and also in isolated follicular cells. In fact, Gsdf1/2 was located in follicular cells surrounding previtellogenic oocytes.

Sea bass *gsdf1* and *gsdf2* are located in the same chromosome, and their coding sequences are placed in different strands and transcription directions. The functional data obtained so far point to common regulatory elements for both genes, but also to a sex-specific regulation for *gsdf1* and *gsdf2* connected to males and females respectively.

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Poster Presentation 39

Effect of dietary n-3 polyunsaturated fatty acids supplementation of *Astyanax lacustris* males on semen quality.

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INTRODUCTION

The success of fish reproduction in captivity depends on the quality of the gametes. High-quality gametes are structurally well-formed, possess fertilization capacity, and generate living descendants. In captivity, the production of these gametes can be controlled by environmental factors, such as photoperiod, water temperature, or spawning substrate; in addition, the nutritional and physiological conditions of the breeders, in particular, have a direct effect on the quality of the gametes and consequently on the performance of the fish reproductive. The yellowtail-lambari, *Astyanax lacustris*, is a small rustic species, with a fast life cycle and high productivity in intensive cultivation due to its ease of handling and high prolificacy. It is considered a model species for research, in addition to having great economic importance in the live fish market and for human food. This study aimed to verify whether the inclusion of omega-3 polyunsaturated fatty acids (PUFAs-ô3) in the diet of *A. lacustris* males improves seminal quality.

METHODS

For this, 500 fish were arranged in 20 boxes of 180 L in the recirculation system. These were fed at a ratio of 32% CP, with four levels of marine fish oil rich in PUFAs-ô3 (AGES-ô3) inclusion (In% = 0, 3, 6, and 9), for 105 days. After this period, males were hormonally induced to sperm, and semen was collected after 226 h/degree. The parameters evaluated were osmolality, seminal volume and color, concentration, morphology, sperm motility, membrane integrity, and sperm kinetic parameters: total and progressive motility, velocity curvilinear linearity and average, linearity and rectilinearity, coefficients of trajectory oscillation, head lateral displacement amplitude, and cross-beat frequency.

RESULTS & DISCUSSION

The seminal color varied from whitish to yellowish. The seminal volume was higher in the GC (0%) and I3% groups. The inclusion of AGEs-ô3 positively influenced the kinetic parameters, as I6% and I9% resulted in higher values for most of these parameters and did not differ statistically from each other, but significantly different from GC and I3%. Thus, as reinforced by other studies, this is an important effect because these kinetic parameters are used as indicators of sperm quality and are highly correlated with fertilization capacity, as they allow the sperm to find and penetrate the micropyle more quickly. Thus, it is concluded that the addition of PUFAs-ô3 to the feed of breeders significantly improved the seminal quality of *A. lacustris* males.

This project was supported by São Paulo State Research Foundation (FAPESP; Proc. 2015/10115-5), National Council for Scientific and Technological Development (CNPq; Proc. 4342832018-5), and the Coordination for the Improvement of Higher Education Personnel (CAPES) for their scholarship granted Proc. 88887.605023/2021.

Poster Presentation 40 (student)

Analysis of body composition of wild male *Mugil cephalus* in association with reproductive stage

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INTRODUCTION

The aquaculture of *Mugil cephalus* (Linnaeus, 1758) presents difficulties mainly due to problems in controlling the reproductive cycle in captivity. Gonadal development is influenced by the nutrients stored in the body of the broodstock and the determination of these nutrients in wild individuals at the various reproductive stages of the gonads maybe one way to establish an appropriate broodstock diet and enhance reproductive development in captivity.

METHODS

We evaluated fat, protein, moisture and ash content of wild males from May to August. A total of 30 reproductively mature individuals (total length >40 cm) were collected in the Ionian Sea (6-10 each month). The classification of the reproductive stages was made using histological sections of the ovaries according to Mañanos et al. (2008) and Mylonas & Zohar (2009).

RESULTS & DISCUSSION

In May, males had mainly immature gonads, and matured gradually during June and July, presenting their full maturation in August, concomitantly with significant increases in gonadosomatic index. Males with immature gonads had the lowest fat content while at the early spermatogenesis and spermatogenesis stages it began to increase, noting its maximum value at the early spermiaton stage ($P< 0.05$) and remaining unchanged afterwards. The protein content did not exhibit any statistically significant changes during the reproductive cycle.

Based on the above results, there was a significant increase in the fat content in the body of flat-head grey mullet during the early spermiation, presumably to support the transfer of nutrients to the gonads during the species migration to the spawning regions. The results of this study can be used to determine the nutritional needs of broodstock in aquaculture and, by extension, to design an appropriate diet.

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Poster Presentation 41 (student)

Evaluation of potential spermatogonia biomarkers genes in the European eel (*Anguilla anguilla*)

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INTRODUCTION

Spermatogenesis is an essential and complex process which begin from spermatogonial renewal and proliferation, and lead to mature spermatozoa fully capable of fertilization. We isolated spermatogonial cells (SPGs) from immature European eel and we aimed to determine potential specific European eel SPG molecular markers, studying four candidate genes: *vasa1, vasa2, dnd1* and *nanos2.*

METHODS

A protocol for the isolation of SPGs in European eel was developed. Testes from immature European eel were collected and incubated in an enzymatic dissociation solution and the early germ cells were separated from somatic cells and debree by Percoll gradient centrifugation. RNA from isolated SPGs, whole testicular tissue and muscle samples was extracted using Trizol reagent and cDNA synthesis was performed using QuantiTect Reverse Transcription kit. qPCRs were carried out using specific primers for European eel *vasa1, vasa2, dnd1* and *nanos2.* An RNAscope Multiplex Fluorescent Reagent kit v2 was used to identify the potential SPG biomarkers in testis sections from immature European eels by *in situ* hybridization (ISH) assays. Probes were created by ACDbio as *vasa1, vasa2, dnd1* and *nanos2* and *ß-actin* probe (positive control). Tissues were labelled with Opal 520 fluorophore and incubated with DAPI. Additionally, immunohistochemistry was used to localize the presence of *vasa* and *nanos2* proteins within the testicular tissue.

RESULTS & DISCUSSION

Percoll gradient centrifugation was valid to purify the SPG cell population and remove somatic cells, but it was not possible to eliminate all somatic cells. The expression of both European eel *vasa1* and *vasa2* was higher in testis than in muscle. However, two expression patterns were observed. Higher expression values of *vasa2* were found in the isolated SPG than in the whole testis tissue, but without reaching a significant difference compared to *vasa1* expression levels. Regarding *dnd1* and *nanos2*, our study revealed a higher expression in the whole testis tissue than in isolated SPG. We hypothesize that higher expression of *dnd1* and *nanos2* in whole testis came from higher number of SPGs in the testis than in isolated SPG in suspension. The cellular distributions of *vasa1, vasa2, dnd1* and *nanos2* in testes were analyzed by ISH. Results revealed that *vasa1* was distributed in more testicular cell types than those expressing *vasa2* and, in turn, than *dnd1* and *nanos2*, while immunohistochemistry displayed that *vasa* and *nanos2* proteins were localized only in SPGs. This confirms that *vasa* (in especial *vasa2*) could be used as a marker of European eel SPGs.

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Poster Presentation 42 (student)

A transcriptomic approach to the onset of spermatogenesis in the European sea bass.

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INTRODUCTION

The European sea bass, Dicentrarchus labrax, is a marine fish species of key economic and cultural relevance in Mediterranean aquaculture. Under farming conditions, European sea bass males exhibit precocious puberty, resulting in negative effects on growth performance. The present study aims to identify molecular markers at the start of spermatogenesis.

METHODS

Fish were reared at the Institute of Marine Sciences (ICM-CSIC) under natural photoperiod and temperature conditions. By the time of samplings and coinciding with the period covering the onset of spermatogenesis, fish were sacrificed and testis subsequently isolated. One part of the testis was snapfrozen in liquid nitrogen for further downstream transcriptomic studies using directional RNA sequencing. Another part was fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, cut at 7um, and finally stained with haematoxylin-eosin for histological studies. The histological analysis was performed to assess testicular development unequivocally and to identify and quantify the type and abundance of germ cells in each of the two spermatogenic stages studied, namely Stage I (immature testis) and Stage II (early recrudescent testis), since their transition mark the onset of meiosis in European sea bass males.

RESULTS & DISCUSSION

This study aims to identify differentially expressed genes (DEGs) and pathways found during the early stages of testicular maturation to select molecular markers triggering the onset of spermatogenesis. The analysis reveals 676 DEGs between Stage I and Stage II. Among them, it is worth mentioning pcna, involved in DNA replication; ar, involved in reproductive development and growth; il-18, a relevant gene for the immune response; cyp26b, a key enzyme regulating the intracellular concentration of retinoic acid and involved in the onset of meiosis; igf1, related with somatic growth and energy store, and amh an important regulator of fish spermatogenesis. Collectively, different gene and enzymatic pathways were found to be involved in the transition from Stage I to Stage II, pointing at their important role in the onset of spermatogenesis. Our results also show an upregulation of the pathway leading to GnRH secretion, known for its relevance in regulating the brain-pituitary-gonad axis and, thus, sex steroid secretion. Also, the dysregulation of the cortisol synthesis and secretion pathway may control the stimulation of spermatogonial differentiation and meiosis and a possible adaptive response to stress. In addition, insulin secretion was also affected, according to its involvement in body growth and pubertal onset.

This work provides new information about molecular markers and pathways involved in the onset of spermatogenesis in European sea bass. Our current studies include all the stages of spermatogenesis to understand better the molecular signatures involved in a complete reproductive cycle.

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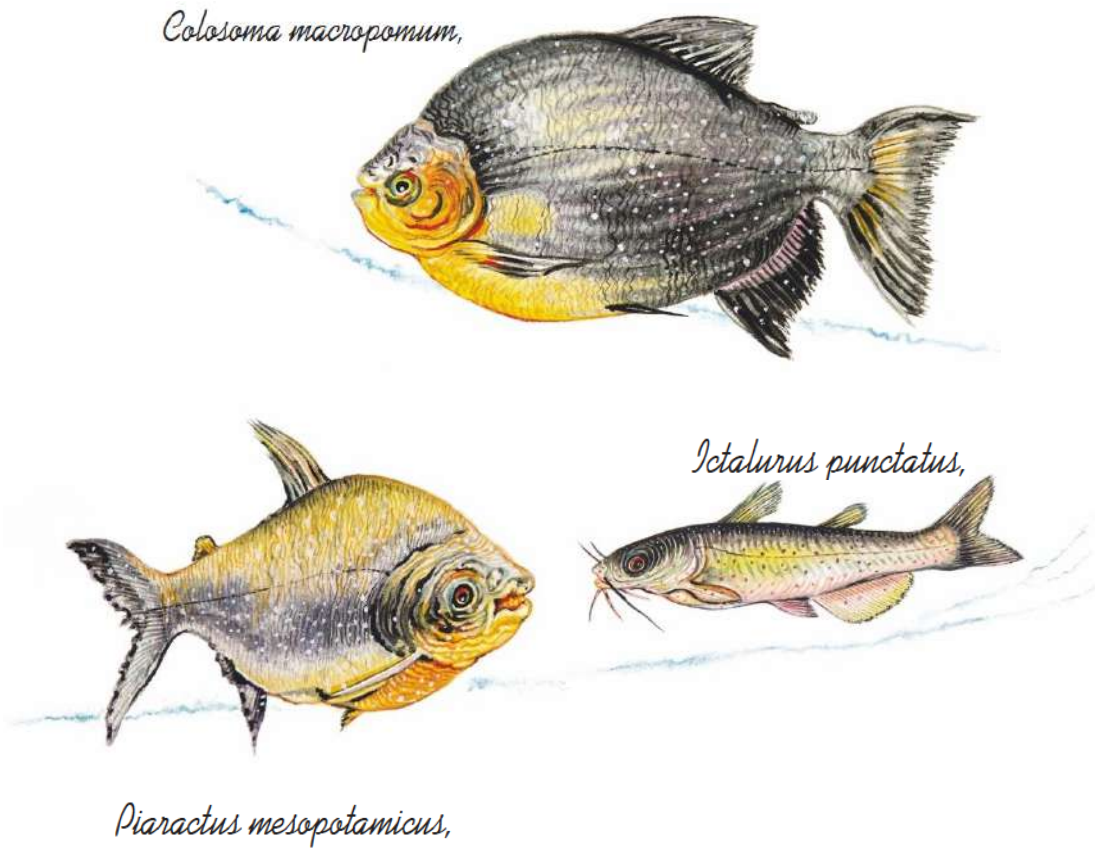
SS5. Climate change and anthropogenic impacts



Poster Presentation 43

ATM and ATR kinases play an important role in sturgeon (*Acipenser ruthenus*) embryo survival and development

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INTRODUCTION

Sturgeons are now categorized as the world's most threatened group of species with almost twothirds population being critically endangered (http://www.iucnredlist.org), partially because of water pollution. Therefore, studies on the impact of genotoxic stress on fertilization and embryo development could contribute to successful conservation efforts. Previous studies showed critical role of ATM and ATR kinases in DNA damage repair and indicated their role in cell cycle regulation. However, no studies have been performed on the role of ATM and ATR in sturgeon. Thus, we assess here how chemical inhibition and morpholino oligonucleotide (MO)-mediated knockdown affect sturgeon embryo development in presence and in absence of genotoxic stress.

METHODS

Sturgeons (*Acipenser ruthenus*) were reared in the aquaculture facility of the Research Institute of Fish

Culture and Hydrobiology at the University of South Bohemia, Vodňany, Czech Republic. After *in vitro* fertilization, we incubated part of the embryos on Petri dishes without (control) or with kinase inhibitors: 10 μM KU55933 (ATM inhibitor), 2 μM VE-821 (ATR inhibitor). Another part of the embryos at 1-cell stage was injected in the animal pole with 250 μM MO to knockdown genes encoding either ATM or ATR. We analyzed embryo survival and hatching rates up to 8-day postfertilization. Exposure to camptothecin (CPT) was used as genotoxic stress following kinase inhibition and gene knockdown. DNA damage response was analyzed by means of western blotting (WB) with antibodies against 53BP1, ATM, phospho-ATM, ATR, phospho-ATR, and anti-cleaved Caspase-3.

RESULTS & DISCUSSION

Overall, the results of WB indicated that MO-mediated gene knockdown and chemical inhibition of ATM and ATR affected protein abundance or kinase phosphorylation status. When **ATM kinase was either chemically inhibited or knocked-down**, embryos showed **higher survival rates** in presence of CPT compared to embryos with intact ATM exposed to the same concentration of CPT. Previous studies showed that ATM plays an important role in DNA damage-induced apoptosis. Therefore, it is possible to speculate that ATM inhibition in sturgeon embryos led to increased survival and hatching rate due to reduced rate of apoptosis at early stages of development. This assumption was confirmed using anti-cleaved caspase-3 antibody, which showed **lower rate of apoptosis in embryos with blocked ATM** at early stages of development. In contrast, knockdown of ATR followed by CPT exposure led to decrease in survival and hatching rates compared to wild-type embryos exposed to CPT. Overall, our results indicated that **ATR knock-down and inhibition may sensitize sturgeon embryos to DNA damage**. It is known that apart from DNA damage response, ATM and ATR control the timing of replication. Therefore, further studies are needed to show in which developmental processes the two kinases are involved and how their disruption may affect later stages of fish development, particularly neurodevelopment and gonad development.

The project was funded by the Czech Ministry of Education, Youth, and Sports, the projects: "CENAKVA" (LM2018099) and Reproductive and Genetic Procedures for Preserving Fish Biodiversity and Aquaculture (CZ.02.1.01/0.0/0.0/16_025/0007370).

Poster Presentation 44

Epigenetic effects of paternal exposure to BPA: DNA methylation in zebrafish germinal cells and F1 embryos

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INTRODUCTION

Bisphenol A (BPA) is an endocrine disruptor widespread used in the manufacturing of plastic devices, resulting in a ubiquitous presence in the aquatic environment. Much evidence supports that fish exposure to BPA leads to a wide range of disorders, including reproductive problems, skeletal malformations or cardiovascular diseases. The observed conditions have been related to changes at transcriptomic and epigenetic level that can be transmitted to further generations. Previous studies in zebrafish males showed that exposure during the late phases of spermatogenesis affects the DNA methylation of spermatozoa. In this study we analyzed the DNA methylation in the germ cells of males exposed to BPA during early spermatogenesis as well as in their F1 embryos.

METHODS

Adult zebrafish males were exposed to the vehicle (0.014% of ethanol, representing control group), 100 µg/L and 2000 µg/L of BPA during two weeks. One week after the treatment had finished, males were mated with non-treated females to obtain F1 embryo or sacrificed to obtain either sperm and testicles. Global DNA methylation was evaluated in testicular cells (UPLC-MS and immunohistochemistry), sperm cells (immunocytochemistry), and in blastomeres of F1 embryos (whole mount immunostaining). The levels of DNA methylation in gene promoters (*vasa, pou5f1 and sox2*) of both sperm and 48hpf-embryo was assessed by bisulfite conversion and sequencing, whereas gene expression was evaluated by RT-qPCR.

RESULTS & DISCUSSION

The assessment of epigenetic landscape of testicular cells and spermatozoa revealed that the global DNA methylation was not modified after exposing males to BPA. Likewise, the levels of 5mC in embryos obtained from control males were similar to those embryos obtained from BPA-exposed males. The analysis of DNA methylation state in specific gene promoters (*pou5f1* and *sox2*) did not reveal any noticeable effect of BPA in sperm, neither did it in the methylation pattern of *vasa* or *pou5f1* promoters on F1 embryos. Nevertheless, it caused a significant increase in the percentage of unmethylated CpGs in *sox2* promoter. This significant demethylation of *sox2* promoter in the progeny of males exposed to BPA could be related to the dysregulated expression observed in this gene. The different pattern of methylation in sperm and embryo after paternal BPA exposure observed in this gene could be a result of a secondary epimutation, an initial genetic change that induces a consequent epigenetic modification, being propagated via genetic, epigenetic or both types of inheritances.

The project received funding from the Spanish Ministry of Economy and Competitiveness (Project AGL2014-53167-C3-3-R)

Poster Presentation 45

Influence of carbamazepine, a contaminant of emerging concern, on the reproductive axis of female lambari (*Astyanax lacustris*)

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INTRODUCTION

Environmental conditions, such as temperature, oxygen, photoperiod, salinity, and anthropogenic activities strongly influence fish reproduction. Due to unfavorable environmental conditions, e.g., increase in the contamination levels, the stimulation of the hypothalamus-pituitary-gonads axis and, therefore, the synthesis of gonadal hormones may be compromised, and reproduction may fail. For teleosts, living in a polluted ecosystem is a threat with regards to the reproductive process since animals may prioritize other physiological adjustments, required for survival, rather than reproduction. Considering those aspects, this research aimed to evaluate the toxicity of carbamazepine (CBZ), a widely used drug, to sexually mature female fish, *Astyanax lacustris*.

METHODS

Fish were exposed to four treatments: 1. Control (dimethyl sulfoxide, DMSO); 2. 250 ng L^{-1}; 3. 500 ng L^{-1}; and 4. 1250 ng L^{-1} of CBZ (DMSO was used as vehicle). The experiment was performed in triplicate and the animals were exposed to the different experimental conditions for 7 days. Before the beginning of the experiments, animals were fed and maintained at constant conditions of oxygen, temperature, and photoperiod. Those conditions were maintained throughout the exposure period. After 7 days of exposure to CBZ, fish were sampled to obtain plasma and pituitary to analyze steroid (testosterone and estradiol) plasma levels and gonadotropin gene expression, respectively.

RESULTS & DISCUSSION

Plasma concentration of testosterone and estradiol were not altered after exposure to CBZ. The gonadotropin gene expression, however, was impacted by the drug. While the mRNA levels of the luteinizing-hormone (*lhb*) were not changed after the exposure to the drug, a significant increase in the mRNA levels of the follicle-stimulating hormone (*fshb*) was observed in fish exposed to 500 ng L^{-1} of CBZ. Considering that *A. lacustris* is a native fish species that inhabits both polluted and clean ecosystems, different strategies for reproduction must be employed. The absence of effects in steroid plasma levels by CBZ in *A. lacustris* suggests that females ensure the reproductive process even in an adverse situation. Moreover, the results observed for *lhb* and *fshb* corroborates, demonstrating that, even though CBZ exposure induces changes in mRNA levels, female fish *A. lacustris* can regulate the hypothalamic-pituitary-gonad axis and maintain the integrity of the reproductive process.

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Poster Presentation 46

Spermatogenesis in the neotropical fish (*Astyanax lacustris*) in response to the environmental changes

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INTRODUCTION

Environmental changes can affect fish physiology and negatively impact health and reproduction of populations, including species with potential for aquaculture. *Astyanax lacustris* is a fish species widely used in fish farming and human consumption and used as live bait for sport fishing. It is a neotropical species used as a study model because of its small size, early sexual maturation and multiple spawning. This study aims to evaluate the effects of pharmaceuticals present in rivers and changes in temperature, which are global concern, on the reproductive cycle and gonadal maturation of *A. lacustris*.

METHODS

The animals were provided by Grupo Votorantim Energia (formerly Estação de Hidrobiologia e Aquicultura de Paraibuna - CESP), Paraibuna, São Paulo, transported to the laboratory, anesthetized in benzocaine solution and then the spinal cord of each animal was sectioned, and the head was separated from the animal's body. The experiment was approved by the Ethics Committee on Animal Use, CEUA, IB, USP (366/2020). In the study of the effect of pharmaceuticals on reproduction, we analyzed the histological changes in the testis of animals that were submitted to a subchronic test for 14 days, with environmentally relevant concentrations of diclofenac and caffeine, isolated and combined. The treatments were: Control (CTRL- without drug), Diclofenac (DCF, 0.4 µg/L), Caffeine (CAF, 27.5 µg/L) and Mixture (DCF + CAF = MIX) at 26ºC. In the study of temperature effect the animals were distributed among 3 temperatures (20°C, 26°C, and 32°C) and will be collected after 4 months, that is when they are already sexually mature. The animals were anesthetized with 0.1% benzocaine solution and their gonads were collected and fixed overnight in Karnovsky solution. The testes were embedded in historesin, sectioned with 3 µm thickness using a Leica Surgipath DB80 LS microtome blade, stained with hematoxylin and eosin and analyzed using an image capture system attached to a Leica DMA 4000B photomicroscope.

RESULTS & DISCUSSION

The analyses carried out evaluating the testicular histology of the animals exposed to the pharmaceuticals showed a lack of pattern in the sexual stages. The animals from the CTRL group were mostly mature since the animals exposed to DCF, CAF and MIX presented different phases of spermatogenesis. The main finding was the presence of developing testes in animals exposed to pharmaceutical, with little sperm production, different from what was seen in the study with these same drugs in acute exposure (Godoi et al., 2020). Since at 27º C the duration of spermatogenesis was approximately 6 days (Quirino et al., 2020) and changes in temperature directly affect spermatogenesis, we suggest that after 14 days exposed to pharmaceutical compounds, the duration of the spermatogenesis will be affected as well as the completion of sperm production.

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Poster Presentation 47

miRNA 29a is downregulated in progenies derived from chronically stressed males

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INTRODUCTION

In aquaculture facilities, fish deal with very different anthropogenic sources of stress that can ultimately affect key subjects in aquaculture production such as the health, growth or reproduction. This work is settled up within a legislative and productive framework that promotes animal welfare and overcoming the adverse effects that stress can have on production batches. Our aim is to evaluate, using *Danio rerio* as a teleost model, the molecular and physiological consequences of zebrafish male progenitor exposition to chronic stress on their unexposed progenies, paying special focus on non-coding RNA modifications in the progeny since these small molecules play a crucial role regulating gene expression in a vast number of pathways.

METHODS

Four biological replicates per experimental group were included in the experiment. Each biological replicate included 50 7dpf larvae born form to two types of crossings depending on the experimental group: 1) CTRL progenies, derived from standard cultured animals and 2) S^+ progenies, derived from crossings involving chronically stressed males and undisturbed females. From each replicate, small RNA populations were extracted using mirVana Isolation Kit and used for RNAseq analysis. A second batch of progenies were evaluated for cranioencephalic development (whole mount alcian blue staining) at 7 dpf.

RESULTS & DISCUSSION

RNAseq analysis revealed dre-miR-29a as the unique downregulated (FDR<0.0200) small RNA in S^+ larvae. We performed a target study of miR-29a contrasting to the published mRNA targets (Target Scan Fish). Collagen and extracellular matrix represented a high percentage of the enriched components with significant enrichment. We performed functional enrichment with g:Profiler using the Reactome database and again, the highest enhanced pathways were related to collagen and extracellular matrix: collagen chain trimerization, collagen biosynthesis, assembly of collagen fibrils, collagen formation and degradation, extracellular matrix organization. Once performed the molecular description of the main targeted pathways, we carried out cartilage staining to explore whether alterations in these molecular pathways were translated at tissue level in cranioencephalic development. We confirmed statistically significant alterations on jaw shape in this group showing large ceratohyal cartilage length, reduced palatoquadrate angle and increased lower jaw length. These measures are considered as high-throughput standard parameter to assess malformations in zebrafish.

