

Book of Abstracts



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ORAL ABSTRACTS

WHEN SPERM WAITS IN THE OVARY: OVARIAN INSEMINATION AS A NOVEL PROPAGATION METHOD FOR EXTERNALLY FERTILISING FISH

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A novel fish propagation method has been developed based on the artificial introduction of sperm into the ovarian lobes of females (ovarian insemination). In externally fertilising fish species, spermatozoa introduced before induced spawning can remain biologically active and be temporarily retained in the ovarian environment until ovulation. At spawning, the retained spermatozoa and ovulated eggs are released together; upon contact with water, sperm activation enables fertilisation.

The procedure is technically simple. The procedure is simple. Previously collected and quality-assessed sperm, whether fresh, pooled, processed or cryopreserved, can be introduced into either ovarian lobe of an anaesthetised female using a steerable catheter or probe; in small fish, an automatic pipette or glass capillary can be used.

The principal findings can be summarised as follows: (i) spermatozoa retained their fertilising capacity for ~40 hours under ovarian conditions; (ii) ovulation and fertilisation were achieved by mixing pituitary- or GnRH analogue-based spawning agents with sperm and introducing the mixture into an ovarian lobe through a catheter, thereby combining hormone treatment and insemination in a single needle-free procedure; (iii) fertilisation was achieved using cryopreserved sperm after specialised post-thaw preparation; (iv) paternal representation and offspring genetic diversity could be increased in pair-spawning species by using inseminated sperm from additional males; (v) following insemination, partial ovulation and spawning occurred in the absence of males, and some released eggs were fertilised and developed into viable offspring; and (vi) controlled interspecific hybrids were successfully produced.

Potential applications include controlled crossing and hybridisation during induced (semi-natural) spawning; increased genetic diversity through pooled sperm; use of processed, cryopreserved or genetically valuable sperm; reduced need to maintain male broodstock or permanently remove males from donor populations, thereby limiting allele loss and ectoparasite transfer; increased egg production per unit spawning area through female-biased broodstock ratios; and genetic rescue or conservation propagation of small, fragmented or inbred populations.

Keywords: *Sperm ovarian lavage, sperm insemination, assisted reproduction; gamete management; conservation breeding; hatchery biotechnology*

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DOES NEST MATING MAKE GAMETES MATCH?

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Pikeperch *Sander lucioperca* is a monogamous nest spawner where each gender mates only once during the spawning season. The process of mating, so-called nest-dance, takes several days and is characterized by the male's aggressive behavior. While developing of a new protocol for artificial reproduction, recognizing the behavioral indicator of ovulation, a rather specific pattern was witnessed, pointing to the potential match and mismatch between the egg and sperm of the male from the nest and a random male, respectively. Accordingly, the trial was set to evaluate the effect of sperm – nester vs. random on embryo survival in pikeperch. Altogether, 11 pairs were hormonally treated and stocked in the spawning chambers supplied with artificial nests and video monitoring. Additional 11 males were hormonally treated and stocked in a separate tank without contact with the females or spawning substrates. Males were not spermiating at the time of hormonal injection, while the sperm collection was in period 5 to 8 days post-injection. Sperm of the male from the nest was stripped 3-9 hours before ovulation, while sperm from random males was stripped 30-60 minutes before fertilization. All the sperm samples were evaluated via the CASA system. Upon egg stripping, half of the eggs were fertilized with milt of the respective male from the nest, while for the fertilization of the other half of the eggs, sperm from a random male was used in the same amount, 1 mL milt per 100g of eggs. The eggs of each replicate (11 per group in total) were incubated in a separate Zug jar. The embryo survival rate was evaluated according to the difference in the egg volumes after swelling and 72 h later, corrected for the share of alive embryos in the 72h sample. The statistics for the evaluation of the difference between the samples were based on the Wilcoxon signed-rank test. Embryo survival was, on average, relatively high in both groups; however, significantly higher in the eggs fertilized with the male from the nest ($P = 0.002$). Respecting that the pairs were fixed without the mate selection possibility, the “gametes fitting” could have taken place during the nest-dance. However, the alternative hypotheses should be tested as well. Perhaps sperm selection during the short storage took place, favoring more viable spermatozoa for fertilization. Likewise, rather than conventional sperm quality features, perhaps some other parameters should be evaluated which nest-dance and pheromonal effect might improve. Testing gametes fitting vs. spermatozoa selection vs. unconventional sperm quality features will improve our understanding of hidden reproductive biology mechanisms.

Keywords: pikeperch; sperm; eggs; embryo survival; gametes fitting.

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BEYOND WATER ACTIVATION: SPERM MEMBRANE PERMEABILITY AND THE HIDDEN VULNERABILITY OF FISH REPRODUCTION

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Fish sperm activation is one of the most rapid and environmentally sensitive events in reproductive physiology. Within seconds after release into water, spermatozoa must detect osmotic and ionic changes, regulate membrane potential, intracellular pH, and water/ion fluxes, initiate and sustain motility, and reach the egg before the fertilisation window closes, making membrane transport a central sperm function. Yet, fish reproduction studies still focus mainly on activation and fertilisation success, while the transport mechanisms remain largely overlooked. This knowledge gap is further complicated by the large diversity of reproductive strategies and signaling pathways among fishes.

Under optimal environmental or standardized hatchery conditions, these activation pathways operate efficiently, ensuring successful fertilisation. However, natural reproductive environments are far more complex than simplified experimental conditions. Factors such as ovarian fluid, egg-derived cues, water turbulence, and rapidly changing osmotic or ionic microenvironments may strongly influence membrane transport, sperm motility, navigation, and fertilisation success. Moreover, aquatic environments are becoming increasingly unstable due to climate change, salinity fluctuations, eutrophication, and contamination by pharmaceuticals, heavy metals, and industrial pollutants. Many of these stressors influence membrane permeability and ion-channel regulation, affecting sperm function and fertilisation. Clarifying how spermatozoa respond to such stressors may help explain reproductive failures, improve artificial reproduction protocols, and provide sensitive indicators of environmental stress in aquatic ecosystems.

In this respect, we discuss why a deeper understanding of water and ion transport mechanisms in fish spermatozoa is becoming critically important not only for reproductive physiology but also for aquaculture sustainability and environmental risk assessment. Particular attention will be given to potassium, calcium, proton, and water transport systems involved in sperm activation, their interactions, and environmental factors that can alter their function. We also address how limited knowledge of membrane transport pathways may restrict our ability to identify causes of reproductive dysfunction under changing environmental conditions.

Keywords: *water transport, ion transport, sperm physiology, environmental stressors.*

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MORPHOLOGICAL AND MOLECULAR SIGNATURES OF OVARIAN AGING IN ZEBRAFISH

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Ovarian aging progressively limits female fertility, yet the mechanisms driving gonadal aging remain poorly understood. Multiple mechanisms have been implicated in the aging process such as genetic damage, oxidative stress, cell death and microenvironmental alterations. In this study, we used zebrafish to investigate the mechanisms underlying ovarian aging and the decline in fertility. To define the cellular and molecular hallmarks of ovarian aging, we compared young (6-month-old) and aged (3-year-old) zebrafish ovaries using histology and bulk RNA sequencing. Young zebrafish ovaries contained abundant secondary growth (SG) oocytes and scattered primary growth (PG) oocytes associated with the germinal epithelium. Morphometric analysis of follicles revealed similar distribution among young ovaries. In contrast, aged ovaries exhibited profound structural remodeling characterized by oocyte depletion, increased ovarian stroma, and infiltration of immune cells. The most notable alteration compared to young ovaries was the increased degeneration of PG oocytes ($p < 0.01$), leading to the formation of fibrotic areas ($p < 0.05$) with immune cell infiltration. Although no differences were observed in the percentage of atretic SG oocytes ($p = 0.97$), differences were detected in the percentage of non-atretic SG oocytes ($p < 0.01$), which may reflect reduced follicle recruitment rather than increased atresia itself in aged females. To explore the molecular basis of ovarian aging, we next conducted bulk RNA sequencing to compare the transcriptomic profiling. Aged ovaries showed pronounced heterogeneity, suggesting divergent trajectories of ovarian aging among individuals. We identified 320 upregulated and 90 downregulated genes in aged ovaries. Gene enrichment analyses using ORA and GSEA revealed terms associated with oxidative stress, immune system processes, cell-to-cell interactions, and several signaling pathways, which have been reported as drivers of aging in other organs. In addition, we identified alterations in components of the steroidogenic machinery, such as the well-known ovarian aromatase (*cyp19a1a*). Reactome and KEGG analyses further revealed enrichment of apoptosis-related and cell signaling pathways. Because ovarian aging is a multifactorial process, we are currently validating several candidate mechanisms using in situ hybridization and immunofluorescence to understand the histological, cellular, and molecular bases of ovarian aging and determining whether all zebrafish ovaries age in the same way. Together, our findings reveal that zebrafish ovarian aging is not a uniform degenerative process, but a heterogeneous remodeling program involving germ cell loss, immune/stromal activation, and altered endocrine signaling. These results establish zebrafish as a tractable vertebrate model for studying the mechanisms driving reproductive aging and fertility decline.

ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF MICROPLASTICS TRANSLOCATE INTO GAMETES IN A SEX-SPECIFIC MANNER, ALTERING THEIR PHYSIOLOGY WITHOUT IMPAIRING MEAN REPRODUCTIVE OUTPUT IN BROWN TROUT

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Microplastic pollution in freshwater systems is increasing, but its effects on salmonid gamete physiology and reproductive output remain poorly resolved. This study assessed whether dietary exposure to polystyrene microplastics (spherical PS-MPs with diameters of 1, 5, and 10 µm), administered at environmentally relevant doses, affects reproductive performance in brown trout (*Salmo trutta*). Fish were exposed to a low PS- MP dose equivalent to 4.8×10^3 particles fish⁻¹ (LMP) or a tenfold higher dose equivalent to 4.8×10^4 particles fish⁻¹ (HMP) during the pre-spawning period. PS-MPs in gametes were determined, as well as broodstock maturation rate, semen and egg quality indicators, and development and performance of offspring derived from microplastic-contaminated gametes.

PS-MP exposure was associated with reduced female spawning participation, with ovulation rate declining from 82.1% in controls to 54.8% and 45.9% in LMP and HMP, respectively, whereas male maturation rate was unaffected. PS-MP particles of 1 µm were detected in semen, whereas 1 and 5 µm particles were detected in eggs; 10 µm particles were not detected in either gamete type, with eggs showing higher detection frequency and abundance than semen. Semen volume was significantly reduced by approximately 40% from the control to the LMP group, whereas sperm density increased from the control to the LMP and HMP groups. Total semen output (product of semen volume and density) was not significantly affected across treatments. Gamete antioxidant responses were altered, including reduced SOD activity in semen and reduced CAT and POD activity in eggs. PS-MP exposure did not produce detectable group-level effects on semen viability or motility. Egg mass, egg hardening capacity, and embryo development to the eyed-egg and hatching stages were also unaffected. No differences were observed in offspring performance (growth and mortality). However, threshold-based analyses showed a higher frequency of below-average reproductive performance in PS-MP treatments across output parameters. These findings indicate that environmentally relevant dietary PS-MPs can induce subtle, gamete-associated physiological responses. In aquaculture, such changes may not reduce mean reproductive output, likely because they are buffered by effective reproductive technologies. In natural environments, where reproduction is unassisted and subject to multiple stressors, these effects may be consequential.

Keywords: *Salmo trutta*; oocyte quality; sperm function; oxidative enzymes; hatching success

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CULTURE ENVIRONMENT INFLUENCES GONADAL REPRODUCTIVE PATHWAYS IN *MUGIL CEPHALUS*

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Reproductive dysfunction and incomplete gonadal maturation remain major bottlenecks for mullet aquaculture, particularly under captive conditions where environmental cues differ from natural habitats. Despite the increasing commercial importance of flathead grey mullet (*Mugil cephalus*), the molecular mechanisms underlying gonadal development and reproductive regulation remain poorly understood. This study evaluated the expression of reproductive- and physiology-related genes in gonadal tissue of male and female mullet reared under different aquaculture environments to better understand how culture conditions influence reproductive development.

All fish belonged to the same batch and age group. Fish were maintained for eight months under three culture systems: recirculating aquaculture systems (RAS), aquaponics, and integrated multitrophic aquaculture (IMTA) in coastal ponds. After this period, fifteen fish per system were sacrificed, and gonads were collected for histological and molecular analyses. Gonadal expression of genes associated with reproductive regulation (*kissr1a*, *aldh1a2*, *mknr2*), metabolic signaling (*lepr*, *igf1r*), inflammatory pathways (*cox2*), and cellular stress regulation (*ARP*) was analyzed by quantitative PCR. The effects of sex, environment, and their interaction were assessed using a GLM.

Strong sex-dependent expression patterns were observed in most analyzed genes. Significant effects of sex were detected for *aldh1a2*, *kissr1a*, *lepr*, *mknr2*, *ARP*, and *cox2* ($p < 0.001$), suggesting sexual regulation of pathways involved in gonadal differentiation, endocrine signaling, metabolism, and tissue remodeling. In contrast, *igf1r* expression remained relatively stable between sexes and culture conditions. Among the analyzed genes, *cox2* exhibited the clearest environmental response, with significant effects of culture system ($p = 0.004$) and interaction. Females reared in IMTA displayed significantly higher *cox2* expression than females maintained in RAS or aquaponics, whereas males showed no environmental differences. Similarly, *lepr* expression was significantly higher in fish reared in IMTA compared to RAS and aquaponics, suggesting a relationship between energetic balance and reproductive regulation. Marginal environmental effects were also observed for *aldh1a2* and *mknr2*. Histological analyses supported the molecular results, with fish reared in IMTA showing significantly more advanced gonadal development than fish maintained in RAS or aquaponics.

Overall, gonadal gene expression in mullet was primarily influenced by sex-specific reproductive physiology, while environmental modulation appeared gene-dependent and particularly relevant for pathways linked to gonadal activity, metabolism, and inflammatory regulation. These findings improve understanding of reproductive molecular mechanisms in flathead grey mullet and support the hypothesis that culture environment may influence gonadal maturation.

Keywords: Flathead grey mullet, IMTA, maturation, aquaculture, molecular modulation.

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WHY TRIPLOID ZEBRAFISH REFUSE TO BECOME FEMALE: REWIRING OF HORMONAL CONTROL IN SEX DIFFERENTIATION

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Sex differentiation in zebrafish is highly plastic, yet triploid individuals consistently develop as males, even under strong estrogenic stimulation. Here, we show that triploidy rewires endocrine and molecular signaling, enforcing a fixed male developmental trajectory. While exposure to 17 α -ethinylestradiol (EE2) induced ovotestes and ovarian features in diploids, triploids remained resistant, exhibiting only increased early germ cell proliferation without feminization.

At the molecular level, triploidy disrupted key regulatory pathways, including downregulation of *fshr* and *amh*, and upregulation of *igf3*, supporting enhanced spermatogenic signaling. Genes involved in retinoic acid metabolism (*cyp26b1*, *dhrs3a*) and steroidogenesis (*star*, *cyp11a1*, *cyp17a1*) were also altered, indicating constrained hormonal responsiveness. Notably, EE2 failed to suppress *cyp11c1* in triploids, suggesting an altered endocrine state that reinforces masculinization.

Together, these findings demonstrate that genomic imbalance overrides hormonal cues, limiting sex reversal and locking zebrafish into a male developmental fate. This work highlights fundamental constraints on reproductive plasticity and provides new insight into endocrine control of vertebrate sex differentiation.

Keywords: *polyploidy, endocrine signaling, transcriptome, ethinylestradiol*

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GAMETE PLOIDY ENGINEERING OVERCOMES INTER-FAMILY REPRODUCTIVE ISOLATION IN TELEOST FISH

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Classically, reproductive isolation intensifies with evolutionary divergence, and viable inter-family vertebrate hybrids are extremely rare. Some hybrids achieve reproduction through unreduced gametes, suggesting that ploidy manipulation might bypass reproductive barriers. Here, we demonstrate that targeted deletion of *rnf212b* (a gene tightly linked to meiotic errors in hybrid triploid loaches) in zebrafish triggered sequential meiotic defects: crossover failure caused premature dissociation of bivalents into univalents, suppressing the first polar body extrusion. Single-cell transcriptomics and mass spectrometry identified Tuba1b, a direct Rnf212b interactor, as a key effector mediating bivalent destabilization. Consequently, *rnf212b*^{-/-} zebrafish produced unreduced eggs, from which we generated gynogenetic lines. Fertilization of *rnf212b*^{-/-} unreduced eggs with sperm from *Gobiocypris rarus*, *Misgurnus anguillicaudatus* and *M. mohoity* yielded morphologically normal inter-subfamily and inter-family triploids, while crosses with *Ctenopharyngodon idella* and *Cyprinus carpio* produced only deformities. These findings uncover asymmetric reproductive isolation in teleosts. Our study establishes a single-gene engineering strategy to generate unreduced eggs in vertebrates, and provides a novel tool to overcome inter-family reproductive barriers.

TEMPORAL REDISTRIBUTION OF GERMLINE PROLIFERATION LINKS DEVELOPMENTAL TIMING TO LIFE-HISTORY STRATEGY IN ANNUAL KILLIFISH

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Life-history strategies shape how reproduction is distributed across the lifespan, yet how this is reflected in germline dynamics remains poorly understood. Here, we show that in the annual killifish *Nothobranchius furzeri*, germline proliferation is temporally redistributed, with a substantial fraction of germ cells established before hatching and only limited proliferation in adulthood.

Using a combination of developmental analyses, cross-species comparisons, and functional genetic perturbations, we find that this pattern is specific to annual species. Despite low adult proliferative activity, nanos-positive germ cells persist in adult gonads, indicating retained stem cell competence. At the same time, AMH signaling actively limits germ cell proliferation, as disruption of *amh* leads to a marked increase in mitotic activity without improving fecundity.

Together, these results show that reproductive output is not directly constrained by germ cell number in this system. Instead, early germline allocation reduces reliance on sustained adult proliferation, while endocrine regulation maintains germline quiescence. These findings link developmental timing to life-history strategy and provide a framework for understanding how vertebrate reproduction is adapted to extreme life histories.

Keywords: germline stem cells; AMH signaling; fecundity; reproductive strategy; endocrine regulation; evolutionary adaptation

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CDK1 DEFICIENCY TRIGGERS PREMEIOTIC ENDOREPLICATION AND UNREDUCED SPERM FORMATION VIA CDK2/CCNE1-FZR1A PATHWAY IN A TELEOST FISH, LOACH

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Polyploidy represents a major driver of eukaryotic genome evolution. In vertebrates, premeiotic endoreplication is considered as an important route to polyploidization, yet the molecular basis governing this process remains elusive. Here, we reported that heterozygous mutation of *cdk1* in the loach, *Misgurnus anguillicaudatus* (Teleostei, Cobitidae) triggered premeiotic endoreplication in spermatogonia, resulting in the production of unreduced diploid sperms. Through integrated proteomic and phosphoproteomic profiling, we uncovered that Cdk1 deficiency led to widespread dysregulation of the phosphorylation of key proteins in the cell cycle and DNA replication pathways in spermatogonia. Mechanistically, Cdk1 deficiency hyperactivated the Cdk2/Ccne1 complex, which phosphorylated Fzr1a at threonine 124 to initiate endoreplication. Inhibition of Cdk2 expression in *cdk1*^{+/-} loach males suppressed this pathway and abolished unreduced sperm formation. Our study elucidates the first molecular pathway for unreduced gamete production in fish and establishes Cdk1 as a key guardian of diploidy before meiosis. Furthermore, leveraging this mechanism, we generated triploid and tetraploid loach lines with unreduced sperms, demonstrating a direct bridge between gene function to polyploid breeding. These findings provide a mechanistic framework for understanding polyploidization in vertebrate evolution and offer a targeted strategy for chromosome set manipulation in aquaculture.

