COOPERATION AGREEMENT

within the international project

Shortening of generation time using surrogate reproduction and in vitro sperm maturation in fish
(hereinafter the „**Agreement**")

Contractual Parties (Partners) of the Agreement:

Organization in the Czech Republic
University of South Bohemia in České Budějovice
VAT Id. No: CZ60076658
Registered seat: Branišovská 1645/31a, 370 05 České Budějovice, Czech Republic
Represented by: Prof. PhDr. Bohumil Jiroušek, Dr., Rector
(hereinafter the "**Czech Beneficiary**")

Organization in Brazil
Universidade Estadual Paulista Júlio de Mesquita Filho, Faculdade de Engenharia de Ilha Solteria, Departamento de Biologia e Zootecnia
CNPJ: 48.031.918/0015-20
Registered seat: Passeio Monção, 226 – Dep. Biologia e Zootecnia Unesp Zona Norte, 153850000 – Illha Solteira, SP – Brasil
Represented by: Prof. Dr. Ricardo Alan Verdú Ramos
(hereinafter the "**Brazilian Beneficiary**")

Preamble

The Contractual Parties cooperate in the implementation of the Project entitled "**Shortening of generation time using surrogate reproduction and in vitro sperm maturation in fish**" (hereinafter the "**Project**") which the Czech Beneficiary submitted to the public tender of the International bilateral grant programme of the Czech Science Foundation of the Czech Republic (the „**Provider**").
Supposing that the Provider enters into the Agreement on Granting of Subsidy to the Project with the Czech Beneficiary (the "Agreement on Granting of Subsidy"), the Contractual Parties undertake herein to cooperate in the Project implementation and in utilizing the results of the Project.
The main **goal of the Project** is to prove or disprove the possibility to speed up the gamete production using transplantation of germ stem cells between later and earlier maturing species and in vitro maturation of sperm on fish models.

Article I
Subject Matter of Agreement

1.1. The subject matter of this Agreement is to describe the roles and rights and obligations of the Contractual Parties relating to the implementation of the Project, in particular, to define the rights and duties of the Contractual Parties concerning (i) rights to intangible property (e.g. intellectual property) necessary for the implementation of the Project, (ii) rights to intangible property created during or in relation to the Project and (iii) regulation of utilizing the results of the Project.

1.2. The nature, purpose, goals, and expected results of the Project are specified (i) in the Project proposal registered with the information/application system of the Provider which forms Annexes hereof.

Article II
Terms and Conditions of Cooperation between Parties

2.1. The Contractual Parties shall cooperate in compliance with the proposed Project and other conditions and documents that are binding for the Project. The Contractual Parties became acquainted with the Project content before signing this Agreement, including the Project application and all Programme conditions.

2.2. The Contractual Parties undertake to use all necessary efforts to achieve the purpose, goals and expected results of the Project as defined in Annexes to this Agreement. Failure to accomplish the purpose, goals, and/or expected results of the Project may only be justified by circumstances generally recognized and defined as force majeure.

2.3. The Contractual Parties undertake to act and perform in a manner that will not jeopardize the implementation of the Project and the interests of the other Contractual Party.

Article III
Structure of the Project –Investigators

3.1. The person responsible for the scientific implementation of the Project by the Czech Beneficiary is the responsible investigator: [redacted] email: [redacted] telephone: [redacted], address: Zatisi 728/II, 389 25 Vodnany, Czech Republic

3.2. The person responsible for the scientific implementation of the Project by the Brazilian Beneficiary: [redacted]D., email: [redacted] telephone: [redacted] address: Passeio Monção, 226 – Dep. Biologia e Zootecnia Unesp Zona Norte, 153850000 – Illha Solteira, SP – Brasil

3.3. The investigators are involved in the activities necessary for the successful completion of the Project in compliance with the approved Project proposal.

Article IV
Project Management, Involvement of Individual Contractual Parties in Project

4.1. The Czech Beneficiary is the Project applicant for the provision of subsidy in the Czech Republic. The Czech Beneficiary shall conclude an Agreement on Granting of Subsidy with the Provider. The Czech Beneficiary provides administrative cooperation with the Provider in the Czech Republic.

4.2. The Brazilian Beneficiary is the Project applicant for the provision of subsidy in the country of its origin under the terms and conditions applicable in Brazil where the subsidy is granted. The Brazilian Beneficiary provides administrative cooperation with the grant provider in Brazil.

4.3. The Contractual Parties undertake to exercise all necessary efforts to implement the Project and to act in a manner that will not jeopardize the implementation of the Project, the Project goals and results, and the interests of both partners.

4.4. The Contractual Parties undertake to perform within the set deadlines and defined extent the activities leading to the Project implementation as specifically determined in the Project proposal
and/or any other activities as necessary or needed for proper Project implementation.

Article V
Course and Evaluation of Project

5.1. For the purposes of verification and evaluation of progress in the Contractual parties´ cooperation during the Project implementation the Brazilian Beneficiary is obligated to provide the Czech Beneficiary all relevant information and documents necessary to prepare:
(a) Interim reports;
(b) Extraordinary reports; and
(c) A final report.

5.2. With respect to the reports referred to in para 5.1. the Brazilian Beneficiary is obligated to adhere to the instructions of the Czech Beneficiary concerning the content and structure of the reports and deadlines for their submission and, further, to submit the reports in such a form that they could be published, as the case may be, either by the Czech Beneficiary or the Provider.

5.3. The Czech Beneficiary is obligated to provide the Brazilian Beneficiary reports on the Project implementation pursuant to the program conditions as required in the country where subsidy is granted to the Brazilian Beneficiary. The Brazilian Beneficiary is obligated to inform the Czech Beneficiary about these conditions for the submission of reports before the start of the Project implementation.

5.4. The Contractual Parties undertake to cooperate on the execution of the implementation plan to the Project results.

Article VI
Rights and Duties of Contractual Parties

6.1. The Contractual Parties are obligated to notify each other about all changes concerning the Project, about any inability to perform obligations under this Agreement duly and on time, and about all material changes and facts that could affect the implementation, expected results, and goals of the Project no later than 4 calendar days from the day on which they become aware of them. The Contractual Parties are further obligated to prove at any time that they remain qualified to participate in the Project implementation.

6.2. The Contractual Parties undertake to archive documents relating to the Project for at least 10 years from the completion of the Project.

Article VII
Intellectual Property, Tangible Property

7.1. This Agreement governs the rights and obligations of the Contractual Parties to the intellectual property of Contracting Parties existing before entering into this Agreement (the "Pre-Existing Knowledge") and sets forth the rules of utilization of such Pre-Existing Knowledge for the purposes of implementation of the Project. Further, the Agreement governs the rights and

obligations of the Contractual Parties to the intellectual property created during the term hereof and that become the property of the Contractual Party having created it.

7.2. Intellectual property for the purposes of this Agreement means any results of intellectual activity, based on which any objectively perceivable intangible property is created. In particular, this includes inventions, technical solutions protected as a utility model, industrial designs, innovations and rationalization proposals, biotechnological inventions, trademarks, copyrighted works, know-how, and other results of intellectual activity.

7.3. Pre-Existing Knowledge which is necessary for the implementation of the Project or the utilization of its results shall remain the property of respective Contractual Party, however, such Contractual Party shall permit the other Contractual Party to use any of its Pre-Existing Knowledge to the extent as necessary for the purposes of implementation of the Project.

7.4. The Contractual Parties agreed that any intellectual property created within or in connection with the Project implementation shall become the property of the Contractual Party whose employees created such intellectual property. The Contractual Parties shall notify each other of every creation of such intellectual property. The Contractual Party having rights to such intellectual property shall bear the costs of filing any applications for protection thereof and costs of relating proceedings.

7.5. If, during or in connection with the Project, any intellectual property is created as a result of the mutual collaboration of employees of Contractual Parties such intellectual property shall become joint ownership of Contractual Parties, whereas the share of respective Contractual Party shall correspond to efforts exerted by each of its employees for creation of such intellectual property. The Contractual Parties shall provide mutual assistance to each other in preparation of applications, including foreign applications, for the protection of such jointly owned intellectual property. The Contractual Parties shall share any costs of filing of applications and costs of relating proceedings in the proportion of their shares.

7.6. If either of the joint owners is not interested in applying for protection of intellectual property, the other joint owner may request to transfer the right to file such an application to it. The joint owners will then negotiate the terms and conditions of such transfer of the right to file an application. The Contractual Parties shall provide mutual assistance to each other in preparation of applications, including foreign applications. The joint owner to which the right to apply has been transferred bears the costs of filing of applications and costs of relating proceedings.

7.7. The Contractual Parties undertake to use or permit the use of the Project results in accordance with their respective interests and the interests of the Provider while respecting the necessary protection of rights to Intellectual property items and confidentiality.

7.8. Provided that intellectual property created during the implementation of the Project belongs jointly to joint owners, the Contractual Parties undertake to use their best efforts to agree to the joint exercise of rights to jointly owned intellectual property. Consent of joint owners is always required for (i) valid granting of a license to a third party, (ii) transfer of rights to jointly owned intellectual property to a third party, and/or (iii) transfer of share to jointly owned intellectual property to a third party.

7.9. Unless agreed otherwise by joint owners, any joint owner is entitled to transfer the intellectual property (Project result) to which is an owner or joint owner at its own expense into practice. In the case of commercial use of the intellectual property by one of the Contractual Parties, the revenues from commercialization will be distributed according to the share of ownership after deducting the costs of commercialization.

7.10. The Contractual Parties are however entitled to use free of charge any intellectual property created within or in connection with the Project implementation for educative, research, and other non-commercial purposes.

7.11. Intellectual property for which the protection is possible (patents, utility models, etc.) cannot be disclosed until the respective application for protection is submitted.

7.12. Any tangible property of Contractual Party which is necessary for the implementation of the Project or the utilization of its results shall remain the property of respective Contractual Party, however, such Contractual Party shall permit the other Contractual Party to use any of its tangible property to the extent as necessary for the purposes of implementation of the Project. If, during or in connection with the Project, any tangible property is acquired or created by the Contractual Party such tangible property shall become joint ownership of respective Contractual Party, whereas the share of respective Contractual Party shall correspond to financial means exerted by each of joint owners for acquiring or creation of such tangible property. Tangible property under this provision shall be used by any Contractual Party for the purposes of implementation of the Project.

Article VIII
Ensuring Protection of Information and Outcomes Obtained in Connection with Project

8.1. The Contractual Parties undertake to provide each other with all information as necessary to carry out the activities hereunder. Unless the Contractual Parties agree otherwise all information obtained from the other Contractual Party, which is not in the public domain is considered to be confidential (the "Confidential Information").

8.2. The Contractual Party that has obtained such Confidential Information is obligated to maintain the confidentiality thereof and ensure sufficient protection against unauthorized access. It must not disclose such Confidential Information to any other person/entity, save for its employees and other persons who are in charge of conducting activities under this Agreement and with whom the respective Contractual Party has concluded a confidentiality agreement with a scope similar to that stipulated for the Contractual Parties by this Agreement, and it shall not use the Confidential Information for any purpose other than the performance of activities under this Agreement.

8.3. Duties under paragraph 8.2. apply without any change and remain valid for a period of 3 years after the termination of this Agreement, notwithstanding the reason for such termination.

Article IX
Liability for Damage

9.1. The Contractual Parties acknowledge that a breach of a duty under this Agreement by Contractual

Party may result in incurring damage to the other Contractual Party and undertake to compensate the other Contractual Party for any caused damage.

Article X
Final Provisions

10.1. This Agreement becomes valid on the date of its signature by both Contractual Parties and effective as of the signature date of the Agreement on Granting of Subsidy. The Agreement is concluded for the duration of the Project and for three years after the completion of the Project. The Contractual Parties have agreed that those provisions of the Agreement which were apparently intended by the Contractual Parties to survive after the termination or expiry of the Agreement shall remain valid and effective (in particular Articles 7.1 - 7.12 and Articles 8.1 - 8.3)

10.2. In case of conflict between the Contractual Parties resulting from the interpretation or the application of this Agreement, or in connection with the activities contained within, the Contractual Parties involved shall make the effort to come to an amicable arrangement rapidly and in the spirit of good cooperation.

10.3. Failing amicable settlement, the Courts of the Czech Republic shall have sole competence to rule on any dispute between the Contractual parties in respect of this Agreement. The competent court shall be the court in the Czech Beneficiary´s seat.