This study was supported by MCIN/AEI/10.13039/501100011033, grant PID2019-108509RB-I00. DGV was funded by MCIN/AEI/10.13039/501100011033 and EU NextGenerationEU/PRTR, grant: IJC2020-044091-I.

Poster Presentation 48

Evolutionary scenario of temperature receptor TRPV (Transient Receptor Potential Vanilloid) family in metazoans with a special focus on "fish" species

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INTRODUCTION

The Intergovernmental Panel on Climate Change reported that ocean surface temperature will increase >1 to 3°C by 2100. The 2021 Nobel prize was attributed to the discovery and study in mammals of Transient Receptor Potential (TRP) channels involved in thermosensing, but data are lacking in other metazoans. TRP Vanilloid (TRPV) family is involved in multiple sensory and physiological functions including thermosensing and temperature-dependent regulations. Fish, as ectothermic organisms, are vulnerable to temperature changes. From a phylogenetic point of view, "fish" form a paraphyletic group, with representatives of cyclostomes, chondrichthyans, actinopterygians and sarcopterygians. We investigated the number, origin and evolution of TRPV among metazoans, with a special focus on the impact of whole genome duplications (WGD), gene-specific duplications and gene losses in "fish".

METHODS

TRPV sequences were retrieved from genome databases using BLAST search, and aligned using Clustal Omega. Phylogenetic analyses were performed using the Maximum Likelihood method, with 1000 bootstrap replicates. Synteny analyses were performed on TRPV genomic regions in species of keyphylogenetic positions.

RESULTS & DISCUSSION

The study revealed a larger number of TRPV genes in vertebrates than currently assumed, with three additional TRPV types TRPV7, 8 and 9. Our evolutionary scenario shows that ancestral vertebrates inherited two TRPV which were duplicated into five types (TRPV1, 4, 5, 7, 8) after the two rounds (1R and 2R) of vertebrate whole genome duplication (WGD) and one local gene duplication that generated TRPV7 and 8. TRPV7 and 8 were lost independently in various vertebrate lineages such as in actinopterygians before the emergence of holosteans, in sauropsids, and in mammals before the emergence of eutherians. Several local gene duplications of TRPV1 generated TRPV3 and TRPV9 in the gnathostome ancestor, and TRPV2 in the tetrapod ancestor. TRPV3 and 9 are present in extant elasmobranchs, while TRPV9 was lost in the osteichthyan ancestor and TRPV3 in the actinopterygian ancestor. Within the sarcopterygian lineage, the actinistians retained TRPV1, 3, 4, 5, 7 and 8 genes, with two additional duplications of TRPV3. Duplications of TRPV5 occurred independently in various lineages, such as in chondrichthyes, anuran amphibians, sauropsids, mammals (where they are called TRPV5 and 6), polypteridae and esocidae. Shortly after the teleost-specific WGD (3R) only TRPV1 retained its two paralogs, whereas TRPV4 and 5 remained as single genes. Both 3R-TRPV1a and b paralogs were kept in some extant teleost species, while TRPV1b paralog was independently lost in others. Both 3R-TRPV1 paralogs were further duplicated in gadiforms. The salmonid-specific WGD (4R) duplicated TRPV1a, 4, and 5 genes leading to six TRPV in salmonids. This study provides a comprehensive evolutionary scenario for the metazoan TRPV family, and proposes a phylogeny-based classification of TRPV across vertebrates including "fishes".

Poster Presentation 49

Paternal exposure to chronic stress impacts on the progeny: a molecular description of the altered biological processes

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INTRODUCTION

Fish reared in captivity are subjected to a range of different husbandry practices that may cause chronic stress to the animals. Here, we describe the impact that paternal chronic stress might have on unexposed to stress offspring at molecular level employing deep-sequencing technologies in the teleost model species *Danio rerio.*

METHODS

Thirty adult male zebrafish were standard maintained until a randomly split into two experimental groups: "control (CTRL)", non-exposed to stress and "stress (S^+)". During 21 days, approximately involving 3 complete rounds of spermatogenesis in this species, S^+ males were exposed twice a day to different stressors while CTRL males were undisturbed. Once stress induction was finished, males from both groups were crossed with non-stressed females. The resulting progenies were standard cultured upon 7 days, when RNA from larvae pools (50 larvae/pool; n=4 pools per condition) was extracted and further on, used as samples for transcriptomic (RNAseq) and qPCR analysis.

RESULTS & DISCUSSION

In total, 20803 protein-coding genes were expressed. Principal component analysis (PCA) of the gene expression showed that control and stressed progenies were clustered suggesting that the stressful conditions of male progenitors have a relevant effect on progeny gene expression scenario at 7 dpf. RNA-seq revealed 180 differentially expressed genes (DEGs) (cutoff filter: >1.5 Fold Change and FDR<0.05), 44 of these DEGs were downregulated and 136 upregulated. A total of 30 upregulated DEGs with FC>2 were link to genes related to cranial and ocular malformations, mineralization genes that favor collagen matrix growth and other genes related to cell cycle and transcriptional regulation. In the case of downregulated DEGs some of them are related to immune system, protein ubiquitination, eye lens development and visual perception. Our results highlighted several biological processes affected on the progenies after male stress induction. Stressed-derived progenies were enriched in "cellular responses to stress" and "cell response to stimuli", both of them associated to stress response, confirming some transmission of the paternal effects in these 7-dpf larvae. Cell cycle, mitosis and DNA repair represented the biological processes with the highest normalized enrichment scores (NES > 2.5). On the other hand, the biological processes link to mRNA translation and their associated pathways were downregulated in zebrafish larvae form stressed males. Some biological processes related to Nonsense Mediated Decay (NMD) pathways scored the largest negative absolute values (NES < -3.2). Our results demonstrate in the zebrafish model, that parental chronic stress, whether of environmental or anthropogenic origin, impact on a deleterious way on the offspring supporting the hypothesis of the vertical transmission of stress effects via sperm.

This study was supported by MCIN/AEI/10.13039/501100011033, grant PID2019-108509RB-I00. DGV was funded by MCIN/AEI/10.13039/501100011033 and EU NextGenerationEU/PRTR, grant: IJC2020-044091-I.

Poster Presentation 50

Ovary structural anomalies in the European sardine (*Sardina pilchardus*) in relation to health status and contaminants exposure.

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INTRODUCTION

The European sardine (*Sardina pilchardus*) represents one of the key species in the Adriatic Sea of both ecologically and socio-economically relevance. During the last years, sardines' catches fluctuation raised the question of the population's future stability. Reproduction is one of the most crucial aspects of fish life cycle and it is strictly related to the environmental condition and animals' welfare. Previous investigations on female specimens highlighted the occurrence of different gonadal alterations during the reproductive period. This study aimed to investigate the relationship between the gonadal and health status of European sardine females during the reproductive period. In addition, gonadal and health impairment were associated with possible exposure to environmental contaminants.

METHODS

Sardines were monthly sampled in collaboration with the local fishermen operating in the waters off the coast of the Marche region within the reproductive period. Histological analysis was performed in both liver and ovary samples to evaluate the presence of histopathological conditions and to quantify the melanomacrophages (MMs). Immunohistochemical analysis of CYP450, MT, CAT, SOD, GPX and HSP70 was performed in liver samples.

RESULTS & DISCUSSION

Histological analysis revealed different structural anomalies in ovaries such as the presence of indefinite structures in the ooplasm, anomalous coalescence of vitellogenin, abnormal presence of previtellogenic oocytes, double oocyte structure-like, necrosis, blood vessels walls thickening and white blood cells infiltration. All these anomalies were observed despite fish age however, their frequency increased with females' age and with the highest spawning activity. Liver samples, from the same animals, were affected by structural alterations such as necrosis at early and advanced status, vacuolization, blood vessel walls thickening, white blood cell infiltration and haemolysis. The MMs analysis highlighted that their increase in number and size is related to age and spawning activity. Considering the immunohistochemical analysis, the expression of selected biomarkers increased with the size and age of females suggesting that fish experienced chronic exposure to different pollutants such as heavy metals, PCB, IPA and NPAH. This hypothesis was supported by the higher expression of oxidative stress enzymes in older females. Our results suggested that sardine females appeared in a compromised health status during the breeding season that reflected a similar situation in the ovary. The pollutants' chronic exposure may be the cause of this health impairment possibly affecting the fertility of this species.

Poster Presentation 51

In vitro evaluation of potential estrogenic compounds on estrogen receptors of European sea bass (_Dicentrarchus labrax_)

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INTRODUCTION

Several hundred tons of chemicals are produced and consumed annually around the world, and this production is increasing due to increased consumption by a constantly growing population. These chemicals may end in the environment as pollutants. Among the emerging pollutants there are many substances that can act as endocrine disruptors. They have the ability to imitate, block, or interfere with the hormones of the endocrine system of organisms, producing harmful effects at the physiological level. Therefore, fish are constantly exposed to several compounds, being some of them known for their potential estrogenic effects. Genomic estrogenic responses follow the classical pathway that involves the binding of estrogens – or structurally similar compounds - to specific transcription factors, the nuclear estrogen receptors. In its turn, rapid non-genomic estrogenic responses were also discovered in fish, as being mediated by membrane receptors such as the G protein-coupled estrogen receptor (Gper). The aim of this study is to functionally characterize European seabass Gpers and evaluate the effects of two potential estrogenic compounds on all five European seabass estrogen receptors (Esr1, Esr2a, Esr2b, Gpera and Gperb).

METHODS

Using the human embryonic kidney cell line HEK293, we have performed transient transfections (1) to characterize the sea bass duplicates of Gper, Gpera and Gperb by using a cAMP response element (CRE)-luciferase reporter gene assay and putative agonists or antagonists, such as estradiol, ethynilestradiol, estriol, testosterone, G1 and G15, and (2) to study the effect of two pollutants, genistein – a phytoestrogen also present in fish meals – and fluoxetine – an antidepressant, mainly constituent of Prozac- on the three subtypes of nuclear estrogen receptors (Esr1, Esr2a and Esr2b) and the two subtypes of membrane estrogen receptors (Gpera and Gperb) from sea bass by using, respectively, transactivation of an ERE- or CRE- luciferase reporter gene assay.

RESULTS & DISCUSSION

Estradiol was able to induce luciferase activity in cells transfected with both membrane receptors, supporting, for the first time, that both teleost gene duplicates are functional. Gpers act via the cAMP signaling pathway and respond differentially to distinct steroid compounds. Our findings indicate that both genistein and fluoxetine differently affect each receptor. In general fluoxetine triggers an antiestrogenic response while genistein behaved as estrogenic in nuclear receptors. In the case of membrane receptors, both genistein and fluoxetine behaved as estrogenic compounds, although Gperb seemed more sensitive to synthetic compounds than Gpera. As a marine species, the characterization of all the sea bass estrogen receptors can become useful for EDC testing into the Marine Strategy Framework Directive in order to achieve a good environmental status of the EU's marine waters.

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Poster Presentation 52 (student)

Effects of Alkylphenol on the regulation of aromatase gene expression in the Indian stinging catfish (*Heteropneustes fossilis*)

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INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a group of chemicals which are present in the environment or food source of an animal and lead to the disturbance of the endocrine system leading to multiple physiological ailments. 4-Nonylphenol (4-NP) is one of the most significant pollutants in aquatic ecosystem arising from anthropogenic sources (industrial affluents, discharge from sewage treatment plans, etc.). The toxicity, bioaccumulation, xenoestrogenecity in various aquatic animals makes it of interest for endocrinologists. Indian stinging catfish (*Heteropneustes fossilis*) is an economically important freshwater teleost in India, the fish is a seasonal breeder and has prominent annual reproductive cycles. Cytochrome P450 Aromatase is the enzyme complex which plays a pivotal role in estrogen synthesis, this enzyme complex converts androgens to estrogens via aromatization and is a product of the cyp19a1b gene. In the present study we investigated the effects of exposure of 4-NP on the aromatase (cytochrome P450) gene expression in the catfish during resting and preparatory phases.

METHODOLOGY

Mature healthy female catfish (50-60g B.W) were purchased from local markets in Varanasi, U.P, India and maintained under laboratory conditions in aquaria. The LC50 value for 4-NP was determined in the resting and preparatory stage catfishes, the values were obtained as 1600ug/L and 1632ug/L respectively. Two sublethal concentrations were selected for the experiment; $1/50^{th}$ (32µg/L) and $1/100^{th}$ (16µg/L). The fish were exposed to the decided doses for 28 days and the blood, brain and ovary samples were collected on days 7, 14, 21 and 28. Total RNA was extracted from the tissue samples and was reverse-transcribed to generate the cDNA. The cDNA was PCR amplified with the help of brain aromatase primer sets. The analysis was done by an in-gel method and quantified with Image Quant TL Plus.

RESULT AND DISCUSSION

In this present study, we have established the estrogen mimicking property of 4-Nonylphenol and its potential as an endocrine disruptor. The modulation of cytochrome P450 aromatase gene (*cyp19a1b*) expression in the brain and ovary by 4-NP, plays a decisive role in regulating reproductive status and steroidogenesis in the catfish. We also have demonstrated that the *cyp19a1b* gene is upregulated by estradiol. We have investigated that there is a positive feedback regulation of *cyp19a1b* by steroidal hormones as reported in other teleosts. GSI and HSI are often applied as an end point of endocrine disruption as positive or negative fluctuation of the parameters usually a product of increased or decreased gonadal mass. In females a reduced GSI is attributed to degeneration of ovaries. Further study will help develop a better understanding of the impact of 4-NP and other alkylphenols on the reproductive status of many aquatic life forms. Through this study we have attempted to demonstrate the potential of teleost fishes as a model for representing the endocrine disruption caused by environmental estrogens.

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Poster Presentation 53

Influence of high temperature on the gonadosomatic and hepatosomatic indices of neotropical fish *Astyanax lacustris*

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INTRODUCTION

Faced with the scenario of climate change and, consequently, changes in global temperature, it is essential to study their effects on the reproduction of teleost, especially freshwater Neotropical ones. Therefore, the objective of this study was to analyze the effects of high temperature on the growth and gonad maturation of *Astyanax lacustris*, a species with important zootechnical and reproductive characteristics. Furthermore, this species has more than one reproductive period throughout the year, making it an excellent experimental model to study the reproductive biology of neotropical teleost.

METHODS

120 males of the *A. lacustris* (4 months old) were used. The animals were divided into two treatments (n=60 each) for a period of seven months; the first group was maintained at an ambient temperature of 27.0 °C (± 1.0) and the second group at a controlled temperature of 32.0 °C (± 0.5). Five replications were used for each treatment, totaling 10 experimental units in a completely randomized design (Ethics Committee on Animal Use 10/2020). Each replicate was kept in 1000 L tanks in a water recirculation system. Three collections were performed: at the beginning (T0), after 30 days (T30) and 210 days (T210) of exposure to temperatures. The animals were euthanized in a benzocaine solution (0.001%) and the total mass (Wt), testicle mass (Wg) and liver mass (Wl) were measured. Hepatosomatic (IHS=Wl/Wt*100) and gonadosomatic (IGS=Wg/Wt*100) indices were calculated.

RESULTS & DISCUSSION

There was no difference in hepatosomatic index (HSI) in the animals at T0 0.62 ±0.21 (P>0.05), demonstrating same zootechnical condition for both groups at the beginning of exposure to different temperatures. After 210 days of cultivation, there was no difference for the HSI on the different days of exposure to the same temperature and the same was observed on the same days of exposure for the different temperatures; 27ºC - T30: 0.93 ±0.25 and T210: 0.77 ±0.21; 32ºC - T30: 0.85 ±0.22 and T210: 0.67 ±0.24 (P=0.62). The gonadosomatic index (GSI) of males at the beginning of exposure was T0 2.21 ±1.00, with no difference between groups (P=0.71). After 210 days of cultivation, there was a difference in the GSI of the animals exposed to 32ºC at T30 (3.61 ±1.10), which was higher than T210 (1.78 ±1.29) (P=0.02). The same was not observed for the animals exposed to 27ºC at T30 (3.45 ±0.76) and T210 (3.01 ±1.07) (P=0.56), but there was difference at T210 between 27 ºC T210 (3.01 ±1.07) and 32 ºC (1.78 ±1.29) (P=0.001). Therefore, we can observe that prolonged exposure of *A. lacustris* males to high water temperature promoted a reduction in GSI values. Similar alterations in GSI values were also observed in *Danio rerio* and in *Oncorhynchus mykiss*, where elevated temperature promoted atrophied and undeveloped gonads or a smaller testis with lower seminal volume (NOBREGA, *et al*., 2010; BUTZGE *et al*., 2021). Accordingly, the reduction of GSI values due to the increase in water temperature can compromise the testicular and reproductive development of *A. lacustris*.

This project was supported by FAPESP [2021/03739-3].

Poster Presentation 54

Influence of high temperature on the testicular development of neotropical fish *Astyanax lacustris*

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INTRODUCTION

Global warming, land use change and habitat loss are the three main threats to aquatic biodiversity worldwide under the influence of anthropogenic disturbances. In the case of freshwater fish, particularly neotropical fish, exposure to these changes puts them in a more vulnerable position than marine fish, because freshwater systems are shallower and have a lower thermal damping capacity. Thus, this study aimed to analyze the effects of high temperature on the testicular development of *Astyanax lacustris*, a species with important zootechnical and reproductive characteristics, as they have more than one reproductive period throughout the year, which makes this species an excellent model. experiment for the investigation of the reproductive biology of neotropical teleosts.

METHODS

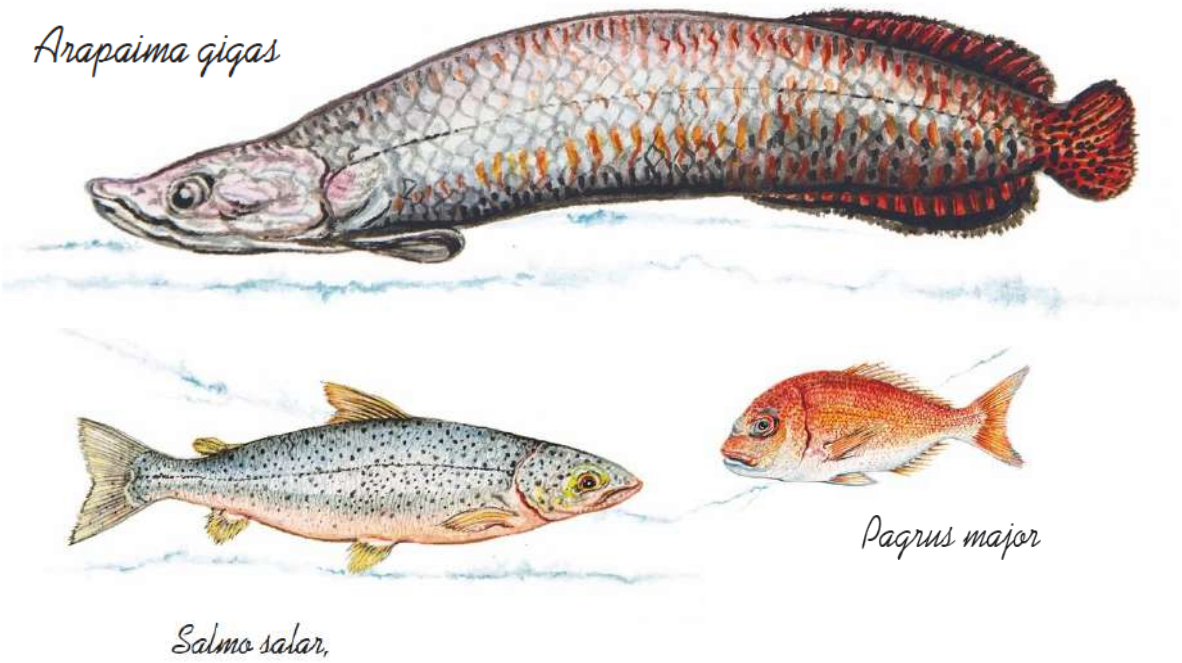
One hundred and twenty male specimens of *Astyanax lacustris*, 4 months old, were used. The specimens were divided into two treatments (n=60 each), for a period of seven months, and kept at room temperature (27.0 ± 1.0°C) and the other at a controlled temperature of 32.0± 0.5°C, with five replications, totaling 10 experimental units in a completely randomized design (Ethics Committee on Animal Use, CEUA-FEIS/UNESP 10/2020). The experiment took place in 1000 L tanks in a water recirculation system, at the Laboratory of Neotropical Ichthyology – LINEO, UNESP - Ilha Solteira, SP, Brazil. Collections were performed after 30 (T30), 120 (T120) and 210 (T210) days of exposure to temperatures. The specimens were euthanized in a benzocaine solution (0.001%), the tests were removed, fixed in a 2% glutaraldehyde solution, 4% paraformaldehyde in Sorensen's phosphate buffer pH 7.2 and processed using the usual light microscopy techniques. The identification of testicular cycle phases followed what was proposed by Brown-Peterson et al. (2011).

RESULTS & DISCUSSION

Throughout the experiment, two phases of testicular development in *A. lacustris* were characterized: the Able to Sperm - Intermediate (AI) and Final (AF) subphase and the Regression phase (R). Most specimens kept at room temperature were able to sperm: T30: 10% AI, 80% AF and 10% R; T120: 10% AI, 70% AF and 20% R; T210: 12% AI, 76% AF and 12% R. However, most species kept at 32ºC were in the Regression phase: T30: 30% AF and 70% R; T120: 20% AI, 30% AF and 50% R; T210: 20% AF and 80% R. The Regression phase was characterized by an expressive increase of vacuoles in Sertoli cells and an atrophy of the germinal epithelium, in addition to the characteristics established by BrownPeterson et al. (2011), for this phase. The same was observed in Oreochromis niloticus, when subjected to temperature extremes (JIN et al., 2019). Thus, the maintenance of *A. lacustris* for a prolonged time at a temperature of 32ºC, resulted in the reduction of specimens able to sperm, compromising Sertoli cells and consequently in the reproductive development of the species.

This project was supported by FAPESP [2021/03739-3].

SS6. Reproduction in aquaculture



Poster Presentation 55

Production regime of Atlantic salmon (*Salmo salar*) broodstock recruits for optimal size at spawning and optimized incorporation of essential nutrients for egg and larvae quality

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INTRODUCTION

Incorporation of essential nutrients to maturing fish is important to assure high fecundity and viable offspring. Atlantic salmon (*Salmo salar*) broodstock recruits are fed a diet high in essential nutrients only 6 months prior to maturation and ceased feeding, and prior to that grower feeds. Over the last decades, the composition of commercial grower feeds has changed to contain less marine ingredients, and thus also less essential nutrients as minerals, vitamins, phospholipids and omega-3 fatty acids. Furthermore, the production of Atlantic salmon bloodstock recruits has lately been shortened by one year to reducing risk of mortality and faster implementation rate of breeding achievements. Production regimes to produce recruits that reach 15 kg size in the spring of the year of spawning and optimization of egg and larvae quality have therefore been targeted by nutritional approaches in the present study.

METHODS

Atlantic salmon (1200 g) at Grieg Seafood (Hylsfjorden, Norway) were given a commercial grower diet (control) or a control with increased levels of essential omega-3 fatty acids and vitamins (test). Feeds were given between February-November the year before spawning (phase 1). Afterwards, fish were fed a broodstock diet with high level of essential nutrients (control) or a broodstock diet with a higher energy content (test) until spring when feeding ceased towards final maturation (phase 2). February, June and November the year before spawning, and April the year of spawning, 10 females and 10 males were sampled. Biometrics, blood and various tissues were sampled for sex steroids and nutrients. In April, gonads were sampled for histological evaluation and for transcriptomics by microarray. In addition, 3 spawning groups were followed, early, normal, and late, adjusted by light and temperature regimes. Fecundity, egg composition and quality measurements, as well as samples of spawning fish were taken, in addition to quality measurements of incubated eggs, and hatching rate.