YOUNG GONADAL ENVIRONMENT REVERSES MALE REPRODUCTIVE AGING

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Reproductive aging is characterized by a decline in gamete quality and germ cell function eventually leads to infertility. This age-related infertility might be due to loss of germ cell stemness, disrupted differentiation signals, or deterioration of the testicular microenvironment. The driving mechanism behind age-related infertility remains elusive as available models do not allow precise dissection of these lineages. Here, the relative contributions of germ cell-autonomous aging and the surrounding somatic niche to infertility are explored using a germline surrogacy approach in zebrafish (*Danio rerio*). Donor germ cells (spermatogonial cells) were isolated from 3 years old Tg(ddx4:egfp) transgenic males exhibiting hallmarks of reproductive aging. These include failure to spawn, absence of milt upon manual stripping, decreased gonadosomatic index, declined proliferation capacity, and biased cell population with reduced spermatozoa. Simultaneously, germ cells isolated from 8 months old Tg(ddx4:egfp) transgenic males were also used for transplantation to compare transplantation efficiency. The isolated germ cells were transplanted into a sterile recipient created by knocking down the *dnd* gene. The transplanted cells from the aged donor successfully migrated and colonized the gonadal anlage of sterile recipients. Although, initial expansion of aged germ cells was slower, the youthful environment restored the young-like spermatogenesis. The chimeras from both donors showed GFP positive developed gonads upon dissection and produced motile sperm confirming the functional spermatogenesis driven by transplanted donor derived cells. Interestingly, after sexual maturation, sperm motility in chimeras derived from old donors (75%) and young donors (68%) was statistically insignificant. In addition, fertility rates showed no significant difference between chimeras from old donors (86.70 ± 14.50%) and those from young donors (85.03 ± 15.16%). Our results demonstrate that germ cells derived from aged, reproductively senescent males retain the intrinsic capacity to undergo complete spermatogenesis cycle. The aged germ cells restore fertility when placed within a young somatic gonadal niche. Thus, suggesting that the aged somatic environment is a primary driver of reproductive aging and age-related germ cell defects are reversible.

Keywords: *germline chimera, spermatogonia, fertility, reproductive aging*

Acknowledgements: *Czech Science Foundation project 26-21447S*

FUNCTIONAL ANALYSIS OF DND1 AND NANOS3 DOMAINS DURING PRIMORDIAL GERM CELL-LIKE CELL INDUCTION IN MEDAKA

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In teleost fish, cells that inherit an assembly of maternal factors, including proteins and mRNAs, collectively known as the germ plasm (Gp), become primordial germ cells (PGCs). Among the Gp component genes, *dnd1* and *nanos3* are core factors that cooperatively regulate PGC formation in medaka (*Oryzias latipes*). Overexpression of these two components is sufficient to induce PGC-like cells (iPGC) from medaka blastomeres (Nishimura and Fujimoto, 2025). However, the molecular mechanism by which DND1 and NANOS3 cooperatively induce iPGCs remains unclear. To address this question, we performed in silico structural prediction and deletion analyses to identify domains involved in DND1-NANOS3 interaction and iPGC induction.

The major functional domains of DND1 include RNA recognition motif (RRM)1, RRM2, and the double-stranded RNA binding motif. NANOS3 contains two zinc finger domains, CCHC1 and CCHC2. In silico analysis using AlphaFold2 predicted close spatial proximity between RRM1 and CCHC1, as well as between RRM2 and the 15-amino acid C-terminal domain (CTD) of NANOS3. When we examined iPGC induction using domain-deleted *dnd1* and *nanos3* constructs, we found that RRM1 and RRM2 of DND1, as well as the CTD and two zinc finger domains of NANOS3, are required for iPGC induction. Furthermore, while co-overexpression of zebrafish NANOS3 and medaka DND1 failed to induce iPGCs, replacing the zebrafish NANOS3 CTD with the medaka CTD successfully induced iPGCs. These results suggest that DND1 and NANOS3 likely function through direct interaction during iPGC induction, involving not only the well-characterized RRM and zinc finger domains but also the less-characterized CTD of NANOS3. In particular, the CTD may contribute to species-specific DND1–NANOS3 interactions, providing new insight into the molecular basis of iPGC induction and germ cell specification.

Keywords: *Primordial germ cells, DND1, NANOS3, medaka*

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References: *Nishimura and Fujimoto, Generation of primordial germ cell-like cells by two germ plasm components, dnd1 and nanos3, in medaka (Oryzias latipes). iScience, 28(3), 111977.*

STURGEON EMBRYOS AS A MODEL FOR GERMLINE SPECIFICATION: MITOCHONDRIAL CONTRIBUTIONS AND EVOLUTIONARY INSIGHTS

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Sturgeons represent an ancient lineage of vertebrates with several unique developmental characteristics, including holoblastic cleavage and vegetal pole localization of germ plasm. Similar features are found in tailless amphibians; however, in sturgeons, germ plasm is spatially separated from precursors of other cell lineages, particularly endoderm. This unique organization provides an exceptional opportunity to investigate primordial germ cell (PGC) specification and the role of maternal determinants during early embryogenesis.

Using a combination of germ plasm visualization, targeted vegetal pole labeling, and PGC tracing approaches, we demonstrated that sturgeon PGCs are specified through a preformation mechanism and that germ plasm is exclusively localized at the vegetal pole. A novel blastomere inhibition system using 2,4-decadienal revealed that cleavage of the vegetal hemisphere is required specifically for germline development, whereas embryos otherwise developed normally in a pseudo-meroblastic manner. These findings suggest that sturgeon embryos may represent an evolutionary transitional state between holoblastic and meroblastic cleavage patterns.

To investigate the role of mitochondria in germline development, germ plasm mitochondria were eliminated by UV irradiation of the vegetal pole. This treatment disrupted germ plasm integrity and prevented PGC development. Transplantation of donor egg mitochondria restored germ plasm function and rescued PGC formation. Mitochondria isolated from different egg regions rescued PGC development equally well, suggesting that germ plasm-associated mitochondria do not possess unique functional properties. In contrast, mitochondria derived from somatic tissues failed to rescue PGC development. Furthermore, purified and cryopreserved egg mitochondria retained their functionality after transplantation.

Together, these findings demonstrate an essential role of mitochondria in sturgeon PGC development and provide new insights into germline specification, evolutionary developmental biology, and potential approaches for preserving matrilineal genetic information in endangered sturgeon species.

IDENTIFICATION OF DOWNSTREAM FACTORS OF DND1 AND NANOS3 INVOLVED IN PGC FORMATION USING TRIBE

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Germ cells are the only cells that transmit genetic information to the next generation. Elucidating the mechanisms underlying germ cell formation is not only biologically important but also expected to contribute to medical and industrial applications. In fish, germ cells are specified through a preformation mechanism, in which only cells that inherit a specialized cytoplasmic structure known as germ plasm, composed of maternally supplied proteins and mRNAs, acquire germ cell fate. It was recently demonstrated that two components of germ plasm, *dead end1* (*dnd1*) and *nanos3*, are cooperatively involved in primordial germ cell (PGC) formation in medaka embryos. However, the molecular mechanisms by which these factors regulate PGC formation remain unclear. Because DND1 and NANOS3 are RNA binding proteins, identification of their target RNAs is essential for understanding their functions. Nevertheless, comprehensive analyses of DND1 and NANOS3 target RNAs have not yet been performed in fish.

In this study, a comprehensive analysis of DND1 and NANOS3 target RNAs was performed using TRIBE (targets of RNA-binding proteins identified by editing). As a result, 409 genes were identified as candidate targets of DND1 and NANOS3. Gene Ontology analysis indicated that these genes are associated with processes such as translation and protein transport. In addition, transcriptome analysis of DND1 and NANOS3 overexpressing embryos was performed using RNA-seq, and the results were integrated with the TRIBE data to extract candidate genes directly regulated by DND1 and NANOS3. Furthermore, *de novo* motif analysis using STREME identified motifs enriched among DND1 and NANOS3 target genes. Among the motif-containing genes that are likely degraded by DND1 and NANOS3, several candidates known to function in somatic cell differentiation were identified. Overexpression of these genes severely impaired PGC maintenance and migration.

Together, these findings identify candidate downstream targets directly regulated by DND1 and NANOS3 which may repress somatic differentiation programs during PGC development.

Keywords: *Medaka, Primordial germ cell, Somatic cell differentiation, RNA binding protein*

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GERM CELLS DERIVED FROM HAPLOID EMBRYOS UNDERGO GENOME DOUBLING DURING THE STEM CELL-LIKE DIVISION PHASE IN MEDAKA (*ORYZIAS LATIPES*)

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Haploid cells contain a single set of chromosomes and provide a useful basis for genetic analysis, because mutating any recessive genes enables phenotypic characterization. However, in vertebrates, haploids are embryonic lethal, making it difficult to obtain gametes directly from haploid embryos. In zebrafish, transplantation experiments have shown that germ cells derived from haploid embryos survive in host embryos and likely undergo genome doubling during spermatogenesis, resulting in the production of isogenic sperm (Nagasaka et al., 2025). In our previous study, germ cells derived from haploid embryos were also shown to undergo genome doubling and differentiate into isogenic eggs or sperm in medaka. However, the mechanism underlying genome doubling remains unknown. To address this issue, we investigated at which stage of germ cell differentiation genome doubling occurs in haploid germ cells.

Medaka haploid embryos were generated by fertilization of eggs with UV irradiated sperm. Before hatching, the nuclear size of germ cells in haploid embryos was approximately half the size of those in diploid embryos. Because haploid embryos die before hatching, we generated haploid-diploid (1n-2n) germline chimeras by transplanting EGFP-labeled germ cells from vasa-EGFP transgenic haploid embryos into sterile diploid embryos. At 6 days post-hatching, when female germ cells in wild-type medaka have already initiated meiosis following stem cell-like division (type I) and cystic proliferation (type II), a subset of germ cells with stem cell-like division in 1n-2n germline chimeras exhibited nuclear sizes comparable to those of diploid controls. These results suggest that genome doubling in germ cells derived from haploids occurs during the stem cell-like division. To further test this possibility, we generated 1n-2n germline chimeras using haploid *meioC* mutants as donors. In these mutants, germ cells remain in stem cell-like division phase, while entry into cystic proliferation and meiosis is arrested. The chimeric testes primarily consisted of 2C germ cells, which mirrored the cell profile of diploid *meioC* mutant gonads. This finding indicates that genome doubling of germ cells derived from haploids occurs during the stem cell-like division phase.

Altogether, our results demonstrate that haploid germ cells undergo genome doubling during the stem cell-like division phase and subsequently produce functional sperm and eggs. We propose that early-stage germ cells, possibly germline stem cells, function as a ploidy checkpoint, ensuring that germ cells acquire one set of homologous chromosomes required for meiotic progression.

Keywords: *genome doubling, germline chimera, ploidy, stem cell division*

Acknowledgements: *JSPS Research Fellowship DC2 (25KJ0487), Hokkaido University EXEX Doctoral Fellowship.*

Reference: *Nagasaka et al., Isogenic fish sperm produced by transplanting gynogenetic haploid-derived germ cells (2025). Biology of Reproduction, 112 (3), 572-582.*

TRIALS FOR THE *IN VITRO* MATURATION OF SMALL-SPOTTED CATSHARK OOCYTES

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In teleosts, the increase of the oocyte diameter and the migration of the nucleus to the periphery of the cell happens during the ovulation. We made some trials to know if these are also good indicators of oocyte maturation in elasmobranchs. The small-spotted catshark, *Scyliorhinus canicula*, was used as a model species to set up *in vitro* techniques, trying to identify indicators of oocyte maturity and evaluating the effects of hormonal inductors.

A biometric analysis was performed on 58 females, from which 16 individuals with mature oocytes were selected to study nuclear position. For the determination of nuclear position, ovaries were extracted, and oocytes with a diameter between 6 and 15 mm were selected, boiled, transversely cut and assigned to different nuclear migration categories (A, B, or C). Oocytes belonging to all three nuclear migration categories were found across all oocyte diameter ranges. Moreover, the mean oocyte diameter was very similar among the three migration categories. These results made evident that the position of the nucleus is not a good indicator of the oocyte stage of maturation in this elasmobranch.

A proper incubation medium (pH 7.7 and 1000 mOsm/kg) was developed for *in vitro* maturation trials after analysing female's plasma pH and osmolality. Preliminary *in vitro* maturation trials were then conducted with 2 females, extracting the oocytes post-mortem and using dihydroxyprogesterone (DHP) at several concentrations (0.1 and 1 µg/mL). Oocytes diameter (registered as a potential maturity indicator) was measured at 1, 6 and 24 h. Secondary trials were then carried out for another 4 females, using DHP at 0.1, 1 and 10 µg/mL and measuring oocytes' diameter for a longer period (every 24h for 3 days, and then every 48 h), trying to evaluate its usefulness as a maturation indicator. However, none of these trials or DHP concentrations appeared to induce a significant increase of the oocytes diameter. New maturation trials are required using different conditions.

Keywords: *assisted reproduction, incubation, conservation, shark, elasmobranch.*

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TOTIPOTENCY WITHOUT A CLEAN SLATE: EPIGENOME DYNAMICS ACROSS THE TELEOST LIFE CYCLE

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In placental mammals, DNA methylation is erased almost completely twice in each generation, once after fertilisation and again in primordial germ cells. This has long been taught as the way vertebrates reset their developmental potential and achieve totipotency. Teleosts such as zebrafish and medaka, however, retain high levels of DNA methylation throughout cleavage, and rather than erasing the parental methylomes, they remodel the maternal epigenomic contribution to match the paternal one, so that by zygotic genome activation the embryo carries a sperm-like methylation landscape. The germline follows the same principle: zebrafish primordial germ cells and male germline cells retain the methylome established during early development rather than resetting it. It therefore appears that retention, rather than erasure, represents the ancestral vertebrate condition, with global epigenome erasure and re-establishment being a mammalian innovation. I will discuss what fish gametes and embryos tell us about how totipotency can be established without erasing what the parents transmit, and what this implies for epigenetic inheritance in species that never wipe the record clean.

REPHEAT PROJECT: MULTI-OMIC APPROACHES TO UNVEIL POTENTIAL TRANSGENERATIONAL EFFECTS AND MECHANISMS OF CLIMATE CHANGE IN ONCORHYNCHUS MYKISS

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Several studies characterized the potential effects of global warming in fish breeders, but little is known about the effects of extreme heatwave (HW) events, the related molecular mechanisms, and how they might be transferred to the resulting offspring. Multi-omic approaches have been conducted to address these questions. Rainbow trout male breeders were exposed or not to a commonly recorded HW in Spain. Hormonal and redox status at blood plasma, sperm quality, and cell proliferation and apoptosis in the testes were assessed in parallel with a mRNA transcriptomic analysis (RNA-Seq). Males exposed to a HW had increased cortisol levels, decreased total antioxidant status and testosterone plasma levels. HW reduced sperm quality, inhibited spermatocyte type I and II differentiation and increased spermatozoa apoptosis. These effects were correlated with the differential expression (DE) of 116 and 1040 (up- and down-regulated, respectively) genes in the testis, reflecting an altered sperm maturation process. We next performed a small non-coding RNA-Seq (snRNA-Seq) in the same testis samples and identified 8 DE miRs (including miR-221-5p and miR-146-3p). A computational prediction of putative mRNAs targets and its cross-dissection with RNA-Seq results revealed that these DE miRs post-transcriptionally regulate 157 genes. In vitro cell transfections using luciferase reporter assays were conducted to validate bioinformatic predictions. Furthermore, although miR-221-5p and miR-146-3p DE was not transferred from the sperm to progenies, miR-221-5p was found DE in the blood plasma of breeders subjected to a HW, being a suitable and less invasive biomarker of reproductive status under HW events. Since miRs does not seem to be the vehicles of transgenerational effects, the DNA methylation profile in progenies was explored. Enzymatic Methyl-sequencing (EM-Seq) analysis from progenies originated from exposed versus non-exposed males was performed in the DNA isolated from gills. Differentially methylated regions (DMRs) in different genomic contexts were identified, and potentially downstream regulated genes unveiled, suggesting that DNA methylation is the transgenerational transmission mechanism. Present study not only unveils the potential effects of the extreme events under a climate change scenario, and the underlying mechanisms, but also the potential adaptive mechanisms (DNA methylation) in future farmed fish.

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SINGLE-CELL TRANSCRIPTOMIC ATLAS REVEALS AN INTERPLAY BETWEEN GERM CELLS AND THE IMMUNE SYSTEM DURING THE RESUMPTION OF SPERMATOGENESIS IN ADULT ZEBRAFISH

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Spermatogenesis is a strictly orchestrated and dynamic process sustained by spermatogonial stem cells, which balance self-renewal and differentiation to ensure continuous sperm production. To better understand the cellular dynamics and molecular mechanisms governing spermatogonial activation and differentiation, adult male zebrafish were exposed to 17 β -estradiol (E2; 10 nM) for 28 days to induce a reversible arrest of spermatogenesis, followed by recovery periods of 0, 2, and 4 days in hormone-free water (REC-0, 2, and 4, respectively). These recovery windows were previously established through histological analyses to ensure the selected time points accurately captured the early stages of spermatogonial reactivation and differentiation. Testes were collected for scRNA-seq using the 10x Genomics Chromium platform and analyzed through an integrated bioinformatics workflow, including clustering, differential expression, RNA velocity, trajectory inference, CellChat, and functional enrichment analyses. RNAscope was used to spatially validate cluster specific genes. Single-cell profiling generated a zebrafish testis atlas during spermatogenic recovery, identifying 19 cell clusters, including germ, stromal, support, and immune cells. Early spermatogonial populations displayed marked heterogeneity and were further resolved into distinct subclusters representing progressive differentiation states. Trajectory inference analyses revealed a continuous transition from undifferentiated to differentiated germ cells, highlighting transcriptional programs associated with lineage commitment and cellular maturation. While REC-0 exhibited higher RNA velocity in later trajectory states, REC-2 showed enhanced state-transition dynamics in intermediate cell states. In contrast, REC-4 displayed lower mean pseudotime values and increased RNA velocity in earlier/less differentiated cell populations, suggesting that germ cell proliferation resumed 4 days after E2 withdrawal. In addition, we identified multiple immune-associated populations in the recovering testis: B cells, mast cells, neutrophils, macrophages, serpin-secreting cells, and T/NK cells. Given that the testis is an immune-privileged environment, we investigated the interplay between these immune populations and spermatogonial cells. Cell-cell communication analyses predicted ligand-receptor interactions involving APP, GNR, SEMA7, CXCL, MIF, TNF, and LT signaling pathways in the cell clusters, suggesting extensive crosstalk between immune and germ cell populations during spermatogenesis resumption. Together, these findings provide for the first time a high-resolution view of zebrafish spermatogenesis recovery, revealing dynamic immune–spermatogonial interactions, transcriptional reorganization, and enrichment of early cellular states. Altogether, these findings advance our understanding of the molecular mechanisms underlying spermatogenesis onset and provide valuable insights into reproductive biology.

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MALE-TO-FEMALE TRANSFORMATION INVOLVES A RETURN TO A NEAR-UNDIFFERENTIATED GONADAL TRANSCRIPTOMIC STATE IN ASIAN SEABASS (*LATES CALCARIFER*)

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Sequential hermaphroditism provides a natural model for studying how a differentiated vertebrate gonad is remodeled into the opposite sexual state. In protandrous Asian seabass (*Lates calcarifer*), a functional testis is transformed into an ovary, yet the timing and hierarchy of the underlying events remain poorly understood. We integrated gonadal histology, cannulation, transcriptome profiling, and small RNA analysis to reconstruct sex change across four consecutive gonadal states: testis, two transforming intermediates, and ovary. Rather than beginning with direct ovarian differentiation, sex change started with a broad transcriptomic shift away from mature testicular identity toward a near-undifferentiated, bipotential-like state. This reset phase preceded overt activation of ovarian pathways, indicating that loss of male identity and acquisition of female identity are temporally separable.

mRNA profiles showed extensive remodeling throughout the transition, whereas small RNA changes were modest. This indicated that a global miRNA-mediated repression is not the dominant driver of the process. Functional enrichment indicated a sequential cascade beginning with immune activation, cytotoxicity, and apoptosis, consistent with clearance of the spermatogenic compartment. Later stages were enriched for oogenesis, chromatin organization, and germline reprogramming, followed by reduced signaling complexity as ovarian identity stabilized. Population-level sampling showed that both timing and outcome of the process are flexible. Across captive cohorts, histology data and cannulation results showed evidence for primary females and long-lived terminal males. Thus, sex change in Asian seabass appears to follow a plastic developmental trajectory rather than a strictly synchronized age- or size-dependent switch.

