A Journey Towards Identification of Paternal-Effect Genes and Exploration of Their Roles During Early Life Stages in Eurasian Perch, Perca fluviatilis

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In loving memory of my Aai,

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Abbreviations

Abbreviation	Definition
ALH	Amplitude of Lateral Head Displacement
ANOVA	Analysis of Variance
ARRIVE	Animal Research: Reporting of *In vivo* Experiments
ATP	Adenosine Triphosphate
BAR	BIN/Amphiphysin/Rvs Family
BER	Base Excision Repair
BLAST	Basic Local Alignment Search Tool
$CaCl_2$	Calcium Chloride
CAEE-UWM	Centre of Aquaculture and Ecological Engineering, University of Warmia and Mazury
CASA	Computer-Assisted Sperm Assessment
CON	Control
CpG	Cytosine–Phosphate–Guanine
CPM	Counts per Million
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats–Associated Protein 9
CV	Coefficient of Variation
DD	Domesticated Females × Domesticated Males
DEG	Differentially Expressed Gene
DNA	Deoxyribonucleic Acid
DPH	Days Post-Hatch
DW	Domesticated Females × Wild Males
EGA	Embryonic Genome Activation
ELH	Early Life Stages
ENCODE	Encyclopedia of DNA Elements
ER membrane	Endoplasmic Reticulum Membrane
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
GC	Guanine–Cytosine
GO	Gene Ontology
GOEA	Gene Ontology Enrichment Analysis
GRNs	Gene Regulatory Networks
H_2O	Water
KCl	Potassium Chloride
LC	Caudal Length
LIN	Linearity
lincRNAs	Long Intergenic Non-Coding RNAs
lncRNAs	Long Non-Coding RNAs
LT	Total Length
MBT	Mid-Blastula Transition
MEG	Maternal-Effect Gene
$MgSO_4$	Magnesium Sulfate

miRNAs	MicroRNAs
mM	Millimoles
MO	Mouth Opening
MOT	Motility
mRNA	Messenger RNA
MS-222	Ethyl 3-Aminobenzoate Methanesulfonate
NaCl	Sodium Chloride
NAD	Nicotinamide Adenine Dinucleotide
NCBI	National Center for Biotechnology Information
NE	Northeast
NGI	Non-Genetic Inheritance
NIFRI	National Inland Fisheries Research Institute
PAS	Polish Academy of Sciences
PCA	Principal Component Analysis
PEG	Paternal-Effect Gene
pH	Power of Hydrogen
piRNAs	Piwi-Interacting RNAs
PTS	Post-Thaw Storage
qPCR	Quantitative Polymerase Chain Reaction
RAS	Recirculating Aquaculture System
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SBIE	Swim Bladder Inflation Effectiveness
sGnRHa	Salmon Gonadotropin-Releasing Hormone Analog
siRNAs	Small Interfering RNAs
sncRNAs	Small Non-Coding RNAs
snoRNAs	Small Nucleolar RNAs
snRNAs	Small Nuclear RNAs
STAR	Spliced Transcripts Alignment to a Reference
SYBR	Cyanine Dye
TMM	Trimmed Mean of M Values
TPM	Transcripts Per Million
UFE	Unfertilized Eggs
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-Line Velocity
WBW	Whole-Body Weight
WD	Wild Females × Domesticated Males
WW	Wild Females × Wild Males
ZGA	Zygotic Genome Activation

Vote of Thanks

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With sincere gratitude,
Abhi

Contents

Abstract

Paternal contributions to early progeny development have long been underappreciated in vertebrate biology. Paternal-effect genes (PEGs) are those whose expression in offspring is altered by paternal factors, molecular signals from genes expressed in the male germline or carried in sperm, without changes to the inherited DNA sequence. This thesis identifies and characterizes PEGs in the Eurasian perch (*Perca fluviatilis*) using a novel outcome-based approach. Instead of tracking specific sperm-derived molecules, we applied a reverse-inference strategy: manipulating sperm via cryopreservation, post-thaw storage, and incorporating extreme phenotypical fish conditions, then assessing resulting larval traits and transcriptomes at the mouth-opening stage. This method captures the functional impact of paternal inputs as expressed in the offspring, rather than inferring it from sperm content alone, using three complementary experimental designs.

In the Fresh-Cryo experiment (Chapter 2), we compared zootechnical parameters and transcriptomic profiles of offspring obtained from fresh versus cryopreserved sperm collected from the same males. Obtained larvae did not differ in most of the physiological parameters analyzed. However, larvae from cryopreserved sperm showed higher wet body weights towards 15 days post hatch. Transcriptomic analysis revealed differential expression of genes involved in visual function and structural development. This study provides, for the first time, the significant influence of the paternal genome on the development of the visual system in fish, highlighting *pde6g*, *opn1lw1*, and *rbp4l* as novel PEGs.

In Post-Thaw Storage experiment (PTS; Chapter 3), we examined whether 30 minutes of sperm storage after thawing affects offspring gene expression and physiology. Post-thaw storage appears to cause increased larval mortality compared to larvae obtained from freshly thawed sperm, while other zootechnical parameters evaluated remained unaffected. These results suggest that PTS stresses the sperm in ways that alter larval survival during their life. Notably, candidate PEGs such as *mfap4*, *gimap4*, *hlag*, and *pigr* showed differential expression across larval stages, whereas genes like *pde6g* and *neo1* did not differ during later larval stages, likely due to compensatory mechanisms. What is interesting, these genes were not just absent in unfertilized eggs but also were related to immune functions, explaining higher mortality rates of the larvae in the PTS group. Overall, these findings highlight that post-thaw semen storage

can have lasting impacts on larval traits, revealing PEGs linked to immune function and developmental fitness.

In the third experiment, Dom-Wild (Chapter 4), we explored evolutionary aspects by comparing progeny sired by wild versus domesticated males. Even though the broodstock and their environment were carefully controlled, the larvae still grew and developed differently. We found that females might selectively control the father's genetic material, including sperm-derived transcripts. Transcriptomic differences between groups point to neurodevelopmental and metabolic pathways selectively shaped by paternal factors but under maternal regulation. Overall, these results support a model of conditional paternal influence, where maternal filtering governs the integration of paternal inputs, creating a dynamic, stage-specific interplay. Maternal effects dominate embryonic development, while paternal influences become more apparent during larval metamorphosis up till the end of larval stages that we recorded. This highlights a temporally coordinated parental contribution to offspring phenotype.

Taken together, all these studies show that sperm-transmitted molecular information, modulated by cryoinjury (cryopreservation), post-thaw storage, or domestication can reliably influence offspring phenotype and gene expression. Across all experimental conditions, genes (e.g., *pde6g*, and *opn1lw1*) showed consistent differences in expression among tested groups, supporting their role as candidate PEGs. Furthermore, observed variation within treatment groups suggests maternal modulation of paternal inputs, highlighting a dynamic, bidirectional axis of inheritance. By redefining sperm as carriers of functional regulatory elements, this doctoral dissertation highlights the significant role of non-genetic paternal inheritance factors in guiding larval development in Eurasian perch. By establishing a robust, reproducible framework to identify PEGs through larval outcomes, it opens new avenues for both basic research in evolutionary biology and applied strategies in aquaculture, including transcriptome-informed broodstock selection.

Streszczenie

Wkład ojcowski we wczesny rozwój potomstwa jest od dawna niedoceniany w biologii kręgowców. Geny wpływu ojcowskiego (PEG, z ang. *paternal-effect genes*) to geny, których ekspresja u potomstwa zmieniana jest przez czynniki ojcowskie, ale bez zmian w odziedziczonej sekwencji DNA. Prezentowana rozprawa doktorska identyfikuje i charakteryzuje PEG u okonia euroazjatyckiego (*Perca fluviatilis*) przy użyciu nowatorskiego podejścia opartego na strategii odwrotnego wnioskowania. Zamiast śledzić konkretne cząsteczki pochodzące z plemników, zastosowaliśmy strategię odwrotnego wnioskowania: modyfikując plemniki poprzez ich kriokonserwację, przechowywanie po rozmrożeniu oraz użycie skrajnych fenotypów rodzicielskich, ocenialiśmy zootechniczne cechy larw i ich transkryptom w momencie otwarcia pyska. Podejście to, poprzez przeprowadzenie trzech komplementarnych eksperymentów pozwoliło uchwycić nam funkcjonalny wpływ wkładu ojcowskiego przejawiający się u potomstwa, zamiast wnioskować o nim wyłącznie na podstawie parametrów plemników.

W eksperymencie Fresh-Cryo (rozdział 2) porównane zostały parametry zootechniczne oraz profile transkryptomiczne potomstwa uzyskanego z użyciem świeżego i kriokonserwowanego nasienia pozyskanego od tych samych samców. Uzyskane larwy nie różniły się pod względem większości analizowanych parametrów zootechnicznych. Jednak larwy pozyskane z użyciem kriokonserwowanego nasienia wykazywały wyższą masę ciała w 15 dniu po wykluciu. Analiza transkryptomiczna wykazała różnice w ekspresji genów związanych z funkcjami wzrokowymi i rozwojem strukturalnym. Badanie to po raz pierwszy ukazuje istotny wpływ genomu ojcowskiego na rozwój układu wzrokowego u ryb, wyłaniając *pde6g*, *opn1lw1* i *rbp4l* jako nowe PEG.

W eksperymencie Post-Thaw Storage (PTS, z ang. *post-thaw storage*; rozdział 3) zbadano czy 30 minutowe przechowywanie nasienia po rozmrożeniu wpływa na ekspresję genów i rozwój potomstwa. Larwy pozyskane z nasienia PTS wykazywały zwiększoną śmiertelność w porównaniu do larw uzyskanych z nasienia bezpośrednio po rozmrożeniu. Pozostałe parametry zootechniczne nie wykazywały zmian. Wyniki te sugerują, że PTS wywołuje stres w plemnikach, który wpływa na przeżywalność larw podczas ich rozwoju. Co istotne, PEGs, takie jak *mfap4*, *gimap4*, *hlag* i *pigr*, wykazywały istotne różnice w ekspresji na różnych etapach rozwoju larw. Natomiast geny takie jak *pde6g* i *neo1*, pomimo istotnych różnic w ekspresji na początkowych etapach rozwoju, nie różniły się w późniejszych etapach rozwoju,

prawdopodobnie z powodu pojawiających się mechanizmów kompensacyjnych. Co ciekawe, geny te były nie tylko nieobecne w niezapłodnionych jajach, ale także powiązane z funkcjami immunologicznymi, co może tłumaczyć wyższą śmiertelność larw w grupie PTS. Uzyskane wyniki podkreślają, że PTS może mieć trwały wpływ na pewne cechy larwalne, ujawniając PEG powiązane z funkcją odpornościową i zdolnością rozwojową.

W trzecim eksperymencie Dom-Wild (rozdział 4) porównano potomstwo pochodzące od skrajnych fenotypów rodzicielskich - dzikich i udomowionych samców okonia. Mimo ścisłej kontroli nad stadem hodowlanym i jego środowiskiem, larwy różniły się tempem wzrostu i rozwoju. Wykazaliśmy, że samice mogą selektywnie regulować wykorzystanie materiału genetycznego ojca, w tym transkryptów plemnikowych. Zaobserwowane różnice w transkryptomach wskazywały na szlaki neurorozwojowe i metaboliczne, które chociaż są zależne od ojca, pozostają pod kontrolą matki. Przedstawione wyniki potwierdzają model warunkowego wpływu ojcowskiego, w którym matczyne mechanizmy regulują wkład ojcowski, tworząc dynamiczną i specyficzną dla danego etapu rozwojowego współzależność. Wpływ matczyny dominuje więc w rozwoju embrionalnym, podczas gdy wpływy ojcowskie stają się bardziej widoczne podczas okresu larwalnego. Podkreśla to skoordynowany wkład rodzicielski w kształtowanie fenotypu potomstwa, przynajmniej na wczesnych etapach jego rozwoju.

Podsumowując, wszystkie przeprowadzone eksperymenty wykazały, że informacja molekularna przekazywana przez plemniki a modulowana za pomocą kriokonserwacji, PTS lub udomowienie tarlaków, może wpływać na fenotyp i ekspresję genów u potomstwa. We wszystkich eksperymentach szereg genów (np. *pde6g*, i *opn1lw1*) wykazywał spójne różnice w ekspresji między badanymi grupami, co potwierdza ich rolę jako kandydatów PEG. Ponadto, obserwowana zmienność wewnątrz badanych grup sugeruje matczyną modulację wkładu ojcowskiego, podkreślając dynamiczną, dwukierunkową naturę dziedziczenia. Redefiniując plemniki jako nośnik funkcjonalnych elementów regulacyjnych, rozprawa doktorska podkreśla znaczenie niegenetycznych mechanizmów ojcowskiego w kierowaniu rozwojem larwalnym u okonia. Przedstawione założenia identyfikacji PEG oparte na danych z podchowu larw otwierają nowe perspektywy zarówno dla biologii ewolucyjnej, jak również zastosowań raktycznych w akwakulturze, w tym np. jak dobierać tarlaki w oparciu o badania transkryptomiczne.

Chapter 1

General Introduction

Spanning knowledge from general inheritance to non-genetic inheritance

1.1 Inheritance: Evolving Perspectives Across Scientific Generations

"Heredity is nothing but stored environment" (Luther Burbank)

Understanding how traits are transmitted from one generation to the next has long been a fundamental question in biology. A trait is considered heritable when offspring resemble their parents more closely than they resemble unrelated individuals within the population (Feldman & Lewontin, 1975). Over the centuries, various thinkers have shaped the foundation of inheritance theory, each building on the understanding of how characteristics persist across generations (**Figure 1.1**). In classical antiquity, early natural philosophers laid the groundwork for reproductive theory. Hippocrates proposed that reproductive "seeds" originated from different parts of the body and were passed to offspring during conception. This idea was one of the first attempts to explain the visible inheritance of traits between parents and their children (Jones WHS, 1923). Aristotle later refined this view by suggesting that conception occurred through the mixing of male and female fluids, with the male contributing the "form" and the female providing the "matter" (Preus, 1970). The 18th and early 19th centuries introduced more formalized concepts of inheritance (**Figure 1.1**). Jean-Baptiste Lamarck proposed that traits acquired during an organism's lifetime could be passed on to its offspring (Lamarck, 1873). This idea, known as Lamarckian inheritance, offered a mechanism by which organisms could adapt and transmit those adaptations across generations. While this theory was influential at the time, it later stood in contrast to Charles Darwin's theory of evolution by natural selection, which emphasized variation and the differential survival of individuals possessing favorable inherited traits (Oldroyd, 1986). Darwin revolutionized the biological understanding of inheritance but did not identify a specific mechanism by which traits were transmitted. That gap was addressed when Gregor Mendel's work on pea plants, initially overlooked, was rediscovered in the early 20th century (**Figure 1.1**) (Mendel, 1865; Bateson & Mendel, 1902). Mendel's experiments revealed that traits are inherited through discrete units, later termed "genes", providing a robust foundation for what would become the field of genetics (Deichmann, 2010; Müller-Wille & Rheinberger, 2014; Patwardhan, 2022; Downes & Matthews, 2024). From these early philosophical and empirical frameworks, inheritance theory has continued to evolve, moving from strictly genetic models to more nuanced views that incorporate environmental influences. This thesis follows the trajectory from classical concepts of trait transmission to the modern exploration of genetic and non-genetic inheritance

mechanisms, particularly in the context of teleost fish. Over the years, researchers have introduced many additional concepts that continue to shape today's narrative of genetic and non-genetic inheritance. Rather, these emerging perspectives have broadened our understanding of how traits can persist across generations beyond classical genetic frameworks. Among the most recent updates, Adrian-Kalchhauser et al., (2020) has synthesized advances in non-genetic inheritance in fish, providing a valuable modern reference point for this evolving field.

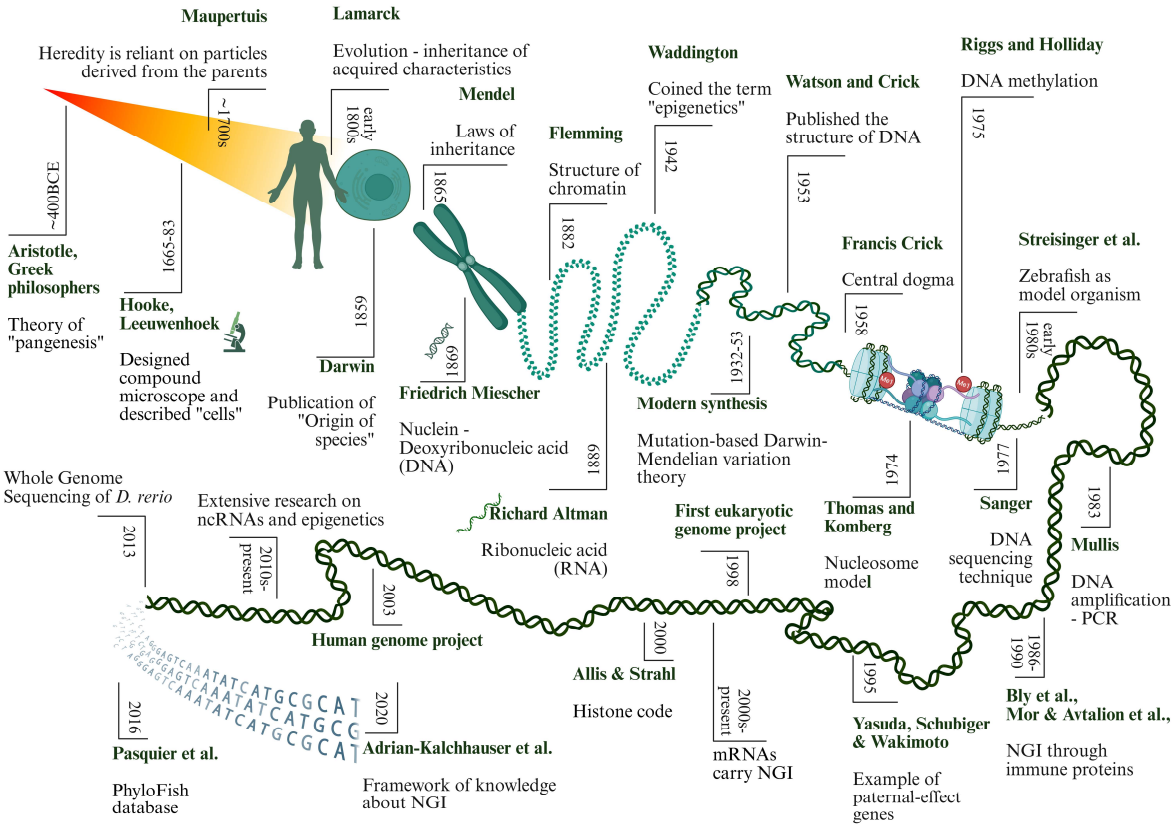

Figure 1.1: *Timeline of key discoveries in genetics, epigenetics, and genomics. This schematic illustrates major historical milestones, beginning with early theories of heredity and culminating in modern genomic technologies. Notable breakthroughs include the discovery of DNA and RNA, the development of the central dogma, the advent of epigenetics, and the introduction of zebrafish (Danio rerio) as a model organism. The figure highlights foundational contributions that shaped our current understanding of gene regulation and inheritance.*

1.2 Concept of non-genetic inheritance

Memes: the new replicators – Robert Dawkins

Although genetic inheritance has long been considered the foundation of biological continuity, it cannot fully explain the vast phenotypic diversity observed within and across generations. For instance, all cells in a multicellular organism contain the same DNA sequence, yet they differentiate into a wide range of specialized cell types with distinct functions. Similarly, individuals sharing identical or near-identical genomes, such as monozygotic twins, full siblings, or inbred laboratory animals, can exhibit marked differences in morphology, physiology, and behavior. These observations suggest that the transmission of information across generations involves more than just DNA sequence, pointing toward the existence of regulatory mechanisms that influence gene expression and phenotype in ways that are not strictly genetic (Waddington, 1942; Lind & Spagopoulou, 2018). This broader category of heritable influences is known as **non-genetic inheritance (NGI),** a term used to describe the transmission of phenotypic traits across generations without changes to the underlying DNA sequence. Numerous concepts have emerged to describe this phenomenon, including soft inheritance, Lamarckian inheritance, transgenerational epigenetic effects, non-Mendelian inheritance, parental effects, carry-over effects, and cell memory (Danchin et al., 2011; Heard & Martienssen, 2014). While each term emphasizes a different nuance, they collectively support the idea that environmental conditions can lead to heritable changes in phenotype through non-genetic pathways.

A widely cited definition by Bonduriansky & Day, (2009) frames NGI as “any effect on offspring phenotype variation that is intergenerationally stable, and could be brought in by environmental differences.” This definition emphasizes the persistence and environmental responsiveness of NGI, highlighting its role in adaptive evolution and development. One of the earliest conceptual frameworks to support NGI came from evolutionary biologist Richard Dawkins. In *The Selfish Gene* (1976), Dawkins introduced the idea of "*memes*", units of cultural transmission that evolve in a manner analogous to genes. Though originally proposed to explain cultural evolution, the meme concept foreshadowed more mechanistic understandings of NGI that are now grounded in molecular biology (Dawkins, 1976). At the molecular level, NGI operates through mechanisms such as DNA methylation, histone modifications, small non-coding RNAs, as well as the transmission of bioactive proteins and lipids (Lind & Spagopoulou,

2018). These mechanisms modulate gene expression in ways that are often responsive to environmental signals and, crucially, can be transmitted across generations. While genetic inheritance involves the stable transmission of DNA sequences, NGI allows for reversible and context-dependent regulation of gene function, enabling organisms to respond more flexibly to environmental conditions. As a result, NGI represents not a replacement but a complement to classical genetic inheritance. Together, they form a more complete picture of heredity, one that accounts for both the enduring blueprint of the genome and the dynamic influences of the environment that shape it across generations.

1.3 Model Organisms, Molecular Messengers: *Fish in Non-Genetic Inheritance Research*

"Fish preserve DNA 'memories' far better than humans" (ScienceDaily, 2009)

In recent years, various model organisms have been employed to investigate non-genetic inheritance and its underlying molecular mechanisms. Among these, fish, particularly externally fertilizing model species such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), have emerged as exceptionally well-suited systems for studying NGI. Their biological characteristics offer unique advantages for dissecting how non-genetic factors are transmitted across generations. The concept of fish as "epigenetic time capsules" has been inspired by studies such as those by (Metzger & Schulte, 2017; Ortega-Recalde et al., 2019), which demonstrated that certain species, unlike mammals, retain DNA methylation patterns across generations. This form of epigenetic memory highlights the ability of fish to transmit environmental information to their offspring through mechanisms that go beyond the DNA sequence. Although the quote is metaphorical, it accurately reflects current scientific understanding: fish can preserve and pass on molecular marks that record environmental exposures, making them ideal for studying epigenetic and broader NGI mechanisms.

Several features make fish particularly advantageous in this field. First, external fertilization removes the confounding influence of post-zygotic parental care. In contrast to mammals, fish embryos develop outside the maternal body, allowing for a clean separation of inherited and environmental factors. This means that all parental influence must occur via the molecular content of gametes, especially the egg and sperm. Second, transparent embryos, rapid development, and short generation times, particularly in zebrafish and medaka, enable researchers to observe developmental changes in real time and across generations (Ishikawa,

2000; Cavalieri & Spinelli, 2017). Moreover, fish typically produce large numbers of gametes, which facilitates high-throughput experimental designs with statistically robust sample sizes. This allows researchers to manipulate specific environmental or molecular factors and observe their effects on offspring phenotype with a high degree of confidence (Anastasiadi et al., 2018). Additionally, the use of controlled breeding and *in vitro* fertilization techniques ensures reproducibility and precision in tracking parental contributions to progeny.

While nematode worms (*Caenorhabditis elegans*) and mice (*Mus musculus*) are also established models for transgenerational epigenetic inheritance (Bohacek & Mansuy, 2015; Baugh & Day, 2020), fish offer a distinct and powerful alternative, particularly in the context of studying gamete-derived information and its impact on development. Their suitability extends to both basic biological research and applied fields such as aquaculture, where understanding parental effects on offspring fitness is of growing importance (Pitcher, 1986; Shekhar, 2025). Taken together, these attributes bring out interest towards fish, especially externally fertilizing teleosts, as outstanding models for investigating NGI (Adrian-Kalchhauser et al., 2020). They enable precise experimental control, reveal intergenerational molecular signals, and offer insights that are not only relevant to evolutionary biology but also critical for improving practices in aquaculture and fisheries management.

1.4 Mechanisms of Non-genetic Inheritance in Fish

The study of NGI in fish has expanded significantly over the past four decades. While early research focused primarily on maternal protein and antibody transfer, more recent efforts have uncovered a multi-layered molecular system that includes RNAs, proteins, lipids, and epigenetic modifications. This section presents these mechanisms in historical context, revealing how our understanding of NGI has grown more comprehensive over time.

1.4.1 Proteins and Immune Factors (1980s–1990s)

The earliest insights into NGI in fish emerged from studies on maternal immunological provisioning. In the 1980s and 1990s (**Figure 1.1**), it was shown that antibodies and immune proteins could be transferred from mothers to offspring through the eggs. For example, maternal immunization in European plaice (*Pleuronectes platessa*) and blue tilapia (*Oreochromis aureus*) resulted in the presence of specific immune proteins in the eggs, influencing the immune responsiveness of the progeny (Bly et al., 1986; Mor & Avtalion, 1990). These findings

highlighted proteins as one of the first empirically validated NGI vectors in fish, acting independently of DNA sequence inheritance.

1.4.2 Messenger RNAs and Developmental Programming (2000s–Present)

As molecular tools advanced, maternally deposited messenger RNAs (mRNAs) were identified as essential for early embryonic development. In the pre-zygotic stage, fish embryos rely on maternal mRNAs for crucial processes such as axis formation, neurogenesis, and metabolic regulation (Lubzens et al., 2016; Winata & Korzh, 2018). Studies in zebrafish and other teleosts show that these mRNAs are selectively packaged into the oocyte and reflect both genetic and environmental inputs from the mother, potentially serving as vehicles for non-genetic information (Ahi et al., 2018).

1.4.3 Non-coding RNAs and Epigenetic Control (2010s–Present)

The 2010s witnessed a surge in research exploring the regulatory roles of non-coding RNAs (ncRNAs) in non-genetic inheritance, revealing a diverse and intricate layer of gene regulation beyond the traditional coding genome (Delihas, 2015). Long non-coding RNAs (lncRNAs) and long intergenic non-coding RNAs (lincRNAs) have been shown to be co-expressed with protein-coding genes in a tissue-specific manner, notably in rainbow trout (*Oncorhynchus mykiss*), suggesting finely tuned regulatory functions (Wang et al., 2016). Among small non-coding RNAs (sncRNAs), microRNAs (miRNAs) have emerged as key modulators of growth, tissue differentiation, and stress responses in fish and other vertebrates (Wu et al., 2020). In turn, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) are essential for mRNA splicing and rRNA modification, forming part of the foundational machinery for post-transcriptional control (Liang et al., 2019). Experimental models in fish have also demonstrated the potential of small interfering RNAs (siRNAs) to silence gene expression and influence developmental pathways (Boonanuntanasarn et al., 2003). While piwi-interacting RNAs (piRNAs) remain underexplored in teleosts, their well-documented role pathway plays a crucial role in defining germ cells, supporting the formation of eggs and sperm, suppressing transposable elements, and maintaining the stability of the genome (Yi et al., 2014). Collectively, these ncRNA classes constitute a sophisticated regulatory network capable of modulating gene expression without altering the underlying DNA sequence, thus offering a compelling molecular framework for understanding transgenerational plasticity in fish and other vertebrates.

1.4.4 DNA Methylation and Phenotypic Outcomes

DNA methylation, particularly at CpG (cytosine–phosphate–guanine) sites, plays a pivotal role in regulating gene expression without altering the underlying DNA sequence. In fish, environmental factors can induce methylation changes that are transmitted to offspring, leading to phenotypic variations. For instance, in Chinook salmon (*Oncorhynchus tshawytscha*), environmental conditions experienced by parents have been shown to alter DNA methylation patterns in gametes (Depincé et al., 2020). These changes correlate with differences in offspring traits such as growth rates and stress responses, suggesting a mechanism for transgenerational plasticity mediated by DNA methylation (Wellband et al., 2021). Similarly, in brook charr (*Salvelinus fontinalis*), parental thermal environments during sexual maturation influenced the DNA methylation landscape of offspring, affecting their development and potentially their fitness in varying thermal habitats (Venney et al., 2022).

1.4.5 Histone Modifications and Phenotypic Expression

Histone modifications, such as methylation and acetylation, influence chromatin structure and gene accessibility, thereby regulating gene expression (Peterson & Laniel, 2004). In fish, these modifications have been linked to phenotypic outcomes. In medaka, specific histone marks like H3K27me3 are retained during early embryonic development, contributing to the regulation of gene expression patterns essential for proper development (Fukushima et al., 2023). Moreover, in threespine stickleback (*Gasterosteus aculeatus*), genome-wide analyses have demonstrated that histone modifications can identify cis-regulatory elements associated with phenotypic variations between ecotypes, such as differences in osmoregulatory functions, highlighting the role of histone modifications in adaptive phenotypic divergence (Okude et al., 2024).

1.4.6 Lipids and Maternal Provisioning

Lipids are crucial components of fish eggs, serving as energy sources and structural elements during embryonic development. Maternal provisioning of lipids can significantly influence offspring phenotype (Rainuzzo, 2020). In medaka, maternal high-fat diets have been shown to alter the lipid composition of eggs, leading to increased embryonic deformities and downregulation of genes involved in transcription and translation during early development (Inoue et al., 2024). Similarly, studies in Atlantic salmon (*Salmo salar*) have demonstrated that variations in egg lipid content affect larval growth and survival rates, emphasizing the

importance of maternal lipid provisioning in shaping offspring fitness (Kaththriarachchi et al., 2025). Furthermore, research on cod (*Gadus morhua*) has shown that changes in egg lipid class composition during embryogenesis can influence larval development and energy utilization, underscoring the role of lipids in early life stages (Fraser et al., 1988). Henrotte et al., (2010) found feeding Eurasian perch broodstock a diet with highly unsaturated fatty acids significantly improves egg and larval quality, comparable to natural diets and superior to commercial salmonid feed. They further show enhancing hatching rates, larval survival, stress resistance, and favorable fatty acid profiles when the species was fed with the diet. These examples underscore the multifaceted mechanisms through which non-genetic factors, such as DNA methylation, histone modifications, and maternal lipid provisioning, contribute to phenotypic diversity in fish. Understanding these processes enhances our comprehension of developmental biology and the potential for transgenerational adaptation in aquatic environments.

1.5 Parental Contributions to Non-genetic Inheritance

In vertebrates, the maternal and paternal contributions to offspring are inherently unequal, both in terms of volume and molecular complexity (Zohar, 2021). The oocyte delivers not only the maternal genome but also cytoplasmic contents such as RNAs, proteins, lipids, and epigenetic regulators (Lubzens et al., 2016), whereas the sperm, though limited in cytoplasm, contributes a highly compact genome along with a growing list of functional regulatory molecules (Krawetz, 2005; Sharma, 2019). This biological asymmetry is a fundamental feature of sexual reproduction and provides a powerful framework for studying how each parent's non-genetic legacy shapes offspring phenotype (Reznick, 1981). Fish, particularly model species like zebrafish, are especially valuable for such studies due to their external development, large clutch sizes, and experimental tractability. Understanding these distinct parental contributions is not only central to developmental and evolutionary biology but also critical for aquaculture, where optimizing breeding strategies and improving larval quality are high priorities (Martínez, 2008; Burt et al., 2011). Furthermore, as environmental pressures intensify, dissecting maternal and paternal effects can help predict how fish populations, and other vertebrates, may respond to challenges such as climate change and pollution, issues of increasing concern to ecologists and resource managers alike.

1.5.1 Maternal Contributions

Due to their large cytoplasmic volume and biochemical richness, fish eggs serve as the dominant vehicle for transmitting non-genetic information from mothers to offspring. The oocyte contains mRNAs, non-coding RNAs, proteins, lipids, organelles, and epigenetic modifications that collectively orchestrate early embryonic development, long before zygotic genome activation (ZGA) occurs. These effects have been elaborated in detail earlier (Sections 1.4.1–1.4.6), and many of them are directly maternally provisioned. From a research perspective, maternal contributions are more accessible and experimentally tractable: eggs can be sampled in high quantity and manipulated with relative ease, enabling mechanistic studies of their contents. As a result, maternal NGI has been more thoroughly characterized, especially in the context of mRNA cargo (Winata & Korzh, 2018), lipid content (Yang et al., 2025), and immune factor transfer (Bly et al., 1986; Mor & Avtalion, 1990). Likewise, maternal epigenetic marks, including DNA methylation, have been shown to persist post-fertilization and contribute to transgenerational effects on physiology and development (Matlosz et al., 2024). However, despite this strong empirical foundation, maternal effects should not be viewed as the default or complete model of NGI. Their apparent dominance reflects both biological reality and methodological convenience, and may unintentionally obscure the emerging significance of paternal contributions.

1.5.2 Paternal Contributions

For a long time, the sperm was thought to contribute only through the haploid genome to the embryo. Yet accumulating evidence now demonstrates that paternal inputs extend well beyond DNA, involving RNAs, chromatin structure, and environmentally modulated epigenetic marks. Small ncRNAs, including miRNAs and piRNAs, are present in teleost sperm and have been implicated in regulating ZGA and early transcriptional programming (Schuster et al., 2016; Joshi et al., 2023; Naveed et al., 2025). The composition of these RNA populations can be influenced by the male's environmental experience, such as temperature or diet, suggesting a route for paternal environmental information to be inherited. Furthermore, sperm epigenetics has gained traction in teleost models. DNA methylation profiles in sperm can reflect environmental cues and have been shown to affect embryonic gene expression in species such as rainbow trout (Zhang et al., 2023). In zebrafish, retained histones and specific modifications such as H3K4me3 are associated with active gene regions and may lead transcription post-

fertilization (Lindeman et al., 2010). Even though awareness is increasing, paternal NGI is still overlooked and not well studied. This is partly because sperm contain much less cytoplasm than eggs, making them harder to investigate, and partly because reproductive biology has traditionally focused more on mothers than fathers. Therefore, teleost fish, with their external fertilization and accessibility of high numbers of both gamete types, offer a rare opportunity to address this gap in a controlled and comparative manner.

1.6 Functional Sperm Biology in the Context of NGI

The sperm cell, despite its minimalist appearance, is increasingly recognized as a functionally rich and environmentally responsive vehicle of paternal inheritance. In externally fertilizing teleosts, where sperm and eggs interact in the absence of maternal buffering during fertilization, the molecular content of sperm plays a critical role in shaping early embryonic development. This section outlines distinct aspects of fish sperm biology that are particularly relevant to the transmission of non-genetic information across generations, a field that remains largely understudied.

1.6.1 Epigenetic Signatures and Environmental Sensitivity

Sperm epigenome is not static. Environmental conditions experienced by males, such as temperature, oxygen availability, or diet, can modify the sperm DNA methylation landscape (Zhang et al., 2023). Along with environmental conditions, domestication too affects sperm epigenetic landscape (Milla et al., 2020). These changes can persist and influence gene expression in the offspring. For example, in Atlantic salmon, sperm from domesticated males carried methylation signatures associated with altered expression of genes involved in development and metabolism (Rodriguez et al., 2019). Similarly, in medaka, exposure to hypoxia caused differential sperm methylation that affected larval transcriptional programs (Wang et al., 2016). Such environmentally induced epigenetic reprogramming of sperm adds a dynamic layer to paternal inheritance and serves as a mechanism by which paternal environmental experiences can shape offspring phenotype without genetic mutations.

1.6.2 Sperm-Borne RNAs and Regulatory Potential

Fish sperm, though highly compacted and lacking substantial cytoplasm, carries diverse sncRNAs, including miRNAs, piRNAs, and lncRNAs (Alata Jimenez et al., 2023). These RNAs are increasingly understood to regulate early developmental events, potentially

modulating zygotic genome activation and transcription factor networks. For example, stress exposure in male zebrafish altered the sperm transcriptome in a way that predicted neurodevelopmental gene expression in the progeny (Ord et al., 2020). Similar observations were made in salmonids, where sperm RNAs were associated with embryonic growth (Cabrita et al., 2014). The sperm RNA cargo, shaped during spermatogenesis and sperm maturation, thus represents a key molecular vector for paternal effects, tightly linked to environmental sensitivity and potentially heritable influence.

1.7 From Molecular Potential to Functional Consequences: *Defining Paternal-effect genes in Fish*

These discoveries have given rise to the concept of **paternal-effect genes (PEGs)**: genes whose expression in the progeny is altered as a result of paternal factors, i.e., molecular signals originating from genes expressed in the male germline or carried in mature sperm, without involving changes to the inherited DNA sequence (Panda et al., 2024). Although the existence of paternal effects is theoretically well supported, the knowledge on functional PEGs in the progeny remains scarce (Curley et al., 2011; Immler, 2018). This is largely due to the technical challenges of tracing specific sperm-delivered molecules into early embryos and mechanistically linking them to phenotypic outcomes. As it was once said "Virtually, nothing is known about the potential transfer and possible role of paternal RNAs in embryo formation in fish" (Bizuayehu & Babiak, 2020). One major obstacle is that variation in the abundance of molecular components in sperm does not necessarily result in detectable phenotypic consequences in the offspring, and thus may not reflect the activity of an ideal PEG. Additionally, isolating sperm cells with differing molecular profiles from the same male is practically unfeasible, making it difficult to attribute offspring variation to differences in sperm content from a single male. As a result, most studies of paternal effects rely on comparisons between genetically distinct males (Wirtz-Ocaňa et al., 2013; Siddique et al., 2017). However, even when such males are used to fertilize eggs from the same female, inherent male-female genetic or epigenetic incompatibilities can confound the results (Ross et al., 2022). These interactions may influence early development in ways that mimic or mask PEG activity, thus introducing bias and potentially leading to false positives or missed effects. **Ultimately, only by maintaining a constant genetic background in the sperm and observing distinct developmental outcomes in the progeny can we begin to reveal genuine PEG activity**. Without this control, it remains difficult to separate paternal genetic effects from defined the

theory of non-genetic inheritance (Crean & Bonduriansky, 2014). Consequently, our understanding of PEGs is still largely based on correlative observations, such as altered RNA or epigenetic profiles in sperm following environmental exposures (Rodriguez et al., 2019; Ord et al., 2020), and their inferred associations with changes in offspring phenotype.

One way to explore PEGs is a reverse-inference strategy: rather than starting from sperm molecules and tracing their effects, one may begin with the phenotypic and transcriptomic consequences observed in the offspring, and relate these to specific conditions experienced by the sperm. Such an approach enables the identification of candidate PEGs based on observed alterations in gene expression or developmental outcomes, potentially narrowing down the pool of genes under paternal influence. This outcome-first model offers a promising pathway to PEG discovery, particularly in systems where molecular tracing remains technically difficult or even out of reach (Crean et al., 2013). Importantly, this approach does not bypass mechanistic investigation, rather it anticipates it. By revealing which genes in the progeny respond consistently to defined sperm manipulations, we can establish a focused set of targets for future studies aimed at uncovering the underlying molecules and regulatory networks through which paternal control is exerted (Immler, 2018). In this way, even in the absence of direct tracing, the identification of PEGs through their developmental consequences provides a valuable and scientifically robust entry point into understanding paternal non-genetic inheritance (Rando, 2012; Perez & Lehner, 2019).

1.8 Towards PEG Discovery: *Experimental Approaches for Identifying Paternal Epigenetic Marks: Insights from Sperm Stress and Wild-Domesticated Comparisons*

Identifying paternal-effect genes requires strategies that enable researchers to isolate paternal-specific regulatory inputs from other confounding variables. One effective approach involves sperm manipulation techniques, such as **cryopreservation** and **post-thaw storage**, which can alter **sperm physiology** and **molecular content** without affecting its genetic background. Additionally, **comparing extreme phenotypes from domesticated and wild populations** has provided novel insights into sperm biology. These controlled perturbations offer a valuable experimental model in which observed variations in offspring development or gene expression can be more confidently attributed to non-genetic paternal factors.

Cryopreservation is well known to induce molecular stress on sperm cells, including oxidative damage, membrane instability, and RNA degradation (Bobe & Labbé, 2010; Cabrita et al., 2010). Post-thaw storage further compounds these effects, leading to time-dependent declines in sperm function and alterations in sperm-carried regulatory molecules (Kopeika et al., 2015). **These manipulations are highly relevant to paternal effect research, as they allow for intra-male comparisons, sperm from the same male can be exposed to different stress levels while maintaining identical DNA.** This preserves the genetic background while allowing the researcher to assess whether changes in sperm condition translate into changes in offspring phenotype, thus providing indirect but powerful insight into the activity of PEGs.

Complementing this, **another promising strategy is to leverage phenotypic extremes by comparing sperm from wild versus domesticated males.** Domesticated fish have undergone generations of artificial selection for desirable traits such as rapid growth, stress resilience, or reproductive efficiency, which often results in systematic shifts in gene expression, physiology, and gamete quality (Teletchea, 2021). This **wild-domesticated contrast has become a commonly used model in non-genetic inheritance studies, offering a natural yet controlled way to probe long-term biological reprogramming** (Lorenzen et al., 2012; Bicskei et al., 2014; Teletchea & Fontaine, 2014)**.** Unlike approaches that rely on acute stressors (e.g., toxins, thermal shock), the wild-domesticated comparison focuses on chronic, evolutionarily relevant divergence, asking how selection under captivity modulates paternal contributions. This makes it possible to study how sperm differences, caused by genes or the environment, play out in more natural conditions. In doing so, we can better understand how paternal epigenetic marks or PEGs matter in both wild populations and managed breeding systems.

Together, these two experimental frameworks, controlled sperm stress within individuals and phenotypic/genetic contrast between populations, offer complementary insights. The first isolates the effects of sperm condition while holding genetics constant, enhancing our ability to detect true non-genetic paternal signals. The second capitalizes on natural or artificial variation to explore broader paternal influences, including those encoded genetically. Used in tandem, these strategies provide a robust platform for identifying candidate PEGs and for probing how paternal biology, through both inherited and non-inherited mechanisms, shapes the next generation.

1.9 Methodological Rationale: *Integrating Transcriptomics and Controlled Larviculture to Explore Paternal-Effect Genes*

The identification of PEGs requires experimental strategies that can capture regulatory outcomes in the progeny attributable to the paternal contribution. For this purpose, we selected transcriptomic profiling of progeny as a primary molecular readout. Transcriptomics provides a direct insight into which genes are actively expressed at a given developmental stage, and therefore is ideally suited to detect the downstream impact of sperm-transmitted molecular signals, whether in the form of RNAs, proteins, or epigenetic marks (Braun & Champagne, 2014; Perez & Lehner, 2019). This approach is particularly appropriate in the context of PEGs, which by definition exert their effects through modulating gene expression in the offspring (Immler, 2018). **To identify these transcriptomic changes at a biologically relevant window, we focused on the mouth-opening stage of larval development**. The mouth-opening stage in teleosts represents a pivotal phase in early larval development, typically occurring shortly after hatching but prior to the onset of exogenous feeding. During this period, larvae rely exclusively on maternally derived yolk and oil droplets for endogenous nutrition, which supports metabolic demands while digestive, sensory, and immune systems continue to differentiate and mature (Gisbert et al., 2004; Hamza et al., 2007; Żarski, et al., 2021b). This transitional window, immediately before first feeding, is marked by low external input and minimal handling, reducing confounding environmental variation. Importantly, sampling at the mouth-opening stage provides a clearer window into parental (especially paternal) influences on gene expression, because the larvae have not yet begun exogenous feeding or experienced significant microbial colonization from live feed or complex rearing systems. Transcriptomic analysis at this stage allows researchers to capture intrinsic developmental signatures, particularly those regulating digestive enzyme ontogeny, neurogenesis, and early immune priming, all of which are transcriptionally active during the mouth-opening stage (Zambonino Infante & Cahu, 2007; Mazurais et al., 2011; Fernández-Díez & Herráez, 2018a). Thus, it provides a biologically relevant and technically controlled window for studying early-life programming and transgenerational effects in teleosts. As a result, selecting this stage maximizes the likelihood of identifying differentially expressed genes linked to paternal inputs.

To complement the molecular data and assess the functional significance of potential PEGs, **we coupled transcriptomic profiling with a quantitative evaluation of larval phenotype under controlled rearing conditions.** This approach, referred to in our study as advanced

zootechnical exploration, allows precise tracking of developmental outcomes, such as growth rate, deformity incidence, behavior, and survival, under standardized and replicable aquaculture environments. As highlighted in previous studies, such phenotypic assays are essential for bridging the gap between molecular signatures and biological function (Bobe & Labbé, 2010; Teletchea, 2021). Taken together, this dual-level strategy, analyzing the larval transcriptome at a critical developmental checkpoint and measuring corresponding performance traits under controlled larviculture, offers a robust and biologically relevant means of uncovering PEG activity.

1.10 Eurasian Perch as a Model for Investigating Paternal-effect genes

The choice of model species is especially important for studying paternal non-genetic inheritance because different species vary in sperm biology, fertilization strategies, and early developmental patterns. These differences can strongly influence how paternal factors are delivered to and affect the offspring. Using a species with well-characterized reproduction and available genomic tools makes it much easier to separate and identify paternal effects without confounding variables (Anderson et al., 2003). The Eurasian perch (*Perca fluviatilis*), a temperate freshwater species native to Northern Europe and Northeast Asia, presents an excellent biological platform to investigate PEGs due to its unique combination of aquaculture relevance, reproductive characteristics, experimental tractability, and a well-annotated genome (Fontaine et al., 1997; Alix et al., 2015). This genomic resource is essential for interpreting transcriptomic data and designing molecular assays central to this study.

Originally targeted for aquaculture in the 1990s, Eurasian perch has steadily gained attention due to its high market value, adaptability to recirculating aquaculture systems (RAS), and suitability for intensive production in temperate climates (**Figure 1.2**; Kestemont & Dabrowski, 1996; Mélard et al., 1996; Fontaine et al., 1997). Over recent decades, the species has transitioned from field trials to fully domesticated production cycles in indoor systems across several European countries, including Belgium, France, and Switzerland (**Figure 1.2**). These advances have not only improved production control but also opened the door to a wide range of biological studies, from reproductive physiology and stress biology to developmental genomics (Teletchea & Fontaine, 2014; Fontaine et al., 2019).

From an experimental standpoint, Eurasian perch offers several key advantages for studying PEGs. Firstly, as an externally fertilizing species, it allows precise experimental manipulation

of sperm prior to fertilization, including cryopreservation and post-thaw storage (Judycka et al., 2019a). This enables tightly standardized conditions while avoiding maternal physiological interference (**Figure 1.2**; (Żarski et al., 2015, 2017a, 2017b, 2019; Palińska-Żarska et al., 2020). Moreover, the large number of eggs released by a single female permits paired evaluations of different treatments on sibling embryos, strengthening within-clutch comparisons (Kucharczyk et al., 1996). Eggs from one female can also be fertilized with sperm from several different males, allowing researchers to control for male-female compatibility effects. Secondly, perch males produce one of the highest sperm outputs among freshwater fish, considering both sperm concentration and semen volume relative to body weight (Alavi et al., 2015). This trait provides ample material for experimental replication, sperm manipulation, and molecular analyses. Thirdly, based on empirical observations, Eurasian perch exhibits a relatively delayed zygotic genome activation compared to many model fish species (Alix et al., 2015). This developmental feature extends the window in which parentally derived RNAs, proteins, and epigenetic marks can act without interference from the zygotic genome, increasing the likelihood of detecting early transcriptional changes linked to paternal factors.

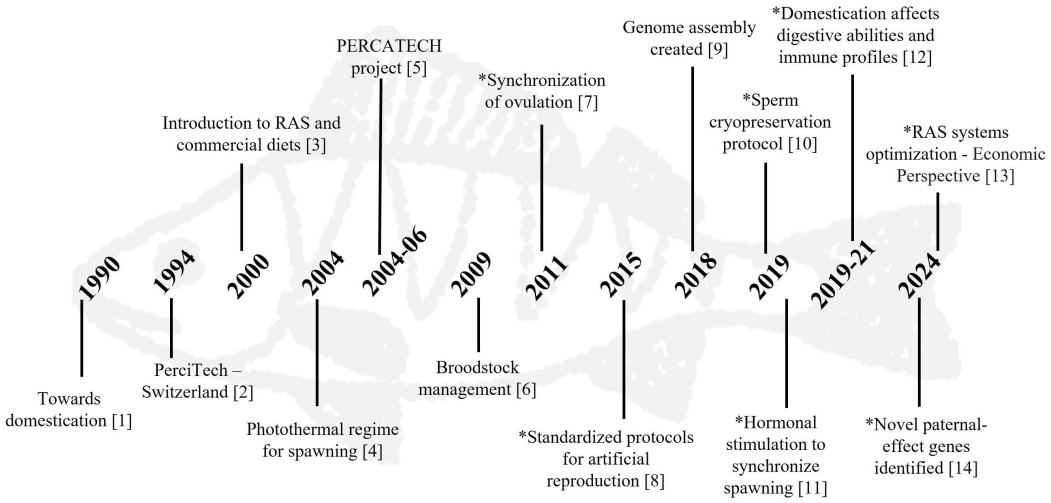

Figure 1.2: *Milestones achieved over the decades for Eurasian perch aquaculture. [1] Initial research on E. perch (Fontaine et al., 1997). [2] PerciTech becomes Europe's first E. perch cultivation and processing company (Fontaine et al., 2019). [3] Beginning of Perch nutrition-based studies (Kestemont et al., 2001). [4] Out-of-season reproduction protocol (Migaud et al., 2004). [5] First European project aiming towards optimizing reproductive protocols (Toner, D. 2008). [6] Domestication and broodstock management applied (Teletchea et al., 2009). [7] Towards controlled reproduction (Żarski et al., 2011). [8] Further progress in broodstock management (Żarski et al., 2015). [9] Genome data (Ozerov et al., 2018). [10] Optimal cryoprotectants and thawing procedures (Judycka et al., 2019). [11] Hormonal stimulation to promote out-of-season spawning (Żarski et al., 2019). [12] Domestication effects development (Palińska-Żarska et al., 2020, 2021). [13] Food security and economic development in EU (Hakuć-Błażowska et al., 2024). [14] Identification of paternal-effect genes pde6g, rbp4l, and opn1lw1 (Panda et al., 2024)* Indicates contributions made to the field by the Team of Reproduction and Development in Fish, PAS Olsztyn. RAS – Re-circulating aquaculture system; EU – European Union*

Eurasian perch also excels as a model for larval phenotyping under controlled rearing, an important consideration when assessing whether paternal molecular differences yield functional phenotypic changes. Its well-described larval stages, group-synchronous spawning, and the availability of controlled photothermal and hormonal protocols allow researchers to generate highly standardized offspring cohorts (Migaud et al., 2004; Żarski et al., 2015). Along with standardized cryopreservation protocol elaborated for this species, allows the investigations of PEGs (Judycka, et al., 2021; Panda et al., 2024). These conditions support robust, replicable evaluation of key developmental traits such as deformity rates, growth, and survival, critical endpoints in any study of paternal inheritance. In summary, the biological characteristics and practical flexibility of Eurasian perch position it as unique yet powerful model for studying PEGs. Its ability to combine molecular precision, experimental control, and phenotypic readouts makes it an ideal system to bridge the gap between sperm biology and offspring development, providing both applied value for aquaculture and fundamental insights into non-genetic inheritance.

Chapter 2

Research aims and hypotheses

2.1 Aims and Objectives

The primary objective of this thesis is to advance our understanding of paternal-effect genes in teleost fish by investigating how sperm manipulation influences offspring development. This study focuses specifically on assessing whether alterations in sperm condition, induced through cryopreservation (Chapter 2; Fresh-Cryo) and post-thaw storage (Chapter 3; PTS), translate into measurable differences in embryonic and larval phenotypes, as well as transcriptomic changes in the progeny. Additionally, the thesis aims to explore how selection pressures imposed by domestication affect the nature and magnitude of paternal effects (Chapter 4; Wild-Dom). This is pursued by comparing progeny sired by wild and domesticated males, a widely used strategy for revealing paternal effects in lower vertebrate models. Unlike stress-induced models commonly employed in rodent studies, this approach leverages naturally evolved phenotypic divergence, thus offering insights into how long-term captive rearing may modulate paternal contributions to offspring phenotype. Furthermore, the thesis seeks to develop and apply an advanced experimental pipeline, including high-resolution larval phenotyping and transcriptomic profiling at the mouth-opening stage, to establish whether altered sperm condition correlates with shifts in gene expression patterns in the progeny. Together, these experimental approaches serve as a foundation for characterizing putative PEGs under biologically and aquaculturally relevant scenarios.