RESULTS AND DISCUSSION

Atlantic salmon males and females in phase 1 showed no dietary differences in the biometrics. The higher content of essential nutrients in the test feed was reflected in the tissues with a higher content compared to analysis in the control group. The higher energy content of the test feed in phase 2 resulted in a higher body weight in April the year of spawning, only significant for males compared to males and females in the control group. On the contrary, females in the test group had significant larger gonad size compared to females in the control group, while males showed a larger variation in gonad sizes at this stage in the maturation. There was no indication of change in the timing of maturation as an effect of the diets, as most of the females were staged to be at late vitellogenesis. Males had mainly spermatocytes at the staging of gonadal histology, irrespectively of dietary treatment, in April the year of spawning. The results of microarray in the gonad showed in general low impact of the dietary treatment, as only 33 and 36 of 44 000 genes were upregulated in females and males, respectively. Interestingly, several cytochrome P450 genes were upregulated due to the dietary treatment both in females and males. These are important enzymes in the biosynthesis of steroids, but at the moment we are waiting for analysis of steroids that can support differences observed in the microarray analysis. We will throughout the winter 2022-2023 collect data and perform analysis of early, normal and later spawners. The project is funded by a R&D license owned by Grieg Seafood.

Poster Presentation 56

Conservation strategy of butter catfish *Ompock bimaculatus*

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INTRODUCTION

The freshwater butter catfish *Ompok bimaculatus* is commonly known as pabda. This species has great consumer preference due to its high nutritive value and good taste. Butter catfish were also considered as an annual breeder. Unlike major carp fish, catfish cannot breed in captivity even after steroid hormone injection. The techniques of induced spawning with stripping would help culturists to increase hatchery production. The fish culturist has to pay attention to the latency period, spawning efficiency, fertilization rate and hatchability along with survival rate among offspring. The reproductive biology and developmental study gives the baseline information for researchers and fish culturist for its seed production, management and conservation. In conservation strategies best strain of butter catfish from wild captivity should propagate by stripped induced breeding to increase population.

METHODS

Adult butter catfish collected from the wild habitat for best strain screening on the basis of reproductive parameters specially gamete quality for induced breeding trials with different hormones (Ovidac: 8-17 IU female/ 3-5 IU male per g body weight, Gonopro-FH: 0.3-1.2 ml female/0.1-0.2 ml male per kg body weight and Ovaprim: 0.5-1.5 ml female/0.2-0.4 ml male per kg body weight). Latency period, fecundity, fertilization and survival-ability were compared for its breeding performance and further development. Embryonic development was studies with different hormone for conservation study.

RESULTS & DISCUSSION

The present study was resulted that all commercially available inducing hormones (Ovidac, GonoproFH and Ovaprim) were able to induce female fish butter catfish ovulation with stripping in one dose only. The current study provides a range of optimum hormone dose in butter catfish with which they can spawn by stripping method (Ovidac: 10-15 IU; Gonopro-FH: 0.5-0.9 ml; Ovaprim: 0.7-1.2 ml per kg body weight). The stripping times were different as per the inducing agents. The latency period was decreasing with increase in hormone dose in the female with all three hormones. The developmental stages were more or less similar in all tried induced breeding groups. The synthetic hormone, Ovaprim gave best results. It may be due to its combination that includes domperidone in addition to gonadotropin. This combination will facilitate oocyte maturation and provide ease in ovulation.

Poster Presentation 57

Induced spawning of silver trevally (*Pseudocaranx georgianus*; Carangidae) in captivity

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INTRODUCTION

The silver trevally or 'araara' *(Pseudocaranx georgianus)* has been selected as a candidate for selective breeding to support the expansion of the New Zealand aquaculture sector. However, initial research into this species shows that it fails to spawn naturally when held under ambient conditions in captivity. Here we report for the first time the induced spawning of wild-caught, captivity-acclimated broodstock using an agonist of gonadotropin-releasing hormone (GnRHa).

METHODS

Wild-caught, captivity-acclimated broodstock (n= 12) were left to spawn naturally without the administration of exogenous hormones for 3 years until 17 December 2018. Broodstock were maintained indoors in a single 13,000-L tank under ambient water temperatures and day length. For the duration of the predicted spawning season (28 October 2018 to 28 February 2019), the effluent drain of the tank was fitted with an external passive egg collector which was checked twice every day (between 0800 and 0900 h and between 1500 and 1700 h). When ambient water temperatures reached 21°C and natural spawning was not evident, fish were mass-sedated, and a gonadal biopsy was taken by inserting a glass cannula connected to a plastic tubing into the ovarian cavity through the genital pore to determine the stage of reproductive development. Females in the advanced stages of oogenesis (oocyte diameters ≥ 400 µm) were subsequently administered GnRHa implants (Ovaplant®) at a target dose of ~100 µg/kg-1 of body weight, while all males were administered a target dose of 50–100 µg/kg-1 of body weight. The broodstock tank was monitored for egg release over the following five weeks. Once spawning started, eggs were collected to estimate fecundity, fertilization and hatching rates.

RESULTS & DISCUSSION

At the time of hormone administration, biopsies revealed that the broodstock population consisted of seven males and five females. All five females (mean body weight of 4.2 kg) had large vitellogenic oocytes with mean oocyte diameters ranging between 470 and 520 µm, and maximum oocyte diameters ranging between 491 and 578 µm. Eggs were first detected in the egg collector in the tank containing GnRHa-treated fish at approximately 44 h post-administration. Egg production was daily, with a total of eight spawning events being observed over a period of seven days post-implantation. The number of eggs collected during each spawning event varied between 149,550 and 5,841,701 eggs (35,607–1,390,881 eggs per kg-1), with an average number per spawn of 1,500,598 eggs (357,285 eggs per kg). Estimates of fertilization and hatching success of the buoyant eggs were variable and ranged from 29 to 95% and 3 to 27%, respectively. The mean egg size was 0.9 ± 0.03 mm diameter (n= 20) and ranged between 0.8 and 0.9 mm. Together these findings provide a future foundation for the development of broodstock management practices to spawn silver trevally in captivity and to facilitate selective breeding efforts for this species.

This research was funded through the New Zealand Ministry of Business Innovation and Employment Endeavour Programme "Accelerated breeding for enhanced seafood production" (#C11X1603).

Poster Presentation 58

Plasma steroid profile following induced sex change of protogynous dusky grouper (*Epinephelus marginatus*) using methyltestosterone and aromatase inhibitor

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INTRODUCTION

Artificial induction of sex change in protogynous teleost species such as the dusky grouper, *Epinephelus marginatus*, under assisted conditions, has been performed with relative success using synthetic androgens and aromatase inhibitors. However, limited information is available on whether or not this reversal is permanent. We used different sex change protocols to follow the plasma level of gonadal steroids in dusky grouper aiming to check the possibility of permanent inversion.

METHODS

Captive-reared females (n=30) were tagged and divided into 6 circular tanks (2000 L) into 3 groups (5 fish/tank; duplicate): control (CTR); methyltestosterone (MT) and aromatase inhibitor (AI). At the beginning of the experimental period, blood samples were collected (initial). Ten females were injected with fish oil (1 ml/kg) (CTR), 10 females with methyltestosterone diluted in fish oil (15 mg/kg) (MT) and 10 females with letrozole diluted in fish oil (100 mg/kg), an aromatase inhibitor (AI). These treatments were repeated after 30, 60, and 90 days, when a blood sample was also collected from the caudal vasculature of each female. The plasma concentrations of estradiol (E2), testosterone (T) and 11ketotestosterone (11-KT) were measured by ELISA using commercial kits (Cayman Chemical Company). A one-way ANOVA test was used to compare the plasma level of steroids throughout the experimental period.

RESULTS & DISCUSSION

Plasma levels of E2 decreased after 60 days of the initial injection with AI and MT, but the initial values were reestablished after 90 days. Plasma levels of T and 11-KT have already increased after 30 days in females injected with AI and these low levels were maintained after 90 days. Plasma levels of T did not change in females injected with MT while 11KT increased after 60 days. The use of AI and MT has been used to induce sex change in dusky grouper, but the profile of plasma steroids after these treatments vary according to season, fish age and ovarian maturation stage. It is a consensus in the literature that plasma E2 level does not necessarily decrease after AI induction, as this estrogen is also important in spermatogenesis, stimulating the renewal of spermatogonia. Regarding the androgens, our data corroborate most studies using AI in protogynous teleosts, with the increase in plasma levels of T and 11-KT, but with a chronology that can vary, while MT results are more controversy.

This project was supported by FAPESP (#2014/16320-7; #2017/06765-0; #2018/18316-8).

Poster Presentation 59 (student)

Development of an enzyme-linked immunosorbent assay (ELISA) in *Sebastes* rockfishes for a serum lipocalin-like protein, a potential novel biomarker to detect reproductive males

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INTRODUCTION

Viviparous rockfishes *Sebastes* spp. are economically important aquaculture species. Prior to the artificial insemination, the testes of mature males are removed because it is generally difficult to obtain sperm solution by squeezing the males. In our previous study, lipocalin-type prostaglandin D_2 synthase homolog proteins (LPGDShp) were identified in the urine of reproductive black rockfish (*Sebastes schlegelii*) males. Subsequently, an LPGDShp-like protein (hereafter, lipocalin-like protein) was immunologically detected in the sera of *Sebastes* rockfish, leading us to develop quantitative immunoassay and explore its potential as a hematological biomarker for selecting reproductive males.

METHODS

We developed a non-competitive sandwich enzyme-linked immunosorbent assay (ELISA) to measure the serum level of the lipocalin-like protein. A recombinant LPGDShp (rLPGDShp) was produced and employed as the assay component (i.e., the assay standard, as well as the antigen for preparing the corresponding polyvalent rabbit IgG: anti-rLPGDShp IgG). The anti-rLPGDShp IgG was labeled with digoxygenin (DIG) and utilized as the primary antibody, while the non-labeled IgG was used for the plate coating. Parallelism between serial dilutions of rLPGDShp and male sera was evaluated for two rockfish species, black rockfish and white-edged rockfish (*S. taczanowskii*). Intra/inter-assay precision tests and a spike-recovery test were performed. The serum levels of the lipocalin-like protein were quantified in black rockfish (sampled at copulation and non-copulation season) and white-edged rockfish (sampled near-monthly interval for a year).

RESULTS & DISCUSSION

All the validation tests exhibited that the developed ELISA was sufficient to measure the lipocalin-like protein in sera of two rockfish species (e.g., inter/intra variations: 2.7%~9.4%/3.8%~9.7%; recovery rate=95.0%~105.5%) with an assay range between 0.391 and 12.5 ng/ml. In black rockfish, serum levels were higher in males during the copulation season than in males during the non-copulation season and in females during both seasons. In white-edged rockfish, the serum levels exhibited synchronous dynamics with the gonadosomatic index (GSI) in males while no such trend was observed in females. In both species, the serum levels in the males had significant positive correlations with the GSI (black rockfish: $r = 0.74$; white-edged rockfish: $r = 0.83$).

CONCLUSION

These results suggest for the first time that a serum lipocalin-like protein can be used in *Sebastes* rockfishes as a non-lethal, hematological indicator to select reproductive males for artificial insemination.

Poster Presentation 60 (student)

Timing of puberty in hatchery-produced greater amberjack (*Seriola dumerili*)

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INTRODUCTION

The process of puberty involves the acquisition of competence of the brain-pituitary-gonad axis in supporting the proliferation, development, and maturation of germ cells. Under aquaculture conditions, the timing of puberty might differ from what is reported in the wild, and fish may either reach precocious or late maturity, while in extreme scenarios they never achieve sexual maturity. Farming of greater amberjack (*Seriola dumerili*) is still in its infancy and information on the reproductive biology of this species has been obtained mostly from wild-caught reared fish. We report here the first attempt to describe the first age of maturity in a first-generation (F1) greater amberjack stock held in sea cages.

METHODS

Hatchery-produced greater amberjack were reared in sea cages from juveniles (0+) to 5 years old (yo). Samplings were conducted every year in June, during the period of reproductive maturation and spawning of cultured reared broodstock in the Mediterranean basin. Fish were bled and plasma levels of eight sex steroids were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) and records were taken of body weight (BW), total length (TL), gonadosomatic index (GSI) and hepatosomatic index (HIS). At the age of 4 and 5 yo, fish were considered reproductively mature and eligible for spawning induction, and were treated with gonadotropinreleasing hormone agonist (GnRHa, 50 μg kg^{-1}) and transferred to tanks for spawning. Egg production was evaluated for 3 weeks.

RESULTS & DISCUSSION

No growth-related sexual dimorphism was observed. The ovaries of 1 and 2 yo females consisted of primary oocytes, while at the age of 3 yo early vitellogenic (Vg) oocytes were also identified. At the age of 4 yo, late Vg oocytes were recorded, but extensive follicular atresia characterized the ovary content of 50% of females. Similarly, at the age of 5 yo, batches of late Vg oocytes were present in the gonads, but atresia was very limited. On the other hand, complete gametogenesis in males was evident already in more than 50% of the examined 2 yo fish. The percentage of males in an advanced spermatogenesis stage reached 100% in 3 yo fish and, therefore, it can be considered as their first age of maturity for male greater amberjack. In regards to the hormonal profile of the monitored F1 fish, low levels of testosterone (T) and 17 β-estradiol (E2) were found in all females that entered vitellogenesis. Moreover, high levels of androstenedione were detected in the plasma of atretic females. In males, high androgen levels were found during advanced spermatogenesis. The administration of GnRHa to 4 and 5 yo fish, induced one and two spawns, respectively, however no fertilized eggs were obtained. The results indicate the male F1 greater amberjack mature well and within the same period observed in wild males, albeit with smaller gonad size. On the contrary, females seem to mature later than in the wild, also with a smaller gonad size. Spawning in response to GnRHa treatment was not effective, contrary to what has been shown with wild-caught culturereared broodstock.

The project was funded by the project NewTechAqua (European Union s´ Programme H2020, GA 862658).

Poster Presentation 61

Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery produced mullet (*Mugil cephalus*)

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INTRODUCTION

In fish, puberty is susceptible to multiple, interacting environmental cues. As a result, farming conditions may have varying influences on the age of first sexual maturity, causing precocious puberty in some species or delayed and even completely blocked puberty in others. Therefore, this study aimed at characterizing pubertal development in grey mullet (*Mugil cephalus*), an important fish candidate for domestication and aquaculture production.

MATERIALS AND METHODS

Two stocks of grey mullet were compared: wild-caught (WC; Apulia, Italy) and hatchery produced (HP; National Center for Mariculture, Eilat, Israel) grey mullet, both subjected to captive conditions consist of ambient seawater salinity (40 ppt; Gulf of Eilat, Red Sea) and photo-thermal regime. The age of the fish was estimated based on their scales. Growth performance and gonadal development were monitored in 2- and 3- year old fish (2y and 3y).

RESULTS AND CONCLUSIONS

Our results revealed that all 2y fish had immature gonads. The majority of 2y females exhibited late perinucleolar oocytes as the most advanced oocyte stage. Yet, HP females had significantly larger oocytes than WC specimens. Testis from an immature age 2y HP specimen showed small seminiferous lobules. Only spermatogonia, along with somatic cells were visible. Gonad section from a 2y HP intersex showed the presence of all stages of spermatogenesis. Scattered perinucleolar stage oocytes were visible, indicating that both WC and HP, had immature testes. Following 3y age category revealed that WC females were larger than cognate males. Although not significant, the HP mullets appear to exhibit a similar trend. Gonadosomatic index (GSI) values in 3y HP females and males were significantly higher than those of the WC of the same age. Undifferentiated gonads were found in 20% of WC fish compared to 5% in the HP fish. While only 33% had reached vitellogenic oocytes level, 54% of the HP females reached this level of development. Males exhibited a significant difference in development: 100% of the WC males showed first stages of spermatogenesis, while 67% of the male population in the HP group produced mature sperm. HP females, sampled during natural spawning season, reached maturity and ovulation. Their GSI exceeded 15%. Interestingly, approximately 50% of the HP females' GSI exhibited markedly developed gonads with GSI values ranging between 10-20% while all the others were lagging far behind, having GSI values between 0.2 to 0.3 %. GSI values in WC females were also divided into 2 sub groups and found to be significantly lower than those of the hatchery produced females (1-6% and <1% respectively). Males also exhibited two GSI groups pattern, higher GSI values were exhibited by HP fish (1.5%-2.75% and <1% compared to 0.2-0.5% and <0.15%). Suggesting hierarchy plays a major role affecting gonadal development in mullets.

In summary, the 3 yr old HP mullet females and males exhibited enhanced gonadal maturation as compared to that of the WC captive-reared fish, probably as a result of domestication. The project received funding from the European Union 7FP (GA 603121, DIVERSIFY)

Poster Presentation 62

Spermatogenesis enhancement in hatchery-produced greater amberjack (*Seriola dumerili*)

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INTRODUCTION

Greater amberjack *Seriola dumerili* (Risso, 1810) is a promising emerging aquaculture species thanks to its rapid growth and consumers' appreciation. Wild-caught greater amberjack males reared in sea cages showed alteration of plasma sex steroid concentrations, high testicular apoptosis, reduced germ cell proliferation and low sperm quality. We report the effects of gonadotropin releasing hormone agonist (GnRHa) and human chorionic gonadotropin (hCG) administration on testis development and male germ cell proliferation in hatchery-produced greater amberjack.

METHODS

Four-year-old hatchery-produced greater amberjack males (F1 generation) reared in a sea cage in Salamina (Greece) were treated with GnRHa, either through EVAc implants (50 μg kg^{-1} body weight) or injections (20 μg kg^{-1} body weight), hCG (1000 IU kg^{-1} body weight) or were left untreated as controls. Two fish per group were treated in mid-May, when testes were in active spermatogenesis. Two weeks after treatments, fish were sacrificed and i) gonadosomatic index (GSI) was calculated as 100 × testis weight/body weight; ii) testis samples were fixed in Bouin's solution and destined to histological analysis and to the immunodetection of the proliferating cell nuclear antigen (PCNA). The effects of the treatments on spermatogonial proliferation and germ cell progression towards meiosis was assessed through the count of the number of PCNA-positive single spermatogonia and the number of spermatocysts (PCNA-positive spermatogonial cysts + spermatocyte cysts).

RESULTS & DISCUSSION

The treatments resulted in an increase of GSI (untreated: 1.0 ± 0.2; GnRHa implant: 2.0 ± 1.4; GnRHa injection: 2.1 ± 0.8; hCG: 2.6 ± 0.7) and seminiferous tubule diameter (untreated: 142.5 ± 23.1 μm; GnRHa implant: 151.0 ± 14.7 μm; GnRHa injection: 196.1 ± 50.8 μm; hCG = 191.4 ± 8.3 μm). According to the subjective histological evaluation, testes of treated fish showed an increase of germinal epithelium height, larger lumina of seminiferous tubules and more abundant luminal spermatozoa compared with untreated controls. Fish treated with hCG showed the most dramatic changes, characterized by confluence of seminiferous tubules in large sperm masses in the internal testicular region. The hormone treatments resulted in both a decrease of proliferating single spermatogonia (untreated: 108.1 ± 1.3; GnRHa implant: 77.9 ± 47.4; GnRHa injection: 37.3 ± 9.2; hCG: 24.7 ± 21.3 cells/mm^2) and an increase of PCNA-positive spermatocysts (untreated: 839.4 ± 12.8; GnRHa implant: 959.2 ± 30.8; GnRHa injection: 868.6 ± 379.5; hCG: 2074.0 ± 38.4 spermatocysts/mm^2).

In conclusion, all the tree treatments were effective in inducing testicular maturation through the stimulation of germ cell progression towards meiosis. Although the injection of hCG showed the most marked overall effects, GnRHa implantation was still able, after two weeks, to support both spermatogonial proliferation and progression towards meiosis, thus suggesting the capacity to sustain spermatogenesis over a longer period compared with the other two treatments.

The project received funding from the ERA-NET Cofund BlueBio program (BESTBROOD project).

Poster Presentation 63

Improving non-toxic fixation of fish gonads, gametes and larvae

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INTRODUCTION

Phosphate buffered formalin (aqueous solution of formaldehyde) (PBF) and paraformaldehyde (PFA) have been universally used for over 100 years as fixatives for histology, immunohistochemistry and ultrastructure analyses with scanning electron microscopy (SEM). However, these reagents are harmful carcinogen, posing serious risk for health and limiting applications in field sampling where powerful ventilation devices and costly disposal procedures are not always available. Despite the recent efforts to obtain alternative fixatives which combine the advantages of traditional fixatives with lack of toxicity, little attention has been devoted so far to optimizing non-toxic fixation protocols for fish tissues, and for fish gametes and larvae in particular. He we address this dearth by testing the fixing performance of a non-toxic acid-free glyoxal fixative (GAF®), originally developed for veterinary pathology, on gonads, gametes and larvae of the European seabass *Dicentrarchus labrax*.

METHODS

GAF® is a neutral fixative, obtained by removing acids from the dialdehyde glyoxal with an ionexchange resin. De-acidification allows to overcome the drawbacks of commercially available glyoxal substitutes of PBF whose strong acidity is probably responsible for some observed detrimental effect on tissues. Here we tested the performance of GAF® as fixative for 1) histology and immunohistochemistry of fish gonads compared to traditional Dietrich's fixative (3:1:0.2:5.8 of ethanol 95%, formaldehyde 40%, glacial acetic acid 99.5%, distilled water), to 30% PBF and to 4% PFA; 2) morphology of fish oocytes compared to 7% PBF routinely used for analyses of fecundity; 3) morphology of fish sperm components (head, midpiece, flagellum) compared to 10% PBF used for optic microscopy and to 4% PFA used for SEM; 4) morphology of larvae compared to traditional 4% PBF and 4% PFA.

RESULTS & DISCUSSION

Here we present results on the fixing performance of GAF® as a non-toxic alternative to traditionally used aldehyde fixatives for histology, immunohistochemistry and SEM of fish gonads, gametes and larvae. Our preliminary results offer important advances for the optimization of non-toxic fixing procedures to be applied in fish reproduction and aquaculture.

Poster Presentation 64 (student)

Elevated rearing temperature increases the occurrence of sexual maturation in male Atlantic salmon (*Salmo salar*) during production of larger sized under-yearling smolts

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INTRODUCTION

In Atlantic salmon aquaculture, early sexual maturation in males is a welfare concern. To increase fish robustness at sea transfer, recent farming regimes include delaying the artificial induction of seawater (SW) tolerance (*i.e.* smolt production) to a point where the fish are at a larger body size (>100 g). However, producing "large smolts" is usually done through daylength and temperature manipulation, two factors that could potentially increase the occurrence of male maturation.

METHODS

We studied two different smolt production regimes, either continuous light throughout (LL regime) or a regime with simulated winter (6 weeks with LD12:12) followed by LL to simulate summer (LDLL regime). Both regimes were examined in two experiments where we used different temperatures: 12˚C (experiment 1, EX1), 8˚C and 16˚C (EX2). In EX1, 216 juvenile Atlantic salmon males and females (mean±SD: 53.3±11.9 g) were reared on 12˚C FW under LDLL or LL throughout the experiment. Sampling occurred one day before all fish were put on LL (0 day*degrees or d˚C) and after 384, 576, 620 and 725 d˚C. Body weight, body condition, and plasma 11-ketotestosterone were measured at each sampling. In EX2, 285 juvenile salmon males (mean±SD: 50.7±8.3 g) were initially reared on LDLL or LL and 12˚C FW for 6 weeks, then, at the same time all fish were put on LL, the temperature was adjusted from 12˚C to 8˚C in half of the tanks, and from 12˚C to 16˚C in the other half. The fish were then kept on these two temperatures for 1000 d˚C. Sampling occurred after 0 (start of the temperature treatments), 384, 672, 832 and 1000 d˚C. Measurements in EX2 included body weight, body condition, gonadosomatic index (GSI) and plasma 11-ketotestosterone. This generated six regimes in total: LDLL12 and LL-12 in EX1, and LDLL-8, LL-8, LDLL-16 and LL-16 in EX2.

RESULTS & DISCUSSION

In EX1, body weight was significantly higher in LL-12 fish compared to LDLL-12 at 384, 576, 620 and 725 d˚C, while body condition was significantly lower in LDLL-12 fish at these timepoints. Plasma 11ketotestosterone increased significantly over time but no difference was found between LDLL-12 or LL-12 fish. In EX2, GSI and plasma 11-ketotestosterone increased in both 16˚C regimes and was found to be significantly higher in LDLL-16 fish compared to LL-16. No elevation in GSI or 11ketotestosterone was observed in 8˚C-fish.

CONCLUSIONS

These results show that elevated rearing temperatures during smolt production exacerbate the problem of male maturation.

The project received funding from the Norwegian Research Council (project no: 299554/F40).

Poster Presentation 65 (student)

Reproductive control and larval culture of flathead grey mullet (*Mugil cephalus*)

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INTRODUCTION

The flathead grey mullet (*Mugil cephalus*) is the most commercially significant omnivorous marine species. However, *Mugil cephalus* in captive conditions do not mature and spawn because apparently the aquaculture environmental does not stimulate the production of gonadotropins- Gths (follicle stimulating hormone-Fsh and luteinizing hormone-Lh) that initiate and control gametogenesis. IRTA has demonstrated that recombinant gonadotropins, (rFsh and rLh) induce gametogenesis and spawning in *Mugil cephalus*. This PhD project aims to: 1) Examine the effect of the environment on the progress of maturation. 2) Examine the effect of rGths doses on ovarian development. 3) Study different conditions in larval rearing to obtain high survivals of juveniles from breeders induced with rGths.