Together, our findings refine the model of protandry by identifying an early reset window before feminization. Our study highlights immune-mediated remodeling, mRNA reprogramming, and chromatin-associated regulation as candidate drivers of sex change, while providing a stage-resolved

resource for understanding vertebrate sexual plasticity and improving sex-ratio management in Asian seabass aquaculture.

Keywords: *barramundi, sequential hermaphroditism, gonadal transformation, natural sex change, sex-ratio management*

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THYROTROPIN-RELEASING HORMONE RECEPTOR AS A KEY DRIVER OF SEXUAL FATE

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Thyrotropin-releasing hormone (Trh) is a peptide that acts in mammals as hormone and plays an important role in regulating thyroid function *via* the hypothalamic-pituitary-thyroid axis. It binds to specific receptors in the pituitary gland (Trhr) to stimulate the release of thyroid-stimulating hormone (Tsh), which in turn stimulates the production of thyroid hormones (T3 and T4). In mammals, Trh receptors are not only found in this classic thyroid pathway, but are also expressed in various parts of the brain and spinal cord, as well as in the pancreatic islets, the stomach, the heart, the prostate and the testicles. In recent studies conducted in our laboratory, we have identified the expression of *trhr* in the undifferentiated gonads of Siberian sturgeon, showing that it is activated in future female gonads at a specific point in time (2.5 months).

This led us to believe that this receptor may be playing an important role in the female sexual differentiation of sturgeons. To investigate this idea, we studied the expression levels of *trhr* in the gonads of two different cohorts of Siberian sturgeon from 2.5 to 6–7 months of age, covering the period of sexual indifferentiation (2.5 to 4 months) and the period of gonadal structuration (4.5 to 6–7 months). Furthermore, its expression was studied after masculinizing treatments with 17methyl-testosterone and after injection of estradiol-17 β using qPCR.

The results show clear activation of *trhr* in future female gonads from 3 to 7 months of age, indicating a participation both during sex differentiation and the period of female gonadal development. In turn, during the masculinisation of genetic females following treatments with 17-methyl-testosterone, a strong suppression of *trhr* is observed at 2.5, 3 and 4 months of age. Conversely, this gene is independent of estrogens, unlike *foxl2*.

By integrating these results with previous data obtained from the Siberian sturgeon, we propose that the gene encoding *trhr* is integrated into the molecular pathway of female gonadal differentiation as follows: *hsd17b1* at 2,5 months of age and *cyp19a1* (aromatase), *foxl2* and *trhr* at 3 months of age.

In turn, repression by 17MT, alongside the repression of other female genes such as *hsd17b1*, *aromatase* and *foxl2*, allows *trhr* to be postulated as another key gene in the female gonadal differentiation pathway.

Keywords: sex differentiation, gonad development, gene expression

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OVULATION-DRIVEN TRANSCRIPTOMIC REORGANIZATION DEFINES MATERNAL RNA PROVISIONING IN PIKEPERCH OOCYTES

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In teleost fishes, developmental competence of the embryo depends critically on maternally inherited transcripts that control early development before zygotic genome activation. Despite their importance for reproductive success and aquaculture production, the precise timing and mechanisms governing maternal transcriptome assembly during final oocyte maturation (FOM) remain poorly understood. This study employed time-resolved RNA-sequencing to characterize transcriptomic dynamics across four successive stages in pikeperch (*Sander lucioperca*): late vitellogenesis (S1), end of vitellogenesis (S2), onset of FOM (S3), and ovulated eggs (S4). To eliminate inter-individual variation, repeated intra-female sampling was performed on high-quality, competent oocytes.

Comparative transcriptomic analysis revealed considerable stability of maternal RNA abundance from late vitellogenesis through the onset of FOM, followed by a pronounced transcriptomic switch right before ovulation. More than 8,000 genes exhibited significant differential abundance between S3 and S4, whereas earlier transitions showed only minor adjustments. Genes enriched in ovulated eggs were predominantly associated with RNA processing, splicing, cytoskeletal organization, cell cycle regulation, and post-transcriptional control mechanisms. Conversely, transcripts related to translation and ribosome biogenesis were markedly decreased at ovulation. Also, we identified distinct gene groups driving this transition, many of which overlapped with previously reported egg-specific genes and egg-quality markers, providing a robust set of maternal-effect candidates which can be used as pikeperch-specific quality markers.

These findings demonstrate that maternal transcriptome formation in pikeperch is finalized abruptly during ovulation rather than progressively throughout oogenesis, identifying ovulation as a critical checkpoint in maternal RNA reorganization. Consequently, this work advances understanding of post-transcriptional regulation during fish oocyte maturation and establishes a genomic framework for investigating molecular determinants of egg competence in teleosts.

Keywords: maternal transcriptome, oocyte maturation, RNA-sequencing, egg quality

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TRANSCRIPTOMIC AND PROTEOMIC SIGNATURES OF EARLY *IN VIVO* POST-OVULATORY AGEING IN PIKEPERCH EGGS

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Egg quality variability remains a major limitation for reproductive success in aquaculture. This study examined the molecular basis of *in vivo* post-ovulatory ageing in pikeperch (*Sander lucioperca*), associated with prolonged egg retention within the ovaries after ovulation. Eggs collected at 1 and 5 h post-ovulation (hPO) were analyzed using integrated transcriptomic and proteomic approaches to identify molecular events accompanying the earliest stages of egg quality decline and candidate biomarkers associated with reduced developmental competence. Despite the absence of visible morphological abnormalities up to 5 hPO, both fertilization and hatching rates were markedly decreased at the later sampling point ($p < 0.01$). Quantitative TMT LC–MS/MS proteomic profiling revealed four proteins associated with early post-ovulatory ageing (gins4, atrx, dnaJB14, and mrpl10; FDR < 0.05), mainly related to chromatin remodeling, DNA replication, mitochondrial activity, and protein homeostasis. Transcriptomic analysis identified 202 differentially abundant transcripts between 1 and 5 hPO, including 82 downregulated and 120 upregulated transcripts in aged eggs. Functional enrichment analysis indicated that the affected genes were primarily involved in RNA processing and cellular component organization, highlighting the importance of these pathways for maintaining egg functionality and developmental potential. In addition, network analysis identified several hub genes, including *ago2*, *ep300*, and *kmt2c*, as putative regulators of egg competence, whereas *ivns1abp* and *setdb1* emerged as promising evolutionarily conserved markers of egg quality deterioration. Overall, the results demonstrate that early *in vivo* post-ovulatory ageing strongly affects the egg transcriptome despite a relatively stable proteome. These findings suggest that egg collection in pikeperch should occur within 4 h after ovulation to maintain optimal reproductive performance and provide novel candidate markers for early molecular assessment of egg quality.

Keywords: pikeperch, reproduction, egg quality, *in vivo* ageing, proteomics, transcriptomics

Acknowledgements: This work was funded by the National Science Center, Poland (NCN) [grant number 2016/22/M/NZ9/00590]

COMBINED PHYSICOCHEMICAL AND PROTEOMIC PROFILING IDENTIFIES BIOCHEMICAL ASSOCIATIONS OF OOCYTE QUALITY AND FERTILISATION OUTCOME IN ATLANTIC SALMON (*SALMO SALAR* L.)

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Introduction

Oocyte quality plays a significant role in fertilisation success and early embryonic development in Atlantic salmon. However, indicators that reflect oocyte quality prior to fertilisation remain limited in aquaculture production systems. This study investigated physicochemical characteristics of oocytes and ovarian fluid (OF) of Atlantic salmon together with proteomic profiling across developmental stages to identify oocyte quality parameters associated with fertilisation outcome.

Materials and Methods

Oocytes and OF were collected from 16 Atlantic salmon females under commercial hatchery conditions. Fertilisation success was assessed at the cleavage stage (4–8 cell stage) and used to classify oocyte batches into high-quality ($\geq 90\%$) and low-quality ($< 90\%$) groups for descriptive comparisons. Physicochemical parameters including OF pH, aspartate aminotransferase (AST), osmolality, oocyte ATP concentration, and relative weight gain in oocytes during water hardening (RWH) were analysed. Proteomic profiling was conducted on ovarian fluid (OF), oocytes, eyed eggs, and hatched larvae from high- and low-quality oocyte batches. Associations between physicochemical parameters, protein abundance, and reproductive outcomes (fertilisation, eyed egg, and hatching success) were evaluated using Pearson correlation and regression analyses.

Results

Oocyte batches classified as high quality (HQ) exhibited significantly higher ovarian fluid (OF) pH than low-quality (LQ) batches (8.70 ± 0.04 vs 8.46 ± 0.16 , $P = 0.039$), whereas AST activity was significantly lower (13.4 ± 5.5 vs 59.8 ± 19.5 milliunits/mL, $P = 0.005$). Relative water hardening was positively associated with eyed egg and hatching success, whereas oocyte ATP concentration and OF osmolality were not significantly associated with fertilisation outcome. Proteomic analysis identified 135 proteins significantly correlated with fertilisation outcome or physicochemical parameters ($FDR < 0.1$), of which 80 were detected in OF. These proteins were primarily associated with proteostasis, translation, lipid metabolism, immune responses, and extracellular matrix organisation.

Discussion

The findings highlight OF as an important biochemical environment associated with oocyte quality and fertilisation performance. Among the ovarian fluid proteins most strongly associated with reproductive outcomes, semaphorin-3ab-like isoform X2, selenoprotein M, and nucleobindin 2 were identified as candidate markers that may help characterise oocyte quality prior to fertilisation. Overall, these findings provide a foundation for further investigation of the molecular and physicochemical factors associated with oocyte quality and reproductive performance in Atlantic salmon.

Keywords: *Ovarian fluid, salmonids, reproductive success*

DEVELOPMENT OF AN *IN VITRO* CULTURE SYSTEM FOR ATLANTIC SALMON GERM CELL PROPAGATION, TRANSPLANTATION AND GENETIC MANIPULATION

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Culture of type A spermatogonia (ASG), including germline stem cells (sperm- and egg-precursor cells), and their manipulation *in vitro* have emerged as a promising biotechnology tool for potential use in both conservation of endangered species and modern aquaculture production systems. When combined with cryopreservation and surrogate broodstock technologies, ASG cultures enable the rapid expansion of germplasm from valuable fish strains/lines, providing a cutting-edge strategy to mass-produce donor-derived gametes carrying desired genetic material. Moreover, beneficial production traits of farmed fish can be induced *in vitro* through precise genetic modifications directly on germ cells.

Here, for the first time in Atlantic salmon, we have achieved prolonged and successful *in vitro* culture of ASG. For that, salmon ASG were co-cultured with rainbow trout Sertoli feeder cells showing clear mitotic activities, as confirmed by immunodetection of a mitosis marker coupled with significantly increasing numbers of ASG. Cultured ASG also showed increasing expression of germ cell-specific gene markers throughout *in vitro* expansion over a period of 41 days. Notably, in addition to ASG proliferation, gene expression analysis of meiosis markers indicated potential spontaneous germ cell differentiation after a prolonged period of culture, a phenomenon that can be advantageous for follow-up studies on *in vitro* spermatogenesis. To assess whether *in vitro* expanded ASG retained their ability to colonize recipients' genital ridges, 21-day cultured germ cells were labelled with PKH-26 red-fluorescent dye and transplanted into F2 *piwil1* mutant (germ cell-free) salmon hatchlings. Evaluation of transplanted fish at 55 days post-transplantation showed successful colonization of fluorescent cells within recipients' genital ridges. Additionally, electroporation of enriched ASG, albeit with residual somatic cells with CRISPR/Cas9 ribonucleoproteins resulted in up to 18% mutation rates. Protocols are being developed for enrichment and clonal expansion of CRISPR-ed spermatogonial cells. Together, we have established a culture system which may be used to accelerate the genetic improvement of key production traits in salmon aquaculture.

Keywords: germline stem cells, surrogate broodstock technology, CRISPR/Cas9.

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SHORT-CHAIN FATTY ACIDS AS GUARDIANS OF FISH SPERMATOGENESIS

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Postbiotics such as short-chain fatty acids (SCFAs), produced through microbial fermentation, are emerging as modulators of reproductive physiology, with butyrate showing promising protective properties¹. In contrast, environmental endocrine-disrupting chemicals (EDCs), frequently present in aquatic systems as complex mixtures, pose a significant threat to fish reproduction and gamete quality. Among them, glyphosate (GLY) and perfluorooctanoic acid (PFOA) are commonly detected in water systems and contribute to chronic low-dose reproductive toxicity in aquatic organisms. This study investigated whether sodium butyrate (SB) can mitigate the potential toxic effects induced by a GLY+PFOA mixture in zebrafish testes using an *ex vivo* paired-organ culture model. For each specimen, one testis served as control while the contralateral was exposed to SB (1 mM), GLY+PFOA (0.5 µg/L each, consistent with EU Drinking Water Directive limits for total pesticides and PFAS²), or GLY+PFOA+SB at previously reported doses. Spermatogenic progression was evaluated histologically, and histone acetylation marks (H3K9ac and H4K12ac) were analyzed in diploid and haploid germ cells by immunohistochemistry. Exposure to the GLY+PFOA mixture disrupted spermatogenesis, leading to an accumulation of type B spermatogonia and a reduction in spermatozoa abundance and cysts area, suggesting impaired germ cell differentiation and compromised gamete production. Notably, co-treatment with SB restored spermatozoa levels to values comparable to controls, highlighting a mitigating action of butyrate on male gametogenesis. At the epigenetic level, SB enhanced H3K9ac in both testicular germ cells and selectively increased H4K12ac in haploid germ cells, consistent with its HDAC inhibitory activity³, whereas GLY+PFOA exposure altered H3K9ac specifically in diploid cells. Importantly, co-exposure of GLY+PFOA with SB returned H3K9ac to control levels.

Overall, these findings demonstrate that environmentally relevant mixtures of GLY and PFOA impair zebrafish spermatogenesis, while they support the potential role of sodium butyrate in safeguarding fish reproductive health under contaminant exposure and highlights the need for further mechanistic and *in vivo* studies.

Keywords: spermatogenesis, endocrine-disrupting chemicals, postbiotics, zebrafish

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UTILIZATION OF MICROFLUIDICS FOR THE SUCCESSFUL ENRICHMENT OF SPERMATOGONIAL STEM CELLS

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The aim of this study was to develop an alternative methodology for the enrichment of fish spermatogonial stem cells (SSCs) by using microfluidics. Currently, main techniques for the enrichment of SSCs entail density gradient centrifugation by using Percoll or Ficoll gradients or fluorescence- or magnetic-activated cell sorting (FACS or MACS). However, gradient density centrifugation leads to very poor cell enrichment, while FACS/MACS necessitate molecular markers specific for the target cells which, in case of SSCs, are not developed. Microfluidic sorting by using spiral microfluidic chips enables separation of cells solely based on their size which takes advantage of the SSCs morphology as the largest testicular cells.

To confirm the utility of cell sorting via spiral microfluidic chips, we first conducted trials on sorting polystyrene beads of 6, 8 and 10 µm in diameter, representing the average diameters of SSCs, spermatogonia type B and early spermatocytes. Chip units of 4 and 8 spirals were used, and we tested different flow speeds from 0.5 to 3 ml/min. Around 80% enrichment of 10-µm beads was accomplished by using the 8-spiral unit and a flow speed of 0.5 ml/min. When testing different bead concentrations from 1 to 10 million beads/ml, the sorting success was comparable. Hence, we dissociated the testicular tissue of *vasa::egfp* zebrafish, as well as African catfish, and conducted a sorting trial as previously optimized on beads. For both species, enrichment of SSCs comparable to the beads tests was observed. These results indicate that microfluidics can be effectively used for sorting of SSCs, and can offer a simpler and cost-effective alternative to currently utilized sorting technologies.

Keywords: *cell sorting, spiral microfluidics, spermatogonia, spermatogonia transplantation, surrogate broodstock production*

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PHOTOPERIOD AND FEEDING ALTER THE TIMING AND INCIDENCE OF ENTERING PUBERTAL SPERMATOGENESIS IN ATLANTIC SALMON GRILSE

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Precocious puberty is a major animal welfare and economic problem in salmon farming. We examined in one sea winter Atlantic salmon in net pens how two feeding levels (normal or restricted [fed on three of seven days]) from September to June, in combination with two photoperiod conditions (natural light or additional continuous light) from mid-January to June, modulated the incidence, timing and progress of puberty. In the normal light-normal feed (NN) group, most males (97%) were maturing in June, half of them having entered puberty at the beginning of December, with complete recruitment by the end of January. Males exposed to normal light and restricted feed (NR group) showed lower body size, condition factor, adiposity and a somewhat reduced (74%) incidence of puberty. Continuous additional light from mid-January while receiving normal feed (CN group) clearly reduced the rate of maturation to 28%. Combining the two restrictive conditions in the CR group resulted in the lowest incidence of maturation of 19%. Analysis of individual, repeatedly taken “live” blood samples for plasma 11-ketotestosterone (11-KT) profiles over time, revealed long-term effects of feeding levels and both, short- and long-term effects of additional continuous light on body weight, androgen levels, and on the incidence, timing of entering, or continuation of already initiated puberty. Analyzing terminal samples taken at 6 times during the experiment revealed differential changes in the four groups regarding GSI, testis histology, proliferation activity and pituitary expression of selected transcripts (*fshb*, *lhb* and *gnrhr2bba*) that reflected treatments and their effects on maturation. In all cases, rising *fshb* transcript and 11-KT plasma levels were closely associated with elevated proliferation activity of Sertoli cells and type A spermatogonia upon entering puberty, taking place one month before the GSI increased. An individual’s chance to enter puberty next spring was positively correlated to the body size in September and increased with the 11-KT plasma level and growth rate in Winter. The effect of continuous light from mid-January onwards varied with the individual’s state of development. Maturation was suppressed in males that did not yet enter, or were on the brink of entering puberty, while an accelerated further development was triggered in males that apparently had passed a point of no return. Overall, restricted feeding exerted relatively mild effects despite resulting in clear growth retardation, while constant light had clear effects, the direction of which depended on the individuals’ state of development when light exposure started.

Keywords: Testicular maturation, caloric restriction, continuous light, follicle-stimulating hormone, androgens, germ and Sertoli cell proliferation.

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DIRECT EVIDENCE OF THE PHAGOCYTIC PROPERTIES OF *IN VITRO* CULTURED ZEBRAFISH SERTOLI CELLS TOWARD SPERMATOGONIA

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BACKGROUND: In teleost fishes, spermatogenesis occurs with coordinated interactions between germ cells and somatic cells, wherein Sertoli cells act as supporting units that encapsulate developing germ cells, controlling their anchoring and chemical environment, facilitating the exchange of nutrients, and regulating the development of germ cells by responding to and translating hormonal signals. As part of maintaining this environment, mammalian Sertoli cells perform phagocytosis on apoptotic germ cells to reduce contamination from necrotic cells and allow for healthy germ cells to continue developing. While phagocytosis of germ cells has been previously described in rodent models, this process remains poorly documented in teleost fishes, with the exception of seasonal species during the regression phase, when aged spermatozoa need to be removed. We recently observed testicular hypertrophy in aged zebrafish, 3D testicular imaging showed meiotic arrests with accumulation of spermatogonia at the periphery of the tubules. Due to the nature of the age-related testicular hypertrophy, we hypothesized that phagocytosis could be much more prevalent in cell cultures from hypertrophic males as opposed to those from younger males, as Sertoli cells are attempting to curb the accumulation of spermatogonia in the tubules. Reporter zebrafish lines have been generated previously to track specific testicular cells including Sertoli and spermatogonial cells.

OBJECTIVE: By culturing fluorescent testicular cells *in vitro* from aged zebrafish (*Danio rerio*) displaying testicular hypertrophy, we aimed to provide direct evidence of the phagocytic function of fish Sertoli cells toward spermatogonia.

METHOD: In order to facilitate the investigation of the Sertoli cell phagocytic properties, two zebrafish reporter lines were crossed to generate offspring expressing GFP in Sertoli cells and DsRed2 in A spermatogonia. Testicular cells were dissociated, co-cultured, and monitored over the course of approximately 15 days using an inverted epifluorescence microscope.

RESULTS: Red vacuoles were observed in the cytoplasm of the green fluorescent Sertoli cells only a few days after the start of the co-culture. These red fluorescent vacuoles constitute a direct indicator of spermatogonial cell phagocytosis.