Rather than pursuing direct mechanistic links between paternal molecular components and offspring traits, this work takes a phenotypic and transcriptomic endpoint approach to identify patterns consistent with paternal influence. Employing this inverse strategy may fill a critical gap in current reproductive biology, where maternal effects have been extensively studied while paternal effects remain comparatively understudied (Braun & Champagne, 2014; Ord et al., 2020).

2.2 Research Hypotheses

This research is grounded in the central hypothesis that alterations in sperm condition, whether due to cryopreservation and post-thaw storage procedures or inherent differences resulting from domestication, can elicit measurable changes in progeny phenotype and gene expression, even when the paternal genetic background remains constant. It is expected that these changes will manifest at both phenotypic and transcriptomic levels, particularly during early larval development, which is a critical period for growth, survival, and morphological patterning.

A secondary hypothesis is that the transcriptomic profiles of larvae sired by males with compromised or environmentally differentiated sperm will reveal distinct patterns of gene expression indicative of paternal-effect genes. These patterns are anticipated to be detectable at the mouth-opening stage, a point at which maternal RNA influence wanes and zygotic transcription becomes dominant. While this study does not seek to establish direct mechanistic links between specific sperm molecules and larval gene expression, it hypothesizes that consistent differences in transcriptomic signatures across experimental groups will reflect paternal influences on offspring development.

Lastly, it is hypothesized that using wild versus domesticated males will not only provide high phenotypic divergence but also help differentiate between environmentally induced and genetically inherited paternal effects. This approach allows the thesis to test whether domestication, through artificial selection and adaptation to captive conditions, modifies the spectrum and magnitude of paternal contributions to offspring developmental trajectories.

Chapter 3

Paternal-effect genes revealed through sperm cryopreservation in Eurasian perch, *Perca fluviatilis*

Also referred as: Fresh-Cryo

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3.1 Introduction

Fish ontogeny, especially at their early life stages, is largely determined by their parents. However, most of the work revolves around the evaluation of maternal contribution (Marteinsdottir & Begg, 2002). This is mostly associated with the well-known fact that the mother provides nutritional and energy reserves (contained in the yolk) utilized by fish during the larval period (Green, 2008). More recently, it became apparent that maternal contribution is far beyond the nutritional reserves, shedding light on the remaining components of egg molecular cargo as a modulator of progeny phenotype (Lubzens et al., 2016). Maternal transcripts are known to be responsible for controlling development at least up to zygotic genome activation (ZGA) (Schulz & Harrison, 2019). Nevertheless, in the past three decades, a piling body of evidence indicates significant paternal influence during the early life history (ELH) (i.e., from fertilization until the end of the larval period in fish) (Vøllestad & TLillehammer, 2000; Rideout, 2004; Krawetz, 2005; Siddique et al., 2017). In addition to reproductive fitness and age of males, other wide variety of progeny traits, such as embryonic developmental rate and larval size upon hatching, have been shown to be contributed by the father (Uusi-Heikkilä et al., 2012). These finding sheds light on the fact that very little is known about the paternal contribution to progeny phenotype in fishes. Understanding paternal contribution to fish ELH is crucial for fish biologists, ecologists and the aquaculture sector, where it can be used to fine-tune selective breeding programs, which may lead to increased production effectiveness and improved welfare of cultured species (Bromage et al., 1992).

Phenotype of the progeny is shaped by the genetic and non-genetic factors (e.g., epigenetic modifications of genome, various molecules – such as RNAs, proteins, metabolites and others – contained in the gametes), with the latter being susceptible to external cues (Adrian-Kalchhauser et al., 2020), including environment the fish lives in as well as any other factors (such as pathogens) the fish may experience (Nynca et al., 2020; Żarski et al., 2020, 2021). There has been lots of discussion about maternal molecular cargo influencing regulation of expression of genes in the progeny. Consequently, genes being modified in the progeny by the maternal molecules contained in the eggs were termed ‘maternal-effect-genes’ (Badyaev & Uller, 2009; Carter & Blizard, 2016; Trapphoff et al., 2016). Research on the gene regulatory effects in offspring resulting from paternal influences, henceforth referred to as ‘paternal-effect genes’ (PEGs), has been relatively scarce. Essentially, PEGs can be understood as genes whose

expression levels in the offspring are influenced by both genetic and non-genetic factors present in the sperm and are transmitted to the progeny.

While standardizing sophisticated reproductive techniques, milt cryopreservation (involving specific procedures enabling effective storage of the viable cells at ultra-low temperatures for a very long period) for fishes has come into limelight over several decades now (Hezavehei et al., 2018). Cryobanking of milt helps to manage the genetic diversity of the fish species, facilitates spawning synchronization, allows selective breeding and much more (Asturiano et al., 2017). Cryopreservation of milt is a big shock to a sperm cell constituting a specific challenge test (Kopeika et al., 2015) which causes irreversible damages to the cryo-sensitive cells which lose fertilizing capacity, in contrast to cryo-resistant cells retaining their functionality (Wang et al., 2022). Importantly, the effect of cryopreservation on spermatozoa functionality depends on the species. On one hand, sperm motility, fertilization rate and the hatching rates were seen to be high and similar with post-thaw milt when compared with use of fresh milt in some Salmoniforms and Esociformes (Nynca et al., 2015; Dietrich et al., 2016). On the other hand, studies done so far on few other teleosts (e.g., Levantine scraper, wild brown trout, Atlantic cod) report reduced motility and viability of sperm after freezing-thawing (Zadmajid et al., 2019). Also, the post-thaw milt from the same fish when used for *in vitro* fertilization, there were significant declination in fertilization success when compared with fresh sperm control, followed by abnormalities like cleavage patterns, hatching success, organogenesis etc. (Ottesen et al., 2012; Nusbaumer et al., 2019). So far studies elaborating implications of sperm cryopreservation on embryonic and larval performance as well as gene expression profile in offspring is very limited (Kopeika et al., 2015; Montague et al., 2023). It has been reported, that along with DNA damage, changes in gene expression, and chromatin integrity in sperm, the transcriptome of the larvae (obtained with frozen-thawed sperm) is also seen to be dysregulated due to sperm cryopreservation (Fernández-Díez & Herráez, 2018b). To add on, the maternal molecular cargo has the ability to repair a certain degree of the DNA damage (Fernández-Díez et al., 2016). Clearly, milt cryopreservation seems to be a very subjective kind of sperm cell stressor.

Eurasian perch is a commercially relevant species farmed in recirculating aquaculture systems (RAS), attaining the 4th level of domestication thus far (Teletchea & Fontaine, 2014). In the last 20 years, it was found to be an excellent model for studies on embryonic development

(Alix et al., 2015), reproduction (Migaud et al., 2004), domestication processes (Palińska-Żarska et al., 2020) and circadian rhythms (Kupprat & Kloas, 2020). In addition, the larvae of this species can be utilized as a complete organism to sequence their RNA repertoire, given their size and developmental advancement. At their mouth opening stage, they are self-sustainable organisms with the ability to adapt to different environments. More importantly, at this stage, they are not yet affected by any human intervention (Palińska-Żarska et al., 2020). In addition, a Eurasian-perch specific, highly standardized sperm cryopreservation procedure was developed by Judycka et al. 2019 the maintenance of high fertilization success with the use of cryopreserved milt. This has brought the tool, enabling much more feasible and sophisticated selective breeding procedures in this species. However, until now, neither molecular nor phenotypical consequences have been investigated following the usage of cryopreserved sperm for the creation of a new generation in this species. In addition, prominently, any consequences passed on to the progeny from sperm subjected to these challenging procedures would be the ones directly linked with a contribution of a male to the overall phenotype of the progeny. This also includes distinguishing PEGs, an important aspect in developmental biology that is highly difficult to identify. Therefore, in our study, we aimed to explore phenotypical and transcriptomic consequences in larvae resulting from the application of sperm cryopreservation technology. Controlled reproduction of Eurasian perch followed by examination of phenotypical performance became a kind of proxy for understanding physiological alterations in progeny, revealing paternally effected genes.

In the present study, we carried out RNA sequencing (RNA-Seq) of RNA obtained from freshly hatched larvae (at the mouth opening stage) to identify the processes being modulated/ affected in the progeny by the usage of cryopreserved sperm for fertilization in Eurasian perch (*Perca fluviatilis*), which is a model for percid fishes, an important group of commercially relevant aquaculture freshwater species. The strength of the present study is its importance around the integration of information on phenotypical performance of the progeny and the transcriptomic profile/repertoire obtained from the whole organism. After all, combining transcriptomics data and associated phenotypic characteristics observed during advanced phenotypical exploration is an excellent approach to link genotype-phenotype relationships (Rey et al., 2021).

3.2 Materials and methods

Ethics statement

The study was conducted according to the European and national legislation for fish welfare and approved by the Local Animal Research Ethics Committee, resolution no 5/2023. The animal study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for animal research.

Broodstock management and controlled reproduction

We crossed 3 female and 6 male wild spawners (see physiological details in **Table s3.1**) from Mikołajki lake and Żurawia fish farm ponds, respectively. The wild fish were caught using fyke nets and transported immediately after in plastic bags filled with water and oxygen (v/v 2:1) to the research facilities of the Centre of Aquaculture and Ecological Engineering of the University of Warmia and Mazury in Olsztyn (CAEE-UWM, NE Poland). The pond-reared fish males were harvested in November, dedicated to oxygenated tanks at the Salmonid Research station of the National Inland Fisheries Research Institute in Rutki (North Poland), where they were overwintered in the flow-through system fed with riverine water (natural photothermal conditions). Males were fed with frozen bloodworms (Chironomidae) by hand during the light phase until apparent satiation (exhibited by lack of reaction of fish to food provided), which depended on the water temperature. Females were caught directly during the spawning season. They were then transported in plastic bags with oxygen to the CAEE-UWM for further controlled reproduction procedures. The females and males were of different origins because the capture of wild males during the spawning season is very difficult, and often these males are prone to have partially participated or even completed the spawning act before being caught. This can have a direct effect on the sperm quality obtained; thus, the males were caught earlier and overwintered. In contrast, the wild females, if overwintered in controlled conditions, tend to have lowered egg quality, affecting the quality of the larvae and therefore causing bias to the results obtained. Wild Eurasian perch females caught during the spawning season are not accepting any type of food while kept in the hatchery (for details see: (Żarski et al., 2017c)), so they were not fed. Nevertheless, as in our study, the overall reproductive protocols did not affect egg quality negatively as we observed very high fertilization rate (over 80%) in all the females (**Figure 3.1**). The males and females were kept separately, according to their gamete maturity stages as recommended by (Żarski et al., 2017b) in RAS with a defined photoperiod (14 hours

light:10 hours dark) and temperature (12°C±0.1) until ready for ovulation and spermiation. To promote and synchronize the spawning act of both sexes, fish were hormonally stimulated using salmon gonadoliberin analog (sGnRHa, BACHEM, Switzerland) (injection at a dose of 50 µg kg^{-1}) (Żarski et al., 2017b). Sperm were collected 7 days post hormonal stimulation (which was within the optimal period of sperm collection of this species) (Żarski et al., 2017d), whereas eggs were collected between 3- and 5-days following injection depending on the maturation stage of the females (Żarski et al., 2011). Prior to any manipulation, such as gamete collection, the fish were anesthetized in MS-222 (Argent, USA) at a dose of 150 mg L^{-1}. Twelve unique families (each family reared in triplicate) were selectively created using 3 females and 6 males. More specifically, eggs from one female were divided into four portions, each fertilized with either fresh (group Fresh) or cryopreserved (group Cryo) sperm from two males, separately (as described in **Figure 3.1a**). Apart from gametes, other information from parents, such as total length (L_T), caudal length (L_C), body weight (before and after gamete stripping), body scales (for estimation of the fish's age) and fin-clip samples, was collected (**Table s3.1**).

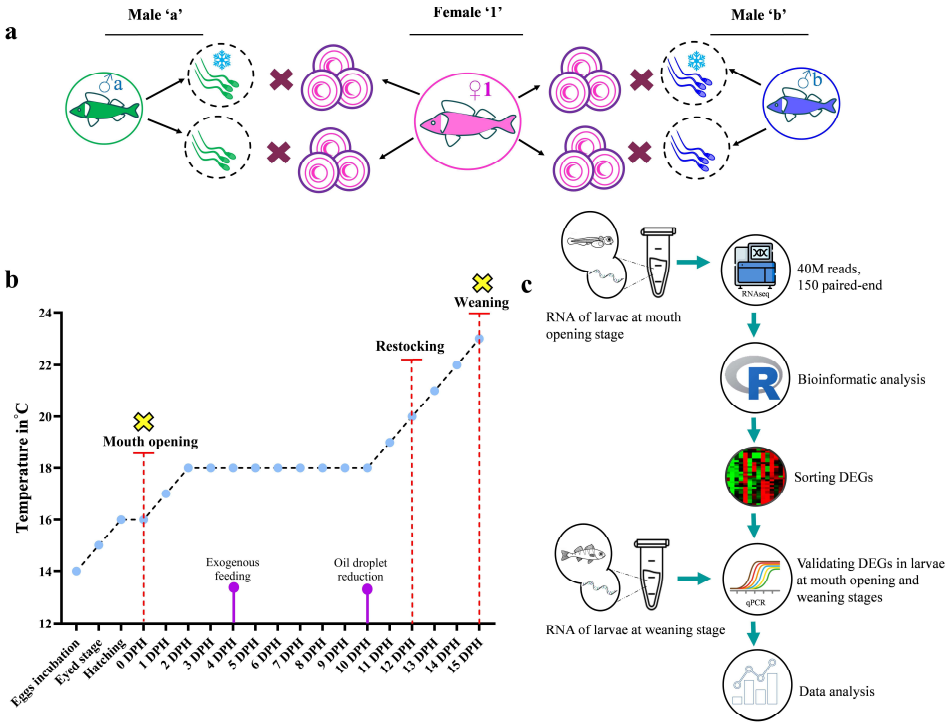


Figure 3.1: *Protocol followed for the experiment.* ***(a)*** *Eggs from one female were fertilized with milt from two males such that eggs from one female were divided into two portions for each male, one fertilized with cryopreserved milt (marked with a snowflake) and the other portion with fresh milt;* ***(b)*** *Rearing schedule and temperature regimen along with sampling points (yellow crosses);* ***(c)*** *RNA extraction and sequencing, followed by sorting DEGs, validations by qPCRs and data analysis. DEGs- Differentially expressed genes.*

Sperm collection and cryopreservation

The 6 males were stripped (with gentle pressure on abdomen) for milt using a catheter (Galmed, Poland) (to avoid contamination of the urine or blood). After collection, each sample was kept on ice. Activation of sperm to check motility was done by a 1:1000 dilution using a two-step procedure. First, the semen was diluted 1:50 (for fresh semen) or 1:5 (for fresh-diluted and frozen/thawed semen) in an immobilizing solution (150 mM NaCl, 5 mM KCl, 1 mM MgSO4 × 7H2O, 1 mM CaCl2 × 2H2O, 20 mM Tris, pH 8.0). Then, the semen was diluted 1:20 in an activating solution (75 mM NaCl, 2 mM KCl, 1 mM MgSO4 × 7H2O, 1 mM CaCl2 × 2H2O, 20 mM Tris, pH 8.0) supplemented with 0.5% bovine serum albumin. From several motility parameters measured using the computer-assisted sperm assessment (CASA) system, the linearity (LIN, %), amplitude of lateral head displacement (ALH, µm), average path velocity (VAP, µm s^{-1}), curvilinear velocity (VCL µm s^{-1}), and straight-line velocity (VSL µm s^{-1}) were also evaluated using the CASA system for both fresh and cryopreserved milt. Alongside, the concentration of fresh milt was measured using NucleoCounter SP-100 (Chemometec, Allerød, Denmark) (Judycka et al., 2021).

One part of the collected milt was used for cryopreservation as described by (Judycka et al. 2021). The milt was diluted with extender (consisting of a final concentration of 0.3 M glucose, 7.5% methanol and 25 mM KCl at 3×10^9/ml spermatozoa). Milt mixed with extender (to final volume of 0.5 ml) was loaded into 0.5 ml plastic straws (IMV Technologies, L'Aigle, France), which were placed on a floating rack. Then, the straws were frozen in liquid nitrogen vapour (3 cm above liquid surface) for 5 min in a Styrofoam box with an isolating Neopor block (Minitübe GmbH, Tiefenbach, Germany) followed by placement in the liquid nitrogen. The other part of the milt was kept on ice to be used directly for the fertilization trials without any manipulations to use as Fresh sperm.

Egg collection and fertilization trials

The chosen females were taken to check their oocyte maturation stages as described earlier (Żarski et al., 2011) by catheterizing a few oocytes, exposing them in Serra's solution (ethanol, formalin, and glacial acetic acid mixed 6:3:1 by volume) and microscopic evaluation of their maturation stages. At ovulation, eggs from females were stripped out into a clean and dry beaker as described earlier (Żarski et al., 2011).

Each egg ribbon with an average weight of 60± 10 g was sampled into 3-5 small portions (<1 g) and weighed, and the eggs per portion were counted. In this way, we could estimate the number of eggs present per gram to aid us in dividing the ribbon into 4 equal portions (2 portions per male). Ribbon(s) from each female were further divided into equal portions (conducted the rearing in triplicate) of ~4 g each and were used to carry out the *in vitro* fertilization (Żarski et al., 2012). Just before fertilization, straws were thawed in a water bath at 40°C for 10 seconds and placed in an Eppendorf tube (Judycka et al., 2021). Then, the eggs were preactivated for 30 s in hatchery water, and milt (fresh or cryopreserved) was added to the eggs at a sperm:egg ratio of 200,000:1. Then, an appropriate amount of milt was added to each egg portion, as previously calculated (separately for each egg sample at a 200,000:1 sperm:egg ratio). The eggs were then stirred for 30 seconds and washed with hatchery water after ~10 minutes to remove excess sperm and any debris.

Incubation of embryos

The fertilized eggs were incubated in 5 L tanks with black walls that functioned within the same RAS. Initially, before hatching, the eggs were placed on mesh (diameter of 3 mm) at a temperature of 14°C. Within 24 hours of fertilization (before embryos reached the mid-blastula transition (MBT)), ~100 random embryos were observed under the microscope to calculate the fertilization rate. Similar counting was again performed after 3 days, while the embryonic development rate was estimated at the neurula stage (when the body of the embryo could be viewed at the animal pole). While incubating the embryos, the photoperiod was maintained at 24 L:0D, and the temperature was raised to 15°C when the embryos reached the eyed-egg stage; then, the temperature was maintained at 16°C as soon as the first hatched larvae were noticed (Palińska-Żarska et al., 2020). We started numbering the age of larvae post hatching as DPH (days post hatching), and to maintain synchronous hatching, the larvae were hatched manually (gentle actuation of the egg ribbons in a bowl). This was done by transferring the egg ribbons to bowls with water from the rearing tanks and stirring gently, and the hatched larvae were put back to their respective tanks. We carried out this operation 4-5 times. That day was named 0 DPH, and the day count had begun.

Larviculture and advanced zootechnics

The hatched larvae in both the Fresh and Cryo groups were reared following the exact same conditions in the RAS system, along the standardized temperature and feeding regimen described (**Figure 3.1b**) (Palińska-Żarska et al., 2020). Beginning from 0 DPH, the temperature was 16°C. At 1 DPH, the water temperature was raised by 1°C, and at 2 DPH, it was at 18°C; this temperature was kept stable up to 10 DPH. Starting from 4 DPH feeds of *Artemia* sp. nauplii *ad libitum* three times per day (first four days of feeding – micro *Artemia* cysts [SF origin], then standard size *Artemia* cysts at 260,000 nauplii per gram [GSL origin]) was insured. At 12 DPH, feeding larvae were restocked equal numbers of larvae in all tanks by counting volumetrically. Here, feeding larvae ensured healthy larvae to a few extents. Subsequently, 11 DPH onwards, the temperature was increased by 1°C per day until 23°C, which is considered the optimal temperature for the growth of perch larvae (Żarski et al., 2019). After the first feeding and before the last feeding, the tanks were cleaned, and dead larvae were counted. In addition, other parameters, such as the oxygen level to 80% and ammonia concentration to <0.02 mg $L^{-1,}$ were maintained. The experiment was conducted for the larvae only until their *Artemia* feeding phase, i.e., the experiment was terminated at the weaning stage, upon sampling.

At the mouth-opening stage (between 0 and 1 DPH, where at least 50% of larvae were found to have reached this stage) and at the time when the protocol envisaged the end of feeding, the larvae with live *Artemia nauplii* (hereinafter referred to as weaning; 15 DPH) were sampled for total length (TL, ±0.01 mm) and wet body weight (WBW, ±0.1 mg). Additionally, samples of larvae were used for extraction of RNA (whole larvae were preserved in RNAlater, Sigma–Aldrich, Germany). During the first sampling, we made sure we collected larvae at the mouth opening (MO) stage, where in-egg embryonic development has been accomplished and larvae were ready to survive in the outer environment but with minimal human intervention applied.

The total length of larvae was determined using a stereoscopic microscope (Leica, Germany). Next, wet body weight measurements using the 'noninvasive method' (Krejszeff et al., 2013) were addressed using a precision laboratory scale (Ohaus, USA). For this purpose, anesthetized larvae were placed on a platform made of nylon mesh (with a mesh size of approx. 200 μm), and excess water was drained out by filter paper. This method minimized possible physical damage to very delicate larvae. Two days after oil droplet reduction, the swim bladder inflation efficiency (SBIE%) was calculated using a stereoscopic microscope by triple counting

perch larvae (with and without a filled swim bladder) randomly caught from each tank on a Petri dish (in total, we determined SBIE from more than 100 larvae from each tank). Before any manipulations, we anesthetized the larvae in a solution of MS-222 (at a dose of 150 mg L^{-1}).

RNA extraction

The total RNA was extracted from snap frozen unfertilized eggs (UFE; ~50 eggs) and a pool of larvae (n=10 for larvae at mouth opening and n=4 for larvae at weaning) using a TotalRNA mini-kit (A&A Biotechnology, Poland) from unfertilized eggs of each female and larvae from each family (for both sampling stages; **Figure 3.1c**), separately. The quantity and purity of extracted RNA were evaluated using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). Samples showed absorbance ratios A260/280 ≥ 2.0 and A260/230 ≥ 2.2. The quality of the extracted total RNA was also evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), and all the samples presented RIN ≥ 9.0. Samples were then outsourced for RNA sequencing.

RNA sequencing and bioinformatics

Libraries (using TruSeq stranded mRNA kit) were sequenced using Illumina's NovaSeq 6000 with standard protocols. Overall, from each sample, more than 40 M reads were obtained, with a 150 bp paired-end sequencing mode.

Differential analysis

The raw reads were quality controlled using FastQC software version 0.11.9 (Andrews, S., 2010). Adapters and low-quality fragments of raw reads (average *Q*Phred score < 20) were trimmed out, and reads were clipped to equal lengths of 100 nt using the Trimmomatic tool ver. 0.40 (Bolger et al., 2014). The resulting read sets of the analyzed samples were mapped to a reference genome *P. fluviatilis* version 11.1.104 obtained from the NCBI database (Sayers et al., 2022) using STAR software ver. 2.7.10a (Dobin et al., 2013) with ENCODE default options.

Transcript count data for the larval samples were filtered to have at least 5 libraries in which there were at least 5 reads. Libraries from before and after the cryopreservation process were compared using the following design: ~ *males* + *condition;* males standing for the 6 males followed during the experiment and condition representing before (fresh) and after

cryopreservation. These analyses were performed in RStudio (version 4.1.3) using the package DESeq2 (Love et al., 2014) and *ashr* for log fold-change shrinkage (Stephens, 2017). Differences were considered significant when corrected p values were inferior to α (α=0.05), and we obtained 11 DEGs.

It should be emphasized that among the 6 families created and used for the entire study, for further analysis, 1 family (from the Cryo group and its counterpart in the Fresh group) was removed because the transcriptomic profile clearly differed from the remaining families and was considered an outlier (see **Supplementary Figure s3.1**).

Gene Ontology Enrichment analysis (GOEA)

GOEA was performed using ShinyGO, version 0.77 platform (Xijin Ge et al., 2020) to test the overrepresentation of GO terms in a list of genes and to understand their biologicaPASl significance as an effective approach (Klopfenstein et al., 2018; Nynca et al., 2022). The 11 DEGs (namely, crystallin beta A2b (*cryba2b*); crystallin beta A4 (*cryba4*); crystallin beta B1 (*crybb1*); crystallin, gamma MX, like 2 (*crygmxl2*); phosphodiesterase 6G, cGMP specific, rod, gamma (*pde6g*); opsin 1, longwave-sensitive, 1 (*opn1lw1*); gamma-crystallin M2-like (*gamma m2*); beta-crystallin A1-like (*cryba1*); gamma-crystallin M3-like (*crygm3*); retinol binding protein 4, like (*rbp4l*); and transforming growth factor beta induced (*tgfbi*)) were fed to the ShinyGO platform, zebrafish was chosen as the best matching species; with the false detection rate (FDR) cutoff of 0.05, and 20 pathways' network was created. A STRING-db, version 12.0 (Von Mering et al., 2005) with functional enrichment of GO biological processes was also performed to retrieve a protein–protein network that also describes the distance between the linked genes.

RT–qPCR validation of differentially expressed genes (DEGs)

Primer design

Primer pairs for all 11 DEGs along with 5 normalizing genes for RT qPCR were designed using NCBI-Primer BLAST, version 1.0.1 (Ye et al., 2012). The sequence that matched the best to *P. fluviatilis* was fed to Primer3Plus software version 3.3.0 (Untergasser et al., 2012; Ye et al., 2012). The best matching pairs with least possibilities to form secondary structures were chosen and checked for GC content and melting temperature (Tm) on µMelt Quartz, version 3.6.2 (Dwight et al., 2011). The sequences of the designed primers are presented in **Table s3.1.**

qPCRs

RT–qPCRs were performed for each gene using a Viia7 (Applied Biosystems) thermocycler. For each qPCR, 10 ng cDNA template was used along with 10 µl (A&A Biotechnology) SYBR RT PCR Master Mix (Cat. No. 2008-100), 0.5 µM forward (1 µl) and reverse (1 µl) primers, 2 µL of starter mix and PCR grade water were added to maintain a final volume of 20 µL. The reactions were performed with the following cycling conditions applied: enzyme activation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and elongation at 60°C for 1 minute. In the analysis of each gene, a standard curve was calculated using a series of 6 two-fold dilutions to determine reaction efficiency (reaction efficiencies between 85% and 110% were considered acceptable). Relative expression for each gene was normalized as the geometric mean of expression values recorded for 5 reference genes (namely, cytochrome c-like, transcript variant X1, *cycs*; tetraspanin 7, *tspan7*; ER membrane protein complex subunit 10, transcript variant X2, *emc10*; pre-mRNA-splicing factor, *syf2* and ER membrane protein complex subunit 3-like, *emc3*), which were chosen from our transcriptomic data on the basis of their stable expression levels and close-to-mean expression values in the RNA-sequencing analysis (Żarski et al., 2021). Each reaction for real-time qPCR validation was performed in triplicate. The data were compared between the Fresh group and Cryo group (at mouth opening and weaning stages).

***In silico* analysis**

Several *in silico* analyses were carried out using tools such as NCBI-BLAST (Altschul et al., 1990), Expression Atlas version 2.0 (Moreno et al., 2022) and PhyloFish (Pasquier et al., 2016). These tools helped us to study the expression levels of our DEGs throughout the early life history in *Danio rerio* as a reference model organism for Eurasian perch. Additionally, it allowed us to explore the expression pattern of DEGs in various tissues in *D. rerio*, and few other evolutionarily close/distant species (namely, *O. mykiss*, rainbow trout; *S. lucioperca*, pikeperch; *A. mexicanus*, surface Mexican tetra; *A. mexicanus* cave Mexican tetra and *A. Anguilla*, European eel) from Eurasian perch. This was done to get hints if a particular gene has tissue-specificity.

Upon sequencing the RNA obtained from UF eggs, we checked for the presence of our DEGs, considering a threshold of TPMs>0.5. While for other species like *D. rerio*, *O. mykiss* and *S. lucioperca*, we used the PhyloFish and Expression atlas platform to check for the

expression values while we used raw data of UF eggs sequenced by Kim et al., (2020) for *M. anguillicaudatus.*

Statistical analysis

The raw data from all the parameters like sperm quality parameters (in %, µm, µm s^{-1}), fertilization and embryonic developmental rates (%), deformities (%), SBIE (%), TL (mm), and WBW (mg) were first fed into GraphPad (version 9.5.1) and paired t-tests ($p<0.05$) for each single parameter to compare between Fresh group and Cryo group were conducted. While calculating then plotting cumulative mortality (%); expression values of our DEGs (in TPMs) after sequencing and normalized expression values after real time qPCRs (mean quantity); transformation of gene replicates in TPMs for presence of genes in tissues were calculated on Microsoft Excel. However, the values were then computed on GraphPad to plot graphs after paired T-tests. All the data were tested with a significance level of 5% (significant differences were considered at p value < 0.05).

2.3 Results

Cryopreservation resulted in a significant decrease in all tested sperm motility parameters compared to fresh milt, except linearity (**Figure 3.2**). However, fertilization and developmental rates were not affected by cryopreservation. Additional analysis of sperm motility parameters in relation to fertilization rate did not reveal any significant correlation (see **Supplementary Figure s3.2**).

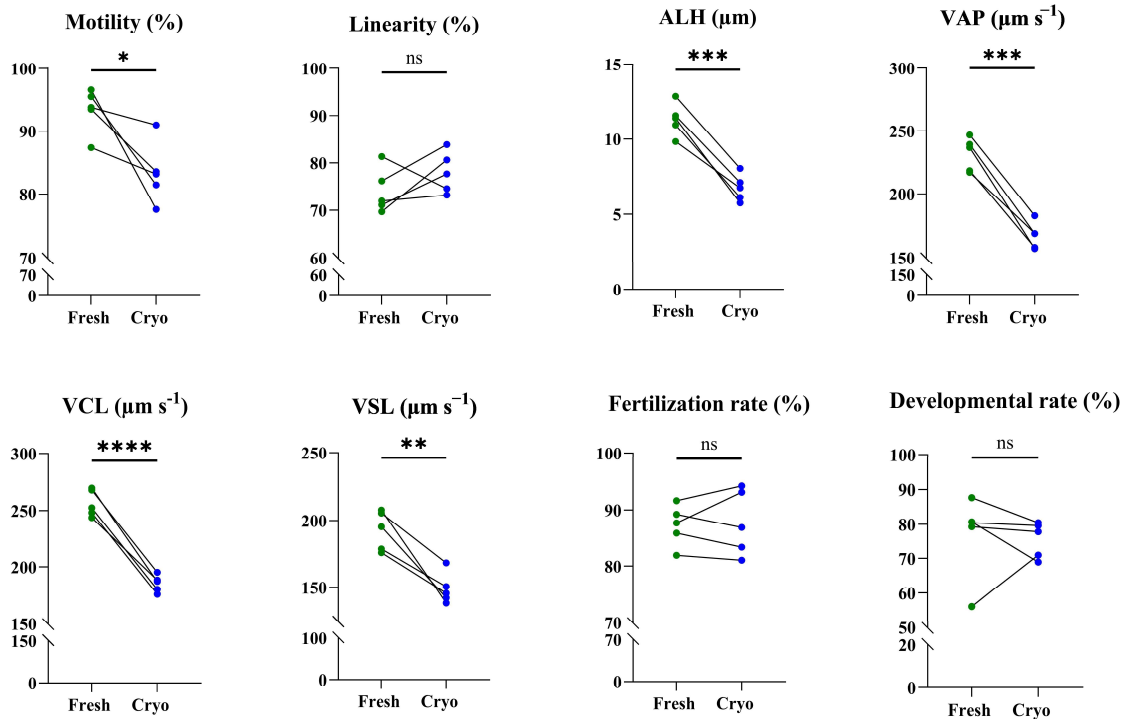


Figure 3.2: *Sperm motility parameters, fertilization and developmental rate between fresh and cryopreserved (Cryo) Eurasian perch (n = 5) milt. The results for statistical analysis are presented as follows: ns nonsignificant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). ALH amplitude of lateral head displacement; VAP average path velocity, VCL curvilinear velocity, VSL straight line velocity.*

Phenotypical parameters:

No significant differences in deformity rate, SBIE rate, TL (both at mouth opening and weaning stages) or mortality were recorded between the Fresh and Cryo groups. However, a significantly higher WBW of the larvae from the Cryo group at the weaning stage was detected (**Figure 3.3**) compared to the Fresh group.

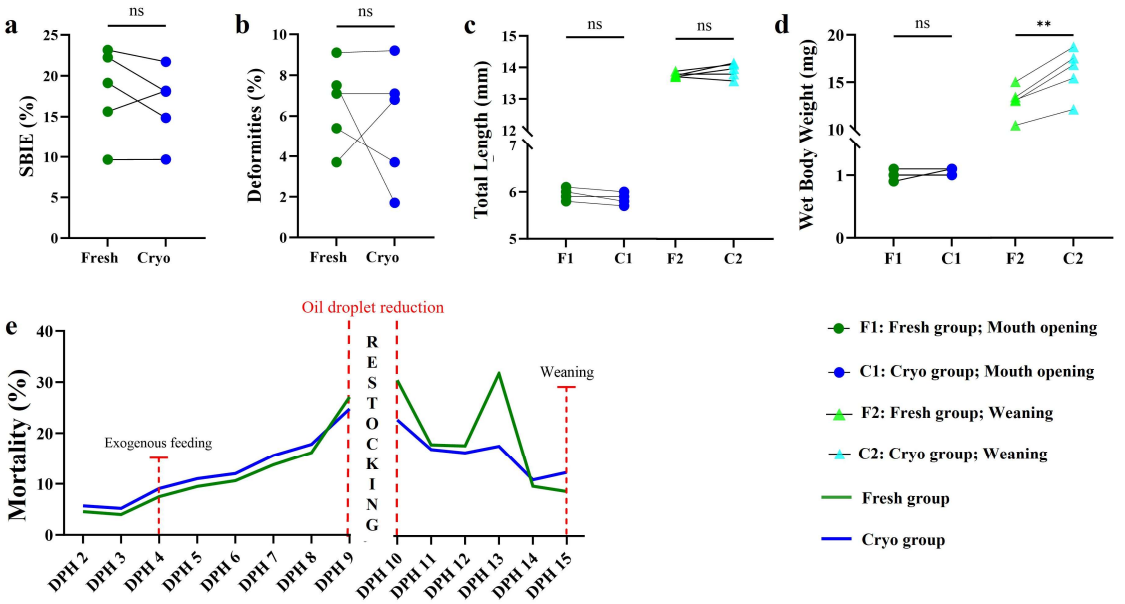


Figure 3.3: *Phenotypical performance of larvae obtained after fertilization of eggs with the use of fresh and cryopreserved milt of Eurasian perch (n = 5).* ***(a)*** *Swim bladder inflation effectiveness (SBIE, %);* ***(b)*** *Deformity rate at mouth opening stage (%);* ***(c)*** *TL of larvae at mouth opening and at weaning stages (mm);* ***(d)*** *WBW of larvae at mouth opening and weaning stages (mg);* ***(e)*** *Cumulative mortality (%) of larvae over the larviculture period, before and after restocking (only eating larvae). The results for statistical analysis are presented as follows: ns nonsignificant, **p < 0.01).*

Differentially Expressed Genes (DEGs)

Analysis of the transcriptomic data enabled the identification of 11 DEGs (**Figure 3.4**) between the Fresh and Cryo groups. The only gene with higher expression in the Fresh group was *tgfbi*, while the remaining 10 genes had higher expression in the Cryo group.

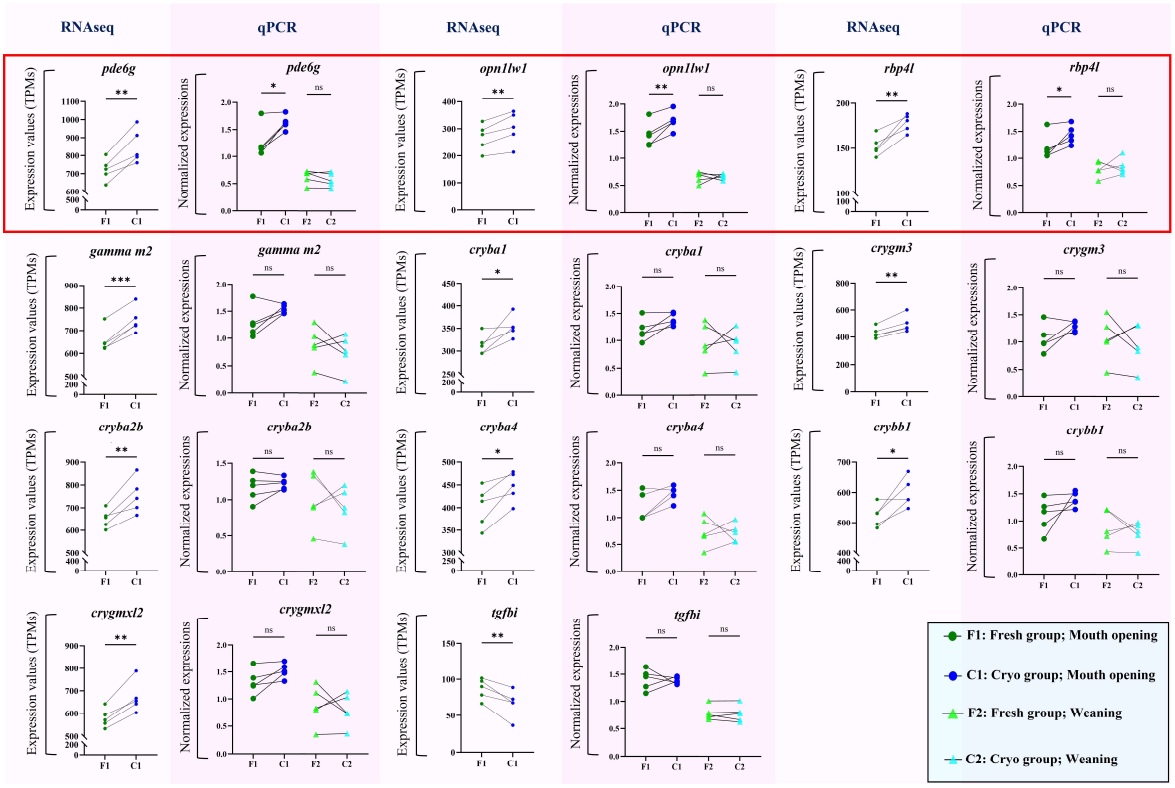
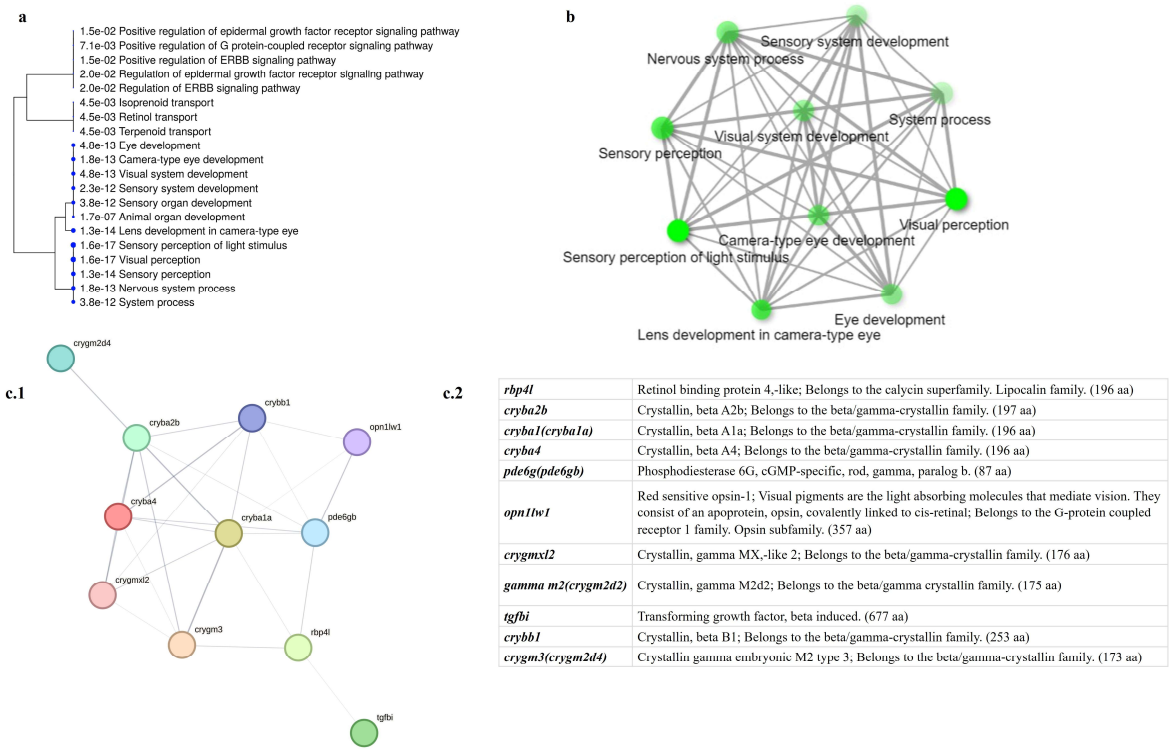


Figure 3.4: *Differentially expressed genes (DEGs). Normalized expression levels of DEGs identified after transcriptomic analysis (RNAseq). Shadowed lane of graphs (with pink color) refers to normalized expression level obtained with qPCRs of larvae at MO stage (F1 and C1) and weaning (F2 and C2) between Fresh and Cryo group (n = 5), respectively. The results of statistical analysis are presented as follows: ns nonsignificant, *p < 0.05, **p < 0.01, ***p < 0.001).*

Functional analysis of identified DEGs suggested common functions in most of them. For instance, *crygmxl2*, *cryba2b*, *cryba4*, *crybb1*, *cryba1* and *crygm3* belong to the *Crystallin* family of genes, which have a major role in early embryonic eye lens development (Cvekl et al., 2015). Clustering analysis of the most enriched gene ontology terms revealed common functions related to eye development, since the remaining genes (*pde6g*, *opn1lw1*, *rbp4l* and *tgfbi*) were found to be responsible for functions of the eyes, such as photorecptors, photoperiodism, and camera-type eyes (**Figure 3.5**).



rbp4l	Retinol binding protein 4,-like; Belongs to the calycin superfamily. Lipocalin family. (196 aa)
cryba2b	Crystallin, beta A2b; Belongs to the beta/gamma-crystallin family. (197 aa)
cryba1(cryba1a)	Crystallin, beta A1a; Belongs to the beta/gamma-crystallin family. (196 aa)
cryba4	Crystallin, beta A4; Belongs to the beta/gamma-crystallin family. (196 aa)
pde6g(pde6gb)	Phosphodiesterase 6G, cGMP-specific, rod, gamma, paralog b. (87 aa)
opn1lw1	Red sensitive opsin-1; Visual pigments are the light absorbing molecules that mediate vision. They consist of an apoprotein, opsin, covalently linked to cis-retinal; Belongs to the G-protein coupled receptor 1 family. Opsin subfamily. (357 aa)
crygmxl2	Crystallin, gamma MX,-like 2; Belongs to the beta/gamma-crystallin family. (176 aa)
gamma m2(crygm2d2)	Crystallin, gamma M2d2; Belongs to the beta/gamma crystallin family. (175 aa)
tgfbi	Transforming growth factor, beta induced. (677 aa)
crybb1	Crystallin, beta B1; Belongs to the beta/gamma-crystallin family. (253 aa)
crygm3(crygm2d4)	Crystallin gamma embryonic M2 type 3; Belongs to the beta/gamma-crystallin family. (173 aa)

Figure 3.5: *In silico analysis of DEGs. (**a**) A hierarchical clustering tree summarizes the correlation among significant pathways based on the gene ontologies. Here 20 most enriched biological processes were clustered, bigger dots indicating more significant p-values. (**b**) Relationship between 10 most enriched biological processes. Two pathways are connected if they share 20% or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes. (**c.1**) STRING-db chart showing proteins (encoded by DEGs) interactions and; (**c.2**) name of the proteins (bracketed protein names are the isoforms of the same proteins in D. rerio).*

qPCR validation of genes

Out of 11 DEGs, in 8 of them, the expression values at both mouth opening (MO) and weaning did not differ between the Fresh and Cryo groups based on the RT–qPCR results (**Figure 3.4**). Moreover, in all these genes, the expression levels decreased with age, and there were no significant differences between the Fresh and Cryo groups. Three DEGs (*pde6g*, *opn1lw1* and *rbp4l*) were confirmed upon validation with qPCR to have higher expression in the Cryo group ($p<0.05$) (**Figure 3.4**) than in the Fresh group at the mouth opening stage. It should be emphasized that for these three genes, similar levels of expression between the Fresh and Cryo groups at the end of the experiment (at weaning) were observed.

In silico verification of DEGs as PEGs

To further investigate if the DEGs are of paternal origin, we checked for their presence in unfertilized (UF) eggs of fish species for which data on the transcriptomic profile of UF eggs were available (**Figure 3.6**). If any genes would have been abundant in UF eggs, we would have to reject the hypothesis that the DEGs are PEGs. As a result, we did not observe any pattern in the expression of DEGs in UF eggs across different species analyzed. For example, from among DEGs identified in our study we did not detect their expression in any percids' UF eggs (i.e. *P. fluviatilis* and *S. lucioperca*). However, 2 out of 11 DEGs were found to be present in *D. rerio,* 4 out of 11 in *O. mykiss* and 1 out of 6 in *M. anguillicaudatus*, and the ones present in different species were always different genes. Next, to additionally confirm their possible paternal influence their expression profile along the embryonic development was examined. This was done in order to check whether their expression starts after ZGA, which could strenghten our assumption that these genes play a role as PEGs since their expression starts after the paternal genome is already playing a role in the embryonic development. The results of this analysis (**Figure 3.7**) allowed us to confirm that these genes are expressed long after ZGA, which additionally supports the hypothesis that their expression could be under the influence of males.

	cryba2b	*cryba4*	*crybb1*	*crygmxl2*	*gamma m2*	*crygm3*	*cryba1*	*tgfbi*	*pde6g*	*opn1lw1*	*rbp4l*
Perca fluviatilis	X	X	X	X	X	X	X	X	X	X	X
Danio rerio	X	X	✓	X	X	X	✓	X	X	X	X
Oncorhynchus mykiss	X	✓	X	✓	X	X	X	✓	✓	X	X
Sander lucioperca	X	X	X	X	X	X	X	X	X	X	X
***Misgurnus anguillicaudatus*	N/A	N/A	X	N/A	X	X	X	X	✓	N/A	N/A

Figure 3.6: *A pictograph for the presence of our candidate genes and genes validated by qPCR (written in pink and shaded in purple, respectively) in UFE in P. fluviatilis, D. rerio, O. mykiss, S. lucioperca, M. anguillicaudatus. (Reference UFE sequencing done as described earlier for P. fluviatilis, from PhyloFish and Expression atlas (TPMs > 0.5 considered); **Results based on TMM counts (Kim et al., 2020); N/A stands for data not available) ✓represents presence while X represents absence of a particular gene in that species.*

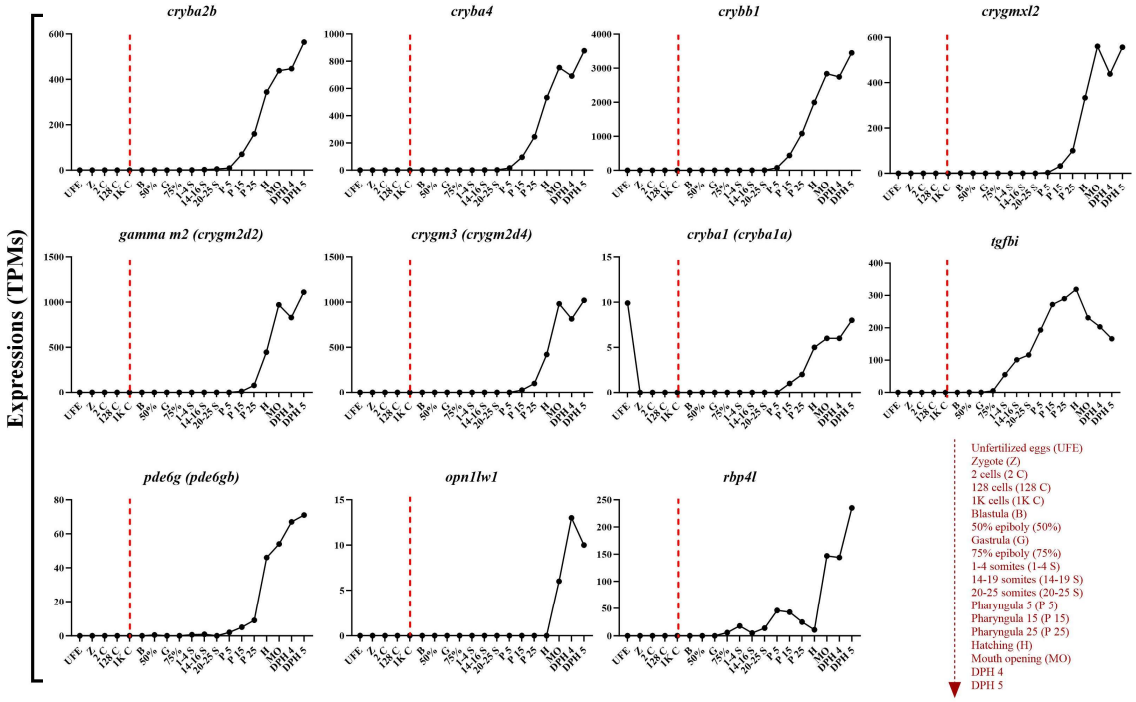


Figure 3.7: *Expression level of identified PEGs along the zebrafish early development (data extracted from White et al., (2017)). Abbreviations used on the x axes are explained on the right-bottom with the broken arrow indicating time course. Broken lines on each graph indicate the moment of zygotic genome activation.*

Tissue distribution of the DEGs

Given the literature background and the results of our experiment, where such important genes are differentially expressed, we wondered whether they are finite to just visual-sensory metabolic pathways or whether they have more to contribute to organism development. In this pursuit, we conducted *in silico* analysis to determine the tissue distribution of these genes across the evolutionarily distinct taxa (**Figure s3.3**). We found that the expression of these genes was not limited to the eyes and/or brain, which could be expected for the genes directly linked with the visual system. These genes in the evolutionarily blind species cave Mexican tetra (*Astyanax mexicanus*), which has adapted to the cave environment, were also found to express these genes in various tissues (Espinasa & Borowsky, 2001; Borowsky, 2008).

3.4 Discussion

In fish reproductive biology research, it is commonly assumed that most of the variation in ELH in fish is attributable to parental genome and the environment provided to the progeny. However, over the years research is adding up to this fact that both the mother and the father

contribute to progeny quality also via the non-genetic mechanisms (Uusi-Heikkilä et al., 2012; Adrian-Kalchhauser et al., 2020). The present study was aimed towards examining paternal effects on ELH traits in Eurasian perch by fertilizing eggs of individual females with either fresh or cryopreserved milt from the same male. Using cryopreservation as a "selection pressure" for sperm 'populations' derived from individual males, we found this had a modulatory effect on progeny's transcriptomic profile and their performance in aquaculture conditions. Consequently, we show, for the first time that paternally inherited factors may have a significant influence on the visual system via targeted gene expression modulation in the progeny.

Results of our study indicate that only the cryo-resistant cells that remained motile after thawing became carriers of genetic and non-genetic information to pass on to the next generation. It has been demonstrated in rainbow trout that sperm cryopreservation did not affect fertilization rates (El Kamouh et al., 2023), and no effects on development and survival during the embryo stage. However, fertilization of eggs using cryopreserved sperm led to significantly reduced larval growth after hatching (Nusbaumer et al., 2019). This is in contrast to our study, where after using cryopreserved milt for fertilization of eggs it resulted in increased WBW of the larvae compared to larvae obtained with fresh milt at the end of our experiment. This allows us to hypothesize, that in Eurasian perch cyopreservation-induced changes are causing permanent alterations to the cryosensitive subpopulation of sperm cells which then become non-functional and are not participating in fertilization. In other words, the effects of cryopreservation observed in our experiment were mediated by changes to the composition of the sperm population, rather than changes to individual sperm.