METHODS

To examine the effect of the environment on the progress of maturation, a total of 144 breeders will be randomly distributed to have 12 males and 12 females in duplicates of three different treatments, recirculating aquaculture systems (RAS, www.irtamar.com), biofloc technology (BFT) and, earth ponds. The RAS and BFT will be in $10m^3$ tanks and $2x50m^3$ earth ponds. A total of 12 fish (6 females and 6 males) per treatment will be sampled, on three times points, immature, mid-maturation, and spawning period to record: body weight, gonad somatic index, description of fresh gametes, blood samples for steroid analysis, samples fixed for histology, samples frozen for biochemical and transcriptome analysis. In addition, a group (6 females and 6 males) from the treatment with the most advanced progression of maturation will be induced to complete maturation. To examine the effect of doses (rGth) on ovarian development, experiments will test different doses of rFsh on oogenesis and rLh on oocyte maturation. Larval quality and rearing conditions: Eggs will be obtained after spawning inductions with IRTA developed protocol using rGths (supplied by www.raraavis-bio.com). The eggs will be incubated under constant conditions to evaluate the hatching rate, larval survival, and yolk consumption. Hatched larvae, will be transferred to different tanks 100 L tanks to assess the effect of light intensity, photoperiod, larval density, and changes in live prey during larval rearing. Finally, this rGth protocol will be presented in a commercial fish farming company to demonstrate induced maturation and spawning of breeders (minimum 6 females and 6 males) in a commercial environment.

RESULTS & DISCUSSION

The effect of the environment on the maturation progress, along with the ovarian response to different doses of rGths treatments, will generate valuable knowledge to improve *Mugil cephalus* management strategies in captivity guaranteeing a cost reduction related to hormone use and breeders handling. In addition to providing knowledge on the role of Gths in maturation, oocyte recruitment, and fecundity. Our results on larval quality aim to demonstrate that rGth induction produces high-quality eggs and larvae and will allow the development of a complete and detailed protocol for the culture of high-quality *Mugil cephalus* larvae, which will guarantee the supply of high-quality seed with high survival for growing out in fish farms. This will contribute to ensuring food security without relying on the induction of mature wild individuals or the capture of wild fry, which presents overfishing problems. Altogether, developing novel biotechnologies can significantly impact the global aquaculture industry.

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Poster Presentation 66 (student)

Stimulation of female sex differentiation of 1+ year old hermaphroditic gilthead seabream (*Sparus aurata*) using 17β-Estradiol treatment with controlled-release delivery systems

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INTRODUCTION

The gilthead seabream (*Sparus aurata*) is a protandrous hermaphrodite, with functional males converting to females for the first time at 3 years of age. The reported percentage of fish converting to females ranges between 30-70%. In selective breeding programmes, it is important to know the sex of an individual at the time of breeding value assignment and its selection to contribute to the next breeding selection cycle of crosses. This is in order to know with which individuals of the opposite sex it can be crossed. In gilthead seabream, uncertainty as to the phenotypic sex of the fish at the expected time of spawning, and failure of an individual to develop as female requires further management of the population and removal of the fish from the spawning stock. The objective of the present study was to develop a method to ensure that all 3-year-old gilthead seabream will be functional females, using shortterm treatment with 17β-Estradiol (E_2). At this stage, we established the optimal E_2 dose and time of administration, that will induce complete feminization in gilthead seabream.

METHODS

The E_2 dose study was undertaken with 1+ year old gilthead seabream treated with an Ethylene-Vinyl Acetate (EVAc) controlled-release delivery system loaded with E_2. The treatment begun in July 2022 using two doses (3 and 6 mg kg^{-1}). Due to heavy mortalities after the first treatment with 6 mg kg^{-1} and after the second treatment with 3 mg kg^{-1}, the study was modified with the addition of two lower doses (1 and 2 mg kg^{-1}) beginning in August 2022. Fish were treated every 30 days. Treatment was interrupted after two implantations in group 2 mg kg^{-1} due to increased mortality rates and was completed after three implantations in group 1 mg kg^{-1}. The feminisation evaluation sampling took place in January 2023, during the normal spawning season for gilthead seabream.

RESULTS & DISCUSSION

Hormonal induction of sex reversal resulted in mortality of 42%, 61% and 56% of the population in the E_2 doses of 2, 3 and 6 mg kg^{-1}, respectively, while the corresponding value for the 1 mg kg^{-1} was 6% after three monthly implantations. Feminization success based on the macroscopic shape of the gonads, was 89% in the 1 mg kg^{-1} group, while it was lower for the doses of 2 and 3 mg kg^{-1} (26% and 22%, respectively), in which fish were treated for a shorter period of time. There was no feminization in the dose of 6 mg kg^{-1}, in the remaining fish, which were given only a single E_2 implant. In addition, significant differences were found between the doses 1, 2 and 3 mg kg^{-1} and the control group in the mean GSI of individuals during the breeding period ($p < 0.05$), while no difference was found between the dose of 6 mg kg^{-1} and the control group for the same index ($p > 0.05$). This study suggests that 1mg kg^{-1} E_2 is the optimal dose to induce feminization of 1+ year old gilthead seabream (*Sparus aurata*) with the treatment duration of three months (Funding by a Grant HCMR 60.70101). It is expected that the same treatment in 2+ year old male gilthead seabream will result in 100% feminization.

Poster Presentation 67 (student)

Cryopreservation of common carp (*Cyprinus carpio*) and Siberian sturgeon (*Acipenser baerii*) sperm in hypotonic cryomedia

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INTRODUCTION

The use of cryobiology techniques and methods for preserving genetic biodiversity is currently a promising approach. Besides, cryopreserved sperm can be widely used for aquaculture practices with various cultivated fish species. For cryopreservation, various extenders in combination with different types of cryoprotectants were used. Usually, cryoprotective agent was used with an isotonic (the similar with seminal fluid) or hypertonic extender. The aim of our study shows how cryopreservation with hypotonic cryomedia influence for motility parameters, spermatozoon volume, and fertilization ability of sperm.

METHODS

Two cryomedia were used for common carp: 200 mM glucose, 30 mM TRIS, 11.1% methanol, pH 8.0 (CM-carp-1), and 60 mM NaCl, 2.9 mM sucrose, 22% methanol, 5% ethylene glycol (CM-carp-2). Three cryomedia were used for Siberian sturgeon: 30 mm TRIS, 0,25 mM KCl, 23,4 mM sucrose, pH 8.0, 15% methanol (CM-sturgeon-1); 1 mM KCl, 22% methanol, 5% ethylene glycol (CM-sturgeon-2); and 1 mM KCl, 15% methanol, 5% ethylene glycol (CM-sturgeon-3). After diluting with each cryomedium, sperm samples were placed into 0.5-ml plastic straws or 4.5-ml cryotube and incubated for 10 min before freezing. Then the straws were frozen using uncontrolled cooling in a styrofoam box, with avarage cooling rate 40°C/min. The cryotubes were frozen by a programmable freezer with cooling rate 2°C/min to -20°C, 20°C/min to -180°C. Phase-contrast video microscopy technique followed by CASA was used to analyze sperm motility before and after cryopreservation. Spermatozoa volume changes after mixing with cryoprotective media were studied using a spectrophotometer equipped with a thermo-controlled chamber in a 1-cm light path cuvette.

RESULTS & DISCUSSION

When CM-carp-1 was used in combination with freezing in 0.5-ml straws, and CM-carp-2 was used in combination with freezing in 4.5-ml cryotubes possible to get a high post-thaw sperm motility percentage (around 50%). The best results after cryopreservation of Siberian sturgeon sperm were obtain with hypotonic cryomedium CM-sturgeon-2 (around 50%) in 0.5-ml straws and in 4.5-ml cryotubes. The spermatozoa had a different reaction to cryomedium when the cell volume was measured. At the end of the incubation, spermatozoa in CM-carp-1 and CM-sturgeon-1 (isotonic cryomedia) were in a compressed state, while in CM-carp-2, CM-sturgeon-2, CM-sturgeon-3 – in a swollen state.

We demonstrate the success of carp and sturgeon sperm cryopreservation with hypotonic cryomedia can be achieved using big volumes of samples and slow cooling rates. Using hypotonic cryomedia and combination with big volume of sample (4.5 cryotube) more suitable for fertilization of large volumes of eggs in aquaculture practice.

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Poster Presentation 68

Photothermal manipulation, induced spawning and early life stages of silver trevally (*Pseudocaranx georgianus*; Carangidae) in Aotearoa/New Zealand

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INTRODUCTION

The silver trevally/araara (*Pseudocaranx georgianus*) possess a range of traits desirable for aquaculture and therefore it has been selected as a potential native finfish species for diversification in New Zealand. Although the species reaches vitellogenesis during spring/early summer in captivity, it fails to naturally spawn, and hormone induction is required. Here, we present the results from a hormone-induced spawning after photothermal manipulation and early life stages of trevally in captivity.

METHODS

In June 2022 (winter), wild-caught captivity-acclimated broodstock (n = 9; 5F:4M) were stocked into a 30,000L tank equipped with a controlled temperature and lighting system, set to mimic winter conditions (10ºC and 09L:15D). After 2 weeks of acclimation, photothermal conditions were manipulated to progressively reach summer conditions (21ºC and 14L:10D) within 7 weeks. After four weeks at 21ºC, the reproductive status of females was assessed by gonopore cannulation. Females in advanced stages of oogenesis (oocyte diameters ≥ 400 µm) were injected with human chorionic gonadotropin (hCG) at a target dose of 600 IU/ kg of body weight. Males received the same dose. Subsequently, four females and three males were re-stocked into a 30,000L tank, while a pair was stocked in a 5,000 L tank. Both tanks were equipped with passive egg collectors and maintained at 21ºC. Tanks were monitored twice a day for the presence of eggs for ~30-days post-injection. When spawning was detected, eggs were collected to estimate fecundity, fertilization, and hatching rates. Fertilized eggs with a homogeneous developmental stage were stocked into a 450 L cylindroconical incubator. Newly hatched larvae were transferred into a semi-commercial 5,000L larval rearing tank and reared at 21ºC. Larvae were sampled periodically and imaged under a microscope to document growth and major development stages.

RESULTS & DISCUSSION

Eggs were first detected 36-h post-injection in both tanks. The single pair only spawned once, while spawning from the 30,000L tank continued for ~five days. Egg and spawning data are currently being analyzed. Newly-hatched larvae were 2.67±0.08 mm total length (TL) and yolk-sacs were completely reabsorbed at 3 days post-hatch (DPH). Larvae developed satisfactorily well under the rearing protocol, assuming an adult-like body shape at 26 DPH (17.17±0.93 mm TL). At 74 DPH fingerlings averaged 85.48±4.59 mm TL and 9.24±1.27 g body weight and deemed ready for transfer to the sea pen at the end of spring. Results indicate that trevally broodstock respond well to photothermal manipulation and that the offspring take approximately 11 weeks to reach a size sufficient for transport to the seapen. Further work is needed to improve the egg quality and refine the larval rearing protocol.

This work was funded by the Royal Society of New Zealand Catalyst Seeding General contract CSGPAF1803 and by the New Zealand Government via the Ngā Pou Rangahau platform, a research framework developed by PFR and supported by a Strategic Science Investment Fund grant from the Ministry of Business, Innovation and Employment.

Poster Presentation 69

Photoperiod manipulation affects differently turbot (*Scophthalmus maximus)* broodstock sperm quality, depending on the season of the year

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INTRODUCTION

Turbot, *Scophthalmus maximus*, is a highly appreciated flatfish species, produced in Southern European countries- Spain, France and Portugal-, and in Iceland, Norway and China, the biggest producer. This species farming is still facing production challenges, namely in terms of captivity reproduction, since this species does not spawn naturally. Females' gonads mature and ovulate, but the eggs are not released. In maternities, photoperiod manipulation is used to produce eggs and sperm on a year-round basis, and larvae are obtained through artificial fertilization. Although this is a standard procedure in the industry, there are no recent studies on the effect of this photoperiod manipulation on sperm quality. Thus, the objective of this study was to evaluate sperm quality in 4 broodstocks under simulated natural photoperiod, displaced in the four seasons of the year.

METHODS

Four captive-reared turbot broodstocks from the company Flatlantic® were used (mean weight 6.08 kg). Each broodstock was reared in a 15 m3 tank at a constant water temperature all year round (14.0 ± 0.5 ºC), fed on a commercial feed (Sparos Lda.) *ad libitum* and under a simulated natural photoperiod. Each photoperiod simulated the natural seasonal oscillations, displaced to overlap the spawning period (15h of light) to the four seasons of the year: spring, summer, autumn and winter. Sperm samples were collected at the corresponding month of 15h light for each broodstock, to evaluate the following sperm quality parameters: sperm motility and cell concentration (CASA system), lipid peroxidation (MDA determination), DNA fragmentation (Comet assay), Cell viability and Reactive Oxygen Species (ROS) (flow cytometer).

RESULTS & DISCUSSION

The results of sperm quality revealed differences among the broodstocks under different photoperiodic manipulations. Progressive motility was higher in the winter group (39.9 %), while velocity was higher in spring (VCL 112.9 μm s^{-1}). Spermatozoa concentration was significantly higher in autumn and winter (9436.7 and 9210.0 Mspz.mL^{-1}), while lipid peroxidation was reduced in summer and winter stocks (22.14 and 22.22 nmoles MDA.$Mspz^{-1}$). The lowest DNA fragmentation was registered in spring (17.42 %) and ROS was increased in winter (46.89% live cells with ROS). Cell viability was high in all four broodstocks. Apparently winter broodstock had the best performance, although sperm presented high levels of ROS. Deficiencies in antioxidants and immune system dysfunctions may alter the oxidant/antioxidant balance, leading to oxidative stress. These results together with a high heterogeneity of sperm traits among broodstocks could compromise the success of larvae production along the year. Nutrition could be a valuable tool to standardize and ameliorate sperm traits, for example with feeds supplementation during reproductive events, using antioxidant and immune stimulating raw ingredients.

The project received funding from "Programa Crescimento Azul (#4)" - PT-INNOVATION-0080 (EEA grants project-BREEDFLAT) and FCT (UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020).

Poster Presentation 70 (student)

Successful cryopreservation of sperm from Mediterranean aquacultured species in biodegradable containers

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INTRODUCTION

The most used sperm cryopreservation containers are straws and cryovials made of polypropylene. Plastic containers are generally not reused, making them waste with a high potential for polluting the environment. We aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative for the cryopreservation of European eel (*Anguilla anguilla*), gilthead seabream (*Sparus aurata*), and European sea bass (*Dicentrarchus labrax*) sperm.

METHODS

European eel (n=12), gilthead seabream (n=12), and European sea bass (n=10) sperm samples with motility >60% were cryopreserved in plastic straws, hard-gelatin, and HPMC biodegradable capsules. Total motility (MOT - %), progressive motility (MOTp - %), and the curvilinear (VCL - µm/s), straight line (VSL - µm/s), and average path (VAP - µm/s) velocities were evaluated by CASA-Mot software. Sperm viability was performed using a Live and Dead KIT and an epifluorescence microscope. To quantify DNA damage, the alkaline comet assay was performed. Head-DNA (%), Tail-DNA (%), Tail Moment, and Olive Moment were evaluated by CaspLab software.

RESULTS & DISCUSSION

European eel sperm cryopreservation in all containers reduced the MOT and MOTp but we found no differences between straws and hard-capsules. Fresh sperm showed higher VCL than sperm cryopreserved using capsules. In addition, fresh and post-thawed sperm stored in straws showed higher VAP than sperm cryopreserved in the HPMC capsules. Cell viability reduction was observed when the sperm was cryopreserved in straws and hard-gelatin capsules. On the other hand, DNA damage was not different between fresh and thawed sperm samples. In gilthead seabream, sperm cryopreservation reduced the kinetic parameters. Samples cryopreserved in straws showed higher VSL than those stored in HPMC capsules. The cryopreservation caused a reduction in the cell viability, and samples cryopreserved in hard-capsules showed higher DNA damage than fresh sperm and samples stored in straws. However, at this level of damage (<10%), the oocytes can repair it after fertilization and are able to continue the embryo development. European sea bass sperm cryopreservation also reduced sperm kinetic parameters. Samples stored in hard-gelatin capsules showed higher velocities than those cryopreserved in HPMC capsules. The cryopreservation process decreased the cell viability but did not increase DNA damage. Biodegradable capsules can maintain sperm quality after cryopreservation, similarly to plastic straws. Thus, hard gelatin and HPMC biodegradable capsules can be considered an alternative to plastic straws for cryopreservation of sperm from all three species.

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Poster Presentation 71

Autophagic interventions on gonadal sexuality in medaka, *Oryzias latipes*

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INTRODUCTION

Autophagy, or cellular self-digestion, is an essential cellular process imperative for energy homeostasis, development, differentiation, and survival. It was recently observed that autophagy genes are relatively abundant in steroidogenic tissues and associated processes are sex biased and estrogen responsive in fish. Previously, we also reported that, autophagy is instrumental for gamete maintenance in adult fish. So, in this investigation, we explored the possibility of autophagic involvement in embryonic germ cell development and sexual assignment in medaka.

METHODS

CRISPR probes were designed in silico, gRNAs were synthesized in vitro, injected @ 25ng/ul/gRNA along with 600ng/ul of CAS9 protein into one cell stage embryos of nanos3`UTR-eGFP transgenic HdRR medaka to generate F_0 founder population. Fish carrying minimal of 4bp alteration resulting in premature stop codon were sequentially bred to produce F_3 heterozygous population, and both F_4 and F_5 embryos were used for germ cell analysis and rescue attempts. Gamete quality and fertilization potential experiments were, respectively, conducted using at least 10 individuals and 8 pairs of fish for a period of 15 days. Transcriptome analyses were conducted using illumina miSEQ protocol.

RESULTS & DISCUSSION

In depth analysis shows that, all candidate autophagy gene are prevalent in the germ cells and homozygous knockout of ULK1b, ATG13, LC3a, SIRT1 and DOR, resulting in complete germ cell depletion and thereby rendering sterility. Interestingly, Beclin1$^{-/-}$, HK4$^{-/-}$ and HK2$^{+/}$/HK4$^{+/-}$ mutants show severe defects in sperm characteristics (motility, etc.) and oocyte development (micropyle formation etc.) and hence affected fertilization and can only be rescued by overexpression of respective gene in concern. However, mutation of HK1, HK2, ULK1a and LC3g does not have any effect on fertility. To understand the autophagy-steroid connection in gonad, we used estrogen receptor knockout fish and found that, apart from significant alterations in autophagic genes, micropyle formation, sperm motility, fertility, and sex ratio (at adulthood) were highly affected in both fish groups and interestingly had a strong correlation with ATG13, LC3a, especially in the ERb2-KO fish gonads. RNA-seq analysis and subsequent validation suggested that autophagy-estrogen-early germ cell development are interlinked. Notably, using ERb2-KO medaka, earlier we found that calcium ion signaling associated alternate (independent of hexokinase/AMPK pathway) autophagy also affects the germ cell health and female development. Cumulatively, our data suggests that germ cell autophagy is critical for early gamete development and further analysis are required to unveil the steroid responsive autophagy regulatory-switches to confirm the gender-skewed autophagy. Expectedly, this study may furnish newer appreciation for fertility management, genderspecific medicine research and therapeutics.

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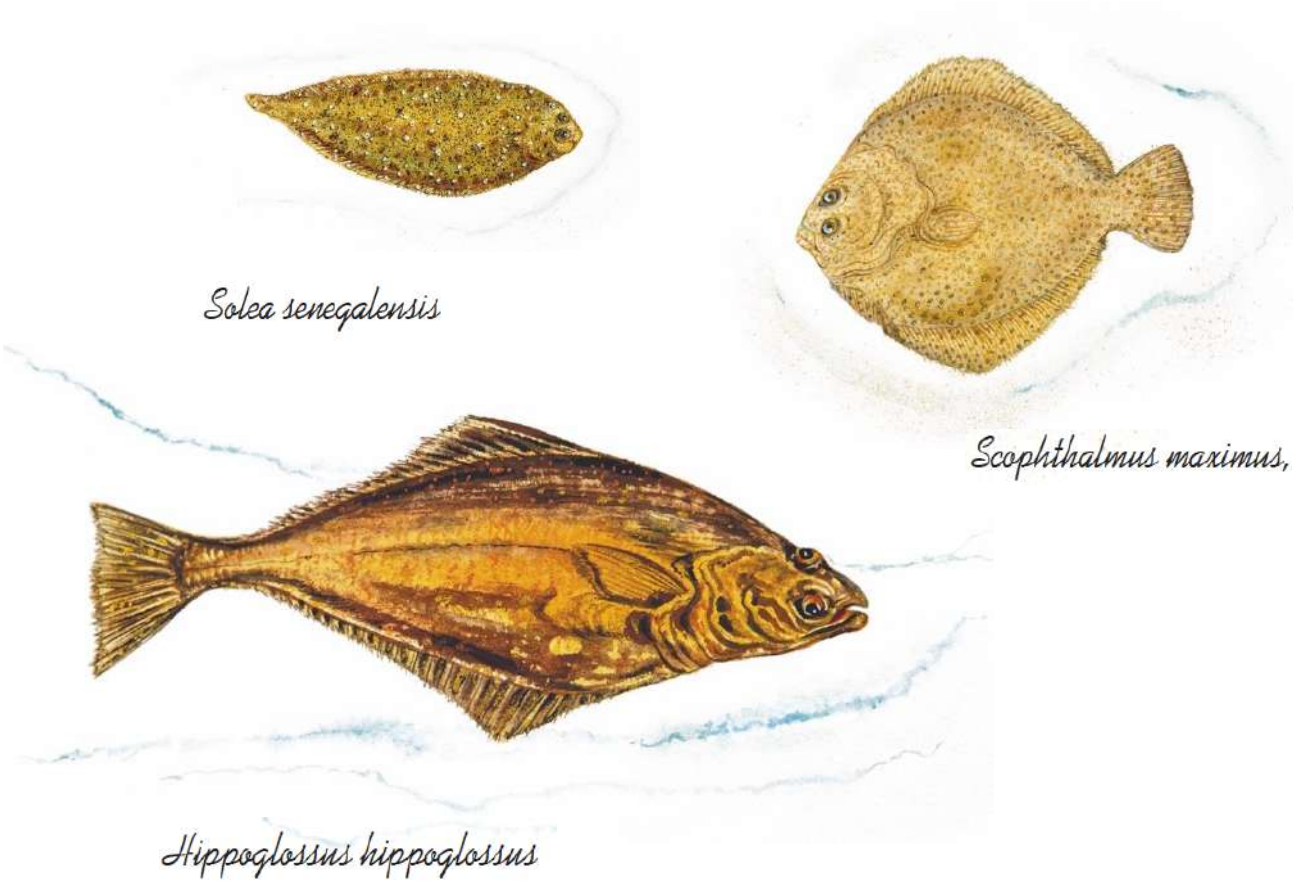
SS7. Gamete and egg quality



Poster Presentation 72

Intracellular pH regulation and sperm motility in the European eel

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INTRODUCTION

pH is an important environmental factor in seawater chemistry and in cell physiology. It is known that maintaining intracellular pH (pH_i) within physiological limits is key for cell function, as most cellular processes are influenced and operate within a narrow pH range. pHi regulation is crucial for motility initiation and chemotaxis in sea urchin sperm, and for sperm capacitation and hyperactivation in mammalian sperm. Sperm activation involves ion fluxes as well as a previous maturation in the seminal plasma, something which has not been studied in depth in marine fish species. pH and potassium are probably involved in sperm maturation and motility in the European eel, as indicated in previous studies.

METHODS

A total of 80 farmed male eels (mean body weight 124 ± 5 g) in two batches of 40 males were transported to our facilities at the Universitat Politècnica de València (Spain). After seawater acclimation, the hormonal treatment started (hCG, 1.5 IU/g fish). Sperm samples were obtained from the 6th week of treatment, and they were checked for motility with CASA system (Proiser, Valencia, Spain), using for experiments only the samples with > 50 % motility. Measuring of intracellular pH was performed by Flow Cytometry, using SNARF-5F AM. The intracellular K^+ concentration was measured with the Flow Cytometer by the null point described in Takai & Morisawa (1995).