CONCLUSION: Our data suggests that the phagocytic activity of zebrafish Sertoli cells toward spermatogonia can be induced *in vitro* following disruption of testicular organization. Co-cultures of fluorescent Sertoli and germ cells constitute a promising *in vitro* tool to investigate molecular mechanisms and regulations involved in the phagocytosis of spermatogonia by Sertoli cells.

Keywords: Zebrafish, spermatogenesis, Sertoli cell, spermatogonia, phagocytosis, testicular hypertrophy

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TEMPERATURE-DEPENDENT REGULATION OF SECRETED NOVEL AID/APOBEC-LIKE DEAMINASES (SNAD1S) IN COMMON CARP (*CYPRINUS CARPIO*) GONADS

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Activation-induced cytidine deaminases (AID/APOBEC) proteins are zinc-dependent enzymes that catalyze cytidine-to-uridine deamination in nucleic acids, thereby contributing to a wide range of genomic, epigenetic, and immune regulatory processes. Secreted Novel AID/APOBEC-like Deaminases (SNAD1s) represent a recently identified family of fish-specific deaminases distinguished by the presence of a signal peptide, a feature absent in classical intracellular AID/APOBEC proteins. Although accumulating evidence suggests that SNAD1 proteins are involved in immune responses, environmental adaptation, and thermal acclimation, their biological functions remain largely unexplored. In particular, little is known about the regulation of *snad1* genes in reproductive tissues. Therefore, the aim of this study was to investigate the effects of thermal acclimation on the expression of all currently identified *snad1* paralogs in the gonads of common carp (*Cyprinus carpio*).

Fish were maintained at 10°C, 18°C, or 30°C, and ovarian and testicular tissues were collected after one and two weeks of acclimation. Expression of 13 *snad1* genes was quantified using multiplex PCR. All analyzed *snad1* paralogs were detected in both gonadal tissues; however, their expression levels differed significantly among genes, temperatures, and sexes. Several paralogs, including *snad_318* and *snad_810*, exhibited consistently high transcript abundance across all experimental groups. Exposure to elevated temperature was associated with significant transcriptional changes, particularly in testes, where increased expression of multiple *snad1* genes was observed. In contrast, ovarian expression profiles remained comparatively stable, indicating sex-dependent regulation of *snad1* transcription in response to thermal conditions.

The pronounced thermal responsiveness of selected *snad1* paralogs supports the emerging hypothesis that SNAD1 proteins participate in physiological adaptation to environmental stress. These findings provide further evidence that SNAD1 enzymes may represent previously unrecognized molecular factors linking environmental sensing with reproductive and immune-related processes in teleost fish.

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AN ACCELERATED SELECTION METHOD YIELDS A PIGMENT-DEFICIENT TRANSGENIC REPORTER LINE IN ZEBRAFISH TO STUDY GONADAL TRANSFORMATION

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The primary objective of our research is to investigate vertebrate sexual development using the zebrafish as a model organism. During zebrafish gonadogenesis, most individuals initially develop a transient “juvenile ovary.” In males, this structure subsequently undergoes apoptosis and is transformed into a testis [1]. Despite its importance, the cellular and molecular mechanisms underlying this transition remain incompletely understood. To visualize gonadal development and transformation *in vivo*, we utilize the *tg{ddx4::egfp}* transgenic zebrafish line (previously *tg{vas::egfp}*; [2–3]), which specifically labels germ cells with Egfp fluorescence.

However, pigmentation in wild-type zebrafish significantly limits the *in vivo* detection of ovarian Egfp signals. To overcome this limitation, we introgressed the transgene into the pigment-deficient double mutant transparent zebrafish line, *casper* [4]. The resulting homozygous transgenic line established on the *casper* background was named *gáspár* (pronounced: ghaashpar).

Conventional introgression of a transgene into a double recessive mutant background generally requires at least three generations [5,6]. To accelerate this process, we developed an efficient selection strategy enabling the identification of homozygous transgenic *casper* individuals within slightly more than two generations. The protocol consists of four sequential selection steps based on: I) absence of black pigmentation; II) absence of reflective pigmentation; III) detection of Egfp fluorescence; and IV) PCR-based genotyping.

To further improve selection efficiency, we identified the genomic integration site of the *tg{ddx4::egfp}* transgene through whole-genome re-sequencing of the transgenic line. Detailed analysis revealed a ~3 kb deletion associated with the integration event. Primers designed against the deleted genomic region enabled direct PCR-based identification of homozygous individuals without the need for additional propagation. Sequencing data also allowed comprehensive characterization of the transgene integration pattern. Bioinformatic analyses suggested the presence of multiple integrated transgene copies, and primers targeting all possible copy junctions (head-to-head, head-to-tail, and tail-to-tail) were designed to determine transgene organization and copy number. Using this approach, we identified three integrated copies of the transgene. Two outer copies were initially detected in opposite orientations, followed by identification of an additional transgene copy located between them.

In parallel, we have initiated development of a novel transgenic reporter construct designed to visualize testis development. Once completed, the construct will subsequently be introduced into the

gáspár line to facilitate investigation of testis formation following the apoptotic phase of the juvenile ovary.

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SCALING UP ATLANTIC SALMON SPERM CRYOPRESERVATION: FROM RESEARCH-SCALE TRIALS TO COMMERCIAL HATCHERY PRODUCTION

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The aquaculture sector increasingly demands genetically specialised products, such as disease-resistant or monosex lines, that must remain available to customers year-round. Sperm cryopreservation allows genetic material from elite males to be stored indefinitely and deployed on demand, but protocols are typically developed and validated at research scale, using small numbers of straws and simple sample tracking. Transferring this technology into commercial Atlantic salmon egg production raises operational questions that research-scale trials rarely address: how to standardise quality across hundreds of straws from many males and multiple sites, how to preserve unambiguous sample identification throughout freezing and storage, and how to thaw and use large straw batches simultaneously without compromising fertilisation performance. This work describes how Benchmark Genetics addressed these challenges while scaling cryopreserved milt from a research tool into a routine production input across its Iceland, Norway and Chile sites, where combined cryobank storage now totals 14 tanks with capacity to hold milt sufficient to fertilize over 1.35 billion eggs from more than 550 production males.

Milt quality was standardised using computer-assisted sperm analysis (CASA) to measure motility, concentration and kinematic parameters before and after freezing, enabling the sperm-to-egg ratio to be refined and cryobank stock to be increased 1.3- to 2-fold from the same milt volume. Between 2016 and 2022, protocol optimisation reduced the straws required per female from 37 to 18 while increasing eggs fertilised per straw from 467 to 750. Post-thaw stability trials across multiple males found no significant decline in fertilisation or survival rate for up to five hours after thawing, supporting simultaneous batch thawing of large straw numbers without a fixed use-immediately constraint.

Continuous monitoring of fertilisation and eyed-egg survival rate as key performance indicators, with corrective actions such as milt re-analysis and dose readjustment, increased the proportion of shipments meeting the quality target (survival >80%) from 84.3% to 95.1%, and reduced out-of-limit shipments from 15.7% to 4.9%, across one production cycle. Together, these results show that combining objective instrument-based quality control, protocol standardisation and systematic performance monitoring allows sperm cryopreservation to be scaled reliably from research-level trials to multi-site commercial hatchery production, while maintaining sample traceability and fertilisation quality.

Keywords: *computer-assisted sperm analysis (CASA); fertilisation rate; milt quality control; protocol standardisation; thawing procedure; hatchery production.*

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RESTORING THE BIOLOGICAL MEANING OF CASA PARAMETERS USING HIGH-FRAME-RATE SPERM TRACKING

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Computer-assisted sperm analysis (CASA) is widely used to describe sperm motility through kinematic descriptors of head trajectories, including VCL, VAP, VSL, LIN, STR, WOB, ALH, and BCF. These parameters are not merely numerical outputs; they contain biologically meaningful concepts related to velocity, trajectory geometry, progression, lateral head displacement, and flagellar beat activity. However, this meaning can be weakened when video acquisition and processing are not transparent or insufficient to resolve the movement being measured. Moderate frame rates, smoothing, and tracking ambiguity may therefore alter measured values and obscure biological interpretation.

We developed an open, fully configurable CASA workflow in Python to revisit the biological meaning of CASA parameters and the logic underlying their calculation. The framework provides stepwise control from sperm head detection and centroid association to trajectory reconstruction, smoothing, visualisation, and parameter calculation. It calculates classical CASA parameters while allowing compatible extension toward beat-related and other biologically informative descriptors. Thus, the workflow clarifies how existing parameters are generated, what aspects of motion they represent, when their biological meaning is preserved or lost, and how CASA can be extended beyond descriptive motility assessment.

Using videos at different frame rates derived from an original high-speed recording, we examined how temporal resolution affects trajectory reconstruction and kinematic outputs. Increasing frame rate reduced displacement between consecutive sperm head positions, decreasing the search radius required for trajectory continuation and lowering the probability of false associations. At the same time, standard CASA metrics and beat-related measures changed systematically with frame rate, indicating that commonly reported values are strongly influenced by acquisition settings and tracking logic.

Overall, classical CASA parameters should be interpreted as sampling-dependent descriptors of reconstructed motion rather than fixed biological constants. High-frame-rate imaging combined with transparent, configurable tracking helps restore their biological significance and extends CASA from a descriptive motility tool toward a flexible experimental platform for analysing sperm performance.

Keywords: *motility assessment; flagellar beat dynamics; head kinematics; temporal resolution; trajectory reconstruction.*

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WHAT DEFINES HIGH AND LOW-QUALITY SPERM SAMPLES? AN INTEGRATIVE ANALYSIS IN THE EUROPEAN EEL

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The European eel is a species of high commercial and ecological value that is currently critically endangered. Although aquaculture is considered a key strategy to support both market demand and restocking programs, closed-cycle production remains unsuccessful, as reproduction still depends on hormonal induction to obtain gametes. However, hormonal treatments in this species often lead to variable sperm quality, compromising reproductive consistency and larval production. In parallel, seminal plasma extracellular vesicles (SP-EVs) have emerged as potential indicators of sperm quality variability through differences in their biophysical profiles; however, their role in fish remains poorly understood. In this context, the present study aimed to identify the features associated with high- and low-quality sperm in the European eel and explore potential correlations with SP-EV biophysical profiles. To generate sperm samples with distinct quality profiles, eel males ($n = 36$) were divided into two groups and subjected to two commonly applied hormonal treatments. Once spermiating, sperm quality was evaluated through an integrative approach combining multiple methods, including sperm motility, viability, oxidative stress markers (lipid peroxidation and DNA damage), and antioxidant enzyme activity (SOD, GPx, and GSR). Seminal plasma was also analysed to determine total antioxidant status (TAS), osmolarity, and pH. Based on these parameters, sperm samples were classified into high- and low-quality categories. Subsequent analyses included the evaluation of antioxidant- and stress-related gene expression (*sod1*, *sod2*, *gpx4*, *gsr*, *hsp70*, and *hsp90*) in sperm cell samples from each group and quality category. In addition, seminal plasma from low- and high-quality samples was separated into SP-EV-containing and EV-depleted fractions by size-exclusion chromatography (SEC). SP-EVs were isolated and characterized based on biophysical parameters, including size distribution and concentration, using tunable resistive pulse sensing (TRPS). Preliminary results suggest distinct functional and molecular patterns across sperm quality categories, while ongoing analyses of SP-EVs are expected to identify specific biophysical profiles associated with sperm performance. Overall, this work provides an integrative characterization of sperm quality in the European eel by combining multivariate analyses with emerging approaches, such as SP-EV profiling. Thus, it sets an innovative framework for improving reproductive management strategies while supporting future analyses, including SP-EV cargo molecular characterization.

Keywords: oxidative stress; antioxidant enzymes; gene expression; extracellular vesicles;

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OVARIAN FLUID ENHANCES SPERM PERFORMANCE BUT SHOWS SPECIES-SPECIFIC EFFECTS ACROSS NATIVE AND INTRODUCED SALMONIDS IN JAPAN

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The coexistence of native and introduced salmonids in Japanese rivers creates opportunities for interspecific reproductive interactions, with potential consequences for hybridization and population integrity. Ovarian fluid (OF), the medium surrounding eggs, plays an important role in shaping sperm behaviour and may influence these processes at the gamete level.

In this study, we examined how OF affects sperm performance across four salmonid species occurring in Japan (*Oncorhynchus mykiss*, *Salmo trutta*, *Oncorhynchus masou*, and *Salvelinus leucomaenis*). We combined computer-assisted sperm analysis, microcapillary chemotactic assays, and untargeted proteomic and metabolomic profiling to capture both functional and molecular aspects of OF-sperm interactions.

Overall, OF increased the proportion of motile sperm and improved swimming performance compared to a standard artificial activation medium commonly used in *in vitro* fertilization protocols. However, these effects varied depending on the combination of sperm and OF. Some OF samples supported more stable sperm performance, while others induced strong but short-lived responses. Differences were also observed between conspecific and heterospecific pairings, suggesting that OF does not affect all sperm equally.

Microcapillary assays showed variable sperm accumulation near OF sources, indicating context-dependent responses rather than a uniform attraction pattern. At the molecular level, OF shared a common biochemical background across species, including amino acids, energy-related metabolites and lipid-associated compounds, but also showed species-specific variation that may underlie functional differences.

Together, these results suggest that ovarian fluid provides a generally supportive environment for sperm, while also introducing subtle, species-dependent effects. Such modulation may influence fertilization outcomes when native and introduced species co-occur, but does not appear to strictly prevent heterospecific sperm activity.

This work provides a step toward understanding how gamete-level interactions contribute to reproductive outcomes in mixed-species systems and highlights the need for further studies linking specific OF components to fertilization success.

Keywords: sperm motility; gamete interaction; fertilization ecology; chemotaxis; proteomics; metabolomics.

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RHEOLOGICAL PROPERTIES OF FERTILISATION MEDIA SHAPE SPERM MOTILITY, INTERACTION BEHAVIOURS, AND DIVERSIFICATION ACROSS FERTILISATION STRATEGIES

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During fertilisation, sperm motility towards the egg is essential, yet sperm performance is commonly assessed in low-viscosity activating media that poorly reflect the rheological complexity of natural fertilisation environments. In both external and internal fertilisation, spermatozoa encounter fluids differing in viscosity, elastic response, fluid motion, and ultrastructure. These properties may influence sperm propulsion, flagellar waveform, activation, aggregation, and interaction behaviour, but their roles in sperm function and diversification remain underexplored. Using comparative motility, flagella and morphological analyses, we investigated viscosity effects on spermatozoa from species with contrasting fertilisation strategies and sperm morphologies. Externally fertilising species, including sterlet, common carp, rainbow trout and African clawed frog, and internally fertilising or spermatophore-associated species, including spotted catfish shark, starry skate and ocellate stingray and axolotl, were exposed to viscosity-modified methylcellulose (MC-M0512, 0-2%) or polyvinylpyrrolidone (PVP-K90, 0-10%) media. In externally fertilising species, progressive motility generally declined with viscosity increase, whereas spermatozoa from internally fertilising species or structurally complex systems maintained or increased progressive motility. Viscosity also altered flagellar waveform, amplitude, wavelength and lateral beating, with species-specific responses reflecting sperm architecture. Fluid viscosity further altered overall movement and interaction behaviours. PVP-K90 $\geq 5\%$ promoted head-pairing in sterlet, whereas increased viscosity altered progression, rotation, clustering, and transitions between helical and planar movement in elasmobranchs, axolotl, and African clawed frog. Elasmobranch and axolotl spermatozoa were also released from spermatozeugmata or clumps in $\geq 0.5\%$ methylcellulose. Thus, rheological properties regulate motility speed, flagellar waveform, sperm activation, release, aggregation, and coordinated movement. To place these results in an evolutionary context, we compared sperm morphology across vertebrates with diverse fertilisation environments to examine whether variations in sperm morphology reflect adaptations to rheological conditions at fertilisation. Recurrent variation in sperm head shape and size, midpiece organisation and flagellar structure was associated with fertilisation mode and mechanical demands of reproductive environments. Together, these findings support the view that spermatozoa have evolved as integrated biomechanical systems shaped to balance hydrodynamic drag, energetic demand and efficient propulsion within species-specific fertilisation microenvironments.

Keywords: *progression, fertilisation environment, morphology, hydrodynamic drag, propulsion, cooperative mechanisms.*

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EFFECTS OF SHORT-TERM STORAGE ON SPERM QUALITY AND EPIGENETIC MODIFICATIONS IN COMMON CARP (*CYPRINUS CARPIO* L.)

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This study investigated the effects of short-term storage on the storage capacity, fertilization ability, and DNA methylation of common carp (*Cyprinus carpio* L.) sperm. Carp sperm loses motility and fertilizing ability when stored without an extender, becoming immotile after 3 days. In this study, sperm was diluted 1:1 with an extender and stored under aerobic conditions (0–2 °C vs 25 °C, with or without antibiotics). Sperm motility was analysed using CASA, live and dead sperm were sorted using a flow cytometric cell sorter. Whole-Genome Bisulfite Sequencing (WGBS) was used to assess DNA methylation. Dilution with an extender and storage at 0–2 °C significantly extended sperm storage capacity, accompanied by a significant reduction in oxygen consumption rate (Ma et al., 2026). Sperm treated with 6 µL/mL antibiotics showed no differences in global DNA methylation compared with sperm stored without antibiotics, either at the whole-genome or chromosome level. However, differences were detected in some specific genes (Ma et al., unpublished). At 1 and 7 days of storage, sperm treated with 6 µL/mL antibiotics showed significantly lower fertilization and hatching rates than sperm stored without antibiotics. Notably, although this treatment maintained sperm motility above 50% for up to 70 days, the fertilization and hatching rates remained below 1% (Ma et al., unpublished). Using a flow cytometric cell sorter, the heterogeneous sperm population was separated into dead and live subgroups, and the live subgroup contained approximately 20% motile sperm. The dead sperm subgroup exhibited significantly lower DNA methylation levels in the CHG and CHH contexts at both the chromosomal and genic-region levels, with the exception of CpG islands. Furthermore, enrichment analysis identified differential DNA methylation in genes associated with sperm motility between the dead and live sperm subgroups (Ma et al., unpublished).

Keywords: Storage capacity, DNA methylation, Extender, Fertilization, Hatching

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POST-MORTEM SPERM KINETICS IN JUNDIÁ (*RHAMDIA QUELEN*): ESTABLISHING COLLECTION WINDOWS BASED ON RIGOR MORTIS PROGRESSION

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The unexpected loss of animals represents a frequent and often unavoidable challenge in animal science and aquaculture. These events may result in losses of selected breeding lines and high-value individuals, and of rare and endangered animals. Although euthanasia of animals is common for gametes collection in many species, when animals are unexpectedly found deceased, there is no control in their post-mortem process. To address this challenge, the recovery of viable gametes from deceased animals could be an important tool to preserve germplasm that would otherwise be permanently lost, being extremely valuable for germplasm banking and aquaculture management. The study aimed to demonstrate the feasibility of collecting and cryopreserving post-mortem sperm from *R. quelen*, evaluating fresh and cryopreserved samples at specific time intervals (0, 4, 8, and 12 hours), which were experimentally established based on prior chronological observations of the species' rigor mortis progression. Previously, the rigor mortis index (RI) was characterized using digital image analysis to determine muscle stiffness over a 48-hour period, revealing that euthanized individuals reach an advanced stage of rigor mortis (exceeding 80% RI) at 12 hours post-mortem. Based on these objective physiological baseline data, an experiment was conducted using sixteen male breeders distributed into four post-mortem collection groups (n = 4 per group): 0 (control, less than 30 minutes), 4, 8, and 12 hours. Animals were euthanized via anesthesia and spinal cord transection, and gonads were collected at the designated intervals. Fresh sperm was evaluated for motility, cryopreserved, and subsequently thawed to assess post-thaw kinetics. Post-mortem storage interval severely affected sperm motility in both fresh and thawed samples. For fresh sperm, total motility dropped sharply from $45.3 \pm 0.23\%$ at 0 hours to $14.6 \pm 0.1\%$ at 4 hours, cascading down to $2.9 \pm 0.03\%$ at 8 hours and $0.7 \pm 0.01\%$ at 12 hours. Post-thaw motility followed a similar downward trend, showing relative stability in the 4-hour group ($12.11 \pm 0.11\%$ compared to $22.33 \pm 0.27\%$ in the control) before collapsing to $4.04 \pm 0.03\%$ at 8 hours and $0.75 \pm 0.01\%$ at 12 hours. In conclusion, post-mortem sperm viability in *R. quelen* decreases rapidly. Utilizing prior rigor mortis progression as an objective baseline, this study demonstrates that sperm collection should be performed within a 4-hour window post-mortem to ensure higher motility for successful cryopreservation.