In the last decades, non-genetic inheritance and transgenerational inheritance is being studied profoundly (Reznick, 1981; Bonduriansky & Day, 2009). Until now the paternal non-genetic inheritance mechamisms has been associated with the methylation pattern of the genome, which is then transferred to the progeny (Jiang et al., 2013). From this perspective the overexpression of the identified PEGs (in Cryo group) in our study suggest heterogeneity of the sperm cells within the same sperm sample representing distinct populations in terms of cryo-resistancy and possibly epigenetic status. It is important to note, that in the same individual fish various subpopulations of spermatozoa with different effect on the progeny's phenotype can be identified (Immler et al., 2014). Additionally, it has already been reported that the

cryopreservation of fish sperm is a selective process indicating existence of various subpopulations of sperm, with different cryo-resistancy, within the same sperm sample (Horokhovatskyi et al., 2018). Therefore, we can suppose that cryo-resistant, and thus functional spermatozoa (the ones which can actually reach the micropyle and contribute to the development of the embryo) from the Cryo group may have different methylation state when compared to functional spermatozoa in the fresh sperm (where generally more spermatozoa are capable of contributing to fertilization, also the ones with modified epigenetic status), allowing us to observe deregulated expression of these genes in the progeny. Considering our results, this brings us closer to the hypothesis that the sperm yielding hypermethylation of these genes are the ones possibly being cryo-sensitive leading to losing their fertilizing capacity. Of course, the confirmation and possible understanding of the exact mechanisms are to be elucidated in the future, but at this point this seems to be among plausible explanation of overexpression of the PEGs identified in our study. However, we cannot dismiss the possibility that also other non-genetic factors, either independently or in combination with the cell's methylation state, that might contribute to the observed differences. Having in mind that the proteomic profile of cryo-resistant spermatozoa before and after cryopreservation is really minor (Horokhovatskyi et al., 2018), the possible alternative mechanisms could include the role of small non-coding RNAs, which have been reported to be sperm-derived carriers of important heritable information, as potential influencers (Gapp et al., 2020).

We observed no significant differences in larval performances at their early life stages from the phenotypical point of view except for one important trait of the offspring, being the WBW, recorded to be higher at the end of the growth trial in Cryo group. This is contradictory to other studies, where fish obtained using cryopreserved sperm for fertilization were characterized by lower phenotypical performance (Nusbaumer et al., 2019). It should be highlighted, that from among all the DEGs identified in our study, 10 genes were related to the visual system development, and all of them were upregulated in the Cryo group. This allows us to hypothesize that upregulation of these genes aids the development of visual organs in Cryo group and, consequently, facilitating catching their prey faster and more efficiently. As compelling as this result may sound, we want to emphasize that it is only a supposition, and observed phenotypic consequences could also possibly stemming from interplay of these genes with the remianing transcriptomic repoertoir or other molecules. Therefore, this hypothesis should be critically tested during a specifically designed future study.

In this study, we have demonstrated that fertilization of eggs with cryopreserved milt resulted in overexpression of genes related with the eye development. Most of these genes (*crygmxl2*, *cryba2b*, *cryba4*, *crybb1*, *cryba1* and *crygm3)* belong to the crystallin superfamily of genes with highly abundant proteins in vertebrate lineages. They are anciently identified in vertebrates and nonchordates, as α-, β-, and γ-crystallins as main sub-families (Slingsby et al., 2013). Underwater, the lens alone provides almost all the focusing power in fish, while in terrestrial species, the cornea provides most focusing power and the lens is mainly used for fine control of image formation (Chen et al., 2001). Certain orthologs of *cryba2b* and *crygmxl2* are directly involved in lens formation. This was demonstrated by Krall et al., (2018), when they used zebrafish model and clustered regularly interspaced short palindromic repeats (CRISPR)/ Cas9 technology to lose the function of lens developing gene regulatory networks (GRNs) in *foxe3* mutant. They observed smaller eyes and defective lens formation 72 hours post fertilization, after the zygotic genome activation has already begun. The *Crystallin* family genes which have turned out to be DEGs in our experiment does not limit only to fishes but expands to higher vertebrates like human cataract lens (Khan et al., 2015). It should be highlighted, that our positively validated candidate genes, *pde6g*, *opn1lw1* and *rbp4l* are found as orthologs in Atlantic salmon where they were found to be responsible for ocular cataract disorders along with other genes, increasing the prevelance of vertebral deformities (Olsvik et al., 2020). The homologs of *pde6g* are been vigorously studied in lower vertebrate model species, as it is one important gene for many retinal degeneration diseases (Mumm et al., 2019). However, the functionality of these genes has not been evaluated basing on the food intake efficiency. Our results, for the first time indicate direct linkage between DEGs responsible for eyes development with weight of larvae which indicates male influence. Moreover, our *in silico* analysis of expressions kinetics along the embryonic development in zebrafish (as shown on **Figure 3.7**) confirmed that all the 11 genes are being extensively expressed long after 1K cell stage i.e., after the zygotic genome activation (Jukam et al., 2017). Lack of considerable expression before that event provides direction that paternally-derived molecular cargo is important in shaping the expression of these genes. Our study provides for the first time, an indirect evidence that this important group of genes, could have role in development of eyes in fishes and other taxa, and are under paternal influence.

As mentioned, major function of the DEGs identified in our study are clearly related to the development of the visual system in fishes. However, the analysis of tissue distribution (see

Figure s3.3) indicates that their function is somehow more complex than only to development of the eyes. This is especially evident when comparing two forms of the same species – eye-less cave Mexican tetra (*Astyanax mexicanus* - cave) and surface Mexican tetra (*Astyanax mexicanus* – surface), where the cave form is having multi-tissue expression of these genes despite not developing eyes at all. This indicates, that paternal effect over the expression of the genes identified in our study may have much more wider consequences, not limited to the formation of the eyes. This also partially explains the compensation of expression of the genes at the whole organism level observed at the end of the study. However, a very important point to note here is that since we checked the gene expression differences in larvae at the weaning stage, subjecting the whole organism to qPCR, this might have masked the differntial expression of our DEGs. In the future, it would be more accurate to study gene expression patterns in tissues-specific manner in developing progeny. It has been reported that the eyes in Eurasian perch at hatching are constituting significant component of the entire body, as the visual system is crucial for survival of the larvae (Kupren et al., 2019). Later the eyes are not growing anymore so rapidly as the rest of the body, especially the organs responsible for the digestion, stating one more reason to maintain tissues-specificity during future analysis. Having in mind, that all the DEGs were found to be expressed in at least some of the digestive-system-related organs in various species (See **Figure s3.3**), we may suggest that the differences in expression of these genes in eyes were simply blurred by the expression of those genes in much more rapidly developing organs. This brings our attention to the fact, that our approach (i.e. studying transcriptome of the whole larvae right after hatching – at mouth opening stage) has been suitable for identification of the novel PEGs, but also has a certain limitations stemming from biases coming from allometric development of various organs during the larval stages. Therefore, a more combined approach, with various research techniques, is recommended in the future studies to explore the paternal contribution in a more holistic way. Nevertheless, thanks to the approach employed in our study we were able to identify novel PEGs and draw prospects for future works focusing on the visual development as a paternally-contributed process.

3.5 Conclusions and future aspects

In the present study, our results clearly demonstrate the robustness of sperm cryopreservation to explore paternal contribution to the progeny in Eurasian perch. Cryopreservation being used as a challenge test here, exhibited the “survival of the fittest” trait in sperm, and we could

identify PEGs. Using phenotypical and transcriptomic approach we observed that the larvae by the end of the rearing period were higher in weights possibly because of the higher expression of genes responsible for the development of eyes in the Cryo group. Here, we refer to DEGs identified, mostly responsible for the visual perception and lens formation that helped the larvae from Cryo group to feed on their prey more efficiently. We also confirmed the absence of expressions of these genes in UFE which means that their expression is not from a maternal genome, but is under paternal effect. Further- more, we learnt that the role of these genes is not just confined to the development of the eyes but also several other tissues of fish species varying on the phylogenetic tree, including blind Mexican tetra. With this study, we identified novel PEGs and a future direction to learn more about how does the father determine gene expression patterns in the progeny. With our findings, and from a fundamental scientific angle, we also show that sperm- selection-mediated phenotypic consequences are clearly an overlooked type of paternal effect which warrants further study about its mechanisms, consequences, and evolutionary importance in different taxa. Additional studies on the basis of current findings would include the behavioural changes in larvae obtained after fertilizng eggs with either fresh or cryopreserved sperm, what would allow to verify the hypothesized here phenotypic consequences in progeny. Additionally, it would be highly interesting to develop specific scientific approach to test whether cryopreservation-induced alteration of intrasperm variability can be selective for certain sperm genotypes in fishes (Alavioon et al., 2017; Sutter & Immler, 2020). This study underscores the significance of our understanding of paternal contributions and encourages to undertake even more challenging endeavors that may bring significant advancements in the fields of fish developmental biology and aquaculture.

3.6 Acknowledgements

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Contributions:

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Chapter 4

Post-thaw storage of semen as a tool towards revealing paternal-effect genes in Eurasian perch, *Perca fluviatilis*

Also referred as: PTS

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4.1 Introduction

Reproduction in fish is a multifaceted process where maternal and paternal contributions jointly determine offspring phenotype. The maternal contribution in fish, through RNA, proteins, and lipids deposited in the eggs is known to control development at least up to zygotic genome activation (ZGA), therefore determining key aspects of the embryo's phenotype and initial developmental trajectory (Lubzens et al., 2016; Debernardis et al., 2025). On the contrary, the paternal contribution, while providing half of the nuclear genotype, has been traditionally underemphasized despite evidence that paternal factors (e.g., sperm-derived RNAs, proteins, and epigenetic modifications) can influence early developmental processes and phenotypic outcomes later in life (Monroe et al., 2021; Renoirt et al., 2023). Historically, the neglect of paternal effects was partly due to the overwhelming influence of the maternal cytoplasmic components and the complexity of teasing apart subtle paternal non-genetic modifications from maternal effects (Siddique et al., 2017). Recent methodological advancements, however, have allowed researchers to begin dissecting both parents' independent and interactive roles in shaping offspring phenotype (Mechaly et al., 2024; Panda et al., 2024).

Emerging research has illuminated that paternal effects extend to epigenetic modifications of sperm, controlled by paternal conditions (like stress, age, nutrition), can alter offspring traits through non-genetic means (Domínguez-Petit et al., 2022). Epigenetic modifications, such as DNA methylation or histone modifications, along with non-coding RNAs present in sperm are transmitted as molecules, recognized as carriers of non-genetic information. These modifications can influence gene expression in the developing progeny, a phenomenon often referred to as non-genetic inheritance (NGI) (Bonduriansky & Day, 2009). In zebrafish (*Danio rerio*), paternal stress exposure altered the sperm small RNA landscape, particularly microRNAs and PIWI-interacting RNAs, which resulted in larvae showing attenuated behavioral and stress responses, indicating a direct influence of environmental conditions on sperm-mediated inheritance (Ord et al., 2020). Also, the paternal methylation pattern serves as a template, while the maternal methylation pattern is largely reprogrammed based on paternal epigenetic information in zebrafish progeny (Jiang et al., 2013; Potok et al., 2013). Thus, a growing body of evidence suggests that paternal contributions may modulate developmental trajectories and adaptive responses (Green & McCormick, 2005; Mashoodh et al., 2018). Yet, the scarcity of studies addressing these effects highlight a significant gap in our understanding, one that is critical for both evolutionary biology and applied fields like aquaculture (Siddique et al., 2017).

The first step towards understanding paternally-controlled traits is the identification of paternal-effect genes (PEGs). They are among such genes whose expression in the offspring is directly influenced by the genotype or non-genetic factors carried by the sperm (Fitch & Wakimoto, 1998). Recently, we have found that cryopreservation, which is a vital technique for long-term storage of sperm, is an ideal tool for the identification of PEGs in Eurasian perch (*Perca fluviatilis*) (Panda et al., 2024). In this study, we have demonstrated that identified PEGs are responsible for the early development of the fish visual system. These findings are linked to the fact that cryopreservation presents specific challenges for sperm cells, leading to structural, molecular, and functional changes within the spermatozoa population and therefore reducing motility and fertilizing ability among cryo-sensitive spermatozoa (Cabrita et al., 2010; Asturiano et al., 2017; Hezavehei et al., 2018; Nusbaumer et al., 2019). Consequently, this technique exerts additional pressure on sperm cells, where only the most robust (cryo-resistant) cells retain their fertilizing capacity, a phenomenon we term "cryo-selection", and having direct contribution to the larval phenotype. Thus, our study showed that cryo-selection is crucial in shaping progeny phenotype, largely related to non-genetic factors (e.g., epigenetic states) conveyed by positively cryo-selected (cryo-resistant) sperm cells. And this was noticeable even with well-standardized cryopreservation protocol for Eurasian perch semen, ensuring high motility and fertilizing ability after thawing (Judycka et al., 2022). To add on to this, we were focusing on freshly hatched larvae transcriptome and their further performance, also seen as early life history of the progeny, which provides a critical window to assess how paternal effects manifest following exposure of the sperm to normal and stressed conditions (Llopiz et al., 2014). Larval phase is relatively short, but very important period in fish ontogeny, where the interplay between genetic inheritance, NGI and environmental factors is highly dynamic. Therefore, examining larval performance along with molecular characterization of their entire body, has been pointed out as a very robust and valuable approach towards exploring complexity of the phenotype stemming from parental contributions (Debernardis et al., 2025).

Although, that cryopreservation protocol is working very well for Eurasian perch, there is still a problem that cryopreserved semen loses its motility in a few minutes after thawing (Bernáth et al., 2015; Judycka et al., 2019; 2022). Therefore, it can be suggested that cryopreserved perch semen should be used for fertilization of eggs immediately after thawing. Thus, post-thaw storage (PTS) period can be considered as an additional selection factor for cryo-resistant spermatozoa adding supplementary challenge test to the sperm. Moreover, with previous studies confined only to the sperm motility (Bernáth et al., 2015; Judycka et al., 2019; 2022), the main mechanistic pathways by which PTS affects progeny remains unclear. It is still

to be elucidated whether PTS imposes a selective pressure favoring more robust sperm, causing sub-lethal sperm damage that manifests in larvae, or involves a combination of both mechanisms. Currently no information regarding effect of PTS on fertilization ability of Eurasian perch spermatozoa as well as its long-term effects on subsequent developmental stages, specifically larval performance and their molecular portrait. Therefore, this study aimed to evaluate the phenotypic and transcriptomic consequences of progeny obtained with cryopreserved sperm being freshly thawed or stored for 30 min after thawing, constituting an integrated approach towards identification of PEGs. Here we focused on Eurasian perch, a freshwater teleost fish species of increasing aquaculture relevance. With well-established protocols for reproduction, the species suits well for molecular studies aiming domestication, adaptability to aquaculture conditions, circadian rhythm and reproductive biology (Żarski et al., 2017d; Palińska-Żarska et al., 2020). Thus, combined with available genomic resources (Ozerov et al., 2018; Debernardis et al., 2025) and larval rearing protocols using Eurasian perch as a model species offers a robust scientific approach for investigating paternal-effect genes in progeny.

4.2 Materials and methods

Ethics Statement

This study was conducted in compliance with European and national regulations regarding fish welfare. The Local Animal Research Ethics Committee approved resolution number 5/2023. The study adheres to the ARRIVE guidelines (https://arriveguidelines.org) for reporting animal research.

Experimental design

To investigate the role of paternal contributions in early development, this study aimed to identify PEGs by applying PTS as an additional stressor in a cryo-selection framework. In this study, we performed *in vitro* fertilizations using cryopreserved sperm that was either used immediately after thawing (control group, CON) or stored post-thaw for 30 minutes at 4°C (PTS group). A total of twelve families were produced. Eggs from each female were divided into four equal portions (~25 g each), ensuring equal distribution of eggs for fertilization trials conducted in triplicate (**Figure 4.1a**). Two portions were fertilized with sperm from the same male, one with CON sperm and the other with PTS sperm. The remaining two portions were fertilized with CON and PTS sperm from a different male (**Figure 4.1a**). This procedure was repeated three times, resulting in three females being crossed with six different males in total.

Specifically, Female 1 was crossed with males a and b to produce families A and B, respectively; Female 2 with males c and d to produce families C and D; and Female 3 with males e and f to produce families E and F. Larviculture was subsequently carried out to monitor zootechnical parameters and collect samples for transcriptomic analysis, enabling a comparison between the CON and PTS groups.

Broodstock management and controlled reproduction

All the manipulation with fish (hormonal injection, gametes collection) were performed under the anesthesia in MS-222 (150 mg L^{-1}; Argent, USA).

Reproduction through males: sperm collection, quality analysis and cryopreservation

For the study, six cultured males (physiological data in Supplementary file, **s4.1**) were used, that were reared in the Department of Salmonid Research of the National Inland Fisheries Research Institute (NIFRI) in Rutki (North Poland), and were overwintered for three months under natural photothermal conditions in a flow-through system supplied with riverine water. Fish were fed with commercial compound feed (AllerAqua Gold). At the early phase of the spawning season, where males were slightly spermiating (a small drop of sperm was possible to be spotted after gentle massage of their abdomen), they were transported in oxygenated water-filled plastic bags, to laboratories of NIFRI in Olsztyn (North-Eastern Poland), where they were placed in recirculating aquaculture system (RAS) at a constant temperature (12°C) and photoperiod (14L:10D). Right after arrival, fish were injected with salmon gonadoliberin analogue (sGnRHa, BACHEM, Switzerland) at a dose of 50 μg kg^{-1} (Żarski, Horváth, et al., 2017b) to promote spermiation.

Semen was collected after 10 days of hormonal stimulation using gentle abdominal pressure and a catheter (Galmed, Poland) to prevent contamination with urine or blood (Sarosiek et al., 2016). Samples were stored on ice immediately after collection. Parameters like sperm motility (**Figure 4.2a**), concentration and viability (with the use of NucleoCounter SP-100, Chemometec, Denmark) of fresh semen were assessed ensuring that good quality of semen was used for cryopreservation (Judycka et al., 2019) (see Supplementary file, **s4.1**). Then, semen was cryopreserved and its motility was further evaluated at 0 and 30 min after thawing using a two-step activation process. Initially, fresh semen was diluted 1:50 and frozen/thawed semen 1:5 in an immobilizing solution (Lahnsteiner et al., 1995) (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, 20 mM Tris, pH 8.0). Activation was achieved by further dilution (1:20) in an activating solution of 75 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1

mM $CaCl_2 \times 2H_2O$, 20 mM Tris, pH 8.0, supplemented with 0.5% bovine serum albumin (Judycka et al., 2019). Various sperm parameters like motility (MOT, %), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), curvilinear velocity (VCL, μm s^{-1}), average path velocity (VAP, μm s^{-1}), and straight-line velocity (VSL, μm s^{-1}), were evaluated for both fresh, cryopreserved and PTS sperm using the computer-assisted sperm assessment (CASA; CEROS II system -Hamilton-Thorne, USA) system.

Cryopreservation of semen was conducted following the standardized protocol (Judycka et al., 2022). The semen with extender was loaded into 0.5 mL plastic straws (IMV Technologies, France), placed on a floating rack, and cryopreserved by exposure to liquid nitrogen vapor (3 cm above the surface) for 5 minutes using a styrofoam box fit with an insulating neopor block (Minitübe GmbH, Germany). The straws were then transferred to liquid nitrogen for their further use, aimed at fertilization of eggs (Judycka et al., 2015).

Reproduction through females: eggs collection

For the experiment, three females caught using fyke nets from Mikołajskie lake (North-Eastern Poland) were used (physiological data in Supplementary file, **s4.1**). They were transported to NIFRI in Olsztyn in the same way as the males. Females were maintained in a RAS with controlled photoperiod 14L:10D and water temperature of 12°C (±0.1°C) until ovulation. Wild fish do not feed under hatchery conditions prior to reproduction operation (Żarski et al., 2017b). Females were stimulated with the same dose of sGnRHa hormone was provided to them as males (50 μg kg^{-1}). Oocyte maturation was evaluated by catheterizing females, exposing collected small sample of oocytes to Serra's clarifying solution (ethanol, formalin, and glacial acetic acid in a 6:3:1 ratio), and assessing their developmental stage microscopically (Żarski et al., 2011). At ovulation, the fish were anesthetized, and eggs were stripped into clean, dry beakers.

Fertilization trials

Cryopreserved sperm straws were thawed at 40°C for 10 seconds in a water bath, and then semen was poured into an Eppendorf, kept at 4°C for 30 minutes (PTS). At the end of this storage period, the same thawing procedure was used for freshly thawed semen (CON) from the same male without providing them a waiting period, enabling fertilizations for both groups simultaneously. For the fertilization of eggs in CON group egg to sperm ratio was 1:100,000, whereas for PTS, 1: 200,000; in order to compensate for the number of motile spermatozoa, which decreased after 30 min of PTS. Just before fertilization, eggs were preactivated in

Woynarovich solution (3g Urea, 4g NaCl in 1L ddH_2O; given in the ratio of 1:5, eggs: solution) for 30 seconds before sperm was introduced (Żarski et al., 2012). After 10 minutes, excess sperm and debris were washed away with hatchery water.

Eggs incubation and larval hatching

The eggs were incubated in 15L tanks with black walls and upper water inflow, which functioned within the same RAS. The eggs were spread on mesh (diameter of around 3 mm) and kept in water at a temperature of 14°C. A piece of the egg ribbon (~100 embryos) was used to monitor under the microscope (Leica, Germany) the different developmental stages after fertilization, like blastula, epiboly, neurula, optic cup, tail detachment, up to full eye pigmentation. This was followed by the hatching larvae count and the deformed larvae count. The photoperiod during all embryos' incubation, and later larvae rearing was maintained at 24L:0D (1500 lux, measured at the water surface). When the embryos reached the eyed-egg stage, the temperature was raised to 15°C; while, as soon as the first hatched larvae were noticed, the temperature in the system was raised to 16°C. To maintain synchronous hatching, the larvae were hatched manually. This was done by transferring the egg ribbons to bowls with water from the rearing tanks and stirring gently (Palińska-Żarska et al., 2020). This operation was repeated a few times until most of the larvae hatched. The day of hatching was considered as 0-day post-hatching (DPH). After hatching, the larvae were left undisturbed for 24 hours. On 1 DPH, the deformed larvae (%) were counted in the subsamples and discarded as they would not survive. On 2 DPH, larvae were counted volumetrically and distributed into tanks at a density of 1500 larvae per tank. Each biological replicate from CON and PTS group were reared separately in triplicate (in total in 36 tanks).

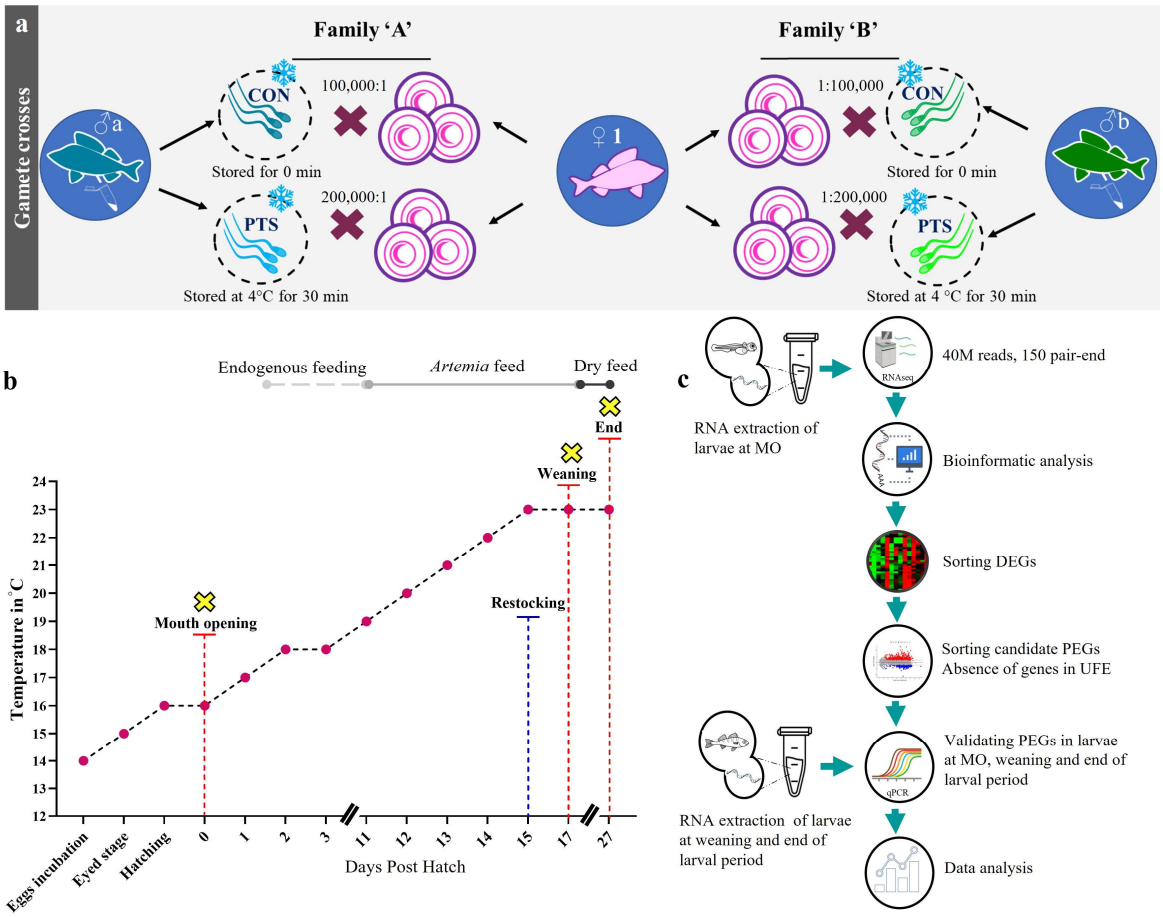

Figure 4.1: *Experimental design and protocol followed.* ***a:*** *Creation of families, where one female (exemplarily female 1) was crossed with two males (exemplarily male a and b here, created families A and B, respectively), with groups as 0 min post-thaw stored (control group; CON) with the sperm: egg ratio 100,000:1 and for the 30 min post-thaw stored (PTS) group being 200,000:1. This gamete crossing operations was done with 3 females and 6 males in the same format.* ***b:*** *Feeding schedule and temperature regime followed for the larval rearing. The yellow crosses represent sampling points.* ***c:*** *Experimental workflow summarizing processing of RNA and data obtained from larvae at various sampling points. MO – Mouth opening; DEGs – differentially expressed genes; PEGs – Paternal-effect genes; UFE – Unfertilized eggs; DPH – days post hatch.*

Larval rearing

Larvae were reared under standardized conditions (Palińska-Żarska et al., 2020) (as shown on **Figure 4.1b**). Feeding with micro Artemia cysts (SF origin) began at 4 DPH. From 4 DPH onwards, feeding success was assessed daily by randomly sampling approximately 100 larvae from each tank and examining their stomach contents under a stereoscopic microscope (Leica, Germany) to check for the presence of food. This was done until 10 DPH when all larvae that do not start to eat died. Starting at 4 DPH, swim bladder inflation effectiveness (SBIE, %) was also counted in the same way as feeding rates and was recorded until 10 DPH. After this time, no increase in the number of larvae with filled swim bladders was observed. The larvae were fed with standard-size Artemia cysts at 260,000 nauplii per gram (GSL origin) since 8 DPH. In

addition, from 12 DPH, dead larvae were examined under the microscope to evaluate the type I cannibalism (when the prey is partially ingested) (Kestemont et al., 2003). Two days before weaning (15 DPH), larvae from each family were counted manually and restocked at a density of 400 larvae/tank. This was done to ensure the same number of larvae in each tank, which varied due to the different mortality rates observed in some families. After weaning (that took place at 17 DPH), larvae were fed exclusively with dry feed (Perla Larva Proactive, Skretting, Norway) three times a day, sprinkling it into each tank in small amounts for ~15 minutes each time (Palińska-Żarska et al., 2020; Santos et al., 2021). Mortality rates (%) were counted twice each day while the tanks were being cleaned. Oxygen level in the tanks was checked every day (with OxyGuard® Polaris, Denmark), and it was never below 80% of concentration along with Ammonia and Nitrites concentration every two days (using DR1900 Portable Spectrophotometer (Hach®, USA), and it was never higher than <0.02 mg L^{-1}). The experiment ended on 27 DPH when more than 50% of the larvae showed no fin fold, therefore, more than half of the fish had already finished the larval period (Palińska-Żarska et al., 2020).

Sampling points

Larvae were sampled at three key time points (**Figure 4.1b**) for RNA extraction, as well as for measurements of total length (TL, mm) and wet body weight (WBW, mg). These stages included: (i) mouth opening (MO; 0 DPH), when the progeny have undergone least human intervention, (ii) at weaning as they transition to commercial compound feed (17 DPH) (Kestemont et al., 2007), and (iii) the end of the larval period (27 DPH). At each time point, 30 larvae per family (10 individuals from each of three replicate tanks) were collected for morphometric assessment. To measure TL and WBW, larvae were first anesthetized. For TL measurement, anesthetized individuals were photographed under a stereomicroscope (Leica, Germany). WBW was determined using a precision balance by placing the larvae on a nylon mesh (approx. 200 μm) and gently blotting away excess moisture with filter paper (Krejszeff et al., 2009). Additionally, 30 larvae per family were preserved in RNAlater (Sigma-Aldrich, Germany) at each sampling point for transcriptomic (at MO) and qPCR (at weaning and at the end of larval period) analysis.

RNA extraction

Total RNA was extracted from larvae at three different time points (**Figure 4.1b**; MO stage, weaning and at the end of larval period) using a TotalRNA mini-kit (A&A Biotechnology, Poland). Specifically, for each family, RNA was extracted from pool of 10 larvae at MO stage (mean WBW 10.0 ± 0.5 mg). For larvae at the weaning, RNA was isolated from pool of four larvae per family (mean WBW 76.6 ± 1.8 mg), and for larvae at the end of larval period, from pool of three larvae per family (mean WBW 180.3 ± 5.6 mg). After extraction the concentration and purity of the RNA were assessed with a DS-11 spectrophotometer (DeNovix, USA), showing absorbance ratios of A260/280 ≥ 2.0 and A260/230 ≥ 2.2. RNA quality was further confirmed using the Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with all samples exhibiting RIN values ≥ 9.0. Importantly, only RNA samples from larvae at the MO stage were then sent for transcriptomic analysis. RNA extracted from MO and other sampling points was used for Real-time qPCR validation.

RNA sequencing

Twelve different libraries were created from larvae at the MO stage (6 families for CON and 6 families for PTS). RNA-seq analysis was outsourced to Macrogen (Amsterdam, Netherlands) using the TruSeq Stranded mRNA kit (Illumina) with a NovaSeq6000 platform, and 40M 150 bp paired-end reads per sample were generated.

Bioinformatic analysis

Raw reads were subjected to quality control using FastQC software version 0.11.9 (Babraham Bioinformatics, 2022). Adapters and low-quality fragments of raw reads (average *QPhred* score < 20) were trimmed, and reads were trimmed to equal lengths of 140nts using Trimmomatic ver. 0.40 (Bolger et al., 2014). The resulting sets of reads of the analyzed samples were mapped to the *P. fluviatilis* reference genome version 11.1.104 obtained from the NCBI database (Sayers et al., 2022) using STAR software version 2.7.10a (Dobin et al., 2013a) with default ENCODE options. Transcript count data for larval samples were filtered to include at least 5 libraries with at least 5 reads. Libraries with 0 min and 30 min PTS were compared using the following scheme: ~ *families* + *condition*; families representing the 6 males observed during the experiment and condition representing 0 min and 30 min PTS. Differential expression analysis was performed in RStudio (version 4.1.3) using the DESeq2 package and *ashr* normalizing the fold change value by logarithm (Stephens, 2017). Results were filtered with thresholds absolute value of log2FC > 1 and q-value < 0.05; differences were considered as significant when adjusted p-values were less than α (α = 0.05).

The transcriptomic expression data (in transcripts per million; TPMs) were first fed to DataMap (version 0.11; (Ge, 2025)), which identified the 100 most variable genes. The dataset was subsequently normalized by z-score transformation, standardizing each row to a mean of 0 and a standard deviation of 1. A heatmap of hierarchically clustered genes was generated using "row clustering" across biological replicates (Families A–F) under the CON vs PTS groups. Thereafter, the differentially expressed genes (DEGs) were similarly transformed and subjected to two heatmap analyses: one utilizing "supervised clustering" and the other "unsupervised clustering." Unsupervised clustering was based on column clustering, internally applying distance metrics and linkage algorithms to group the columns using DataMap. In contrast, in the supervised approach, manual clustering was applied to organize samples into two groups, ensuring that each male under CON and PTS conditions remained paired. A Principal Component Analysis (PCA) plot (Ge et al., 2018) using iDEP, and clustering of genes using GeneMANIA/ Cytoscape (Mostafavi et al., 2008; Ono et al., 2025) were generated for visualization.

GOEA analysis

Gene Ontology Enrichment Analysis (GOEA) has been employed to elucidate the biological significance of DEGs. For assessment of gene functions, we used ShinyGO platform (Ge et al., 2020), preceded by BLAST search of Eurasian perch transcriptome obtained against the human Swiss-Prot protein dataset. Only the top match for each protein was taken, which provided gene names and UniProt accession numbers for the aligned proteins. In the absence of enriched biological pathways among the DEGs using the ShinyGO platform, we extracted the gene table that was grouped by functional categories that were defined by high-level GO terms (see Supplementary file, **s4.2**). Furthermore, GeneMANIA platform (Mostafavi et al., 2008) was utilized to infer putative gene functions and to construct interaction networks by integrating data from publicly available databases. Among the 31 identified genes, *mfap4* appeared in four instances, each localized to distinct chromosomal regions, while *gimap* was represented by two orthologs, *gimap4* and *gimap7*. GeneMANIA successfully networked 27 genes that network with physical interactions, available co-expression datasets, and shared protein domains. The interactions were further visualized using Cytoscape.

qPCR validation

Among DEGs, genes chosen for validation based on their consistency in expression patterns, meaning all biological replicates following similar trends of their relative expressions (either upregulated or downregulated). Next, the selected genes were checked for their maternal origin, meaning whether they were expressed in unfertilized eggs (UFE) (based on our previously obtained transcriptome of UFE, with threshold of expression >1TPM; ((Panda et al., 2024), see Supplementary file, **s4.3**), and as PEGs only genes not expressed in UFE genes were considered. Primers were designed for such genes (**Table 4.1**), and once they were found to be positively validated using RT qPCR at MO stage, the expression of those genes was also checked for larvae at weaning and at end of larval period.

Primers for 16 PEGs chosen for validation, along with 5 normalizing genes for RT qPCR, were designed using Primer3Plus software version 3.3.0 (Untergasser et al., 2012). The primers were confirmed for their specificity using NCBI-PrimerBLAST, version 1.0.1 (Ye et al., 2012). The predicted amplicon sequence was then checked for melting temperature (Tm) on µMelt Quartz, version 3.6.2 (Dwight et al., 2011). The normalizing genes were chosen according to their lowest coefficient of variation (CV = standard deviation/ mean expression *100) within the transcriptomic data obtained (Żarski et al., 2021). The best matching families were chosen

based on their least possibilities to form secondary structures and GC content. For the four *mfap4* genes chosen for validation, we used Clustal Omega (Madeira et al., 2024), a platform to align multiple sequences, and primers were designed on the most unique regions possible (see Supplementary file, **s4.4**). For easier understanding of the gene paralogs, they have been named differently, with their chromosome numbers (chr) provided along with their names. The primer's specificity was then verified using the NCBI Primer-BLAST tool.

RT–qPCRs were performed for each gene using a Viia7 (Applied Biosystems) thermocycler. For each qPCR, 10ng cDNA template was used along with 10 µl SYBR RT PCR Master Mix (A&A Biotechnology), 0.5 µM forward (1 µl) and reverse (1 µl) primers, 2 µL of starter mix and PCR grade water were added to maintain a final volume of 20 µL. The reactions were performed with the following cycling conditions applied: enzyme activation for 10 minutes at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing and elongation at 60 °C for 1 minute. For each gene, first, a standard curve was performed using serial dilutions (six 2-fold dilutions) of the mixture of cDNA templates to verify the reaction efficiency. Reaction specificity has been assessed with the melt-curve analysis and compared to µMelt prediction. Next, the quantification of expression has been performed for each gene and for each sample separately, which was then followed by analysis of their relative expression using the delta delta Ct (2–ΔΔCt) method (Livak & Schmittgen, 2001). Relative expression for each gene was normalized as the geometric mean of expression values recorded for 5 normalizing genes (namely, lysine demethylase, *kdm1a*; stathmin 1b, *stmn1b*; ubiquinone biosynthesis, *coq9*; ATPase H+ Transporting V1 Subunit B2, *atp6v1ba*; and TATA-Box Binding Protein Associated Factor 13, *taf13*).

Table 4.1: *Primers designed for the chosen genes for validation, along with normalizing genes.*

	Genes	Accession number	Reference	Forward (5'-3')	Reverse 3'-5')	Size (bp)
Normalizing	*kdm1a*	XM_039786859.1	NC_053131.1	GAAGCAGGTCATCCCTCCAC	GCCTCCTGGGATGTCATACG	132
	stmn1b	XM_039786859.1	NC_053118.1	AAACTGGAAGCGGCAGAAGA	TTGAGAGCTGCCATCCTTGC	203
	coq9	XM_039809994.1	NC_053119.1	GGCTTCCAGGCTAAACACCA	CCAGTGTCTCAGCACCGATT	164
	atp6v1ba	XM_039783937.1	NC_053130.1	TTTGCCATTGTCTTCGCAGC	GTCATTGGCCAGGTTGAGGA	120
	taf13	XM_039799669.1	NC_053116.1	GGAGCTCCGGTGTATGATGT	CTTTGTGGGTCATTTCCGTGA	113
Differentially Expressed Genes	*sxt(chr13)*	XM_039820350.1	NC_053124.1	TCCTGGTCTGGTCTGTCACT	TGTGACCATGACCGTCTTGT	113
	pde6g	XM_039820828.1	NC_053112.1	AAGAAGGCCCCTCCCAGATT	GCAGAGGGAAGAGGGCTAAA	210
	trim16	XM_039787215.1	NC_053132.1	CCCAGGTTTGACATTGTGGG	CAGGACACTGGCCTTCAGAG	154
	alox15b	XM_039822191.1	NC_053125.1	AGGACTATCGCTGGCATGTG	CAGTGGCTGCAGTGAAGAGA	132
	plaat4	XM_039787023.1	NC_053131.1	GGAAAGTGGTCGGCAATGATA	AGTGCTCGCAGTTGCTATTCAC	150
	ralgapb	XM_039798363.1	NC_053115.1	ACGGCTTGACTCTCCCATTG	GGTTGGGCTCTCTGCTGATT	126
	neo1	XM_039804040.1	NC_053117.1	CAGAGCACCCACTGGTTTCT	TCACGTGTCTGATGCAGCAT	127
	gimap4	XM_039783037.1	NC_053130.1	TCCTTGTAGGCCTAGTGTCTGA	TGTTGGAGCCATATGGACCA	113
	mfap4(chr10)	XM_039813507.1	NC_053121.1	GCAGGAAAGTAGGGCCCATA	ACTGGATGTCCTGAACTGCA	164
	mfap4(chr11)	XM_039815040.1	NC_053122.1	CCTGCTCGGTCTCTGTTTCA	TGACACGCCAGCACTTTTTG	135
	mfap4(chr18)	XM_039781790.1	NC_053129.1	CGGTAGACTGCGGTGACATT	CACAGTACACCTGGACAGCA	102
	mfap4(chr21)	XM_039788849.1	NC_053132.1	TCAGGCACCGTTTAGGAACC	TTTTCTTCCACGTTGCAGCG	144
	fads2	XM_039808830.1	NC_053119.1	ACAGTACCTACAAGGACTGCTG	GTGACGTCCCTGACACCATAC	216
	pigr	XM_039821933.1	NC_053125.1	AGCTCTGAGTTCCTCTAGGGT	TCTCTGATGCAACTACTACTCG	132
	dmbt	XM_039781339.1	NC_053129.1	CTGGCCCAATATGGCTGGAT	ATGAGCCAGGCCTCTTACCTA	144
	hlag	XM_039820208.1	NC_053124.1	GGCTGCATCATCCTCTGGTTA	TCTGTGTGCAGGATGGGTTTA	134

Data analysis and statistics

All the data passed Shapiro Wilk test for normal distribution, and thus they were proceeded to conduct parametric tests. The obtained data from sperm motility parameters (in %, µm, µm s^{-1}), were analyzed using one-way ANOVA between fresh, CON and PTS groups. Fertilization and embryonic developmental rates (%), deformities (%), SBIE (%), TL (mm), and WBW (mg) were analyzed using paired t-tests between CON and PTS groups. Other parameters like cumulative mortality and cannibalism rates (%) during larviculture were subjected to 2-way ANOVA. All analyses were performed at a significance level of $p<0.05$ using GraphPad Prism Software (USA). Similarly, relative expression values after RT-qPCRs were first analyzed with paired t-tests with GraphPad and later plotted using the Matplotlib package (Ari & Ustazhanov, 2014) in Python.

4.3 Results

Cryopreservation and PTS Impair sperm motility parameters

The semen quality was ensured using sperm motility parameters, sperm concentration (23.7±3 x 10^9 spermatozoa/mL) and sperm viability (84.2±3.4%). Cryopreservation resulted in decreasing of all the sperm motility parameters, except linearity (**Figure 4.2a-f**) when comparing with Fresh semen. Further upon storing the semen in 4°C conditions for 30 minutes decreased all of the tested CASA parameters compared both to fresh and CON semen.

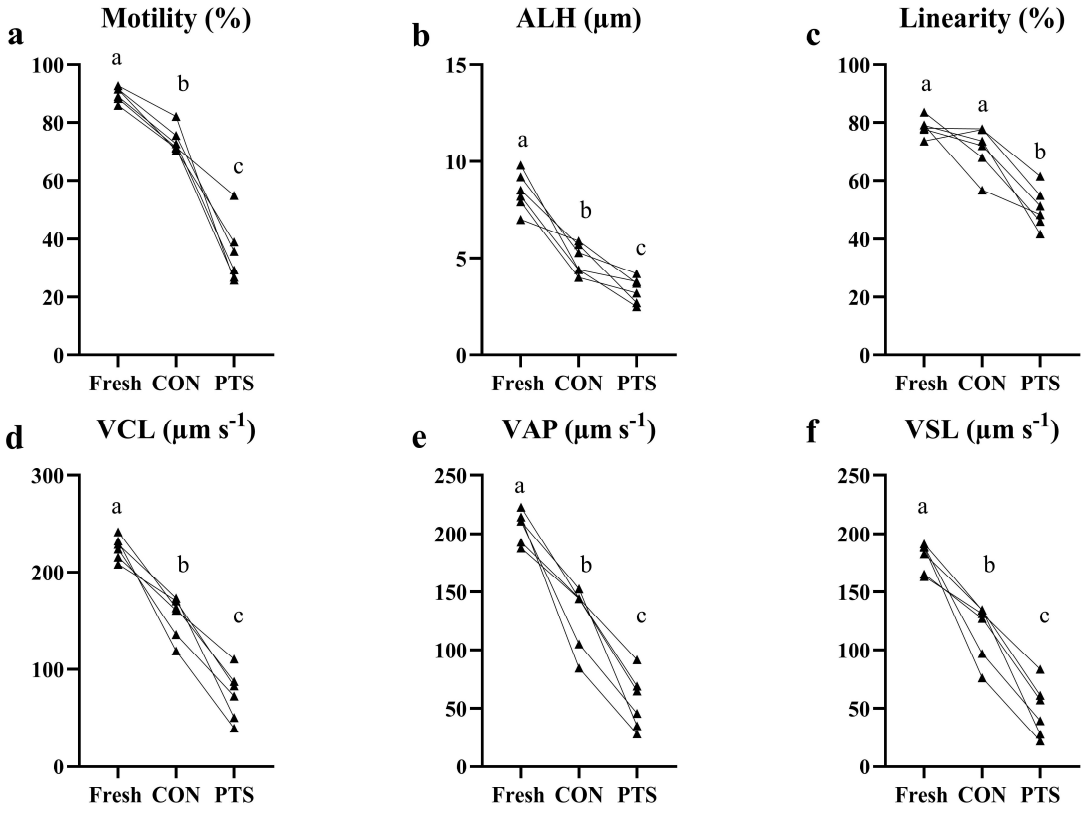

Figure 4.2: *Sperm motility parameters in fresh, CON and PTS groups. Different superscripts indicate significant differences (p <0 .05) between Fresh, CON and PTS sperm. 0 min post-thaw (control group; CON) and 30 min post thaw-stored (PTS group) sperm.*

PTS increases larval mortality

No significant differences (p>0.05) were found in the fertilization rates (**Figure 4.3a**), embryo developmental stages (**Figure 4.3b-g**), hatching rates (**Figure 4.3h**), and larval deformity between CON and PTS groups (**Figure 4.3i**). Lack of significant differences were also noted in terms in TL and WBW throughout the larviculture period (**Figure 4.4a-b**). There were significant differences (p<0.05) in SBIE between CON and PTS groups on 5 DPH, 8 DPH, and 9 DPH (**Figure 4.5a**), unlike foraging rates between families in CON or PTS conditions (**Figure 4.5b**). Mortality rates in the PTS group were statistically higher (p<0.05) compared to the CON group, starting from oil droplet reduction (9 DPH) until the end the larval period (**Figure 4.6**). There was no significant difference (p>0.05) in the case of cannibalism between the CON and PTS groups during larviculture (**Figure 4.7**).