10.4. The law applicable to this Agreement shall be the law of the Czech Republic.
The terms and conditions of subsidy granted to the Brazilian Beneficiary by its country of origin shall be governed by valid laws and regulations of the country granting such subsidy.

10.5. The Contracting Parties understand that this Agreement is subject to mandatory publication in the Register of Contracts pursuant to Act No. 340/2015 Coll., On Special Conditions of Effectiveness of Certain Contracts, Publication of These Contracts and the Register of Contracts (Contract Register), as amended. All actions related to the publication of the Agreement will be provided by the Czech Beneficiary.

10.6. The Agreement may cease to exist upon full discharge of all obligations by the Contractual Parties arising hereunder, and/or by a written agreement of the Contractual Parties in which the Contractual Parties agree upon the terms and conditions of the termination of the Agreement.

10.7. Changes and amendments to the Agreement may be made solely by agreement of the Contractual Parties in the form of written numbered amendments to the Agreement. The Partners are not entitled to transfer rights and duties hereunder to a third party without the prior written agreement of the other Beneficiary.

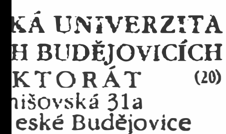
10.8. The Agreement is made in two copies with the validity of the original, with each Contractual Party receiving one copy.

10.9. The Contractual Parties hereby declare that they have read through the whole Agreement, agree with the text, and further represent that this Agreement has been concluded in full compliance with their internal policies and that they are fully aware of the obligations they assume by concluding this Agreement.

Annexes:
Annex 1 – Part C of the project proposal submitted to the Czech Science Foundation
Annex 2 – Research project FAPESP-GACR 2021 submitted to the Brazilian Sao Paulo Research Foundation

On behalf of the Czech Beneficiary:

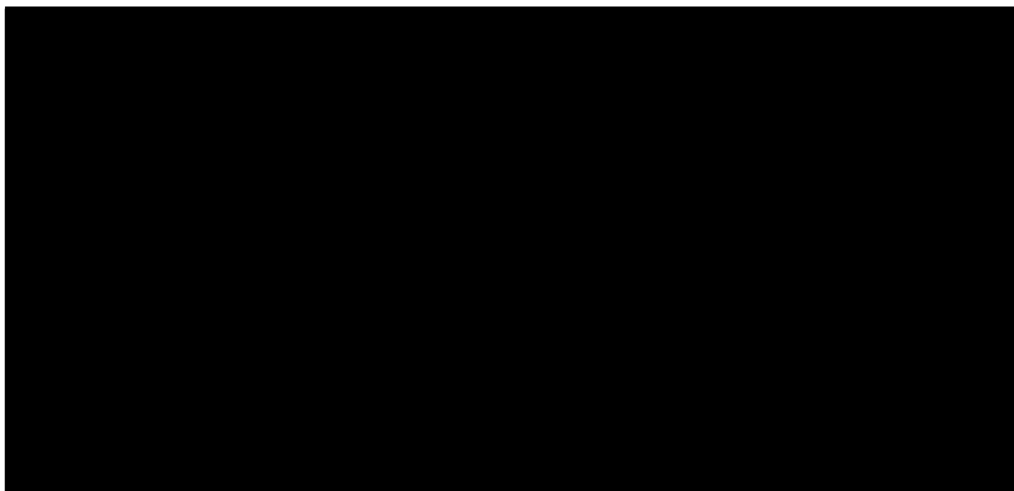
In České Budějovice on 0 2 -05- 2022



Name: Prof. PhDr. Bohumil Jiroušek, Dr.
Position: Rector

On behalf of the Brazilian Beneficiary:

In Ilha Solteira (SP), on 02/18/2022.



Title: Shortening of generation time using surrogate reproduction and *in vitro* sperm maturation in fish

Applicant (Czech Republic): [redacted]
Applicant (Brazil): [redacted]

1. Introduction

Fish is the most diverse group of vertebrates displaying a large number of species with economic and ecological relevance. On the other hand, it is also the group with an increasing number of endangered species. Besides the diversity of other characteristics, fishes also represent the fastest and the slowest maturing group of vertebrates. In this regard, turquoise killifish (Actinopterygii) is considered the fastest maturing known vertebrate taking two weeks until first reproduction (Vrtílek et al 2018), while Greenland shark (*Chondrichthyes*) is the slowest one with first reproduction estimated to 150 years (Nielsen et al. 2016). The maturation time is one of the main bottlenecks seen among the broodstocks of commercially important species and also consists a limitation for restoration of endangered species. For example, Atlantic Bluefin tuna (*Thunnus thynnus*) and beluga sturgeon (*Huso huso)* are both commercially very important fishes, but at the same time also endangered, and mature at 8 and 25 years, respectively.

The animal is considered mature when its germ cells go through all steps of gametogenesis. In fish, six main stages can be distinguished: a) embryonic primordial germ cells (PGCs) differentiating from totipotent blastomeres; b) undifferentiated gonial cells (bipotent germ stem cells) which can self-renew themselves; c) differentiated spermatogonia or oogonia which undergo species specific number of mitotic cycles; d) primary spermatocytes or oocytes entering meiosis (the oocytes start the growing phase at this stage), e) secondary spermatocytes or oocytes ending with maturation; and d) gametes (see review Schulz et al 2010).

The embryonic PGCs or subsequently gonial stem cells can be used for cryopreservation of genetic resources and surrogate reproduction via germ-line chimera in fish. The procedures differ in accordance with host stage and donor cell type. For example: a) transplantation of blastomeres containing PGCs into embryo (Lin et al. 1992), transplantation of single PGCs into embryos, which enabled transplantation even between different Cyprinid family members (Saito et al., 2008, 2010) and, b) transplantation of gonial stem cells isolated from juveniles or adults into larvae (Takeuchi et al. 2003, Okutsu et al., 2006), juveniles or adults (Lacerda et al., 2006). It was shown that after transplantation of rainbow trout testicular cells into masu salmon larvae, the trout germ cells undergo differentiation and produce both sperm and eggs via surrogate salmon parent (Okutsu et al., 2007). Nowadays, gonial stem cell transplantation into the body cavity of larva seems to be the easiest and most efficient method of surrogate reproduction in fish. The advantage is high number of cells, which can be isolated from adult donor and robustness of recipient being already in non-sensitive larval stage. The main benefit of the method is cryobanking of gonial cells from commercially or ecologically important species and surrogate reproduction via species with advantageous traits. One of the most important traits is definitely the maturation time. **However, until now, there is no convincing evidence that the transplanted germ cells follow the developmental speed of the recipient.** Generally, the gametogenesis is controlled by extrinsic (recipient) and intrinsic (donor) regulations. Extrinsic regulation includes endocrine (e.g. gonadotropin hormones) and paracrine regulation (e.g. steroids and growth factors) in so called brain-pituitary-gonadal axis (Schulz et al. 2010). To clarify the importance of extrinsic and intrinsic regulations, rat testicular cells were transplanted into mouse male. The rat germ cells always differentiated and underwent meiosis with cell cycle timing characteristic of the rat, while mouse germ cells transplanted into mouse (control) always retain their physiological speed (França et al. 1998). Thus, it was suggested that extrinsic regulation does not implement a step-wise regulation of germ cell differentiation, but rather is responsible for regulating the balance and transition between the different micro-environments that are required for the germ cells to execute their autonomous developmental program. **We hypothesise that this autonomous regulation relates to germ cell epigenetic marks.**

The shortening of the maturation time is partially possible via extrinsic regulation using common zootechnical approaches such as manipulation of light regime or temperature in poikilothermic fishes

(Wang et al 2010). In this context, Hamasaki et al (2017) showed that it is possible to shorten the maturation time by using transplantation of tiger puffer testicular cells into grass puffer recipients. The male recipients reached maturity at 11 months and females at 2 years, while a previous study revealed the generation time of male and female tiger puffer to be at least 2 and 3 years, respectively (Chuda et al. 1997). However, a comparative study describing gametogenesis of grass puffer control with grass puffer/tigger puffer germ-line chimera was not performed. Thus, the level on which the recipient affects the maturation speed is still unclear.

In past decades, establishments of germ cell culture condition proved that although fish spermatogonia exhibit potential for continuous proliferation, their long-term culturing depends strongly on culture conditions (see review Xie et al 2020). Under specific *in vitro* culture conditions, cultured germ cells can undergo meiosis and spermiogenesis to generate motile spermatozoa. Germ cell differentiation occurred spontaneously in long term culture conditions (Hong et al. 2004; Neumann et al. 2011; Kawasaki et al. 2016) and has been initiated in some fish species such as medaka (Hong et al. 2004) and zebrafish using supplementation with factors and steroid hormones (Sakai 2002; Kurita et al. 2004; Wong and Collodi 2013). **To date, no *in vitro* maturation has been performed on germ cell of late maturing species such as sturgeon.** Recently, interest in 3D organoids as an intermediate system between *in vivo* animal models and more conventional 2D *in vitro* culture models has surged (Sato et al 2019). An organoid is a miniaturized and simplified version of an organ produced *in vitro* in three dimensions that shows realistic micro-anatomy. They are derived from one or a few cells from a tissue, embryonic stem cells or induced pluripotent stem cells, which can self-organize in three-dimensional culture owing to their self-renewal and differentiation capacities. Sakib et al (2019) has developed mammalian testicular organoids and proved that viable spermatozoa could be produced from cultured organoids. Moreover, organoids can serve as an *in vitro* platform to study cell–cell interactions and tissue development. We believe that using this system with preserved cell-cell interactions and testicular niche, it is possible that germ cells from long maturing species (e.g. sturgeon that has a complex acromosome formation) can undergo differentiation and spermiogenesis *in vitro* with less effort.

2. Applicant's research related to the project

A long-term aim of the applicant's laboratory is surrogate reproduction of critically endangered sturgeon. The strategy is to use a small sturgeon species with short generation cycle (e.g. sterlet) as a surrogate parent for a bigger, late maturing and critically endangered sturgeons (e.g. beluga or Atlantic sturgeon). For this purpose, we have studied sturgeon PGC labelling and development (Saito et al 2014, Saito and Pšenička 2015). We found that the PGCs are formed from specific germinal cytoplasm (so called preformation), which is maternally supplied from vegetal pole of embryo. Then we optimized all necessary methods for surrogate reproduction such as isolation, cryopreservation, and transplantation of sturgeon gonial cells (Pšenička et al. 2015, Pšenička et al 2016). We developed several methods for sterilization of the recipients using antisense morpholino oligonucleotide (MO dnd1) or CRIPSR/Cas9 targeting germline specific *dnd1* gene (Linhartová et al 2015, Baloch et al 2019a), using iron oxide nanoparticles delivered to the germinal cytoplasm (Baloch et al, 2019b), and using UV targeting the germinal cytoplasm at the vegetal pole of embryo (Saito et al 2018). In addition, Linhartová et al (2018) studied possibility of production of sterile sturgeon hybrids. More recently, we have also optimized *in vitro* culture of sturgeon germ stem cells (Xie et al. 2019). Interestingly, the *in vitro* amplified cells showed a very good transplant capability and proliferation in the recipients. Our laboratory also works with germ cell of teleostean species. We established *vasa::gfp* transgenic strain expressing enhanced green fluorescent protein (EGFP) exclusively in germ cells under the control of *ddx4* (*vasa*) promotor. The zebrafish strain has been already used in several experiments. Saito et al (2008, 2010) isolated and successfully transplanted single PGCs from the *vasa::gfp* zebrafish into different cyprinid fishes including goldfish. The goldfish then produced zebrafish sperm. Franěk et al (2019) optimized the production of sterile triploid zebrafish for purpose of surrogate reproduction. Then we compared the development of spermatogonia transplanted into zebrafish recipient prepared using different sterilization methods: triploidization, hybridization, and MO *dnd1 (Franěk et al in preparation)*. Franěk et al (2021) produced common carp offspring via goldfish surrogate. At this work, we used MO *dnd1* for sterilization of goldfish recipients, and carp

testicular cells (spermatogonia). Noteworthy, the estimated evolutionary distance between goldfish and common carp is about 34 mya. To the best of our knowledge, this is the furthermost successful interspecific surrogate reproduction using spermatogonia to date. We have also developed a novel method of surrogate reproduction using transplantation of blastomeres including PGCs into larva (Franěk et al in preparation). The method will be used in the proposed project.