Keywords: Post-mortem recovery, motility, catfish, aquaculture

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POST-THAW SPERM STORAGE AS A TOOL FOR IDENTIFYING PATERNAL-EFFECT GENES IN EURASIAN PERCH (*PERCA FLUVIATILIS*)

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The influence of paternal factors on offspring quality, particularly during early developmental stages, has historically received limited attention. Recent evidence, however, indicates that altered sperm phenotypes can serve as effective tools for identifying paternal-effect genes (PEGs), whose expression in progeny is shaped by genetic and non-genetic information carried by spermatozoa. In the present study, we investigated post-thaw sperm storage (PTS) as a stress-inducing factor to assess its impact on larval performance and to identify candidate PEGs in Eurasian perch (*Perca fluviatilis*). *In vitro* fertilization was performed using cryopreserved sperm applied either immediately after thawing (0 min) or following 30 min of post-thaw storage at 4°C. Although PTS resulted in a substantial reduction in sperm motility, fertilization success remained unaffected, enabling evaluation of downstream paternal effects on progeny phenotype. Larvae derived from the PTS group exhibited significantly elevated mortality beginning at 9 days post-hatch, suggesting a pronounced paternal influence on early larval viability. Transcriptomic analysis conducted at the mouth-opening stage identified 41 differentially expressed genes (DEGs), many of which were associated with immune-related pathways. Among the identified DEGs, *mfap4*, *gimap*, *hlag*, *pigr*, *neo1*, and *pde6g* emerged as strong candidate PEGs. In addition to transcriptomic changes associated with paternal effects, our results also revealed interactions between paternal contributions and maternal modulation of gene expression, highlighting the complexity of parental regulation during early development. We demonstrate that even short-term, 30-minute PTS not only impairs sperm motility but also induces persistent effects on offspring performance and survival. Furthermore, transcriptomic profiling revealed that maternal background can either enhance or buffer paternal effects, providing further evidence for complex parental interactions underlying early developmental processes.

Keywords: cryopreservation, transcriptomics, larvae, non-genetic inheritance

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IS POST-THAW AGGLUTINATION REALLY A PROBLEM IN COMMON CARP SPERM CRYOPRESERVATION?

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In this study we have investigated if agglutination of DNA following cryopreservation of common carp (*Cyprinus carpio*) sperm affects its post-thaw motility and fertilizing capacity. DNA agglutination is commonly observed phenomenon following sperm cryopreservation which is normally treated by post-thaw application of DNase. In cyprinid sperm agglutination is often observed when sugar-based extenders are used for cryopreservation. In our experiments, agglutination of varying degree was observed following thawing. Application of 100 µg/ml DNase resulted in immediate liquefaction of the agglutinated matter. Nevertheless, there was no significant difference between the motility of partially agglutinated ($57 \pm 12\%$) and liquefied ($62 \pm 12\%$) sperm ($P = 0.235$). Similarly, no significant difference was observed in the embryonic development of eggs fertilized with agglutinated ($56 \pm 17\%$) or DNase-treated non-agglutinated ($69 \pm 10\%$) sperm ($P = 0.056$). Addition of DNase at concentrations of 10-100 µg/ml to the extender prior to cryopreservation also successfully prevented agglutination of sperm resulting in post-thaw motility percentages ranging from $64 \pm 3\%$ (at 10 µg/ml) to $47 \pm 16\%$ (at 100 µg/ml) with $62 \pm 12\%$ observed in the non-DNase treated partially agglutinated group, again without a significant effect of DNase concentration (ANOVA $P = 0.193$) on sperm motility. It was also observed that higher concentrations of DNase were detrimental to spermatozoa resulting in membrane disruption and disintegration of cells 10-15 minutes post-thaw. Thus, cryopreservation-induced agglutination of common carp sperm does not have an effect on the post-thaw motility and fertilizing capacity of common carp sperm, however if needed, it can successfully be prevented by pre-freezing application of DNase at concentrations of 10-20 µg/ml.

Keywords: *cyprinid sperm, cryopreservation, agglutination, DNase treatment, motility, fertilization*

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CONNECTING CONTINENTS THROUGH AQUATIC GERMPLASM: THE AGGRC MISSION IN A GLOBAL CONTEXT

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Preservation and commercialization of genetic resources for aquatic species have become urgent priorities as aquaculture continues to grow and numerous factors exert pressure on natural aquatic systems worldwide and threaten genetic diversity. Development of germplasm repositories is a powerful tool to safeguard gene pools, yet efforts remain fragmented and lack standardization, accessibility, and long-term impact. The Aquatic Germplasm and Genetic Resources Center (AGGRC) was established in 2014 in Baton Rouge, Louisiana to address these challenges through intersectoral integration of research, technology development, industrial-scale approaches, and outreach. The AGGRC interdisciplinary research and development model can be effectively introduced and integrated into the European research landscape to strengthen global collaboration in germplasm cryobiology and repository development. Adoption of an open-technology approach in which inexpensive devices can be shared, modified, standardized, especially as all-inclusive kits would greatly expand community-level capability and broaden participation.

To address this, we present an overview of the AGGRC mission, infrastructure, and operational framework, alongside case studies demonstrating application in germplasm collection, cryopreservation, quality management, and repository development at multiple organizational levels. Interdisciplinary methodologies include generalized pathways for sperm cryopreservation across multiple species, processing of workflows, simulation modelling, and user-driven systems designed for long-term storage and distribution. Exploration of existing European initiatives could highlight opportunities for harmonization, technology sharing, and co-creation of standards. In our experience, a network approach to the protection of aquatic species, along with open-access resources and training programs would significantly enhance efficiency, reproducibility, and collaboration across sectors and continents.

Interactive models of this type can provide scalable and adaptable frameworks for advancing aquatic genetic resource preservation globally. Given the thousands of aquatic species and their tremendous diversity, this is clearly an immense global problem beyond the resources of single agencies or countries. Continued partnership will be essential to develop global repositories, standardized protocols, and coordinated strategies that ensure the long-term preservation and utilization of aquatic genetic biodiversity.

Keywords: *germplasm repositories, open technology, cryopreservation, quality management*

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DEMOCRATIZING ACCESS TO CRYOPRESERVATION TECHNOLOGIES: A SOUTH AMERICAN PERSPECTIVE ABOUT OPEN-HARDWARE SOLUTIONS

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Sperm cryopreservation is a valuable biotechnology for fish reproduction in many sectors including aquaculture, wild fisheries, and conservation. Implementation of cryopreservation depends on making freezing technologies more accessible and replicable. However, this remains limited in many contexts because conventional freezing systems can be expensive, difficult to transport outside of research stations, and challenging to reproduce across users. Nitrogen vapor shipping dewars are widely used in research and production for cryopreservation, being less expensive than programmable freezers and possible to use in the field. However, they remain costly and difficult to acquire in South America and other regions. Open hardware is a solution that can improve access and standardization in cryopreservation. The CryoKit, is an open-hardware 3D-printed device developed by the Aquatic Germplasm and Genetic Resources Center to provide simple, inexpensive, and reliable access to cryopreservation in any environment. The CryoKit floats on liquid nitrogen within a foam box, exposing samples in straws or vials to nitrogen vapor at an adjustable height to control cooling time to -80°C. The goal of this study was to evaluate the CryoKit as a representative open-hardware approach for expanding access to fish sperm cryopreservation in South America and other regions. The feasibility was tested in silver catfish (*Rhamdia quelen*), a South American model species for fish reproductive biotechnology. Sperm from three males was frozen using a conventional approach with either a shipping dewar or the CryoKit as the freezing platform. Post-thaw sperm motility and kinetics were assessed, and the freezing curves were recorded to compare the cooling behavior of both systems to -80°C. Total motility was similar between methods, with 51% for the CryoKit and 50% for shipping dewar freezing ($P > 0.05$). Likewise, curvilinear velocity results of 33 $\mu\text{m/s}$ for CryoKit and 32 $\mu\text{m/s}$ for the shipping dewar showed no differences ($P > 0.05$). The freezing curve shapes were similar, with mean cooling rates to -80°C of 24.9 °C/min for the CryoKit, and 24.2 °C/min for the shipping dewar. These results demonstrate that the CryoKit can achieve cryopreservation outcomes similar to conventional field freezing methods for *R. quelen*. Therefore, this open-hardware 3D-printed device represents a promising approach to expand access to fish sperm cryopreservation in different environments, especially where economic constraints limit the use of industrial equipment. Open hardware can help democratize access to cryopreservation technologies and can lay a foundation for community-level standardization.

Keywords: open hardware; germplasm repositories; sperm kinetics; biotechnology; aquatic genetic resources.

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SCALABLE LARGE-VOLUME CRYOPRESERVATION OF FISH SPERM USING SIMPLIFIED HYPOTONIC MEDIA

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Despite considerable progress in fish sperm cryopreservation, its broader use in aquaculture is still limited by the need for highly species-specific protocols. Conventional approaches have typically been developed around the osmotic and physiological characteristics of individual species, with media designed to mimic seminal plasma in terms of ionic composition and high osmolality. This is particularly important in externally fertilising fishes, where changes in environmental osmolality can initiate sperm motility. However, although such species-tailored strategies can be effective, they often remain difficult to scale for hatchery practice, especially when large volumes of sperm are required.

In this study, we tested a simplified approach to large-volume sperm cryopreservation in three phylogenetically and physiologically distinct fish groups: common carp, sturgeons, and rainbow trout. The media were intentionally adapted to each group while maintaining a shared hypotonic principle and the same combination of permeable cryoprotectants, methanol and ethylene glycol. For common carp, the medium contained NaCl and sucrose; for sturgeons, KCl alone; and for rainbow trout, glucose alone. This design demonstrates that a common cryobiological concept can be adjusted to species-specific requirements without relying on complex media formulations. For all species, sperm were diluted 1:1 with the cryoprotective medium and frozen in 4.5 ml cryotubes. In sturgeons, the approach was further successfully extended to 10 ml cryotubes. Across all tested taxa, the simplified hypotonic media prevented premature motility activation and supported effective cryopreservation under large-volume conditions. These findings indicate that the success of this approach is not restricted to a single species or reproductive model, but may reflect a broader principle applicable to fish sperm cryopreservation. The practical significance of this strategy lies in its scalability. Larger containers and reduced dilution complexity allow greater sperm volumes to be processed with fewer individual units, lower handling demands, and potentially reduced plastic consumption. Although the use of larger volumes is associated with lower freezing rates and therefore longer processing times, it offers clear advantages for aquaculture, particularly in large-scale fertilisation, where substantial sperm quantities are required to fertilise large egg batches.

Overall, this work supports a shift from narrowly species-specific optimisation towards a more scalable and application-oriented framework for fish sperm cryopreservation, while still allowing targeted adaptation of medium composition to the biology of individual species.

Keywords: *methanol; ethylene glycol; fertilisation; cryobanking; aquaculture*

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SPERM CRYOPRESERVATION PROTOCOLS IN SERRASALMIDAE, THE MAIN SOUTH AMERICAN NATIVE AQUACULTURE GROUP: A SYSTEMATIC REVIEW

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Brazilian aquaculture has expanded substantially in recent decades, with Serrasalminae, particularly tambaqui (*Colossoma macropomum*), representing one of the most economically important native groups. Given their strategic relevance, preserving genetic resources is essential, and sperm cryopreservation is a key tool for both aquaculture and conservation. This study systematically reviewed sperm cryopreservation protocols developed for South American Serrasalminae over nearly four decades. Following PRISMA guidelines, a literature search was conducted in Scopus, Web of Science, PubMed, and SciELO. From 122 records, 40 studies met the eligibility criteria. Brazil accounted for 82.5% of publications, highlighting the regional concentration of research, while tambaqui was the most studied species (60%). Dimethyl sulfoxide was the predominant permeating cryoprotectant, with glucose and BTS as the most common extenders. Egg yolk was the main non-permeating cryoprotectant, although nearly half of the studies did not use extracellular supplementation. The most common storage devices were 0.25–0.5 mL straws. Historically, sperm quality assessment relied on subjective motility evaluation, with CASA-based analyses only emerging in 2010. Despite substantial operational progress, the field remains marked by methodological heterogeneity, fragmented validation criteria, and limited technological harmonization, restricting replicability and large-scale implementation in germplasm repositories. Advancing ex situ conservation of South American aquatic genetic resources will require standardized functional validation, objective sperm quality assessment, and protocols aligned with conservation genetics principles.

Keywords: Aquatic Genetic Resources; Conservation Genetics, Cryoprotectants, Germplasm banking

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ROBUST MAINTENANCE OF THE PATERNAL METHYLOME FOLLOWING SPERM CRYOPRESERVATION AND ITS TRANSMISSION TO EARLY EMBRYOS IN COMMON CARP

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Sperm cryopreservation is widely used in aquaculture to support selective breeding, broodstock management, and the conservation of genetic resources in common carp (*Cyprinus carpio*). While its effects on sperm quality are well documented, its potential impact on the paternal epigenome remains poorly understood. In teleost fish, paternal DNA methylation patterns are largely maintained after fertilisation, raising the question of whether cryopreservation-induced alterations in sperm could affect epigenetic information transmitted to the next generation. To address this, we compared fresh and cryopreserved sperm from the same males using whole-genome bisulfite sequencing and analysed embryos produced with these sperm at the mid-blastula transition stage using enzymatic methyl-seq. Cryopreservation significantly reduced sperm motility and fertilisation success, confirming a clear impairment of sperm function. However, no global differences in CpG methylation were detected in sperm, and no differentially methylated cytosines were identified between conditions. Inter-individual methylation variability also remained highly similar between treatments. In embryos, global CpG methylation patterns were similarly stable between treatments. Nevertheless, 1,521 differentially methylated cytosines were detected in embryos derived from cryopreserved sperm. These differences were modest in magnitude and scattered across the genome, without aggregation into consistent regional patterns. Overall, these results indicate that while sperm cryopreservation strongly impairs sperm performance in common carp, it does not induce widespread disruption of the paternal methylome. Any detectable methylation effects in embryos appear modest in magnitude, distributed across CpG sites, and not indicative of coordinated or large-scale changes.

Keywords: sperm cryopreservation; DNA methylation; embryo; epigenetics; aquaculture; *Cyprinus carpio*

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POSTER ABSTRACTS

P-01 | ENVIRONMENTAL STRESS SENSING THROUGH TRPV CHANNELS IN MAGALLANA GIGAS GONADS UNDER A REALISTIC SCENARIO: SILVER EXPOSURE AND THERMAL ANOMALIES

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The convergence of increasing thermal anomalies such as marine heatwaves (HW) and chemical pollution threatens marine broadcast spawners. In this context, *M. gigas* serves as a biological model due to its high sensitivity to environmental fluctuations. Previous work evidenced that silver exposure significantly compromises sperm motility (Fernández-García et al., 2023), but the underlying molecular mechanisms related to reproductive sensitivity under multi-stressor scenarios remain unclear. This study provides an integrative analysis of the transcriptional dynamics of Transient Receptor Potential Vanilloid (TRPV) channels, key cellular thermo-sensors, in the gonads under thermal stress and silver contamination.

Oysters were acclimated to the average spring temperature (AST: ~16.3 °C) in Ria de Aveiro, Portugal, and later subjected to a 6-day experimental period simulating a HW (~22.1 °C). Within each thermal condition, oysters were exposed to environmentally relevant concentrations (0.25 µg/L) of either silver nanoparticles (Ag NPs) or ionic silver (Ag⁺), both previously established as reprotoxic (Fernández-García et al., 2023). At the onset of the experiment, gills, hepatopancreas, and gonads were dissected (n=10) to establish a baseline profile of TRPV tissue distribution. Following the experimental period, gonadal tissue (n=10 per treatment/temperature) was collected.

Phylogenetic analysis identified 4 distinct sequences, reclassified as TRPVA, TRPVB, TRPVC/D-1, and TRPVC/D-2. Notably, TRPVB emerged the most stress-responsive in the gonad, exhibiting a divergence in signaling: while Ag NPs upregulated TRPVB expression under HW conditions, Ag⁺ triggered a significant downregulation. This suggests that silver forms activate distinct signaling pathways that are differentially modulated by thermal stress, highlighting the complexity of combined contaminant and climate pressures. These findings reveal the physiological plasticity of *M. gigas* and indicate that climate change may alter the dynamic profile of emerging contaminants in oyster reproductive tissues.

Keywords: Phylogeny, Marine heatwave, Emerging contaminants, Climate change

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Fernández-García et al. Silver nanoparticles and silver ions indistinguishably decrease sperm motility in Pacific oysters (*Magallana gigas*) after short-term direct exposure. *Environmental Toxicology and Pharmacology* 101 (2023): 104202. doi: 10.1016/j.etap.2023.104202

P-02 | MELATONIN AND AFMK IN PLASMA, OVARIAN FLUID, AND EGGS DURING REPRODUCTION IN FARMED RAINBOW TROUT: AN ANTIOXIDANT ROLE

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Melatonin (Mel; N-acetyl-5-methoxytryptamine), recognized in fish as both a biological timekeeper and a regulator of physiological processes, exhibits strong antioxidant properties. Mel acts by directly scavenging reactive oxygen species to form N¹-acetyl-N²-formyl-5-methoxykynuramine (AFMK) or by enhancing antioxidant enzyme activity. This study investigated the distribution of Mel and AFMK in plasma, ovarian fluid, and eggs of farmed rainbow trout (*Oncorhynchus mykiss*; spring-spawning Rutki-selected strain) and evaluated their potential role in protecting post-ovulated eggs against oxidative stress.

The study included three- and four-year-old fish from the same broodstock. Blood plasma was repeatedly collected from four-year-old females (n = 12) 14 days before ovulation, at ovulation, and 12 days post-ovulation. Eggs were collected at ovulation, which was identified through daily monitoring and confirmed by manual stripping. In addition, eggs from three-year-old females (n = 5) were analysed to assess age-related hormone differences and fertilisation success. Before analysis, eggs were rinsed with a 1:10 solution of OvaFish™ extender (IMV Technologies, France) to remove residual ovarian fluid, minimise its influence on the results, and allow evaluation of the effect of rinsing on survival rate. Mel and AFMK levels were determined by HPLC with fluorescence detection following solid-supported liquid extraction (SLE), while superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities were measured with commercial colorimetric assays.

Plasma Mel levels remained stable throughout the reproductive stages (0.51 - 0.63 pmol/ml), with no significant differences among sampling points ($P = 0.34$) and were not correlated with Mel levels in ovarian fluid ($r = 0.45$). AFMK was undetectable in both plasma and ovarian fluid, whereas both Mel (0.58 ± 0.09 pmol/mg protein) and AFMK (0.18 ± 0.09 pmol/mg protein) were detected and positively correlated ($r = 0.82$) in eggs of four-year-old fish. Mel concentrations in eggs from both age groups did not correlate with antioxidant enzyme activities. Mel and AFMK levels were lower in eggs from four-year-old than that from three-year-old females (Mel: 1.07 ± 0.16 pmol/mg protein; AFMK: 0.58 ± 0.1 pmol/mg protein), whereas SOD activity showed the opposite trend, suggesting compensatory upregulation of enzymatic defenses with age-related Mel decline. The strain showed an 85–95% survival rate at the eyed-egg stage.

Overall, the results support the hypothesis that melatonin contributes to the antioxidant protection of rainbow trout eggs through non-enzymatic mechanisms, with AFMK formation reflecting direct ROS scavenging by melatonin. As Mel levels in eggs were about three times higher than in plasma or ovarian fluid, hormone synthesis in oocytes cannot be excluded.