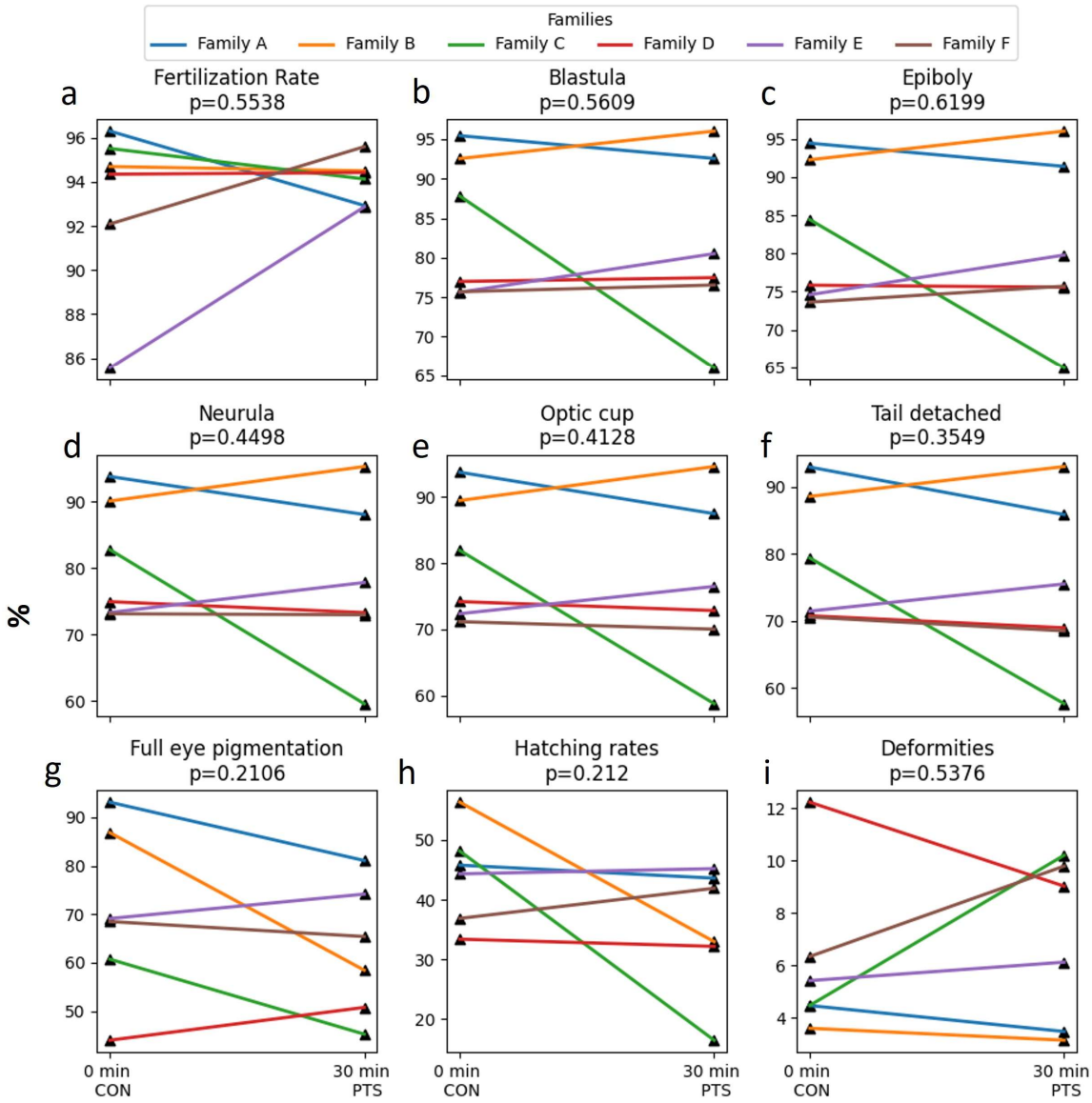

Figure 4.1: *Early life history traits.* ***(a)*** *Fertilization;* ***(b-g)*** *embryonic developmental;* ***(h)*** *hatching and* ***(i)*** *hatched larvae deformity rates recorded in Eurasian perch embryos and larvae obtained with cryopreserved sperm either 0 min post-thaw (control group; CON) or with 30 min post thaw-stored (PTS group) sperm. No statistical differences were observed.*

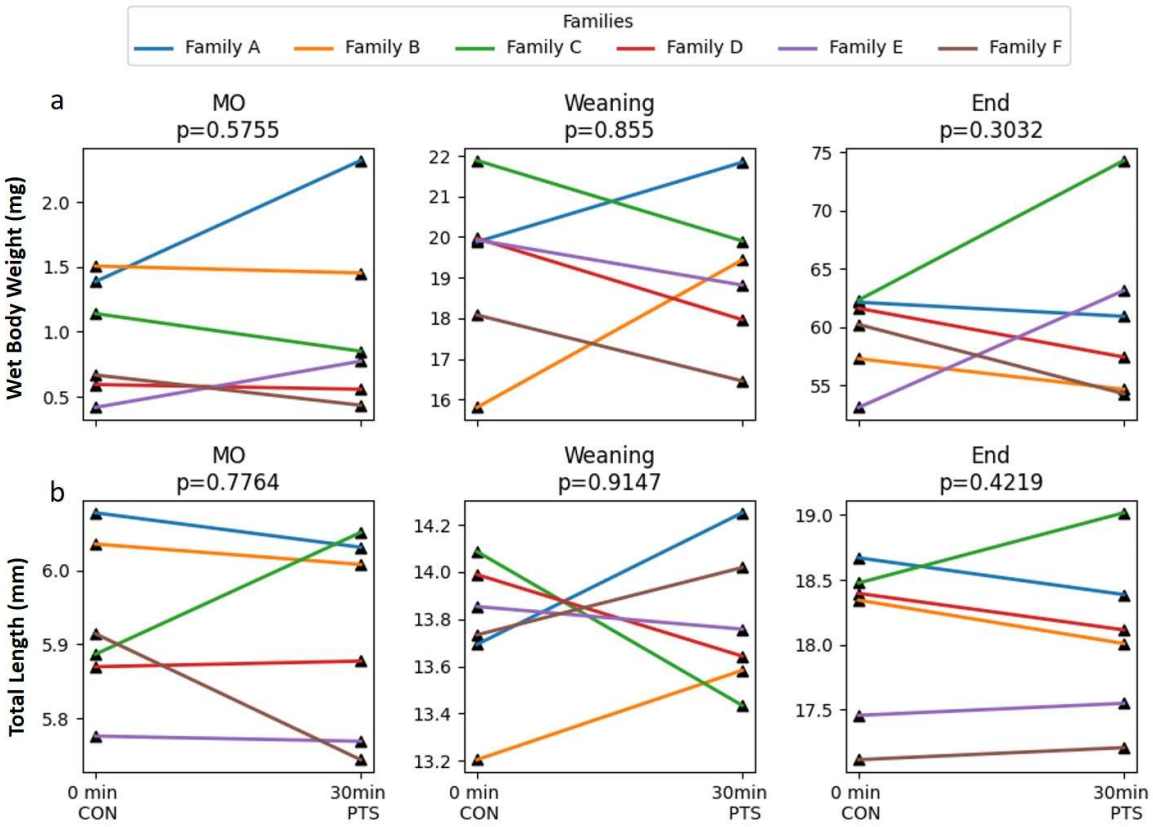

Figure 4.4: *Zootechnical parameters - Growth indicators. Wet body weights (mg) and total lengths (mm) of larvae at mouth opening (MO) stage, weaning and end of larval period. 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm. Lack of superscripts indicate no significant differences among the groups.*

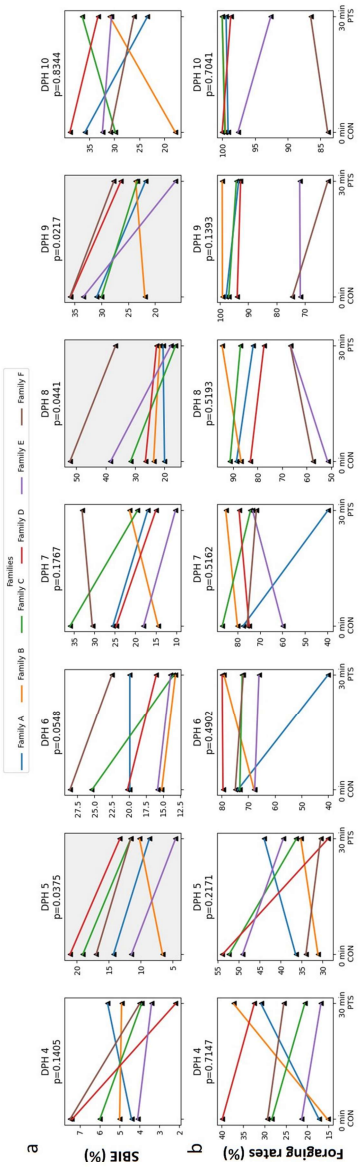

Figure 4.5: *Zootechnical parameters – Functional performance.* ***a:*** *Swim bladder inflation effectiveness (SBIE, %) measured during early larval development. Data in bounding boxes in grey for certain days with their p values (<0.05) highlighted were significantly different.* ***b:*** *Foraging rates (%) of larvae from the first day of exogenous feeding (4 DPH) until 10 DPH. 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

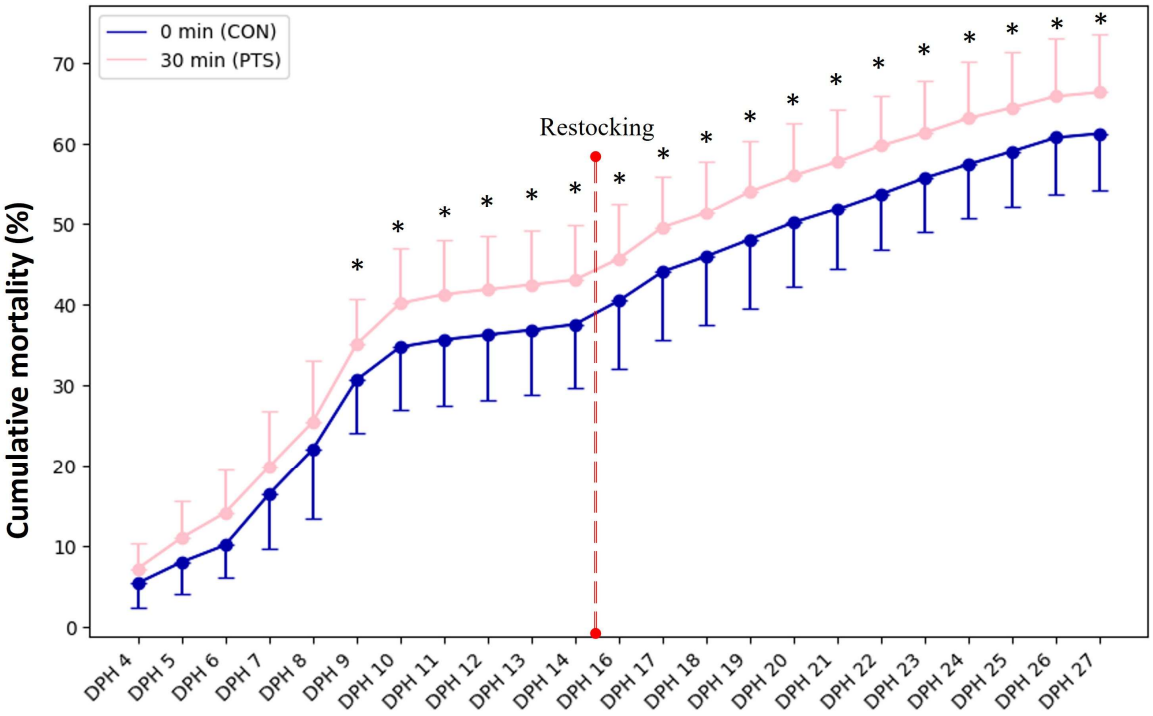

Figure 4.6: *Cumulative mortality (%) recorded along the larvicultural period. On day 15 larvae were restocked to equalize the number of larvae in each tank before weaning them into commercial compound feed. Data marked with an asterisk for each day indicates statistical difference between CON and PTS ($p<0.05$). 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

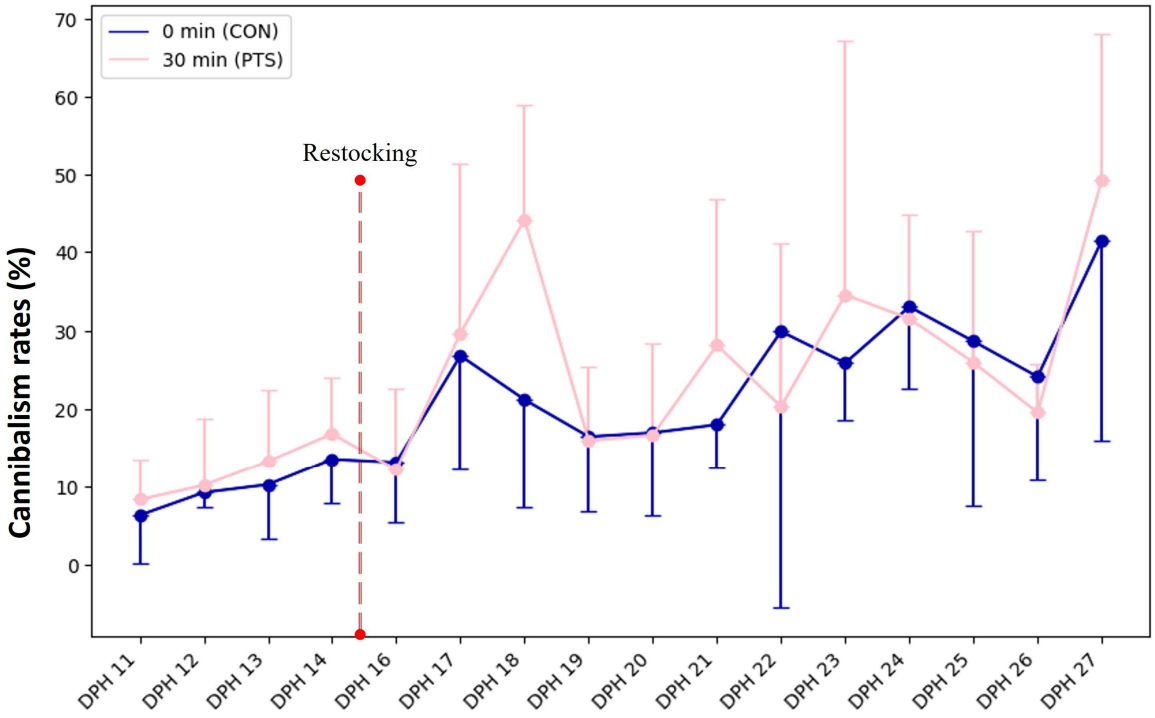

Figure 4.7: *Type 1 cannibalism rates (%) of larvae recorded during the larvicultural period. On day 15 larvae were restocked to equalize the number of larvae in each tank before weaning them into commercial compound feed. Data between the groups for each day of rearing were not statistically different (p>0.05). 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

Transcriptomic profiling

A transcriptomic profiling of larvae at mouth opening was conducted to identify DEGs between CON and PTS groups. Following RNA-seq analysis, a total of 30,744 genes were initially identified. After applying filtering criteria for expression level, 20,447 unique protein-coding genes remained, which were then used for the differential analysis to identify potential PEGs. Heatmap showing hierarchical clustering of 100 most variable genes in the transcriptome of freshly hatched larvae clearly showed differences between CON and PTS (**Figure 4.8a**). The heatmaps show three distinct clusters formed when not supervised, also indicating a maternal driven pattern (**Figure 4.8b**). PCA of transcriptomic data revealed a total explained variance of 61%, with PC1 accounting for 43% and PC2 for 18% (**Figure 4.9a**). In most male-female combinations (families A–D), samples from the CON and PTS groups showed clear separation along PC1. In contrast, families E and F exhibited limited separation along PC1 but greater dispersion along PC2. The genetic background within each pair was constant, and treatment conditions were identical, yet variation in transcriptomic profiles differed in both direction and

magnitude across families. The families are seen to be driven in a female-driven manner as the families seem to vary based on their paired female.

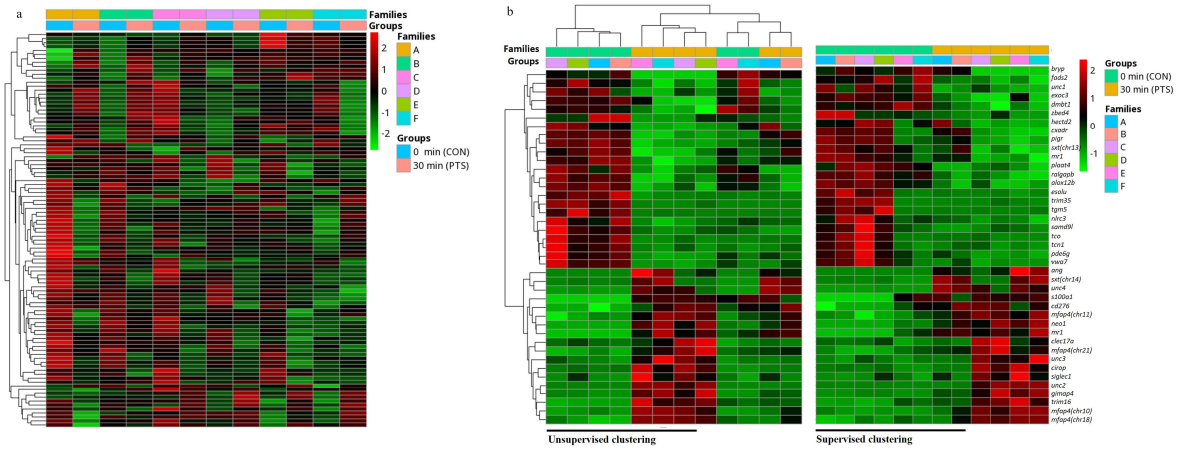

Figure 4.8: *Transcriptomic profiling of Eurasian perch larvae at MO.* ***a:*** *Heatmap showing hierarchical clustering of 100 most variable genes in the transcriptome of Eurasian perch larvae at MO (red and green bricks represent over- and under-expression levels, respectively while black color represents median variability of the gene).* ***b:*** *Heatmaps showing unsupervised and supervised clustering of the obtained 41 differentially expressed genes (DEGs; red and green bricks represent over- and under-expression levels, respectively while black color represents median variability of the gene). MO – Mouth opening; 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

Gene ontology analysis revealed lack of enrichment in any biological processes, however, they did group into some high-level Gene Ontology categories (see Supplementary file, **s4.3**). Out of 41 DEGs, for 31 genes human protein ortholog has been found. The highest number of genes (n=9) is responsible for 'regulation of response to stimulus', followed by 'immune response' processes (n=8). Upon checking physical interactions among DEGs (**Figure 4.9b**) the genes seem to form a dense network connecting *mr1*, *gimap*, *plaat*, *samd9l* and *siglec1*, along with their orthologs in humans, among many other such clusters. The genes clustering around our DEGs in the GeneMANIA/ Cytoscape network likely represent functionally associated genes that may contribute to or modulate the biological processes regulated by the DEGs.

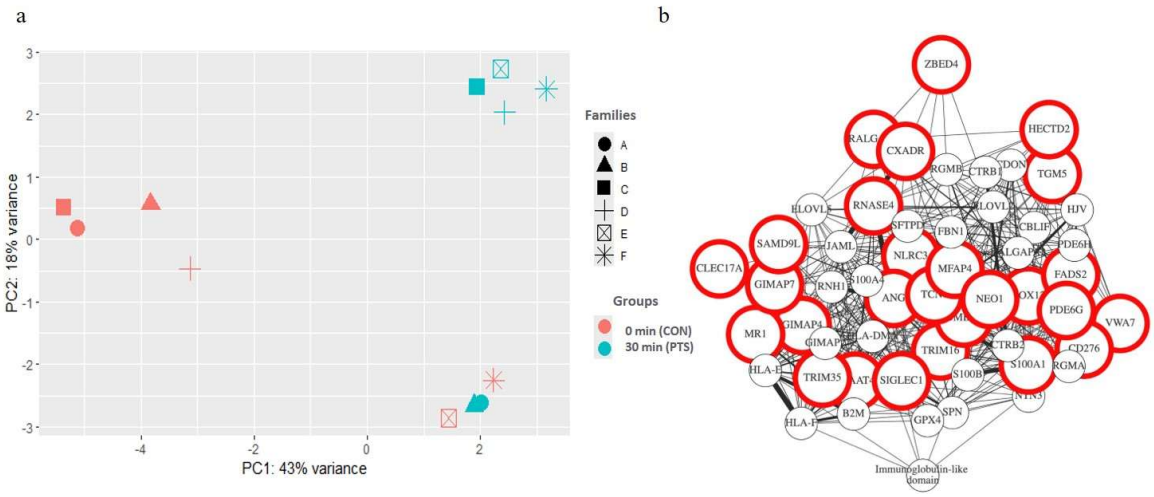

Figure 4.9: *Multivariate and network analysis for transcriptional signatures in CON and PTS groups.* ***a:*** *Principal component analysis of CON: 0 min and PTS: 30 min group showing the dispersion of families created, represented using symbols, while colors represent the groups (CON and PTS).* ***b:*** *Genes clustered according to their co-expressions, shared protein domains and physical interactions with other genes. The gene names encircled in red are our differentially expressed genes. 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

From overall 41 DEGs, 16 genes were chosen for RT-qPCR validation (**Figure 4.10**), among them, 8 genes were positively validated at MO stage (**Figure 4.11, 4.12**). Genes that were upregulated in the PTS group and positively validated only at MO stage include *mfap4* (chr21), *mfap4* (chr10), and *neo1*, on contrary to downregulated in the PTS group is *pde6g* (**Figure 4.11**). The expression level of these was similar in both groups at weaning and end of the larval period (27 DPH) suggesting their expression compensation along the larval metamorphosis. The genes that maintained differential expression also at weaning, and 27 DPH include 3 upregulated genes in the PTS group [*mfap4*(chr18), *gimap4* and *hlag*], and 1 downregulated gene in the PTS group - *pigr* (**Figure 4.12**). The genes selected for validation but not confirmed at the mouth opening stage are presented in Supplementary file, **s4.5**.

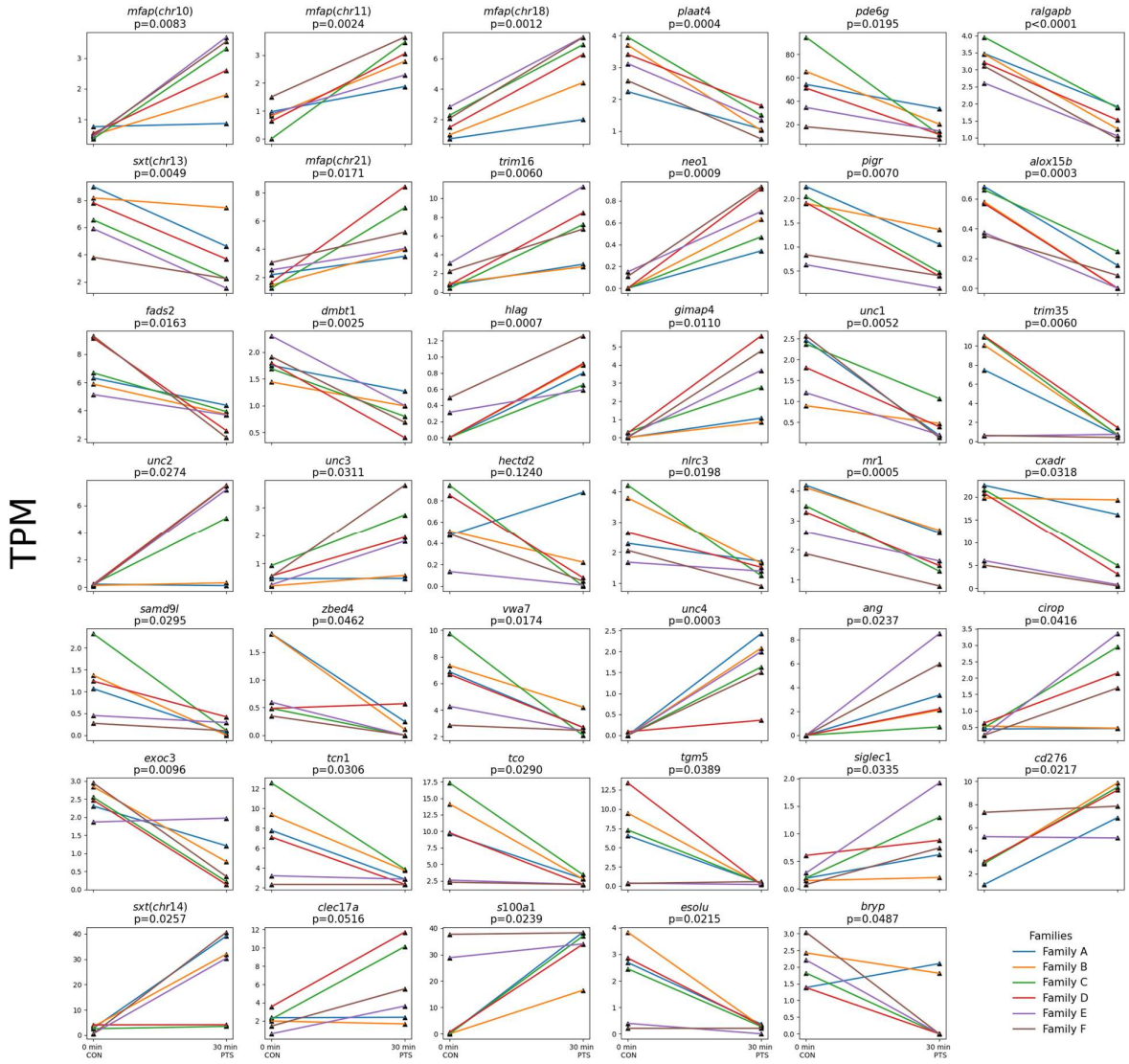

Figure 4.10: *Graphs showing gene expression changes (up/downregulated) relative to CON group in normalized TPM values. For mfap4 genes in the parentheses the gene name has been supplemented with information on number of chromosomes it originates from. 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm; unc – uncharacterized gene; chr - chromosome; TPM – Transcripts per million.*

Microfibril-associated glycoprotein 4 (*mfap4*) gene is present on 5 different chromosomes (chr10, chr11, chr18, chr19 and chr21) in Eurasian perch, and so we considered them as paralogs, that occupy different loci, on different chromosomes, and may evolve to take on new or specialized functions over time. Out of those 5 different loci, the gene transcripts from 4 chromosomes have turned up to be PEGs. Furthermore, 2 of them, *mfap4*(chr21), and *mfap4*(chr10), were validated positively and have significant differences until the MO stage, and third one, *mfap4*(chr18), is not just positively validated but also is seen to maintain significantly different expression along the larval period. Importantly, the three (validated)

differentially expressed paralogs of *mfap4* have higher expression in the PTS group, showcasing the strong candidature of the gene as PEGs.

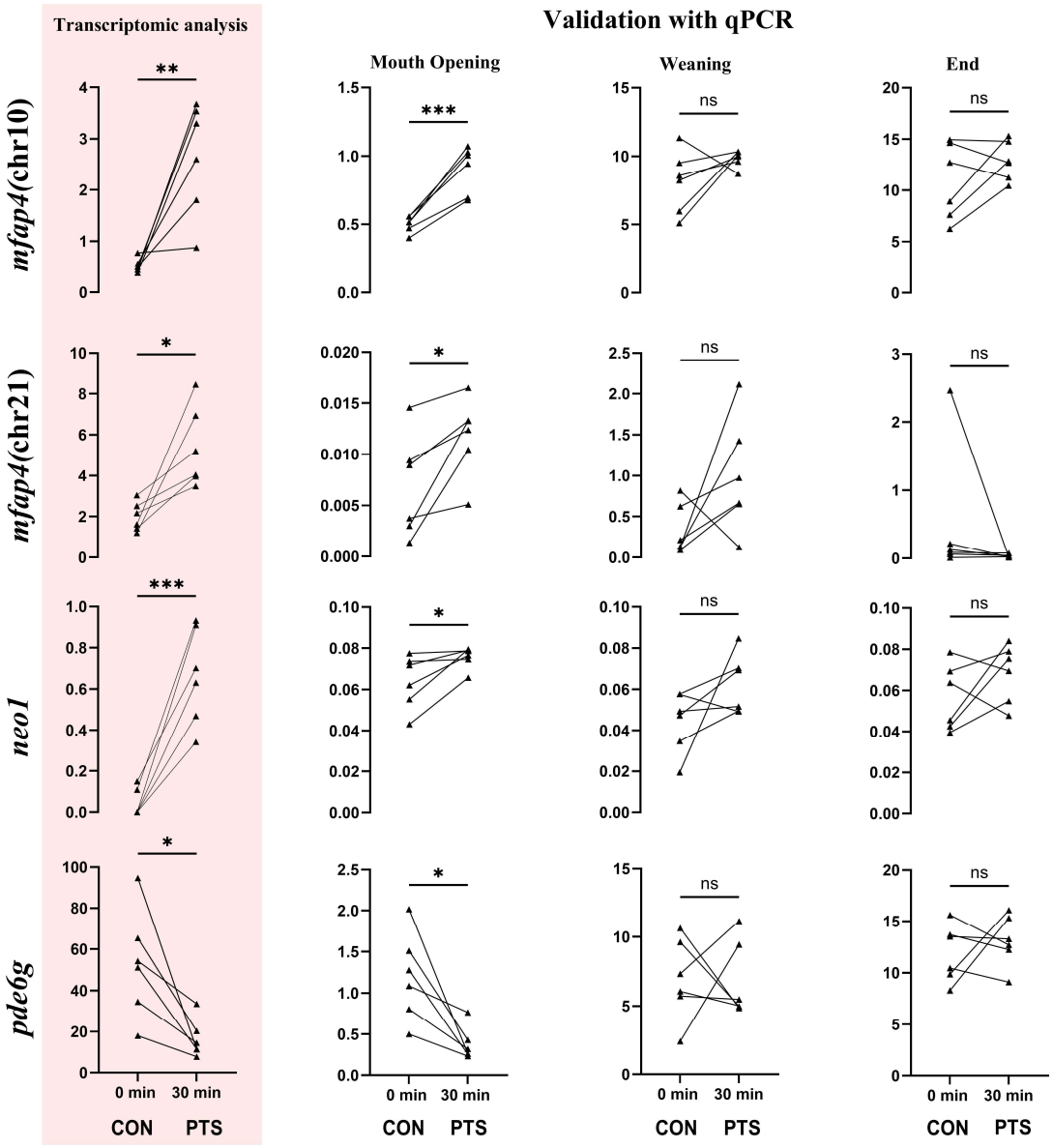

Figure 4.11: *Graphs in pink panel represent the candidate paternal-effect genes chosen for validation after transcriptomic analysis. Validation using RT-qPCR was done for RNA from larvae at mouth opening, weaning, and end of larval period. Significant differences in expression among the groups was seen only at the MO stage. (p<0.05 = *, p<0.01 = **, p<0.001= ***). 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

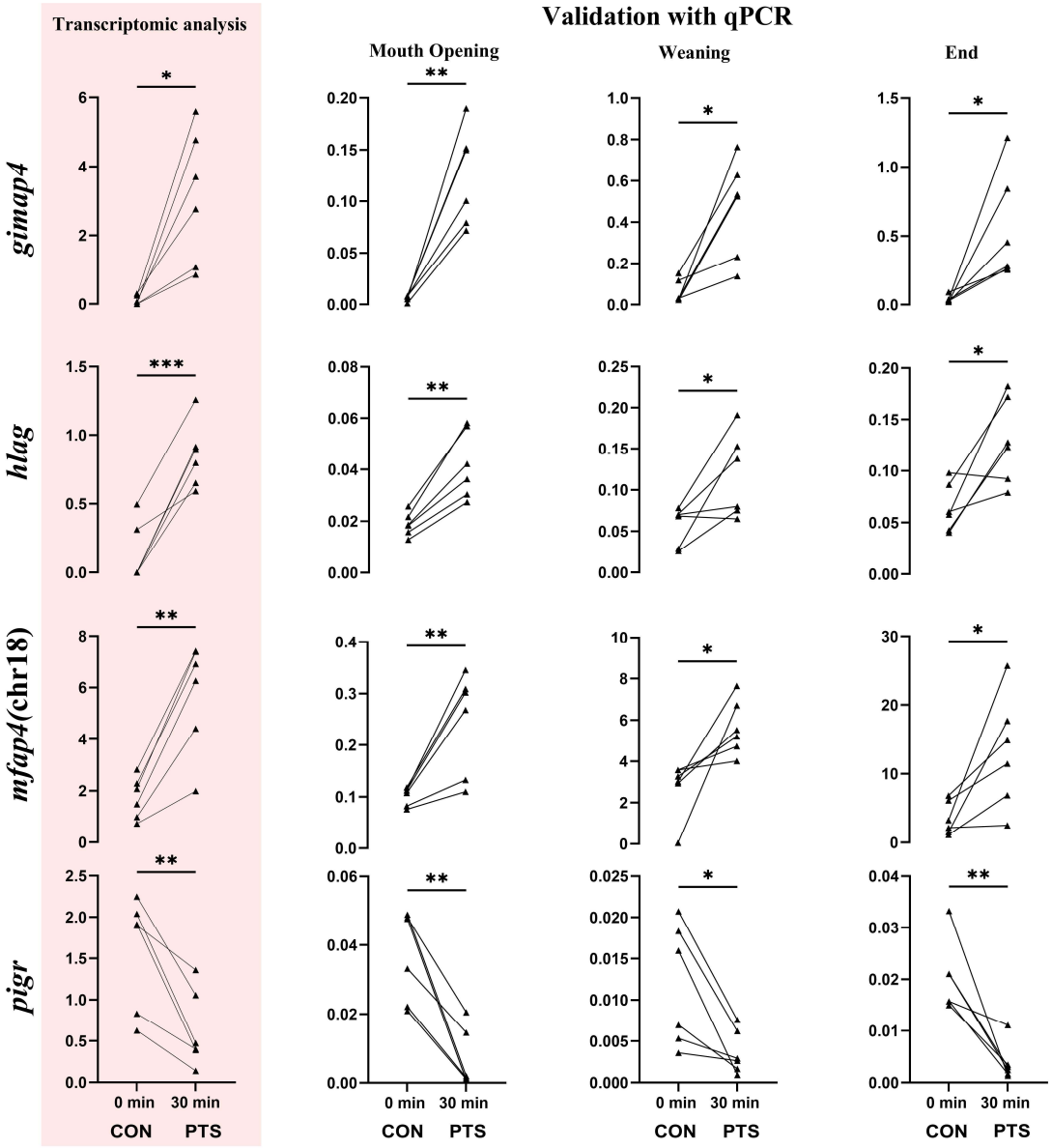

Figure 4.12: *Graphs in pink panel represent the candidate paternal-effect genes chosen for validation after transcriptomic analysis. Validation using RT-qPCR was done for RNA from larvae at mouth opening, weaning, and end of larval period. Significant differences in expression among the groups was seen at all three considered stages. (p<0.05 = *, p<0.01 = **, p<0.001= ***). 0 min post-thaw (control group; CON); 30 min post thaw-stored (PTS group) sperm.*

4.4 Discussion

In this study, we show that even short-term post-thaw storage of cryopreserved sperm can induce significant changes in both gene expression and phenotype in Eurasian perch offspring. Using a controlled, paired fertilization design, we observed consistent transcriptomic responses

to PTS across progenies from different families, enabling the identification of several novel candidate paternal-effect genes. These findings suggest that PTS imposes selective pressure on sperm, thereby differentially programming the resulting offspring. Interestingly, the magnitude and pattern of these responses appeared to be female-dependent, indicating that maternal identity may modulate how PTS influences non-genetic inheritance factors carried by sperm.

There is currently limited research on the effects of PTS of fish sperm, and studies assessing its impact on offspring development or larval viability are virtually absent. The majority of existing work evaluates short-term sperm quality metrics, particularly motility, within minutes to hours after thawing (Gallego et al., 2017; Nynca et al., 2021), while fewer studies examine fertilization success, and almost none extend assessments to later developmental stages. In this context, our study presents novel findings, demonstrating that larvae derived from post-thaw stored semen in Eurasian perch exhibit significantly increased mortality beginning at 9 days post-hatching. This suggests that sub-lethal cryo-induced damage, while not necessarily impeding fertilization, may have delayed effects on embryonic or larval survival. We also documented a progressive decline in sperm quality, as reflected by motility parameters, from fresh semen to 0 minutes post-thaw and further to 30 minutes post-thaw. These observations are consistent with previous findings (Judycka et al., 2020; 2022) who reported that even under optimized cryopreservation conditions, perch sperm undergoes a rapid decline in motility within the first few minutes post-thaw, reaching approximately 54% after 30 minutes. Such early post-thaw deterioration is atypical among fish, and to our knowledge, no comparable rapid decline has been reported in other species. In salmonids such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), or Adriatic grayling (*Thymallus thymallus*), post-thaw sperm can maintain high fertilizing ability for up to 1 hour, with gradual declines observed thereafter (Horváth et al., 2015). Even in species such as African catfish (*Clarias gariepinus*), sperm stored post-thaw for 24 hours can retain fertilization capacity, although rates drop markedly by 96 hours (Kovács et al., 2010). These differences emphasize that PTS tolerance is highly species-specific and affected by sperm physiology, cryoprotectants, and extender composition (Nynca et al., 2016). Despite the physiological deterioration observed, cryopreserved sperm has been successfully used in controlled fertilization in various species for research or genetic conservation (Yang & Tiersch, 2008; Viveiros et al., 2012). However, our findings go a step further, demonstrating for the first time that short-term PTS can affect larval survival, providing compelling evidence that conventional quality assessments (e.g., motility, fertilization rate) are not sufficient to ensure progeny viability. Importantly, this short post-thaw window of sperm functionality in perch, which has often been viewed as a limitation,

can be repurposed as an experimental tool. The predictable and rapid decline in semen quality offers a unique opportunity to experimentally manipulate sperm condition, enabling researchers to investigate the role of paternal-effect genes and non-genetic paternal contributions to early development. To our knowledge, this is the first study to link PTS with altered larval phenotype, representing a significant step forward in understanding the long-overlooked role of paternal quality in fish development.

We have demonstrated that, many parameters of the early life stages of larvae, like hatching, deformity, feeding rates, etc., do not seem to be affected significantly by the PTS of semen compared to CON group. Though significant differences in SBIE which is a crucial zootechnical parameter (Honryo et al., 2022), there is no strict pattern being followed to claim this parameter to be paternally driven. Similarly, we did not observe any differences in Type 1 cannibalism (sibling cannibalism) between our comparison groups, despite this behavior being a common trait in the Percidae family (Kestemont et al., 2003). This trait is typically influenced by ecological conditions rather than parental control, particularly under captive conditions. In our previous study, we speculated that foraging effectiveness has been modified by the cryo-selection of the sperm and attributed to the modified visual system as paternally-controlled trait (Panda et al., 2024). However, in this study we did not see the differences in foraging until 10 DPH, what suggests that higher mortality after the yolk sac depletion can stem from impaired digestion capacity of exogenous food or metabolism (Le et al., 2016). Thus, larval mortality can stand alone to be a very strong result from this experiment, after all, mortality is a powerful phenotype to assess larval quality (Franz et al., 2021).

The overview on transcriptomic data demonstrate that PTS introduces consistent, condition-driven gene expression changes in a subset of larval transcriptomes, supporting the use of PTS as a stressor for identifying paternal contributions to early gene regulation in the progeny. For instance, the visualization of 100 most variable genes (**Figure 4.8a**) already shows clear partitioning between most CON and PTS samples, suggesting a strong effect of PTS on global gene expression. When focusing on the 41 DEGs the supervised clustering maintains the female-based family structure and reveals that, in several families, PTS samples display consistent shifts in expression relative to their CON counterparts. This indicates that, despite shared genetics, sperm exposed to PTS undergo changes that alter larval transcriptional profiles (Valcarce et al., 2023). In contrast, the unsupervised clustering organizes samples based solely on expression similarity, and here, we observe that CON and PTS samples often group separately, highlighting that PTS introduces a reproducible transcriptomic signature (Wang et

al., 2022). However, some variability remains, particularly in families E and F, where the distinction between treatments is less pronounced. This suggests that not all males' sperm respond equally to post-thaw storage stress, hinting at male-specific resilience (Yang et al., 2009; Wang et al., 2022). Further analysis of transcriptomic data revealed substantial intergroup variability, and low number of DEGs observed. PCA analysis showed that most families (A–D) exhibited a shared, time-dependent transcriptional response to PTS, with clear separation along PC1. In contrast, families E and F showed minimal separation along PC1 but greater dispersion along PC2, indicating a distinct source of variation. Since the genetic background was held constant within each pair, this divergence could reflect individual differences in how sperm from these males responded to post-thaw storage stress (**Figure 4.9a**). Notably, families sharing the same female (A–B, C–D, E–F) dispersed similarly along the principal components, suggesting a potential maternal effect modulating the offspring's response to PTS-altered sperm (Andree et al., 2015; Paul et al., 2023). This variability may arise from interactions between sperm-borne non-genetic changes, such as epigenetic modifications or small RNA profiles, and female-specific oocyte environments (Labbé et al., 2017; Godden et al., 2025). Although the exact mechanisms are still unclear, these findings suggest that both paternal tolerance to PTS stress and maternal factors work together to influence early gene expression in the offspring. This highlights complex, context-dependent parental effects observed under controlled experimental conditions.

The transcriptomic profiling of CON and PTS groups of larvae at the MO stage is essential as the larvae would have been least manipulated by human intervention. Results indicate that the female may be an important component influencing how paternal effects are expressed. While the sperm's main contribution to the embryo is its compacted DNA, it also delivers, for example, small RNAs and epigenetic marks that can influence early development (Lubzens et al., 2016). However, when sperm DNA is damaged – for example due to oxidative stress, membrane instability, or fragmentation during PTS (Cabrita et al., 2005), the embryo must rely on internal repair systems to maintain developmental potential. In fish and other vertebrates, these repair mechanisms come mainly from the oocyte cytoplasm and play a central role in recognizing and fixing such damage, especially before or during zygotic genome activation (Dey et al., 2023). For instance, in rainbow trout, when the base excision repair (BER) pathway was inhibited using a PARP inhibitor (3AB), it caused increased embryonic mortality and genome instability (Fernández-Díez et al., 2015). Similarly, studies in zebrafish showed that moderate levels of DNA damage in sperm can be tolerated if the maternal repair machinery is functioning properly (Fernández-Díez & Herráez, 2018b). But these systems are not perfect, if

the damage is too strong or not repaired correctly, it may lead to apoptosis or developmental arrest. Also, while DNA can often be repaired, epigenetic changes caused by cryopreservation, such as abnormal methylation or histone modifications, may not be fully corrected and could still be passed to the offspring (Cabrita et al., 2005; Dey et al., 2023). So, although the egg can compensate for some damage, this alone does not explain the full variation we observed in transcriptomic profile. Despite the mechanism of such maternally-shaped variability in paternal contribution to offspring transcriptomic repertoire is unclear, our results suggest that the paternal effect is shaped not only by sperm-derived factors, but also by how the egg responds to it indicating a complex interaction between maternal and paternal factors in shaping the larval phenotype.

Despite the variability in transcriptomic profile obtained, we still were able to identify 41 candidate PEGs among the CON vs PTS groups. Gene ontology analysis revealed, that this is relatively random set of genes, without shared biological function. Given that functionally heterogeneous gene sets can still reveal regulatory complexity (Li et al., 2020), we focused on genes with consistent expression patterns across PTS biological replicates, either uniformly upregulated or downregulated, and absent in unfertilized eggs, suggesting their paternal origin. This led us to identify 16 candidate PEGs with shared expression pattern constituting valuable candidate PEGs in Eurasian perch. Within this set we could identify genes involved in immune regulation, cell signaling, and developmental processes. Additionally, these genes exhibited notable patterns of differential expression, including both converging and diverging trajectories, across developmental stages. Such expression dynamics enhance the relevance of these genes as candidates for downstream validation and potential PEGs.

The validation performed in this study shed light on several important immune-related genes. For instance, the *mfap4* gene which encodes a microfibril-associated protein that plays a role in organizing the extracellular matrix. In zebrafish, a common teleost model, *mfap4* is expressed in a subset of macrophages, making it a useful marker for these immune cells. This expression suggests that *mfap4* not only contributes to the structural integrity of tissues (for instance, in vascular and connective tissues) but may also be involved in innate immune responses and tissue remodeling during development (Niu et al., 2011). Another noteworthy gene found is *gimap4,* which is showcasing their higher expression in all the stages of the PTS group larvae, along with being absent in the UFE transcriptome. The GTPase domain of the immune-associated nucleotide binding protein (*gimap*) genes are rather huge family and are seen to be upregulated in response to infections (Limoges et al., 2021; Rives et al., 2024). Another line of

evidence of paternally-controlled expression of immune-related genes comes from the consistently high expression of major histocompatibility complex class I-related (*mhc*), also called as human leukocyte antigen (*hla-g*) gene, in the PTS group across the stages. Among many genes encompassed in the region, *hla-g* is a nonclassical class I molecule known to protect the progeny from maternal immune rejection (Grimholt & Dixon, 2016). However, functions of this gene are yet to be clearly understood in fish. We also found the continuous under-expression of the *pigr* gene in PTS group in Eurasian perch across larval period. The gene is again a part of the polymeric immunoglobulin receptor (*pigr*) superfamily, and is said to be critically involved in IgM antibody-based local mucosal activity (Stosik et al., 2023). This, along with the other positively validated genes at MO stage, but compensated with age across other stages, include *mr1*, a paralog of *hla-g*; *neo1*, *neogenin*, which constitute a subgroup of the immunoglobulin superfamily (Shen et al., 2002) and are key components of the innate and adaptive immune system in vertebrates (Gao et al., 2023). All of this is in contrast to the commonly known fact that, as reported for cod (*Gadus morhua*), immune transcripts are often maternally inherited (Caipang & Fagutao, 2015), and provides evidence on paternal contribution to progeny immune system, with its functional validation still yet to be further explored. Additionally, the immune-related PEGs identified at this stage may, like the observed early-life mortality, represent consequences of PTS rather than direct causes. However, the possibility that weakened immune function contributed to mortality cannot be excluded.

Among the validated candidate PEGs, a specific attention should be paid to *pde6g*. This gene produces the gamma subunit of the cyclic GMP-phosphodiesterase (pde6) enzyme which plays a critical role in regulating the enzyme's activity, ensuring proper visual signal transmission and amplification (Dvir et al., 2010). This gene has already been reported in our previous experiments as putative PEG (Panda et al., 2024). In the experimental setting, eggs were fertilized with either fresh or cryopreserved sperm, and upregulation of *pde6g* in progeny from cryopreserved sperm was observed. This finding suggested that sperm cryopreservation, by selecting for a subpopulation of sperm with distinct epigenetic (e.g., methylation) profiles, can reveal paternal-effect genes, thereby influencing early eye development and potentially other sensory functions in the offspring. In the present study we found, where PTS became an additional selection pressure caused under-expression of this gene in the progeny. This indicates that while the paternal control over this gene is associated with positive cryo-selection, is negatively associated with further selection consequences during the PTS. Despite the exact mechanism need to be further elucidated, our data constitute valuable additional evidence of paternal-control on expression of this gene.

4.5 Conclusion

Our findings provide further evidence that paternal contributions, even when arising from subtle variations in sperm quality, can exert measurable effects on offspring phenotype and gene expression, functioning in concert with and under the modulation of maternal influences. We demonstrate that PTS of sperm acts as a dual selection pressure by eliminating sensitive spermatozoa while enriching sperm subpopulations that are more resilient. Beyond delivering the paternal genome, sperm carry regulatory RNAs, including mRNAs and microRNAs, that can modulate early embryonic development. Thus, selective filtering during PTS has the potential to alter progeny phenotype. Importantly, our previous results indicate that cryo-selection can serve as a positive filtering force (Panda et al., 2024), yet the associated storage-related stress imposes an additional layer of selection, with most likely detrimental consequences. Notably, both the higher early-life mortality and the predominance of immune-related PEGs may be parallel consequences of PTS, though impaired immune function could still have contributed to mortality. Along with the transcriptomic data we also show the interplay between paternal effects and maternal modulation of gene expression which highlights the inherent complexity of parental contributions to developmental regulation. Finally, by integrating phenotypic assessments with transcriptomic analyses, we identified new candidate PEGs and corroborated earlier reports of *pde6g* as a PEG, thereby extending the current understanding of molecular mechanisms underlying paternal influences in fish reproduction.

4.6 Data availability

Raw data from the analysis of different families of freshly hatched larvae can be accessed via the NCBI BioProject database under the PRJNA1265076 accession number.

4.7 Acknowledgement

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication

Not applicable.

CRediT Authors contribution

Abhipsa Panda - writing original draft, involved in visualization, methodology, investigation, validation, data analysis, data curation. **Sylwia Judycka -** visualization, methodology, investigation, data analysis, data curation, writing - review and editing, conceptualization, supervision. **Katarzyna Palińska-Żarska -** conceptualization, methodology, investigation, resources, writing - review and editing, supervision. **Rossella Debernardis -** investigation, data curation. **Joanna Nynca -** investigation, writing - review and editing. **Rafał Rożyński -** resources. **Anna Małgorzata Majewska -** investigation**. Jan Paweł Jastrzębski -** formal analysis, data curation. **Daniel Żarski -** conceptualization, methodology, validation, investigation, resources, data curation, writing - review and editing, supervision, project administration and funding acquisition.

Chapter 5

Dynamic interplay of maternal and paternal contributions to offspring phenotype in Eurasian perch, *Perca fluviatilis*

Also referred as: Dom-Wild

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Contribution: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, writing – Original draft, writing – Review and Editing, Visualization.

5.1 Introduction

Early development is shaped by the combined influences of maternal and paternal contributions, each playing distinct roles in determining offspring phenotype (Uller, 2008; Badyaev & Uller, 2009). This contribution goes beyond genetics, extending to non-genetic inheritance (NGI) factors (influenced by external conditions affecting parental experience) like mRNAs, small RNAs, proteins, metabolites, and epigenetic modifications (Adrian-Kalchhauser et al., 2020). It has been widely documented that, alongside offspring genotype, NGI factors constitute a significant component of parental investment and collectively are pivotal in shaping progeny traits, impacting on development, survival, and evolutionary success from early life stages (Rideout, 2004; Bougas et al., 2013; Cortese et al., 2022; Domínguez-Petit et al., 2022).

To date, research has predominantly focused on maternal effects and their role in offspring phenotypic variability (McCormick, 2006; Wolf & Wade, 2009; Colson et al., 2019; Domínguez-Petit et al., 2022). However, there are also several evidences that fathers too play significant roles in offspring development (Rideout et al. 2004; Macartney et al. 2018; Panda et al., 2024). Parental contributions include direct impact on offspring traits including behaviour, mediated through gene expression levels (Domínguez-Petit et al., 2022), adding a further layer of complexity to the mechanisms underlying offspring phenotype. Despite growing awareness of these parental effects, research continues to focus on maternal and paternal influences separately. A more integrated understanding of how both parents - in the current study considered as their genetic and non-genetic contributions via oocytes and spermatozoa - interact to shape offspring development is crucial to delve into the mechanisms affecting phenotypic variability and long-term evolutionary outcomes. In this context, transcriptomics is a valuable tool for examining how parental effects influence gene expression and, consequently, progeny phenotype (Chandhini and Rejish Kumar 2019; Debernardis et al. 2025). Therefore, by leveraging transcriptome analysis, it is possible to gain deeper insights into how parents play their role in affecting offspring development and performance.

Parental effects have been studied across various taxa (Badyaev & Uller, 2009), yet fish are particularly suitable for investigating the complexities of parents' interactions due to their high fecundity and developmental plasticity (Karjalainen et al., 2016). Also, majority of fishes exhibit external fertilization (Sutton and Wilson 2019) allowing straightforward monitoring of the development from the moment of fertilization. Up to now, studies have primarily focused on investigating how certain parental traits in fish (i.e., length, weight, colour, egg size and content) affect offspring development and performance (Green & McCormick, 2005; Van

Leeuwen et al., 2016; Cortese et al., 2022). On the contrary, very few investigations have explored the impact of parental effects on progeny transcriptome (Monroe et al., 2021; Banousse et al., 2024), and even fewer have examined the potential correlation between larvae's zootechnical traits and molecular profiles, in relation to the influence of parental contributions (Debernardis et al. 2025). Despite some progress, many gaps in knowledge still remain. One of these lies in the complex interactions between mothers and fathers (Bonzi et al., 2024), whether through cooperation or context-dependent contributions that ultimately determine the fate of their offspring. In fact, the understandings of the roleplay of maternal and paternal effects to offspring, as well as quantifying each parent's specific impact on the offspring's gene expression profile, remains poorly understood (Van Leeuwen et al., 2016). This presents a valuable opportunity for further exploration into how both maternal and paternal contributions shape the phenotype of future generations, thereby contributing to the broader understanding of evolutionary processes.

One way to study parental effect is by crossing individuals from two extreme phenotypes, yielding viable progeny. In the case of fish, it has been well documented that domestication is a dynamic process of physiological and behavioural adaptation to man-controlled environment (Teletchea & Fontaine, 2014), that significantly impacts the fish phenotype. Several studies have highlighted considerable differences between domesticated and wild fish, which seem to vary depending on the species. For example, studies on cyprinids have revealed a higher reproductive capacity in domesticated individuals than in wild ones (Krejszeff et al. 2009, 2010), whereas in percids, reproductive performance was higher in wild fish (Khendek et al. 2017). Also, molecular analyses have shown that domestication affects eggs gene expression profiles (De Almeida et al. 2019; Nynca et al. 2020; Żarski et al. 2020) and impacts expression levels of genes related to digestion and immune functions in offspring from the earliest developmental stages (Palińska-Żarska et al. 2020, 2021). Importantly, these phenotypic changes can arise very early in the domestication process, even in the first generation, as a result of significant epigenetic modifications (Rodriguez, et al., 2019). These variations can alter traits such as growth and immunity, affecting organism’s performance and fitness (Podgorniak et al., 2022). Taking all of this into account, domestication significantly influences the molecular composition of gametes in comparison to wild, impacting the molecular profile of offspring upon crosses. This makes this approach viable and powerful to study parental effects.

In this study, we created unique paired reciprocal crosses between wild and domesticated Eurasian perch (*Perca fluviatilis*) spawners to separately analyze maternal and paternal

contributions to offspring phenotype and gene expression. While the broodstock originated from wild and domesticated sources, our objective was not to examine domestication effects per se. Rather, these groups served as biologically distinct parental backgrounds, enabling us to explore how parental origin – irrespective of domestication – shapes offspring traits. A subset of freshly hatched larvae from each family was used for whole-organism transcriptome profiling at the mouth-opening stage, while the remaining larvae were reared separately under controlled conditions to evaluate growth and survival during the larval period. This design allowed us to identify maternal- and paternal-effect traits and genes, offering novel insights into the complexity of early developmental inheritance. By disentangling these effects, our study contributes to a deeper understanding of transgenerational trait transmission in fishes, with potential implications for both aquaculture breeding strategies and evolutionary biology.

5.2 Materials and methods

Ethics statement

The study was carried out in compliance with European and national regulations on fish welfare and received approval from the Local Animal Research Ethics Committee (resolution no. 5/2023). The animal research is documented following the ARRIVE guidelines (https://arriveguidelines.org) for reporting animal studies.

Experimental design

Eurasian perch has been chosen as a model for this study due to its growing commercial importance as a valuable freshwater, non-salmonid teleost species. It serves as an ideal subject for research on fish domestication (Fontaine & Teletchea, 2019), reproduction (Żarski et al. 2021; Panda et al. 2024), development (Król et al., 2019; Kupren et al., 2019), and physiology (Henrotte et al. 2010; Palińska-Żarska et al. 2020), with easy access to wild and domesticated stocks (Teletchea & Fontaine, 2014). Additionally, the establishment of standardized protocols for reproduction (Żarski et al. 2017) and larviculture (Palińska-Żarska et al. 2020), alongside recent advancements in genomic research (Ozerov et al. 2018; Panda et al. 2024) in this species, have enabled comprehensive investigations.

For this study, we created reciprocal crosses between wild (**W**) and domesticated (**D**) Eurasian perch, which represent biologically divergent parental types with well-documented phenotypic differences known to influence offspring characteristics (Palińska-Żarska et al. 2020, 2021). While these broodstocks differ in life history, our comparisons focused on

maternal and paternal effects, not domestication per se. Their divergence served as a practical model to probe parental contributions to offspring phenotype and gene expression. To accommodate natural and artificial reproductive cycles, the experiment was conducted in two phases: domesticated females were crossed in February under artificial photothermal conditions, while wild females were crossed in May during their natural spawning season. Cryopreserved milt from wild and domesticated males allowed consistent pairwise fertilizations across both time points. Each female's egg ribbon was divided in two and fertilized with milt from a domesticated or wild male, generating four experimental groups: DD (dom ♀ × dom ♂), DW (dom ♀ × wild ♂), WW (wild ♀ × wild ♂), and WD (wild ♀ × dom ♂) (**Figure 5.1a**). This produced 24 families (6 per group). Pairwise analyses allowed us to isolate maternal effects (e.g., DD vs. WD; WW vs. DW) and paternal effects (e.g., DD vs. WD; WW vs. WD), with shared male or female partners across comparisons (**Figure 5.1b**).

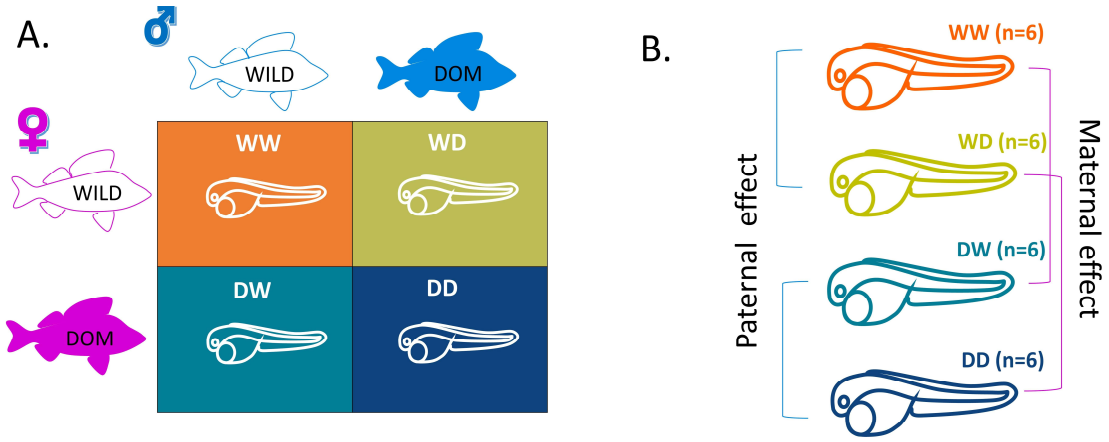

Figure 5.1: *Experimental design and Groups comparisons scheme.* ***(a)*** *Crossing matrix of Wild (W) and Domesticated (D) fish, resulting in four offspring experimental groups: wild females and wild males (WW), wild females and domesticated males (WD), domesticated females and wild males (DW), and domesticated females and domesticated males (DD). A colour scheme for each group has been kept consistent in the data visualization throughout the article.* ***(b)*** *Overview of the analysis strategy used for comparing groups to investigate maternal and paternal effects. DOM: domesticated*

Broodstock management and collection of gametes from domesticated and wild spawners

The broodstock characteristics for both wild and domesticated individuals are described in Supplementary file, **table s5.1**.