From the above mentioned, it is evident that the applicant masters all the techniques required for successful project solving.

3. Project plan

The main aim of this project is to study mechanisms of how the germ cell developmental speed is controlled and prove or disprove the possibility of shortening of maturation time using fish models. We intend to employ interspecific germ cell transplantation methods using earlier and later maturing species (WP1) and *in vitro* germ cell culture system by establishing a 3D testicular organoid (WP2).

3.1. Experimental animal

a) Teleostean: Zebrafish (*Danio rerio*) *vasa::gfp* and *vasa::rfp* strains with labelled germ cells and goldfish (*Carassius auratus*) will be used. Both animals from Cyprinidae family are well-established model species in the applicant's laboratory. While zebrafish mature within 3 months, goldfish maturation time is about 2 years, which is 8-fold more. The project will benefit from their significant difference in maturation time. Moreover, a member of applicant's laboratory has already successfully transplanted goldfish germ cells into zebrafish recipient and obtained functional sperm (Saito et al. 2008). However, the speed of gametogenesis was not the aim of this research.

b) Chondrostean: sterlet (*Acipenser ruthenus*), and beluga (*Huso huso*) belonging to the same family of Acipenseridae, will be used. Sterlet is one of the smallest and fastest maturing sturgeons and is a well-established model in the applicant's laboratory. Sterlet is adult at 4-5 years and has about 1-3 kg. On the other hand, beluga is one of the biggest and latest maturing fishes. It matures at about 20-25 years with a weight of about 80 kg. The slow developmental rate will be used to study differences in early gametogenesis (proliferation phase).

3.2. WP1 - Interspecific germ cell transplantation

We intend to perform interspecific transplantation of germ cells from long maturing species to earlier maturing species and *vice versa*. In the embryos, PGCs undergo through an erasure of their epigenetic marks acquired during the first steps of embryo development. Then, upon gametogenesis, new epigenetic marks are acquired in relation to gamete function (Fig.1).

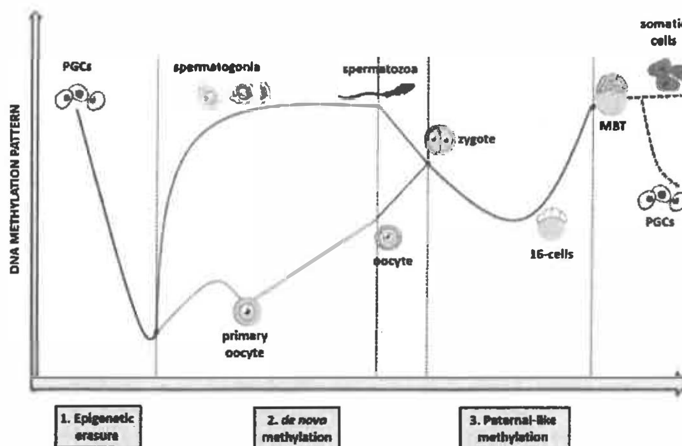


Fig. 1 shows scheme of DNA methylation profiles during development and gametogenesis. Methylation in male germ cells is shown in blue and in female germ cells in orange (Labbe et al 2017).

We hypothesize that PGCs which just underwent epigenetic erasure will develop according to the extrinsic signals from their recipient microenvironment (approach 1). In approach 2, we expect that the transplanted gonial cells with a certain level of epigenetic signature will be developing intermediately between recipient and donor or can display some developmental difficulties leading from transplantation between far related species (approach 2). If the hypothesis will be confirmed, the epigenetic analysis of different germ cells will be topic of the further research.

Approach 1: Transplantation of primordial germ cells (PGCs)

According to the stage of recipient and donor, there are generally two possible methods of PGC transfer: a) blastomeres including future PGCs into an embryo at the blastula stage (Lin et al. 1992) and b) single PGCs from an embryo at the somitogenesis stage into an embryo at the blastula stage (Saito et al 2008) (Fig 2). However, this approach cannot be used in sturgeons due to the holoblastic cleavage pattern of their embryos.

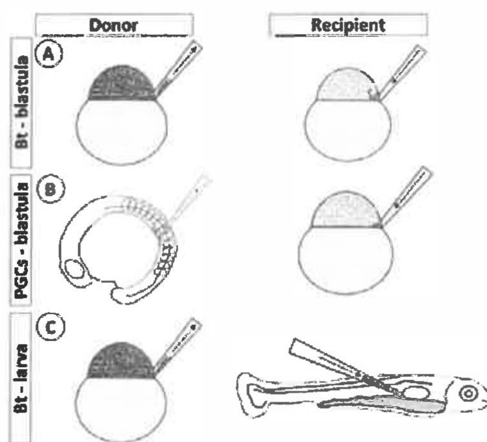


Fig. 2 shows different PGCs transplantation methods. A) blastomeres (Bt) into embryo at blastula stage, B) single PGCs to embryo at blastula stage, and C) blastomeres into larva.

The transplantation of blastomeres or PGCs into an embryo at the blastula stage is relatively difficult methods. It requires a culture of embryos without chorion which results in a low survival. Thus, we developed a new method for a purpose of this project, which will be preferably used. In this method, the earliest PGCs will be transferred into larva, which is already very robust and attains almost no mortality after transplantation (Fig. 2c, Franěk et al., in preparation). The PGCs will be visualized either using *vasa::gfp* or *vasa::rfp* transgenic strain (in the case of zebrafish) or by injecting GFP-*nanos3* 3'UTR mRNA or rhodamine-dextran into embryo at 1-4 cell stage according to Saito et al. (2006).

Approach 2: Transplantation of gonial stem cells

The gonial cells will be isolated from gonads of juvenile or adults. Although the transplanted cells will be undifferentiated, they are supposed to have already a certain level of epigenetic signature, which can partially override cues controlling distinct phase of gametogenesis. The cells will be isolated according to Pšenička et al (2015). Briefly, the gonads will be minced by scissors, enzymatically dissociated, and purified using differential plating and density gradient. The cells will be labelled using cell tracers (e.g. PHK26, CellTrace CFSE, CellTrace Far Red) and injected into the peritoneal cavity of the recipient at larval stage.

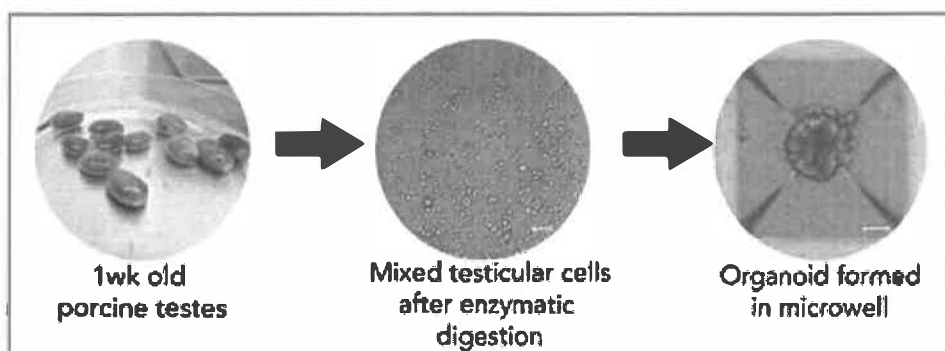
For both approaches, the sterilization of recipients will be ensured by injection of MO *dnd* into embryo at 1-4 cell stage (Linhartová et al 2015). To compare the developmental speed with endogenous germ cells, part of the recipients will remain untreated and their germ cells will be labelled with a different colour. The speed of proliferation and differentiation will be evaluated using histology and BrdU/EdU Assays (Yoshizaki et al 2016) at different stages of gametogenesis. The

obtained gametes and offspring from teleostean models will be tested by species-specific PCR products to confirm the donor's origin.

3.3. WP2 - *in vitro* germ cell culture system

In previous research, we developed an in *vitro* culture system for the propagation of sturgeon germ stem cells (Xie et al 2019). Here, we intend to extend this research and establish an *in vitro* culture system so called organoid. Considering that spermatogenesis is a multifactorial process that requires proper coordination between germ cells and testicular somatic cells, *in vitro* models that maintain the 3D structure, such as organoids, are ideal tools to study factors involved in germ cell development. Moreover, organoids could be used to produce gametes *in vitro*, which would be very useful for species that have a long period of sexual maturation, such as sturgeons. **To the best of our knowledge, there is no study on testicular organoids in fish to date.**

In WP2, we will stimulate spermatogenesis by 1) cultivating functionally distinctive testicular cell lines to support male germ cell differentiation; 2) supplementing with hormones which are related to spermatogenesis, such as 11-KT, Igf3 and androgens; and finally 3) establishing 3D organoids to imitate the *in vivo* environment according to Sakib et al (Fig.3, 2019). To generate the testicular organoids, immature testes of sterlet (n = 10/species) will be digested as previously described methods (Pšenička et al 2015). Testicular organoids will be generated according to methods described by Sakib et al. (2019). For this purpose, AggreWell 400 plates (STEMCELL Technologies Inc, Vancouver, Canada, cat # 34450) will be prepared according to the manufacturer's instructions and washed with phosphate buffer. Then, 0.5 mL of organoid-forming medium will be added to each microwell. This medium consists of Dulbecco Modified Eagle Medium F12 supplemented with insulin 10 ug/mL, transferrin 5.5 ug/mL, selenium 6.7 ng/mL, 20 ng/mL epidermal growth factor and 1 % penicillin-streptomycin. The plate will be centrifuged at 2,000 xg for 2 minutes to remove bubbles, and then 500 or 1000 testicular cells, resuspended in 0.5 mL of medium, will be added to each well. Then, the plate will be centrifuged at 500 g for 5 minutes. The culture medium will be supplemented with 1:100 of Matrigel. The formed organoids will be kept in microwells (1 mL medium/well) for 4 to 30 days at 27°C for zebrafish and 22 °C for sturgeon, 5% CO_2, with the medium being changed every two days. In WP2, we also plan to stimulate spermatogenesis by supplementing with hormones which are related to spermatogenesis such as gonadotropins, 11-KT, Igf3 (insulin-like growth factor 3) under the same conditions described above. After 4, 10, 20 and 30 days of cultivation, the organoids will be collected for immunofluorescence (e.g. germ cell specific anti-DDX4 antibody) and histological analyses to identify the cell types present in the organoids under stimulation. Additionally, organoids will be collected for gene expression to confirm the expected effects. In advance, we plan to meet the Brazilian applicant in Rennes (France) this year (2021) and start developing of organoids in collaboration with Dr Lareyre – expert in fish spermatogenesis.



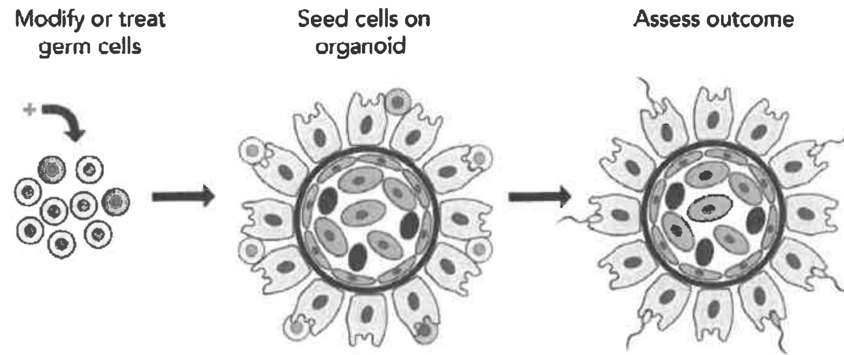


Figure 3. Formation of testicular organoids from prepubertal swine testicular cells using microwell and a centrifugal aggregation system. Organoids are spherical structures in which the inside is composed of Leydig cells and endothelial cells inside (interstitial compartment), while the outer surface is formed by a seminiferous epithelium composed of Sertoli cells and germ cells. Taken from Sakib et al (2019).