Keywords: Antioxidant defense, Oxidative stress, AFMK formation, Post-ovulatory ageing (POA), Egg quality, Antioxidant enzyme activity

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P-03 | ANCESTRAL SIGNALS OF SEX: EARLY MALE GENE EMERGENCE IN STURGEON DEVELOPMENT

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Sex differentiation is the process by which an undifferentiated, bipotential embryo develops into either a male or a female, making it a critical foundation for reproduction. In fish males, despite the lack of conservation in sex-determining genes, a number of fairly conserved genes that act as regulators of early male gonadal development and induce their differentiation have been identified in teleost fish. The most relevant genes are *amh*, *dmrt1*, *sox9*, *dhh*. This topic has received little attention in non-teleost actinopterygian fish as sturgeons. The limited data available suggest that genes traditionally regarded as classic male regulatory genes in teleost fish are not activated in the gonads of future male sturgeons during sexual differentiation. In very recent studies, our laboratory observed the activation of a set of male genes at a specific point in time (2.5 months). These genes are little known or completely unknown in fish and vertebrates in general during sex differentiation (*t-box transcription factor*, *neurexin 3a* (*nrx3a*), *neurexin 3b* (*nrx3b*), *perilipin 1* (*plin1*), *hatching enzyme 1* (*he*), and *sodium/hydrogen exchanger 3-like* (*sech3*)). This study focuses on them to determine whether they are activated early in male gonadal development (2.5 to 6-7 months of age) and regulated by steroid hormones. The first genes activated in male gonads at very early stage (2.5 months of age) were *nrx3b* and *sech3*, followed by the activation of *nrx3a* 15 days later (3 months of age), and the emergence of *tbx1* over the following 15 days (3.5 months of age). *plin1* was activated at 4 months of age and *he* did not show sex-specific activation at early stages.

We have identified a potential pathway for male differentiation in sturgeons (*nrx3b/sech3-nrx3atbx1*) that differs from that described during testicular differentiation of other vertebrates. Furthermore, this pathway is independent of estrogens; consequently, its suppression in females is not linked to the early production of estrogens that drive the development of a future female organism. Further research is needed to understand how this masculine gene are regulated.

Keywords: *sex differentiation, gonad development, gene expression*

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P-04 | ISOLATION AND PROTEOMIC CHARACTERIZATION OF EXTRACELLULAR VESICLES FROM CARP (*CYPRINUS CARPIO*) SEMINAL PLASMA

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Extracellular vesicles (EVs) are membrane-bound particles involved in intercellular communication and play important roles in reproductive processes. However, the presence and characteristics of EVs in fish seminal plasma remain poorly understood. The aim of this study was to isolate and characterize EVs from carp seminal plasma. Seminal plasma was obtained from carp males (n = 5) following centrifugation of milt. EVs were isolated using two isolation approaches ultracentrifugation (UC) and size-exclusion chromatography (SEC). Particle concentration and size distribution were analyzed using fluorescence nanoparticle tracking analysis (F-NTA). Vesicular membrane integrity was evaluated using MemGlow™ 488 staining and EV morphology was examined using cryogenic transmission electron microscopy (cryoTEM). Proteomic analysis was performed using data-independent acquisition mass spectrometry (DIA-MS). Both UC and SEC enabled successful isolation of EVs from 2 mL of carp seminal plasma. Ultracentrifugation yielded 7.9×10^{11} particles/mL, whereas SEC isolation resulted in 3.3×10^{11} particles/mL. The isolated particles exhibited a mean diameter of 112-113 nm, consistent with the size range of small extracellular vesicles. Fluorescent membrane staining confirmed the presence of lipid-bilayer particles and CryoTEM confirmed the presence of membrane-bound microvesicle-like particles. Assessment of sample purity showed strong reactivity of the plasma protein transferrin in UC-derived fractions, while only trace levels were detected in SEC fractions, indicating reduced contamination with abundant seminal plasma proteins. Therefore, SEC-derived EVs were selected for proteomic analysis and DIA-MS identified 1,654 proteins. Among them were well-established EV markers including CD81, CD63, CD9, PDCD6IP and TSG101, further supporting the vesicular origin of the isolated particles. Functional enrichment analysis revealed overrepresentation of proteins involved in vesicle organization, membrane docking, vesicle-mediated transport, the ESCRT complex and SNARE-dependent vesicular trafficking, suggesting active mechanisms involved in EV biogenesis and vesicle transport. In addition, EVs proteins included metabolic enzymes (creatine kinase B and adenylate kinase), enzymes involved in protein modification (glycosylation-related enzyme OGT), molecular chaperones (HSPA/HSP90 family), antioxidant proteins (peroxiredoxins, SOD1) and components of the ubiquitin–proteasome system, suggesting that carp seminal plasma EVs may carry proteins involved in metabolic regulation, redox balance, protein modification and protein homeostasis.

Keywords: EVs, proteomics, vesicle-mediated transport, size-exclusion chromatography

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P-05 | SHORT-TERM PRESERVATION TECHNIQUES FOR ZEBRAFISH (*DANIO RERIO H.*) SPERM

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Reproductive efficiency is fundamental to sustainable aquaculture and the maintenance of biomedical model organisms. Short-term storage of zebrafish (*Danio rerio H.*) sperm is critical for routine breeding and spermatology research, as existing extenders typically preserve usable sperm quality for ≤ 24 hours. This study established a physiology-based framework for zebrafish sperm preservation by integrating seminal plasma characterization with systematic extender optimization. Zebrafish seminal plasma analysis revealed a mean osmolality of 251.4 ± 5.2 mOsmol kg^{-1} , dominated by Na^+ (98 mM) and Cl^- (122 mM), with moderate K^+ (29.4 mM), low Ca^{2+} (0.66 mM), and pH 7.85. These values provided the physiological basis for extender formulation. Due to the limited sperm volume produced by individual males, pooled sperm samples were first validated as experimental units using five independent pools, each prepared from six males; these pools showed no significant differences in motility or velocity during 36 h of storage. Twenty preliminary extenders (E1–E20) and seven redesigned formulations (E21–E27) were subsequently evaluated during cold storage (0–2 °C), followed by warming to 28 °C for 10 minutes prior to sperm motility and velocity evaluation. The optimized formulated extender E27 (150 mM NaCl, 40 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 20 mM Tris; pH 7.5; ~ 385 mOsmol kg^{-1}) showed superior performance, maintaining approximately 52% motility after 36 h together with significantly higher VCL and VSL values than all other treatments. Delayed extender addition caused irreversible losses in sperm quality, demonstrating that immediate ionic and osmotic stabilization is critical. This work provides the first seminal plasma reference dataset for zebrafish and establishes a biologically grounded framework for zebrafish sperm preservation by introducing a novel extender capable of extending functional storage beyond current practical limits, thereby supporting fish reproductive technologies, gamete management, and the maintenance of biomedical model organisms.

Keywords: Seminal plasma; Extender optimization; Sperm motility; Gamete management

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P-06 | CRISPR/CAS9-MEDIATED KNOCKOUT OF THE SPERM-SPECIFIC CNGK CHANNEL TO INVESTIGATE ITS ROLE IN ZEBRAFISH SPERM MOTILITY AND FERTILITY

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Sperm motility is essential for successful fertilisation and is tightly regulated by ion-signalling pathways involving multiple ion channels. Among these, potassium (K⁺) channels play crucial roles in the activation and maintenance of sperm motility across vertebrates. The recent discovery of the sperm-specific Cyclic Nucleotide-Gated Potassium Channel (CNGK) in zebrafish has highlighted the complexity of K⁺ signalling in fish sperm physiology. Although the research demonstrated the importance of CNGK in sperm motility, its precise functional role remains unclear. The study also found that zebrafish CNGK channels were activated by alkaline intracellular pH (pH_i) rather than by cyclic guanosine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP), a finding that differs markedly from that in sea urchin CNGK channels. Interestingly, the functional characteristics of zebrafish CNGK channels are comparable to those of mammalian SLO3 (sperm-specific K⁺ channels), which are also activated by pH_i. To address these ambiguities and investigate the deeper physiological roles of CNGK channels in zebrafish spermatozoa, we used CRISPR/Cas9-mediated gene editing to disrupt *cngk* expression. Guide RNA (gRNA) target sites were selected for Cas9-mediated editing within exon 9 (gRNA1) and exon 10 (gRNA2) of the *cngk* gene. Preliminary genotyping of injected embryos revealed that gRNA 1 targeting exon 9 produced more pronounced polymorphisms, indicating higher efficiency in generating *cngk* mutants. These findings confirm the feasibility of CRISPR/Cas9-mediated genome editing of the *cngk* locus and provide a strong foundation for establishing stable zebrafish knockout lines to investigate the role of this channel in sperm physiology. This study is a first step to determine how the loss of *cngk* expression affects sperm motility, fertilisation capacity, spermatogenesis, and sperm maturation, as well as whether alternative signalling mechanisms can compensate for CNGK channel function. Overall, this study provides new insights into the molecular regulation of fish sperm motility and fertility, and improves our understanding of the functional roles of ion channels in fish spermatozoa.

Keywords: *ion channel, gene editing, mutants, sperm physiology, reproduction.*

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P-07 | IMPROVING EUROPEAN EEL SPERM PRESERVATION WITH A NEW EXTENDER

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European eel (*Anguilla anguilla*) is a critically endangered species. P1 extender has been used for eel sperm preservation for more than two decades; however, its formulation does not include a buffering system. This study evaluated the performance of P2 (including TAPS) for both short-term refrigerated storage and long-term cryopreservation of European eel sperm. Males were hormonally induced with weekly injections of rhCG (1.5 IU/g), and sperm samples were collected after 12 weeks of treatment. Only samples showing >50% initial motility were used. Sperm quality was assessed using CASA (Computer Assisted Sperm Analyzer), evaluating total motility (MOT), progressive (MOTp), curvilinear (VCL), straight line (VSL), and average path (VAP) velocities.

For short-term storage, sperm samples from 14 males were diluted (1:25) in P1 (in mM: NaCl 125; NaHCO₃ 20; MgCl₂ 2.5; CaCl₂ 1; KCl 30), P1+TAPS (in mM: NaCl 120; NaHCO₃ 20; MgCl₂ 2.5; CaCl₂ 1; KCl 30; TAPS 10), or P2 (in mM: NaCl 80; NaHCO₃ 20; MgCl₂ 2.5; CaCl₂ 1; KCl 30, TAPS 60) all extenders with pH adjusted at 8.5 and 325 mOsm/kg. Then, the samples were stored at 4 °C and analyzed with CASA 24, 48 and 72 h after the dilution. Kinetic parameters decreased progressively during storage at 4 °C. However, after 72 h, samples diluted in P2 (57 ± 5%) and P1+TAPS (55 ± 4%) showed significantly higher MOT than those diluted in P1 (51 ± 5%). Regarding sperm velocities, samples diluted in P2 showed significantly higher VCL (143 ± 5 µm/s), VSL (80 ± 4 µm/s), and VAP (96 ± 4 µm/s) than those diluted in P1 (VCL: 133 ± 5; VSL: 73 ± 4; VAP: 89 ± 4 µm/s). Samples diluted in P1+TAPS showed intermediate velocities results.

For long-term storage, samples from 8 males were individually cryopreserved in the proportion of 1:8:1 (sperm: P1 or P2, + 5% egg yolk: methanol) into plastic straws (0.5 mL). The straw freezing was performed in liquid nitrogen vapor and were stored in a liquid nitrogen tank. After one week, the samples were thawed in water-bath at 40 °C for 13 s and evaluated using the CASA. For cryopreservation, the use of P1 and P2 as extenders did not affect the MOT and velocities of the thawed sperm. Thus, the results indicate that the new extender P2 improves the maintenance of sperm quality during short-term storage and can be used as extender for European eel sperm cryopreservation. Further experiments are currently underway to optimize sperm density per straw when using P2 in the cryopreservation process.

Keywords: European eel; sperm preservation; short-term storage; cryopreservation; CASA

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P-08 | SUCCESSFUL USE OF SEMINAL PLASMA AS EXTENDER DURING EUROPEAN SEA BASS SPERM SHORT AND LONG-TERM STORAGE

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We aimed to evaluate the effectiveness of natural seminal plasma as an extender compared to a synthetic non-activating medium (NAM) for short-term storage and cryopreservation of European sea bass (*Dicentrarchus labrax*) sperm. Sperm samples from 10 males were diluted in seminal plasma and NAM (in mM: NaCl 59.83, KCl 1.47, MgCl₂ 12.91, CaCl₂ 3.51, NaHCO₃ 20, and glucose 0.44, supplemented with 1% BSA, 310 mOsm/kg, and pH 7.7). For experiment 1, the samples were diluted at a ratio of 1:25, and for experiment 2 at 1:5.7 (sperm: extender).

In experiment 1, samples were stored at 4 °C for up to 144 h. Sperm quality was assessed at 0, 24, 48, and 144 h using CASA (Computer Assisted Sperm Analyzer) to measure sperm total motility (MOT), progressive motility (MOTp), curvilinear (VCL), straight line (VSL), and average path (VAP) velocities. During the storage, only the samples diluted in seminal plasma maintained quality up to 48h. They showed significantly higher MOT (69 ± 5%), MOTp (49 ± 14%), and velocities than samples diluted in NAM (MOT 52 ± 4%, MOTp 25 ± 3%) at 48h. At 144 h, samples diluted in both extenders showed a substantial decrease in all the parameters, whereas those diluted in seminal plasma showed significantly higher values.

In the experiment 2, diluted samples received the addition of 5% Me₂SO, resulting in a final ratio of 1:6 (sperm: cryoprotectant solution). Then, samples were loaded in straws (0.25 mL), cryopreserved in liquid nitrogen vapor (6.5 cm/15 min). After one week, the samples were thawed (35 °C/15 s) to evaluate the sperm kinetic parameters with CASA and to assess DNA fragmentation by the alkaline comet assay. Samples cryopreserved using seminal plasma as extender showed significantly higher kinetic parameters (MOT 52 ± 4%, MOTp 37 ± 3%, VCL 135 ± 4 µm/s, VSL 107 ± 4 µm/s, VAP 119 ± 5 µm/s) than those used NAM extender (MOT 35 ± 3%, MOTp 21 ± 3%, VCL 101 ± 4 µm/s, VSL 72 ± 4 µm/s, VAP 82 ± 4 µm/s). However, no significant differences were observed for DNA fragmentation.

Thus, natural seminal plasma is a more effective extender than NAM for maintaining European sea bass sperm quality during short-term storage and cryopreservation, although further optimization is required to improve long-term preservation outcomes.

Keywords: Marine fish; aquaculture; cryopreservation; CASA; DNA integrity

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P-09 | LARGE-SCALE ASSISTED FERTILIZATION FOR TAMBAQUI (*COLOSSOMA MACROPOMUM*) USING CRYOPRESERVED SEMEN IN A BREEDING IMPROVEMENT PROGRAM

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Tambaqui (*Colossoma macropomum*), the leading native species in Brazilian aquaculture, holds major socioeconomic importance. Semen cryopreservation is a key strategy for germplasm banking and genetic improvement; however, thawing and fertilization protocols still require standardization. This study aimed to evaluate the use of cryopreserved semen and to establish a large-scale assisted fertilization protocol for the species within a Breeding Improvement Program (BIP).

Semen was frozen (Jan–Feb/2025) in a solution containing 5% glucose (65%), methyl glycol (10%), and egg yolk (5%), using 2 and 5mL cryotubes. In 2026, females were selected via cannulation and oocyte clearing with Serra’s solution (criterion: >75% germinal vesicle migration/peripheral position). Broodstock were hormonally induced with pituitary extract, and mating pairs were selected to avoid inbreeding and produce families for a BIP at Embrapa Fisheries and Aquaculture.

Thawing was performed at 50 °C for 90 s (2 mL) and 120 s (5 mL). Oocytes (50 g) were fertilized with 10 mL of thawed semen mixture and activated with 1.05% NaHCO₃ for 2 min. In the control group (fresh semen), 50 g of oocytes were fertilized with 500 µL of semen and activated with water. All batches were incubated in 200L systems. Fertilization rates were assessed after 8 h.

Fresh semen (76.3%; n=7) outperformed cryopreserved semen (55.5%; n=6) in fertilization rate ($p < 0.01$), survival, and normal morphology ($p < 0.05$). Simple linear regressions of fertilization versus spermatozoa (spz) survival ($R^2 = 0.058$; $p = 0.429$) and versus normal spz ($R^2 = 0.038$; $p = 0.524$) were not significant. PCA (PC1) clearly separated fresh from cryopreserved samples, with positive loadings for survival and normal morphology and a negative loading for tail defects.

Although cryopreservation caused morphological damage, sperm survival was maintained, and fertilization was still achieved despite of the presence of tail defects. In assisted reproduction, direct deposition of semen onto oocytes prior to activation minimizes sperm travel distance, enabling successful fertilization even by spermatozoa with tail injuries. The established protocol demonstrates the feasibility of large-scale use of cryopreserved semen for tambaqui, validating its practical application in Breeding Improvement Programs.

Keywords: Cryobanking; fish gametes; fertilization; theriogenology.

Acknowledgements: Brazilian Development Bank (BNDES), Project BRS Aqua – EMBRAPA.

P-10 | POST-OVULATORY ASSEMBLY OF DND1/NANOS3-CONTAINING GERM PLASM REVEALS A DISTINCT MECHANISM OF GERM PLASM FORMATION IN MEDAKA (*ORYZIAS LATIPES*)

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In many animals, germ cells are formed through inheritance of maternally provided germ plasm (Gp). Gp is a specialized cytoplasm enriched in maternal mRNAs and protein complexes. However, the mechanisms underlying its assembly remain largely unknown.

In zebrafish and *Xenopus*, the Balbiani body (Bb), a conserved structure formed during early oogenesis, is thought to act as a precursor structure for Gp in embryo (Divyanshi *et al.*, 2024). Gp components first accumulate in the Bb during oogenesis and are later reorganized into Gp after fertilization. In zebrafish, Bucky ball (Buc) localizes to the Bb and is required for both Bb formation and the subsequent reassembly of Gp components in early embryos. However, it remains unclear whether this Bb-based model is conserved in other fish species. Recently, we found that two Gp components, *dnd1* and *nanos3*, synergistically contribute to germ cell formation in medaka (*Oryzias latipes*) (Nishimura *et al.*, 2025). However, it remains unknown whether Gp containing *dnd1* and *nanos3* is continuously formed from the Bb and whether this process requires Buc. To address these questions, we analyzed the spatiotemporal relationship between Buc and *dnd1/nanos3* from oogenesis to early embryogenesis in medaka.

We analyzed the localization of Buc, *dnd1*, and *nanos3* during oogenesis. Similar to zebrafish, Buc localized to the Bb in early-stage oocytes. In contrast, *dnd1* and *nanos3* were unexpectedly absent from the Bb and were distributed throughout the oocyte cytoplasm. Notably, during mid- to late oogenesis, Buc became enriched on the presumptive vegetal pole, whereas *dnd1* and *nanos3* localized to the presumptive animal pole, indicating that these factors were spatially segregated during oogenesis. After ovulation, however, Buc-positive granules appeared around the presumptive animal pole in unfertilized eggs. At this stage, *dnd1* and *nanos3* clearly co-localized with Buc, suggesting that Gp components separated during oogenesis are reorganized into Gp after ovulation. Furthermore, in *buc* (–/–) mutants, enrichment of *dnd1* and *nanos3* at the animal pole was lost in mid- to late-stage oocytes, and their localization to Gp was not detected in embryos. These results show that, although Buc is spatially separated from *dnd1* and *nanos3* during oogenesis, it is required for their localization to the animal pole and subsequent assembly into Gp.

Together, these findings suggest that Gp containing *dnd1* and *nanos3* is assembled after ovulation rather than being directly inherited from the Bb, representing a previously unrecognized mechanism of Gp formation in vertebrates.

Keywords: oogenesis, Balbiani body, Bucky ball, Germ cells

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References: Divyanshi, Yang J. Germ plasm dynamics during oogenesis and early embryonic development in *Xenopus* and zebrafish. *Mol Reprod Dev.* 2024;91(8):e23718.

Nishimura T, Fujimoto T, Generation of primordial germ cell-like cells by two germ plasm components, *dnd1* and *nanos3*, in medaka (*Oryzias latipes*) *iScience.* 2025;29:111977.

P-11 | SPERMATOZOA MORPHOLOGY IN MEDITERRANEAN ELASMOBRANCHS

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Elasmobranchs play a crucial role in ecosystem regulation, but they are highly vulnerable to rapid environmental changes, particularly those driven by anthropogenic activities. Therefore, elasmobranchs are among the most threatened vertebrate groups worldwide, with overfishing and habitat degradation representing the primary threats to their survival. To address these challenges, *in situ* and *ex situ* conservation programs are complementary approaches. The implementation of assisted reproductive technologies, still poorly developed for elasmobranchs, represents a critical component of these *ex situ* strategies. The main goal of this work was to get a better understanding of the sperm cells morphologies of different Mediterranean elasmobranch species.