The domesticated fish used in the study belonged to the 8th generation bred under fully controlled (in indoor recirculating aquaculture system -RAS-) conditions at the Percitech fish farm in Switzerland. While, wild spawners were captured from lake Mikołajki (Poland) during

the spawning season. The captured wild fish were transported in plastic bags with oxygen to Center of Aquaculture and Ecological Engineering of the University of Warmia and Mazury in Olsztyn (CAEE-UWM, NE Poland) where they were placed in the RAS. In both cases, fish underwent consistent and standardized hormonally induced reproductive procedures described by Żarski et al. (2019). Briefly, both populations, during spawning procedure were kept at a controlled photoperiod with 14 hours of light and 10 hours of dark (14 L:10 D) and temperature (12 °C) until gametes collection. Fish were hormonally stimulated with a salmon gonadoliberin analogue (sGnRHa, BACHEM, Switzerland) with domesticated females being treated with two doses (10 and 25 μg kg^{-1} injection with 7-day interval) to promote and synchronize spawning in both sexes (Żarski et al., 2019). Wild females, were treated with single hormonal injection (50 μg kg^{-1}). In both populations, milt was collected 7 days post hormonal stimulation (25 μg kg^{-1}), which was within the optimal period of milt collection of this species (Żarski et al. 2017). Finally, eggs of domesticated fish were collected on day 9 following priming injection, whereas eggs of wild females on day 4 after injection (Żarski et al., 2011). Prior to any manipulation fish were anesthetized in MS-222 (Argent, USA) at a dose of 150 mg L^{-1}.

The timing of spawning for domesticated (February) and wild (May) fish reflects the established reproductive biology of these two broodstocks. Domesticated perch were maintained under long-established artificial photo-thermal regimes used in commercial aquaculture, which induce predictable spawning windows aligned with hatchery operations. At Percitech, the breeding facility involved in this study, six genetically distinct broodstocks are maintained, each optimized to reproduce at different times of the year via tailored light and temperature cycles. In contrast, wild fish were spawned during their natural reproductive season in May. Importantly, the reproductive protocols used for both wild and domesticated fish have been independently optimized over many years by members of our research team, ensuring best-practice gamete handling and fertilization success tailored to each broodstock's biology. While these protocols differed in timing and hormonal induction, they are not interchangeable. Applying a unified protocol across both groups would likely compromise gamete quality, fertilization rates, and embryonic viability.

Milt collection and cryopreservation

For this study, from both populations cryopreserved milt was used to ensure procedural consistency. As mentioned earlier, this technique also enabled the novel approach of conducting pair-wise comparisons between the different families created.

Milt was stripped from 12 males, 6 domesticated (average weight 490.2 ± 91.5 g) and 6 wild (average weight 221 ± 86.6 g), by gently applying pressure to the abdomen, using a catheter (Galmed, Poland) to prevent contamination with urine or blood. After collection, each milt sample was kept on ice. Spermatozoa motility was first assessed using a two-step activation procedure. For fresh milt, samples were first diluted 1:50, and for frozen/thawed milt, they were diluted 1:5 in an immobilizing solution (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, 20 mM Tris, pH 8.0). Then, milt was then diluted 1:20 in an activating solution (75 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, 20 mM Tris, pH 8.0) with 0.5% bovine serum albumin.

Cryopreserved milt was essential to ensure controlled, repeatable fertilizations with the same male pairs across cross types, minimizing variation in gamete quality. This also allowed us to select families with the highest fertilization success, consistent with our prior studies emphasizing quality-controlled comparisons (Żarski, et al., 2021). While domesticated males were larger, this reflected natural population differences and collection constraints. It is often difficult to collect wild males in sufficient number and size during their limited spawning season, and we used the best available individuals while aiming to minimize disparities. Rather than a confounder, size variation contributed to the desired contrast in parental traits, supporting our "extreme phenotype" approach.

Various motility parameters were measured using the computer-assisted sperm analysis (CASA) system, including motility (MOT, %), linearity (LIN, %), amplitude of lateral head displacement (ALH, μm), average path velocity (VAP, μm s^{-1}), curvilinear velocity (VCL, μm s^{-1}), and straight-line velocity (VSL, μm s^{-1}) for both fresh and cryopreserved milt. Additionally, the concentration of fresh milt was determined using the NucleoCounter SP-100 (Chemometec, Allerød, Denmark) (Judycka, et al., 2019). Ensuring high-quality milt and accurately determining its concentration was crucial, as the cryopreservation procedure depends on the final sperm concentration, which is subsequently used to maintain a consistent sperm:egg ratio during *in vitro* fertilization. Milt cryopreservation was carried out using the method

developed by our Team Judycka et al. (2021) with a final concentration of 0.3 M glucose, 7.5% methanol and 25 mM KCl at 3 × 10^9/ml spermatozoa.

Egg collection and *in vitro* fertilization

Females were chosen on the basis of their oocyte maturation stages, in order to maintain synchronicity during rearing trials. Oocyte maturation stage was evaluated according to the technique described by Żarski et al. (2017) by catheterizing sample of oocytes, exposing them in clarifying Serra's solution (ethanol, formalin, and glacial acetic acid mixed 6:3:1 by volume) and microscopic assessment of their maturation stages based on 6-stage classification (Żarski et al., 2011).

Eggs were collected from 12 selected females: 6 domesticated (average weight 466 ± 81.4 g) and 6 wild (average weight 434.5 ± 102 g), using gentle abdominal pressure into a clean, dry beaker. Each egg ribbon, averaging 113 ± 25 g in weight (see Supplementary file **s5.1**), was then split into two equal parts. One part was fertilized with cryopreserved milt from a single domesticated male (e.g., ♂1_D), and the other with cryopreserved milt from a single wild male (e.g., ♂1_W). Importantly, each fertilization used milt from an individual male, not a pooled sample. The same male pairs were used to fertilize eggs from both domesticated and wild females (**Figure 5.2**), ensuring consistency in paternal origin. This process was repeated for all 12 females, using different male combinations to create fully reciprocal crosses and paired samples for comparative analysis.

Just before *in vitro* fertilization, straws with milt were thawed in a water bath at 40°C for 10 seconds and placed in an Eppendorf tube. Then, the eggs were preactivated for 30 seconds in Wyonarovich solution (1:5) (Żarski et al., 2012), and milt was added to the eggs at a previously optimized sperm:egg ratio of 100,000:1 (Judycka et al., 2019). Upon introducing the thawed milt, eggs were then stirred for 30 seconds and washed with hatchery water after ~10 minutes to remove excess spermatozoa and any debris. Additionally, around 1g of unfertilized eggs (UFE) were snap frozen in liquid nitrogen for further RNA extraction.

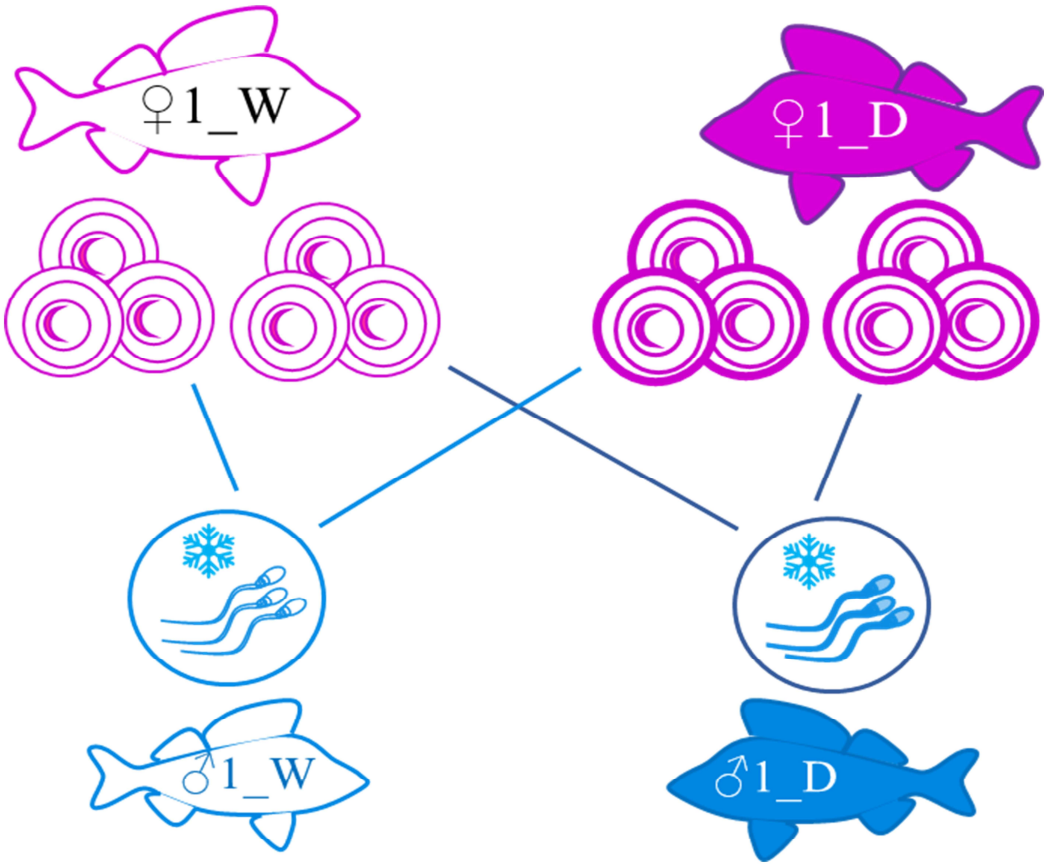

Figure 5.2: *The diagram illustrates a paired fertilization method, where eggs from a specific domesticated female (♀1_D) were fertilized in two ways: half with milt from a domesticated male (♂1_D) and the other half with wild male milt (♂1_W). Same combination of domesticated and wild males was used to fertilize eggs coming from a specific wild female (♀1_W). The same approach was followed for all the domesticated and wild females, using different combinations of males. W: wild. D: domesticated.*

Incubation of embryos

The rearing trials for progeny derived from both wild and domesticated fish were carried out in the exact same way, with the exception that the fertilized domesticated eggs were transported in sealed plastic bags filled with water (70%) and oxygen (30%) and placed inside styrofoam boxes containing 500 g of ice to prevent overheating. The transport lasted approximately 16 hours and covered a developmental window from mid-blastula to ~80% epiboly. Temperature was monitored at regular intervals and remained stable throughout, matching conditions used for wild embryos. Handling during transport was minimal, and the protocol followed procedures validated in previous studies involving perch (e.g., Palińska-Żarska et al. 2020,

2021). While a minor influence of transport on early development cannot be entirely excluded, we consider it negligible given the short duration, stable conditions, and the biological consistency of transcriptomic and phenotypic outcomes. Next, upon arrival the fertilized eggs were further treated in exactly the same way as the eggs from wild fish. All the eggs were incubated in 15L tanks with black walls and upper water inflow, that functioned within the same RAS. The eggs were spread on mesh (diameter of around 3 mm) and kept in a water at a temperature of 14°C (**Figure 5.3a**). Fertilization rate (before embryos reached the mid-blastula transition) was calculated for each family separately (in duplicate), by counting ~100 embryos under the microscope. The photoperiod during all embryo's incubation, and later larvae rearing was maintained at 24L:0D (24 hours light: 0 hours dark; 1500 lux, measure at the water surface). When the embryos reached the eyed-egg stage the temperature was raised to 15°C; while, as soon as the first hatched larvae were noticed the temperature in the system was raised to 16°C. To maintain synchronous hatching, the larvae were hatched manually. This was done by transferring the egg ribbons to bowls with water from the rearing tanks and stirring gently. This operation was repeated few times until most of the larvae hatched. The day of hatching was considered as 0-day post-hatching (DPH).

After hatching, the larvae were left undisturbed for 24 hours. On 2 DPH, they were counted volumetrically and distributed into three tanks (for each experimental group) at a density of 1500 larvae per tank.

Larval rearing

At 1 DPH, the water temperature was raised to 17 ˚C, and at 2 DPH to 18 ˚C, which was kept stable up to 10 DPH. From 11 DPH onward, the water temperature was gradually increased by 1˚C per day until 23 ˚C, considered the optimal temperature for the growth of Eurasian perch larvae (Kestemont et al. 2003; Debernardis et al. 2025). Starting from 3 DPH the mortality of the larvae was counted twice a day after each cleaning. From 4 DPH larvae were started to be fed with Artemia sp. nauplii *ad libitum* three times per day (first four days of feeding – micro Artemia cysts [SF origin], then standard size Artemia cysts at 260,000 nauplii per gram [GSL origin]) (Santos et al., 2021) (**Figure 5.3a**). The deformity rate was counted at 2 DPH (**Figure 5.3b**). Also, from 4 DPH feeding rates started to be monitored by randomly collecting ~100 larvae from each tank and examining their stomachs under a stereoscopic microscope (Leica, Germany) to determine whether they contained food. Starting at 6 DPH, swim bladder inflation effectiveness (SBIE %) was measured in the same way as feeding rates. Both feeding rates and SBIE were recorded until 10 DPH, at which larvae had finally yolk sac fully utilized. After this

time, no increase in the number of larvae with filled swim bladders was observed, while larvae that had not begun eating by this time already died. In addition, from 12 DPH, dead larvae were observed under the microscope to evaluate the type I cannibalism (when the prey is partially ingested) (Kestemont et al. 2003).

Two days before weaning (15 DPH) larvae from each family were counted manually and restock in the density of 500 larvae/tank. This was done to ensure the same number of larvae, which varied due to the different mortality rates observed in some families. After weaning (that took place at 17 DPH) larvae were fed exclusively with dry feed (Perla Larva Proactive, Skretting, Norway) three times a day, sprinkling it into each tank in small amounts for ~15 minutes each time. Oxygen level in the tanks was checked every day (with Aquaculture oximeter Polaris) and it was never below 80% of concentration along with Ammonia and Nitrites concentration every two days (using DR1900 Portable Spectrophotometer), and it was never higher than <0.02 mg L^{-1}. The experiment ended on 27 DPH when more than 50% of the larvae showed no fin fold, therefore more than half of the fish finished already the larval period.

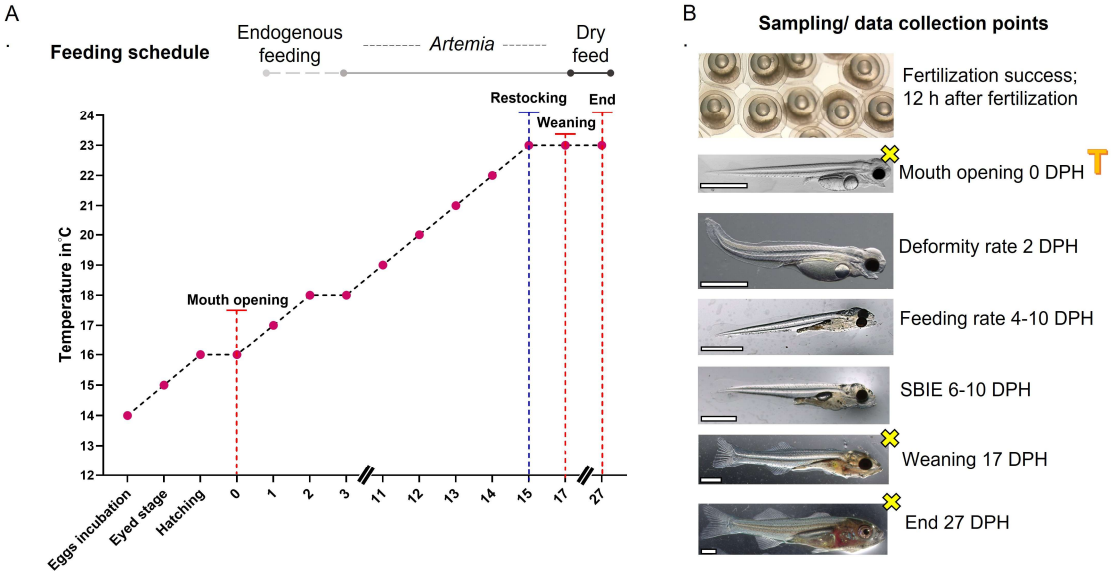

Figure 5.3: *Protocol of eggs incubation and larvae rearing followed for the experiment.* ***(a)*** *Rearing schedule and temperature regimen used for embryos and larvae.* ***(b)*** *Sampling points for zootechnical data collection. Marked with yellow crosses are the sampling points for both zootechnical data collection and molecular analysis. T= describe the moment when collected larvae were used for transcriptomic analysis. DPH: Days post hatch*

Sampling points

Zootechnical traits of larvae were evaluated based on data obtained during three precisely chosen developmental moments following Palińska-Żarska et al. (2020) and Debernardis et al. (2025):

1. At the mouth opening stage (0 DPH) - where at least 50% of larvae were found to have their mouth open. This is the point at which the larva is ready for independent life, while still being subjected to minimal manipulation by humans and the conditions in which it lives.

2. At the moment of weaning (17 DPH) - the moment when larvae start to be fed with compound diets,

3. At 27 DPH – considered as the end of the larval period (when at least 50% of larvae finish their larval stage) and the end of the experiment.

At each of these sampling points, n= 30 larvae per family (n=10 from each tank) were collected to measure total length (TL, ±0.01 mm) and wet body weight (WBW, ±0.1 mg). The larvae were first anesthetized using MS-222 at a concentration of 150 mg L^{-1}, then photographed under a stereoscopic microscope (Leica, Germany) for TL measurement, while WBW was determined using a precision laboratory scale by placing the anesthetized larvae on a nylon net (mesh size approx. 200 μm) and gently blotting excess water with filter paper (Krejszeff, et al., 2013). At each sampling point, additional n=30 larvae from each family were collected and preserved in RNAlater (Sigma-Aldrich, Germany) for subsequent molecular analyses.

RNA extraction

Total RNA was extracted from snap frozen UFE (~ 50 eggs) and larvae at three different developmental stages (mouth-opening stage, weaning and at the end of larval period) using a TotalRNA mini-kit (A&A Biotechnology, Poland). Specifically, for each family, RNA was extracted from pool of 10 larvae at mouth opening stage (10.12 ± 0.17 mg). For larvae at the weaning stage, RNA was isolated from pool of four larvae per family (77.62 ± 4.29 mg), and for larvae at the end of larval stage, from pool of three larvae per family (208 ± 17 mg). After extraction the concentration and purity of the RNA were assessed with DS-11 spectrophotometer (Denovix), showing absorbance ratios of A260/280 ≥ 2.0 and A260/230 ≥ 2.2. Quality of the RNA was further confirmed using the Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with all samples exhibiting RIN values ≥ 9.0.

Importantly, only RNA samples from larvae at mouth opening stage were then sent for transcriptomic analysis. RNA extracted from UFE and other sampling points were used for Real-time qPCR validation.

RNA sequencing and library preparation

Twenty-four different libraries were created. RNA-seq analysis was performed by Macrogen (Amsterdam, Netherlands) using the TruSeq Stranded mRNA kit (Illumina) with a NovaSeq6000 platform, and over 40 M 150 bp paired-end reads per sample were generated. Read files have been processed with nf-core/rnaseq v3.12.0 (Patel et al. 2023) using GCF_010015445.1_GENO_Pfluv_1.0_genomic.fna as reference genome and GCF_010015445.1_GENO_Pfluv_1.0_genomic.gtf as reference annotation with "--skip_biotype_qc" and "--aligner star_rsem" parameters. Briefly, the reads were checked with fastqc (Babraham Bioinformatics, 2022) and trimmed with trimgalore (Krueger, 2022) then aligned to the reference genome with STAR (Dobin et al., 2013b) and quantified with RSEM (B. Li & Dewey, 2011). A summary table with general statistics of the RNA-Seq data is provided in Supplementary file, **s5.2**.

Differential expression genes (DEGs) analysis

RNA-seq data were analysed by performing comparisons both between and within subjects using the edgeR package in RStudio, following the authors' recommendations (Chen et al., 2016; Robinson et al., 2009). Low-expressed genes were filtered by *filterByExpr* function, as advised. To analyse maternal effects, females were treated as the variable factor, while males, whether domesticated or wild, were kept constant (DD vs WD and WW vs DW). Likewise, for the assessment of paternal effects, males were considered the variable factor, with females held constant throughout the analysis (DD vs DW and WW vs WD). Differences were considered significant when false discovery rate (FDR) was inferior to α ($\alpha = 0.05$) and specific differentially expressed genes (DEGs) were found for maternal and paternal effects comparisons.

Next, the DEGs found for the different experimental groups were compared to identify specific genes unique to each comparison as well as those shared between them and the results were visualized using a Venn diagram (Bardou et al., 2014).

To explore the variability in transcriptomic data, a heatmap showcasing the top 100 most variable genes and a Principal Component Analysis (PCA) plot have been generated using iDEP 2.0 for visualization (Ge et al., 2018).

Gene Ontology (GO) analysis

GO analysis was conducted following the method described by Żarski et al. (2021). In brief, Eurasian perch transcriptome was first mapped to the human proteome using the Swiss-Prot database. Sequence alignment was then performed using BLASTX, and only the top match for each protein was selected, providing gene names and UniProt accession numbers for the aligned proteins. These were subsequently utilized for GO analysis using the ShinyGO platform (Ge et al., 2020). GO analysis was carried out on DEGs found, separately for each comparison. The 20 most enriched biological processes were identified based on an FDR < 0.05.

***In silico* identification of perch-specific maternal-effect genes**

The direct transfer of maternal RNAs from egg to progeny remains a topic of ongoing debate. This study focuses on RNAs, with one of the aims being to investigate the presence of maternal RNAs in offspring. To explore this, we sought to identify gene candidates that could serve as maternal effect genes specific for Eurasian perch. In detail, out of the 573 identified purely maternal-effect genes (for details see the Results section), 324 DEGs were found to be present in the UFE transcriptome of Eurasian perch (Panda et al. 2024), what enabled to identify maternal genes (i.e., they were deposited as maternal transcripts in the egg) (**Figure 5.4**). To explore how selected maternal-effect genes (MEGs) behave throughout early development, we carried out a biologically informed *in silico* filtering to select a subset of candidates for further expression trajectory analysis. Among the 573 purely maternal-effect genes identified by RNA-seq, we first retained 324 genes also present in the unfertilized egg (UFE) transcriptome (Panda et al. 2024), suggesting direct maternal RNA deposition. To identify perch-specific MEGs, we compared these transcripts to zebrafish expression profiles (Expression Atlas; Moreno et al. 2022), focusing on expression between zygote and mid-blastula stages. Genes with no detectable expression in zebrafish (TPM < 0.5) were considered potentially perch-specific and maternally derived. This yielded 28 candidate genes, of which the six most abundant in perch eggs (>100 TPM) were selected for developmental expression profiling across larval stages (**Figure 5.4,** Supplementary file**, s5.3**). These genes were not selected to validate RNA-seq outcomes statistically, but to assess whether such perch-specific maternal transcripts exhibit distinct persistence or regulation during early development.

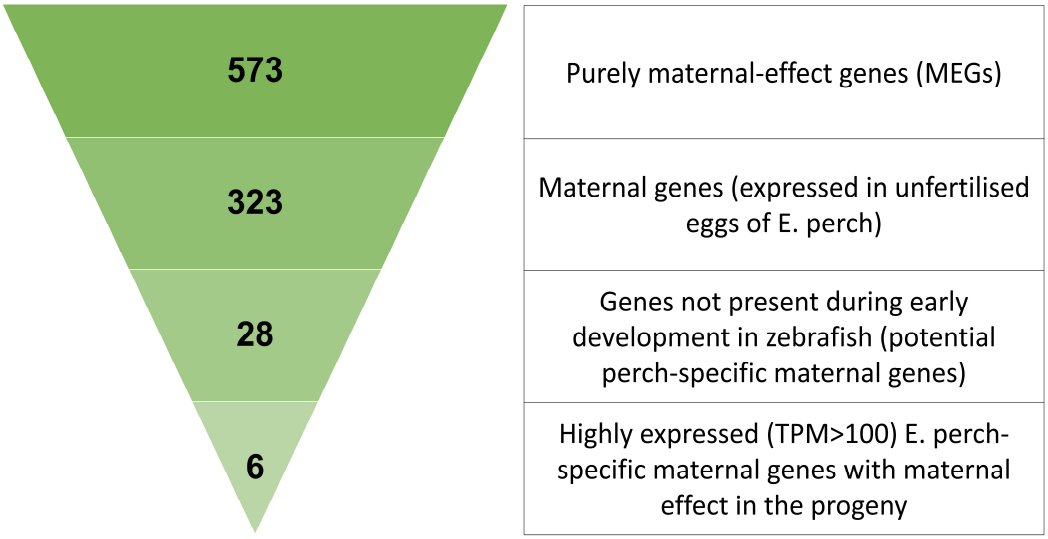

Figure 5.4: *Graphic representation of the filtering cascade followed used to select the six Eurasian-perch-specific maternal genes with documented maternal-effect candidates.*

Exploration of expression profile of candidate maternal-effect genes during early development by RT-qPCR

Total RNA from eggs and larvae were reverse transcribed using the TranScriba kit (A&A Biotechnology, Poland) with oligo(dT)18 primers according to the manufacturer's instructions. Briefly, 1 µg of total RNA was mixed with 4 µl of 5x reaction buffer, 0.5 µl of RNAse inhibitor, 2 µl of dNTP mix and 4 µl of TranScriba reverse transcriptase. The reaction was conducted for 60 min at 42°C and then completed by heating at 70 °C for 5 min.

Primers for the 6 selected genes along with 6 reference genes for RT- qPCR were designed using Primer3Plus software version 3.3.0 (Untergasser et al., 2007). For egg samples, two common reference genes were employed: beta actin (*b-actin*) and ribosomal protein L8 (*rpl8*). For larval samples, the reference genes included: ATP Synthase Peripheral Stalk-Membrane Subunit B (*atp5pb*), Nascent Polypeptide Associated Complex Subunit Alpha (*naca*), ATP Synthase F1 Subunit Gamma (*atp5f1c*), Isocitrate Dehydrogenase (NAD (+)) 3 Non-Catalytic Subunit Beta (*idh3b*). These reference genes were selected based on their lowest coefficient of variation (CV) recorded in our transcriptomic data (Żarski et al. 2021), using TPMs calculated for all the biological replicates. The sequences of the designed primers are presented in Supplementary file, **s5.4**.

Real-time qPCR was then conducted using a Viia7 thermocycler (Applied Biosystems). For each qPCR reaction (20 µL total volume), 10 ng of cDNA template was combined with SYBR Green qPCR Master Mix (A&A Biotechnology, Poland) and 0.5 µM of both forward and reverse primers. The cycling conditions consisted of a 10-minute enzyme activation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing and elongation at 60°C for 1 minute. After amplification, the efficiency of each primer was calculated using the Real-time PCR Miner program (Zhao & Fernald, 2005). Then, the changes in gene expression were analysed using the delta delta Ct (2–ΔΔCt) method (Schmittgen & Livak, 2001). Data were normalized using geometric mean of reference genes.

Data analysis and statistics of zootechnical traits

The analysis of zootechnical data was performed using IBM SPSS Statistics for Windows (Version 29.0.2.0, IBM Corp, 2023) (Čaplová & Švábová, 2020). Paired t-tests were then conducted for each parameter and for all the comparisons (DD vs WD and WW vs DW for maternal effect whereas DD vs DW and WW vs WD for paternal effect). Differences were considered statistically significant when $p < 0.05$. Graphs were generated using GraphPad Prism (version 9.4.1).

5.3 Results

Zootechnical data

No significant differences in fertilization rates were observed when comparing the influence of both paternal- and maternal-effects across groups (**Figure 5.5**). However, when analysing hatching rates, significant differences were only observed in the maternal-effect analysis, with wild females consistently exhibited the highest hatching rates, regardless of the male's origin (**Figure 5.6**). Also, no differences were observed for deformity rate (**Figure s5.5a,** in Supplementary file, **s5.5**). For Type 1 cannibalism (where the prey is not fully digested), the only significant difference emerged for maternal effect analysis, specifically while comparing groups where females were paired with wild males (**Figure s5.5a,** in Supplementary file, **s5.5**).

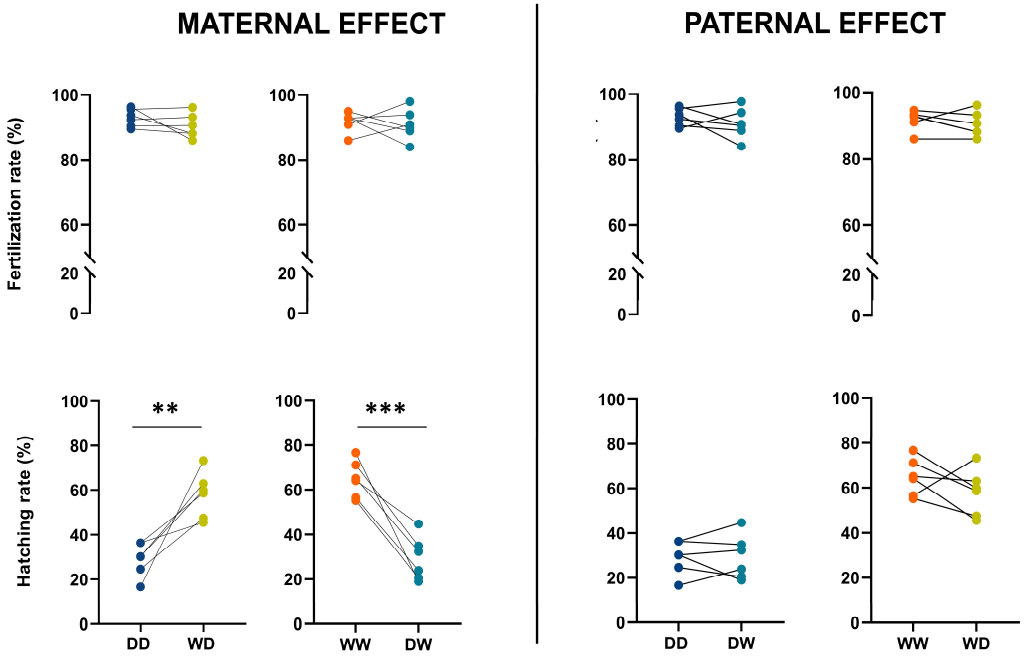

Figure 5.5: *Fertilization rate and hatching rate for all the families of Eurasian perch. The asterisks (**p < 0.01, ***p < 0.001) show significant differences between the groups. DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

A significant difference in mortality rates before weaning was observed when groups were compared as WW vs DW (for maternal effect) and WW vs WD (for paternal effect), with the WW group always showing a significantly higher mortality rate (**Figure 5.6**). Indeed, mortality rates were higher in larvae with a predominantly wild phenotype compared to those originating from domesticated parents as shown also in the cumulative mortality graphs (**Figure s5.5b,** in Supplementary file **s5.5**).

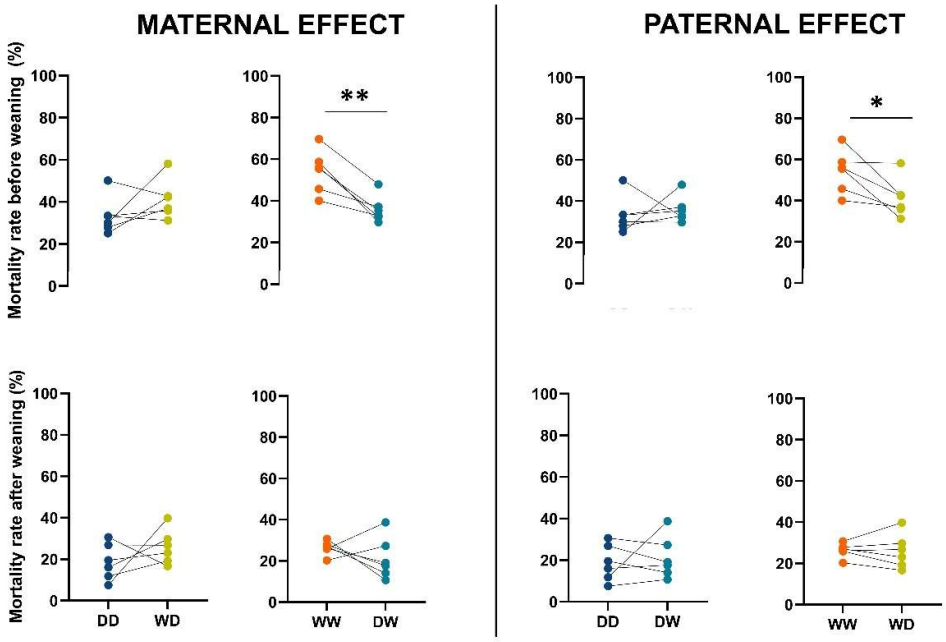

Figure 5.6: *Mortality rate before and after weaning for all the families of Eurasian perch. The asterisks (*p< 0.05, **p < 0.01) show significant differences between the groups. DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

The feeding rate was evaluated over a 7-days period (from 4 DPH to 10 DPH) to study kinetics of feeding onset (**Figure 5.7 and 5.8**). Significant differences were mainly observed when males were crossed with domesticated females (**Figure 5.8**). In contrast, the analysis of SBIE over a 5-day period (from 6 DPH to 10 DPH) revealed significant daily differences tied to maternal influences (**Figure 5.7**). Moreover, the larvae coming from domesticated females consistently showed a higher SBIE, regardless of the phenotype of the male they were paired with.

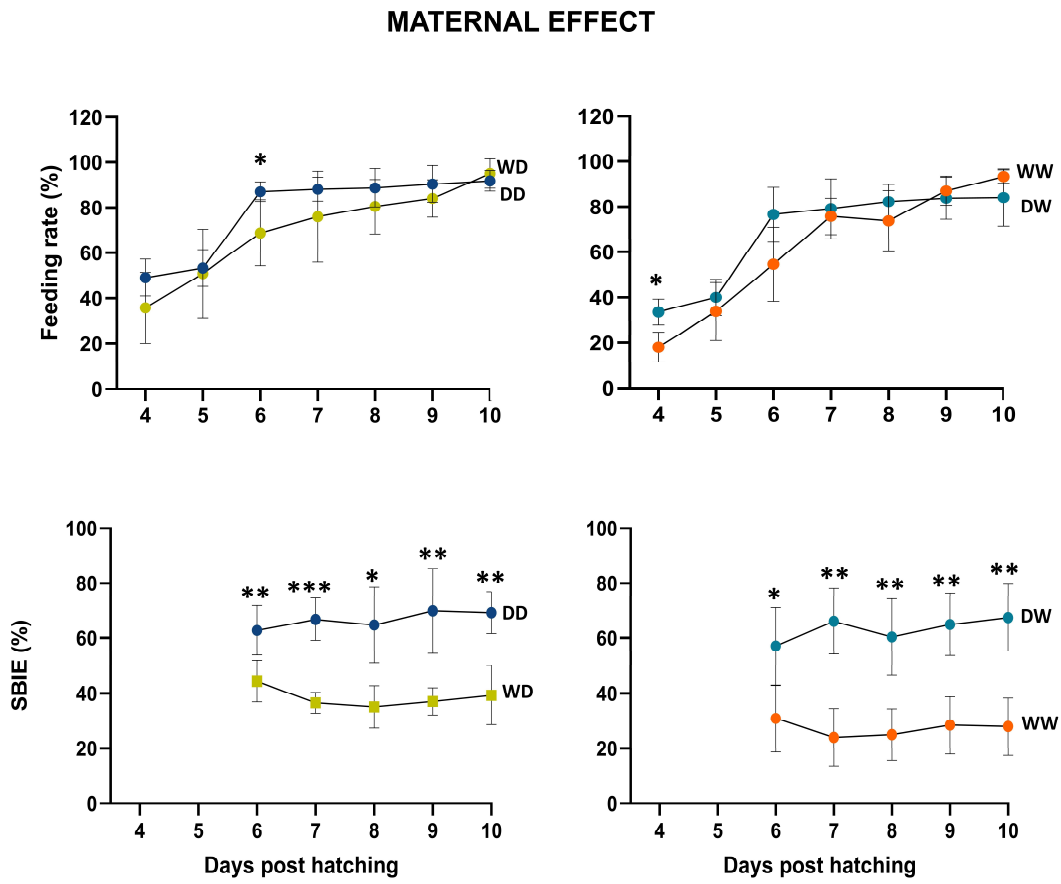

Figure 5.7: *Feeding rate and SBIE kinetics (%) for all Eurasian perch families analyzed for maternal-effect. The asterisks (*p< 0.05, **p < 0.01, ***p < 0.001) show significant differences between the groups over time. SBIE – swim bladder inflation effectiveness DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

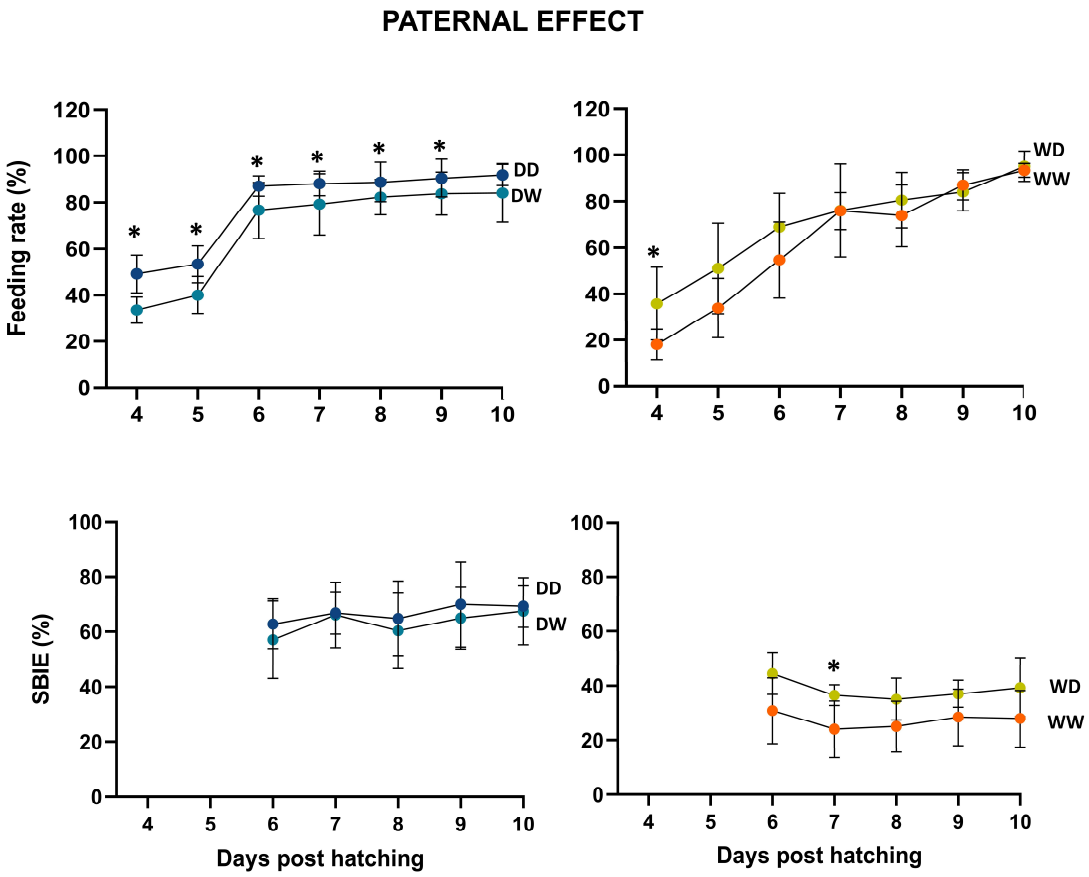

Figure 5.8: *Feeding rate and SBIE kinetics (%) for all Eurasian perch families analyzed for paternal-effect. The asterisks (*p< 0.05) show significant differences between the groups over time. SBIE – swim bladder inflation effectiveness; DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

Significant differences in total length (TL) (**Figure 5.9**) were observed across the groups both at the mouth-opening stage and at the end of the larval period (referred to as end sampling in the graphs), exclusively in relation to the maternal-effect analyses. No significant differences were observed between groups analysed for paternal-effect.

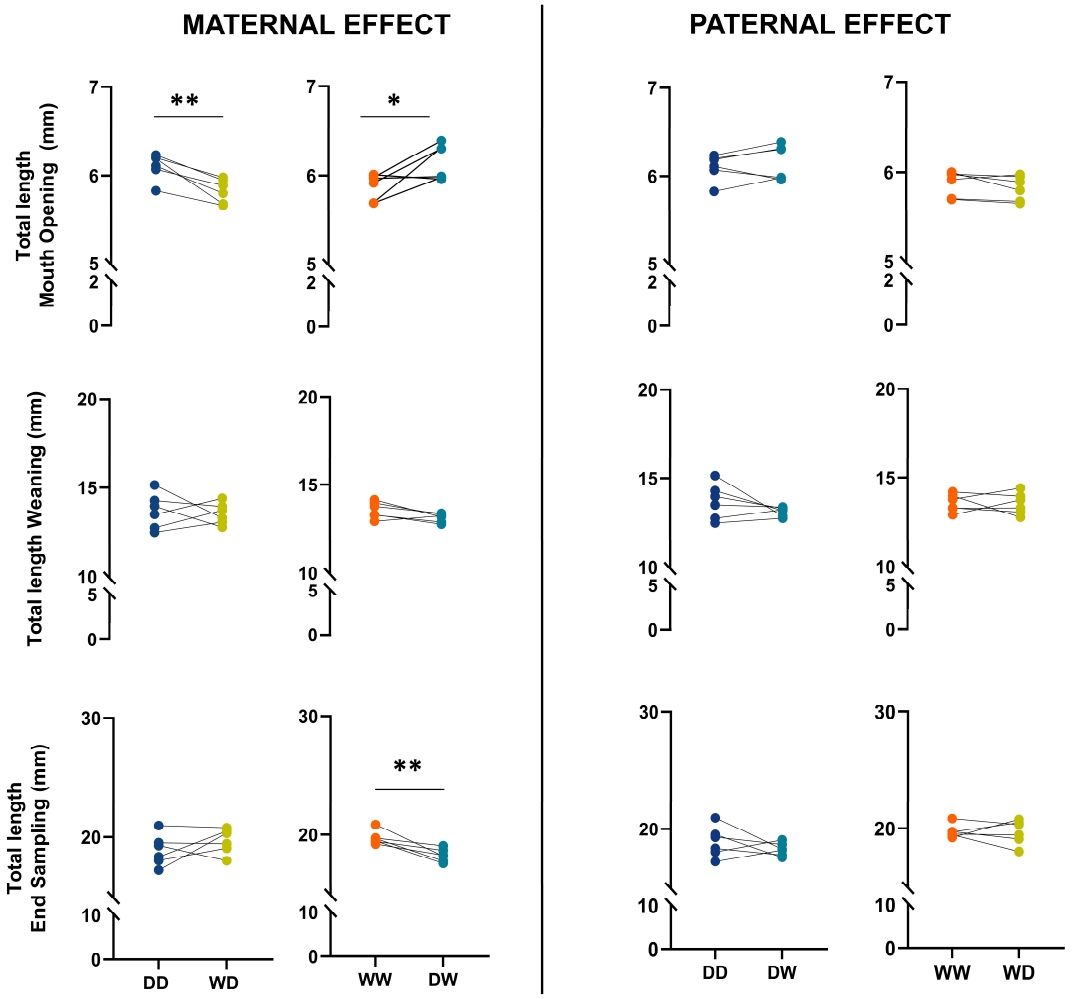

Figure 5.9: *Total length (TL; mm) of larvae measured at different developmental stages for all Eurasian perch families. The asterisks (*p< 0.05, **p < 0.01) show significant differences between the groups over time DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

For wet body weight (WBW) (**Figure 5.10**), most significant differences were observed in the maternal-effect analysis. Although larvae from domesticated females were characterized by higher WBW at the mouth-opening stage, by weaning and at the end of the experiment, larvae from wild females exhibited higher WBW. Regarding paternal-effects, a significant difference was only observed at weaning, specifically when comparing the WW and WD groups.

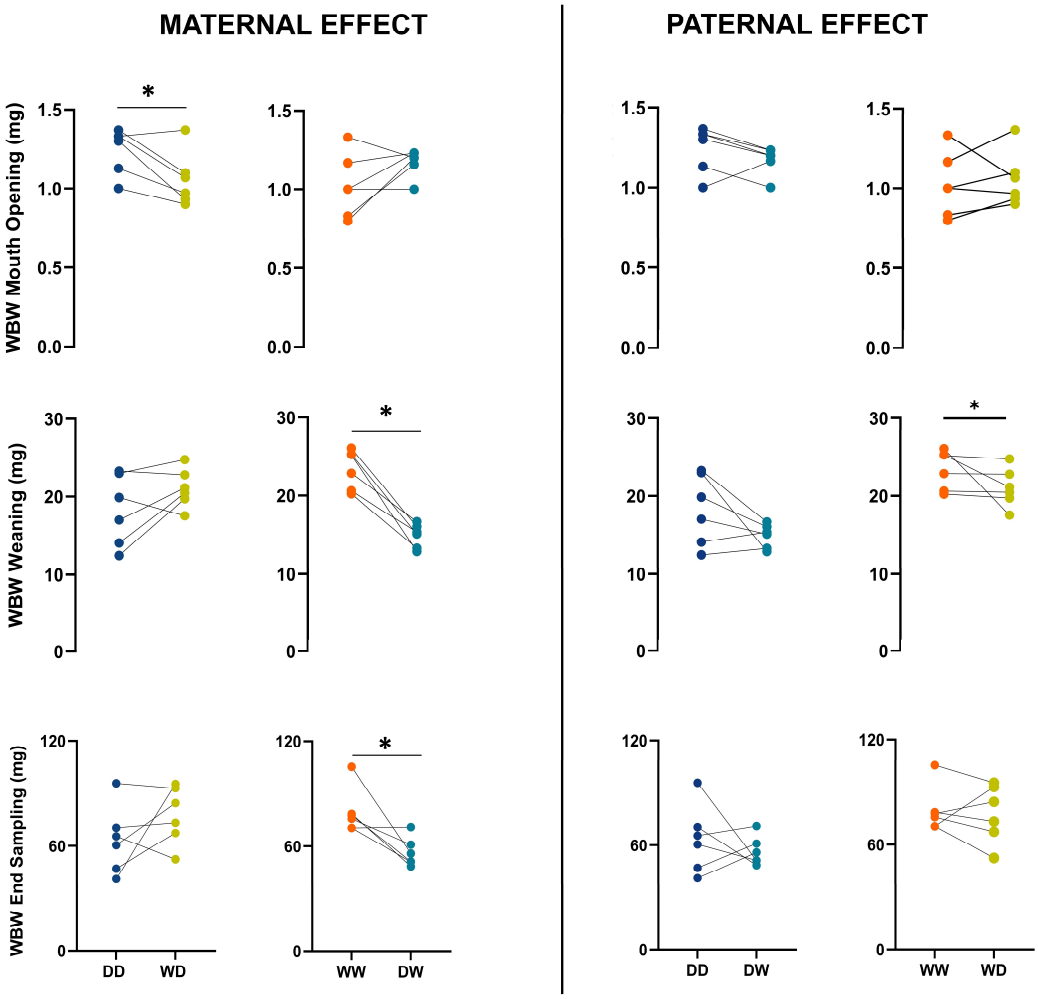

Figure 5.10: *Wet body weight (WBW; mg) of larvae measured at different developmental stages for all Eurasian perch families. The asterisks (*p< 0.05) show significant differences between the groups over time. DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

In summary, zootechnical data analysis revealed that females seem to play a dominant role compared to males, across several parameter related to offspring growth, from hatching success to early development traits (such as length, weight, SBIE). Additionally, differences in foraging capacity observed when males were crossed with domesticated females suggest that these variations are more closely tied to domestication conditions, with males potentially playing a key role in influencing this phenomenon.

Transcriptomic data

A differential expression gene (DEG) analysis was conducted to investigate maternal- and paternal-effects across various comparisons. Following RNA-seq analysis, a total of 30,744

genes were initially identified. After applying filtering criteria for expression level, 20,447 protein-coding genes remained, which were then used for the DEGs analysis, to identify potential specific maternal- and paternal-effect genes (for full list of DEGs see Supplementary file, **s5.3**). Visualization of 100 most variable genes (**Figure 5.11a**) shows significant transcriptomic diversity among the experimental groups and the PCA (**Figure 5.11b**) reveals that the family distribution is clearly influenced by the female origin.

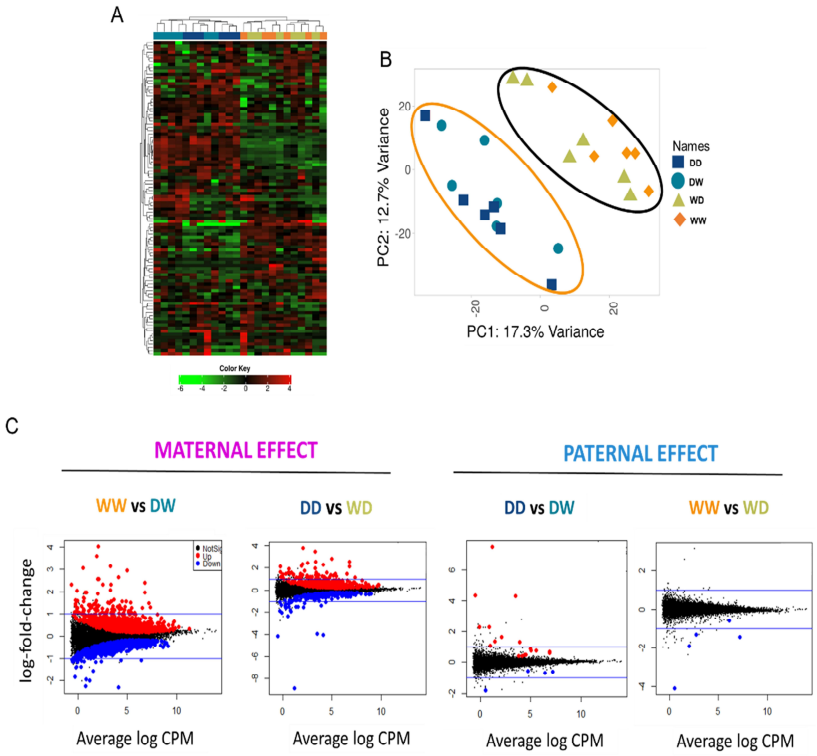

Figure 5.11: (a) *Heatmap showing hierarchical non-supervised clustering of 100 most variable genes in the transcriptome of freshly hatched Eurasian perch larvae.* ***(b)*** *Principal component analysis for all the Eurasian perch families created. The analyses were done on the basis of female origin - the orange ellipse depicts families created using domesticated mother, while the black ellipse depicts females from the wild origin.* ***(c)*** *Volcano plot of DEGs (FDR < 0.05) for all the 4 comparisons made for maternal- and paternal-effect analysis. CPM: counts per million; DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

Around 17,000 identified and expressed genes were found to be non-differentially expressed, indicating that these genes consistently contribute to shaping the larvae's profile, regardless of parental origin and can be named as “conserved genes”. A Gene Ontology (GO) analysis revealed that many of these non-differentially expressed genes are primarily associated with nervous system development and intracellular transport (**Figure s5.5d,** see Supplementary file, **s5.5**).

For maternal effect analysis (with females as the variable and males as the constant) 2,259 DEGs (FDR < 0.05) were found when females were crossed with wild males, and 765 DEGs (FDR < 0.05) when paired with domesticated males (**Figure 5.11c**). The analysis for paternal effect, with males being the variable and females as the constant, revealed 22 DEGs (FDR < 0.05) when males were crossed with domesticated females, and only 5 DEGs (FDR < 0.05) when paired with wild females (**Figure 5.11c**). To check for any overlap between groups, a Venn intersection analysis was performed (**Figure 5.12**). This approach led to the identification of two categories of DEGs:

- purely maternal- or paternal-effect genes: which are common across groups regardless of the male's (or females') origin;

- conditionally maternal- or paternal-effect genes: these genes show expression differences based on the specific paternal (or maternal) lineage. Their expression is influenced by interaction with a parent of a particular origin, indicating a conditional effect dependent on the paternal (or maternal) phenotype.