4. Gantt Diagram

Year 1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Transplantation of gonial cells from beluga to sterlet and *vice versa*. Observation of the germ cell proliferation	■	■	■	■	■	■	■	■	■			
Transplantation of PGCs and gonial cells from zebrafish to goldfish and *vice versa*. Observation of the gonadal development.	■	■	■	■	■	■	■	■	■	■	■	■
Trip to Brazil – transplantation of germ cells in Brazilian model *Astyanax altiparanae and Colossoma macropomum*						■	■	■				
Establishment of preliminary 2D *in vitro* culture of zebrafish and sturgeon spermatogonia							■	■	■	■	■	■
Writing a paper on novel PGCs transplantation method into larva (preliminary data)							■	■	■	■	■	■
Year 2	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Repetition and completion of transplantation experiments	■	■	■	■	■	■	■	■	■	■	■	■
Establishment of organoids of zebrafish and sturgeon spermatogonia according to preliminary results from Brazilian partner	■	■	■	■	■	■						
Writing an extensive manuscript on influence of surrogate reproduction on speed of gametogenesis							■	■	■	■	■	■
Year 3	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Repetition or completion of *in vitro* culture experiments	■	■	■	■	■	■						
Writing a manuscript on generation of zebrafish and sturgeon sperm *in vitro*							■	■	■	■	■	■

We expect to publish our results in at least three manuscripts generally in higher impact journals and to present our results at international conferences. A part of the results produced within the project will be involved in a PhD thesis.

5. Social relevance

Fish is the most consumed and commercialized source animal protein in the world (FAO, 2021). Global marine catch has stagnated since the early 1990s and more pressure is created on aquaculture production. However, many aquaculture species have a long period of sexual maturation, making cultivation of these species costly. The germ stem cells, as the precursors of gametes, have exceptional importance for reproduction. From a basic science point of view, they offer a unique developmental pattern to study specification, migration and differentiation; from the applied science perspective, the germ stem cells present the possibility of using new biotechnological approaches such as reproduction via surrogate parents in order to improve breeding strategies. Considering that cryopreservation of fish oocytes or embryos has not been mastered yet, they are practically the only cells having the potential to be cryopreserved and subsequently be useable to restore both paternal and maternal genetic information.

6. Justification for international collaboration

Both, the main applicant from Czech Republic and the main applicant from Brazil started their research on fish germ cells more than 15 years ago. Last year, during preparation of a collective review on development of germ stem cells (Xie et al 2020), we identified a significant gap in the germ cell research, which is regulation of developmental speed of germ cells. Both groups have a long-term effort to shorten the generation cycle of their economically and ecologically important species via surrogate reproduction. However, to date, researchers in the field have brought only contradictory results about the possibility. The Czech applicant has been devoted to study germ cells on the cellular level. The laboratory has developed methods for isolation and transplantation of germ cells in several fish species including sturgeons. On the other hand, the Brazilian applicant has much experience with gonadal maturation, morphological characterization of germ cells and endocrine/paracrine regulation of gametogenesis. Thus, the joint proposal focused on accelerating germ cell development will obviously benefit from the synergy of these two partners. The Brazilian partner will support the Czech partner in *in vitro* experiments (WP2), specifically generation of the testicular organoids. They will perform transcriptome and steroid analyses of gonads of their model species *Colossoma macropomum* in order to discover candidate players involved in accelerated gonadal maturation. Then they will establish gonadal organoids to drive *in vitro* gametogenesis using candidate factors previously obtained from the analyses. On the other hand, the Czech partner will lead the transplantation experiments (WP1) and support Brazilian experiments regarding germ cell transplantation in their models *Astyanax altiparanae and Colossoma macropomum.* In addition, Brazilian researchers intend to enrich the WP1 with a different transplantation technique so called xenografting. In this experiment, pieces of testes from immature male will be grafted under skin of an adult recipient. Brazilian research will also take advantage of the applicant's expertise on the sturgeon model. As long maturing fish, sturgeon data will help to understand which factors could be silent during gonadal maturation. At the end, the collaborative research will expand our knowledge on the mechanisms that govern germ cell development in species relevant to Czech Republic and Brazil. Additionally, this research will contribute by developing biotechnological applications to improve aquaculture of both countries, and also important for the restoration of endangered species.

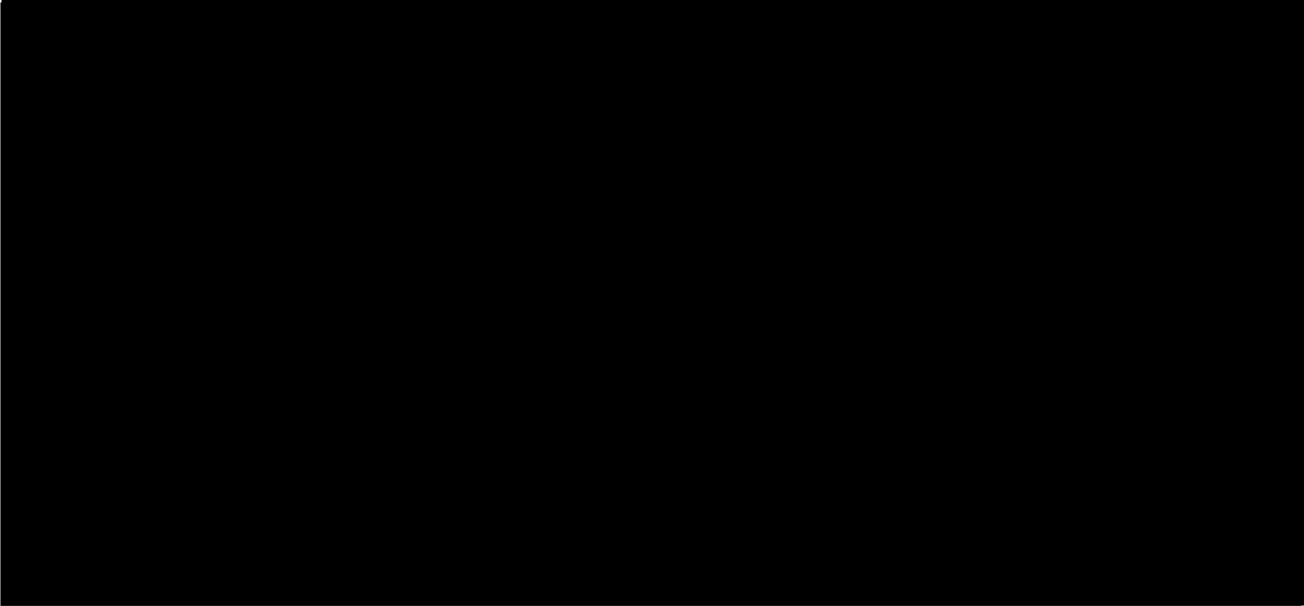
7. Other international collaboration

The applicant, ███, spent one year as postdoc at the Fisheries Faculty of Science of Hokkaido University in Japan (███). The laboratory focuses mainly on embryogenesis, genome manipulation and germ cell transfer. At present, our group has a close scientific relationship also with Laboratory of Fish Physiology and Genomics, INRA, France ███) and Institute of Marine Research, Bergen, Norway ███ ███). These laboratories aim to speed up maturation in salmonids (rainbow trout and Atlantic salmon, respectively) and they support objectives of the proposed project.

The applicant has been involved in international COST Action "AQUAGAMETE" (a networking project) and Innovative Training Network of the Marie Sklodowska-Curie Actions "IMPRESS", both

funded by the EU Research and Innovation Programme Horizon 2020. Besides, the applicant organized 2nd Workshop on Diversification in Inland Finfish Aquaculture in 2013 (118 participants) and 6th International Workshop on the Biology of Fish Gametes in 2017 (100 participants), which is an evidence of applicant's international collaboration activities.

8. Team structure



9. Readiness of the teams

Czech applicant: The Laboratory of Germ Cells is part of Research Institute of Fish Culture and Hydrobiology in Vodnany, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice. The research on fish germ cell is main activity of the applicant's laboratory, since it was established in 2015. The supervisor's and junior researcher's expertise deals with fish embryo development, gene expression inhibition, genome editing CRISPR/Cas9 system, micromanipulation and grafting, and they will train a new PhD student joining this project. The faculty keeps many sturgeon species, such as sterlet including albinotic form, beluga, American paddlefish, Russian, Siberian, Atlantic, shortnose, white and stellate sturgeon. Regarding model fish species, the applicant owns several wild type and transgenic zebrafish strains, which are kept in zebrafish housing system ZebTec. The workplace is equipped with histological laboratory, fluorescent stereomicroscopes, 7x sets of micromanipulators and microinjectors, motorized manipulator, inverted motorized fluorescent microscope, spectrophotometers, qPCR cycler, a number of thermocyclers, flow cytometer and cell sorter, various systems for electrophoresis including microchip electrophoresis system, numerous common microscopes and stereomicroscopes, centrifuges, etc.

Noteworthy, the South Bohemia university (the applicant's workplace) has received the HR Excellence in Research Award. The applicant supports gender equality and implements it at all levels of career path and in recruitment process and follows Code of Ethics complied with the European Charter for Researchers and is committed to create favourable working environment.

Brazilian applicant: The Brazilian applicant coordinates the Neotropical Ichthyology Laboratory (LINEO), and develop research in fish reproduction, focused on production, conservation and impacts of climate change, with emphasis on gamete morphology, reproductive cycle, germ cell transplantation and gamete cryopreservation. The Brazilian team is composed by 5 research members and several students among them undergrad, masters and PhD students. The research team has expertise on different aspects of fish reproductive physiology and biotechnology using zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and Neotropical species such as lambari (*Astyanax altiparanae)*, tambaqui

(*Colossoma macropomum*) among others. Several methodologies have been employed by the group, e.g. tissue culture techniques (testicular explants), histology, immunofluorescence, cell proliferation assays (BrdU), gene expression (RT-qPCR), *in situ* hybridization, *in silico* analysis, transcriptome and others. The workplace is equipped with StepOne Plus, hybridization oven, biofreezer, thermocyclers, electrophoresis, thermoblocks, microtome, microscopes, cell culture room, horizontal and vertical laminar flow hoods, and others. These equipments were acquired through funds obtained by Brazilian funding agencies (São Paulo Research Foundation/FAPESP, National Council for Scientific and Technological Development/CNPq and Brazilian Coordination for Improvement of Higher Education Personnel/CAPES). In addition, the group has intense collaboration with researchers from different countries (Germany, Argentina, Canada, Spain, Holland, Japan, Portugal and the Czech Republic).

10. References

Baloch, A.R., Franěk, R., Tichopád, T., Fučíková, M., Rodina, M., Pšenička, M., 2019a. Dnd1 knockout in sturgeons by CRISPR/Cas9 generates germ cell free host for surrogate production. Animals 9: 174.

Baloch, A.R., Fučíková, M., Rodina, M., Metscher, B., Tichopád, T., Shah, A.M., Franěk, R., Pšenička, M., 2019b. Delivery of Iron Oxide Nanoparticles into Primordial Germ Cells in Sturgeon. Biomolecules 9: 333.

Chuda H, Matsuyama M, Ikeda Y, Matsuura S. 1997. Development of the maturation- and ovulation-induction method in cultured tiger puffer *Takifugu rubripes* by hormone treatments. Nippon Suisan Gakkaishi 63:728–733.

FAO, 2021. FAO Fisheries & Aquaculture - National Aquaculture Sector Overview - Brazil [WWW Document]. URL http://www.fao.org/fishery/countrysector/naso_brazil/en (accessed 4.8.21).

França, L.R., Ogawa, T., Avarbock, M.R., Brinster, R.L., Russell, L.D., 1998. Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol. Reprod. 59, 1371–1377.

Franěk, R., Kašpar, V., Shah, MA., Gela, D., Pšenička, M., 2021. Production of common carp donor-derived offspring from goldfish surrogates. Aquaculture 534, 736252.

Franěk, R., Tichopád, T., Fučíková, M., Steinbach, C., Pšenička, M. 2019 Production and use of triploid zebrafish for surrogate reproduction. Theriogenology 140: 33-43.

Hamasaki, M. Takeuchi, Y. Yazawa, R. Yoshikawa, S. Kadomura, K. Yamada, T. Miyaki, K. Kikuchi, K. Yoshizaki G. 2017. Production of tiger puffer *Takifugu rubripes* offspring from triploid grass puffer *Takifugu niphobles* parents. Mar Biotechnol, 19: 579-591.

Hong Y., Liu, T., Zhao H., Xu H., Wang W., Liu R., Chen T., Deng J., and Gui J. 2004. Establishment of a Normal Medaka fish Spermatogonial Cell Line Capable of Sperm Production *in vitro*. Proc Natl Acad Sci 101 (21): 8011–16.

Kawasaki T., Siegfried K.R., Sakai N. 2016. Differentiation of Zebrafish Spermatogonial Stem Cells to Functional Sperm in Culture Dev 143 (4): 566–74.