RESULTS & DISCUSSION

In this work, the absolute intracellular concentration of potassium in European eel sperm has been determined for the first time, being 124 ± 30 mM K^+. In addition, the intracellular pH (pHi) of quiescent eel spermatozoa was determined by two methods (nigericin and null point) that gave similar results, 7.47.6. The natural pHi range of sperm samples in the quiescent stage was 7.4-8.0, with no evident relationship with sperm motility. However, a linear correlation was seen between sperm motility and the pH of the diluent or extracellular pH (pHe), as well as between the pHi and the pH of the diluent. The pHi change post-activation in seawater (ASW) depended on the initial pHe of the diluent medium. Activation with ASW (pH 8.2) induced an internal alkalinization of the cells when the sample had previously been diluted in a pHe < 8.0; an acidification when pHe > 8, and no pHi variation when pHe was 8.0. These experiments indicated that a careful selection of the diluents should be performed before measuring natural pHi changes in sperm cells. Thus, studies on the specific seminal plasma composition of marine fish species are necessary before studying their physiology. Furthermore, our study indicates that intracellular alkalinization is not a universal fact during sperm activation.

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Poster Presentation 73 (student)

Senegalese sole short-term sperm quality can be influenced by different collection media

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INTRODUCTION

Compared to wild Senegalese sole (*Solea senegalensis*), F1 males present lower sperm volume and quality, being important to optimize the methods for sperm collection to apply further reproductive technological uses such as long- or short-term storage. Commercial dilution media, like marine freeze (IMV), can improve sperm quality during short storage but do not allow the usage of those samples for quality control due to fluorescence interference on a flow cytometer and also on colorimetric assays. In this study, a comparison of three different methods for sperm collection and maintenance was done.

METHODS

Six F1 male broodstocks were maintained at Ramalhete station (University of Algarve, Faro, Portugal) in fiber-glass tanks on a semi-closed system kept with a 2:1 sex ratio (male:female). Before sampling, fish were anesthetized with 300 ppm phenoxyethanol, the genital pore was cleaned to avoid any contamination, and sperm from the same male (n=7) was collected with a sterile 1 mL syringe using three different methods: syringe without any dilution media (Ctr), a syringe filled with 100 µL of marine freeze (MF), or filled with 100 µL of Mounib (125 mM sucrose, 100 mM $KHCO_3$, 6.5 mM reduced glutathione). Immediately after collection, sperm motility was assessed by CASA (Computer Assisted Sperm Analysis) system, registering different parameters: total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN), average path velocity (VAP), and spermatozoa fast, medium and slow subpopulations. Sperm samples were placed in a Makler chamber, activated with artificial seawater and motility was measured 15 s post-activation, the time of highest motility in this species. Motility was also measured 24 h later to evaluate if sperm maintained its quality after storage at 4 ºC.

RESULTS & DISCUSSION

Results revealed that short-term sperm motility can be influenced by the type of dilution medium used for sperm collection. In fresh samples, TM was significantly higher in samples collected with a dilution media (70% in Mounib, 74% in MF) compared with control (47%), although after 24 h TM decreased drastically on Mounib treatment (7%), it was maintained on MF (63%). The same pattern was observed for PM, LIN and VAP, and on VCL and VSL the MF treatment also had a significant decrease after 24 h. Regarding the spermatozoa subpopulations, the MF treatment showed the highest percentage of fast spermatozoa (53%), followed by Mounib (45%), and the lowest was obtained in the Ctr (18%). After 24 h only Mounib treatment showed a significant decrease in fast spermatozoa population (2%). Differently from the commercial marine freeze, the Mounib sperm extender is a solution with a known composition that does not produce any type of interference when sperm is diluted. Overall, the results demonstrated that Mounib can replace MF during sperm collection, allowing to perform fresh sperm quality analysis using flow cytometry, not interfering with the quality analysis performed. This can be important when samples need to be selected for artificial fertilization or cryopreservation procedures.

This work was funded by Programa Crescimento Azul (#4) - PT-INNOVATION-0080 (EEA grants project-BREEDFLAT), and by FCT through the PhD fellowship SFRH/BD/148280/2019 to F.F, and CCMAR Strategic Project - UIDB/04326/2022.

Poster Presentation 74

Effects of multiple vitellogenins on egg quality in Atlantic halibut (*Hippoglossus hippoglossus*) and European plaice (*Pleuronectes platessa*)

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INTRODUCTION

A clear link between vitellogenin (Vtg) abundance and egg quality in fish has not been established yet. The main purpose of this study was to assess processing and utilization of multiple Vtgs in relation to egg quality in a commercially established aquaculture species, the Atlantic halibut (*Hippoglossus hippoglossus*), and in an alternative flatfish species with potential for aquaculture, the European plaice (*Pleuronectes platessa*).

METHODS

Good and poor-quality egg batches were collected from ~15 halibut females during 4 consecutive reproductive seasons. Quality assessment of egg batches was based on embryo survival prior to hatching. Part of each batch was incubated for egg quality assessment and sampling at different developmental stages from 1-hour post fertilization (hpf) eggs up to 50 days post fertilization (dpf), prior to first feeding. Additionally, postvitellogenic stage oocytes were collected from halibut via ovarian biopsies. Developmentally comparable stages to halibut were targeted for samplings in plaice (from unfertilized eggs to 25 dpf). Collected samples were subjected to parallel monitoring-based liquid chromatography mass spectrometry to quantify heavy and light chain lipovitellins (Lvs; LvH and LvL) from three different Vtgs in both species in addition to free amino acid (FAA) levels, cathepsin (CAT) gene (*cts*) expression and CAT enzyme activity.

RESULTS & DISCUSSION

Except for LvHC being less abundant in poor quality postvitellogenic oocytes, none of the Lvs significantly differed in quantity between good and poor quality halibut eggs at any stage. Poor quality unfertilized plaice eggs had less abundant LvHAa and LvHC. By 10 dpf VtgAa- and VtgAb-derived Lvs (heavy and light chain) contents of poor-quality plaice eggs were significantly lower than in good quality eggs. LvLAa levels remained low in poor quality plaice eggs at 15 dpf and 20 dpf. LvHC levels were also reduced in poor quality plaice eggs at 15 dpf. Good quality halibut eggs were richer in FAAs especially at 12 dpf. No significant differences were detected in FAA levels between good and poor-quality eggs from plaice. The disparate profiles of Lvs in good versus poor quality eggs and offspring between halibut and plaice show that variability of yolk protein utilization can exist even among closely related species. Despite lack of differences in Lv quantities at any developmental stage, differences in FAA levels between good and poor-quality halibut eggs and offspring suggest some impairment of Lv processing. In this regard, quantifications of CAT gene expression and enzyme activity were not very informative of causation, revealing only higher levels of *ctszl* mRNA, and lower *ctsh* mRNA levels in poor versus good quality postvitellogenic halibut oocytes. CATD activity levels were significantly higher in good quality halibut offspring, but only at 20 dpf. In plaice, the depressed profiles of several Lvs seen in poor quality eggs, without significant changes in FAA profiles, suggest a robustness in ability to cope with impaired deposition of Vtg-derived yolk proteins. Further studies are needed to elucidate the details of yolk protein processing in this species.

Poster Presentation 75 (student)

Effect of vitamin C and b-glucan on Senegalese sole sperm quality and larval reprogramming

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INTRODUCTION

Vitamin C and b-glucans have a high antioxidant activity. They are usually used as supplement in diets of fish to increase welfare and immune status. Moreover, they have shown priming effects in fish. Senegalese sole broodstock are normally fed with fresh diets and optimization of dry feeds is a priority. Also, evaluation of these compounds to prime larvae could benefit the aquaculture industry. In this study, we evaluated the effect of vitamin C and b-glucan on sperm quality and larval plasticity to reprogram growth performance.

METHODS

Two diets supplemented with 1% vitamin C and 1% b-glucans (Macrogard) were prepared using as reference a commercial diet (Sparos, Portugal). Diets were supplied to F1 broodstocks and sperm quality evaluated for two spawning seasons under natural conditions using a CASA system. In order to evaluate the effects on larval plasticity, embryos in pharyngula and larvae close at hatch were exposed to three doses of vitamin C and b-glucans (0.5, 2 and 4 g L^{-1}) for 6 hours and DNMT's gene expression was quantified. An untreated group was kept as a control. In a second experiment, larvae at hatched were exposed to selected dose of vitamin C and β-glucans (2 and 1 g L^{-1}, respectively) and cultivated using standardized protocols for sole, evaluating metamorphic index and weight. To evaluate long term effects of initial treatments, fry soles were individually tagged with PIT and cultivated following a common garden strategy and growth evaluated after 76 days.

RESULTS & DISCUSSION

Percentages of running males for the two seasons ranged between 19.8 and 53.8%. Majority of quantitative and quality sperm parameters as determined by CASA showed a great temporal variation through the season although no differences associated with the diets were found. No differences were found with respect to biochemical parameters levels of reactive oxygen species (ROS), DNA fragmentation and cell viability. With respect to the reprogramming effects, embryos activated transcription of *dntm3aa* and *dnmt3bb2* after vitamin C treatments. In larvae close to hatch, expression of *dnmt1*, *dnmt3aa* and *dnmt3bb2* was reduced after b-glucans treatment and *dnmt3ab* increased mRNA levels after the vitamin C treatment. To evaluate the long-term effects on growth, hatched larvae were newly exposed to vitamin C and b-glucans confirming *dnmt3aa* reduced significantly the expression in both exposures. When larvae were cultivated, reprogrammed larvae with vitamin C grew significantly faster than control and b-glucans groups. Also, reprogramed juveniles with vitamin C showed faster growth rates than control and b-glucans groups without modifying shape parameters indicating a positive effect of Vitamin C to reprogram sole for growth performance.

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Poster Presentation 76 (student)

A model species is not a model species! The importance of choosing the appropriate strain of zebrafish

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INTRODUCTION

In zoology, animal models have always been used to improve our knowledge. Among aquatic models, zebrafish is used for a large number of applications including medicine, ecotoxicology or development and reproduction. Consequently, several strains are available with their own history of domestication, potentially generating intra-species variabilities for specific biological functions. Up to now, information regarding comparisons of several strains in the same conditions are not available. Our objective is to compare key parameters linked to the growth and reproduction between classically used strains in order to detect potential differences and commonalities between studied strains.

METHODS

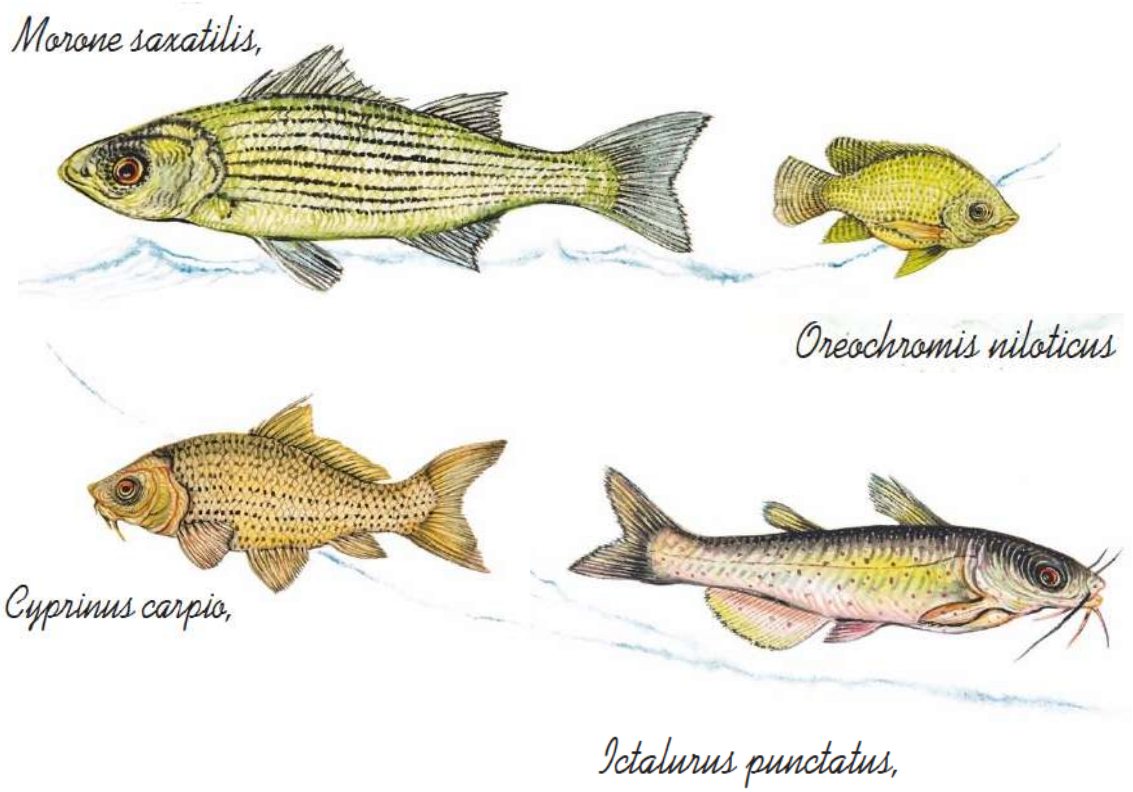
Four Wild-Type strains (AB, TU, WIK and SJD) were provided by the European Zebrafish Resource Center and maintained in recirculating water systems from the larval stage to the adult stage. Growth control was performed every 20 days from 10 dpf (days post fertilization). Age of puberty in females was determined by observing the occurrence of the first spawning from 86 dpf. A measurement of whole-body DHP (assumed Maturation Inducing Hormone) content was performed at 100 dpf and 160 dpf using ELISA test. In males, sperm analyses were performed using a Computer Assisted Sperm Analysis (CASA) system at 367dpf.

RESULTS & DISCUSSION

SJD had a significantly higher standard length than WIK at 70 and 110 dpf. No difference in the females weight was detected at 100dpf but, at 160dpf, WIK weight was significantly higher than AB one. First spawning occurred at 86 dpf for AB and SJD with better reproductive performance (number of eggs released, higher fertilization rate) for AB over the whole period tested. For TU, first spawning occurred at 93 dpf while for WIK, it occurred at 107 dpf. At 100 dpf, DHP was detectable in several individuals in AB, SJD and TU but none in WIK. Nevertheless, no significant difference appeared, probably due to the fact that DHP was not detectable in all females. At 160 dpf, all strains except TU expressed DHP, without any significant differences between the strains. Regarding males, sperm analyses showed that AB sperm was significantly more concentrated than SJD and TU ones. Percentages of immobile and progressive spermatozoa were homogeneous between the strains but for some sperm velocity parameters such as VCL, VSL and VAP, AB strain showed significantly better results than SJD or TU strains.

In the literature, it is assumed that the onset of puberty in zebrafish is positively correlated to body size. This assumption contradicts our data as AB individuals were smaller compared to all other strains, while they had the most precocious puberty. Moreover, WIK, which was the last strain to spawn, showed similar growth kinetics as AB. Furthermore, DHP was not detectable in any WIK individuals at 100 dpf, in contrast to the other strains, which strengthens the hypothesis that only WIK was not yet mature at this stage. Interestingly, AB females released more eggs with a higher quality than SJD; also AB males showed a better sperm quality than SJD. Our results suggest that AB strain displayed better reproductive performance than the other strains even if a slower growth rate was monitored. These results could guide researchers in their choice of strains depending on the purpose of their research.

SS8. Behaviour and pheromones



Poster Presentation 77

Public aquariums as an environment to study the biology and ecology of marine fishes. The case of reproductive behavior of grey triggerfish (*Balistes capriscus*) in captivity

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INTRODUCTION

Public aquariums may contribute to the field of experimental biology and ecology of marine fauna, including behavioral studies, since they have the infrastructure to maintain a wide range of living organisms in tanks that resemble their natural environment. The understanding of reproductive behavior is required for various practical bio-manipulation and ecological applications, but also for successful maintenance under optimal welfare conditions and breeding. We report the reproductive behavior of grey triggerfish (*Balistes capriscus*) as observed at Cretaquarium.

METHODS

Three wild grey triggerfish were displayed in the largest exhibition tank of Cretaquarium (600m^3 volume) along with 14 dusky groupers (*Epinephelus marginatus*), 3 common dentex (*Dentex dentex*), 1 meagre (*Argyrosomus regius*), two brown meagre (*Sciaena umbra*) and one greater amberjack (*Seriola dumerilii*). The aquarium was set as a semi-closed circulation system with mechanical and biological filtration and a daily water renewal rate of 20%. Semi-transparent roof panels provide an ambient photoperiod regime. Water temperature, dissolved oxygen, pH and salinity remain constant throughout the year at 21.5 ± 0.5ºC, > 85%, 7.8 ± 0.1 and 35ppt respectively. The reproductive behavior of grey triggerfish was monitored daily and a sample of the egg mass was sampled by diving.

RESULTS & DISCUSSION

During the first half of May 2022, the male triggerfish started constructing multiple nests in the sandy bottom of the tank, aggressively defending the surrounding area against the other male gray triggerfish and the other fishes of the tank but not the divers. On two occasions (May 15th and June 5th), the female gray triggerfish laid in one nest a gelatinous matrix of unfertilized eggs of 0.64 ± 0.02mm diameter containing multiple lipid droplets. The female remained in the nest grooming the eggs as well as defending them against other fish for 48h.

This is the first instance of reproductive behavior of the gray triggerfish in captivity. The present observations are consistent with studies in the wild that showed spawning period peaks during June and July in the northern hemisphere. The behavioral pattern of a dominant, territorial male responsible for the construction and maintenance of the nests and a female that observed the nests pre-spawning chose one and provided maternal care post-spawning is similar with the pattern described in the wild. Although both the male and female gray triggerfish demonstrated the distinct reproductive behavioral pattern, the produced eggs were unfertilized both times. Since the timing of the egg laying events in captivity, matched the seasonality in the wild, it could be assumed that the ambient photoperiod serves as a cue for the initiation of reproductive behavior by both male and female grey triggerfish. However, the applied constant temperature and the lack of thermoperiod may be responsible for the unfertilized eggs since temperature is a factor controlling sperm physiology at many levels such as membrane permeability, enzymatic activity or metabolism.

Poster Presentation 78

Reproductive behaviour and fertilized spawns in cultured Senegalese sole (*Solea senegalensis*) broodstocks that had different social learning opportunities before puberty.

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INTRODUCTION

Senegalese sole (*Solea senegalensis*) cultured males (hatchery reared) do not participate in reproductivebehaviour to fertilise spawns resulting in a reproductive failure. Cohabitation of cultured breeders with wild breeders, has increased cultured male participation in reproductive behaviours including a low participation in fertilised spawning that was dominated by the wild breeders. The aim of the study was to describe the reproductive behaviour and spawning of cultured sole breeders without the presence of wild breeders that were organized in groups that had different social learning opportunities before puberty.

METHODS

The experiment was carried out over three years, testing four groups of cultured Senegale se sole breeders, during each annual reproductive season. The broodstocks were kept in IRTA La Rápita, (Catalonia, Spain), in tanks (14 m^3) with natural temperature and photoperiod. The experimental groups were: W1 and W2 groups, two replicate tanks of cultured breeders that were reared with spawning wild breeders before the experiment; CP group, was a positive control of cultured breeders that were reared before the experiment with cultured breeders that liberated unfertilised eggs; and CN group, that was a negative control of cultured breeders that were reared before the experiment in isolation as a single year class and had no contact with adult breeders during rearing. The spawning quality, paternity analysis of larvae, and behavioural analysis (locomotor activity and reproductive behaviours) were evaluated for each experimental group. The behaviours analysed were Rest the Head (the head of a fish was rested onanother fish), Guardian (a fish guards a fish from another fish), Follow (fish follow each other in a procession) and Couple (two fish swimming coupled to release gametes).

RESULTS & DISCUSSION

During the three years, spawning was obtained from all tanks. Generally, the first year had the highest egg production and the third year the lowest. However, fertilised eggs were only obtained from W1 in the first year. A total of eight fertilised spawns were collected that had a fertilisation rate of 28.02 ± 13.80 % and a hatching rate of 15.04 ± 10.40 %. The mean number of larvae obtained per spawn was 7,683 ± 5,947 and the total number of larvae from all eight spawns was 61,468. The paternity analysis assigned 64.3% of larvae to a single couple of breeders, while 34.3 % of larvae were not assigned to a single family, but inconclusively to more than three parents. The highest locomotor activity was observed in group W1, while W2 and CP were intermediate and CN the lowest. The reproductive behaviours during the peak of locomotor activity (19:00-20:00) showed that the main behaviours observed were Rest the Head and Follow, while the Guardian behaviour was low and Coupled behaviour was only observed in W1. Over time the reproductive behaviours decreased, except for Follow. The social learning opportunities provided by cohabitation with wild fish before the experiment increased activity and fertilised spawning. However, the number of successful spawns was low and over time stopped in association with a decrease in reproductive behaviour, suggesting that other mechanisms of behavioural learning could be involved in reproductive success. The low success was similar to cohabitation with wild fish during the spawning period raising questions on learning and dominance.

Poster Presentation 79 (student)

Automatic fish detection and tracking for analysis of reproductive behaviours in Senegalese sole (*Solea senegalensis*) using neural networks and the DeepSORT algorithm.

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INTRODUCTION

Senegalese sole (*Solea senegalensis*) is an emerging aquaculture species with good aquaculture potential. However, sustainable development has been hindered by a reproductive failure of cultured broodstocks, hatched and reared in captivity. Cultured males exhibit a behavioural dysfunction and do not participate in courtship to fertilise eggs. The aim of the present project was to automate the detection of reproductive behaviours based on captured videos and a machine learning system.

METHODS

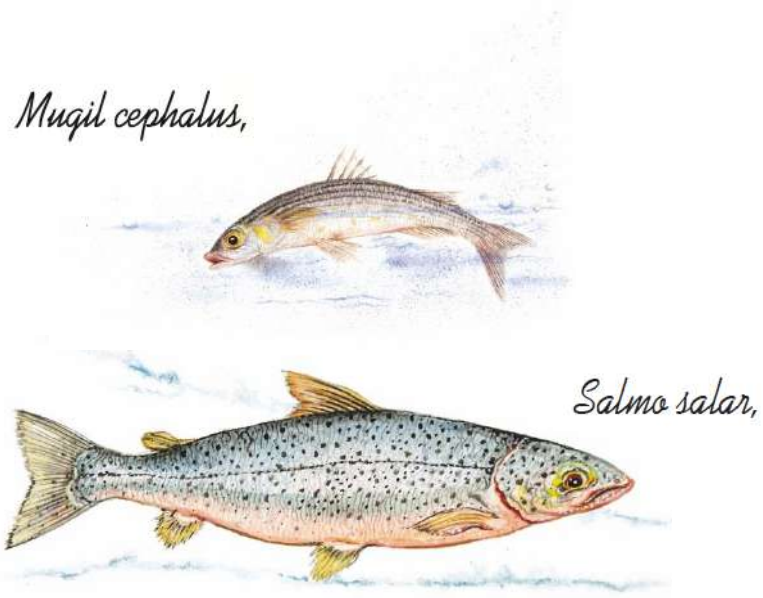
Three broodstocks, two treatment groups with cohabitation of wild and cultured breeders and a control of cultured broodstock were monitored for four spawning seasons to determine reproductive behaviour and spawning output. Behaviour was video recorded with submersible cameras during the peak hour (19:00-20:00) of reproductive behaviour on nights with spawning during April, the peak of the reproduction season. Four different behaviours were registered from the videos by human observers: rest the head (an individual rests it's head on another sole), guardian (a sole covers and guards another sole), follow (two or more individuals following each other) and couple (two sole swim to the surface to spawn). To develop the automated detection of reproductive behaviours the same video frames analysed by human observers were used in different types of neural networks that were trained and tested to define a machine learning scheme that predicted the classification of reproductive behaviours.

RESULTS & DISCUSSION

The method's main scheme was divided into two parts: a neural network for detecting static behaviour in fish, and deepSORT algorithm for tracking dynamic behaviour in fish across multiple frames. There were two steps for implementation. A) Each video frame was divided into a grid of class with labels, positions, and bounding boxes for each object. The classified dataset was fed into a Neural Network, which detected multiple behaviours in single frame with position information. B) For dynamic behaviour, the deepSORT method was used for fish tracking in multiple frames. Using deepSORT the detected fish from Neural Network with position information was assigned unique id and calculated the distance between all fish for analysing the follow behaviour of fish, along with the past information from the other frames to deduce the final behaviour. This two-step method provided information on fish detection and tracking in single and multiple frames. The method was applied to an initial data set of cropped images from a one-hour video that had been trimmed into 10 seconds for each behaviour as used for the human observer analysis. Each frame was annotated with a bounding box containing objects, which were typically an individual fish, rest the head and guardian behaviour and were trained on YOLO, a regression-based object detection algorithm. The model predicted five behaviours: static solo fish, rest the head, guardian and follow. The automated detection of behaviours was compared to the human observer classified behaviours in a confusion table that demonstrated only 8% error between the two analyses. Therefore, automated recognition performs faster, with reduced human workload.