This analysis revealed that 573 genes were commonly shared across the maternal effect groups, regardless of the males' origin; these genes were categorized as purely maternal-effect genes. Additionally, 179 genes were specific to groups where females were crossed with domesticated males, while 1,677 genes were specific to groups where females were crossed with wild males. These genes were classified as conditionally maternal-effect genes. GO analysis revealed that purely maternal- effect genes are primarily involved in stress response processes, sterol and lipid biosynthetic processes (**Figure s5.5e,** see Supplementary file, **s5.5**). In contrast, conditionally maternal effect genes are mainly associated with regulation of cellular component biogenesis, cellular response to stress, DNA replication and regulation of molecular functions (**Figure s5.5f** in Supplementary file, **s5.5**).

For the paternal-effect groups, no purely paternal-effect genes were identified. However, 7 genes were found when males were crossed with domesticated females and these were classified as conditionally paternal-effect genes, listed here: *supt5h* (homolog, dsif elongation factor subunit); *znf648* zinc finger protein 648; *lamtor4* (late endosomal/lysosomal adaptor, mapk and mtor activator 4); *nlrc3* (nlr family card domain containing 3); *tim3* (t-cell immunoglobulin and mucin domain-containing protein 3); *pnmt* (phenylethanolamine n-methyltransferase); *LOC120549683* (interferon alpha-inducible protein 27-like protein 2A).

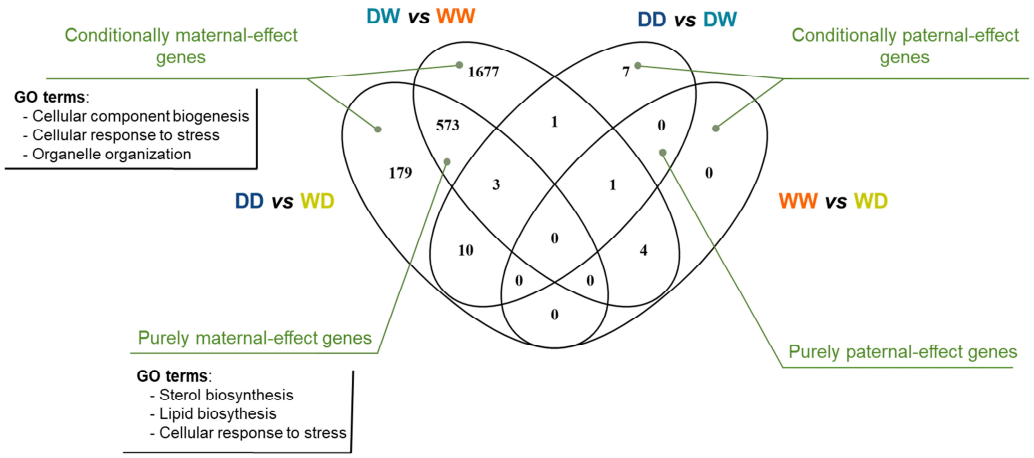

Figure 5.12: *Venn diagram illustrating the overlapping genes classified as purely maternal-effect and paternal-effect genes, as well as those exclusive to conditionally maternal- and paternal-effect genes across different comparisons. Additionally, the figure lists the top three most enriched Gene Ontology (GO) terms associated with purely maternal-effect genes (n=573) and all conditionally maternal-effect genes (n=1856). DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

Developmental expression profiles of perch-specific maternal-effect genes

We focused on a subset of the 573 purely maternal-effect genes to explore their maternal origin and potential perch-specific regulatory roles. Six candidate genes were selected based on their presence in the unfertilized egg transcriptome, their perch-specificity compared to zebrafish early development, and their high expression in perch eggs (**Table 5.1, Figure 5.13**).

Table 5.1: *Genes identified as Eurasian perch-specific maternal genes with maternal-effect documented in our study.*

Gene_id	Human orthologs	Danio rerio orthologs	Transcript_id	Full genes' name
crtac1a	CRTAC1A	*crtac1a*	XM_039784574.1	Cartilage acidic protein 1
LOC120558149	SLC16A7	*slc16a7*	XM_039799069.1	Monocarboxylate transporter 2
si:ch211-79k12.1	KDR	*kdr*	XM_039806878.1	Vascular endothelial growth factor receptor 2
LOC120547834	COX5B	*cox2b*	XM_039783533.1	Cytochrome c oxidase subunit 5B, mitochondrial
lsp1a	CALD1	*cald1b*	XM_039809086.1	Caldesmon
bin2b	BIN2	*bin2a*	XM_039797340.1	Bridging integrator 2

All six genes were detected in unfertilized eggs, supporting their maternal origin. Expression profiles tracked across developmental stages revealed diverse dynamics. For instance, *slc16a7* was highly expressed in eggs and mouth-opening larvae, then declined, suggesting an early developmental role. Interestingly, *cox5b* expression was initially higher in wild eggs compared to domesticated ones. However, in domesticated samples, expression progressively increased throughout larval development, peaking at weaning - suggesting a sustained maternal influence. Other genes, such as *crtac1a*, *kdr*, and *cald1*, displayed stage- or cross-specific modulation, including late-stage increases or lineage effects.

Three of the six genes (*slc16a7*, *cald1*, *crtac1a*) showed expression at the mouth-opening stage consistent between RNA-seq and qPCR. Although full concordance was not observed across all genes, the multi-stage qPCR analysis offered insight into the temporal dynamics of these maternally inherited transcripts.

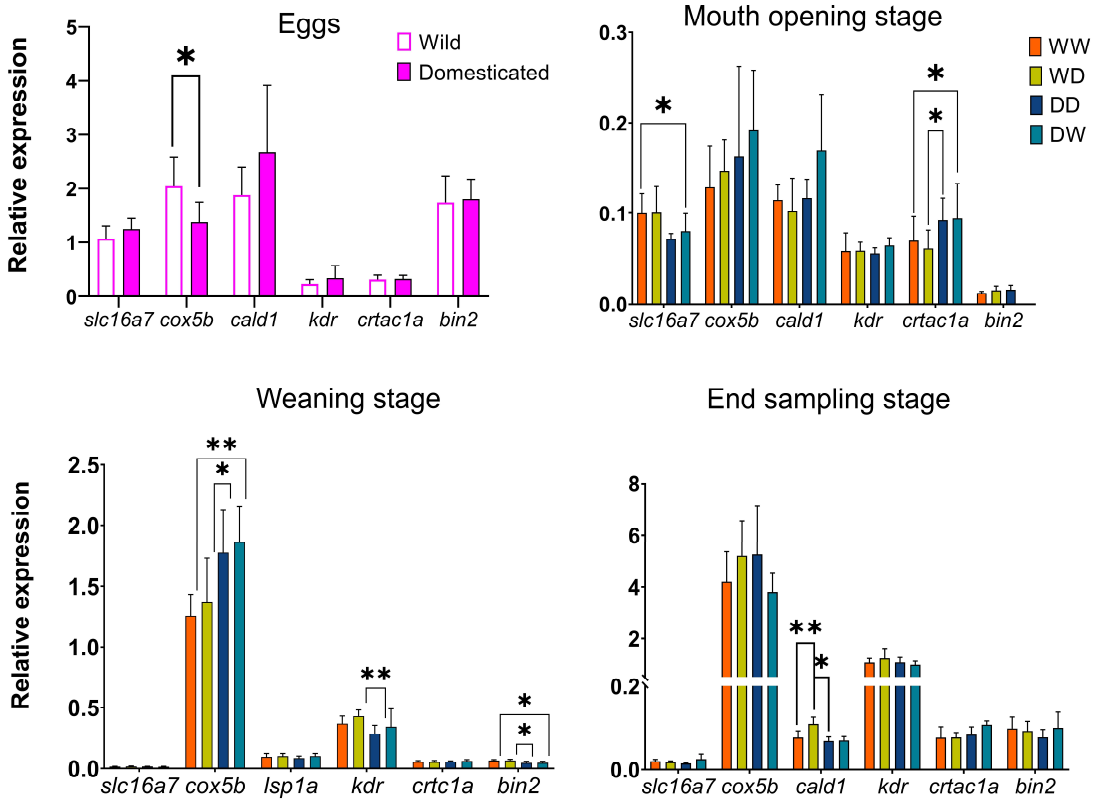

Figure 5.13: *RT-qPCR for the 6 genes selected across the egg stage and various larval stages (from mouth opening to the end of the larval period, here referred as end sampling). The results of statistical analysis are presented as follows: *p < 0.05, **p < 0.01. DD: Domesticated female x Domesticated male; DW: Domesticated female x Wild male; WW: Wild female x Wild male; WD: Wild female x Domesticated male (First letter stands for females followed by males)*

5.4 Discussion

Understanding the respective contributions of maternal and paternal origins in shaping early life traits in Eurasian perch offers valuable insights into the mechanisms of larval development and performance. While parental origin influenced certain zootechnical traits, maternal effects consistently emerged as the primary driver of offspring phenotype, particularly during early developmental stages. This is likely due to the maternal provisioning of molecular and nutritional components within the egg, which directly support early growth, feeding capacity, and larval survival.

In contrast, the transcriptomic analysis of larvae at the mouth-opening stage revealed a more nuanced dynamic. While maternal influence remained predominant, reflected in the greater number of DEGs attributable to maternal origin, our results also point to subtle but detectable paternal contributions. Importantly, although wild and domesticated individuals were used to establish contrasting parental combinations, reflecting their distinct life histories. Taken together, our findings suggest a regulatory framework in which maternally inherited transcripts establish the developmental foundation, while paternally inherited factors may modulate or refine gene expression, contributing to offspring condition in a context-dependent manner.

Zootechnical traits

The results reveal that mothers have a dominant influence across several key zootechnical traits, such as hatching rates, growth-related traits (i.e., weight, length) and swim bladder inflation, while paternal effects has been limited to foraging kinetics in only one group. This strong maternal impact is likely due to the female provisioning the egg with vital nutrients, hormones, and cytoplasmic components that sustain the larval development (Green, 2008). Consistent with previous research (Bang et al., 2006; Bougas et al., 2013), we found that mothers play a critical role in shaping early larval performance with length of larvae at mouth-opening (an important predictor of future performance and adaptability) emerging as a key example of a maternal-effect trait (Debernardis et al., 2025). Notably, this strong maternal influence is particularly evident until oil droplet reduction stage. After this point, it appears to diminish as yolk's oil droplets - containing the nutrients provided by the mother - are consumed, signalling a transition when larvae begin independent feeding (Debernardis et al., 2025). This may explain why initially smaller or lower-weight wild larvae can compensate their growth once they shift to exogenous feeding. Overall, our results underscore the importance of maternal provisioning in early life stages, which appears to set the stage for growth and development, even if its direct influence seem to fade as larvae become self-sufficient. Further exploration could investigate whether these early maternal effects have any lasting impact on later stages of development or long-term performance outcomes.

Phenotypic differences between domesticated and wild populations are well-documented, with parental origin having a significant influence on offspring traits, particularly during the early post-hatching period (De Almeida et al., 2019; Palińska-Żarska et al., 2021, 2020). In this context, some of the zootechnical traits analyzed appear to be affected by the parental experience. For instance, eggs from wild females often exhibit better hatching rates compared to those from domesticated females (Khendek et al., 2017). Conversely, larvae from

domesticated parents exhibited higher feeding rates and more effective swim bladder inflation (SBI), compared to those from wild parents, with the latter experiencing higher mortality rates, particularly during early development. Indeed, domesticated larvae also demonstrated enhanced feeding activity right after the introduction of *Artemia sp. naupli*, a likely result of improved swim bladder inflation (SBI), which has been linked to better feeding capabilities (Czesny et al., 2005; Gebauer et al., 2024). In contrast, the elevated mortality in larvae from wild parents may be linked to their lower foraging efficiency once their endogenous yolk-derived reserves are depleted (China & Holzman, 2014). On the other hand, Palińska-Żarska et al. (2020) observed that differences between domesticated and wild larvae emerged primarily after weaning, with domesticated larvae showing superior digestive capabilities, leading to higher WBW gains by the end of the larval period. In our study, larvae from domesticated parents were initially larger than those from wild-type parents. However, by weaning, the wild-type larvae exhibited faster growth rates, ultimately surpassing the domesticated ones. This suggests that while domesticated larvae benefit from early advantages in feeding and growth, in this case wild larvae may exhibit adaptive advantages that allow them to catch up in later stages, potentially reflecting the resilience and growth potential of wild phenotypes under certain conditions.

Overall, zootechnical results proved that the use of these two distinct populations (domesticated and wild), exhibiting strong phenotypic differences, served as a robust approach helping to better understand parental influence in shaping the offspring's traits. These findings emerged despite the logistical and temporal differences in the spawning of wild and domesticated broodstocks. Rather than being confounding factors, these differences reflect biologically appropriate, highly optimized protocols that align with the long-term reproductive history of each stock. Importantly, these procedures were developed and refined over many years by the team members of this study. The resulting variation (particularly between the commercial domesticated males' line and wild fish) provided an opportunity to study parental effects under contrasting life-history conditions. We deliberately embraced this divergence to capture "extreme phenotypes," thereby increasing our ability to detect meaningful parental contributions within a rigorously structured cross-design.

Transcriptomics

In animals, parental contribution to the transcriptome is unequal and so is the control over early embryogenesis (Zhao et al., 2020). Recently, the transcriptomic profile of fish larvae at mouth-opening has been highlighted as a window to effectively reflect parental influences on offspring phenotypes (Debernardis et al., 2025). In our study, we also focused our transcriptomic analysis on this developmental point, as it represents the end of embryogenesis and minimizes interfering effects from post-hatch environmental exposure. This allowed us to assess whether maternal dominance extends to the molecular level by analyzing gene expression patterns with attention to independent maternal and paternal contributions.

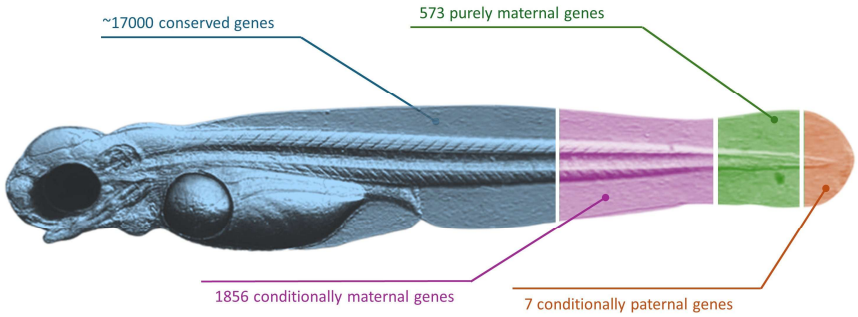

***Figure 5.14**: Presumed transcriptomic portrait of a freshly hatched Eurasian perch larva considering parental contributions.*

Based on the results, a profile of a freshly hatched larva (which in Eurasian perch coincide with the mouth-opening stage) would consist of approximately 80% of a conserved core of around 17,000 genes, displaying stable expression (**Figure 5.14**). These genes represent essential components for larval development, indicating that their expression at certain level is crucial regardless of external factors. Many of these genes are linked to nervous system development and intracellular transport, both essential for larval growth and survival (Quinlivan & Farber, 2017; Nelson & Granato, 2022). Genes linked to neurogenesis are consistently expressed in the transcriptomes of larvae across diverse fish species (Ferraresso et al., 2013; Marisaldi et al., 2021; Nelson & Granato, 2022), emphasizing the essential role of this process in early life history. The development of the nervous system is crucial, as it governs motor functions, sensory processing, and behavioural responses, all of which are vital for the proper adaptation and survival of fish larvae in their early stages (Nelson & Granato, 2022). Also, neurogenesis has been recognized as a non-genetically inherited process for embryonic development, with Colson et al. (2019) and Żarski et al. (2021) indicating a significant maternal

influence on this pathway. The importance of neurogenesis-related genes underscores the importance of maternal inheritance, extending beyond genetic contributions to include regulatory non-genetic factors such as maternal mRNAs, which seem to play a key role in early neural development and functioning. Therefore, these findings suggest that a significant portion of the larval transcriptome remains robustly conserved, highlighting the stability of essential biological pathways crucial for embryonic and larval development. Additionally, the results indicate that suggested in the previous studies maternally derived modifications to nervous system development are more specific for particular neurogenesis pathways and processes, warranting further exploration.

Despite the conservation of a large percentage of the larval transcriptome, approximately 20% exhibits variability influenced by parental effects. Maternal effects, in particular, show a significantly higher number of DEGs compared to paternal effects. Notably, 573 genes were identified as purely maternal-effect genes, enriched in functions related to stress response, cholesterol biosynthesis, and ribosome biogenesis, suggesting vital maternal role in preparing offspring for environmental challenges, metabolic demands, and growth. Similarly, conditionally maternal-effect genes (n = 1856) are predominantly involved in regulation of cellular component, cellular response to stress, DNA replication and regulation of molecular functions. Their expression, however, appears to be adjusted by the experience (shaping non-genetic factors, such epigenetic state) of the mating partner. This indicates a dynamic regulatory mechanism, where maternal inputs lay the foundation, while paternal inputs seem to refine gene expression adjusting offspring phenotypes to suit specific environmental conditions or physiological challenges. Moreover, these set of genes can be seen as maternal-effect genes only when the male does not claim to governs them to act. This indicates that paternal contribution to gene expression is generally limited during early development, but may become active under specific conditions, potentially when paternal-origin signals (e.g., epigenetic marks or small RNAs acquired during the male's life) influence gene regulation. Such effects might reflect environmentally mediated paternal programming or a form of conditional paternal influence that becomes relevant only under particular developmental contexts. Such dynamic interaction points to a complex regulatory mechanism, where both maternal and paternal influences interact in shaping offspring traits, highlighting the importance of both parents in early larval development and survival. These findings suggest a dynamic interplay scenario, where maternal and paternal factors interact to shape offspring characteristics.

The analysis of paternal-effect genes revealed 7 *conditionally paternal effect genes*. While these genes do not cluster under a single GO term, they are involved in critical biological processes, including stress responses, cellular growth, immune system regulation, and gene expression control, further suggesting that paternal influences also play a role in shaping the offspring's phenotype, albeit in a more context-dependent manner. This confirms the notion that both maternal and paternal factors are integral to shaping early life stages, contributing to a balanced and adaptive developmental process.

Although this study did not identify any purely paternal effect genes, likely reflecting the dominant role of maternal factors in the earliest stages of larval development, recent research suggests that paternal influences can still play a role. Specifically, sperm methylation patterns may affect offspring phenotypes, even if these effects are not immediately apparent in transcriptomic data (Jiang et al., 2013; Rodriguez Barreto et al., 2019; Panda et al., 2024). For example, sperm DNA methylation patterns in Atlantic salmon have been shown to reflect environmental conditions experienced by males in captivity, with altered methylation correlating with fitness-relevant traits in F_1 offspring (Wellband et al., 2021). Similarly, brook charr exhibit paternal-line methylation influences that persist into fry stages and affect offspring growth and phenotype, particularly in response to parental thermal environment (Venney et al., 2022). Moreover, in zebrafish, parts of the paternal methylome are retained through zygotic reprogramming, suggesting that sperm-derived epigenetic marks can functionally contribute during early development (Skvortsova et al., 2019). These findings collectively indicate that paternal effects may not manifest at the specific larval stage we analyzed, but could emerge either later or under environmental triggers. Based on these studies, our observation – that purely paternal-effect genes are not present at the mouth-opening stage – does not exclude meaningful paternal influence later on. We therefore recommend future work explore epigenetic and gene expression patterns at earlier embryonic stages and during prolonged larval development, to fully capture the timing and impact of paternal contributions.

Purely maternal genes

Maternal RNAs deposited in the eggs are critical to early embryonic development, yet the precise role and transfer mechanisms of specific maternal mRNAs from egg to offspring remain under investigation (Harry & Zakas, 2023). Our further validation strategy focused on following expression patterns of purely maternal-effect genes to pinpoint candidate Eurasian perch-specific maternal genes with further effect on offspring. This approach helped to elucidate various scenarios of maternal contributions from egg to juveniles.

One of the candidate genes, *crtac1*, is known for its role in chondrocyte differentiation and cartilage formation. It is evolutionarily conserved gene across species, including fish (Redruello et al., 2010). Meanwhile, *slc16a7* encodes a monocarboxylate transporter (Mct2), vital for lactate and pyruvate transport (Felmlee et al., 2020), underscoring its importance in early larval metabolism when energy demands are high. Our study is the first to shed light on these genes as strong candidates for maternal-effect genes. Since we did not detect differential expression of these genes in UFE it may be suggested that maternal control starts later in the development with possible involvement of other genes interacting with them. This, however, require further investigation by, at first, studying the kinetics of their expression along the embryonic development.

In the eggs, *cox5b*, which encodes a subunit of cytochrome c oxidase critical for mitochondrial respiration (Silva-Marrero et al., 2017), showed higher expression in wild individuals compared to domesticated ones. Interestingly, this pattern reversed from the mouth-opening stage onward, with *cox5b* expression progressively increasing in domesticated larvae and peaking at weaning. This post-hatch upregulation suggests that domesticated individuals progressively enhance mitochondrial activity to support higher energy demands associated with rapid growth and the transition to exogenous feeding. These stage-specific shifts suggest a prolonged maternal influence on metabolic regulation, positioning *cox5b* as a strong candidate maternal-effect gene. Additionally, this supports the notion that transcriptomic profiling can serve as a predictive tool, providing insights into both the past conditions (maternal environment) and future developmental outcomes of offspring (Debernardis et al., 2025).

At the weaning stage, the *kdr* gene, also known as *vegfr2* (vascular endothelial growth factor receptor 2), showed a significant difference in expression, particularly in groups where females were crossed with domesticated males. This gene plays a crucial role in the early developmental stages of fish larvae, contributing to the formation of vascular networks essential for nutrient delivery and overall growth (Vogrin et al., 2019). Considering our results, it is possible to speculate that vascular development may be particularly important during the weaning stage, as larvae transition from feeding on Artemia to dry feed, a dietary shift that imposes increased metabolic demands and necessitates efficient nutrient distribution via an enhanced vascular system. The observed higher expression of *kdr* in offspring from wild females compared to domesticated ones might relate to the offspring of wild females exhibiting accelerated growth starting at the weaning stage.

The expression of the *cald1* gene showed significant statistical differences only at the latest juvenile stage. This gene encodes caldesmon, a protein that plays a significant role in the regulation of actin and myosin interactions (Meyer-Rochow & Royuela, 2002). During larval period, teleost exhibit rapid growth and extensive changes in muscle structure (Johnston et al., 2011), therefore this gene may play a crucial role in regulating actin-myosin interactions, essential for muscle contraction. Interestingly, in wild phenotypes, *cald1* expression in juveniles appears to be primarily under maternal control, whereas in the domesticated phenotype, it shifts during the larval period to paternal influence, highlighting another dimension of dynamic interplay between maternal and paternal effects during larval metamorphosis.

The *bin2* gene is a member of the BIN/amphiphysin/Rvs (BAR) family of proteins, which are involved in membrane dynamics, endocytosis, cytoskeletal interactions, neural network formation and immune cell regulation (Rao & Haucke, 2011; Mallik et al., 2022). Although its specific functions in fish larvae are not well characterized, the maternal influence observed at the weaning stage may relate to the critical physiological and immune system adjustments occurring during this period. Maternal factors, provided during oogenesis, seem to equip the larvae to better navigate developmental and environmental challenges encountered during the weaning transition. This highlights the importance of maternal contributions in preparing the progeny to overcome future developmental challenges.

This study aimed to explore how a subset of perch-specific maternal-effect genes behave throughout early ontogeny. While qPCR and RNA-seq were consistent for three of the six genes at the mouth-opening stage, the goal of the qPCR analysis was not to validate RNA-seq per se, but to gain insight into developmental expression patterns. Discrepancies between these platforms are well known and often reflect differences in sensitivity, transcript isoform detection, and reference normalization (Everaert et al., 2017; Coenye, 2021). Rather than a technical replication, the qPCR results allowed us to identify varying trajectories, from transient early expression (e.g., *slc16a7*), to persistent upregulation during the larval stages (*cox5b*), to reactivation at later stages (*kdr*). These findings add a dynamic, temporal layer to our understanding of how maternally derived transcripts may shape offspring development in perch.

In conclusion, the identification of candidate maternal-effect genes highlights the diverse physiological pathways influenced by maternal inheritance, ranging from metabolism and vascular development to muscle growth and immune regulation. These findings not only

enhance our understanding of the molecular mechanisms underlying maternal effects but also provide a foundation for exploring their broader implications in aquaculture and evolutionary biology. In a practical context, understanding maternal contributions to traits such as early growth and survival can inform broodstock selection and spawning strategies in perch culture, potentially improving larval performance and hatchery success.

5.5 Conclusions

Basic rules of developmental process may seem straightforward - each parent provides genetic material to the progeny, equally contributing to its phenotype. However, as our results illustrate, this mechanism is far from simple. Equal genetic contribution does not always correspond to equal influence over the development of offspring. The analysis of the zootechnical performance highlights contributions showing dominant maternal control, shaping key aspects of progeny growth and survival. A deeper look into our results suggests that the shaping of progeny phenotype involves a dynamic and stage-dependent interplay between maternal and paternal contributions. While maternal inputs dominate early development, likely due to preloaded transcripts and egg provisioning, paternal influences appear later and may play a modulatory role in gene expression and phenotype refinement. This raises intriguing questions for selective breeding strategies: whether the selection of mothers over fathers should be prioritized, given their greater influence on offspring development. However, our findings reveal that parental effects are dynamic and growth-wise regulated, with maternal contributions playing a pivotal role during early stages, while paternal influences, though initially subtle and somewhat dormant, become increasingly significant during larval metamorphosis and beyond. This highlights a complex and dynamic interplay between maternal and paternal traits in shaping developmental trajectories. Rather than pointing to strict parental coordination (Annett et al., 1999; Itzkowitz et al., 2002), our results suggest a temporally structured division of influence, where maternal factors dominate early development, while paternal contributions may gradually emerge later in ontogeny. Future studies incorporating transcriptomic profiling of multiple developmental timepoints would be essential to capture these later-emerging paternal contributions and better understand the timing and transition of parental influence. Especially, that our results clearly suggest that this dynamic parental interplay over the progeny's phenotype appears to begin at fertilization and persists throughout the embryonic and larval stages, continuing far longer than anticipated.

5.6 Data availability

Raw data from the analysis of different families of freshly hatched larvae can be accessed via the NCBI BioProject database under the PRJNA1196822 accession number.

5.7 Authors contribution

Rossella Debernardis*: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing – Original Draft; **Abhipsa Panda****: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing – Original Draft; **Sylwia Judycka**: Conceptualization, Methodology, Investigation, Writing – Review & Editing; **Katarzyna Palińska-Żarska**: Conceptualization, Methodology, Investigation, Supervision, Writing – Review & Editing; **Christophe Klopp**: Data Curation, Writing – Review & Editing; **Tainá Rocha de Almeida**: Data Curation, Writing – Review & Editing; **Sylwia Jarmołowicz**: Investigation; Piotr Hliwa: Resources; **Daniel Żarski**: Conceptualization, Methodology, Investigation, Supervision, Project Administration, Funding Acquisition, Writing – Review & Editing

***Rossella Debernardis -** has been responsible for experiment; laboratory works and data analysis related to maternal effect.

****Abhipsa Panda -** has been responsible for experiment; laboratory works and data analysis related to paternal effect.

Acknowledgments

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Chapter 6

General discussion

A move towards the exploration of paternal effect on offspring

6.1 Shifting the Paradigm of Inheritance

Modern inheritance research is moving beyond a strictly gene-centered framework to include non-genetic signals transmitted through gametes (Labbé et al., 2017). In fish, this represents a substantial shift from the long-standing emphasis on maternal effects toward a more balanced view that also recognizes the paternal contribution (Herráez et al., 2017). Such a perspective is important because it broadens our understanding of how traits can persist, adapt, or change across generations, especially in species where parents have no post-fertilization contact with their offspring.

This paradigm shift matters in several ways. First, it can help explain variation in offspring performance that is not accounted for by DNA sequence alone (Reznick, 1981). Second, it may allow aquaculture programs to improve breeding practices by recognizing and managing paternal influences on early development, beyond just sperm counts and motility (Rex Dunham, 2023). Third, it opens the door to a better understanding of how environmental conditions faced by males before spawning might shape their offspring, with potential applications in both conservation and commercial fish production (Jonsson & Jonsson, 2014).

Adopting this broader perspective highlights several key areas that warrant further investigation. The stability of paternal epigenetic marks after fertilization remains unclear, particularly in the context of their persistence and potential influence during early embryonic development. These paternal signals are likely to interact with maternal cytoplasmic factors, possibly shaping gene expression dynamics in the zygote. In externally fertilizing fish, identifying paternal-effect genes presents unique methodological challenges, requiring carefully designed experimental models. Understanding these mechanisms is essential not only for uncovering the functional role of non-genetic paternal contributions but also for assessing their evolutionary significance.

This thesis is positioned to contribute to answering some of these questions by exploring paternal-effect genes and sperm-carried non-genetic factors in Eurasian perch. It will combine knowledge of perch reproductive biology with emerging frameworks in epigenetics and non-genetic inheritance to highlight the role of the paternal gametes as a carrier of developmental signals. Altogether, recognizing paternal non-genetic inheritance reshapes our understanding of how traits are transmitted and how fish populations adapt to their environment, moving the field toward a more integrative and realistic model of inheritance in aquatic species.

We address the knowledge gap in paternal contribution to progeny, through a series of experiments designed to identify and characterize PEGs in Eurasian perch. By applying sperm manipulation techniques such as cryopreservation, post-thaw storage stress, and comparing wild versus domesticated broodstock, we aimed to understand how sperm molecular integrity and origin influence offspring development at both phenotypic and transcriptomic levels. Importantly, the experimental system employed strict maternal control, allowing us to isolate paternal effects without the confounding influence of maternal variability.

In doing so, this research challenges the long-held assumption that maternal provisioning is the dominant determinant of early fish development (Armstrong & Nislow, 2006). Instead, it provides compelling evidence that fathers contribute molecular regulators, delivered via sperm, that shape larval phenotype, developmental success, and gene expression patterns (Siddique et al., 2017; Immler, 2018). Moreover, this thesis demonstrates how integrating zootechnical observations (e.g., larval survival, growth, swim bladder inflation) with transcriptomic profiling yields a powerful systems-level view of paternal influence.

Taken together, the work presented here not only enhances our understanding of PEGs and non-genetic paternal effects in fish, but also lays a foundation for novel applications in aquaculture, such as precision broodstock selection and molecular diagnostics for sperm quality. As such, it represents a significant contribution to the emerging field of paternal epigenetics and adds a critical dimension to our understanding of inheritance in vertebrates.

6.2. Synthesis of Experimental Findings

The experimental framework of this thesis was designed to probe paternal contributions to early life development in Eurasian perch through multiple, complementary approaches. Across three studies, we manipulated sperm integrity and origin to uncover the presence and functional roles of PEGs. These manipulations included cryopreservation (Fresh-Cryo), post-thaw storage (PTS), and contrasting wild versus domesticated males (Dom-Wild). **Each experiment used controlled maternal input and standardized fertilization procedures to isolate paternal factors**. Importantly, we coupled zootechnical performance metrics, such as hatching rates, deformity rates, growth parameters, swim bladder inflation and mortality during development, with whole-body transcriptomic analysis of larvae at mouth-opening stage. The insights gained from these experiments form a cohesive narrative regarding how paternal molecular contributions shape progeny outcomes.

6.2.1. Cryopreservation as a Functional Selection Tool (Fresh-Cryo)

Cryopreservation is widely used in aquaculture for long-term sperm storage, synchronization of reproduction, and genetic resource management (Asturiano et al., 2017). However, its application also introduces a potent selection pressure on sperm cells. Exposure to freezing in liquid nitrogen temperatures, osmotic shock, and oxidative stress damages cellular structures and functions, allowing only the most robust spermatozoa to survive and retain fertilizing capacity (Cabrita et al., 2010; Nusbaumer et al., 2019). In this study, we exploited this property to investigate whether cryo-selected spermatozoa carry distinct molecular cargo with implications for offspring development.

Using cryopreserved versus fresh sperm from the same males, we conducted controlled *in vitro* fertilizations, ensuring that oocyte quality and environmental conditions were identical across treatments. The obtained larvae exhibited significant phenotypic differences in wet body weight. Transcriptomic profiling at the mouth-opening stage revealed differential expression of genes associated with phototransduction and visual system formation, most notably *pde6g*, *opn1lw1*, and *rbp4l* (Panda et al., 2024). These genes are involved in early eye and neural development, suggesting that the cryopreservation process selectively influences the expression of PEGs that regulate essential larval functions. The repetition of gene expression effects across multiple replicates, together with consistency in the known functions of affected genes, supports using cryopreservation as a precision tool to identify sperm-transmitted regulators of development. In this context, genes such as *pde6g*, *opn1lw1*, and *rbp4l* may represent candidates linking paternal gamete integrity with offspring phenotype, warranting further functional investigation.

6.2.2. Post-Thaw Storage (PTS) as a Precision Stressor

While cryopreservation serves as a binary filter (viable vs. non-viable sperm), post-thaw storage introduces a more advanced challenge. In the PTS study, thawed semen was stored at 4°C for 30 minutes before fertilization. Although this treatment resulted in a sharp decline in sperm motility, as expected in Eurasian perch (Judycka et al., 2022), whose sperm motility diminishes within minutes after thawing, it did not reduce fertilization success. This created an opportunity to test whether the sublethal deterioration of sperm function would affect larval performance and gene expression. Indeed, larvae from the PTS group exhibited markedly higher mortality rates, a sensitive indicator of compromised development and reduced aquaculture viability (Bobe & Labbé, 2010). Transcriptomic profiling revealed 41 differentially

expressed genes (DEGs), many involved in immune function (*gimap*, *mfap*, *hlag*, *pigr*) and developmental regulation (*trim*, *plaat*, *ang*, *cxadr*). Particularly notable was the recurring downregulation of *pde6g*, also affected in the Fresh-Cryo study, suggesting a robust paternal imprint on sensory development. These findings support the idea that post-thaw sperm resilience reflects deeper molecular integrity, which in turn influences progeny phenotype. The subtlety of the PTS design, manipulating only the duration of sperm storage, also provided a controlled and repeatable model for uncovering paternal molecular contributions without introducing new environmental or maternal variability.

6.2.3. Wild vs. Domesticated Crosses as a Window into Evolutionary Epigenetics (Dom-Wild)

The third experimental system utilized the divergence between wild-caught and domesticated Eurasian perch males to investigate paternal effects embedded in natural and aquaculture environments. These two groups differ in numerous ways, including genetic background, stress exposure, diet, and reproductive conditioning (Teletchea & Fontaine, 2014; Fontaine et al., 2019; Milla et al., 2020). By fertilizing oocytes from a shared identical maternal source with sperm from each group, we isolated paternal influences under standardized fertilization and rearing conditions. Higher feeding rates observed in the DD group compared to the DW group further suggest that domestication effects may be emerging through paternal influence. No other parameters compared show significant differences across the crosses.

The larvae sired by wild and domesticated males exhibited differences in early development, growth trajectories, and gene expression profiles. Transcriptomic comparisons revealed gene sets related to neurodevelopment, metabolic plasticity, and stress resilience, echoing prior findings in salmonids where hatchery vs. wild males showed contrasting sperm methylation and RNA profiles (Bicskei et al., 2014; Le Luyer et al., 2017). The occurance of the gene *pde6g* in this experiment was conditionally maternal form, indicating the paternal involvement of the gene expression too. Results suggest that paternal origin is shaped by environmental history and can modulate offspring gene regulation, possibly through stable epigenetic marks in sperm. **However, the experiment demonstrated also that the maternal genome can influence the transcriptional activity of paternal genes.** Unlike the cryopreservation and PTS experiments, the Dom-Wild comparison introduced greater biological complexity, due to variation in male genetics and environmental exposure. However, this complexity also enhanced ecological relevance (Lorenzen et al., 2012). It reinforced the idea that paternal effects are not artifacts of

artificial manipulation, but instead reflect biologically meaningful variation rooted in evolutionary and life-history processes.

6.3. Overview

Together, these three experiments present a cohesive and complementary picture of paternal influence in fish development. Cryopreservation and PTS offer mechanistic clarity, revealing how sperm integrity modulates offspring phenotype via transcriptomic regulation. The wild-domesticated contrast adds ecological context, demonstrating that PEGs can reflect long-term adaptation and environmental conditioning. Crucially, in all three experiments, larval phenotypes correlated with changes in gene expression, affirming the value of integrating zootechnical data with transcriptomics. Moreover, the recurrence of key genes such as *pde6g* across experiments supports their classification as candidate PEGs with functional significance. The next sections of this discussion will further explore how these findings demonstrate the power of a combined molecular and phenotypic approach, how cryopreservation functions as an effective research tool, and how maternal factors might interact with paternal contributions to shape early developmental outcomes.

6.4. Integrative Power of Zootechnical and Transcriptomic Assessment

Significant differences between the test and the control groups were observed in several sperm motility parameters such as velocity, linearity, and motile percentage. This enabled, for instance, to reveal the adverse effect of sperm cryopreservation on motility characteristics (Muchlisin et al., 2025). However, these impairments minorly compromised fertilizing ability in our model species, as fertilization rates between fresh and cryopreserved semen remained statistically indistinguishable though similar results may or may not be seen for some other fish species (Yang et al., 2009; Gallego et al., 2017; Santana et al., 2020). To not limit ourselves here, one of the central conceptual and methodological strengths of this thesis lies in the integration of classical phenotypic (zootechnical) assessments with molecular (transcriptomic) analysis, providing a multifaceted view of paternal effects in fish. While each of these approaches offers valuable insights in isolation, their combination enables the identification of mechanistic links between paternal gamete condition and the developmental trajectory of offspring. This dual strategy represents a significant step forward in the study of non-genetic inheritance and paternally influenced developmental regulation in teleosts (Braun & Champagne, 2014).

6.4.1. Why Zootechnics Alone Is Not Enough

Traditional reproductive and developmental studies in aquaculture have long relied on phenotypic traits such as fertilization rate, hatching success, larval growth, deformity incidence, swim bladder inflation, and survival (West, 1990; Gallego et al., 2013). These traits form the backbone of zootechnical assessment and provide valuable benchmarks for hatchery practices and breeding decisions. However, phenotypic data alone are often insufficient to resolve the biological complexity underlying early life development, particularly when it comes to subtle or dynamic traits shaped by non-genetic inheritance. Across diverse teleost species, traits such as length, body mass, deformities, and behavior show variable sensitivity to parental identity and environmental conditions (Burt et al., 2011; Jonsson & Jonsson, 2014). For example, in our study, larval total length remained consistent across groups, despite significant differences in body mass. This suggests that larval length may be more strongly buffered by maternal effects, while traits like overall growth and behavior are more plastic and responsive to paternal inputs. Similar patterns have been observed elsewhere like in Atlantic cod. Kroll et al., (2013) showed that paternal identity significantly affected early traits like yolk-sac volume and standard length. In bluegill sunfish (*Lepomis macrochirus*), paternal effects were evident in larval foraging behavior but not in size or mass (Lister & Neff, 2006), while in Arctic charr (*Salvelinus alpinus*), larval body length interacted in complex ways with skin pigmentation and other morphological features (Eilertsen et al., 2008). These results point to species-specific physiological patterns and selective expression of paternal effects, which can be further elaborated with studying their molecular evidence.

Swim bladder inflation effectiveness (SBIE) offers a particularly vivid example of the need for molecular resolution. Though traditionally scored in binary terms (inflated or not), SBIE is in fact a multifactorial trait involving visual and vestibular sensory cues, neuromuscular coordination, muscular strength, and timely behavioral activation (Peruzzi et al., 2007). In Eurasian perch (*Perca fluviatilis*), the swim bladder initially develops during early larval stages as a physostomous organ connected to the gut via a pneumatic duct, allowing larvae to gulp atmospheric air at the water surface (Jacquemond, 2004). This is essential for initial swim bladder inflation. As development progresses, the pneumatic duct closes, and the swim bladder becomes physoclistous, relying on gas secretion and resorption to regulate buoyancy. While some physoclistous fish species may also gulp air bubbles present in the water column rather than at the surface, in perch, surface air-gulping is the predominant mechanism for first swim bladder inflation (Zaccone et al., 2012). This developmental transition from a transient

physostomous state to a physoclistous condition is characteristic of perch and should be considered when discussing fish swim bladder (Schwebel et al., 2018; Pelster, 2021). Impaired inflation can result from failures in muscle development, phototaxis, swim trajectory, or even timing (Winata et al., 2009). Our results did not reveal SBIE to be strictly governed by the paternal treatment, particularly sperm cryopreservation and post-thaw storage, but there could be a subtle role for sperm-transmitted regulators of early physiology and sensory development. This aligns with studies in zebrafish and other species, where genes like *nkx2.1*, *foxa2*, and *hoxa5*, regulated by Fibroblast growth factor (FGF) and Hedgehog pathways, govern swim bladder morphogenesis (Winata et al., 2009; Cass et al., 2013). Here, by combining phenotype with gene expression profiling can these physiological complexities be disentangled, revealing the contributions of sperm-derived molecular signals to swim bladder development.

Mortality, often considered a blunt but essential phenotype, is also strongly modulated by both parental and environmental factors. For instance, paternal exposure to genotoxicants increased mortality and malformation in offspring of brown trout (*Salmo trutta*) and Arctic charr (Devaux et al., 2011). In guppies (*Poecilia reticulata*), paternal diet influenced offspring body size, a trait closely tied to survival prospects (Evans et al., 2017). In contrast, studies in haddock (*Melanogrammus aeglefinus*) found no measurable paternal effects on survival under starvation, with maternal effects dominating (Rideout et al., 2004). In our Eurasian perch experiments, paternal effects on mortality were striking, aligning with previous findings in ide (*Leuciscus idus*) and northern pike (*Esox lucius*), where paternal identity accounted for a significant portion of embryonic variation, often surpassing maternal contributions (Siddique et al., 2017). These contradictory results highlight that mortality cannot be interpreted as a monolithic trait; it must be dissected into its mechanistic drivers, some of which are paternally regulated at the gene expression level.

Even behavioral and social traits such as cannibalism require a more nuanced framework. In our study, cannibalism did not correlate with paternal treatment, suggesting that it may not be a strongly heritable trait from the male side, or that it is highly context-dependent. Supporting this, previous research in species like African sharptooth catfish (*Clarias gariepinus*) has shown that cannibalism is more responsive to environmental factors, such as food availability, stocking density, and shelter, than to parental genetic background (Hecht & Appelbaum, 1988). Such behavioral traits, while observable and practically important, reveal little about underlying regulatory mechanisms unless paired with transcriptomic or epigenetic data.

In sum, zootechnical traits reflect complex, multifactorial processes that are often species-specific and regulated at multiple biological levels. While decades of aquaculture research have yielded rich phenotypic datasets, these remain incomplete without a mechanistic link to gene regulation. Transcriptomic analysis provides that missing layer, revealing which pathways are altered by sperm origin, treatment, or environmental exposure, and connecting observable phenotypes to their molecular foundations. Only through such integration can we fully understand the developmental outcomes of paternal contributions and harness this knowledge for both basic science and aquaculture innovation.

6.4.2. Transcriptomics as a Molecular Lens

Transcriptomic profiling offers a high-resolution view into the molecular architecture of early development (Vesterlund et al., 2011; Chandhini et al., 2019). By capturing stage-specific gene expression patterns in whole-body larval samples, we were able to detect DEGs associated with paternal treatment (e.g., cryopreservation, PTS, Dom-Wild origin) and refer to these with functional traits. RNA-sequencing at the mouth-opening stage revealed differential expression of genes associated with key developmental and physiological processes. Specifically, we identified 11 DEGs between fresh and cryopreserved sperm, 45 DEGs after post-thaw storage, 20 DEGs between domesticated female × domesticated male (DD) vs domesticated female × wild male crosses (DW), and 5 DEGs between wild female × wild male (WW) vs wild female × domesticated male (WD) crosses. Interestingly, certain genes emerged as potential common regulators across these experimental comparisons (**Figure 6.1**). For example, *pde6g* was shared between the Fresh-Cryo and PTS treatments, *nlrc3* and *trim16* were common to both PTS and DD vs DW groups, and *opn1sw1* was the sole overlapping DEG between the DD vs DW and WW vs WD comparisons. The comparisons include genes involved in visual development and phototransduction, such as *pde6g*, *rbp4l*, and *opn1lw1*; immune function genes like *pigr*, *hlag*, *gimap*, and *mfap*; and genes related to metabolism and stress regulation, including *neo1* and *dnmt3a*. This suggests that parental effects may influence a broad spectrum of biological pathways beyond gross morphology, potentially shaping offspring fitness through molecular regulation. These DEGs are not random background noise; they are highly conserved, developmentally regulated genes whose differential expression aligns with known phenotypic outcomes. The gene *pde6g*, which is upregulated in larvae sired by cryopreserved sperm but downregulated following prolonged post-thaw storage, plays a key role in photoreceptor function and visual signal transduction (Dvir et al., 2010). These expression patterns suggest subtle yet functionally significant impairments that may impact hatchery performance (Lagman

et al., 2016). Similarly, changes in immune-related transcripts in PTS-derived larvae, despite uniform rearing conditions, imply that sperm-transmitted molecules may influence immune system priming during early development. For example, studies on the sex-role reversed pipefish (*Syngnathus typhle*) have demonstrated that paternal immune experience can affect the immune gene expression of offspring (Keller & Roth, 2020). Specifically, grandparental immune challenges led to altered expression of immune-related genes in grandoffspring, suggesting that paternal effects can have lasting impacts on the immune system across generations (Beemelmanns & Roth, 2017). This has profound implications not only for developmental biology but also for aquaculture, where early-life stress resistance is critical for reducing mortality and improving productivity (Vindas et al., 2016).

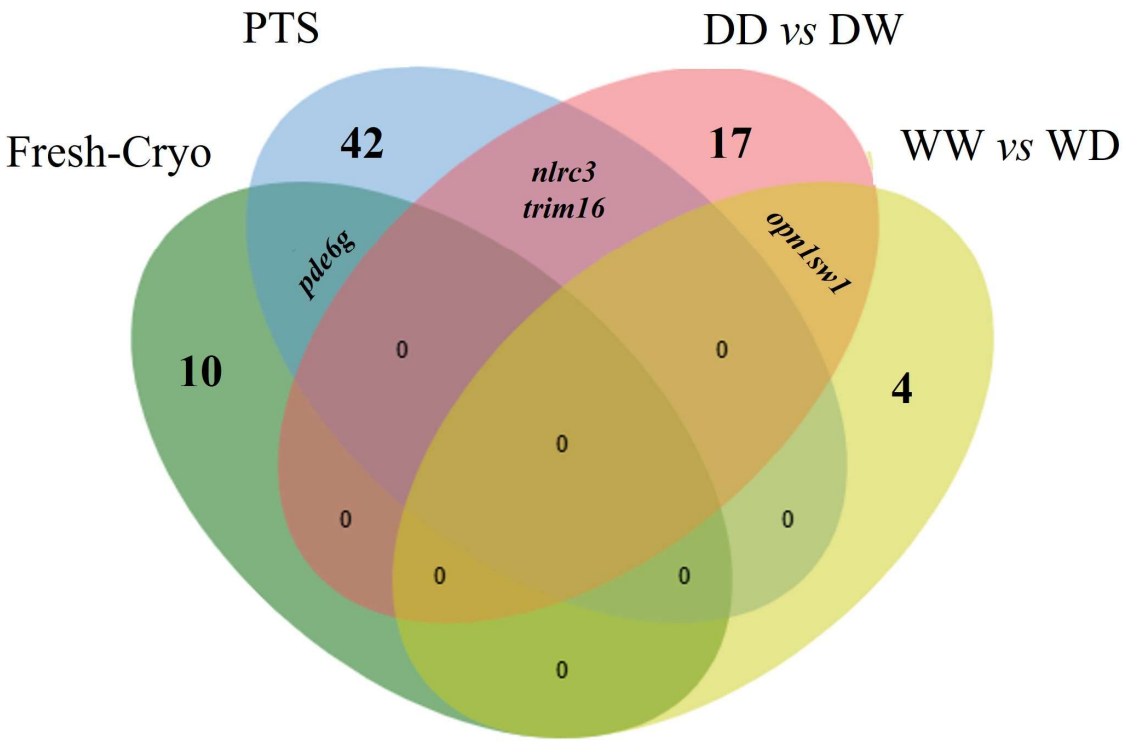

Figure 6.1: *Venn indicating genes common to different experiments (Fresh-Cryo, PTS, DOM-WILD) performed for this thesis. WW- Wild ♀ x Wild ♂; WD- Wild ♀ x Dom ♂; DD – Dom ♀ x Dom ♂; DW – Dom ♀ x Wild ♂*

6.4.3. Linking Molecular and Phenotypic Data: *A Systems Approach*

What makes the present work distinctive is not merely the application of transcriptomic tools, but the explicit integration of molecular and zootechnical data (Davis & Hetzel, 2000).

For instance, the upregulation of the *pde6g* gene, associated with improved visual function, may enhance prey capture efficiency in the progeny, potentially explaining the observed increase in wet body weight (Panda et al., 2024). The *sox2* gene in zebrafish plays a crucial role in the development of the swim bladder as well as sensory systems, including the visual network. Its proper expression is essential for both buoyancy control and sensory function in developing fish larvae (DeOliveira-Mello et al., 2019; Cao et al., 2023). By identifying these links across replicate experiments and experimental models, we strengthen the inference that paternal condition, and by extension, PEGs, play a functional role in shaping early ontogeny.

Using a systems biology perspective can help move beyond descriptive findings and begin to build testable models of inheritance (Jablonka & Noble, 2019). This approach is particularly useful for exploring how transcriptomic changes in sperm might persist across later stages of offspring development (Mohanty et al., 2019). Investigating how these changes interact with environmental factors like diet or temperature may eventually help clarify their relevance for offspring performance and fitness, especially under natural or semi-natural conditions (Johnsson et al., 2014; Larsen et al., 2015).

6.4.4. Implications for Broodstock Management and Breeding

From an applied perspective, the integrated approach used during realization of experiments included in this thesis can revolutionize broodstock management. Traditional criteria for selecting males, like age, body condition, sperm motility etc., may fail to predict trans-generational developmental success. While fertilization capability is often regarded as the primary indicator of sperm quality (Bobe & Labbé, 2010), it does not necessarily guarantee high-quality progeny if the molecular composition of the sperm, such as its RNA, protein, or epigenetic content, is compromised. Thus, successful fertilization alone may not reflect the sperm's full developmental potential or its contribution to offspring fitness. The identification of functional PEGs linked to key traits such as immune competence, visual development, and growth potential suggests that molecular screening should be incorporated into selective breeding programs.

In particular, transcriptomic signatures in early stages of larvae could serve as biomarkers for paternal gamete quality, especially under suboptimal conditions like cryopreservation. For instance, in blue catfish (*Ictalurus furcatus*), researchers employed RNA sequencing to profile gene expression in testicular tissues of males categorized by sperm velocity. This approach led to the identification of specific mRNA transcripts correlated with sperm motility (Wang et al.,

2022; Hess et al., 2024). Similarly, studies on gilthead seabream (*Sparus aurata*) and zebrafish have revealed that the abundance of certain mRNAs in spermatozoa and testicular cells can serve as indicators of male breeder performance (Guerra et al., 2013). In both the species *bdnf* and *kita* transcripts were more abundant in good male breeders or high-quality samples. Further, the goal is to help hatcheries not only preserve genetic diversity but also improve larval robustness and survival of Eurasian perch, and maintain sustainable aquaculture.

6.5. Cryopreservation vs. Wild-Dom Approach: *Methodological Perspectives*

The methodological contrast between fresh-cryopreserved and wild-domesticated origin gametes offers a unique lens through which to examine the interplay of technology, biology, and aquaculture practice. Cryopreservation provides standardized, readily available germplasm, minimizing seasonal or spatial variability but introducing potential risks of reduced viability or altered fertilization dynamics. In contrast, the "wild-dom" approach harnesses freshly collected gametes from wild broodstock, maintaining natural variability and potentially higher immediate performance, yet facing challenges of logistics, disease risk, and sustainability. Evaluating these methods side by side illuminates critical trade-offs in laboratory protocols, handling, and post-thaw assessments, ultimately shaping how reproductive management is approached in both conservation and commercial settings.