Kurita K., Burgess S.M., Sakai N. 2004. Transgenic Zebrafish Produced by Retroviral Infection of in Vitro-Cultured Sperm. Proc Natl Acad Sci USA 101 (5): 1263–67.

Labbé C., Robles V., Herraez M.P. 2017. Epigenetics in fish gametes and early embryo. Aquaculture, 472, 93-106.

Lacerda S.M.S.N., Batlouni S.R., Silva S.B.G., Homem C.S.P., França L.R., 2006. Germ cells transplantation in fish: the Nile-tilapia model. Anim. Reprod. 3, 2,146-59

Lin, S., Long, W., Chen, J., Hopkins, N., 1992. Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. Proceedings of the National Academy of Sciences U. S. A. 89: 4519-4523.

Linhartová, Z., Saito, T., Kašpar, V., Rodina, M., Prášková, E., Hagihara, S., Pšenička, M., 2015. Sterilization of sterlet *Acipenser ruthenus* by using knockdown agent, antisense morpholino oligonucleotide, against dead end gene. Theriogenology 84 (7): 1246-1255.

Linhartová, Z., Havelka, M., Pšenička, M., Flajšhans, M. 2018. Interspecific hybridization of sturgeon species affects differently their gonadal development. Czech J. Anim. Sci., 63, 1.

Neumann J.C., Chandler G.L., Damoulis V.A., Fustino N.J., Lillard K., Looijenga L., Margraf L., Rakheja D., Amatruda J.F. 2011. Mutation in the Type IB Bone Morphogenetic Protein Receptor

Alk6b Impairs Germ-Cell Differentiation and Causes Germ-Cell Tumors in Zebrafish. Proc Natl Acad Sci 108 (32): 13153–58.

Nielsen J., Hedeholm R.B., Heinemeier J., Bushnell P.G., Christiansen J.S., Olsen J., Ramsey C.B., Brill R.W., Simon M., Steffensen K.F., Steffensen J.F. 2016. Eye lens radiocarbon reveals centuries of longevity in the Greenland shark (*Somniosus microcephalus*) 353, 6300.

Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y., Yoshizaki, G., 2007. Production of trout offspring from triploid salmon parents. Science 317, 1517.

Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G., 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc. Natl. Acad. Sci. U. S. A. 103, 2725–9.

Pšenička, M., Saito, T., Rodina, M., Dzyuba, B., 2016. Cryopreservation of early stages of germ cells in sturgeon, comparison between whole tissue and dissociated cells freezing. Cryobiology, 72(2), 119–122.

Pšenička, M., Saito, S., Linhartová Z., Gazo, I., 2015. Isolation and transplantation of sturgeon early-stage germ cells. Theriogenology 83: 1085-1092.

Saito, T., Fujimoto, T., Maegawa, S., Inoue, K., Tanaka, M., Arai, K., Yamaha, E., 2006. Visualization of primordial germ cells in vivo using GFP-nos1 3'UTR mRNA. Int. J. Dev. Biol. 50, 691–9.

Saito, T., Goto-Kazeto, R., Arai, K., Yamaha, E., 2008. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol. Reprod. 78, 159–66.

Saito, T., Goto-Kazeto, R., Fujimoto, T., Kawakami, Y., Arai, K., Yamaha, E., 2010. Inter-species transplantation and migration of primordial germ cells in cyprinid fish. Int. J. Dev. Biol. 54, 1481–86.

Saito, T., Pšenička, M., Goto, R., Adachi, S., Inoue, K., Arai, K., Yamaha, E., 2014. The origin and migration of primordial germ cells in sturgeons. PLoS One 9, e86861.

Saito, T., Pšenička, M., 2015. Novel technique for visualizing primordial germ cells in sturgeons (*Acipenser ruthenus*, *A. gueldenstaedtii*, *A. baerii*, and *Huso huso*). Biology of Reproduction 93: article no. 96.

Saito, T., Güralp, H., Iegorova, V., Rodina, M., Pšenička, M. 2018. Elimination of primordial germ cells in sturgeon embryos by ultraviolet irradiation. Biology of Reproduction 99(3), 556–564.

Sakai, N. 2002. Transmeiotic differentiation of zebrafish germ cells into functional sperm in culture. Development 129 (14): 3359–65.

Sakib S., Uchida A., Valenzuela-Leon P., Yu Y., Valli-Pulaski H., Orwig K., Ungrin M., Dobrinski I. 2019. Formation of organotypic testicular organoids in microwell culture. Reprod Biol 1; 100 (6): 1648-1660.

Sato T., Vries R.G., Snippert H.J., van de Wetering M., Barker N., Stange D.E., van Es J.H., Abo A., Kujala P., Peters P.J., Clevers H. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature, 459 (7244): 262 –265.

Schulz R.W., França L.R., Lareyre J. LeGac F., Chiarini-Garcia H., Nobrega R.H, Miura, T. 2010 Spermatogenesis in fish. General and Comparative Endocrinology 165: 390–411.

Takeuchi, Y., Yoshizaki, G., Takeuchi, T., 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol. Reprod. 69, 1142–9.

Vrtílek, M., Žák, J., Pšenička M., Reichard, M. 2018. Extremely rapid maturation of a wild African annual fish. Current Biology 18(15): R822-R824.

Wang N., Teletchea F., Kestemont P. Fontaine P. 2010 Photothermal control of the reproductive cycle in temperate fishes. Reviews in Aquaculture 2(4):209 – 222.

Wong T.T., Collodi P. 2013. Dorsomorphin promotes survival and germline competence of zebrafish spermatogonial stem cells in culture. PLoS One 8 (8).

Xie, X., Nóbrega, R., Pšenička, M. Spermatogonial stem cells in fish: Characterization, isolation, enrichment, and recent advances of in vitro culture systems. Biomolecules 2020, 10, 644.

Xie, X., Li, P., Pšenička, M., Ye, H., Steinbach, Ch., Li, Ch., Wei, Q. 2019. Optimization of *in vitro* culture conditions of sturgeon germ cells for purpose of surrogate production. Animals 9, 106.

RESEARCH PROJECT - FAPESP-GACR 2021

Morphophysiological elements involved in early and long gonadal maturation in fish and biotechnological applications

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UNESP - Ilha Solteira

2021

Abstract

The first gonadal maturation in teleosts is under multifactorial control of neurohormones, pituitary gonadotropins, as well as a number of gonadal hormones including sex steroids and growth factors. The speed of this process varies among vertebrates and is controlled by extrinsic and intrinsic regulations. There are species such as *Nothobranchius furzeri* (Actinopterygii), which is considered the fastest maturing known vertebrate taking two weeks until first reproduction, whereas other species as *Salmo salar* (Actinopterygii) take 3 years until first reproduction. In general, cultivation of long-maturing species has a high cost in aquaculture until the first reproduction takes place. Therefore, development of strategies to shorten generation time has become necessary for a more efficient production. Thus, this project aims to evaluate extrinsic and intrinsic factors that could accelerate the first gonadal maturation of *Astyanax altiparanae*, a well established Neotropical model. Specimens of *A. altiparanae* will be reared at different temperatures, and gonadal histology, pituitary gonadotropin expression and plasma steroid levels will be assessed. Based on the treatment that induced rapid maturation, transcriptome analyses (gonad and pituitary) will be carried out to identify candidate genes involved in the acceleration of the first gonadal maturation. Subsequently, we propose the development of biotechnological strategies to accelerate gonadal maturation of economically and ecologically important species such as tambaqui, *Colossoma macropomum* (Brazil) and sturgeons as *Acipenser ruthenus* (Czech Republic). These strategies consist of developing a 3D testicular organoid to drive *in vitro* sperm maturation and xenografting pieces of immature testes from long maturing species into *A. altiparanae* reared at different temperatures. Altogether this project will contribute to basic knowledge on fish gonadal maturation and developing strategies to accelerate gonadal maturation in species with economical and ecological relevance in Brazil and Czech Republic.

1. Introduction

Fish is the most consumed and commercialized animal protein in the world (FAO, 2021). In Brazil, the cultivation of native species represents 35% of the national production, and the most cultivated species are considered of long gonadal maturation, such as the round fishes, pacu (*Piaractus mesopotamicus*), tambaqui (*Colossoma macropomum*), their hybrid tambacu and the native catfish cachara (*Pseudoplatystoma fasciatum*) and 'pintado' (*Pseudoplatystoma coruscans*) (IBGE, 2021). In the world, many species of commercial

value have a long period of sexual maturation, making cultivation of these species costly until reproduction takes place. The identification/understanding of the factors that differentiate short and late-maturing species become strategic for a more efficient production. However, the understanding of the determining factors for gonadal maturation is still incipient.

It is known that gonadal maturation is governed by a complex scheme involving the brain-pituitary-gonadal axis, which is susceptible to external stimuli (such as light, temperature and rainfall) (Tsutsui and Ubuka, 2020; Zahangir et al., 2021). Nevertheless, there are important gaps regarding the understanding of the dynamic physiological processes associated with gonadal maturation. Knowledge of neuroendocrine and molecular signaling mechanisms is extremely important for basic knowledge about fish reproduction, providing subsidies for the future elaboration of fish reproductive protocols.

In this regard, species with early gonadal maturation become excellent models to understand the determining factors that trigger gonadal maturation. In the last few years, *Astyanax altiparanae* has been established as a neotropical model species (Chehade et al., 2020; Godoi et al., 2020; Pinheiro et al., 2021). This species presents early maturation at 4 months after hatching, being able to reproduce practically throughout the year. Moreover, studies indicate that some species may have their first accelerated maturation and spermatogenesis according to the breeding temperature (Quirino et al., 2020; Rahman et al., 2019; Shahjahan et al., 2017). These factors make the species ideal for the study of gonadal maturation, as it allows comparing neuroendocrine and molecular signaling within the species itself, and to identify differences in the expression of the genes involved in the acceleration of gonadal maturation.

Organoids

In the last decades, the advances in 3D cell culture technologies have enabled tissue-like structures to be created *in vitro* (Clevers, 2016). In this regard, organoids, *in vitro* miniature organ structures, have been developed from primary tissues, embryonic stem cells, induced pluripotent stem cells (iPSCs), adult stem cells or tumor cells (Clevers, 2016; Xu et al., 2018; Sakib et al., 2019a). In this 3D culture system, the stem cells can differentiate into various cell types, and recapitulate the cell architecture and functionality of their original tissue as *in vivo* (Clevers, 2016; Xu et al., 2018; Sakib et al., 2019a). This advent has opened up enormous possibilities in fundamental science as well as in more applied areas. For example, organoids can be used to study normal tissue and organ morphogenesis, development of infectious diseases and tumors, to assess the toxicity of drugs in high

screening performance, and replace non-functional tissue as therapeutic strategy in degenerative diseases (Clevers, 2016; Xu et al., 2018). In reproductive science, there are few studies showing the development of gonadal organoids, in particular the testicular organoids (Baert et al., 2017; Alves-Lopes et al., 2017; Pendergraft et al., 2017; Strange et al., 2018). In this regard, the research group of Dr. Ina Dobrinski (University of Calgary, Canada) was able to develop a method using microwell and a centrifugal aggregation system to obtain swine testicular organoids (**Figure 1**) (Sakib et al., 2019b). Through this method, the developed organoids consisted of spheres composed of Leydig cells and endothelial cells in their inner side, while the outer surface was formed by a seminiferous epithelium composed of Sertoli cells and germ cells (Sakib et al., 2019b). A basement membrane separates the outer surface (seminiferous epithelium) from the inner sphere.