The study was funded by the European Union's Programme H2020, project NewTechAqua GA862658 and the Spanish project INIA – FEDER (RTA2014-00048). The participation of AQ was supported by a Marti-Franquès COFUND EU Fellowship.

SS9. Reproductive biotechnologies



Poster Presentation 80 (student)

Establishment of 3D testicular organoid system as a novel tool to study spermatogenesis in fish

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INTRODUCTION

Spermatogenesis is a stem-cell driven system which requires a complex multicellular interaction and intricate signaling. The established 2D culture systems, such as organotypic cultures, do not reflect the complex cell-cell interaction and signaling as observed *in vivo*. Recently, the establishment of scaffoldfree or scaffold-based testis cell culture systems would provide a 3D microenvironment to support testis cells growth and development, facilitating the *de novo* testis organogenesis and functioning. This artificial system would permit a more detailed investigation of physiological testicular functions, including the interactions between germline stem cells and somatic cells, as well as mechanisms of involved in male puberty or infertility. The current study aimed to develop a scaffold-based and scaffoldfree testis culture system in fish using sterlet sturgeon (*Acipenser ruthenus*) as model species.

METHODS

We tested two approaches to generate 3D testis culture; the first one by encapsulating the testicular cells inside a hydrogel (scaffold-based) that mimics the biological and mechanical properties of the extracellular matrix, and the second, by employing a centrifugal forced aggregation with microwells which generate a large number of size and composition-controlled spheroids (scaffold-free). For the first approach, we used a commercially available hydrogel, named Vitrogel ORGANOID (The Well Bioscience), a synthetic hydrogel that mimics the extracellular matrix, and has four formulations (Vitrogel 1,2,3 and 4) with different stiffness (50 to 300Pa). In the second approach, we used Aggrewell (StemCell Technologies) plates which can generate up to 1200 spheroids/well. Testes from 2 years old sterlet sturgeon were enzymatically dissociated and the testicular cells were either encapsulated in four different types of Vitrogel and incubated in specific medium for 20 days. Testicular cells were also placed into Aggrewell plates, centrifuged, and incubated for 7 days in a specific medium. Cell aggregates were observed under microscope, and some were processed for histological analysis.

RESULTS & DISCUSSION

Our results showed that testicular cells were able to form aggregates in all types of Vitrogel, although round shaped aggregates could be observed only in Vitrogel 1. In the other types of Vitrogel, cell aggregates formed disorganized, grape shaped structures which probably reflects the influence of different stiffness on the cell structure. Focusing on Vitrogel 1, we observed that cell aggregates formed organized structures with an epithelium at the periphery resembling most likely testicular cord as found *in vivo*. During culture period, cell aggregates increased in size and cellular density, although the growth ratio remained stable from day 17. We also observed cellular buds which also increased in size and cellular density during the period of culture. Histological analysis confirmed that cell aggregates showed similar structure of a germinal compartment as observed *in vivo*, although cellular markers are needed for a better cellular characterization. We also demonstrated that that Aggrewell was more efficient in generating aggregates in shorter time (7 days), with higher number, large size and more uniform shape. Both systems support 3D testicular cell culture, but cellular complexity and development need to be further investigated as well as their potential applications to aquaculture.

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Poster Presentation 81

Tiger trout as a recipient in surrogate production of Balkan salmonids

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INTRODUCTION

Intra- or interspecific transplantation of germline stem cells (GSCs) such as spermatogonial and oogonial stem cells (SSCs and OSCs) has been recognized as an efficient tool for the conservation of genetic resources in fish. Having sterile recipients is one of the most important aspects in GSC transplantation as it warrants production of solely donor-derived gametes and progeny by the recipients. In this study we aimed to test whether the hybrid and sterile tiger trout (interspecific hybrid of a browntrout (*Salmo trutta m. fario*) female and a brook trout (*Salvelinus fontinalis*) male) could be used in surrogate production of other salmonid species.

METHODS

Donor rainbow trout (*Oncorhynchus mykiss*) males were euthanized and their testes were dissected. Approximately 15000 GSCs were injected into each recipient tiger trout larva (5-7 dph). To assess the success of the conducted transplantations, we specified three endpoints: (1) production of functional gametes; (2) development of recipient gonads past the baseline development assessed through histological analyses; and (3) detection of donor cells inside of recipient gonads through molecular analyses. Recipient fish were checked at 22 months posttransplantation (pt).

RESULTS & DISCUSSION

With regard to larvae recipients, rainbow trout SSCs were transplanted into 371 tiger trout larvae. Although immediate survival of transplanted larvae was close to 100%, high mortality was observed among them during later growth, thus, 55 individuals (~15%) were alive 3 months pt and only 17 (~5%) reached the age of 22 months. At 22 months pt, gametes were stripped from only one male recipient. Following dissection, both testes were visible and displayed characteristics of normal salmonid testes during spermiation. Following fertilization with the sperm stripped from the donor tiger trout, 2328 rainbow trout eggs were incubated in the hatchery. Of these, 75% hatched and 58% emerged. At the ageof 9 months, all individuals displayed phenotypical traits characteristic of the rainbow trout. Molecular analyses (sequencing of the lactate dehydrogenase (*LDH-C1**) gene) corroborated that the progeny wasindeed donor-derived (rainbow trout).

Present study displayed that the tiger trout can be successfully used as a surrogate parent for trout species. Rainbow trout is phylogenetically distant from brown trout and brook trout, however, production of rainbow trout was still possible from tiger trout surrogate parents. Therefore, we can presume that tiger trout can be used for the conservation of endangered Balkan trouts such as *Salmo marmoratus* or *Salmo obtusirostris* which are phylogenetically much closer. However, the very high mortality rate in the early developmental stages does hinder its performance as a good surrogate candidate, and other candidates such as the triploid or MO-sterilized rainbow trout should be explored.

This research was supported by the Ministry of Innovation and Technology of Hungary within the framework of the Thematic Excellence Programme 2020, National Challenges Subprogramme (TKP2020-NKA-16) as well as the National Research, Development and Innovation Office of Hungary (projects K138425 and FK142933).

Poster Presentation 82

Development of a surrogate broodstock technology in mackerel by germ cell cryopreservation and transplantation

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INTRODUCTION

Chub mackerel (*Scomber japonicus*) is distributed in Japanese coastal waters and is a significant target of capture fisheries. It has recently become a critical aquaculture target fish species in Japan, as individuals in this species provide high-quality fillets with higher lipid content than wild fish. Therefore, the breeding of new strains with beneficial traits is required. In this study, we developed surrogate broodstock technology for chub mackerel as a supporting technology for the breeding program by combining cryopreservation of mackerel germ cells by slow freezing and germ cell transplantation with germ cell-less hybrid mackerel as the recipient.

METHODS

The testes of 1-year-old chub mackerel were equilibrated in 1 mL cryomedium (1.3 M DMSO, 10% egg yolk, 0.1 M trehalose in L15 medium) on ice for 60 min. Then, they were frozen at a rate of −1ºC/min for 90 min before being preserved in liquid nitrogen. After six months of cryopreservation, testes of chub mackerel were enzymatically dissociated and transplanted into hybrid mackerel larvae 10 days post-hatching. Hybrid recipients were reared for one year, and then milt was collected from male recipients. DNA was extracted from the milt of each recipient to identify donor-derived sperm by PCRRFLP analysis of mtDNA. Milt from hybrid recipients was used to fertilize eggs stripped from a wild female chub mackerel to confirm the fertilizability of donor-derived sperm. Microsatellite analysis was performed to examine whether each resultant larva carried donor-derived sperm nDNA.

RESULTS & DISCUSSION

While 7.6×10^6 live spermatogonial cells/g testes were obtained when fresh testes were dispersed, 5.6×10^6 live spermatogonial cells/g of testes (77.2% of fresh testes) were obtained from cryopreserved testes. It was shown by these results that the number of spermatogonia that could be used for subsequent transplantation was not drastically reduced by cryopreservation. Five males matured after one year by rearing the hybrid recipients transplanted with cryopreserved chub mackerel germ cells. It was revealed by PCR-RFLP of mtDNA that all five of these hybrid recipients produced sperm derived from chub mackerel. Furthermore, parentage assignments by microsatellite analysis of the F1 larvae produced by the progeny tests using sperm from the hybrid recipients revealed that 100% (n = 8) of the F1 offspring possessed one allele derived from the donor chub mackerel and another allele derived from the female chub mackerel. Thus, we successfully produced functional chub mackerel sperm derived from cryopreserved testes using surrogate broodstock technology. This method is expected to significantly reduce the labor and space required to maintain various strains, which previously required the rearing and mating of many individuals, by cryopreserving the testes in liquid nitrogen. Furthermore, it is possible to avoid genetic deterioration and inbreeding that could occur with mating for the production of the next generation. It is expected to be a useful application for breeding programs in future mackerel aquaculture.

Poster Presentation 83

Vitrification reduces the *in vitro* maturation capacity of zebrafish (*Danio rerio*) ovarian follicles

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INTRODUCTION

Vitrification is a cryopreservation technique, which consists of freezing different cell lineages by exposing them to high concentrations of cryoprotectants solutions and subsequently to extremely low temperatures. Although there are established protocols of vitrification for ovarian follicles in stage III of development in zebrafish, little is known about the ability of the ovarian follicles to complete the follicular development after the vitrification, that is the goal of this study.

METHODS

Mature females were euthanized on a lethal dose of tricaine (0.6 mg/dL) and decapitated to ovaries collection, which were placed in a Petri dish containing L-15 culture medium to identify the follicles in stage III. Part of these follicles were exposed to an equilibrium solution of L-15, methanol 1.5 M, DMSO 2.25 M and sucrose 0.25 M for 15 minutes. After that period, they were exposed to the vitrification solution containing L-15, methanol 1.5 M, DMSO 5.5 M and sucrose 0.5 M for 90 seconds and subsequently submerged in liquid nitrogen. The vitrified follicles were thawed by remaining at 28ºC for 30 seconds and using three warming solutions of sucrose: 1 M for 1 minute, 0.5 M for 3 minutes and 0.25 M for 5 minutes; and then submitted to the *in vitro* culture. The *in vitro* maturation was performed using L-15 medium containing 0.8 µg/mL of 17α,20β-Dihydroxy-4-pregnen-3-one (16146-1, Cayman), 30% fetal bovine serum and 100 µg of gentamicin in oven at 27ºC for 270 minutes. A fraction of the follicles vitrified was incubated in Trypan Blue to check the integrity of the membranes.

RESULTS & DISCUSSION

After thawing, the vitrified follicles were cultured with fresh follicles collected on the day of cultivation as control. 120 follicles from each group were evaluated, repeated 4 times, which were arranged in groups of 10 follicles in a 12-well culture plate. Only follicles with intact membrane after thawing were used to compose the culture plate of the vitrified group. At the end of 270 minutes, the follicles were observed and those that showed maximum transparency of the oocyte, the disappearance of the germinal vesicle and micropyle were mature. We observed a decrease in the *in vitro* maturation capacity in 4 cultures, 82% in the control group versus 52% in vitrified group (P=0.05); and an increase in the amount of damaged/dead follicle in the vitrification group (64% versus 18% of control group, P=0.03) after *in vitro* culture. Although the follicles could complete their development after thawing, morphological differences in size after *in vitro* maturation were observed, when compared to the control group. This could be associated with the increase of the membrane fluidity, which may have been caused by release of proteases and/or by changes in the ionic transport mechanisms. Even with these differences, it was possible to observe the micropyle in the vitrified group after *in vitro* culture, indicating the resumption of meiosis. However, we do not know if they can be fertilized or generate viable individuals, therefore, further studies must complement these results. Thus, despite decreasing *in vitro* maturation capacity by about 30%, vitrification allows preservation of zebrafish ovarian follicles and subsequent *in vitro* maturation, until the micropyle formation, reinforcing its application in the reproductive management of fish.

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Poster Presentation 84 (student)

Functional sperm production from frozen germ cells of a fish on the verge of extinction: the case of the Tokyo bitterling

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INTRODUCTION

The Tokyo bitterling (*Pseudorhodeus tanago*) is a small Cyprinid fish endemic to Japan. It lays its eggs in freshwater mussels' gill chambers that drastically decreased their numbers recently. For this reason, it is on the verge of extinction and is strictly protected as a "natural monument" in Japan. Although habitat conservation has been attempted to protect this species, its efficacy has been limited. Therefore, this study aimed to establish a new method for long-term preservation of their genetic resources by combining germ cell cryopreservation and transplantation. In this study, cryopreservation conditions of their testes were optimized and Chinese rosy bitterling (*Rhodeus ocellatus*) suitability was evaluated as a recipient because it is easy to raise and reproduce in captivity.

METHODS

As cryoprotectants, cell membrane-permeating reagents (dimethyl sulfoxide [DMSO], propylene glycol, glycerol, ethylene glycol, and methanol), non-permeating ones (glucose, trehalose, and sucrose), and 1.5% (wt/vol) BSA were added to Leibovitz's L-15 solution. Their effectiveness on testicular cell survival was evaluated. Further, equilibration times with the cryomedium were optimized. Under each of these conditions, the testes were slowly cooled down to −80°C by using a freezing container, followed by plunging into liquid nitrogen and stored for at least 24 h. Each sample was thawed and the live testicular cell recovery rate was examined. Tokyo bitterling gametes were then attempted to be produced by transplanting their cryopreserved germ cells into Chinese rosy bitterling recipients. Newly hatched larvae sterilized by *dnd* gene knockdown were used as recipients. Donor germ cells (3000 each) labeled with PKH26 were injected into the hatchlings' abdominal cavity and the transplantation success was evaluated by fluorescent observation of the recipients' genital ridges 16 days post-transplantation. The remaining recipients were raised to maturity. Semen was obtained by adding abdominal pressure gently. As sperm was collected, DNA fingerprinting using sperm DNA was performed to identify their genetic origin. Lastly, sperm produced by the recipients were inseminated to Tokyo bitterling eggs to confirm their functionality.

RESULTS & DISCUSSION

Freezing the testes in a cryomedium containing 1.3 M DMSO and 0.1 M trehalose after 15 min equilibration on ice was found to be the suitable cryopreservation condition. Under this condition, the live testicular cell recovery rate was 50.6 ± 1.5%. In the transplantation experiment, PKH26-labeled cells were incorporated into the recipient's genital ridges at a frequency of 67.1 ± 3.6% in the fresh group and 65.8 ± 11.2% in the frozen group, with no significant differences. The remaining recipients produced sperm in 6/10 in the fresh group and 7/7 in the frozen group. Because of sperm DNA analysis, 5/6 in the fresh group and 1/7 in the frozen group were revealed to produce Tokyo bitterling sperm. The eggs fertilized with these sperm's hatching rate was 90.1 ± 3.4% in the fresh group and 91.7% in the frozen group. Thus, it was shown that Chinese rosy bitterling is capable of producing sperm derived from the Tokyo bitterling's frozen germ cells. Although improvements in sperm production efficiency and egg production technology development are needed, the technology developed in this study has made it possible to preserve the male genetic resources of immature Tokyo bitterling and individuals in non-spawning seasons for a long period of time.

Poster Presentation 85 (student)

Hormonal profile and reproductive performance of male spotted wolffish (*Anarhichas minor*) in response to two methods of GnRHa administration

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INTRODUCTION

An ideal candidate for aquaculture diversification, the spotted wolffish (*Anarhichas minor*) inhabiting the Northern Atlantic coasts, has an outstanding growth rate at low temperature, tolerance to highstocking densities, and reasonable market value. However, in commercial or experimental aquaculture facilities, males exhibit reproductive dysfunctions requiring manipulations to improve spermiation and milt quality. To aid reproduction in captivity, hormonal therapies (*e.g.* GnRHa) are a classical practice in many fish species. For the first time, we describe the response of male spotted wolffish to GnRHa preparations (implants vs. injections) with or without the addition of a dopamine antagonist (DA).

METHODS

The study was reviewed and approved by the Norwegian Animal Research Authority (FOTS #28744). Spotted wolffish broodstock were maintained in a single fabricated 30.000-L tank supplied with continuous flow deep-sea water and fed daily with a commercial marine fish diet. Thirty individually tagged males with body weights and standard lengths ranging between 10.0±1.4 kg and 84.1±3.5 cm, respectively, were assigned randomly into six treatment groups (n=5): control-saline solution injection; DA (1 mg/kg BW) injection; GnRHa (200 µg/kg BW) injection; GnRHa (200 µg/kg BW) + DA (1 mg/kg BW) injection; GnRHa (100 µg/kg BW) EVAc implants; and GnRHa (100 µg/kg BW) EVAc implants + DA (1 mg/kg B.W.) injection. On days 0, 4, 7, 14, and 21, blood, skin mucus, and sperm samples were collected by manual striping. Sperm quality parameters were monitored using the CASA system while LC-MS/MS was used to measure sex steroid hormones (Testosterone, T and 11-ketotestosterone, 11-KT).

RESULTS & DISCUSSION

An extended high variability in the measured parameters was recorded at the different sampling points among the treated animals, but sperm quality was enhanced by the combination of DA with GnRHa. This effect was especially conspicuous in the GnRHa-injected group compared to the implanted animals. Unexpectedly, we recorded an increased number of spermiating breeders throughout the trial in those groups supplemented with DA regardless of the delivery system. In support of the spermiation results described above, plasma 11-KT and T levels followed similar tendencies. Overall, these results indicate that the induction of spotted wolffish with GnRHa in combination with DA could permit an enhanced spermiation in this species, which may eventually allow robust and controlled hatchery production. Our group is currently investigating the dosage optimization of both GnRHa and DA, as well as a detailed description of the mechanisms inducing the response.

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Poster Presentation 86

Cryopreservation protocols for chondrichthyan sperm cryobanking: new *ex situ* conservation tools for sharks, rays, and chimaeras.

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INTRODUCTION

Chondrichthyans (commonly named sharks, rays, and chimaeras) are one of the most threatened groups of vertebrates. Given this situation, aquaculture could play a key role in their conservation through *ex situ* conservation strategies used for multiple species. However, to ensure the success and sustainability of these strategies, breeding programs and reproductive techniques should be implemented. Among these reproductive techniques, sperm preservation is a potential tool that is almost never used in chondrichthyans. In fact, there were no existing widespread preservation protocols for chondrichthyans sperm, and shark sperm cryopreservation had not yet been achieved.

METHODS

Here we present a series of successful cryopreservation protocols for chondrichthyans sperm, tested on 13 species. We have formulated a sperm extender (in mM; 433 Urea, 376 NaCl, 120 Trimethylamine N-oxide, 8.4 KCl, 50 Glucose, 7 $CaCl_2$-$2H_2O$, 3.5 $NaHCO_3$, 0.08 Na_2SO_4, 1.4 $MgSO_4$; pH 6.5; Osmolality 1000 mOsm/kg) that is useful for different species, where sperm maintains its motility for up to 36 days at 4 °C. The cryopreservation of sperm was achieved by supplementing our extender with different combinations of cryoprotectants: methanol, dimethyl sulfoxide (DMSO), and fresh egg yolk. Samples were frozen in cryotubes inside a Styrofoam box using liquid nitrogen vapor. Pre-freezing and post-thawing sperm quality was assessed by analyzing spermatozoa motility and membrane integrity.

RESULTS & DISCUSSION

In rays, the use of 10% DMSO or 10% methanol produced post-thawing motility values higher than 40%. In sharks and chimaeras, the combination of 5% DMSO, 5% methanol, and 10% egg yolk produced mean values close to 35%, with the notable exception of the bluntnose sixgill shark (*Hexanchus griseus*) with values over 70%. Overall, the addition of egg yolk increased the post-thawing motility values, by up to 42.1% in samples with initial motility values of 70%.

For the first time, shark, skate, and chimaera sperm cryopreservation has been reported, including species considered “Critically Endangered” according to IUCN criteria, such as the blue shark (*Prionace glauca*) and the bull ray (*Aetomylaeus bovinus*). This deepens our knowledge of the reproductive techniques that can be applied to chondrichthyans. Such results enable the creation of cryobanks for elasmobranch sperm, which become new tools for their conservation, complementing *ex situ* conservation efforts developed by public aquaria worldwide. Besides gene conservation and reproductive research projects, a regular supply of frozen sperm will reduce the problems that result from the long-distance transport of specimens, inbreeding, or the lack of synchronized reproductive cycles in captivity. Therefore, the use of aquaculture techniques for the conservation of these threatened species has become a new path to be explored.

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Poster Presentation 87 (student)

SDF-1/CXCR4 signal is involved in the induction of Primordial Germ Cell migration in a model marine fish, Japanese anchovy (*Engraulis japonicus*)

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INTRODUCTION

Japanese anchovy (JA) is an ecologically relevant and extremely important marine fish. JA matures in just four months and can continue to spawn throughout the year in a controlled condition. In our laboratory, we have established an experimental system of inhouse culture, breeding and gene manipulation, with an aim to develop this fish as a marine model fish for various research. The knowledge of primordial germ cells (PGCs), the source of germ cells, is highly essential for the controlled reproduction. However, the biology of PGCs are scarcely documented and hence is major hurdle for reproductive manipulation experiments like gonad sterilization and surrogacy. So, in this study, we have focused on the SDF-1/CXCR4 responsive PGC migration in JA and attempted to induce gonadal sterility by tapping into the abovementioned signaling pathways.

METHODS

To clarify PGC migration in JA, we microinjected *egfp-nanos3* 3'UTR mRNA into just fertilized eggs and performed live fluorescence microscopic analysis. Next, we conducted phylogenetic and early developmental analysis of the PGC related genes i.e., *vasa*, *sdf-1a*, and *cxcr4b*. Overexpression of *sdf1a* mRNA and CXCR4 antagonist immersion treatment were used to disrupt normal migration of PGCs. Analysis of PGC mismigration, gonadal gene transcription and gonadal histology were also performed.

RESULTS & DISCUSSION

In the injected embryos, at around 32 hpf (hour post fertilization), most of the PGCs were observed to be gathered around the lateral line and further migrated to the gonadal ridge. Phylogenetic analysis of *vasa*, *sdf-1a*, and *cxcr4b* showed high homology to closely related Atlantic herring (*Clupea harengus*, 85.14%, *vasa*) and distant zebrafish (*Danio rerio*, 80.93%, *vasa*). Quantitative analysis of SDF1/CXCR4 showed highest expression at 8-16 hpf, suggesting the initiation of PGC migration. *sdf-1a* mRNA was injected at 50, 100, and 250 ng/µl, and the negative effect on PGC migration could be observed at all concentrations. As a result of measuring the distance between the farthest PGCs (DFP), 100 ng/µl had the greatest effect, but there was no significant difference between various experimental concentrations of *sdf-1a* mRNA. On the other hand, the embryos immersed into CXCR4 antagonist (concentrations ranging from 1 to 20 µg/ml) showed increased DFP and decreased PGC settlement in the gonadal analgen. In addition, some of the CXCR4-antagonist treated adults had smaller gonads and delayed maturation. Although neither of the above two experiments could induce complete sterilization, but both had an inhibitory effect on PGC migration of JA. Cumulatively, our study suggests that SDF1/CXCR4 responsive chemokine actions are active in JA but may be partially involved in regulating PGC migration. Further analysis is needed to clarify the migration biology and decipher the reproductive ability, to uplift Japanese anchovy into a complete marine model fish status.

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Poster Presentation 88 (student)

Induced infertility in farmed Atlantic salmon (*Salmo salar)*

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INTRODUCTION

Two major reproduction-related issues are currently raising sustainability concerns for the Atlantic salmon aquaculture industry: I) genetic introgression, and II) male precocious puberty. Both of these problems can be addressed by inducing infertility in farmed salmon through ablation of either germ cells (GCs) or germ cell-supporting cells (GCSCs). Germ cell-free (GCF) farmed Atlantic salmon would not be able to mate with wild type conspecifics, would not enter puberty before harvest and remove the risk of premature osmoregulatory changes that are currently leading to substantial economic losses.

METHODS

Two methods are being developed to achieve induced infertility:

I) Precision medicine: nanobodies against specific membrane proteins of GCSCs are being selected and nanobody-drug conjugates (NDCs) are being developed, to be applied as a single dose together with routinely administered vaccines, given to all juvenile farmed salmon. After binding, internalization, and cell-specific drug release, GCSCs will be eliminated, yielding GCF salmon as a consequence. To our knowledge, this is the first ever application of nanobody-based drugs in teleosts, and along with achieving the end goal of induced infertility or delayed sexual maturation, we hope to map pharmacokinetics, explore relevant half-life extension strategies, efficacy of conjugates etc. that will inform the field for any future applications of similar drug modalities.