6.5.1. Cryopreservation and Post-Thaw Storage: *Mechanistic Precision and Experimental Control*

Standardized protocols for sperm cryopreservation have been developed with potential for high-throughput applications in many species along with zebrafish, a model organism for biomedical research (Yang et al., 2007; Cabrita et al., 2010; Judycka et al., 2019b). Cryopreservation imposes extreme physiological stress on sperm, including osmotic imbalance, reactive oxygen species (ROS) production, membrane destabilization, and protein denaturation (Cabrita et al., 2010; Asturiano et al., 2017). Only a subset of sperm cells with sufficient structural integrity and metabolic robustness can survive this process, especially in species such as Eurasian perch, where high post-thaw sperm motility values were obtained, however post-thaw motility declines within minutes (Bernáth et al., 2015; Judycka et al., 2019a, 2022). This stress response functions as a powerful biological filter, allowing researchers to selectively utilize cryo-resistant sperm for fertilization and observe their functional outcomes.

From a methodological perspective, cryopreservation provides several advantages that make it a powerful tool for studying paternal effects in fish. First, cryopreservation enhances experimental design by allowing sperm from a single male to be stored and used across multiple fertilizations, including at different times or in combination with egg batches. This enables greater control over paternal input and facilitates complex experimental setups that would otherwise be constrained by the availability of fresh gametes (Cabrita et al., 2008). Second, it facilitates the isolation of paternal contributions (Immler, 2018). Studies confirm that sperm cryopreservation when combined with constant maternal inputs and tightly controlled environmental conditions, any phenotypic or molecular differences observed in progeny can be more confidently attributed to variations in the male gamete (Hezavehei et al., 2018; Nusbaumer et al., 2019; Panda et al., 2024). Third, cryopreservation protocols are scalable and can be adapted across diverse fish species, supporting high-throughput applications in both aquaculture and basic developmental biology (Cabrita et al., 2010; Beirão et al., 2019a). Protocols have been established for wide range of freshwater fish species including percids (Judycka, et al., 2019) which facilitate genetic improvement programs and broodstock management in aquaculture (Asturiano et al., 2017). This scalability and standardization are particularly valuable in contexts where large-scale screening of male reproductive traits or gamete-derived molecular signals is required (Beirão, et al., 2019b).

The PTS protocol extends this precision by introducing a quantitative stress gradient, allowing finer discrimination of sperm quality than Fresh-Cryo comparisons. As demonstrated in our study, even brief post-thaw storage (30 min at 4 °C) impaired functional sperm integrity in ways that manifested not only in larval phenotype (e.g., mortality) but also in transcriptomic profiles, particularly among genes related to immune response and sensory development. Together, these approaches constitute a mechanistically interpretable model for PEG discovery.

6.5.2. Wild vs. Domesticated Males: *Ecological Context and Biological Relevance*

By contrast, the wild-domesticated male comparison offers ecological realism and captures the consequences of long-term environmental and selective pressures (Coman et al., 2006). Stressors experienced by males drive physiological and behavioral adaptations that may be encoded epigenetically and carried into the sperm (Schreck et al., 2001). Domesticated males, reared in RAS, experience stable photothermal conditions, controlled nutrition, and selection for traits such as growth, stress tolerance, and tameness (Teletchea & Fontaine, 2014; Fontaine et al., 2019). Wild males, on the other hand, encounter unpredictable temperature fluctuations,

food availability, and predation, leading to different physiological and potentially epigenetic adaptations (Beirão et al., 2019b). This evolutionary divergence is likely to be encoded, at least in part, in the molecular composition of sperm, including RNAs, DNA methylation, histone modifications, and protein content. In our study, larvae obtained from wild and domesticated males showed significant differences in gene expression, especially in pathways related to neural development, metabolism, and environmental responsiveness, suggesting that domestication alters the paternal molecular imprint transmitted during fertilization (Milla et al., 2020).

Despite promising findings from studies comparing wild and domesticated male fish, this approach introduces significant interpretive challenges. First, wild-caught males inherently exhibit greater biological variability than their domesticated counterparts (Carvalho, 1993). This includes differences in genotype, environmental history, and overall health status, which complicate efforts to attribute observed developmental or molecular outcomes in progeny specifically to paternal factors (e.g., Sutter & Immler, 2020; Judycka et al., 2024). Second, the absence of genetic uniformity in above mentioned studies introduces additional complexity in attributing observed effects. Without genetic standardization, it's difficult to discern whether developmental or transcriptomic differences in offspring arise from genomic, epigenomic, or even microbiomic divergence between the males (Lorenzen et al., 2012; Milla et al., 2020). Finally, wild males may harbor latent infections, environmental contaminants, or chronic stress markers that go undetected yet significantly influence sperm quality and the early embryonic environment (Cabrita et al., 2010). These uncontrolled confounders can bias results and undermine causal inference in developmental studies relying solely on wild-vs-domesticated contrasts. Nevertheless, these limitations are balanced by the naturalistic insight the design provides. PEGs revealed through Dom-Wild comparisons may better reflect evolutionarily relevant variation than those exposed through artificial selection pressures. Moreover, this contrast is directly applicable to aquaculture, where the integration of wild germplasm is often considered for enhancing genetic diversity and resilience.

6.5.3. Complementarity of Approaches

Cryopreservation-based methods and wild-domesticated male comparisons should be viewed as complementary approaches, each offering unique yet reinforcing insights into paternal effects and sperm-mediated inheritance. Cryopreservation and post-thaw storage provide highly controlled, mechanistically interpretable conditions under which paternal

gamete integrity can be perturbed and its developmental consequences observed. These approaches allow researchers to isolate functional aspects of sperm quality, such as resistance to oxidative and osmotic stress, and link them to specific changes in offspring phenotype and gene expression (Cabrita et al., 2014). Whereas, wild versus domesticated male comparisons introduce a broader ecological and evolutionary context, capturing the consequences of long-term environmental conditioning, artificial selection, and captive rearing on sperm-mediated inheritance. These males differ not only in environmental history but also in stress physiology, epigenetic landscape, and reproductive timing (Teletchea & Fontaine, 2014; Fontaine et al., 2019). Larvae sired by these males displayed distinct transcriptomic profiles, especially in genes related to metabolism and neurodevelopment (Eachus et al., 2021). These patterns parallel observations in domesticated salmonids, where hatchery-induced methylation marks have been shown to be stably inherited (Le Luyer et al., 2017; Gavery et al., 2018). Thus, while cryopreservation enables reductionist precision, the wild-domesticated contrast offers biological realism, highlighting how paternal effects unfold across an evolutionary continuum. Taken together, these experimental systems allow for cross-validation of candidate PEGs and support a hierarchical hypothesis-building framework in which mechanistic insights derived from controlled sperm manipulation can be tested and contextualized within ecologically relevant variation.

6.6. The Mother as a Gatekeeper of Paternal Effects

Although the focus of this thesis has been the exploration of paternal contributions to early development in Eurasian perch, the data and experimental outcomes point to a broader interpretive framework in which maternal influence cannot be ignored. Indeed, while our designs are meticulously controlled for maternal identity to isolate paternal effects, the observed outcomes strongly suggest that the mother serves not only as a contributor to early embryonic development but also as a crucial modulator, or gatekeeper, of paternal influence. This idea aligns with emerging views in developmental biology and epigenetics, where parental effects are not merely additive or parallel but instead interact in complex, context-dependent ways to shape the phenotype of the offspring (Donelson et al., 2008; Jorgensen et al., 2011). Despite the maternal dominance in early gene expression, our analyses revealed experiment-specific transcriptomic shifts in larvae, particularly in response to sperm condition (e.g., cryopreservation, post-thaw storage) and male origin (wild vs. domesticated). This confirms that paternal factors do influence the larval transcriptome in a detectable and reproducible way (Debernardis et al., 2025).

In externally fertilizing fish, maternal provisioning of the egg has traditionally been considered the dominant factor affecting early life history traits (Armstrong & Nislow, 2006). The oocyte supplies not only the maternal genome but also the entire cytoplasmic apparatus necessary to support zygotic development until embryonic genome activation (EGA) begins (Lubzens et al., 2016). Maternal RNAs, proteins, hormones, and organelles establish the intracellular environment in which sperm-derived contributions must function (Jensen et al., 2014). This environment determines the efficiency with which the sperm's nuclear material is decondensed, its epigenetic marks are interpreted or reprogrammed, and its RNA cargo is either preserved, degraded, or translated. Thus, even under standardized fertilization protocols, the maternal cytoplasm is an active participant in interpreting paternal inputs (Kojima et al., 2025). The evidence from our PTS and Dom-Wild experiments supports this notion. Despite having controlled for sperm treatments and environmental rearing conditions, larval performance outcomes often varied not just by paternal treatment but also between families sharing the same male treatment. This suggests that inter-female variation in ooplasm composition may have influenced how sperm-derived information was processed (Waghmare et al., 2021). This could have led to differences in gene expression and phenotype that could not be solely attributed to paternal origin. While the design of the PTS study minimized this variation by using consistent female partners across treatments, minor fluctuations in maternal RNA profiles or cytoplasmic factors may still have impacted the developmental potential of the sperm used.

The concept of maternal buffering or amplification of paternal effects is increasingly recognized in other taxa (Champagne, 2020). In mammals, oocyte factors such as histone chaperones and reprogramming enzymes influence whether sperm-derived epigenetic marks and RNAs are retained or reprogrammed during early embryogenesis (Rando, 2012; Zhou & Dean, 2015; Labbé et al., 2017). In fish, studies in zebrafish and medaka have shown that the timing and nature of epigenetic reprogramming vary by species, with zebrafish embryos maintaining more of the paternal methylome through early cleavage stages (Wang & Bhandari, 2020, 2024). These differences suggest that the maternal machinery not only contributes its own set of regulatory inputs but also exerts control over the interpretation of paternal epigenetic and transcriptomic content.

In our Dom-Wild study, the use of a shared maternal background was essential to isolate paternal effects, yet the observed gene expression differences in offspring sired by wild versus domesticated males may still have been shaped by maternal responsiveness to sperm-origin differences. For instance, certain paternal molecules, particularly those differing as a function

of domestication, may have provoked variable maternal responses during fertilization or zygotic activation, potentially altering the regulatory environment in subtle but biologically meaningful ways. The maternal ooplasm could act as a sensor or filter, selectively stabilizing or repairing paternal RNAs or proteins depending on their sequence, modification state, or evolutionary compatibility (Fernández-Díez et al., 2016; Dey et al., 2023; Schumacher et al., 2023). This possibility is especially relevant given the evolutionary divergence between wild and domesticated stocks, where incompatibilities in parental epigenetic or molecular landscapes could lead to complex developmental outcomes.

Moreover, the timing of EGA in teleosts, which typically occurs around the mid-blastula transition, marks a critical window during which interactions between maternal and paternal transcripts are most dynamic (Ing-Simmons et al., 2022). Prior to EGA, maternal RNAs drive development, but the transition to zygotic control depends on the coordinated integration of signals from both parents (Schulz & Harrison, 2018). The findings from our transcriptomic analyses, which captured gene expression just as the larvae reached the mouth-opening stage, suggest that early paternal effects, conveyed via sperm-derived RNAs or epigenetic signals, must have acted prior to or during this transition. Their persistence into later developmental stages implies that maternal factors either preserved these inputs or failed to fully erase them, reinforcing the concept of maternal permissiveness or constraint as a shaping force in paternal-effect gene expression.

Taken together, these findings suggest that maternal contributions extend beyond their own roles in developmental programming (Mashoodh et al., 2018). They may also modulate paternal influence through cytoplasmic interactions, selective molecular degradation or stabilization, and epigenetic reprogramming mechanisms (Curley et al., 2011). Understanding these interactions is essential for a complete model of inheritance and development, particularly in systems such as externally fertilizing fish, where both parents contribute their effects at a single, synchronized moment: fertilization. Future studies in this area could benefit from reciprocal crossing designs, where oocytes from multiple maternal lines are fertilized with identical sperm treatments, allowing for a deeper examination of maternal modulation effects. In addition, profiling of maternal RNA pools and cytoplasmic components in parallel with paternal transcriptomic and epigenetic data could help clarify how these two sources of information converge, or conflict, during early development. Such work would not only advance our understanding of teleost reproduction but also enrich broader theories of parental interaction and transgenerational inheritance.

Chapter 7

Conclusions

This thesis set out to explore the molecular and developmental consequences of paternal contributions in Eurasian perch, with a particular focus on identifying paternal-effect genes (PEGs) and understanding how sperm condition and paternal origin influence early larval outcomes. Through a series of controlled experiments, including cryopreservation, post-thaw sperm storage, and wild versus domesticated male comparisons, it was shown that the father's role in early development extends well beyond the transmission of nuclear DNA. The integration of zootechnical assessments with transcriptomic profiling was instrumental in revealing this complexity. Conventional phenotypic metrics such as growth, mortality, and swim bladder inflation provided visible evidence of developmental divergence across paternal treatments. However, it was through molecular analysis that specific genes, such as *pde6g*, *opn1lw1*, *nlrc* and *trim16* were repeatedly implicated in key developmental pathways, including sensory development, immune regulation, and stress response. The use of sperm cryopreservation and post-thaw storage as controlled stressors provided a novel and reproducible platform for probing the functional consequences of sperm quality. These methods enabled the identification of PEGs under well-defined, mechanistically interpretable conditions. The comparison of wild and domesticated males further underscored that paternal molecular influences are ecologically relevant, shaped by environmental history and long-term domestication pressures. Crucially, this work also revealed that the maternal cytoplasm plays a regulatory role in modulating paternal effects. Even under standardized female conditions, variation in embryonic and larval outcomes suggests that maternal factors influence the stability and interpretation of sperm-derived signals, highlighting an underappreciated maternal gatekeeping role. Taken together, these results support a multifactorial and bidirectional model of inheritance in fish, wherein both parents contribute a suite of genetic and non-genetic factors that guide early embryogenesis. The findings affirm that paternal contributions are molecularly active and functionally consequential from the earliest stages of life.

Chapter 8

Implications and recommendations

8.1 Implications

From a theoretical standpoint, this thesis challenges traditional, genome-centric views of inheritance by demonstrating that sperm carries more than just DNA, it carries regulatory signals capable of influencing gene expression, cellular behavior, and phenotype in the offspring. This contributes to the expanding field of non-genetic inheritance and highlights the need for evolutionary and developmental models that account for epigenetic and transcriptomic paternal inputs. The repeated identification of PEGs in a non-model vertebrate like Eurasian perch opens new avenues for comparative and functional genomics in fish and beyond. It raises compelling questions about how environmental cues, including domestication and cryopreservation, become biologically encoded in gametes and transmitted across generations. From an applied perspective, the findings have direct relevance to aquaculture. The ability to detect and interpret paternal molecular signals offers a potential toolkit for improving broodstock selection, optimizing sperm cryopreservation protocols, and enhancing offspring quality through more informed reproductive management. For instance, transcriptomic or epigenetic markers associated with sperm quality could be integrated into routine assessments, thereby increasing the efficiency, sustainability, and robustness of breeding programs. Moreover, the study reinforces the importance of including both parental origins in the evaluation of reproductive performance, especially as domestication continues to shape the biology of cultured species. High feeding rates observed in DD groups compared to DW groups, for instance, suggest emerging domestication effects mediated via the paternal line, reinforcing the value of paternal phenotype and history in broodstock decisions. Ultimately, this work calls for a paradigm shift in how we conceptualize inheritance and reproduction in fish: not as a unidirectional flow of genetic information, but as a molecular conversation between gametes, shaped by history, environment, and biological context.

8.2 Broader Significance and Future Directions

The findings of this thesis extend beyond the specific context of Eurasian perch to raise broader questions about the nature of inheritance, developmental regulation, and the emerging role of paternal factors in shaping offspring phenotype. By identifying PEGs that influence

early larval performance and by demonstrating their modulation via sperm manipulation and paternal origin, this work contributes to a growing shift in developmental biology, one that emphasizes bidirectional parental influence and recognizes that sperm can serve as a vector for heritable information far richer than a haploid genome. From a scientific perspective, this research supports and expands on the conceptual framework of non-genetic inheritance. NGI encompasses a wide array of phenomena, epigenetic modifications, parental RNA transfer, gametic imprinting, and cytoplasmic inheritance, that allow phenotypic traits and developmental trajectories to be influenced by the experience and condition of the parents, independently of changes in DNA sequence (Bohacek & Mansuy, 2015; Bonduriansky & Day, 2009). While NGI has been widely documented in mammals and model invertebrates, its presence and significance in fish, especially in economically important but genetically complex species such as Eurasian perch, have been less thoroughly explored. The results presented here help to fill this gap, offering empirical evidence that epigenetic and transcriptomic variation in sperm can influence the gene expression and performance of offspring in teleosts.

One of the most compelling outcomes of this work is the consistent identification of genes, such as *pde6g*, *pigr*, and *gimap*, whose expression is altered in progeny depending on the condition or identity of the paternal sperm. These genes are not abstract markers; they play crucial roles in processes such as vision, immune function, and developmental timing. Their altered expression in response to cryopreservation stress, and post-thaw storage suggests that PEGs are functionally meaningful and responsive to paternal physiological state, rather than being fixed or randomly distributed traits.

Moreover, the results point to the potential application of PEG research in aquaculture and fisheries management. Current methods for evaluating male broodstock quality often emphasize sperm motility, fertilization success, and morphological traits, which, while useful, provide only a limited picture of the sperm's developmental competence. The discovery that specific gene expression signatures in offspring can reflect paternal sperm condition introduces the possibility of developing molecular diagnostics for male fertility and offspring viability. For example, early detection of downregulated *pde6g* or altered immune-related gene expression in larvae could serve as a proxy for compromised sperm integrity, allowing hatchery managers to make informed decisions about broodstock use or cryopreservation protocol optimization.

In addition to these practical implications, the results also suggest directions for future fundamental research. One clear avenue is the incorporation of epigenomic profiling into PEG

studies. While transcriptomic data revealed functional consequences in the offspring, the precise molecular carriers, whether small ncRNAs, histone modifications, or methylation patterns, remain to be identified. Technologies such as bisulfite sequencing for DNA methylation, ChIP-seq for histone marks, or small RNA-seq could help map the epigenetic features of sperm under different treatment regimens and trace their persistence in embryos and larvae.

Another important direction involves functional validation of candidate PEGs. Although correlational data suggest strong involvement of genes like *pde6g* in paternal effects, definitive proof requires experimental manipulation. The development of gene editing technologies in fish, such as CRISPR-Cas9, offers the possibility of knocking out or modifying suspected PEGs in embryos to directly test their role in developmental processes and survival. Such work would provide causal evidence for the functions inferred here and deepen our understanding of how non-genetic inheritance operates at the molecular level. It is also worth considering the ecological and evolutionary relevance of PEGs. The observation that wild and domesticated males transmit different regulatory signals to their offspring raises questions about the evolution of paternal contributions in response to environmental conditions. If sperm-transmitted signals are shaped by a male's experience, health, or ecological niche, they may serve as vehicles for rapid adaptive responses across generations, potentially influencing population dynamics, stress resilience, or fitness in fluctuating environments. In this light, PEGs represent not only a mechanistic phenomenon but also a bridge between environmental plasticity and evolutionary change.

Finally, this research opens conceptual space for the development of more holistic models of inheritance. The classical genetic paradigm, while foundational, is insufficient to fully explain the intergenerational transfer of complex traits and the early programming of developmental trajectories. The integration of paternal non-genetic effects with maternal provisioning, environmental conditions, and zygotic regulation offers a richer and more accurate depiction of early life biology. Such models have the potential to unify disciplines as diverse as reproductive physiology, developmental genomics, evolutionary biology, and aquaculture science.

In summary, the broader significance of this thesis lies not only in identifying PEGs in Eurasian perch but also in demonstrating how paternal contributions, previously underestimated, can be measured, manipulated, and linked to meaningful outcomes in

offspring. It affirms the value of combining transcriptomic data with phenotypic traits, highlights the promise of sperm manipulation as an experimental and diagnostic tool, and contributes to a growing recognition that inheritance is a dynamic, multi-layered process shaped by the interactions of both parents and their environments.

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Supplementary files

Supplementary table 3.1: *Physiological details collected during the experiment.*

Table s3.1a: *Broodstock characteristics used for the controlled reproduction.*

	Population	Date of spawning	W(fish) in g	W(ribbon) in g	eggs count	L (total) in cms	L (caudal) in cms	Age (in years)
Female 1	Mikołajki	20.4.22	382	60	607	28.7	26	4+ (~5)
Male 1	Pond	20.4.22	111			27.4	24	5+ (~6)
Male 2	Pond	20.4.22	148			22.5	17.8	4+ (~5)
Female 2	Mikołajki	20.4.22	341	70	522	29.6	26.8	5+ (~6)
Male 3	Pond	20.4.22	132			21.2	18.4	4+ (~5)
Male 4	Pond	20.4.22	96			20	17.4	3+ (~4)
Female 3	Mikołajki	21.4.22	457	110	442	35	28.9	5+ (~6)
Male 5	Pond	21.4.22	120			20.5	17.6	4+ (~5)
Male 6	Pond	21.4.22	249			26.4	23.4	4+ (~5)

Table s3.1b: *Concentration and motility parameters of milt used for the experiment.*

	Concentration of fresh sperm (in billions)	Motility %		Linearity %		ALH (µm-1)		VAP (µms-1)		VCL (µm-1)		VSL (µms-1)	
		Fresh	Cryo	Fresh	Cryo	Fresh	Cryo	Fresh	Cryo	Fresh	Cryo	Fresh	Cryo
Male 1	22.1	93.8	91	71.9	73.2	12.9	8.0	239.4	169.0	269.7	187.2	195.2	142.3
Male 2	20.2	96.6	77.6	69.7	80.6	11.4	5.7	218.5	158.2	248.3	176.0	175.8	145.6
Male 3	19.2	95.5	81.5	71.1	77.7	11.6	7.1	217.1	169.3	243.9	188.4	178.7	150.6
Male 4	19.4	87.4	83.2	81.3	74.5	9.8	6.7	236.8	157.4	252.5	180.1	207.6	138.5
Male 6	26.9	93.5	83.6	76.1	83.8	10.9	6.1	246.9	182.9	267.9	195.3	205.4	168.2

Table s3.1c: *Fertilization and developmental rates of embryos.*

Fertilization rates	Fresh Avg		Cryo avg	
	% PRE MBT	% POST MBT	% PRE MBT	% POST MBT
Family 1	85.90814213	83.38405797	79.24857462	77.79218679
Family 2	89.25286336	86.92357065	80.65815626	79.24119768
Family 3	81.93084693	81.06979268	56.03459051	70.97637795
Family 4	87.73463536	93.1082743	80.43892693	79.6001805
Family 6	91.64303889	94.23893655	87.62023002	80.40637128

Table s3.1d: *Deformity rates of larvae upon hatching.*

Deformed larvae after hatching (%)		
	Fresh	**Cryo**
Family 1	3.7	6.8
Family 2	5.4	3.7
Family 3	7.5	1.7
Family 4	7.1	7.1
Family 6	9.1	9.2

Table s3.1e: *Swim bladder inflation effectiveness on DPH 11.*

Swim Bladder Inflation Effectiveness (%)		
	Fresh	**Cryo**
Family 1	19.13	14.87
Family 2	23.15	21.71
Family 3	15.63	18.17
Family 4	22.26	18.08
Family 6	9.65	9.67

Table s3.1f: *Length of larvae at mouth opening and weaning stages.*

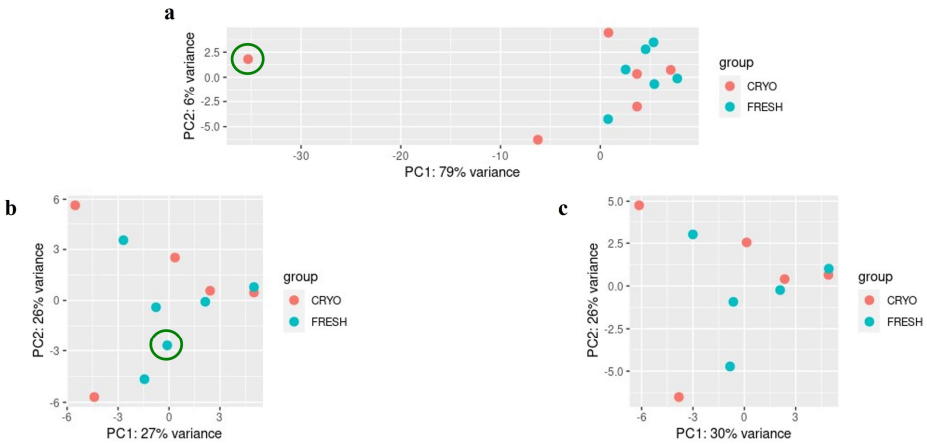
Length of larvae after hatching and at weaning stages (in mm)				
	Mouth opening		**End sampling**	
	Fresh	**Cryo**	**Fresh**	**Cryo**
Family 1	5.8	5.7	12.2	12.2
Family 2	6.0	5.8	12.0	12.6
Family 3	5.9	5.9	12.4	12.9
Family 4	6.1	6.0	12.1	13.0
Family 6	5.9	5.9	12.0	11.7

Table s3.1g: *Weights of larvae at mouth opening and weaning stages.*

Weights of larvae after hatching and at weaning stages (in mg)				
	Mouth opening		**End sampling**	
	Fresh	**Cryo**	**Fresh**	**Cryo**
Family 1	1.0	1.0	13.0	16.8
Family 2	0.9	1.1	13.4	17.5
Family 3	1.0	1.0	15.0	18.7
Family 4	1.1	1.1	13.1	15.4
Family 6	1.0	1.0	10.4	12.1

Table s3.1h: *Cumulative motality of larvae during the entire rearing period.*

	FRESH GROUP		CRYO GROUP	
	Cumulative mortality	**non cumulative stdev**	**Cumulative mortality**	**non cumulative stdev**
DPH 2	4.549	0.013	5.696	0.021
DPH 3	3.976	0.016	5.170	0.015
DPH 4	7.490	0.025	9.070	0.016
DPH 5	9.474	0.032	11.014	0.015
DPH 6	10.600	0.029	12.009	0.007
DPH 7	13.765	0.031	15.603	0.013
DPH 8	16.169	0.042	17.802	0.011
DPH 9	27.124	0.113	24.772	0.040
RESTOCKING				
	Cumulative mortality	**non cumulative stdev**	**Cumulative mortality**	**non cumulative stdev**
DPH 10	30.619	0.109	22.862	0.041
DPH 11	17.938	0.140	17.013	0.070
DPH 12	17.738	0.170	16.345	0.022
DPH 13	31.930	0.366	17.650	0.085
DPH 14	9.756	0.365	11.021	0.017
DPH 15	8.705	0.368	12.485	0.038
DPH 16	6.396	0.364	7.127	0.020
DPH 17	9.219	0.361	11.537	0.032



Supplementary figure s3.1: *Principal component analysis (PCA) of transcriptomic profile of the larvae obtained using either fresh or cryopreserved sperm. a: PCA plot of all the samples/families (n=12) used in the study. Data point encircled in green has been identified as an outlier family from the Cryo group. b: PCA plot of 11 samples used in the transcriptomic analysis after removal of the outlier. Encircled (in green) data point indicates family which has been later removed from the data set as it constituted counterpart to the outlier (from panel a). c: PCA plot of the final data set used in the analysis of current study (n=10).*

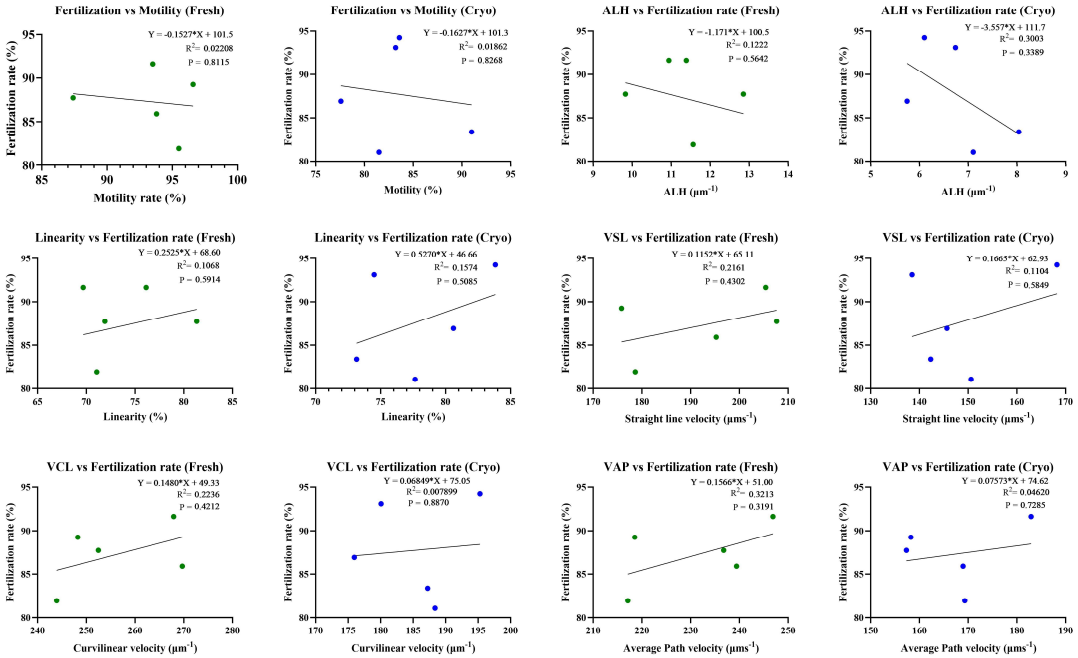


Fig. s2: Linear regression analysis between sperm motility parameters and fertilization rate.

Supplementary figure s3.2: *Linear regression analysis between sperm motility parameters and fertilization rates.*

Supplementary table s3.2: *Primers designed for RT qPCR for the experiment.*

Gene name	Sequences	Amplicon length (bp)
crystallin, beta A2b [cryba2b]	F: ACAAGATCCGCTCCATCAAG	167
	R: GATGGGTCTGAAGGAAAGCA	
crystallin beta A4 [cryba4]	F: GCTATGAGCACGCCTCCTAC	173
	R: CTCACGCTCGTAGATGGTCA	
crystallin beta B1 [crybb1]	F: CATGATGTTCGACCAGGAGA	153
	R: TCCCCACGGAAGTTAGTCTG	
crystallin, gamma MX, like 2 [crygmxl2]	F: TAACTGCTGCAACTCCATGC	134
	R: AGTTGTTGAAGCCCATCCAG	
retinal cone rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit gamma-like [pde6g]	F: AGACCGGACACAAACTGACC	140
	R: GGTCCTCTGCTTGAACTTGG	
red-sensitive opsin-like [opn1lw1]	F: CCAGGCGGTACAATGAAGAT	105
	R: GCGGAGCAATGTGGTAATTT	
gamma-crystallin M2-like [gamma M2]	F: GGGCAACCAGTACTTCCTGA	188
	R: CCATGACGTTGTCACAGTCC	
beta-crystallin A1-like [cryba1]	F: TACAGCGGTTCCCTCTCCTA	218
	R: AGCCAACTTCAGGCATCATC	
gamma-crystallin M3-like [crygm3]	F: GGAGAACTTCGGTGGTCAGA	138
	R: CCTCTGTAGTTGGGCTGCTC	
retinol binding protein 4, like [rbp4l]	F: TTTGACCCCAAGAGGTATGC	165
	R: ACACAACCCAGAAGCCAAAC	
transforming growth factor beta induced [tgfbi]	F: CTGAAGGAGCGTCTGTCCTC	146
	R: AAACGTCCGGTCTTATCGTG	
tetraspanin 7 [tspan7]	F: CACCAACTGCTCACCAGAGA	179
	R: ACAAGCAGCAGGACAGGAAT	
cytochrome c-like, transcript variant X1 [cycs]	F: TGTGGAGAATGGAGGAAAGC	124
	R: ATTCCAGACAATGCCTTTGC	
ER membrane protein complex subunit 10, transcript variant X2 [emc10]	F: GCCCAGCGTCTCACTAACTC	172
	R: GGCTCTGACAAATGCTGTGA	
pre-mRNA-splicing factor [syf2]	F: GGAAGTTGTGGAGGAGGACA	167
	R: CTGCATCGTCAGCAGTGATT	
ER membrane protein complex subunit 3-like [emc3]	F: AACTGGGCCTTCTCTGGATT	150
	R: CCCAAACACGTTGAGGAAGT	

Supplementary figure s3.3: *Expression values, measured in TPMs and expressed as log fold changes, of differentially expressed genes (DEGs) identified in larvae of the Eurasian perch, obtained using either cryopreserved or fresh sperm, across various tissues in evolutionarily distinct fish species.* ***Due to large size of the file, the figure(s) can be accesed following https://doi.org/10.1038/s41598-024-56971-w***

Supplementary table s4.1: *Physiological data of broodstock used in this experiment.*

	Population	WBW (fish) in g	Weight (ribbon) in g	eggs/ gram	TL (total) in cm	TL (caudal) in cm	Sperm concentration (×10⁶/mL)	Sperm viability (%)
Female 1	Mikołajskie	545.7	184		35.5	31.1		
Male a	Flow-through system	62.4			16.6	13.8	21.8	80.0
Male b	Flow-through system	61.3			17.8	15.8	23.4	86.2
Female 2	Mikołajskie	543.4	183.5		36	31		
Male c	Flow-through system	66.4		500	18.2	15.5	28.7	83.0
Male d	Flow-through system	46.5			16	13.8	25.8	87.6
Female 3	Mikołajskie	647.4	197.5		37	31		
Male e	Flow-through system	43			16	13.8	20.4	87.7
Male f	Flow-through system	34.3			14.5	12.5	22.1	80.6

Supplementary table s4.2: *Genes grouped by functional categories defined by high-level GO terms on ShinyGO platform. Ge SX, Jung D & Yao R, Bioinformatics 36:2628–2629, 2020* ***Due to large size of the file, the tables can be provided upon request.***

Supplementary table s4.3: *Transcript ID obtained from NCBi database; abbreviations used to denote the gene; full name of the gene in Eurasian perch; and Presence of genes in unfertilized eggs (UFE) in amount of >1TPM is marked as Y referring to presence and N refers to absence of the gene.* ***Due to large size of the file, the tables can be provided upon request.***