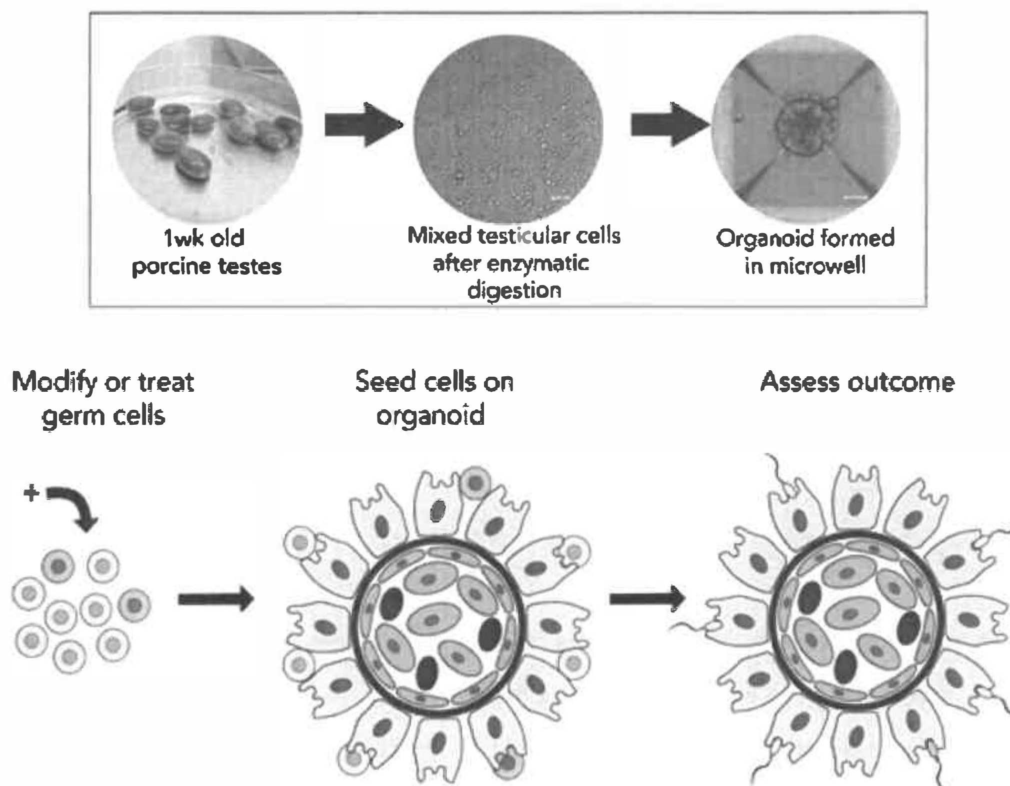

Figure 1. Formation of testicular organoids from prepubertal swine testicular cells using microwell and a centrifugal aggregation system. Organoids are spherical structures in which the inside is composed of Leydig cells and endothelial cells inside (interstitial compartment), while the outer surface is formed by a seminiferous epithelium composed of Sertoli cells and germ cells. Taken from Sakib et al. (2019b).

This model has been the only one so far to recapitulate testicular morphogenesis and testis structure *in vivo*, being widely applicable to several species, including rodents, primates and men (see review in Sakib et al., 2019b). Considering that spermatogenesis is a multifactorial process that requires proper coordination between germ cells and testicular somatic cells, *in vitro* models that maintain the 3D structure, such as organoids, are perfect tools to study germ cell development and drive gamete formation *in vitro*. To date, there are no testicular organoids developed for fish. Once established, the organoids would be useful to understand the factors involved in germ cell maturation and expand this knowledge to improve *in vivo* reproduction techniques. Moreover, organoids could be used to produce gametes *in vitro*, which would be very useful for species that have a long period of sexual maturation, such as pirarucu (*Arapaima gigas*), tambaqui (*C. macropomum*) or economically important species of Czech Republic, such as sturgeon, which can present maturation period of 10 to 25 years.

Xenografts

Germ cell transplantation and testicular graft can be used for restoration of endangered species or effective breeding in fish. In fish, germ cell transplantation was first demonstrated in salmonids, in which primordial germ cells (PGC) from rainbow trout (*Oncorhynchus mykiss*) were microinjected into coelomic cavity of masu salmon larvae (*Oncorhynchus masou*) (Takeuchi; Yoshizaki; Takeuchi, 2004). Subsequently, functional oocytes and offspring of rainbow trout were produced by triple-colored masu salmon (Okutsu et al., 2007). From then on, surrogate reproduction using germ cell transplantation technique has been used in other fish species such as kingfish (*Odontesthes bonariensis*) (Majhi et al., 2009, 2017), Nile tilapia (*Oreochromis niloticus*) (Farlora et al., 2014; Silva et al., 2016), yellow tail tuna (*Seriola quinqueradiata*) (Morita et al., 2012, 2015), medaka (*Oryzias latipes*) (Seki et al., 2017), fugu (*Takifugu rubipres*) (Hamasaki et al., 2017). In particular, our research group has developed a germ cell transplantation technique using a Neotropical species piracanjuba (*Brycon orbignyanus*) as donor, and *A. altiparanae* as recipient (Siqueira-Silva et al., 2019). Another alternative for surrogate gamete production is the testis xenograft. This transplantation method consists of grafting gonadal tissue into another individual, generally, under the skin (Bhatta et al., 2012). Testicular grafts in isogenic rainbow trout were capable of maturing and producing viable sperm (Nagler et al., 2001). Interesting to note that grafted ovaries are able to detach from their original position and restore vascularity and maintain their normal development (Cloud, 2003).

2. Project plan

The main aim of this study is to unravel the endocrine and paracrine components of brain-pituitary-gonadal axis involved in the accelerated gonadal maturation of *A. altiparanae* reared under different temperatures (Workpackage [WP] 1). The understanding of these factors will be used as a basis to drive spermatogenesis *in vitro*, from the establishment of testicular organoids using zebrafish (*Danio rerio*) and *A. altiparanae* as models (WP 2). The knowledge obtained in WP 1 and 2 will be used to analyze *in vivo* the shortening of the long-maturing species cycle using testis xenograft (WP 3). WP3 will be carried out using the expertise of Czech partner.

2.1 WP1: To evaluate the effects of extrinsic factors (different temperatures) in the first gonadal maturation of *Astyanax altiparanae*, a Neotropical species with a short maturation cycle. Transcriptome analyses (gonad and pituitary) will attempt to identify candidates involved in the acceleration of the first gonadal maturation. This information will be essential for WP2, WP3 and also for the Czech partner in his experimental tasks.

Task 1: Animal breeding

Task 1 will be carried out at the Laboratory of Neotropical Ichthyology (LINEO) at UNESP, campus of Ilha Solteira-SP, Brazil. Specimens of *A. altiparanae* at 4 days post-hatching (dph) (undifferentiated larva) will be randomly distributed among 3 replicate tanks for each evaluated temperature (20°C, 26°C and 32°C). In this species, the first sign of morphological ovarian differentiation was observed at 58 dph, whereas the first sign of testis formation was found at 73 dph (Adolfi et al., 2015). The animals will be raised in 180 L polyethylene boxes in a closed system, using a biological filter, a UV filter and a chiller to maintain temperatures. The animals will be fed *ad libitum* using a commercial food (Nutripiscis®, 40%). The parameters of temperature, concentration of dissolved oxygen (DO) and electrical conductivity (HANNA HI98194 DiST®6) will be measured daily throughout the experimental period. Animals will be sampled for histology and plasma steroid analysis (see below) every 5 days from 50 dph until 90 dph, and then monthly for one year.

Task 2: Gonadal histology and pituitary gonadotropin gene expression analysis

Gonadal histology analysis and pituitary gene expression analysis of luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) will be evaluated at different time

intervals as mentioned above. Gonads will be dissected out and fragmented into several pieces. Some fragments will be snap frozen (RNAseq - see Task3), while others will be fixed in modified Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]) for at least 24 h at room temperature, and subsequently dehydrated, embedded in resin (Leica HistoResin) and sectioned at 3 μm thickness for staining with hematoxylin and eosin. Additional pieces will be fixed in 4% paraformaldehyde solution in phosphate buffered saline (PBS) for at least 24 h at room temperature, dehydrated, embedded in Paraplast High Melt (Leica, Wetzlar, HE, Germany) and sectioned with 5 μm thickness for staining with hematoxylin and eosin. Gonadal maturation will be assessed and classified into reproductive stages according to Brown-Peterson et al (2011).

Pituitaries will be removed and immediately stored in liquid nitrogen for RNA isolation using TRI Reagent (TR118, Euromedex). Frozen tissues will be lysed with Precellys Evolution Homogenizer (Ozyme, bertin technologies) and the “nucleospin RNA” kit (740955, Macherey Nagel) will be used to access the total RNA extracted. 1 μg of total RNA will be transcribed into cDNA for expression analysis of the specific *lh* and *fsh* genes of *A. altiparanae* (Jesus et al., *2017;* Jesus et al., *unpublished data*).

Task 3: Steroid analysis

Based on the gonadal histological analysis and pituitary gonadotropin expression (Task 1), plasma steroid levels will be measured by an enzyme linked immunosorbent assay (ELISA), using commercial kits (Cayman Chemical®) according to manufacturer’s protocol. All analyses will be performed according to the method validated for each species and absorbances will be measured using a microplate reader (Spectra Max 250 Molecular Devices) in the Laboratory of Metabolism and Reproduction of Aquatic Organisms at the Institute of Biosciences at University of São Paulo.

Task 4: RNA-seq

Based on the gonadal histological analysis and pituitary gonadotropin expression (Task 1) RNA-seq will be carried out in testes and pituitaries to identify candidate genes involved in the acceleration of *A. altiparanae* first gonadal maturation. Total RNA of testes and pituitaries from Task 1 will be extracted as conventional methods. Total RNA will be extracted and samples with RNA integrity number > 8 will be used for library preparation. The resulting RNAseq libraries will be sequenced on an Illumina HiSeq2500 sequencer (Illumina, Inc.).

The sequencing service will be done by Genohub (https://genohub.com/). This dataset would identify potential genes and pathways involved in accelerated gonadal maturation.

2.2 WP 2: Development of 3D testicular organoid as an *in vitro* approach for shortening the gonadal maturation. To establish this novel culture method, species such as zebrafish and *A. altiparanae* will be used. Once standardized, we will establish testicular organoids to drive sperm maturation *in vitro* for species of zootechnical interest, such as tambaqui (*C. macropomum*) (Brazil) and sterlet (*A. ruthenus*) (Czech Republic).

Task 1: Animal breeding

Sexually mature males of zebrafish and *A. altiparanae* will be used in this study. The WP2 will be carried out at the Department of Structural and Functional Biology of the Institute of Biosciences - UNESP/Botucatu. For native species, immature tambaqui (*C. macropomum*) (3 months) will be collected from fish farms in the state of São Paulo. The experimentation with sterlet (*A. ruthenus*) will be performed in Dr. Martin Psenicka's laboratory at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice. All procedures adopted will be in accordance with the rules of the Ethics Committee on Animal Experimentation at São Paulo State University and the experiments will be also registered according to SISGEN.

Task 2: 3D testicular organoids

Testes (n = 10/species) will be digested with 0.2% collagenase and 0.12% dispase as previously described (Nóbrega et al., 2010). After digestion, the obtained cell suspension will be counted by conventional methods before generating the organoids. In the work of pigs, about 1.2 x 10^6 testicular cells were able to generate 1000 organoids (Sakib et al., 2019a). Testicular organoids will be generated according to methods described by Sakib et al. (2019a). For this purpose, AggreWell 400 plates (STEMCELL Technologies Inc, Vancouver, Canada, cat # 34450) will be prepared according to the manufacturer's instructions and washed with phosphate buffer. Then, 0.5 mL of organoid-forming medium will be added to each microwell. This medium consists of Dulbecco Modified Eagle Medium F12 supplemented with insulin 10 ug/mL, transferrin 5.5 ug/mL, selenium 6.7 ng/mL, 20 ng/mL epidermal growth factor and 1% penicillin-streptomycin. The plate will be centrifuged at 2,000 xg for 2 minutes to remove bubbles, and then 500 or 1000 testicular cells, resuspended in 0.5 mL of medium, will be added to each well. Then, the plate will be centrifuged at 500

xg for 5 minutes. The culture medium will be supplemented with 1:100 of Matrigel. The formed organoids will be kept in microwells (1 mL medium/well) for 4 to 10 days at 27°C, 5% CO_2, with the medium being changed every two days.