Male individuals of both endangered and non-endangered species were sampled (coming mainly from bycatch or stranding events, but also targeted scientific tagging campaigns), thanks to the cooperation with local fishers from the Port of Valencia and Xàbia, the University of Murcia and TRAGSA. Other samples were obtained thanks to collaboration with the Valencian Community's stranding network. Sperm samples were collected and preserved. A subset of samples was cryopreserved and incorporated into a germplasm bank, while others were fixed in 2.5% glutaraldehyde for morphological analyses. Spermatozoa ultrastructure was examined using scanning electron microscopy. In total, images of 7 different species were obtained: *Raja radula*, *Raja asterias*, *Raja clavata*, *Mobula mobular*, *Rhinobatos rhinobatos*, *Scyliorhinus canicular* and *Alopias vulpinus*. Different measurements were taken: sperm head length (HL), amplitude of the head helix (AH), length of the head helix (LH) and number of helices (HN). Additionally, when a male specimen was available for a necropsy, its internal reproductive anatomy was recorded and preliminarily sketched.

Keywords: sperm cells, assisted reproduction, ultrastructure, conservation.

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P-12 | ESTABLISHMENT OF A 3D TESTICULAR ORGANOID SYSTEM USING AGGREWELL AS A NOVEL TOOL TO STUDY SPERMATOGENESIS IN FISH

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Spermatogenesis is a stem cell–driven developmental process requiring precise spatial organization of germ cells and somatic supporting cells within the testicular niche. Conventional two-dimensional (2D) culture systems fail to replicate the structural complexity necessary for proper cell stratification and tissue morphogenesis. Three-dimensional (3D) organoid systems enable cellular self-organization and represent promising tools for modelling gonadal development in vitro. The present study aimed to generate scaffold-free 3D testicular organoids in sterlet sturgeon (*Acipenser ruthenus*) and evaluate their structural organization, with particular focus on the formation of testis cord-like arrangements and germ cell niche architecture.

Testicular tissue from two-year-old males was enzymatically dissociated and cultured using AggreWell plates (StemCell Technologies), which promote controlled spheroid formation through centrifugal forced aggregation. Cells were cultured for 15 days under optimized conditions supporting aggregation, compaction, and differentiation. The AggreWell system enabled efficient production of uniform organoids with reproducible size and morphology.

Histological evaluation using H&E staining revealed compartmentalised tissue-like structures; enclosed organoids, with successful organoid formation in 96% of samples and 100% positive detection of the germ cell marker DDX4, including cell stratification patterns resembling early testis cord-like organisation observed in vivo. Immunohistochemical analyses and RNAseq further confirm a distinct and organised cell population within the organoids, supporting the hypothesis of the establishment of a structured germ cell niche. These results demonstrate that AggreWell-derived organoids can possibly resemble testicular tissue organisation, highlighting their utility as an in vitro model to study spermatogenesis, testicular morphogenesis, and germ cell–somatic cell interactions in fish, with potential applications in reproductive biology and aquaculture biotechnology.

Keywords: *Sterlet sturgeon, Spermatogenesis, Testicular organoids, Testis cord-like structures, AggreWell, Germ cell niche*

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P-14 | CELLULAR AND MOLECULAR ASPECTS OF TESTIS REGENERATION IN ZEBRAFISH

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Regeneration has been extensively studied in vertebrate somatic organs, yet remarkably little is known about the regenerative capacity of the adult gonad. To address this gap, the study is investigating the cellular and molecular mechanisms underlying testicular regeneration using zebrafish.

For examining the regeneration process, an appropriate gonadectomy procedure was required, and hence a partial gonadectomy procedure was successfully generated with a 100% survival rate of the operated fish. In this process, a small portion of the zebrafish testis is removed surgically, and the fish were maintained under standard laboratory conditions. Then the amputated testis from different operated fish were collected at different time periods (after 1,2,4,8 weeks) to investigate the rate of regeneration and the contribution of distinct cell types during the regenerative process. Hence, the testes were processed for histological studies (Gill's Hematoxylin and Eosin Staining), which showed that there was an accumulation of somatic, immune and progenitor-like cells at the site of injury. It revealed that somatic cells responded to the injury in the first wave as compared to germ cells. Furthermore, to characterise the transcriptomic signatures for elucidating molecular pathways during the regeneration process, the samples (regenerating parts) were subjected to bulk RNA sequencing after 1,2,4 and 8 weeks post-amputation. Collectively, this study presents a novel surgical model for investigating testicular regeneration and identifies key cellular responses and transcriptomic changes associated with regenerative progression.

Keywords: *Partial Gonadectomy, Histology, Immune Cells, Bulk RNA Sequencing.*

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P-15 | *IN VIVO* PHARMACOLOGICAL MODULATION OF NOTCH SIGNALING IN THE TESTES OF TELEOST FISH

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The Notch signaling pathway regulates germ cell fate and gonadal maturation in teleost fish, and recent single-cell RNA sequencing (scRNA-seq) analysis in zebrafish (*Danio rerio*), have revealed that this pathway is highly enriched in spermatogonia. Driven by these findings, we aimed to investigate its role in balancing spermatogonial stem cell (SSC) maintenance and differentiation, studying the *in vivo* pharmacological modulation of the Notch pathway within a commercially relevant aquaculture species, the rainbow trout (*Oncorhynchus mykiss*). Fish received intracoelomatic injections every 48 hours over a 7-day period with the γ -secretase inhibitor DAPT (10 mg/kg; Notch antagonist), valproic acid (VPA, 50 mg/kg; Notch agonist), or a vehicle control (1.5% DMSO). Testicular responses were subsequently evaluated using histology (H&E), multiplexed *in situ* hybridization chain reaction-Ethylene Carbonate (HCR-EC) combined with immunohistochemistry (IHC), and RT-qPCR. Histological analysis revealed that short-term pharmacological modulation neither induced acute cytotoxicity nor disrupted the overall seminiferous tubule architecture. Although endogenous basal expression of Notch components (*notch3*, *aph1b*, *jag2b*) remained below the visual detection threshold of *in situ* HCR-EC, quantification of *nanos2* mRNA and Vasa protein demonstrated that the proportion of *nanos2*-expressing undifferentiated spermatogonia remained stable across all groups. Conversely, RT-qPCR revealed that Notch blockade via DAPT induced a significant downregulation ($p < 0.05$) of global *nanos2* mRNA expression. Classical Notch downstream effectors (*her6*, *hey1*) and other somatic/germ cell markers (*vasa*, *sycp3*, *amh*) exhibited non-significant fluctuations, potentially buffered by transient expression kinetics and intrinsic negative feedback loops within the pathway. Together, these findings suggest a specific temporal sequence following Notch pathway inhibition: systemic blockade reduces the transcriptional output of the key self-renewal marker *nanos2* prior to any physical depletion of the undifferentiated spermatogonial pool. This highlights the subtle yet fundamental role of Notch signaling in sustaining the molecular identity of teleost spermatogonial stem cells before morphological changes become apparent.

Keywords: spermatogenesis, Notch signaling, *in situ* hybridization chain reaction-ethylene carbonate, spermatogonial stem cells.

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P-16 | SEEING BEYOND THE SLICE: MACHINE LEARNING APPROACHES FOR SERTOLI AND SPERMATOGONIAL STEM CELL DETECTION AND QUANTIFICATION IN 3D IMAGING OF ZEBRAFISH TESTES

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Since the 1960s, quantitative analyses of Sertoli cells and spermatogonial stem cells have primarily relied on histological examination of serial sections of the testis. Although this approach has provided important insights into germ cell population dynamics, it remains labor-intensive, and more importantly, it lacks the resolution required to reliably distinguish rare isolated spermatogonial stem cells from neighboring spermatogonial doublets or cyst-forming populations. In contrast to germ cell populations, Sertoli cell dynamics have received comparatively little attention and remain poorly characterized. Likewise, the three-dimensional (3D) spatial distribution of spermatogonial stem cells and Sertoli cells in non-restricted testes remains poorly explored at large scale in fish and warrants further investigation. In the present study, we leveraged recent advances in optical tissue clearing and high-resolution confocal imaging to achieve deep 3D visualization of adult zebrafish testes. The modified CUBIC protocol enabled image acquisition at depths of up to 900 µm with a z-step of 0.5 µm, while preserving tissue integrity and fluorescent signals from reporter transgenes specifically expressed in spermatogonia (DsRed2) and Sertoli cells (eGFP). We employed a deep learning approach based on the nnU-Net framework to enable automated segmentation of spermatogonial cysts and Sertoli cells in 3D confocal image stacks. nnU-Net was previously trained on six independent datasets, each comprising 100 manually annotated images of 750 × 750 pixels. This annotation was performed using the integrated tools of Napari, an interactive multi-dimensional image viewer. nnU-Net predictions were further refined using a watershed algorithm to reduce cyst fusion. To date, our attempts to identify cell nuclei within segmented cysts using tools such as Stardist or Cellpose have been unsuccessful due to weak DNA staining in these poorly chromatin-condensed cells. As an alternative, we manually annotated a large number of cysts containing 1, 2, 4, or 8 cells to train a random forest classifier to identify spermatogonial stem cells based on multiple metrics, including cyst volume and fluorescence intensity. Overall, this integrative computational approach combining deep learning-based segmentation and machine learning-based classification provides a robust framework for high-throughput and high-precision quantification of spermatogenic populations in three-dimensional imaging datasets.

Keywords: Zebrafish, spermatogenesis, spermatogonial stem cell, artificial intelligence.

Acknowledgements: This work was supported by the European project AQUAEXCEL3.0 (871108) and FAPESP (2021/06742-5).

P-17 | REPRODUCTIVE CYCLE OF SCYLORHINUS CANICULA IN THE WESTERN MEDITERRANEAN SEA: WHAT WE KNOW

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The small-spotted catshark (*Scyliorhinus canicula*) is one of the most abundant shark species in the Northeast Atlantic and Mediterranean Sea, and it is listed by the IUCN as Least Concern globally and in Europe. As well as that, because of its size and easy maintenance in captivity, it comes out as a perfect candidate for becoming a model elasmobranch species. However, its reproductive cycle is still not so well known. The aim of this study was therefore to fill in the gaps in our knowledge on the reproductive cycle of this species.

Monitoring of populations was conducted along the coast of the Valencian Community from 2018 to the present day. This monitoring was carried out through a biometric survey of animals caught as bycatch by trawlers. The main data collected were: month of capture, sex, total length, weight, liver weight, gonads weight, presence/absence of sperm, presence/absence of encapsulated eggs. Gonadosomatic (GSI) and hepatosomatic (HSI) indexes were also calculated. In addition, in the case of females, measurements of the diameter of the 25 bigger oocytes per female were taken as well. For some of the animals, blood samples were taken and blood plasma levels of testosterone (T) and estradiol (E2) were also determined by ELISA, in males and females respectively.

The monthly changes in the different parameters were examined. Significant differences were found between months for GSI and HSI, and different profiles of variation were observed in males and females. Two female GSI significant peaks were observed in March and August-October, while male GSI variations were not so clear. Regarding HSI, two main female significant peaks were observed in March and September, concurring with their GSI peaks. Male HSI significant peaks were observed in June and October-December, being much more evident than their GSI peaks. Some of the biggest oocyte diameters found in the ovaries (>16 mm) coincided with the GSI peak in August. Encapsulated eggs were mainly found following the autumn GSI peak. In contrast, sperm was present all year long. Different sex ratios of the captured sharks were found depending on the month, suggesting a seasonal sexual segregation and potential migrations. E2 blood plasma levels followed the ovaries GSI variations, while T in males showed less evident changes through the year. Considering the whole, we have obtained a preliminary outline of the annual reproductive cycle of this species.

Keywords: *reproductive cycle, somatic indexes, conservation.*

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P-18 | PRELIMINAR TRANSCRIPTOMIC CHARACTERIZATION OF TESTICULAR SINGLE NUCLEI OF THE NEOTROPICAL BRYCON AMAZONICUS

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Brycon amazonicus (matrinxã), a Neotropical characid, is highly relevant to aquaculture in the Amazon, in spite of the poor understanding of its molecular mechanisms of male reproduction and spermatogenesis. Single-nuclei RNA sequencing (snRNA-seq), which allows a detailed analysis of complex tissues, emerges as a valuable tool for investigating reproductive physiology in fish. This study aimed to develop a preliminary transcriptomic characterization of individual testes nuclei of *B. amazonicus* and to generate a high-quality dataset for future cell annotation analyses, eventually leading to a construction of a cellular map of the matrinxa testis. Testicular tissue was mechanically macerated in cooled TST buffer, filtered through a 40 µm mesh, centrifuged at 500 × g, and diluted in PBS with BSA. Nuclei were counted and checked for integrity and contamination before library preparation using the Illumina PIPSeq™ T20 kit. Sequencing was conducted on the Illumina NextSeq 2000 platform. Quality controls showed reasonably uniform distributions of nFeature_RNA and nCount_RNA, with most nuclei exhibiting between 200 and 1000 detected genes. Few outliers and almost no mitochondrial gene expression indicated high-quality nuclei and minimal contamination by cytoplasmic RNA or damaged cells. A strong positive correlation between nCount_RNA and nFeature_RNA ($r = 0.99$) confirmed the biological and technical consistency of the data. Principal component analysis suggested a gradual decrease in explained variance after components 10 to 15, considered the most useful for subsequent clustering and UMAP analyses. Preliminary analyses revealed nine distinct transcriptomic clusters in the testicular tissue of matrinxa. Ongoing investigations seek to identify marker genes in each group and assist in future cell annotation and characterization of transcriptional pathways linked to spermatogenesis. These findings provide a solid foundation for understanding reproductive biology in *B. amazonicus* and may benefit future applications in aquaculture and reproductive biotechnology.

Keywords: spermatogenesis; snRNA-seq; aquaculture; clustering; reproductive biology; Neotropical fish.

Acknowledgements: Embrapa Pesca e Aquicultura, Aquagenômica and CNPq.

P-19 | EXTRACELLULAR VESICLES IN TROUT SEMINAL PLASMA: PREPARATION AND PRELIMINARY PROTEOMIC FEATURES

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Extracellular vesicles (EVs) are important actors of intercellular communication, and thus potential biomarkers of organ functions. Their study in the fish seminal plasma represents a major interest in gamete biology, because they result from EVs production over the whole genital tract (testis and sperm ducts) during the whole spermatogenetic process. They stand as potential markers of i) the intercellular communication between Sertoli cells and germ cells within the testicular cysts during spermatogenesis, ii) the cytoplasmic material released by budding during spermiogenesis, including that of mitochondrial material, iii) the communication between the epithelial cells of the sperm ducts and the spermatozoa during their storage prior to fertilization, and iv) the extent of apoptotic events during spermatogenesis. Any environmentally-triggered alteration in spermatogenesis is also expected to be reflected in the seminal plasma extracellular vesicles (SPEVs). The SPEVs are therefore potentially bearing the signature of the male environmental and physiological history during its reproductive period. Besides, the known ability of extracellular vesicles to fuse with spermatozoa also makes them potential candidates to edit spermatozoa quality, and eventually to control the transfer non-coding RNAs to the offspring.

In this work, we set up a procedure to prepare and characterize SPEVs from rainbow trout sperm. After the elimination of spermatozoa using 800 g centrifugation, we showed that loose flagella contaminate the seminal plasma, and a specific filtration step was added and validated. Large EVs and small EVs were collected after 2 h of a 15 600 g and 100 000 g centrifugation respectively. Nanoparticles Tracking Analysis revealed a strikingly large overlapping of the EVs size distribution between the two EV types, indicating the need to proceed to further size exclusion chromatography. Seminal plasma storage at -80°C diminished the large EVs size but small EVs were unaffected. All vesicles were processed for transmission electron microscopy in order to visualize their morphology, integrity, and purity. The proteomic profile of this set of SPEVs was preliminarily assessed by high resolution liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS).

This preliminary study on SPEVs characterization in rainbow trout will allow us to study SPEVs variability between rainbow trout males in relation to the strain, the collection time during the season, and the male environment. It is expected to characterize a set of protein markers that can be used to qualify male history and the resulting sperm quality.

Keywords: spermatogenesis, intercellular communication, spermatozoa, sperm quality.

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P-20 | DOES SEQUENTIAL CRYOPRESERVATION OF SPERMATOZOA IN F0 AND F1 COMPROMISE REPRODUCTIVE PERFORMANCE IN A NEOTROPICAL CATFISH?

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The exponential increase in fish consumption and the progressive degradation of their habitats have led to an alarming decline in fish populations. To prevent irreversible biodiversity losses, implementing effective conservation measures has become a priority. Among the available strategies, sperm cryopreservation has emerged as a key tool for the *ex-situ* conservation of germplasm with ecological and economic importance. However, this technique can induce structural and physiological alterations in spermatozoa, significantly compromising their fertilizing capacity and offspring's viability and performance. Thus, the main objective of this study was to evaluate whether sequential sperm cryopreservation—first in the parental generation (F0) and then in the F1 progeny—affects sperm quality and reproductive efficiency. The F1 males were grouped according to the state origin of the (F0) sperm used for their conception: fresh sperm (T1) or cryopreserved sperm (T2). From each group, 12 males were selected, and their sperm samples were cryopreserved and analyzed to determine sperm quality (concentration, motility, morphology, and DNA integrity) and reproductive efficiency (fertilization, hatching, and normal larval rates). No significant differences were observed between treatments for most parameters, with both treatments showing optimal percentages of normal spermatozoa (T1: $60.42 \pm 9.68\%$; T2: $65.87 \pm 16.87\%$), post-thaw sperm motility (T1: $58.6 \pm 29.7\%$; T2: $59.7 \pm 25.0\%$), fertilization rate (T1: $56.5 \pm 10.3\%$; T2: $51.4 \pm 8.2\%$), and DNA fragmentation (T1: $23.09 \pm 9.28\%$; T2: $16.30 \pm 11.72\%$). However, the hatching rate showed a significant difference ($p=0.049$) between treatments (T1: $46.8 \pm 8.0\%$ and T2: $38.8 \pm 6.7\%$). Nevertheless, these outcomes could be mitigated by minor artificial fertilization protocol adjustments. In summary, the results of this study suggest that sperm cryopreservation in the parental (F0) generation does not compromise the sperm quality or reproductive efficiency of the cryopreserved sperm from the F1 generation of the silver catfish *R. quelen*. However, we emphasize the imperative need to incorporate omics tools in future studies assessing the effects of sperm cryopreservation beyond the F1 generation.

Keywords: sperm cryopreservation, sperm quality, reproductive efficiency, first generation (F1)

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P-21 | POST-MORTEM SPERM CRYOPRESERVATION IN THE ENDANGERED GROOVEBELLY STINGRAY (*DASYATIS HYPOSTIGMA*)

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The Groovebelly Stingray (*Dasyatis hypostigma*) is a species endemic to the south-western Atlantic currently classified as Endangered by the IUCN, due to population declines primarily driven by overfishing. Given the species' vulnerability and the absence of established reproductive protocols, this study aimed to assess the feasibility of sperm cryopreservation as a tool for its ex situ conservation. Wild stingray specimens (n=9) were obtained through donations from local fisheries in the Praia dos Ingleses community, Florianópolis, Brazil, where individuals were accidentally caught as bycatch during commercial and artisanal fishing activities. Semen was collected by stripping and processed for short-term refrigerated storage and cryopreservation. Fresh sperm motility was analysed through manual cell-by-cell counting using a high-speed camera attached to a microscope. Initial motility was 86 % which decreased to 66 % after 72 hours. Cryopreservation was tested using nine cryoprotectant solutions: (1) 10% dimethyl sulphoxide (DMSO) (2) 10% methanol (MET); (3) 5% MET + 5% DMSO; (4) 10% DMSO + 10 % egg yolk (EY); (5) 10% MET + 10% EY; (6) 5% DMSO + 5% MET + 10% EY; (7) 10% DMSO+ 10% powdered milk (PM); (8) 10% MET + 10% PM; (9) 5% DMSO + 5% MET + 10% PM. Post-thaw sperm motility and viability were analysed using Computer-Assisted Sperm Analysis (CASA) and flow cytometry, respectively. Variables were analysed using a one-way

ANOVA and compared using Tukey's test. Non-parametric data were analysed using KruskalWallis analysis, followed by Dunn's test. All analyses were performed considering a significance of 5% ($p < 0.05$). Among the cryoprotectant solutions tested, DMSO+PM ($16.65 \pm 4.72\%$) and MET+PM ($17.11 \pm 3.57\%$) resulted in higher ($p = 0.022$) percentages of motile cells compared to DMSO ($7.37 \pm 2.22\%$) and DMSO+MET ($7.04 \pm 2.62\%$). In the viability analysis sperm cryopreserved with DMSO+PM exhibited better performance ($28 \pm 9.24\%$, $p = 0.0221$) compared to the DMSO ($18.73 \pm 7.79\%$). However, no significant differences were observed between DMSO+PM and the other treatments. The results indicated that formulations containing nonpermeable cryoprotectants provided better preservation of motility and viability than those using permeable agents alone. This study is the first to successfully apply post-mortem sperm cryopreservation on *D. hypostigma*, demonstrating that bycatch animals are also suitable for germplasm banking. These findings provide a critical foundation for future reproductive biotechnologies and conservation strategies aimed at preserving genetic diversity in this and other threatened elasmobranch species.