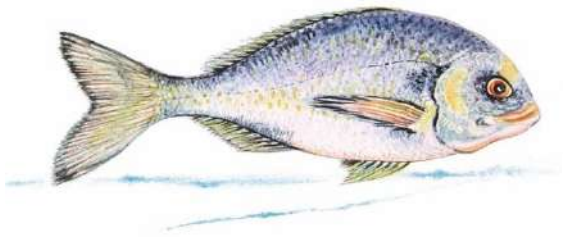
II) As GCSC stem cell populations may lack the membrane proteins for targeting with NDCs, a genome modification approach is pursued to induce infertility. To this end, two salmon strains need to be developed. Crossing fish within strains will be used to propagate the strains, while crossing fish between strains will produce progeny in which GCs or GCSCs will ablate, yielding GCF fish.

RESULTS & DISCUSSION

For the nanobody work stream, a range of salmonid and mammalian cells lines expressing the targets has been established, and while llama immunization and binder selection using whole cell strategy and refinement of such strategies such as FACS-enabled phage sorting has not yet yielded specific highaffinity binders, switching workflow to recombinantly produced targets is projected to improve phage pool specificity, enable to find specific binders and progress to *in vitro* and *in vivo* testing of the concept. Initial results revealed specific promoter expression of a number of selected salmon promoters in Atlantic salmon. Testing effector molecules for cell ablation in zebrafish is under way. Transgenesis of new constructs is planned for GC- and GCSC-specific expression of the effector molecules to produce GCF salmon offspring. Potential beneficial and adverse side effects of the methodologies on yield, welfare, growth rate and survival of these GCF salmon will be studied.

The project is funded by the Netherlands Research Council (NWO) and the Institute for Marine Research (IMR), Norway.

Additional Abstracts submitted late or excluded from the printed program



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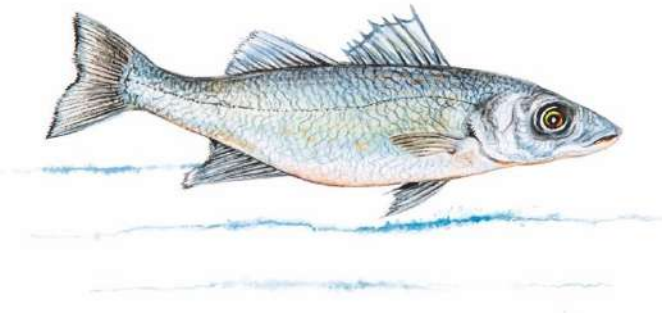

Dicentrarchus labrax

Poster Presentation 101

Composition of the rainbow trout testicular extracellular matrix and progress towards the development of testicular organoids

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INTRODUCTION

Testicular organoids are promising 3D cell culture models to study direct and indirect cell to cell interactions between germ stem cells (GSC) and somatic cells, as well as mechanisms leading to male infertility (disease, ecotoxicology). Testicular organoids could also allow the amplification of GSC for the conservation of genetic resources and the regeneration of the cohorts of interest. The production of organoids based on the use of heterologous (natural and synthetic) extracellular matrix (ECM) has been commonly reported in mammals, but endogenous scaffold could be more suitable. The present study aimed to first initiate the production of testicular organoids from adult zebrafish using commercial hydrogels. In parallel, we investigated the composition of the rainbow trout testicular extracellular matrix (tECM) to acquire knowledge and improve the quality of the scaffolds used in the 3D cultures.

METHOD

Two different zebrafish transgenic lines named *gsdf*:GFP (Gautier et al., 2011 doi:10.1095/biolreprod.111.091892) and *vasa*:rfp (Fan et al, 2008, doi:10.1089/scd.2007.0178) were used in this study to demonstrate cell reaggregations and GSC survival in a 3D microenvironment. Zebrafish testicular cells were encapsulated into commercial hydrogels (Vitrogel or Matrigel) and cultured up to 19 days in a specific medium. Testes collected from immature rainbow trout were decellularized using sodium dodecyl sulfate. The quality of the decellularized tECM was investigated using transmission and scanning electron microscopies, and immunohistochemistry. High-throughput mass spectrometry-based proteomics were carried out on non-decellularized and decellularized testicular samples using DAA-PASEF or DIA-PASEF.

RESULTS & DISCUSSION

We demonstrated that spontaneous reaggregation was possible using combined testicular cells freshly dissociated from two different zebrafish reporter lines. The cell clusters encapsulated into Matrigel presented a more regular and round shaped morphology as compared to Vitrogel. We also observed that cell clusters changed in morphology, size and cell density although exhibited a limited growth at the end of the culture. We successfully obtained tECM from rainbow trout testes without affecting its 3D structure. Proteomics analyses unraveled the protein complexity of non-decellularized and decellularized testes including growth factors and components of the basal lamina, respectively.

CONCLUSION

Our data provide new perspectives for improving the scaffolds and media used for the production testicular organoids.

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Poster Presentation 102

Nuclear progestin receptor–mediated linkage of blood coagulation and ovulation

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INTRODUCTION

Ovulation is a dramatic remodeling process that includes rupture of blood capillaries and clotting, but coagulation is not thought to directly regulate this process. Therefore, we take zebrafish as the research object to systematically study the expression, regulation, and function of coagulation factors *f5* and *f3a* in the ovulation process of zebrafish at both *in vivo* and *in vitro* levels.

METHODS

Fully-grown immature follicles or mature follicles were collected at different time points for examination of spatial and temporal distribution of f5 and f3a mRNA during a daily spawning cycle. Ovarian follicles at different stages were collected from wildtype (*wt*) or nuclear progesterone receptor (Pgr) knockout ($pgr^{-/-}$) females and incubated with various doses of DHP or various exposure times to investigating the effect of DHP, a native ligand of zebrafish Pgr, on f5 and f3a mRNA levels. A dual-luciferase reporter assay was applied to verify that a direct regulation of f5 and f3a promoter activity via Pgr. A double transgenic line *Tg(pgr:eGFP/fli1:DsRed)* was generated for observation of Pgr expressing cells and capillary vessel networks. The numbers of erythrocytes within the surface capillaries of mature follicles were visualized using o-dianisidine staining as follicles progressing towards rupture and ovulation. Effects of anticoagulants on ovulation were examined in vitro and in vivo. Females of f5+/- were chose for the examination of female fertility in a consecutive spawning test.

RESULTS & DISCUSSION

There are remarkable increases of *f5* (~3145-fold) and *f3a* (~120-fold) in zebrafish ovarian follicle cells during ovulation. This increase was mediated through Pgr, which is essential for ovulation in zebrafish, and was totally abolished in ovarian follicular cells from $pgr^{-/-}$ mutants. In addition, promoter activities of *f5* and *f3a* were significantly enhanced by DHP via Pgr. Similar regulation of human F5 promoter activity was induced via human PGRB, suggesting a conserved mechanism. Site-directed mutagenesis of the zebrafish *f5* promoter further demonstrated a direct regulation of coagulation factors via progestin response elements. All the follicular cells with strong Pgr expression were located adjacent to capillary vessel networks on the surface of the IVa follicles in this dual transgenic zebrafish line *Tg(pgr:eGFP/fli1:DsRed)*. Moreover, a stark increase of erythrocytes occurred in capillaries meshed in wild-type preovulatory follicles but was absent in $pgr^{-/-}$ mutants. Interestingly, anticoagulants significantly inhibited ovulation both *in vitro* and *in vivo*, respectively. Furthermore, reduced fecundity was observed in $f5^{+/-}$ female zebrafish. Taken together, our study provides plausible evidence for steroid regulation of coagulation factors, and a new hypothesis for blood clotting–triggered ovulation in vertebrates.

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Poster Presentation 103 (student)

In vitro responsiveness of pikeperch (_Sander lucioperca_) ovarian follicles as a potential indicator of their maturational competence

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INTRODUCTION

Under *in vitro* conditions, isolated fish ovarian follicles retain the ability to resume meiosis and progress through the last stages of oogenesis when exposed to maturation-promoting stimuli. Assessing the dynamic response of follicles in culture can help estimate the reproductive state of the broodstock and potential outcome of artificial hormone-induced ovulation. Here we report a protocol for *in vitro* maturation of pikeperch (*Sander lucioperca* L.) ovarian follicles. Human chorionic gonadotropin (hCG) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) were used to evaluate the maturational competence of follicles isolated during the pre-spawning period, which enabled observing their response prior to any endogenous stimulation of maturation.

METHODS

Adult pikeperch females (TL: 46 ± 3 cm; W: 772 ± 115 g; GSI: 7 ± 3%) were sampled during the chilling period of the photo-thermal spawning induction (6 °C, 8 h light/16 h dark cycle at sampling moment), prior to any hormonal stimulation. The maturation medium consisted of 90% Leibovitz L-15 media, supplemented with antibiotics, 0.1% Bovine Serum Albumin and 15 mM HEPES (pH 7.5), with the addition of either 100 ng/ml DHP, 5 to 20 IU/ml hCG or a combination of the two (100 ng/ml DHP and 20 IU/ml hCG). Incubations were done at 12°C. Resumption of meiosis and maturation were evaluated regularly by scoring the percentage of follicles that underwent germinal vesicle breakdown (GVBD) and ooplasm clearing, compared to the control group with no hormonal stimulation.

RESULTS & DISCUSSION

In the pre-spawning period, most of the fish contained follicles in late vitellogenic stage of development that have not yet attained maturational competence (Ø 839 ± 6 µm). This was confirmed *in vitro*, as these follicles did not respond to stimulation with DHP alone. Maturation outcome was significantly improved with the addition of hCG, which together with DHP induced complete GVBD in 47 ± 16% and ovulation in 31 ± 12% of follicles, in less than 96 hours. Stimulation with only hCG resulted in comparable final GVBD rates; however, the length of incubation notably increased (up to 150 hours), regardless of hormone concentration. Slower response during hCG treatment enabled gradual and synchronized nuclear and cytoplasmic events, resulting in proper lipid droplet formation in all mature follicles, as well as evident hydration – increase in diameter was 112 ± 10 µm, as opposed to 32 ± 15 µm in the DHP+hCG group. In contrast, most of maturing follicles in the latter group had issues with precocious ovulation and lipid droplet fragmentation. Although the overall quality of maturation was higher in hCG-only groups, the rate of ovulation was significantly lower (9 ± 8%), which necessitates either changes in the culture conditions, or inclusion of additional ovulation stimulation, to support the complete *in vitro* process of final maturation in pikeperch follicles.

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Poster Presentation 104

Gametogenic pathways in diploid and triploid asexual hybrids from *C. hankugensis-longicorpa* fish complex

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INTRODUCTION

The cellular and molecular machinery ensuring sexual reproduction is largely conserved among all eukaryotes but can be easily disrupted by hybridization. One of the most intriguing outcome of hybridization is the emergence of asexual reproduction. Asexuals exploit a various gametogenic alterations to prevent recombination between orthologous chromosomes. Nevertheless, in polyploids full or partial restoration of sexual reproduction is possible. To investigate the transition between sexual and asexual reproduction, we selected di- and triploid hybrids from Korean loach hybrid complex. This complex includes two parental species *Cobitis hankugensis* (HH) and *Iksookimia longicorpa* (LL) as well as their diploid (HL) and triploid (HHL, LLH) hybrids. Diploid hybrids produce diploid eggs while triploid hybrids form haploid eggs. However, the underlying mechanisms of gamete formation in diploid and triploid hybrids remain unknown.

METHODS

To investigate gametogenic pathways of diploid and triploid hybrids, we firstly analyzed oocytes during pachytene and diplotene stages of meiosis. We identified ploidy level of oocytes using FISH with chromosome specific marker. To assess fertility of hybrid females, we examined gonadal morphology of adult hybrid females using confocal microscopy. Afterwards, using whole mount FISH, we identified genome composition of gonocytes, pachytene and diplotene cells in intact gonadal fragments.

RESULTS & DISCUSSION

In diplotene oocytes of diploid hybrids, we found duplicated number of chromosomes with genomes of both parental species forming bivalents. In contrast to diplotene oocytes, we found that pachytene oocytes are represented by two population of cells, the majority of oocytes had nonduplicated genome while small portion of oocytes had duplicated genome. Similarly to pachytene oocytes, the majority of gonocytes have diploid genome size while only small portion of gonocytes is tetraploid. We suggest that only in the small portion of the gonocytes can endoreplicate their genomes before meiosis. Such oocytes form bivalents during pachytene and proceed to diplotene and lead to the formation of diploid gametes. Pachytene oocytes with unduplicated genome are unable to proceed beyond pachytene.

In diplotene oocytes of triploid HHL hybrids, we observed 24 bivalents of *C. hankugensis*. In pachytene, we observed several populations of oocytes: oocytes with both *C. hankugensis* bivalents and *I. longicorpa* univalents, oocytes with *C. hankugensis* bivalents and oocytes with *I. longicorpa* univalents. Among gonocytes, we found diploid and triploid cells. We assume that some portion of diploid gonocytes emerged after premeiotic genome elimination of *I. longicorpa* genome. In meiosis such oocytes have pairing between *C. hankugensis* chromosomes causing the haploid gamete formation. Oocytes without genome elimination enter meiosis but unable to proceed beyond pachytene. Thus, we showed a transition from asexual reproduction in diploid hybrids to partial restoration of sexual reproduction in triploids.

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Poster Presentation 105

Aspects of the reproductive biology of the tropical mullet, *Mugil incilis*

Medina, Katrina[(1)]; Moscarella, Carlos[(1)]; Felip Alicia[(2)] and Rodríguez Forero, Adriana[(1)]

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INTRODUCTION

The mugilids are a group of euryhaline and eurythermic fish with great ecological and economic importance due to their potential as a bioremediator in their natural habitat or in aquaculture for their market value. In this sense, mullets are highly appreciated as food and they have been recently considered to play a relevant role in the blue economy to diversify species and marine aquaculture product development. In addition, female fish roe is a food delicacy in many countries. The mullet, *Mugil incilis*, is a species traded in tropical coastal areas where highly vulnerable fishing populations live from their fishing. The reproductive biology of this species is poorly known and there is no certainty about their spawning grounds. Accordingly, our goal is to evaluate the gametogenic development of *M. incilis* in four different habitats of the Colombian Caribbean Sea over the course of the annual reproductive cycle in natural conditions.

METHODS

A total of 80 adult *M. incilis* (>20cm in total length) were captured for gonad characterization and seasonality of gametogenesis in the region of Magdalena (Colombia) as follows: 1) Close to a coastline of the estuary called the Ciénaga Grande de Santa Marta (CGSM), 2) The "Puente de la Barra", which divides the sea from the estuary (CGSM), 3) The mouth of the Piedras river and 4) The open sea area two miles from the coast of the CGSM. To analyze the reproductive performance of the animals, some indicators such as growth and body indexes (gonadosomatic (GSI), hepatosomatic and visceral fat index) were evaluated. Sex ratio and the stage of gonad development were determined by histological examination. Blood samples were collected for hormonal analyses of sexual steroids (estradiol and testosterone).

RESULTS & DISCUSSION

Our results indicated that tropical female mullet remained in the sea during their reproductive period (December-February), while there was a greater abundance of males in the mouth of the Piedras river. However, sperm activation is believed to occur in seawater, since it is immotile in freshwater. Tropical mullet is a gonochoristic species with a male:female sex ratio of 1:2 found under natural conditions. Identification and characterization of the gonad stages in males could be divided into four stages of testicular development: a) Stage I, immature testis containing spermatogonia, b) Stage II, mid recrudescence (early maturing testis) with abundant cysts containing spermatocytes, c) Stage III, late recrudescence (maturing testis) with spermatids transforming into spermatozoa and d) Stage IV, full spermiating testis with the lumen of the lobules filled with sperm. In females, ovarian stages could be divided into six stages according to the oocyte diameter: a) stage of chromatin nucleolus, b) perinucleolar stage, c) cortical alveoli, d) vitellogenic oocytes, e) maturation and f) resorption or post-ovulatory stage. The increase of GSI was consistent with the elevation of estradiol and testosterone for each sex. These findings contribute to a better understanding of the reproductive physiology of this tropical species for further control of its reproduction in captivity.

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Poster Presentation 106

Semen characterization and sperm structure of the tropical lisa (*Mugil incilis*)

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INTRODUCTION

Mugilids are species of interest for feeding populations located in coastal areas, often distributed in developing countries. Its gonads are a delicacy and are also widely marketed in some European countries as a "Bottarga" also called "eoran" or "avgotaraho, which is salted, pressed and dried. This species presents a population decline due to overfishing and to water pollution. *Mugil incilis* is an ecologically and economically valuable estuarine/marine fish with a natural range from West Indies and the Atlantic coasts of Central and South America to southeastern Brazil and is currently considered a vulnerable species in Colombia. There is no knowledge about the seminal characteristics or reproduction patterns of this species. Therefore, the evaluation of the sperm physiology of the species will contribute to the implementation of reproductive biotechnologies aimed at conservation and production for food security purposes.

METHODS

The characterization of fresh sperm (n = 30 males; total length:17.07 ±1.72 cm and body weight 42.83 ± 9.98 g) was performed through analyzes of sperm volume, motility rate (5), sperm concentration, pH and sperm morphology. Spermatogenesis was also studied by histology and transmission electron microscopy.

RESULTS AND DISCUSSION

Preliminary results indicate that the records for this species are similar to those found in other mugilids and that its sperm quality enables it for *in situ* breeding programs. Fresh sperm motility was activated in sea water (35UPS) at room temperature of 26ºC. Fresh sperm presented a sperm volume of 0.02 ± 0.03 spermatozoa mL^{-1}; a motility rate (%) of 10.86 ± 24; a time of motility 0.54 ± 1.07 spermatozoa activity min^{-1}; a total time of motility of 2.69 ± 5.99 spermatozoa activity min^{-1}; a sperm concentration of 6,392,667 ± 7,875,930 spermatozoa mL^{-1}. The spermatozoon of *M. incilis* is uniflagellated. The testicular development presented as: a) Stage I, immature testis containing spermatogonia, b) Stage II, mid recrudescence (early maturing testis) with abundant cysts containing spermatocytes, c) Stage III, late recrudescence (maturing testis) with spermatids transforming into spermatozoa and d) Stage IV, full spermiating testis with the lumen of the lobules filled with sperm. Results from this study form a baseline to describe the male gonad dynamics and reproduction of this fish, considered an emerging species for fish culture on the Caribbean region.

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Poster Presentation 107

Seminal plasma composition and sperm motility in tāmure/Australasian snapper *(Chrysophrys auratus)* after short – term storage

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INTRODUCTION

The tāmure/Australasian snapper *(Chrysophrys auratus)* is a highly prized fish for both commercial and recreational fisheries and a new candidate for aquaculture in New Zealand. However, there is a knowledge gap about its reproductive biology. As part of an initiative to cryobank sperm from selectively bred broodstock, this study aimed to characterize for the first time sperm quantity and quality, and seminal plasma parameters, and to examine effects of cold storage on fresh sperm.

METHODS

During spring 2023, fresh milt was collected from captive-bred seven-year-old snapper (n = 10; mean body weight 1.6 kg (±0.06 SE) maintained under ambient conditions at The New Zealand Institute for Plant and Food Research Limited (PFR) in Nelson. Upon sedation, total milt was collected from each fish into individual 15-mL centrifuge tubes to estimate volume and pH. Computer-assisted sperm analysis (CASA, Sperm Class Analyzer®, Microptic, Spain) was applied to quantify sperm quality using 100 µL/sample stored at 4ºC. To estimate spermatocrit, 1-mL aliquots of each sample were centrifuged at 15,000 rpm. Thereafter, osmolarity and pH of the seminal plasma were tested before samples were frozen at −80°C. To test the effects of cold storage on quality, CASA was used to assess kinematic and motility parameters. Milt quality was analysed on the day of collection (time 0) before being stored at 4°C and periodically sampled until the percentage of rapidly active sperm was <5%.

RESULTS & DISCUSSION

The average total milt volume expressed from fish was 5 mL/kg^{-1} of body weight. The average pH was 7.3 (±0.04 SE), while the average percentage of spermatocrit was 68.9% (±3.4 SE). Seminal plasma osmolarity ranged between 308 and 319 mmol/kg. Cold storage of 1–2 days resulted in a significant decrease of sperm kinematic and motility parameters, curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP). Motility, assessed as the percentage of rapidly active sperm, showed a similar pattern of a significant decrease after 2 days of cold storage, reducing from ~80% rapid motility to < 5% after 9 days. Combined, our findings show that the short-term cold storage for 1–2 days has no significant impact on kinematic or rapid motility parameters tested, indicating that this species may be a good candidate for short-term milt storage. Storage of milt is a crucial enabler for undertaking selected crosses of elite breeders, to ensure the best mating combinations are used to maintain genetic diversity while enhancing economically important traits. This knowledge adds to the ongoing body of work to enable snapper as a new species for aquaculture.

This work was funded by the Royal Society of New Zealand Catalyst Seeding General contract CSGPAF1803 and by the New Zealand Government via the Ngā Pou Rangahau platform, a research framework developed by PFR and supported by a Strategic Science Investment Fund grant from the Ministry of Business, Innovation and Employment.

Fish species represented in the symposium

Common English name	Scientific name
amur sturgeon	*Acipenser schrenckii*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
bamboo leaf wrasse	*Pseudolabrus silboldi*
black rockfish	*Sebastes schlegelii*
blue gourami	*Trichogaster trichopterus*
butter catfish	*Ompock bimaculatus*
chub mackerel	*Scomber japonicus*
cobaltcap silverside	*Hypoatherina tsurugae*
common carp	*Cyprinus carpio*
dusky grouper	*Epinephelus marginatus*
European eel	*Anguilla anguilla*
European plaice	*Pleuronectes platessa*
European sardine	*Sardina pilchardus*
European seabass	*Dicentrarchus labrax*
flathead mullet	*Mugil cephalus*
gilthead seabream	*Sparus aurata*
greater amberjack	*Seriola dumerili*
grey triggerfish	*Balistes capriscus*
murrel	*Channa punctatus*
Indian freshwater/stinging catfish	*Heteropneustes fossilis*
Japanese anchovy	*Engraulis japonicus*
lambari	*Astyanax lacustris*
largehead hairtail	*Trichiurus japonicus*
meagre	*Argyrosomus regius*
medaka,	*Oryzias latipes*
mosquito fish	Gambusia holbrooki
Mozambique tilapia	*Oreochromis mossambicus*
Nile tilapia	*Oreochromis niloticus*
Pacific bluefin tuna	*Thunnus orientalis*
Pacific halibut	*Hippoglossus stenolepis*
pikeperch	*Sander lucioperca*

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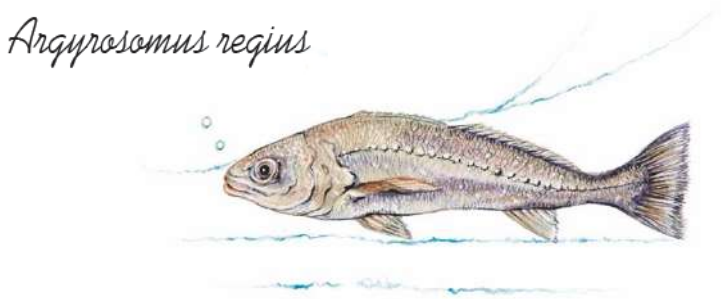
Common English name	**Scientific name**
rainbow trout	*Oncorhynchus mykiss*
red stingray	*Hemitrygon akajei*
ricefield eel	*Monopterus albus*
Senegalese sole	*Solea senegalensis*
Siberian sturgeon	*Acipenser baerii*
silver trevally	*Pseudocaranx georgianus*
spined loach	*Cobitis taenia*
spined loach	*Cobitis hankugensis-longicorpa*
spotted wolffish	*Anarhichas minor*
stinging catfish	*Heteropneustes fossilis*
sturgeon	*Acipenser ruthenus*
tiger trout	*Salmo trutta* × *Salvelinus fontinalis*
Tokyo bitterling	*Pseudorhodeus tanago*
Tropical mullet	*Mugil incilis*
turbot	*Scophthalmus maximus*
zebrafish	*Danio rerio*



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MISHRA	ABHA	BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY	India
NARWAL	RITU	DEPARTMENT OF ZOOLOGY, UNIVERSITY OF DELHI	India
SAHU	UMA BHARATI	DEPARTMENT OF ZOOLOGY, UNIVERSITY OF DELHI	India
SEHGAL	NEETA	UNIVERSITY OF DELHI	India
GOPABANDHU	SNEHASHISH	BANARAS HINDU UNIVERSITY	India
ASHKENAZI-MEIRI	IRIS	NCM-IOLR	Israel
COHEN	OMER	HEBREW UNIVERSITY	Israel
COHEN-ROTHSCHILD	NOAM	THE HEBREW UNIVERSITY OF JERUSALEM	Israel
DEGANI	GAD	MIGAL—GALILEE RESEARCH INSTITUTE	Israel
GOLAN	MATAN	ARO - VOLCANI CENTER	Israel
GOTHILF	YOAV	TEL AVIV UNIVERSITY	Israel
LEVAVI-SIVAN	BERTA	THE HEBREW UNIVERSITY OF JERUSALEM	Israel
MAGNUS	YOAV	DAGON	Israel
ROSENFELD	HANNA	NCM-IOLR	Israel
BERTOTTO	DANIELA	UNIVERSITY OF PADOVA	Italy
CARNEVALI	OLIANA	UNIVERSITÀ POLITECNICA DELLE MARCHE	Italy
CHEMELLO	GIULIA	UNIVERSITÀ POLITECNICA DELLE MARCHE	Italy
CONCU	DANILO	INTERNATIONAL MARINE CENTRE - IMC	Italy
DALLA VALLE	LUISA	UNIVERSITY OF PADOVA	Italy