Task 3: Characterization of testicular organoids

Testicular organoids of all species will be collected after 4, 7 and 10 days of culture and analyzed using a confocal microscope from the Electron Microscopy Center of the Institute of Biosciences, UNESP/Botucatu. For this purpose, the organoids will be fixed in 4% buffered paraformaldehyde and placed on histological slides by cytospin centrifugation. The slides will be washed with phosphate buffer, blocked with CAS-Block and incubated overnight with the following antibodies specific for zebrafish: anti-Pou5f3, anti-Nanog, anti-Nanos, anti-Gfrα1a to identify undifferentiated spermatogonia; anti-Fsh receptor to identify Sertoli cells and Leydig cells; anti-α-SMA (alpha-Smooth Muscle Actin) to identify myoid peritubular cells; anti-BrdU (Bromodeoxyuridine) for cell proliferation. Anti-Pou5f3, anti-Nanog, anti-Nanos antibodies, anti-Gfrα1a and anti-Fsh receptor were produced by RHEABIOTECH (Campinas) and were previously tested in zebrafish (JP-FAPESP - grant number 14/07620-7). The anti-α-SMA antibody (Abcam) was used to identify peritubular myoid cells as previously described (Sakib et al., 2019a). Anti-BrdU (BD Biosciences) is used to detect proliferating cells (S phase of the cell cycle) (Nóbrega et al., 2015). In the case of proliferation activity, BrdU (100 µg/mL) will be added in the last 6 hours of culture, as previously described (Nóbrega et al., 2015). The slides will be incubated with secondary antibodies conjugated with streptavidin AlexaFluor 594 or streptavidin DyLight 488. The material will be counterstained with Hoescht or propidium iodide (PI) and evaluated under a confocal microscope. Testicular organoids will also be characterized by light microscopy. For this purpose, the organoids will be collected by centrifugation (cytospin) and fixed in a 4% glutaraldehyde buffered solution, for 4 hours at 4°C. After fixation, the organoids will be dehydrated in an increasing series of alcohol (70% for 4 hours; 95% for 4 hours), infiltrated and included in Technovit 7100 plastic resin (HeraeusKulzer, Wehrheim, Germany). Subsequently, sections of 3 µm thickness will be obtained using a microtome equipped with glass knives. The histological sections will be stained with 0.2% toluidine blue and analyzed under a Leica microscope model DMI4000, series 346051 of the Reproductive and Molecular Biology group (Department of Structural and Functional Biology, UNESP-Botucatu).

Task 4: Spermatogenesis in vitro using testicular organoids

To drive *in vitro* spermatogenesis, zebrafish testicular organoids will be cultured in the presence of the zebrafish recombinant hormone Fsh. In this species, the effects of Fsh and Lh on regulation and promoting zebrafish spermatogenesis are already known (Chauvigné et al., 2014; Nóbrega et al., 2015). The organoids will be grown in the presence or absence of these gonadotropins for 4 to 10 days, under the same conditions described above. The medium will be changed every two days. After 4, 7 and 10 days of cultivation, the organoids will be collected for immunofluorescence and histological analyses to identify the cell types present in the organoids under gonadotropin stimulation. Additionally, organoids will be collected for gene expression to confirm the expected effects. For that, the total RNA of the organoids will be extracted using the commercial kit RNAqueous-Micro Kit (Ambion, TX, USA), followed by the synthesis of cDNA according to usual procedures and quantitative real-time PCR reaction (qPCR) using specific primers (forward and reverse). Transcriptome analysis and plasma steroid profiling from accelerated gonadal maturation of *A. altiparanae* will provide information on endocrine/paracrine factors or steroid concentration that could be used to drive spermatogenesis using testicular organoids of other species. If the hormonal treatments were successful in generating spermatozoa *in vitro*, fertilization tests will be performed to confirm spermatozoa viability.

2.3 WP3: Development of testis xenograft as an *in vivo* approach for shortening the gonadal maturation. Testis xenograft experiments will be performed using pieces of *C. macropomum* testes that will be grafted under the skin of *A. altiparanae* under different extrinsic conditions (WP1).

Task 1: Collection of donor testis pieces and testis xenograft

Immature males of C. *macropomum* will be used. Males will be submitted to anesthesia with tricaine in the proportion of 1:10000, and testes will be dissected and fragmented into six fragments of approximately 5 mm thick. Two fragments will be grafted on each side of the dorsal region of 1 year old *A. altiparanae* reared in 3 temperatures (see WP1) (**Figure 3**). Samples from the graft region will be collected in the first, third and fifth weeks after grafting for histological analysis, in order to assess the integrity of the grafted testis.

Task 2: Histological analysis of testis grafts

Samples from the graft region will be collected, fixed in modified Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]) for at least

Figure 3. Scheme methodology used for subcutaneous xenograft testis. Taken from Bhatta et al., 2012.

24 h at room temperature, and subsequently dehydrated, embedded in resin (Leica HistoResin) and sectioned at 3 µm thickness for staining with hematoxylin and eosin. Additional pieces will be fixed in 4% paraformaldehyde solution in phosphate buffered saline (PBS) for at least 24 h at room temperature, dehydrated, embedded in Paraplast High Melt (Leica, Wetzlar, HE, Germany) and sectioned with 5 µm thickness for staining with hematoxylin and eosin. Gonadal maturation will be assessed and classified into reproductive stages according to Brown-Peterson et al (2011).

3. Schedule

	2022											
Workpackage 1	01	02	03	04	05	06	07	08	09	10	11	12
Task 1: *Astyanax altiparanae* breeding and rearing under different temperatures	X	X	X	X	X	X	X	X	X	X	X	X
Task 2 and 3: Animal sampling, gonadal histology and plasma steroid levels measurements	X	X	X	X	X	X	X	X	X	X	X	X
Task 4: Total RNA extraction from gonads of different experimental conditions (Brazil/ Czech Republic). Transcriptome analysis. Bioinformatic analyses.						X	X	X	X	X	X	X
	2023											
Workpackage 2	01	02	03	04	05	06	07	08	09	10	11	12
Task 1: zebrafish and *A. altiparanae* breeding and gonads collection from	X	X	X	X	X	X	X	X	X	X	X	X

Colossoma macropomum for experimentation at Dr. Martin Psenicka's laboratory (Czech Republic)												
Task 2 and 3: Developing testicular organoids using zebrafish. Characterization of testicular cell types within the organoid. Functional viability using hormones.	X	X	X	X	X	X						
Task 2 and 3: Developing testicular organoids using *Astyanax altiparanae*. Characterization of testicular cell types within the organoid. Functional viability using hormones and data from transcriptome analysis.				X	X	X	X	X	X			
Task 4: Spermatogenesis *in vitro* using testicular organoids. Functional viability. Fertility tests.						X	X	X	X	X	X	X
	2024											
Workpackage 3	01	02	03	04	05	06	07	08	09	10	11	12
Task 1: Collection of *C. macropomum* testis pieces and testis xenograft	X	X	X	X								
Task 2: Animal sampling, gonadal histology of testis grafts		X	X	X	X	X	X					
Workshop at Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice. (Czech Republic)										X		
Final Report and manuscripts preparation										X	X	X

4. Significance and motivation

Fish is the most consumed and commercialized animal protein in the world (FAO, 2021). However, many aquaculture species have a long period of sexual maturation, making cultivation of these species costly. To shorten the reproductive cycle using either surrogate reproduction or *in vitro* cultivation could make the aquaculture more efficient.

5. Justification for international collaboration

Both, the main applicant from Czech Republic and the main applicant from Brazil started their research on fish germ cells more than 15 years ago. Last year, during preparation of a collective review on development of germ stem cells (Xie et al., 2020), we identified a significant gap in the germ cell research, which is regulation of developmental speed of germ cells. Both groups have a long-term effort to shorten the generation cycle of their economically and ecologically important species via surrogate reproduction. However, to date, researchers in the field have brought only contradictory results about the possibility. The Czech applicant has been devoted to studying germ cells on the cellular level. The laboratory

has developed methods for isolation and transplantation of germ cells. On the other hand, the Brazilian applicant has much experience with gonadal maturation, morphological characterization of germ cells and endocrine/paracrine regulation of gametogenesis. Thus, the joint proposal focused on accelerating germ cell development will obviously benefit from the synergy of these two partners. Brazilian partner will support Czech partner in *in vitro* experiments (WP2). They will perform transcriptome analysis of gonads in order to discover candidate players involved in accelerated gonadal maturation. Then they will establish gonadal organoids to drive in vitro gametogenesis using candidate factors previously obtained from transcriptome analysis. On the other hand, Czech partner will lead the transplantation experiments (WP1) and support Brazilian experiments regarding germ cell transplantation. In addition, Brazilian research will also take advantage of the applicant's expertise on the sturgeon model. As long maturing fish, sturgeon data will help to understand which factors could be silent during gonadal maturation. At the end, the collaborative research will expand our knowledge on the mechanisms that govern germ cell development in species relevant for Czech Republic and Brazil. Additionally, this research will contribute by developing biotechnological applications to improve aquaculture of both countries, and also important for restoration of endangered species.

6. Readiness of the teams

Brazilian team: The Brazilian applicant coordinates the Neotropical Ichthyology Laboratory (LINEO), and develop research in fish reproduction, focused on production, conservation and impacts of climate change, with emphasis on gamete morphology, reproductive cycle, germ cell transplantation and gamete cryopreservation. The Brazilian team is composed by 5 research members and several students among them undergrad, masters and PhD students. The research team has expertise on different aspects of fish reproductive physiology and biotechnology using zebrafish (*D. rerio*), medaka (*O. latipes*), and Neotropical species such as lambari (*A. altiparanae*), tambaqui (Colossoma macropomum) among others. Several methodologies have been employed by the group, e.g., tissue culture techniques (testicular explants), histology, immunofluorescence, cell proliferation assays (BrdU), gene expression (RT-qPCR), in situ hybridization, in silico analysis, transcriptome and others. The workplace is equipped with StepOne Plus, hybridization oven, biofreezer, thermocyclers, electrophoresis, thermoblocks, microtome, microscopes, cell culture room, horizontal and vertical laminar flow hoods, and others. These equipment were acquired through funds obtained by Brazilian funding agencies (São Paulo

Research Foundation/FAPESP and National Council for Scientific and Technological Development/CNPq). In addition, the group has intense collaboration with researchers from different countries (Germany, Argentina, Canada, Spain, Holland, Japan, Portugal and the Czech Republic).

Czech applicant: The Laboratory of Germ cells, Research Institute of Fish Culture and Hydrobiology in Vodnany, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice. The supervisor's and junior researcher's expertise deals with fish embryo development, gene expression inhibition, genome editing CRISPR/Cas9 system, micromanipulation and grafting, and they will train a new PhD student joining this project. The faculty keeps many sturgeon species, such as sterlet including albinotic form, beluga, American paddlefish, Russian, Siberian, Atlantic, shortnose, white and stellate sturgeon. Regarding model fish species, the applicant owns several wild type and transgenic zebrafish strains, which are kept in zebrafish housing system ZebTec. The workplace is equipped with histological laboratory, fluorescent stereomicroscope, puller and grinder for preparation of needles, 7x sets of micromanipulators and microinjectors, motorized manipulator, inverted motorized fluorescent microscope Olympus IX83, spectrophotometers, a number of thermocyclers, qPCR cycler, flow cytometer and cell sorter, various systems for electrophoresis including microchip electrophoresis system, numerous common microscopes and stereomicroscopes, centrifuges, etc.

7. Other international collaboration

This research would have intense collaborations with researchers in the EU, such as Dr. Rüdiger W Schulz and Dr. Jan Bogerd (Utrecht University, the Netherlands); Dr. Jean-Jacques Lareyre (INRA, Farance); Dr. Manfred Schartl (University of Würzburg, Germany); Dr. Adelino Canário (Universidade do Algarve); Dr. Francisco Prat (Instituto de Ciencias Marinas de Andalucía, Spain); Dr. Ana Gómez (Instituto de Acuicultura de Torre la Sal, Spain); Dr. Francesc Piferrer (Institute of Marine Sciences, Spain); and non-EU, such as Dr. Gustavo Somoza (INTECH, Argentina); Dr. Luiz Renato de França (Federal University of Minas, Brazil); and Dr. Hamid Habibi (University of Calgary, Canada).

8. Expected results and impact. Dissemination and exploitation plans

This research will expand our knowledge to unravel the endocrine and paracrine components of the brain-pituitary-gonadal axis involved in teleost gonadal maturation, allowing the use of this information, acquired in model species, in teleost species that have

economical potential in aquaculture, both, in Brazil and Czech Republic. Additionally, this research will contribute by developing biotechnological applications to improve aquaculture of both countries. The results of this project will be of high interest for several stakeholder types (different research institutes from Brazil and Czech Republic, scientific and local community, fish farms, among others), and some of them, at the end of the project, will benefit from the results and technologies generated (e.g., transcriptome, organoids, xenografts). The main tools used to disseminate the project outcomes will be the following:

- website/Facebook/Instagram: it will serve as a communication and dissemination allowing a wider public and interest groups to learn about the project existence and goals;
- publication of articles in scientific journals and on specific technical/scientific magazines, as well as on national/local press, illustrating the main project findings and implication;
- participation at dissemination events: national and international conferences and workshops;
- participation at events of scientific divulgation for the general community (universities, schools, among others) and fish farmers.