Keywords: germplasm repositories; elasmobranch conservation; fish reproduction; cryobiology.

Acknowledgements: *We would also like to thank the National Council for Scientific and Technological Development (CNPq) for financial support through projects: 444572/2024-4 and 403157/2024-2. Also, the Rio Grande do Sul Research Support Foundation (FAPERGS - Research and Development Program focused on Climate Disasters - Grant: 24/2551-0002156-6). Researchers D.P. Streit Jr (305387/2022-7) and JL Benato (20172/2025-3) are CNPq research fellows. NS Teixeira acknowledges CNPq (n° 153681/2025-0).*

P-22 | SPERM CRYOPRESERVATION DOES NOT ALTER LARVAL OR JUVENILE BEHAVIOR IN RHAMDIA QUELEN

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Sperm cryopreservation is an important assisted reproduction technique widely used for fish aquaculture breeding programs, germplasm repositories, and conservation strategies. However, during the freezing-thawing process, cryoinjuries may affect the cells and compromise their viability. While post-thaw sperm quality and fertilizing capacity are routinely assessed, the effects of cryopreserved sperm on offspring behavioral development remain unclear. This study evaluated whether sperm cryopreservation affects early and juvenile behavioral responses in silver catfish *Rhamdia quelen*. Fresh and cryopreserved sperm were compared for kinetic parameters, membrane integrity, and reproductive performance. Fertilization was performed using oocytes from hormonally induced females, and offsprings derived from fresh or cryopreserved sperm were evaluated during larval development and at the juvenile stage. Larval behavior was assessed at 5, 10, 15, and 20 days post-hatching using video tracking to quantify locomotor activity, including distance traveled, mean velocity, and spatial use. At 90 days post-hatching, juveniles were subjected to behavioral assays adapted for fish models, including a novel tank test for locomotor activity, a social interaction test, and a mirror test to estimate aggressive approach behavior. Cryopreservation reduced sperm motility compared to fresh sperm, from $94.92 \pm 3.85\%$ to $64.75 \pm 6.43\%$ ($p < 0.0001$), and affected selected kinetic parameters, including lower curvilinear velocity ($p = 0.0060$) and progressivity ($p = 0.0001$). Despite these post-thaw alterations, membrane integrity, fertilization rate, hatching rate, and the proportion of morphologically normal larvae did not differ between the groups. Larval locomotor responses changed with development, with older larvae showing increased exploratory activity. However, offspring behavioral patterns showed no difference between the groups. Also, at 90 days post-hatching, no differences were observed between the groups in locomotor activity, social interaction, or aggressiveness-related responses. These findings indicate that the sperm cryopreservation protocol used for *R. quelen* did not impair offspring behavioral development up to the juvenile stage in the first generation. This technique supported normal reproductive outcomes and preserved key behavioral responses in the resulting progeny, strengthening its application as a reproductive biotechnology.

Keywords: germplasm repositories; offspring development; fish reproduction; locomotor activity; fish behavior; cryobiology.

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P-23 | THE USE OF EXTRACELLULAR VESICLES (EVs) IN FISH SPERM CRYOPRESERVATION: THE GILTHEAD SEABREAM MODEL

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Extracellular vesicles (EVs) carry bioactive molecules, including proteins, lipids, antioxidant compounds and microRNAs, acting as mediators of cellular processes, intercellular communication and cell survival. Studies in mammalian sperm cryopreservation have demonstrated EVs cryoprotective effects on sperm motility, while reducing MDA content and maintaining the integrity of sperm plasma membrane. Commercially available EVs, such as milk-derived EVs have demonstrated protective effects against oxidative stress. Although no studies have examined these EVs on fish sperm cryopreservation, it is reasonable to propose that it can mitigate sperm damage induced by cryopreservation. The present study aimed to test two different concentrations of milk-derived EVs in gilthead seabream (*Sparus aurata*) sperm cryopreservation. Before cryopreservation, sperm samples were incubated with EVs for 1h to check for motility changes. During the experiment, sperm from ten males was collected during the breeding season and samples were cryopreserved using the protocol described by Cabrita et al., (2005) with 5% DMSO as cryoprotectant. Two milk-derived EVs concentrations were incorporated (1×10^8 or 4×10^8 particles/ml) as supplementation for 1h. Treated and control post-thaw sperm quality was assessed by CASA, measuring motility parameters (total motility, progressive motility, VCL, VSL and linearity), DNA fragmentation by comet assay, lipid peroxidation through MDA quantification, cell viability and reactive oxygen species using flow cytometry. Fertilization assays (n=5) were performed in all treatments using eggs from the same females. Preliminary results demonstrate that milk-derived EVs can be incorporated in the extender media without deleterious effects of sperm quality. However, no significant improvement was observed regarding the control. The highest EVs concentration showed higher percentage of motile cells as well as VCL and VSL compared to the other concentration. Contrarily, lipid peroxidation was higher in cells with the highest EVs concentration, which may indicate oxidation of some remaining EVs, even after a washing process. Fertilization results confirm that EVs do not negatively affect fertilization success. This study represents the first approach to the use of milk-derived EVs in fish cryopreservation extenders. More research need to be conducted to test different EVs concentrations as well as different EVs sources to explore their potential for cryopreservation.

Keywords: DNA damage, Lipid peroxidation, sperm motility, cell cryodamage, oxidative stress.

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Cabrита et al. (2005). Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. *Cryobiology* 50, 273-284.

P-24 | A TISSUE-RESOLVED TRANSCRIPTOMIC ATLAS OF THE REPRODUCTIVE AXIS IN PIKEPERCH (*SANDER LUCIOPERCA*)

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Comprehensive tissue-specific transcriptomic resources are essential for understanding reproductive physiology and developing molecular biomarkers in aquaculture species; however, such resources remain limited for pikeperch (*Sander lucioperca*). To overcome this limitation, we generated a tissue-resolved transcriptomic atlas of adult pikeperch, with particular emphasis on organs involved in the reproductive axis, including the brain, pituitary, gonads, and liver. RNA sequencing was performed on 13 adult tissues using sex-balanced pooled samples composed of three males and three females for somatic tissues and six individuals per sex for gonadal tissues. Alignment against the pikeperch reference genome detected 32,987 mRNA transcripts, of which 26,166 showed consistent expression levels (> 1 TPM). Principal component and hierarchical clustering analyses based on the 500 most abundant transcripts demonstrated strong tissue-dependent transcriptomic separation. Using a stringent tissue-specificity threshold ($\text{Tau} \geq 0.9$), we identified pronounced transcriptional specialization within the reproductive axis. The highest number of tissue-specific transcripts was observed in the brain (2,315), followed by female gonads (1,419), male gonads (762), liver (539), and pituitary (418). Functional enrichment analyses confirmed distinct biological roles for each organ. Brain- and pituitary-specific genes were primarily associated with neuronal communication and endocrine regulation, including trans-synaptic signaling and dopaminergic pathways. Liver-specific transcripts were linked to amino acid metabolism and coagulation-related processes, whereas gonadal transcriptomes reflected sex-specific reproductive functions, including DNA replication and mitotic regulation in ovaries and meiotic progression in testes. Among the most highly expressed tissue-specific transcripts were hepatic apolipoproteins (*apoa1b*, *apoc1*, *apoa2*) and *fgb*, brain-associated genes such as *epd* and *tfa*, and pituitary-associated genes including *gh1*, *smtla*, *cga*, *prl*, and *lhb*. In addition, gonads displayed highly sex-specific candidate markers, including a natectin-like lectin transcript in females and a myelin-associated glycoprotein-like transcript in males. This study provides the comprehensive tissue-resolved transcriptomic atlas in pikeperch, revealing strong organ-specific transcriptional programs across the reproductive axis, and candidate biomarkers for future studies on reproductive physiology.

Keywords: pikeperch, transcriptomic atlas, RNA-seq, tissue-specific expression.

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P-25 | EXPLORING SURROGATE BREEDING IN EUROPEAN PERCID SPECIES

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Establishing reliable protocols for spermatogonial stem cell (SSC) isolation and transplantation remains a major prerequisite for the development of surrogate breeding technologies in percid fish species. In the present study, SSC isolation efficiency and interspecific transplantation success were evaluated in Eurasian perch (*Perca fluviatilis*), pikeperch (*Sander lucioperca*) and Volga pikeperch (*Sander volgensis*) using triploid larvae as recipients. Three transplantation trials evaluated donor-recipient combinations and developmental stages. Triploid recipient larvae were generated by hydrostatic pressure shock (8000 PSI for 10 min at 5 minutes post-fertilization). In Trial I, 28-day post-hatch (DPH) triploid pikeperch (N=100) and triploid hybrids (*S. lucioperca*♀×*S. volgensis*♂, N=100) received approximately 3000 perch-derived SSCs each; control siblings were left untreated. In Trial II, 9 DPH hybrid larvae (N=96) were injected with perch SSCs. In Trial III, 9 DPH (N=125) and 16 DPH (N=276) triploid pikeperch larvae were injected with Volga pikeperch SSCs; 200 triploid larvae were kept as non-injected controls. Post-transplant survival ranged from 24-49% depending on the trial and timing. In Trial I, survival to 50 days post-transplantation (DPT) was 39.3% (pikeperch) and 49.2% (hybrid) in treated groups, versus 34.4% and 57.0% in controls (differences not significant). Trial II survival was 39.6% at 50 DPT. In Trial III, survival was 24% for the early group and 49% for the late group recipients. About 24% of injected larvae retained labeled cells in extragonadal sites (peritoneum or fat), indicating cell survival without gonadal colonization. These results demonstrate that while the optimized enzyme combination effectively isolates viable SSCs, and microinjection of up to 3000 cells is tolerated by triploid percid larvae, donor SSCs failed to home to or colonize the host gonads under the tested conditions. In conclusion, we achieved technical success in SSC isolation and transplantation protocols, but germline chimerism was not realized.

Keywords: germ cell transplantation, spermatogonia, triploidization, aquaculture biotechnology

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P-26 | PSYCHOBOTIC SUPPLEMENTATION ALLEVIATES ANXIETY-LIKE BEHAVIOUR IN ZEBRAFISH WITHOUT ALTERING OVARIAN GENE EXPRESSION

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Routine husbandry procedures can induce stress responses in farmed fish. Minimizing stress is essential for improving animal welfare and may contribute to enhanced reproductive performance. Psychobiotics, represent a promising nutritional strategy to mitigate stress while preserving productivity. This study used zebrafish (*Danio rerio*) to evaluate the effects of Probinerv® (*Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* Rosell®-175, inulin and vitamin B6) through three independent trials. Trial 1 assessed adult behavioural responses, intestinal morphology and brain gene expression; Trial 2 evaluated juvenile behavioural responses and growth performance; and Trial 3 examined ovarian gene expression in adult females, in order to assess the potential of this psychobiotic to modulate the gut–brain–gonad axis and improve reproductive performance. Adult zebrafish (3–4 months post-fertilisation) received a control diet (CTRL) or the same diet supplemented with Probinerv® via *Artemia franciscana* bioencapsulation and water-surface administration (PSYCHOB) for 42 days. Anxiety-like behaviour was assessed by the Novel Tank Test. Brain gene expression analysis included markers of monoaminergic signalling, GABAergic neurotransmission, synaptic plasticity, stress and neuroendocrine signalling, and neuroimmune regulation. Ovary expression analysis included genes involved in antioxidant defence, pro-inflammatory signalling, glucocorticoid signalling, and ovarian differentiation and steroidogenesis. Midgut histology was analysed and goblet cells were quantified with NIS-Elements AR v.6 software (Nikon). Exploratory behaviour in juvenile zebrafish was evaluated at 21 dpf using a Novel Object Test adapted for early developmental stages and growth performance was studied and growth performance was evaluated through biometric measurements. Our results demonstrate that psychobiotic supplementation promotes behavioural modulation consistent with reduced anxiety-like responses in adult zebrafish and increased intestinal goblet cell abundance without altering intestinal architecture. Among the genes analysed, it significantly increased the expression of *gabra1*, *syn1* and *creb1b*. In contrast, genes involved in serotonin and dopamine pathways (*tph2*, *mao* and *drd1b*) showed no statistically significant differences. Similarly, inflammatory and stress-related markers displayed limited modulation, although *il10* and *tgfb1a* exhibited an upward trend in supplemented animals. In females, none of the ovarian genes examined differed between CTRL and PSYCHOB groups ($p > 0.05$). The absence of modulation in this panel indicates that, although Probinerv® acts centrally on the brain and intestine, it does not perturb the principal molecular checkpoints of ovarian stress-sensing and steroidogenesis, supporting its compatibility with reproductive performance in broodstock nutrition strategies. Juvenile fish exhibited enhanced growth but no behavioural alterations in response to psychobiotic supplementation. In conclusion, Probinerv® modulates behaviour and brain gene expression and improves intestinal and growth parameters without affecting ovarian molecular markers, supporting its role as a gut–brain axis modulator compatible with reproductive function.

Keywords: psychobiotic, zebrafish, gut-brain axis, ovary

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P-27 | INFLUENCE OF BROODSTOCK ORIGIN ON POST-THAW SPERM QUALITY OF *POGONIAS COURBINA*

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Pogonias courbina is a Sciaenidae species native to the South American coast, currently classified as Critically Endangered in Brazil and Vulnerable by the IUCN. Due to its conservation status, studies on its reproductive biology and conservation strategies have been conducted. Among these approaches, sperm cryopreservation stands out as a promising tool for the development of germplasm repositories and long-term genetic conservation. However, information on the influence of broodstock origin on the efficiency remains scarce. The aim of this study was to evaluate the influence of broodstock origin on the efficiency of a sperm cryopreservation protocol for *P. courbina*. Sperm samples with above 80% subjective motility of wild ($n = 4$) and farm-raised males ($n = 7$) were cryopreserved using HBSS supplemented with 5% egg yolk and 10% DMSO. Post-thaw evaluations included sperm morphology, sperm concentration, and sperm kinetics assessed by CASA. Wild individuals showed a higher frequency of short-tail spermatozoa ($19 \pm 4\%$), whereas farm-raised fish presented a greater frequency of strongly coiled tails ($23 \pm 9\%$). Total motility (MOT) was significantly higher in samples obtained from farm-raised ($71 \pm 11\%$) compared to wild fish ($37 \pm 17\%$). Differences between broodstock origins may be associated with physiological and management factors. Overall, the cryopreservation protocol preserved key sperm quality parameters, quality parameters, supporting its use for germplasm conservation and reproductive management of *P. courbina*.

Keywords: germplasm repositories; sperm cryopreservation; sperm kinetics; ex situ conservation; marine fish.

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P-28 | EFFECTS OF DIETARY A-TOCOPHEROL-ENRICHED MICROALGAE ON MALE REPRODUCTIVE TRAITS IN F1 SENEGALESE SOLE

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First-generation (F1) Senegalese sole (*Solea senegalensis*) males exhibit reproductive dysfunctions under captivity, limiting sustainable production. Broodstock nutrition and functional ingredients are increasingly explored to improve male reproductive performance, with algae being promising sources of nutrients and antioxidant compounds. Previous studies showed that dietary algae can modulate sperm quality and mitigate oxidative damage in F1 Senegalese sole (Félix et al., 2025). Therefore, the present study evaluated whether the microalgae *Phaeodactylum tricornutum* enriched with α -tocopherol during cultivation could improve sperm quality and oxidative status in Senegalese sole F1 broodstock. The enriched biomass was produced and incorporated into diets at 1% and 3% (SPAROS), alongside a control, and tested in duplicate tanks (n= 20) at the Ramalhete Experimental Station (CCMAR, Portugal). Sperm was collected during the breeding season and quality was evaluated by CASA, including total (TM) and progressive motility (PM), curvilinear (VCL) and straight-line velocity (VSL), and linearity (LIN) at 15, 30, 45, and 60 s post-activation. Lipid peroxidation (MDA), DNA fragmentation (Comet assay), viability, and ROS status were also assessed. Results showed no differences among diets in TM or PM. However, significant differences in sperm kinetics were detected: VCL and VSL were higher in the 3% group than in the 1% group at 15 and 30 s postactivation ($P < 0.05$). MDA progressively decreased in the supplementation groups, reaching significantly lower values in the 3rd sampling ($P < 0.05$). DNA fragmentation was lower in both 1% and 3% groups than in the control ($P < 0.05$), with no differences between inclusion levels; no differences were detected in the third sampling. No dietary effects were observed in the percentage of viable cells, positive or negative to ROS. Overall, α -tocopherol-enriched *P. tricornutum*, particularly at 3% inclusion, may enhance sperm kinetics and reduce oxidative damage, supporting its potential for functional broodstock nutrition. Further research needs to confirm the effects of long periods of feeding using this supplementation approach.

Keywords: *Phaeodactylum tricornutum*; sperm quality; oxidative stress; broodstock nutrition

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P-29 | INDUCED REPRODUCTION ON NILE TILAPIA USING DIFFERENT HORMONES

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The Nile tilapia is a fish that present spontaneous reproduction in adequate conditions and the hormonal induction is not common, although, can be strategically employed when the control of spawning time is required, such as in synchronizing reproduction and biotechnological applications. However, current protocols remain limited in effectiveness and must be optimized according to the hormonal inducers available. This study aimed to perform assisted reproduction of Nile tilapia using different hormonal inducers. Forty-five broodstock (25 males and 20 females) were selected and distributed in a completely randomized design with four treatments and five replicates. The treatments included crude carp pituitary extract (CPE – 5.5 mg/kg), sGnRHa + domperidone (Ovaprim® – 0.5 mL/kg), mGnRHa + metoclopramide (Ovopel® – 0.5 pellet/kg), human chorionic gonadotropin (hCG – CHORULON® – 1.5 IU/g), and a control group (without hormonal induction). After induction, the fish were kept in 25 L tanks (one fish per tank) until the onset of spontaneous spawning, which was performed manually through extrusion. For males, seminal evaluation included sperm kinetics, concentration, morphology, and membrane integrity. For females, fertilization rate, hatching, and fecundity were analysed. Sperm parameters using CASA showed no significant differences between treatments at 10 and 50 seconds after activation ($p > 0.05$). A significant difference in sperm concentration was observed between the control and Ovopel® ($p < 0.05$), with greater values ($1,43 \times 10^9 \pm 9,6 \times 10^8$ sperm/mL) to control. For normal sperm morphology was verify that control and HCG presented higher values ($p < 0,05$), with $43,43 \pm 6,11$ and $39,57 \pm 2,08\%$ of normal sperm, respectively. Regarding fertilization, only one experimental replicate yielded positive results, with CPE, hCG, and Ovopel® achieving 89%, 90%, and 90% of fertilization, respectively. However, only CPE (0.3%) and Ovopel® (31%) resulted in hatching. In this sense, considering that males produce semen of satisfactory quality, it is recommended not to induce, and if necessary, to use HCG. In females, considering the success in larval production with Ovopel®, it was found that this could be an alternative inducer for the species.

Keywords: fish gamete quality; hormonal inducers; *Oreochromis niloticus*; ovulation semen production.

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