GIOACCHINI	GIORGIA	UNIVERSITÀ POLITECNICA DELLE MARCHE	Italy
GIOMMI	CHRISTIAN	UNIVERSITÀ POLITECNICA DELLE MARCHE	Italy
MARADONNA	FRANCESCA	UNIVERSITÀ POLITECNICA DELLE MARCHE	Italy
SODAGAR	ANAHITA	UNIVERSITY OF PADUA	Italy
VALLAINC	DARIO	FONDAZIONE IMC	Italy
ZUPA	ROSA	UNIVERSITY OF BARI ALDO MORO	Italy
AMANO	YUICHI	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan
CHAKRABORTY	TAPAS	KYUSHU UNIVERSITY	Japan
HAYASHIDA	TAKAO	JAPAN FISHERIES RESEARCH AND EDUCATION AGENCY	Japan
GALOTTA	MARIEL	KYUSHU UNIVERSITY	Japan
ICHIDA	KENSUKE	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan
KOBAYASHI	YASUHISA	KINDAI UNIVERSITY	Japan
MOHAPATRA	SIPRA	KYUSHU UNIVERSITY	Japan
MORIYA	NATSUKO	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan
MURATA	RYOSUKE	NAGASAKI UNIVERSITY	Japan
OHTA	KOHEI	KYUSHU UNIVERSITY	Japan
SOYANO	KIYOSHI	NAGASAKI UNIVERSITY	Japan
SURUGAYA	RYOHEI	HOKKAIDO UNIVERSITY	Japan
TOKUMOTO	TOSHINOBU	SHIZUOKA UNIVERSITY	Japan
WATANABE	NOZOMI	HOKKAIDO UNIVERSITY	Japan
YAHIRO	ISSEI	KYUSHU UNIVERSITY	Japan
YAMAGUCHI	YO	HOKKAIDO UNIVERSITY	Japan
YAMAKAWA	KOHJU	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan
YAMAMOTO	YOJI	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan

YAZAWA	RYOSUKE	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan
YOSHIZAKI	GORO	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOLOGY	Japan
YUSUKE	KATO	TOKYO UNIVERSITY OF MARINE SCIENCE AND TECHNOROGY	Japan
BOGERD	JAN	UTRECHT UNIVERSITY	Netherlands
DOVGAN	TATIANA	UTRECHT UNIVERSITY	Netherlands
JEHANNET	PAULINE	WAGENINGEN UNIVERSITY & RESEARCH	Netherlands
LUKSENBURG	MARTIJN	UTRECHT UNIVERSITY	Netherlands
PALSTRA	ARJAN	WAGENINGEN UNIVERSITY & RESEARCH	Netherlands
SCHULZ	RÜDIGER	UTRECHT UNIVERSITY and INSTITUTE OF MARINE RESEARCH	Netherlands
FURTADO RIBEIRO	FLAVIO	NEW ZEALAND INSTITUTE FOR PLANT AND FOOD RESEARCH	New Zealand
LEWIS	BLAKE	THE UNIVERSITY OF OTAGO	New Zealand
WYLIE	MATTHEW	NEW ZEALAND INSTITUTE FOR PLANT AND FOOD RESEARCH	New Zealand
ALIX	MAUD	INSTITUTE OF MARINE RESEARCH	Norway
ALVES MUSIALAK	LUCIANA	NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY	Norway
ANDERSSON	EVA	INSTITUTE OF MARINE RESEARCH	Norway
BOGEVIK	ANDRÉ S	NOFIMA AS	Norway
CRESPO	DIEGO	INSTITUTE OF MARINE RESEARCH	Norway
KATHTHRIARACHCHI	SONALI	INLAND NORWAY UNIVERSITY OF APPLIED SCIENCES	Norway
KJÆRNER-SEMB	ERIK	INSTITUTE OF MARINE RESEARCH	Norway
KLEPPE	LENE	INSTITUTE OF MARINE RESEARCH	Norway
KRIVENJEVA SINANI	VALENTINA	INLAND NORWAY UNIVERSITY OF APPLIED SCIENCES	Norway

MYKLATUN	LARS EIRIK	INSTITUTE OF MARINE RESEARCH	Norway
NIEPAGEN	NILS	NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY	Norway
NORBERG	BIRGITTA	INSTITUTE OF MARINE RESEARCH	Norway
SANCHEZ MATIAS	GABRIELA ANGELICA	NORWEGIAN UNIVERSITY OF LIFE SCIENCES	Norway
SUPERIO	JOSHUA	NORD UNIVERSITY	Norway
YILMAZ	OZLEM	INSTITUTE OF MARINE RESEARCH	Norway
JUDYCKA	SYLWIA	POLISH ACADEMY OF SCIENCES	Poland
ŻARSKI	DANIEL	POLISH ACADEMY OF SCIENCES	Poland
ASHOORI	SAMYAR	UNIVERSIDADE DE LISBOA	Portugal
CABRITA	ELSA	CCMAR - CENTRO DE CIÊNCIAS DO MAR DO ALGARVE	Portugal
CANARIO	ADELINO	CCMAR - CENTRO DE CIÊNCIAS DO MAR DO ALGARVE	Portugal
CHEN	JIE	CCMAR - CENTRO DE CIÊNCIAS DO MAR DO ALGARVE	Portugal
FATSINI	ELVIRA	CCMAR - CENTRO DE CIÊNCIAS DO MAR DO ALGARVE	Portugal
FÉLIX	FRANCISCA	CCMAR - CENTRO DE CIÊNCIAS DO MAR DO ALGARVE	Portugal
OLIVEIRA	RUI	ISPA	Portugal
OLIVEIRA	CATARINA	CCMAR - CENTRO DE CIÊNCIAS DO MAR DO ALGARVE	Portugal
MARTA	ANATOLIE	IAPG, CZECH REPUBLIC	Romania
DE MELLO	PAULO	KAUST - KING ABDULLAH UNIVERSITY OF SCIENCE AND TECHNOLOGY	Saudi Arabia
MOON	HYE-NA	JEJU NATIONAL UNIVERSITY	South Korea
NAMGUNG	JIN	JEJU NATIONAL UNIVERSITY	South Korea
ASTURIANO	JUAN F.	UNIVERSITAT POLITECNICA DE VALENCIA	Spain
BEATO	SILVIA	INSTITUTE OF MARINE SCIENCES (ICM-CSIC)	Spain
BLANES-GARCIA	MARTA	UNIVERSITAT POLITÈCNICA DE VALÈNCIA	Spain

BLAZQUEZ	MERCEDES	INSTITUTE OF MARINE SCIENCES (ICM-CSIC)	Spain
CERRUD	GIANCARLO	IRTA AND UNIVERSIDAD AUTONOMA DE BARCELONA	Spain
CHAUVIGNÉ	FRANÇOIS	INSTITUTE OF MARINE SCIENCES (ICM-CSIC)	Spain
DE LA FUENTE CARBALLO	MONICA	STOLT SEA FARM	Spain
DÍAZ	NOELIA	INSTITUTE OF MARINE SCIENCES (ICM-CSIC)	Spain
DUNCAN	NEIL	IRTA	Spain
FELIP	ALICIA	INSTITUTO DE ACUICULTURA TORRE DE LA SAL (IATS-CSIC)	Spain
FERRÃO	LEONOR	UPV - DPTO. DE CIENCIA ANIMAL	Spain
FERREIRO	ISABEL	STOLT SEA FARM, S.A.	Spain
GAYO	PATRICIA	IFAPA EL TORUÑO	Spain
GIMÉNEZ	IGNACIO	RARA AVIS BIOTEC S.L.	Spain
GÓMEZ	ANA	INSTITUTO DE ACUICULTURA TORRE DE LA SAL (IATS-CSIC)	Spain
HERRAEZ	PAZ	UNIVERSIDAD DE LEON	Spain
LOMBÓ	MARTA	UNIVERSIDAD DE LEON	Spain
MAÑANOS	EVARISTO	INSTITUTO DE ACUICULTURA TORRE DE LA SAL (IATS-CSIC)	Spain
MASCOLI	ALESSIA	INSTITUTO DE ACUICULTURA TORRE DE LA SAL (IATS-CSIC)	Spain
MORINI	MARINA	UNIVERSITAT POLITÈCNICA DE VALÈNCIA	Spain
MUÑOZ-CUETO	JOSE A.	UNIVERSITY OF CADIZ	Spain
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ROBLES	VANESA	UNIVERSIDAD DE LEÓN	Spain
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SÁNCHEZ-BAIZÁN	NÚRIA	INSTITUTE OF MARINE SCIENCES (ICM-CSIC)	Spain
SANCHIS	NEREA	INSTITUTE OF MARINE SCIENCES (ICM-CSIC)	Spain

SARIH	SAMIRA	UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA	Spain
SEERRATOSA	FRANCESC	UNIVERSITAT ROVIRA I VIRGILI	Spain
SEMPERE BEA	LAURA	INSTITUTO DE ACUICULTURA TORRE DE LA SAL (IATS-CSIC)	Spain
VALCARCE	DAVID G.	UNIVERSITY OF LEÓN	Spain
ZAPATER	CINTA	INSTITUTO DE ACUICULTURA TORRE DE LA SAL (IATS-CSIC)	Spain
JEUTHE	HENRIK	AQUACULTURE CENTRE NORTH	Sweden
PALAIOKOSTAS	CHRISTOS	SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES	Sweden
CHANG	CHING-FONG	NATIONAL TAIWAN OCEAN UNIVERSITY	Taiwan
TSENG	PENG-WEI	NATIONAL TAIWAN OCEAN UNIVERSITY	Taiwan
PANTHUM	THTIPONG	KASETSART UNIVERSITY	Thailand
SRIKULNATH	KORNSORN	KASETSART UNIVERSITY	Thailand
XU	LAN	IMET- UNIVERSITY OF MARYLAND BALTIMORE COUNTY	USA
BHANDARI	RAMJI	UNIVERSITY OF NORTH CAROLINA GREENSBORO	USA
HUERTAS	MAR	TEXAS STATE UNIVERSITY	USA
LI	WEIMING	MICHIGAN STATE UNIVERSITY	USA
LUCKENBACH	ADAM	NOAA – NORTHWEST FISHERIES SCIENCE CENTER	USA
PLANAS	JOSEP	INTERNATIONAL PACIFIC HALIBUT COMMISSION	USA
SEALE	ANDRE	UNIVERSITY OF HAWAII	USA
SORENSEN	PETER	UNIVERSITY OF MINNESOTA	USA
STUBBLEFIELD	JOHN	IMET- UNIVERSITY OF MARYLAND BALTIMORE COUNTY	USA
WONG	TEN-TSAO	IMET- UNIVERSITY OF MARYLAND BALTIMORE COUNTY	USA
ZMORA	NILLI	IMET- UNIVERSITY OF MARYLAND BALTIMORE COUNTY	USA

ZOHAR	YONATHAN	IMET- UNIVERSITY OF MARYLAND BALTIMORE COUNTY	USA

Dedications

"**Ithaka***", by **Constantine Cavafy**, 1911 (translated by Edmund Keely)

As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon—don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians, Cyclops,
wild Poseidon—you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbors you're seeing for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind—
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean

Dedicated to my "Ithaka", and the "Ithaka" of all persons and scientists (must) have in their lives!

** "**Ithaka**" is the name of the island of Odysseus -the hero of Homer's epic poems "Heliad" and "Odyssey"- who fought for 10 years to conquer Troy (using his Trojan Horse) and then wondered for 20 years in the Seas, before succeeding to return home.*

Post symposium information

Concluding remarks and summary of the symposium

First of all – a big THANK YOU Dinos for hosting us all on the beautiful island of Crete, and congratulations for arranging a fantastic conference! The dinner and party last night were amazing – I am sure we could have continued all night long! Also, you made me sit and listen to every single presentation, which was quite an achievement ☺.

My own first "Fish Reproduction" meeting was in St John´s, Canada, in 1987 – the third in this series that has now reached number 12. I remember being young, new and somewhat starstruck by names such as the power couple Robin Wallace (my big hero at the time) and Kelly Selman, and also Dick Peter, Larry Crim, Nancy Sherwood, Roland Billard, Sandy Scott, Zvi Yaron – the list goes on and on…. Even Yoni Zohar was there – I was sorry to hear Yoni is ill, please send him my best regards and I hope he has a speedy recovery – this meeting is not the same without him!! Anyway, I also remember the friendly atmosphere of my first meeting in St John´s and the attention to students, to make us in the younger generation feel included. This atmosphere of inclusion remains one of the most important and unique aspects of the ISRPF family.

This week, we came to Crete from 28 countries, from Asia, Australia/New Zealand, North America, South America and Europe. "Fish" are diverse, and not really possible to classify, as Sylvie Dufour explained to us, and presentations have included well over 40 different species – model fish such as zebrafish and medaka, established aquaculture species such as Atlantic salmon, rainbow trout, tilapia, common carp, European sea bass and gilthead sea bream, new and emerging species for aquaculture and conservation purposes, such as flathead mullet, greater amberjack, Atlantic halibut, Senegalese sole and the notoriously difficult European eel, ornamental fish such as the Siamese fighting fish, and studies of wild species such as Atlantic cod, cobaltcap silverside, marine stickleback and sea lamprey, as well as invasive species such as rainbow trout and silver carp.

Through the 9 Special Sessions, we have been taken from the gene and micromolecule regulation of reproduction, all the way into the actual bedrooms of the fish, watching them mate in todays´ session!

We had two both interesting and thought-provoking plenary lectures. Sylvie Dufour put our work in perspective by showing us the history of fish reproductive physiology – dating back to 6000 years BC – but also the important applications to our current environmental situation! Daniel Pauly presented a new and very interesting perspective on the relationship between growth and maturation, and what limits the decision to mature in fish. The importance of oxygen availability for attainment of reproductive competence certainly merits more attention by our community!

Now, I will try to summarize some highlights and thoughts of the conference, but this is a major and somewhat overwhelming task, and it is unfortunately impossible to mention everyone – that would require several hours and I will not put you through that kind of torture (though it has been done before!) – In addition, that would not really be fair on the presenters of todays' sessions as they literally just ended… so I decided to not go into each session in detail and, with one exception, not mention any names! Having said that, it is great to see that a new (or newer) generation has stepped up to give us all the excellent State-of-the-art presentations that we have heard this week! But instead of going through all the sessions separately, I will try to summarize some of my thoughts (after communicating with some

of you), on the overall highlights of this conference and developments since we met in Brazil five years ago:

First, the general increase in applications of very basic science and advanced techniques from model species to actual aquaculture and wild species is incredibly encouraging and it genuinely warms my heart (as someone who has done mostly applied work over the years) – we obviously need model species that are easy to keep and make it possible to study mechanisms over many generations without having to wait for years – but as we have seen, every species is different, not everything is immediately transferable from one species to another and eventually the knowledge that we gain from the models needs to be applied and validated in real life!

The apparent increase in reporting of negative results is another interesting aspect of this meeting. It is discouraging to get negative results, and it is tempting to just not report them. However, if those negative results are not reported as they are, someone may repeat the work and may get the same results again. This is a waste of time, energy and resources and it is so important to remember that by putting them out there to be discussed, you may actually get new input and ideas on how to proceed and get a better success in the next experiment. Sometimes, just pursuing the work through to the next generation of animals can give the results you hope for!

Another thing that stands out is how the development from new species to established aquaculture species changes the research questions we need to ask: The first thing we need to do, whether it be a new species in aquaculture or for conservation is to close the lifecycle. For that, we must understand reproduction – how to have fish mature and spawn in captivity, and how to obtain a viable egg, and healthy offspring. Many species still require spawning induction by hormones, and gamete and egg quality can be highly variable, as we have seen. This calls for an understanding of both reproductive physiology and behaviour. To optimize production, we need to understand embryonic and larval requirements for nutrition, we need to understand the relationship between growth, puberty and maturation and we need to understand how all of this is regulated through the Brain-Pituitary-Gonad (BPG)-axis (and also, the GH-Igf-axis). It is safe to say that our insight into the networks of the BPG axis has improved significantly this week! Breeding programs need availability of sperm and eggs, and we have seen some encouraging first steps this week to be able to cryopreserve eggs and embryos in addition to sperm. However, as we move to producing fish for the market, we want to prevent maturation to happen – it reduces efficiency of feed utilization, growth and flesh quality, and increases the costs for the farmer. As we breed fish through many generations and change their genetic traits, production numbers increase and especially in open cage aquaculture, we need to make sure that fish cannot escape and interact genetically with wild populations – so the need for research changes from induction of maturation to actually producing sterile fish. Here, we need knowledge both on sex determination and differentiation and regulation of the earliest stages of puberty. This can be a challenge and can sometimes take more than one generation to achieve. Importantly, new sterility models are currently being developed, while germ cell transplantation has applications both for large-scale sterile fish production, conservation of wild fish stocks and, possibly, as we saw this morning, as a tool for handling and eliminating invasive species.

There has been a significant advancement in analytical methods and their availability. The development of various imaging techniques that allow us to understand in detail important physiological processes and how they are regulated in the cell are particularly impressive. Single cell sequencing is another very powerful tool that is of enormous importance in understanding physiological and endocrine regulatory mechanisms at a deeper level.

The increase in the use and application of epigenetics (and genetics) to improve our understanding of many, if not all aspects of reproduction is a stunning development, which will certainly continue in the years to come. The understanding of micro-RNAs and their role in reproduction is also increasing. Overall, there is a development from just showing analytical data to using these techniques to understand important mechanisms and pathways in regulation of reproduction and gamete quality. The same can be

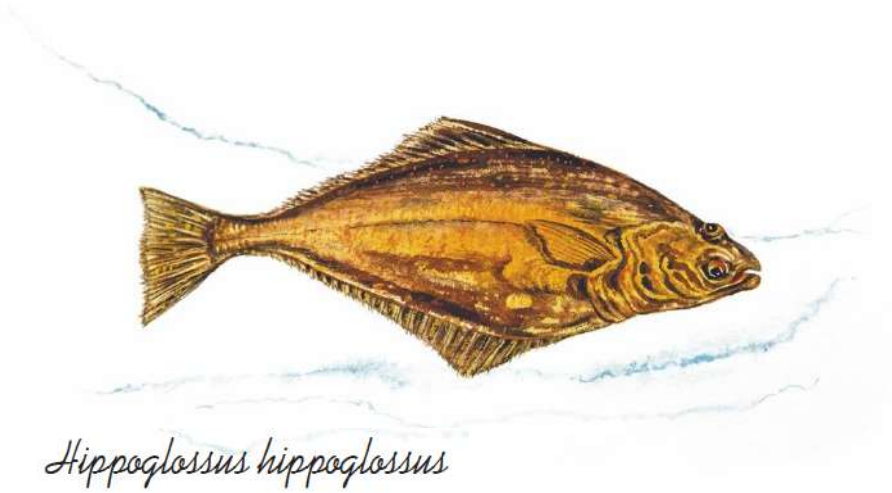
said about the development in the use of omics – proteomics, transcriptomics - from just showing the analytical data to actually using them as tools to increase our understanding of regulatory mechanisms and networks, and being able to pinpoint, for example, markers for gamete quality.

Climate change is very real, and we are experiencing the consequences more and more. As water temperatures increase, oxygen availability is reduced and pH decreases. This in itself affects reproduction and species that can, will migrate to cooler water – thus affecting the overall ecosystem balance and foodwebs. We are already seeing this and are beginning to understand how it can affect reproduction. In addition, these changes alter the sensitivity to, and toxicity of, pollutants in the water. This is arguably the greatest challenge we are facing today, and I am convinced that this will have a profound effect on our work and that we will have many more presentations addressing this at the next meeting!

But, to end on a more optimistic note, all the student presentations have been of excellent quality – my congratulations to each and every one of you! After five days and nine sessions, the last presentations are of course easiest to remember. Having said that, I must mention the presentation today by Natsuko Moriya on Lh gene overexpression to induce sperm production in juvenile trout, it really stood out. We know that new graphic tools are available for making presentations, but I am still very impressed by all the student presentations - you are the future!

Finally, as I said in the beginning, and I think I speak for everyone, we have had a fantastic week. Have a safe trip home everyone - so long, and thanks for all the fish!

Dr. Birgitta Norberg,
Reproduction and Early Development Research group,
Institute of Marine Research,
Austevoll Research Station,
5392 Storebø, Norway



Student presentation awards

Four student awards were given, for best posters (2) and for best oral presentations (2), and included a monety award as well. Three different 3-member committees evaluated the Oral presentations, and the odd-numbered and even-numbered poster presentations. In the table on the right, the evaluation criteria considered by the committtees are shown.

Criteria	Weight	Score*	Total (W x Sc)
Overall appearance, layout and visual impact: Oral presentation/poster was "eye catching", with nice graphs, tables and drawings, pleasantly arranged (not too crowded/excessive information) in space and time (oral presentation) and well presented (orally or as a poster)	4	1-5	20
Originality of work: Novelty of subject as a new idea and note merely a continuation/repetition of existing or on-going research area.	4	1-5	20
Cohesion: Concise, clear presentation of objectives, methods and results. Easy to understand by non-specialists.	2	1-5	10
Validity: The interpretation of results and the conclusions are valid and well-supported. Statistics were appropriate and followed. No overinterpretation of impact.	5	1-5	25
Applicability & Impact: The findings have a significant contribution to the field of Fish Reproduction, and advance our basic knowledge with immediate or potential applications and solutions to societal problems.	5	1-5	25
Sum Total			**100**

* **Poor = 1, Satisfactory = 2, Good = 3, Very good = 4, Excellent = 5**

From left to right, Mrs Natsuko Moriya, Mr Yuichi Amano, Dr Constantinos C Mylonas, Mr Kohju Yamakawa and Mr Issey Yahiro.

Student Poster Presentation awards

Yahiro, Issey (P87)
Kyushu University, Japan
"SDF-1/CXCR4 signal is involved in the induction of Primordial Germ Cell migration in a model marine fish, the Japanese anchovy (*Engraulis japonicus*)"

Yamakawa, Kohju (P84)
Tokyo University MST, Japan
"Functional sperm production from frozen germ cells of a fish on the verge of extinction: the case of the Tokyo bitterling"

Student Oral Presentation awards

Amano, Yuichi (O56)
Tokyo University MST, Japan
"Using surrogate fish for eradicating invasive fish: Can surrogate triploid rainbow trout mate with their wild-type counterparts and produce lethal hybrids?"

Moriya, Natsuko (O64)
Tokyo University MST, Japan
"Luteinizing hormone gene over-expression in pre-pubertal rainbow trout can induce sperm production within a short period"

Special Participation Award

As the president of the Scientific Program Committee of the 12th ISRPF, I would like to acknowledge the presence and contribution to this symposium, of the **only person who attended all twelve (12) ISRPF**, beginning with the first one in Paimpont, France (1977).

Professor Yonathan (Yoni) Zohar (Institute of Marine and Environmental Technology and University of Maryland Baltimore County, USA)

Professor Yonathan Zohar received a Museum replica of the discus of Festos (the logo of the 12th ISRPF), the earliest form of a transportable document, coming from the Minoan Civilization in Crete, excavated from the Palace fo Festos, and dating back to 1500 BC. Unfortunately, Professor Zohar had to leave the day before the closing of the symposium, due to a sudden illness, so his award ceremony was done using a "WhatsApp" connection with the help of Dr. Nilli Zmora (IMET), who will take the award to him in Baltimore.

See you next time - «Στο επανιδείν»


διαζώμα


WELCOME TO THE
12th International Symposium on Reproductive Physiology of Fish
P49
P50
P63
CONGRESS HALL
Goro Yoshizaki
Institute for Reproductive Biotech
for Aquatic Species
Tokyo University of Marine Science and
WELCOME TO THE

Seriola dumerili,

The drawings that decorated the pages of the 12th ISRPF Program and Book of Abstracts are a kind contribution from ***Viotopos Publishers*** *(https://www.viotopos.com), and represent some of the major fish species used in aquaculture around the world. They were prepared specifically for the 12th ISRPF by* ***Mrs Thalia Zeimpeki****, whom we thank sincerely!*