9. References

ADOLFI, MATEUS C, CARREIRA, A. C., JESUS, L.W., BOGERD, J., FUNES, R.M., SCHARTL, M., BORELLA, M.I. 2015. Molecular cloning and expression analysis of dmrt1 and sox9 during gonad development and male reproductive cycle in the lambari fish, *Astyanax altiparanae*. Reproductive Biology and Endocrinology, 13(1), 2. https://doi.org/10.1186/1477-7827-13-2

ALVES-LOPES JP, SODER O, STUKENBORG JB. 2017. Testicular organoid generation by a novel in vitro three-layer gradient system. Biomaterials 130: 76–89.

BAERT Y, DE KOCK J, ALVES-LOPES JP, SODER O, STUKENBORG JB, GOOSSENS E. 2017. Primary human testicular cells self-organize into organoids with testicular properties. Stem Cell Rep 8: 30–38.

BHATTA, S.; IWAI, T.; MIURA, C.; HIGUCHI, M.; SHIMIZU-YAMAGUCHI, S.; FUKADA, H.; MIURA, T. Gonads directly regulate growth in teleosts. Proceedings of the National Academy of Sciences, v. 109, n. 28, p. 11408–11412, 10 jul. 2012

BROWN-PETERSON, N.J., WYANSKI, D.M., SABORIDO-REY, F., MACEWICZ, B.J., LOWERRE-BARBIERI, S.K., 2011. A Standardized Terminology for Describing Reproductive Development in Fishes. Marine and Coastal Fisheries 3, 52–70. https://doi.org/10.1080/19425120.2011.555724

CHAUVIGNE F, ZAPATER C, GASOL JM, CERDA J. 2014. Germ-line activation of the luteinizing hormone receptor directly drives spermiogenesis in a nonmammalian vertebrate. PNAS 111: 1427–1432.

CHEHADE, C., AMARAL, F.G., BRANCO, G.S., CASSEL, M., DE JESUS, L.W.O., COSTA, F.G., BORDIN, S.A., MOREIRA, R.G., BORELLA, M.I., 2020. Molecular characterization of different preproGnRHs in *Astyanax altiparanae* (Characiformes): Effects of GnRH on female reproduction. Mol Reprod Dev 87, 720–734. https://doi.org/10.1002/mrd.23351

CLEVERS H. 2016. Modeling Development and Disease with Organoids. Cell 16; 165 (7): 1586-1597.

CLOUD, J. G. Surgical transplantation of sexually immature ovaries in rainbow trout (*Oncorhynchus mykiss*). Journal of experimental zoology. Part A, Comparative experimental biology, v. 298, n. 1, p. 73–6, 2003.

FAO, 2021. FAO Fisheries & Aquaculture - National Aquaculture Sector Overview - Brazil [WWW Document]. URL http://www.fao.org/fishery/countrysector/naso_brazil/en (accessed 4.8.21).

FARLORA, R.; HATTORI-IHARA, S.; TAKEUCHI, Y.; HAYASHI, M.; OCTAVERA, A.; ALIMUDDIN; YOSHIZAKI, G. Intraperitoneal Germ Cell Transplantation in the Nile Tilapia *Oreochromis niloticus*. Marine Biotechnology, v. 16, n. 3, p. 309–320, 6 jun. 2014.

GODOI, F.G.A., MUÑOZ-PEÑUELA, M., GOMES, A.D.O., TOLUSSI, C.E., BRAMBILA-SOUZA, G., BRANCO, G.S., LO NOSTRO, F.L., MOREIRA, R.G., 2020. Endocrine disruptive action of diclofenac and caffeine on *Astyanax altiparanae* males (Teleostei: Characiformes: Characidae). Comp Biochem Physiol C Toxicol Pharmacol 231, 108720. https://doi.org/10.1016/j.cbpc.2020.108720

HAMASAKI, M.; TAKEUCHI, Y.; YAZAWA, R.; YOSHIKAWA, S.; KADOMURA, K.; YAMADA, T.; MIYAKI, K.; KIKUCHI, K.; YOSHIZAKI, G. Production of Tiger Puffer *Takifugu rubripes* Offspring from Triploid Grass Puffer *Takifugu niphobles* Parents. Marine Biotechnology, p. 1–13, 23 set. 2017.

IBGE, 2021. Produção da aquicultura [WWW Document]. URL https://sidra.ibge.gov.br/Tabela/3940 (accessed 4.8.21).

JESUS LWO, BOGERD J, VIECELI FM, BRANCO GS, CAMARGO MP, CASSEL M, MOREIRA RG, YAN CYI, AND BORELLA MI. “Gonadotropin Subunits of the Characiform *Astyanax Altiparanae*: Molecular Characterization, Spatiotemporal Expression and Their Possible Role on Female Reproductive Dysfunction in Captivity.” *General and* Comparative Endocrinology 246 (May 15, 2017): 150–63. https://doi.org/10.1016/j.ygcen.2016.12.004.

MAJHI, S. K.; HATTORI, R. S.; YOKOTA, M.; WATANABE, S.; STRÜSSMANN, C. A. Germ Cell Transplantation Using Sexually Competent Fish: An Approach for Rapid Propagation of Endangered and Valuable Germlines. PLoS ONE, v. 4, n. 7, p. e6132, 2 jul. 2009.

MAJHI, S. K.; KUMAR, S. Germ Cell Transplantation: A Potential Tool for Propagation of Endangered Fishes. Annals of Aquaculture and Research, 2017.

MORITA, T.; KUMAKURA, N.; MORISHIMA, K.; MITSUBOSHI, T.; ISHIDA, M.; HARA, T.; KUDO, S.; MIWA, M.; IHARA, S.; HIGUCHI, K.; TAKEUCHI, Y.; YOSHIZAKI, G. Production of Donor-Derived Offspring by Allogeneic Transplantation of Spermatogonia in the Yellowtail (*Seriola quinqueradiata*)1. Biology of Reproduction, v. 86, n. 6, p. 1–11, 2012.

NAGLER, J. J.; CLOUD, J. G.; WHEELER, P. A.; THORGAARD, G. H. Testis transplantation in male rainbow trout (*Oncorhynchus mykiss*). Biology of reproduction, v.64, n. 2, p. 644–646, 1 fev. 2001.

NOBREGA RH, GREEBE CD, VAN DE KANT H, BOGERD J, DE FRANCA LR, SCHULZ RW. 2010. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. PLoS One Sep 5 (9): e12808.

NOBREGA RH, MORAIS RD, CRESPO D, DE WAAL PP, FRANCE LR, SCHULZ RW, BOGERD J. 2015. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. Endocrinology 156 (10): 3804-17.

OKUTSU, T.; SUZUKI, K.; TAKEUCHI, Y.; TAKEUCHI, T.; YOSHIZAKI, G. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proceedings of the National Academy of Sciences, v. 103, n. 8, p. 2725–2729, 21 fev. 2006.

PENDERGRAFT SS, SADRI-ARDEKANI H, ATALA A, BISHOP CE. 2017. Three dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity in vitro dagger. Biol Reprod 96: 720–32.

PINHEIRO, J.P.S., LIMA, J., ASSIS, C.B. DE, BRANCO, G.S., GOMES, A.D., MOREIRA, R.G., 2021. Paternal exposure to aluminum, acidity, and temperature affects fatty acid seminal profile, embryonic and larval development of Astyanax altiparanae. Chemosphere 266, 128935. https://doi.org/10.1016/j.chemosphere.2020.128935

QUIRINO, P., DA SILVA RODRIGUES, M., DA SILVA CABRAL, E.M., DE SIQUEIRA-SILVA, D.H., MORI, R.H., BUTZGE, A.J., NÓBREGA, R.H., NINHAUS-SILVEIRA, A., VERÍSSIMO-SILVEIRA, R., 2020. The influence of increased water temperature on the duration of spermatogenesis in a neotropical fish, *Astyanax*

altiparanae (Characiformes, Characidae). Fish Physiol Biochem. https://doi.org/10.1007/s10695-020-00869-7

RAHMAN, M.L., ZAHANGIR, M.M., KITAHASHI, T., SHAHJAHAN, M., ANDO, H., 2019. Effects of high and low temperature on expression of GnIH, GnIH receptor, GH and PRL genes in the male grass puffer during breeding season. Gen Comp Endocrinol 282, 113200. https://doi.org/10.1016/j.ygcen.2019.06.004

SAKIB S, UCHIDA A, VALENZUELA-LEON P, YU Y, VALLI-PULASKI H, ORWIG K, UNGRIN M, DOBRINSKI I. 2019a. Formation of organotypic testicular organoids in microwell culture. Reprod Biol. 1; 100 (6): 1648-1660.

SAKIB S, VOIGT A, GOLDSMITH T, DOBRINSKI I. 2019b. Three-dimensional testicular organoids as novel in vitro models of testicular biology and toxicology. Environ Epigenet. 31; 5 (3): dvz011.

SHAHJAHAN, M., KITAHASHI, T., ANDO, H., 2017. Temperature affects sexual maturation through the control of kisspeptin, kisspeptin receptor, GnRH and GTH subunit gene expression in the grass puffer during the spawning season. Gen Comp Endocrinol 243, 138–145. https://doi.org/10.1016/j.ygcen.2016.11.012

SEKI, S.; KUSANO, K.; LEE, S.; IWASAKI, Y.; YAGISAWA, M.; ISHIDA, M.; HIRATSUKA, T.; SASADO, T.; NARUSE, K.; YOSHIZAKI, G. Production of the medaka derived from vitrified whole testes by germ cell transplantation. Scientific Reports, v. 7, n. March, p. 43185, 3 mar. 2017.

SILVA, M.A.; COSTA, G.M.J.; LACERDA, S.M.S.N.; BRANDÃO-DIAS, P.F.P.; KALAPOTHAKIS, E.; SILVA JÚNIOR, A.F.; ALVARENGA, E.R.; FRANÇA, L.R. Successful xenogeneic germ cell transplantation from Jundia catfish (*Rhamdia quelen*) into adult Nile tilapia (*Oreochromis niloticus*) testes. General and Comparative Endocrinology, v. 230–231, p. 48–56, maio 2016.

SIQUEIRA-SILVA, D., SANTOS-SILVA, A., COSTA, R., SENHORINI, J., NINHAUS-SILVEIRA, A., VERÍSSIMO-SILVEIRA, R., 2019. Preliminary study on testicular germ cell isolation and transplantation in an endangered endemic species *Brycon orbignyanus* (Characiformes: Characidae). Fish Physiology and Biochemistry 1–10. https://doi.org/10.1007/s10695-019-00631-8

STRANGE DP, ZARANDI NP, TRIVEDI G, ATALA A, BISHOP CE, SADRI-ARDEKANI H, VERMA S. 2018. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. Emerg Microb Infect 7:82.

TAKEUCHI, Y.; YOSHIZAKI, G.; TAKEUCHI, T. Biotechnology: Surrogate broodstock produces salmonids. Nature, v. 430, n. 7000, p. 629–630, 5 ago. 2004.

TSUTSUI, K., UBUKA, T., 2020. Discovery of gonadotropin-inhibitory hormone (GnIH), progress in GnIH research on reproductive physiology and behavior and perspective of GnIH research on neuroendocrine regulation of reproduction. Mol Cell Endocrinol 514, 110914. https://doi.org/10.1016/j.mce.2020.110914

XU H, JIAO Y, QIN S, ZHAO W, CHU Q, WU K. 2018. Organoid technology in disease modeling, drug development, personalized treatment and regeneration medicine. Exp Hematol Oncol. 5; 7: 30.

ZAHANGIR, M.M., MATSUBARA, H., OGISO, S., SUZUKI, N., UEDA, H., ANDO, H., 2021. Expression dynamics of the genes for the hypothalamo-pituitary-gonadal axis in tiger puffer (*Takifugu rubripes*) at different reproductive stages. Gen Comp Endocrinol 301, 113660. https://doi.org/10.1016/j.ygcen.2020.113660