Supplementary table s4.4: *Primers designed for mfap gene. The underlined sequences represent forward primers while the reverse primers are not underlined.*

```
CLUSTAL O (1.2.4) multiple sequence alignment

XM_039788849.1      ------------------------------------------------------------          0
XM_039813507.1      ------------------------------------------------------------          0
XM_039815040.1      ATATATGTAGTAGGGGGTCCCTGCTCGGTCTCTGTTTCAGTTAAGGGGTTCTTGGTTTAA          60
XM_039781790.1      ------------------------------------------------------------          0

XM_039788849.1      ------------------------------------------------------------          0
XM_039813507.1      ------------------------------------------------------------          0
XM_039815040.1      AAAAACATTGAAGACCTCTGCTTTACTCCAGATTACAAAGTTGTCTCTTTGGTCCATTAA          120
XM_039781790.1      ------------------------------------------------------------          0

XM_039788849.1      ----------------------CGCGGTGTGTCAGAGTTGT--CCCTCCATGGCAAATCT          36
XM_039813507.1      ------------------------------------------------------------          0
XM_039815040.1      TGGAGGCGAGTCCCCAAAAAGTGCTGGCGTGTCAGATTTGGGTGCCAACATTGTGTAAGC          180
XM_039781790.1      ------------------------------------------------------------          0

XM_039788849.1      CTGC-GCCTGCAGCTCCAGCTGTCAGAAGGTATGGCCAATCATTTTCCTTTGAAAAGG--          93
XM_039813507.1      ------------------------------------------------------------          0
XM_039815040.1      CAATGGCTGGCAGACACACACTCAAGGACATGCTTCCACCCGTTTATAGTGTTATCGGTG          240
XM_039781790.1      ------------------------------------------------------------          0
XM_039788849.1      TGTTGATCAGCTGCCAGCAGCTCATTCTGCCGGTGGACTGCAGTGAGATTCATAATAATG          466
XM_039813507.1      TGTTGAGCAGCTGCCATAAGCTCTTTCTGCCGTTTGACTGCAGTGACATTTACCGCAATT          227
XM_039815040.1      TGTTGACCAGCTGCCTGCCGCTCATTCTGCCGGGAGACTGCAGTGACATTTACCACAATG          649
XM_039781790.1      TGTTGACCAGCTGCCTGCCGCTCATTCTGCCGGTAGACTGCGGTGACATTTACCACAATG          165
                    ****** ********    **** ********   ****** **** *** *    ***
XM_039788849.1      ACAACAGCCGCCCCAGTGGAGTGTACACCATCTATCCCATTGGAGCCACGTCTGCTGTCC          526
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XM_039815040.1      ACAACAGCCGACCCAGTGGAGTATACACTATCTATCCCATCGGAGCCACGTCTGCTGTCC          709
XM_039781790.1      ACAACAGCCGACCCAGTGGAGTGTACACCATCTATCCCATCGGAGCCACGTCTGCTGTCC          225
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XM_039788849.1      AGGTGTACTGTGACATGGGTTCACAAGGAGGACGGTGGACGGTGTTCCAGAGGAGGATGG          586
XM_039813507.1      AGGTGTACTGTGACATGAATTCAGAAGGAGGACGGTGGACGGTGATGCAGAGGAGGATGG          347
XM_039815040.1      AGGTGTACTGTGACATGGTCTCAGAAGGAGGACGGTGGACGGTGTTCCAGAAGAGGATGG          769
XM_039781790.1      AGGTGTACTGTGACATGGTCTCAGAAGGAGGACAGTGGACGGTGTTCCAGAGGAGGATGG          285
                    *****************   *** ********* ********** * **** ********
XM_039788849.1      AGACCAAATAAC---AACGAAAATTTCGGTGCCTGACAATTTTTTTTCAGAAGACACGCT          1338
XM_039813507.1      CCAGTTGGGCGGCTGCAGCAGGAAAGTAGGG-CCCATAATCTTTAAATGGAAAAA--CAT          1121
XM_039815040.1      AT---AAATAAATAAGCTGAACATTAA----------------------------------          1503
XM_039781790.1      CCAGTTTATCTGTACGGTGACA----------------------------------------          1014
                                          *
XM_039788849.1      GTACGGGGCACTAAGTTACCGTTAGCTAGTAGCTGTATTAAACCCAATGGTTAAATTGCT          1398
XM_039813507.1      ATATAGT-CTGATTGGTAGTGTTAATCACGGC-----------------ATGAACTGAT          1162
XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014

XM_039788849.1      AACAGCTAACGTTAAGCGGTGTAAAGTGTGACTGTATTATCCTGTAGAGGATCCCGACAC          1458
XM_039813507.1      AACACAAGTCTAAAAGAGAGAAAGAAGTAAAGAAAATGAAAATGAAAAGTATAAAAAGA-          1221
XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014

XM_039788849.1      CGGGACGTAACAGTCTGTCTGCAGCCGCCGCTGTCGGAAAAACAACACAGACGGTGCGTT          1518
XM_039813507.1      ---------------TCTTTGCAGTTCAGGACATC---------CAGTTGACGTTTGGTG          1257
XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014
XM_039788849.1      TTATTTACATTATAAATATATTATATATATAGTATTTCAGTTCAGGCACCGTTTAGGAAC          1818
XM_039813507.1      ------------------------------------------------------------          1262
XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014

XM_039788849.1      CAGCACCATTTTAAAAGTATCGTTTTAGCACCGGTATCGGAAAAAACCCAAAAGATACCC          1878
XM_039813507.1      ------------------------------------------------------------          1262
XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014

XM_039788849.1      AACCCTACTTGTTACAGTCTGTGGTGGTTTAAATCAGTCTCAGCTCGCTGCAACGTGGAA          1938
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XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014

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XM_039815040.1      ------------------------------------------------------------          1503
XM_039781790.1      ------------------------------------------------------------          1014
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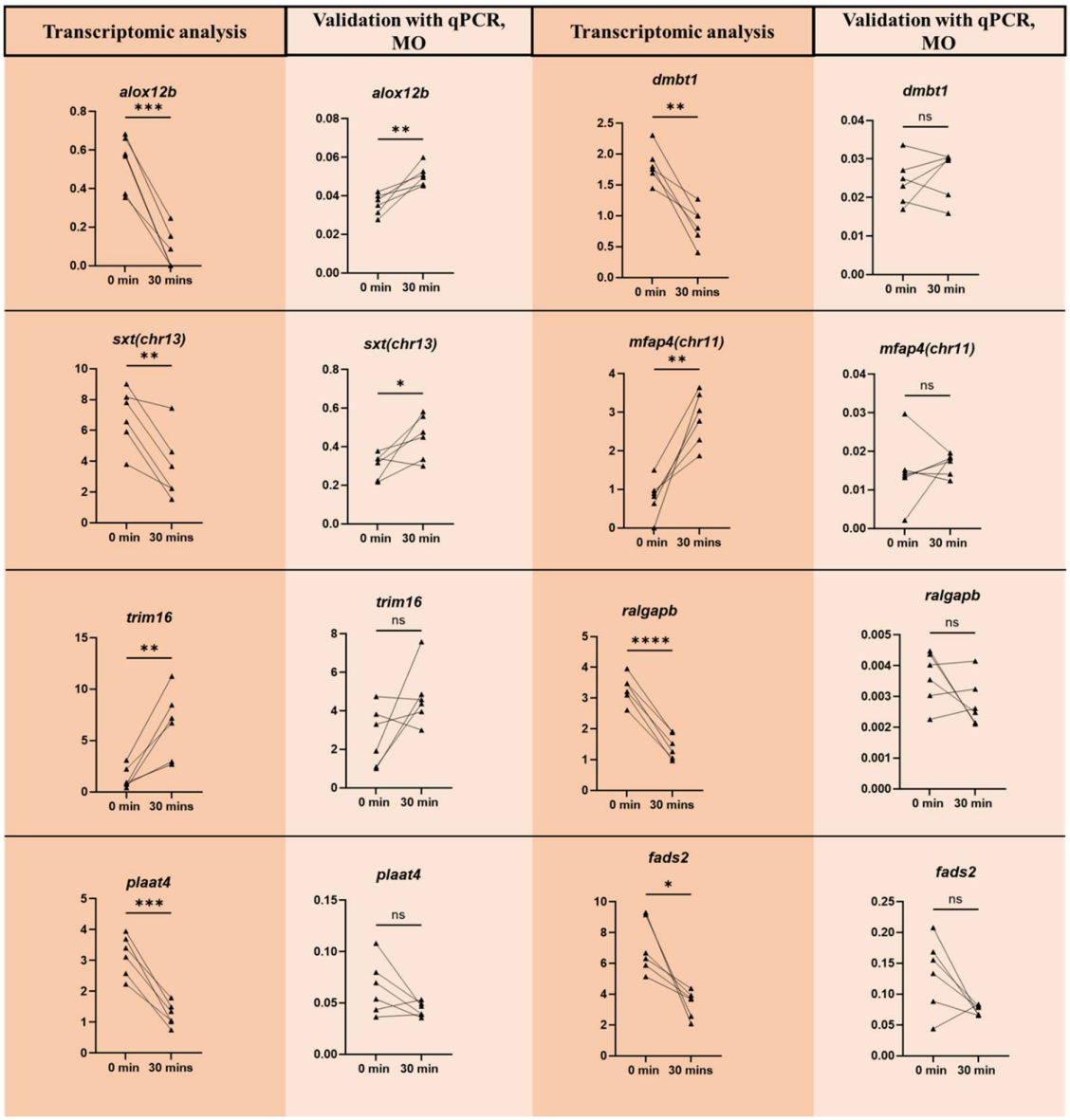
mfap4(chr11): XM_039815040.1	*mfap4*(chr18): XM_039781790.1



Supplementary figure s4.5: *Genes that were not positively validated.*

Suplementary table 5.1: *Broodstock characteristics for both wild and domesticated individuals and milt evaluation with CASA after cryopreservation. WBW: Wet Body Weight, LT: Total Length; W: Weight; DOM: Domesticated.*

MALES									
WILD	**Wight body weight (g)**	**Total length (cm)**	**Concentration of fresh semen (x 10^9)**	**Motility (%)**	**Linearity (%)**	**Amplitude of lateral head displacement (μm^{-1})**	**Average path velocity (μms^{-1})**	**Curvilinear velocity (μm^{-1})**	**Straight line velocity (μms^{-1})**
Male 1	170	23.4	30.9	78.4	80.7	6.0	172.2	187.5	155.9
Male 2	178	24.7	27.5	76.0	77.0	7.5	175.2	192.2	152.8
Male 3	335	31.2	27.7	79.6	75.1	8.3	188.0	203.5	162.3
Male 4	324	31.1	27.9	79.9	73.9	8.1	175.4	190.5	147.6
Male 5	190	25.2	21.2	77.3	72.4	9.9	187.2	204.0	151.9
Male 6	129	22.8	32.8	80.5	73.5	10.4	205.1	223.5	169.7
DOM									
Male 1	666	355	26.4	82.4	75.9	5.7	134.1	157.9	123.2
Male 2	467	337	25.9	69.6	68.5	4.8	105.9	137.0	97.2
Male 3	468	340	34.2	73.9	72.1	4.8	115.8	144.5	105.6
Male 4	450	335	29.2	87.0	75.0	5.7	134.1	160.2	121.9
Male 5	491	329	28.3	73.5	68.2	5.0	116.2	145.1	105.1
Male 6	399	319	37.5	85.6	56.4	5.3	78.4	120.6	66.8

FEMALES				
WILD	**Wight body weight (g)**	**Total length (cm)**	**Weight of ribbon (g)**	**Eggs count per 1g**
Female 1	470	28.7	28.7	545
Female 2	603	32.8	124	517
Female 3	409	30	84	557
Female 4	438	33.7	130	518
Female 5	293	28	75	504
Female 6	394	30	84	463
DOM				
Female 1	369	311	92	404
Female 2	384	314	142	479
Female 3	549	306	159	485
Female 4	563	335	106	552
Female 5	447	322	150	601
Female 6	484	327	88	514

Supplementary table s5.2: *General RNA-seq Statistics. DupInt: Intercept value from DupRadar; % Dups: Mark Duplicates - Percent Duplication; M Aligned: Reads Aligned (millions); % Alignable: % Alignable reads; % Proper Pairs: % Reads mapped in proper pairs; Error rate: Error rate: mismatches (NM) / bases mapped (CIGAR); M Non-Primary: Non-primary alignments (millions); % Mapped: % Mapped Reads; % Proper Pairs: % Properly Paired Reads; M Total seqs: Total sequences in the bam file (millions); M Reads Mapped: Reads Mapped in the bam file (millions)*

Sample Name	dupInt (%)	Dups (%)	5'-3' bias	M Aligned	Alignable (%)	Proper Pairs (%)	Error rate	M Non-Primary	M Reads Mapped	Mapped (%)	Proper Pairs (%)	M Total seqs	M Reads Mapped
1DD	0.10%	29.00%	1.26	21.3	87.10%	56.30%	0.25%	3.6	42.6	89.20%	89.20%	47.7	46.1
1DW	0.13%	32.10%	1.3	26.1	89.80%	55.10%	0.26%	4.5	52.2	91.60%	91.60%	57	56.7
1WD	0.11%	27.40%	1.28	23	89.10%	59.10%	0.27%	3.6	46	91.10%	91.10%	50.4	49.6
1WW	0.11%	27.10%	1.3	25.6	89.70%	59.50%	0.29%	4.2	51.1	91.80%	91.80%	55.7	55.4
2DD	0.10%	27.60%	1.28	22.8	90.80%	59.70%	0.25%	3.6	45.5	92.50%	92.40%	49.2	49.1
2DW	0.12%	30.90%	1.28	27.2	89.90%	56.40%	0.27%	4.5	54.4	91.80%	91.80%	59.3	58.9
2WD	0.11%	28.80%	1.24	22	87.90%	57.20%	0.28%	3.5	44.1	90.00%	90.00%	49	47.6
2WW	0.11%	26.90%	1.27	23.8	89.40%	59.80%	0.29%	3.8	47.6	91.50%	91.50%	52	51.4
3DD	0.10%	29.30%	1.25	27	88.70%	57.60%	0.26%	4	54	90.80%	90.70%	59.5	58
3DW	0.13%	31.80%	1.25	26.3	88.60%	55.40%	0.27%	4.1	52.6	90.70%	90.70%	58	56.7
3WD	0.10%	26.00%	1.26	24.6	89.90%	60.80%	0.28%	3.9	49.2	92.00%	92.00%	53.4	53.1
3WW	0.11%	27.20%	1.24	22.8	89.00%	59.00%	0.28%	3.8	45.6	91.10%	91.10%	50	49.3
4DD	0.11%	27.90%	1.27	25.6	89.40%	59.10%	0.26%	3.9	51.2	91.40%	91.40%	56	55.2
4DW	0.12%	30.10%	1.2	25.9	88.60%	56.80%	0.27%	4	51.8	90.70%	90.60%	57.1	55.7
4WD	0.10%	27.80%	1.29	22.5	90.10%	59.10%	0.26%	3.6	44.9	91.80%	91.80%	48.9	48.5
4WW	0.10%	27.90%	1.28	22.3	89.80%	59.00%	0.29%	3.5	44.6	91.60%	91.60%	48.7	48.2
5DD	0.10%	26.50%	1.25	21.9	89.80%	60.50%	0.26%	3.3	43.9	91.70%	91.70%	47.9	47.1
5DW	0.09%	25.20%	1.27	21.8	90.30%	61.80%	0.27%	3.4	43.7	92.20%	92.20%	47.3	47
5WD	0.10%	25.50%	1.26	22.6	90.70%	61.60%	0.27%	3.6	45.2	92.60%	92.60%	48.8	48.8
5WW	0.08%	24.50%	1.28	21.9	90.50%	62.30%	0.30%	3.5	43.8	92.50%	92.40%	47.4	47.3
6DD	0.10%	27.80%	1.29	26.5	91.20%	59.80%	0.29%	4.3	53.1	92.90%	92.90%	57.1	57.4
6DW	0.11%	31.80%	1.26	32.1	90.40%	56.00%	0.26%	5.2	64.3	92.20%	92.20%	69.7	69.5
6WD	0.12%	29.80%	1.19	28.2	89.60%	57.50%	0.27%	4.5	56.3	91.60%	91.60%	61.5	60.8
6WW	0.11%	28.10%	1.27	26.3	90.60%	59.30%	0.29%	4.2	52.7	92.40%	92.40%	57	56.9

Supplementary table s5.3: *Lists of genes from the differentially expressed genes (DEG) analysis. This file contains the results of DEG analyses under various crossing conditions: **A**. List of 22 DEGs (FDR < 0.05) from the paternal effect analysis where males were crossed with domesticated females. **B**. List of 5 DEGs (FDR < 0.05) from the paternal effect analysis where males were crossed with wild females. **C**. List of 765 DEGs (FDR < 0.05) from the maternal effect analysis where females were crossed with domesticated males. **D**. List of 2,259 DEGs (FDR < 0.05) from the maternal effect analysis where females were crossed with wild males. **E**. Candidate maternal effect genes. A table showing the expression patterns of 28 genes in zebrafish, from the zygote stage to the early larval stage. The final column presents the average expression levels of these genes in the E. perch unfertilized egg transcriptome. Six candidate maternal effect genes display notably high expression levels (>100 TPM) in E. perch eggs compared with zebrafish.* ***Due to large size of the file, the tables can be provided upon request.***

Supplementary table s5.4: *Details of the primers used for qRT PCR in the study. *=housekeeping genes*

Gene name	Sequences	Amplicon length (bp)
*beta actin [XM_039824642.1]	F: ACCTTCTACAACGAGCTGAGAGT	153
	R: AGTGGTACGACCAGAGGCATA	
*rpl8 [XM_039817090.1]	F: CGTGACCCATACCGCTTCAA	110
	R: CTCTTCCACGCAGCAGATGA	
*idh3b [XM_039797081.1]	F: TCATGTGAATAGCCTGCCGG	130
	R: TTCGATCACACCCGTCACAC	
*atp5f1c [XM_039792329.1]	F: AAGCGCCAGAAGACAAGTCA	181
	R: TGCAGCAGGTTTCTCAGCTT	
*naca [XM_039799779.1]	F: GCCAAGAATGTGCAGGGTTC	123
	R: GGGGGTAGTGGATTTTGGAGT	
*atp5pb [XM_039800828.1]	F: CCAGGCGGTACAATGAAGAT	128
	R: GCGGAGCAATGTGGTAATTT	
crtac1a [XM_039784574.1]	F: CGTCGTGGTGTCGCTAATCA	120
	R: CTCTAAGAGGGCGTGGGGAC	
slc16a7 [XM_039799069.1]	F: TACGGGCTTTGTGGACATGT	204
	R: GCGAAAACCATGCCGAAACT	
kdr [XM_039806878.1]	F: GTACACTTGCATAGCCACGC	131
	R: CCGAGGTCATCAACACGAGA	
cox5b [XM_039783533.1]	F: GGCCCTCAAACATGGAAAGG	122
	R: CACAAAGACAGCCAACCAGC	
lsp1a [XM_039809086.1]	F: GCCTTCAGTCCCTTCAGTCC	135

	R: GTCGTCTATCTTGGGCAGCA	
bin2b [XM_039797340.1]	F: CAAAGTCAGAGGCGGGAAGT	152
	R: GTCTCCTCCTGCACCTTAGC	

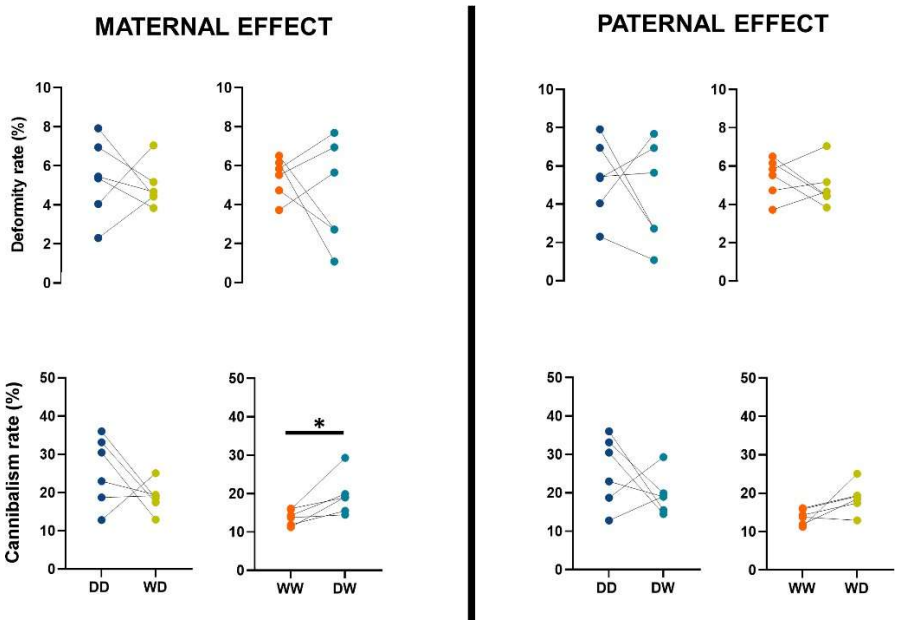


Figure s5.5a: *Deformity and Cannibalism rate (%) measured for all the Eurasian perch families. Asterisk show significance difference (*p< 0.05)*

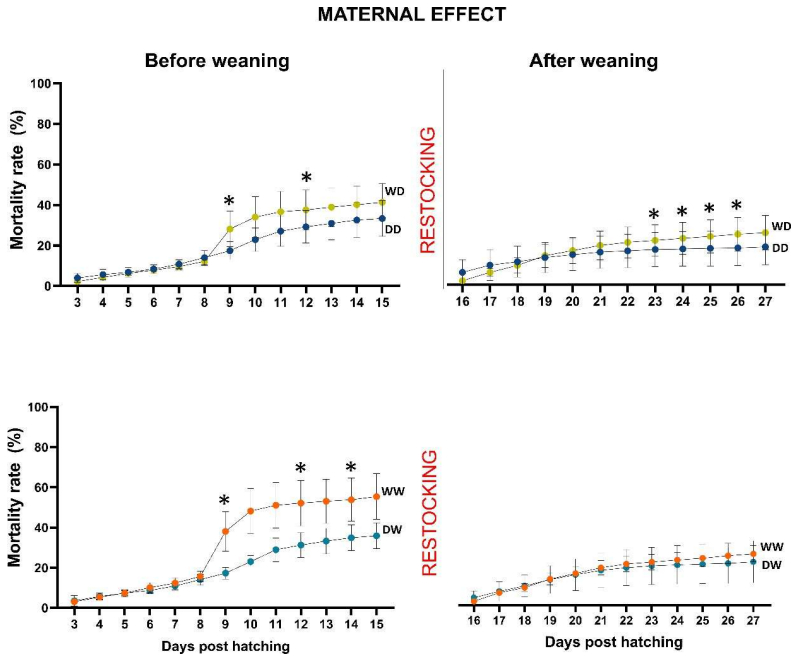


Figure s5.5b: *Cumulative mortality (mean ± SD) before and after restocking for all crossings of Eurasian perch larvae analysed for maternal-effect.*

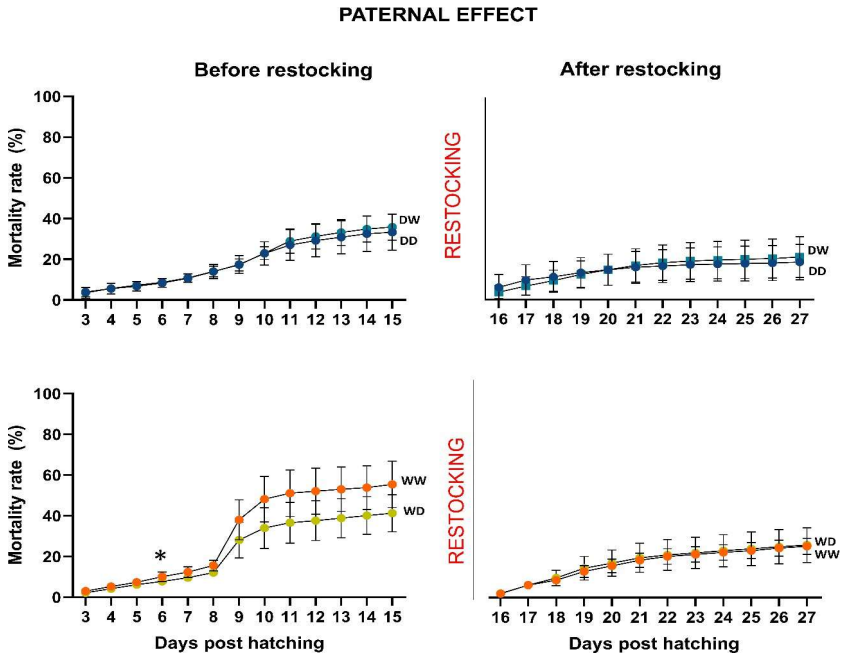


Figure s5.5c: *Cumulative mortality (mean ± SD) before and after restocking for all crossings of Eurasian perch larvae analysed for paternal-effect.*

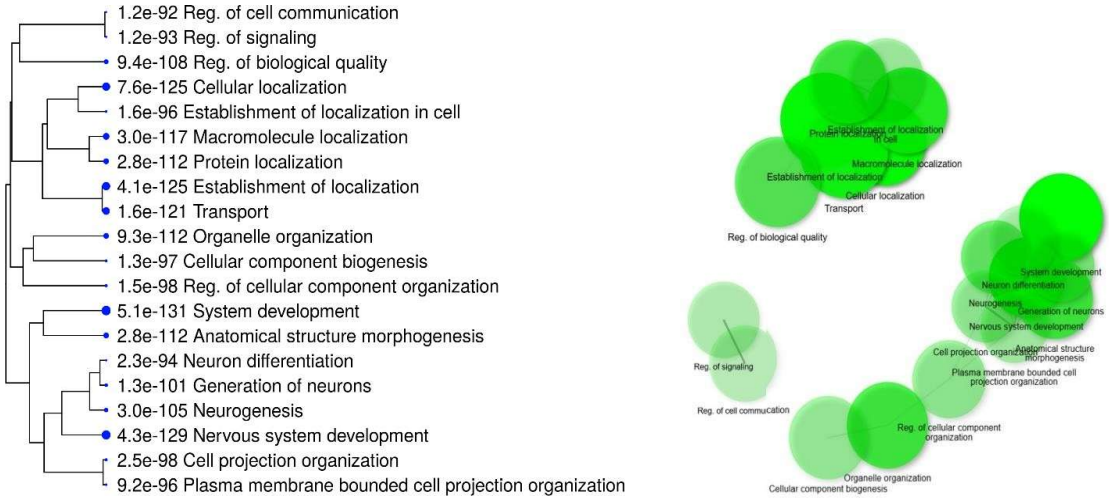


Figure s5.5d: *Tree view and network visualization showing the 20 most significantly enriched GO (biological process) for non-differentially expressed genes.*

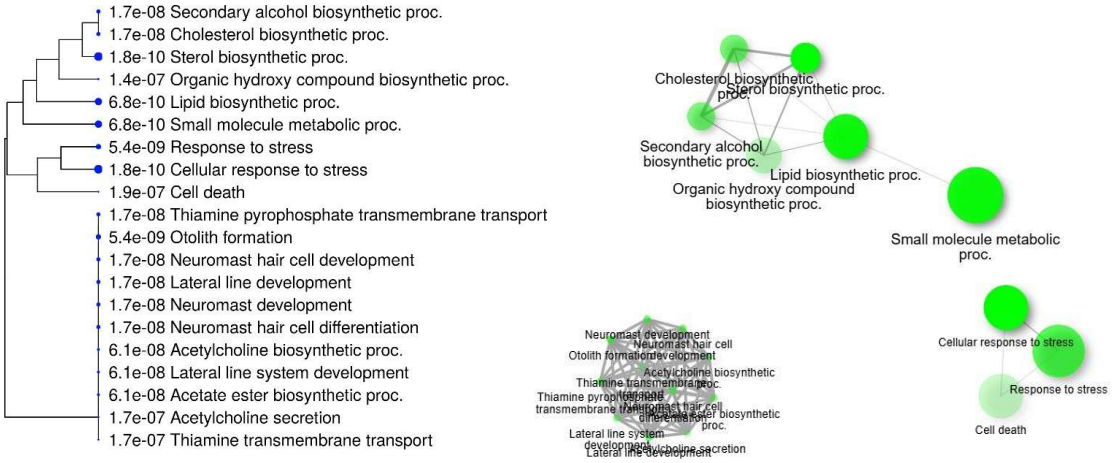


Figure s5.5e: *Tree view and network visualization showing the 20 most significantly enriched GO (biological process) for purely maternal-effect genes.*

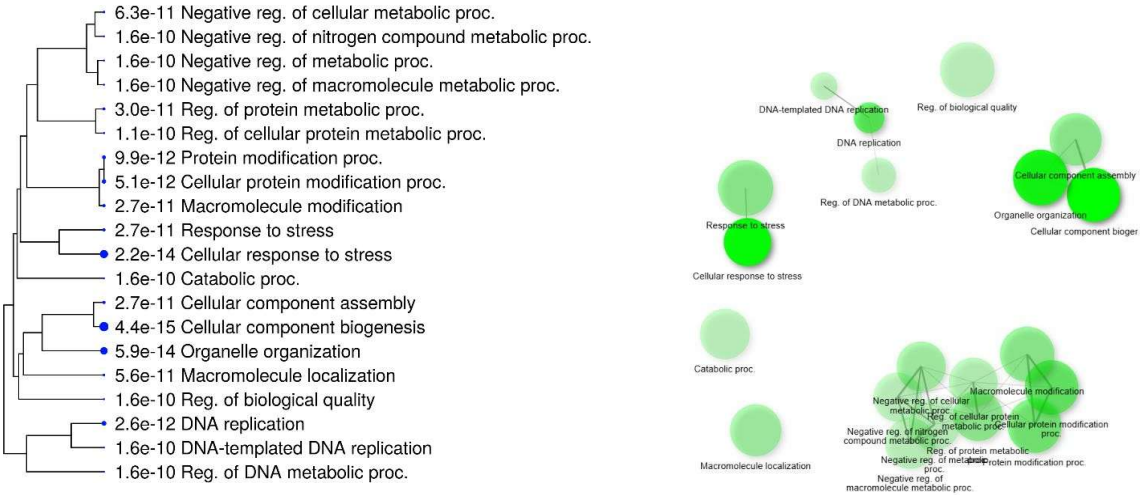


Figure s5.5f: *Tree view and network visualization showing the 20 most significantly enriched GO (biological process) for all the conditionally maternal-effect genes.*

Chapter 11

Authors statements

Załącznik nr 10. Oświadczenie kandydata o jego merytorycznym udziale w powstanie pracy
***Annex No. 10.** Statement of the candidate on their substantive contribution to the creation of a work*

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Oświadczenie
Statement

Niniejszym oświadczam, że w pracy „Paternal-effect-genes revealed through sperm cryopreservation in *Perca fluviatilis*", Panda Abhipsa; Judycka Sylwia; Palińska-Żarska Katarzyna; Debernardis Rossella; Jarmołowicz Sylwia; Jastrzębski Jan P.; de Almeida Taina R.; Błażejewski Maciej; Hliwa Piotr; Krejszeff Sławomir; Żarski Daniel; *Scientific Reports* 2024, 14: art. no 6396 (1-16) (10.1038/s41598-024-56971-w) mój udział polegał na konceptualizacji, metodologii, walidacji, analizie danych, badaniach, zasobach, opracowaniu danych, redakcji i przygotowaniu pierwszej wersji manuskryptu, przygotowaniu odpowiedzi na recenzje, wizualizacji.[7]

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[7]Określenie merytorycznego udziału kandydata w powstanie artykułu naukowego/monografii naukowej (https://credit.niso.org/)

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- **Zasoby:** Zapewnienie materiału badawczego, odczynników, narzędzi badawczych, pacjentów, próbek laboratoryjnych, zwierząt doświadczalnych, sprzętu, zasobów obliczeniowych lub innych narzędzi analitycznych.
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- **Data curation:** Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later re-use.
- **Formal analysis:** Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data.
- **Funding acquisition:** Acquisition of the financial support for the project leading to this publication
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- **Methodology:** Development or design of methodology; creation of models.
- **Project administration:** Management and coordination responsibility for the research activity planning and execution.
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KARTA PODPISÓW

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Załącznik nr 10. Oświadczenie kandydata o jego merytorycznym udziale w powstanie pracy
Annex No. 10. *Statement of the candidate on their substantive contribution to the creation of a work*

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Olsztyn, 22.07.2025
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Oświadczenie
Statement

Niniejszym oświadczam, że w pracy „Dynamic interplay of maternal and paternal contributions to offspring phenotype in Eurasian perch, *Perca fluviatilis*", Debernardis, Rossella; Panda, Abhipsa; Judycka, Sylwia; Palińska-Żarska, Katarzyna; Klopp, Christophe; de Almeida Taina R.; Jarmołowicz, Sylwia, Hliwa, Piotr; Żarski, Daniel; przedłożonej do recenzji w czasopiśmie BMC Biology mój udział polegał konceptualizacji, metodologii, walidacji, analizie danych, badaniach, opracowaniu danych, redakcji i przygotowaniu pierwszej wersji manuskryptu, wizualizacji.[7]

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- **Zasoby:** Zapewnienie materiału badawczego, odczynników, narzędzi badawczych, pacjentów, próbek laboratoryjnych, zwierząt doświadczalnych, sprzętu, zasobów obliczeniowych lub innych narzędzi analitycznych.
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Chapter 12

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Paternal-effect-genes revealed through sperm cryopreservation in *Perca fluviatilis*

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Knowledge about paternal-effect-genes (PEGs) (genes whose expression in the progeny is influenced by paternal factors present in the sperm) in fish is very limited. To explore this issue, we used milt cryopreservation as a specific challenge test for sperm cells, thus enabling selection amidst cryo-sensitivity. We created two groups of Eurasian perch (*Perca fluviatilis*) as a model – eggs fertilized either with fresh (Fresh group) or cryopreserved (Cryo group) milt from the same male followed by phenotypic-transcriptomic examination of consequences of cryopreservation in obtained progeny (at larval stages). Most of the phenotypical observations were similar in both groups, except the final weight which was higher in the Cryo group. Milt cryopreservation appeared to act as a "positive selection" factor, upregulating most PEGs in the Cryo group. Transcriptomic profile of freshly hatched larvae sourced genes involved in the development of visual perception and we identified them as PEGs. Consequently, larvae from the Cryo group exhibited enhanced eyesight, potentially contributing to more efficient foraging and weight gain compared to the Fresh group. This study unveils, for the first time, the significant influence of the paternal genome on the development of the visual system in fish, highlighting *pde6g*, *opn1lw1*, and *rbp4l* as novel PEGs.

Fish ontogeny, especially at their early life stages, is largely determined by their parents. However, most of the work revolves around the evaluation of maternal contribution[1]. This is mostly associated with the well-known fact that the mother provides nutritional and energy reserves (contained in the yolk) utilized by fish during the larval period[2]. More recently, it became apparent that maternal contribution is far beyond the nutritional reserves, shedding light on the remaining components of egg molecular cargo as a modulator of progeny phenotype[3]. Maternal transcripts are known to be responsible for controlling development at least up to zygotic genome activation (ZGA)[4]. Nevertheless, in the past three decades, a piling body of evidence indicates significant paternal influence during the early life history (ELH) (i.e. from fertilization until the end of the larval period in fish)[5–8]. In addition to reproductive fitness and age of males, other wide variety of progeny traits, such as embryonic developmental rate and larval size upon hatching, have been shown to be contributed by the father[9]. These finding sheds light on the fact that very little is known about the paternal contribution to progeny phenotype in fishes. Understanding paternal contribution to fish ELH is crucial for fish biologists, ecologists and the aquaculture sector, where it can be used to fine-tune selective breeding programs, which may lead to increased production effectiveness and improved welfare of cultured species[10].

Phenotype of the progeny is shaped by the genetic and non-genetic factors (e.g. epigenetic modifications of genome, various molecules – such as RNAs, proteins, metabolites and others – contained in the gametes), with the latter being susceptible to external cues[11], including environment the fish lives in as well as any other factors (such as pathogens) the fish may experience[12–14]. There has been lots of discussion about genes forming molecular cargo of the oocyte and subsequently affecting offspring development via regulation of gene expression in the

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progeny, which were termed as 'maternal-effect-genes'[15–17]. Research on paternal genes encoding inherited factors (including RNAs, proteins or epigenetic modifications) that influence the offspring, henceforth referred to as 'paternal-effect-genes' (PEGs)[18], has been relatively scarce. Here, we use the term 'paternal-effect-genes' to refer to genes whose expression in the progeny is influenced by paternal factors carried in the sperm.

While standardizing sophisticated reproductive techniques, milt cryopreservation (involving specific procedures enabling effective storage of the viable cells at ultra-low temperatures for a very long period) for fishes has come into limelight over several decades now[19]. Cryobanking of milt helps to manage the genetic diversity of the fish species, facilitates spawning synchronization, allows selective breeding and much more[20]. Cryopreservation of milt is a big shock to a sperm cell constituting a specific challenge test[21] which causes irreversible damages to the cryo-sensitive cells which lose fertilizing capacity, in contrast to cryo-resistant cells retaining their functionality[22]. Importantly, the effect of cryopreservation on spermatozoa functionality depends on the species. On one hand, sperm motility, fertilization rate and the hatching rates were seen to be high and similar with post-thaw milt when compared with use of fresh milt in some Salmoniforms and Esociformes[23,24]. On the other hand, studies done so far on few other teleosts (e.g. Levantine scraper, wild brown trout, Atlantic cod) report reduced motility and viability of sperm after freezing-thawing[25]. Also, the post-thaw milt from the same fish when used for *in vitro* fertilization, there were significant declination in fertilization success when compared with fresh sperm control, followed by abnormalities like cleavage patterns, hatching success, organogenesis etc.[26,27]. So far studies elaborating implications of sperm cryopreservation on embryonic and larval performance as well as gene expression profile in offspring is very limited[21,28]. It has been reported, that along with DNA damage, changes in gene expression, and chromatin integrity in sperm, the transcriptome of the larvae (obtained with frozen-thawed sperm) is also seen to be dysregulated due to sperm cryopreservation[29]. To add on, the maternal molecular cargo has the ability to repair a certain degree of the DNA damage[30]. Clearly, milt cryopreservation seems to be a very subjective kind of sperm cell stressor.

Eurasian perch is a commercially relevant species farmed in recirculating aquaculture systems (RAS), attaining the 4th level of domestication thus far[31]. In the last 20 years, it was found to be an excellent model for studies on embryonic development[32], reproduction[33], domestication processes[34] and circadian rhythms[35]. In addition, the larvae of this species can be utilized as a complete organism to sequence their RNA repertoire, given their size and developmental advancement. At their mouth opening stage, they are self-sustainable organisms with the ability to adapt to different environments. More importantly, at this stage, they are not yet affected by any human intervention[34]. In addition, a Eurasian-perch specific, highly standardized sperm cryopreservation procedure was developed by Judycka et al.[36] enabling the maintenance of high fertilization success with the use of cryopreserved milt. This has brought the tool, enabling much more feasible and sophisticated selective breeding procedures in this species. However, until now, neither molecular nor phenotypical consequences have been investigated following the usage of cryopreserved sperm for the creation of a new generation in this species. In addition, prominently, any consequences passed on to the progeny from sperm subjected to these challenging procedures would be the ones directly linked with a contribution of a male to the overall phenotype of the progeny. This also includes distinguishing PEGs, an important aspect in developmental biology that is highly difficult to identify. Therefore, in our study, we aimed to explore phenotypical and transcriptomic consequences in larvae resulting from the application of sperm cryopreservation technology. Controlled reproduction of Eurasian perch followed by examination of phenotypical performance became a kind of proxy for understanding physiological alterations in progeny, revealing paternally effected genes.

In the present study, we carried out RNA sequencing (RNA-Seq) of RNA obtained from freshly hatched larvae (at the mouth opening stage) to identify the processes being modulated/affected in the progeny by the usage of cryopreserved sperm for fertilization in Eurasian perch (*Perca fluviatilis*), which is a model for percid fishes, an important group of commercially relevant aquaculture freshwater species. The strength of the present study is its importance around the integration of information on phenotypical performance of the progeny and the transcriptomic profile/repertoire obtained from the whole organism. After all, combining transcriptomics data and associated phenotypic characteristics observed during advanced phenotypical exploration is an excellent approach to link genotype-phenotype relationships[37].

Materials and methods

Ethics statement

The study was conducted according to the European and national legislation for fish welfare and approved by the Local Animal Research Ethics Committee, resolution no 5/2023. The animal study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for animal research.

Broodstock management and controlled reproduction

We crossed 3 female and 6 male wild spawners (see physiological details in Table s1) from Mikołajki lake and Żurawia fish farm ponds, respectively. The wild fish were caught using fyke nets and transported immediately after in plastic bags filled with water and oxygen (v/v 2:1) to the research facilities of the Centre of Aquaculture and Ecological Engineering of the University of Warmia and Mazury in Olsztyn (CAEE-UWM, NE Poland). The pond-reared fish males were harvested in November, dedicated to oxygenated tanks at the Salmonid Research station of the National Inland Fisheries Research Institute in Rutki (North Poland), where they were overwintered in the flow-through system fed with riverine water (natural photothermal conditions). Males were fed with frozen bloodworms (Chironomidae) by hand during the light phase until apparent satiation (exhibited by lack of reaction of fish to food provided), which depended on the water temperature. Females were caught directly during the spawning season. They were then transported in plastic bags with oxygen to the CAEE-UWM for further controlled reproduction procedures. The females and males were of different origins because the capture of wild

males during the spawning season is very difficult, and often these males are prone to have partially participated or even completed the spawning act before being caught. This can have a direct effect on the sperm quality obtained; thus, the males were caught earlier and overwintered. In contrast, the wild females, if overwintered in controlled conditions, tend to have lowered egg quality, affecting the quality of the larvae and therefore causing bias to the results obtained. Wild Eurasian perch females caught during the spawning season are not accepting any type of food while kept in the hatchery (for details see: Żarski et al.[38]), so they were not fed. Nevertheless, as in our study, the overall reproductive protocols did not affect egg quality negatively as we observed very high fertilization rate (over 80%) in all the females (Fig. 2). The males and females were kept separately, according to their gamete maturity stages[39] in RAS with a defined photoperiod (14 hours light:10 hours dark) and temperature (12 °C±0.1) until ready for ovulation and spermiation. To promote and synchronize the spawning act of both sexes, fish were hormonally stimulated using salmon gonadoliberin analog (sGnRHa, BACHEM, Switzerland) (injection at a dose of 50 μg kg^{-1})[39]. Sperm was collected 7 days post hormonal stimulation (which was within the optimal period of sperm collection of this species)[40], whereas eggs were collected between 3- and 5-days following injection depending on the maturation stage of the females[41]. Prior to any manipulation, such as gamete collection, the fish were anesthetized in MS-222 (Argent, USA) at a dose of 150 mg L^{-1}. Twelve unique families (each family reared in triplicate) were selectively created using 3 females and 6 males. More specifically, eggs from one female were divided into four portions, each fertilized with either fresh (group Fresh) or cryopreserved (group Cryo) sperm from two males, separately (as described in Fig. 1a). Apart from gametes, other from information parents, such as total length (L_T), caudal length (L_C), body weight (before and after gamete stripping), body scales (for estimation of the fish's age) and fin-clip samples, were collected (Table s1).

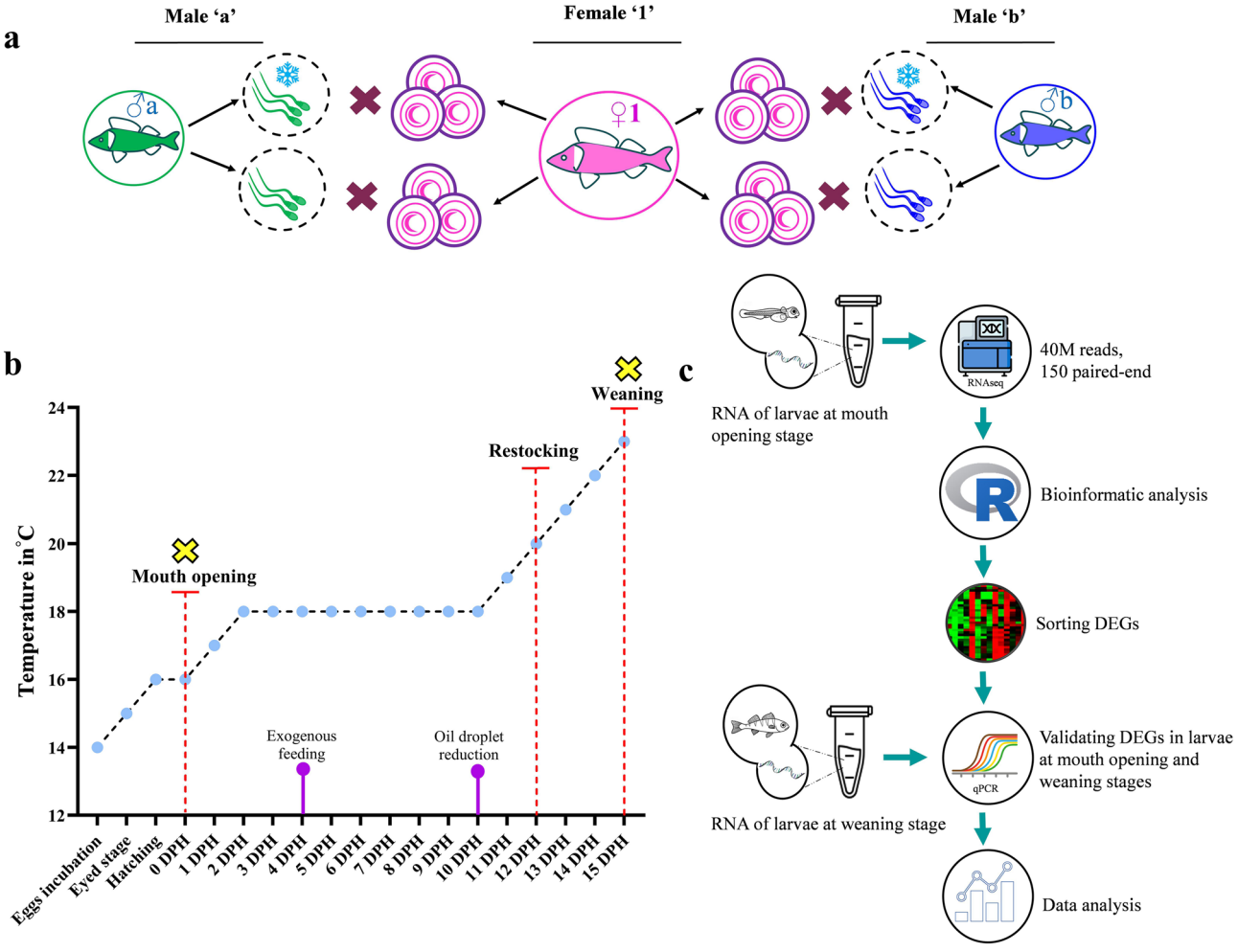


Figure 1. Protocol followed for the experiment. (**a**) Eggs from one female were fertilized with milt from two males such that eggs from one female were divided into two portions for each male, one fertilized with cryopreserved milt (marked with a snowflake) and the other portion with fresh milt; (**b**) Rearing schedule and temperature regimen along with sampling points (yellow crosses); (**c**) RNA extraction and sequencing, followed by sorting DEGs, validations by qPCRs and data analysis.

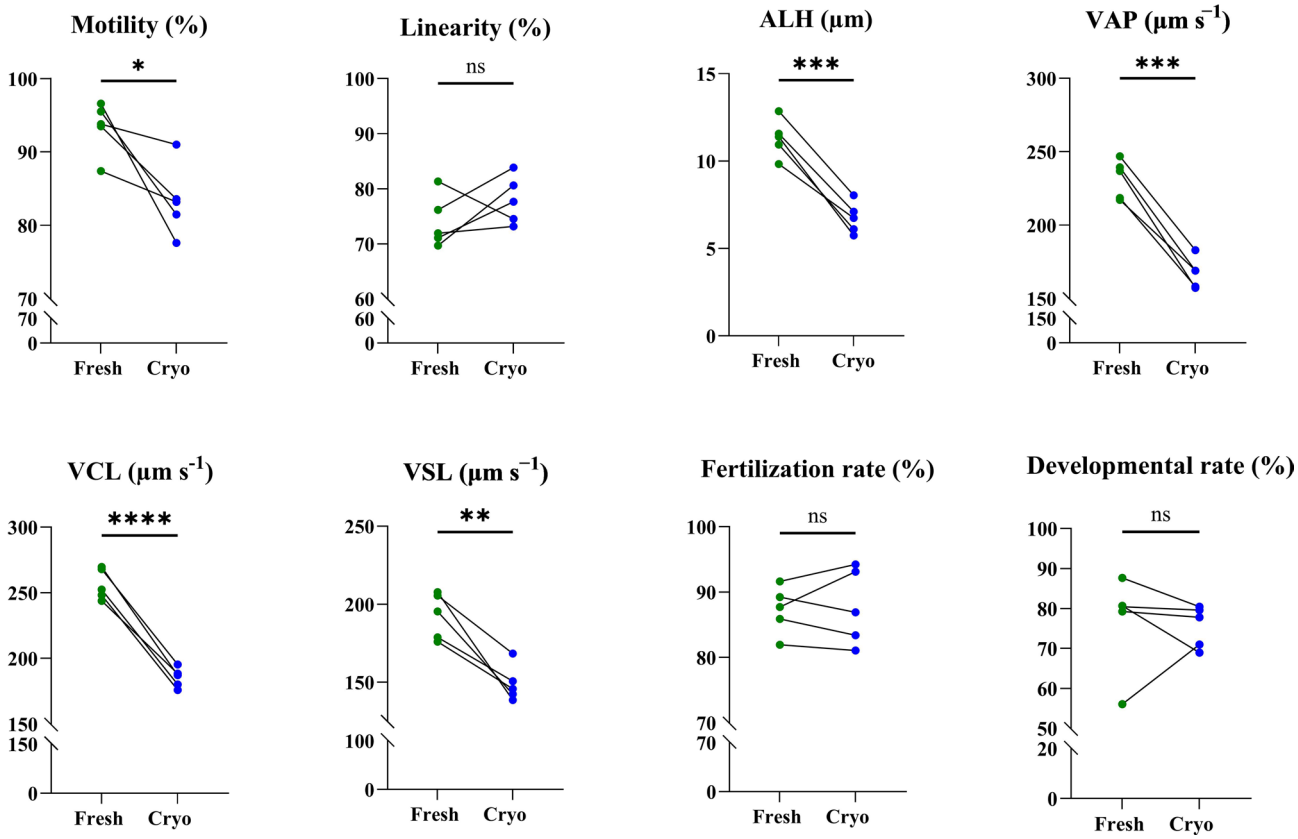


Figure 2. Sperm motility parameters, fertilization and developmental rate between fresh and cryopreserved (Cryo) Eurasian perch (n = 5) milt. The results for statistical analysis are presented as follows: *ns* nonsignificant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). *ALH* amplitude of lateral head displacement; *VAP* average path velocity, *VCL* curvilinear velocity, *VSL* straight line velocity.

Sperm collection and cryopreservation

The 6 males were stripped (with gentle pressure on abdomen) for milt using a catheter (Galmed, Poland) (to avoid contamination with the urine or blood). After collection, each sample was kept on ice. Activation of sperm to check motility was done using a two-step procedure. First, the semen was diluted 1:50 (for fresh semen) or 1:5 (for frozen/thawed semen) in an immobilizing solution (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, 20 mM Tris, pH 8.0). Then, the semen was diluted 1:20 in an activating solution (75 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, 20 mM Tris, pH 8.0) supplemented with 0.5% bovine serum albumin. From several motility parameters measured using the computer-assisted sperm assessment (CASA) system, the linearity (LIN, %), amplitude of lateral head displacement (ALH, µm), average path velocity (VAP, µm s^{-1}), curvilinear velocity (VCL, µm s^{-1}), and straight-line velocity (VSL, µm s^{-1}) were evaluated for both fresh and cryopreserved milt. Alongside, the concentration of fresh milt was measured using NucleoCounter SP-100 (Chemometec, Allerød, Denmark)[42].

One part of the collected milt was used for cryopreservation as described by Judycka et al.[42]. The milt was diluted with extender (consisting of a final concentration of 0.3 M glucose, 7.5% methanol and 25 mM KCl at 3×10^9/ml spermatozoa). Milt mixed with extender) was loaded into 0.5 ml plastic straws (IMV Technologies, L'Aigle, France), which were placed on a floating rack. Then, the straws were frozen in liquid nitrogen vapour (3 cm above liquid surface) for 5 min in a Styrofoam box with an isolating Neopor block (Minitübe GmbH, Tiefenbach, Germany) followed by placement in the liquid nitrogen. The other part of the milt was kept on ice to be used directly for the fertilization trials without any manipulations to use as Fresh sperm.

Egg collection and fertilization trials

The chosen females were taken to check their oocyte maturation stages as described earlier[41] by catheterizing a few oocytes, exposing them in Serra's solution (ethanol, formalin, and glacial acetic acid mixed 6:3:1 by volume) and microscopic evaluation of their maturation stages. At ovulation, eggs from females were stripped out into a clean and dry beaker as described earlier[41].

From each egg ribbon with an average weight of 60± 10 g, 3-5 small portions (<1 g) were sampled and weighed, and the number of eggs per portion were counted. In this way, we could estimate the number of eggs present per gram to aid us in dividing the ribbon into 4 equal portions (2 portions per male). Ribbon(s) from each female were further divided into equal portions (conducted the rearing in triplicate) of ~4 g each and were used

to carry out the *in vitro* fertilization[43]. Just before fertilization, straws were thawed in a water bath at 40 °C for 10 seconds and placed in an Eppendorf tube[42]. Then, the eggs were preactivated for 30 s in hatchery water, and milt (fresh or cryopreserved) was added to the eggs at a sperm:egg ratio of 200,000:1. Then, an appropriate amount of milt was added to each egg portion, as previously calculated (separately for each egg sample). The eggs were then stirred for 30 seconds and washed with hatchery water after ~10 minutes to remove excess sperm and any debris.

Incubation of embryos

The fertilized eggs were incubated in 5 L tanks with black walls that functioned within the same RAS. Initially, before hatching, the eggs were placed on mesh (diameter of 3 mm) at a temperature of 14 °C. Within 24 hours after fertilization (before embryos reached the mid-blastula transition (MBT)), ~100 random embryos were observed under the microscope to calculate the fertilization rate. Similar counting was again performed after 3 days, while the embryonic development rate was estimated at the neurula stage (when the body of the embryo could be viewed at the animal pole). While incubating the embryos, the photoperiod was maintained at 24L:0D, and the temperature was raised to 15 °C when the embryos reached the eyed-egg stage; then, the temperature was maintained at 16 °C as soon as the first hatched larvae were noticed[34]. We started numbering the age of larvae post hatching as DPH (days post hatching), and to maintain synchronous hatching, the larvae were hatched manually. This was done by transferring the egg ribbons to bowls with water from the rearing tanks and stirring gently, and the hatched larvae were put back to their respective tanks. We carried out this operation 4-5 times. That day was named 0 DPH, and the day count had begun.

Larviculture and advanced zootechnics

The hatched larvae in both the Fresh and Cryo groups were reared following the exact same conditions in the RAS system, along the standardized temperature and feeding regimen described (Fig. 1b)[34]. Beginning from 0 DPH, the temperature was 16 °C. At 1 DPH, the water temperature was raised by 1 °C, and at 2 DPH, it was at 18 °C; this temperature was kept stable up to 10 DPH. Starting from 4 DPH feeds of *Artemia* sp. nauplii *ad libitum* three times per day (first four days of feeding – micro *Artemia* cysts [SF origin], then standard size *Artemia* cysts at 260,000 nauplii per gram [GSL origin]) was insured. At 12 DPH, feeding larvae were restocked in equal numbers of larvae in all tanks by counting volumetrically. Here, feeding larvae ensured healthy larvae to a few extents. Subsequently, 11 DPH onwards, the temperature was increased by 1 °C per day until 23 °C, which is considered the optimal temperature for the growth of perch larvae[44]. After the first feeding and before the last feeding, the tanks were cleaned, and dead larvae were counted. In addition, other parameters, such as the oxygen level to 80% and ammonia concentration to <0.02 mg $L^{-1,}$ were maintained. The experiment was conducted for the larvae only until their *Artemia* feeding phase, i.e. the experiment was terminated at the weaning stage, upon sampling.

At the mouth-opening stage (between 0 and 1 DPH, where at least 50% of larvae were found to have reached this stage) and at the time when the protocol envisaged the end of feeding the larvae with live *Artemia nauplii* (hereinafter referred to as weaning; 15 DPH) were sampled for total length (TL, ±0.01 mm) and wet body weight (WBW, ±0.1 mg). Additionally, samples of larvae were used for extraction of RNA (whole larvae were preserved in RNAlater, Sigma−Aldrich, Germany). During the first sampling, we made sure we collected larvae at the mouth opening (MO) stage, where in-egg embryonic development has been accomplished and larvae were ready to survive in the outer environment but with minimal human intervention applied.

The total length of larvae was determined using a stereoscopic microscope (Leica, Germany). Next, wet body weight measurements using the 'noninvasive method'[45] were addressed using a precision laboratory scale (Ohaus, USA). For this purpose, anesthetized larvae were placed on a platform made of nylon mesh (with a mesh size of approx. 200 µm), and excess water was drained out by filter paper. This method minimized possible physical damage to very delicate larvae. Two days after oil droplet reduction, the swim bladder inflation efficiency (SBIE%) was calculated using a stereoscopic microscope by triple counting perch larvae (with and without a filled swim bladder) randomly caught from each tank on a Petri dish (in total, we determined SBIE from more than 100 larvae from each tank). Before any manipulations, we anesthetized the larvae in a solution of MS-222 (at a dose of 150 mg L^{-1}).

RNA extraction

The total RNA was extracted from snap frozen unfertilized eggs (UFE) (~50 eggs) and a pool of larvae (n = 10 for larvae at mouth opening and n=4 for larvae at weaning) using a TotalRNA mini-kit (A&A Biotechnology, Poland) from unfertilized eggs of each female and larvae from each family (for both sampling stages; Fig. 1c), separately. The quantity and purity of extracted RNA were evaluated using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). Samples showed absorbance ratios A260/280 ≥ 2.0 and A260/230 ≥ 2.2. The quality of the extracted total RNA was also evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), and all the samples presented RIN ≥ 9.0. Samples were then outsourced for RNA sequencing.

RNA sequencing and bioinformatics

Libraries (using TruSeq stranded mRNA kit) were sequenced using Illumina's NovaSeq 6000 with standard protocols. Overall, from each sample, more than 40 M reads were obtained, with a 150 bp paired-end sequencing mode.

Differential analysis

The raw reads were quality controlled using FastQC software version 0.11.9[46]. Adapters and low-quality fragments of raw reads (average QPhred score < 20) were trimmed out, and reads were clipped to equal lengths of 100 nt using the Trimmomatic tool ver. 0.40[47]. The resulting read sets of the analyzed samples were mapped to

a reference genome *P. fluviatilis* version 11.1.104 obtained from the NCBI database[48] using STAR software ver. 2.7.10a[49] with ENCODE default options.

Transcript count data for the larval samples were filtered to have at least 5 libraries in which there were at least 5 reads. Libraries from before and after the cryopreservation process were compared using the following design: ~ *males* + *condition;* males standing for the 6 males followed during the experiment and condition representing before (fresh) and after cryopreservation. These analyses were performed in RStudio (version 4.1.3) using the package DESeq2[50] and *ashr* for log fold-change shrinkage[51]. Differences were considered significant when corrected p values were inferior to α (α = 0.05), and we obtained 11 DEGs.

It should be emphasized that among the 6 families created and used for the entire study, for further analysis, 1 family (from the Cryo group and its counterpart in the Fresh group) was removed because the transcriptomic profile clearly differed from the remaining families and was considered an outlier (see Fig. s1).

Gene ontology enrichment analysis (GOEA)

GOEA was performed using ShinyGO, version 0.77 platform[52] to test the overrepresentation of GO terms in a list of genes and to understand their biological significance as an effective approach[53,54]. The 11 DEGs (namely, crystallin beta A2b (*cryba2b*); crystallin beta A4 (*cryba4*); crystallin beta B1 (*crybb1*); crystallin, gamma MX, like 2 (*crygmxl2*); phosphodiesterase 6G, cGMP specific, rod, gamma (*pde6g*); opsin 1, longwave-sensitive, 1 (*opn1lw1*); gamma-crystallin M2-like (*gamma m2*); beta-crystallin A1-like (*cryba1*); gamma-crystallin M3-like (*crygm3*); retinol binding protein 4, like (*rbp4l*); and transforming growth factor beta induced (*tgfbi*)) were fed to the ShinyGO platform, zebrafish was chosen as the best matching species; with the false detection rate (FDR) cutoff of 0.05, and 20 pathways' network was created. A STRING-db, version 12.0[55] with functional enrichment of GO biological processes was also performed to retrieve a protein−protein network that also describes the distance between the linked genes.

RT−qPCR validation of differentially expressed genes (DEGs)

Primer design

Primer pairs for all 11 DEGs along with 5 normalizing genes for RT qPCR were designed using NCBI-Primer BLAST, version 1.0.1[56]. The sequence that matched the best to *P. fluviatilis* was fed to Primer3Plus software version 3.3.0[56,57]. The best matching pairs with least possibilities to form secondary structures were chosen and checked for GC content and melting temperature (Tm) on µMelt Quartz, version 3.6.2[58]. The sequences of the designed primers are presented in Table s2.

qPCRs

RT−qPCRs were performed for each gene using a Viia7 (Applied Biosystems) thermocycler. For each qPCR, 10 ng cDNA template was used along with 10 µl (A&A Biotechnology) SYBR RT PCR Master Mix (Cat. No. 2008-100), 0.5 µM forward (1 µl) and reverse (1 µl) primers, 2 µL of starter mix and PCR grade water were added to maintain a final volume of 20 µL. The reactions were performed with the following cycling conditions applied: enzyme activation for 10 minutes at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing and elongation at 60 °C for 1 minute. In the analysis of each gene, a standard curve was calculated using a series of 6 two-fold dilutions to determine reaction efficiency (reaction efficiencies between 85 and 110% were considered acceptable). Relative expression for each gene was normalized as the geometric mean of expression values recorded for 5 reference genes (namely, cytochrome c-like, transcript variant X1, *cycs*; tetraspanin 7, *tspan7*; ER membrane protein complex subunit 10, transcript variant X2, *emc10*; pre-mRNA-splicing factor, *syf2* and ER membrane protein complex subunit 3-like, *emc3*), which were chosen from our transcriptomic data on the basis of their stable expression levels and close-to-mean expression values in the RNA-sequencing analysis[14]. Each reaction for real-time qPCR validation was performed in triplicate. The data were compared between the Fresh group and Cryo group (at mouth opening and weaning stages).

In silico analysis

Several in silico analyses were carried out using tools such as NCBI-BLAST[59], Expression Atlas version 2.0[60] and PhyloFish[61]. These tools helped us to study the expression levels of our DEGs throughout the early life history in *Danio rerio* as a reference model organism for Eurasian perch. Additionally, it allowed us to explore the expression pattern of DEGs in various tissues in *D. rerio*, and few other evolutionarily close/distant species (namely, *O. mykiss*, rainbow trout; *S. lucioperca*, pikeperch; *A. mexicanus*, surface Mexican tetra; *A. mexicanus* cave Mexican tetra and *A. Anguilla*, European eel) from Eurasian perch. This was done to get hints if a particular gene has tissue-specificity.

Upon sequencing the RNA obtained from UFE, we checked for the presence of our DEGs, considering a threshold of TPMs>0.5. While for other species like *D. rerio*, *O. mykiss* and *S. lucioperca*, we used the PhyloFish and Expression atlas platform to check for the expression values while we used raw data of UFE sequenced by Kim et al.[62] for *M. anguillicaudatus*.

Statistical analysis

The raw data from all the parameters like sperm quality parameters (in %, µm, µm s^{-1}), fertilization and embryonic developmental rates (%), deformities (%), SBIE (%), TL (mm), and WBW (mg) were first fed into GraphPad (version 9.5.1) and paired t-tests ($p<0.05$) for each single parameter to compare between Fresh group and Cryo group were conducted. While calculating then plotting cumulative mortality (%); expression values of our DEGs (in TPMs) after sequencing and normalized expression values after real time qPCRs (mean quantity); transformation of gene replicates in TPMs for presence of genes in tissues were calculated on Microsoft Excel. However,

the values were then computed on GraphPad to plot graphs after paired t-tests. All the data were tested with a significance level of 5% (significant differences were considered at p value < 0.05).

Results

Cryopreservation resulted in a significant decrease in all tested sperm motility parameters compared to fresh milt, except linearity (Fig. 2). However, fertilization and developmental rates were not affected by cryopreservation. Additional analysis of sperm motility parameters in relation to fertilization rate did not reveal any significant correlation (see Fig. s2).

Phenotypical parameters

No significant differences in deformity rate, SBIE rate, TL (both at mouth opening and weaning stages) or mortality were recorded between the Fresh and Cryo groups. However, a significantly higher WBW of the larvae from the Cryo group at the weaning stage was detected (Fig. 3) compared to the Fresh group.

Differentially expressed genes (DEGs)

Analysis of the transcriptomic data enabled the identification of 11 DEGs (Fig. 4) between the Fresh and Cryo groups. The only gene with higher expression in the Fresh group was *tgfbi*, while the remaining 10 genes had higher expression in the Cryo group.

Functional analysis of identified DEGs suggested common functions in most of them. For instance, *crygmxl2*, *cryba2b*, *cryba4*, *crybb1*, *cryba1* and *crygm3* belong to the *Crystallin* family of genes, which have a major role in early embryonic eye lens development[63]. Clustering analysis of the most enriched gene ontology terms revealed common functions related to eye development, since the remaining genes (*pde6g*, *opn1lw1*, *rbp4l* and *tgfbi*) were found to be responsible for functions of the eyes, such as photoreceptors, photoperiodism, and camera-type eyes (Fig. 5).

qPCR validation of genes

Out of 11 DEGs, in 8 of them, the expression values at both mouth opening (MO) and weaning did not differ between the Fresh and Cryo groups based on the RT−qPCR results (Fig. 4). Moreover, in all these genes, the expression levels decreased with age, and there were no significant differences between the Fresh and Cryo groups. Three DEGs (*pde6g*, *opn1lw1* and *rbp4l*) were confirmed upon validation with qPCR to have higher expression in the Cryo group ($p < 0.05$) (Fig. 4) than in the Fresh group at the mouth opening stage. It should be emphasized that for these three genes, similar levels of expression between the Fresh and Cryo groups at the end of the experiment (at weaning) were observed.

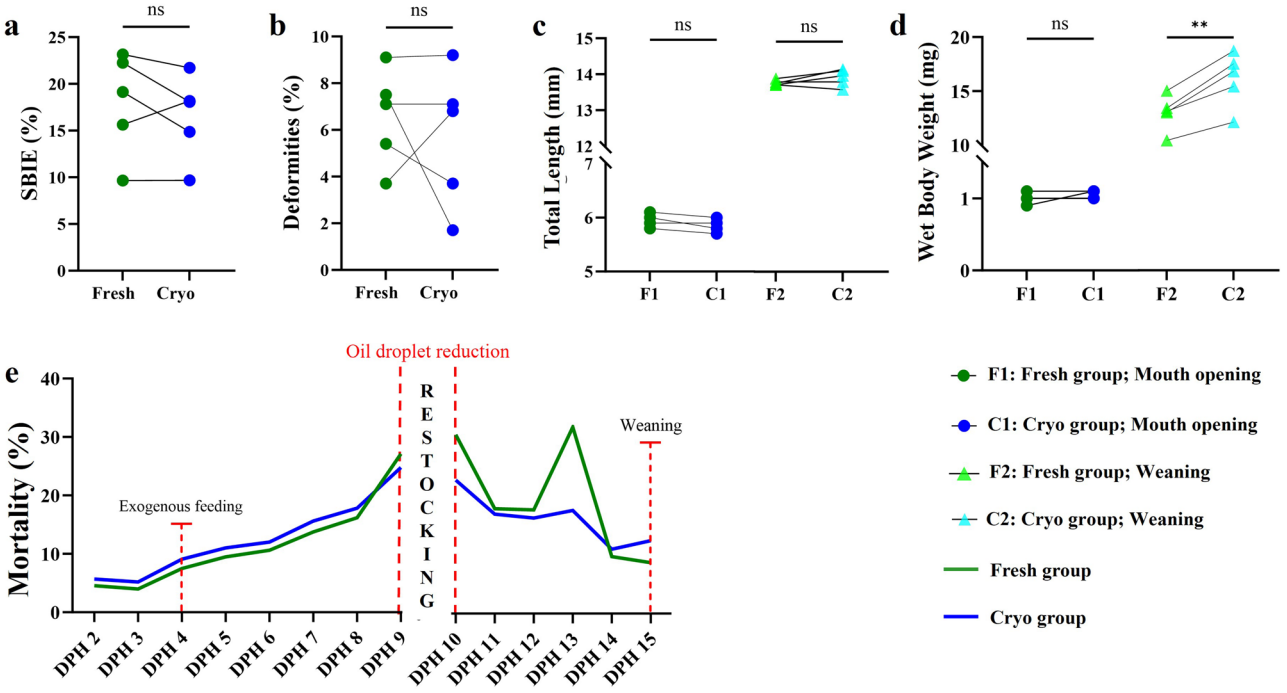


Figure 3. Phenotypical performance of larvae obtained after fertilization of eggs with the use of fresh and cryopreserved milt of Eurasian perch (n = 5). (**a**) Swim bladder inflation effectiveness (SBIE, %); (**b**) Deformity rate at mouth opening stage (%); (**c**) TL of larvae at mouth opening and at weaning stages (mm); (**d**) WBW of larvae at mouth opening and weaning stages (mg); (**e**) Cumulative mortality (%) of larvae over the larviculture period, before and after restocking (only eating larvae). The results for statistical analysis are presented as follows: *ns* nonsignificant, $^{**}p < 0.01$).

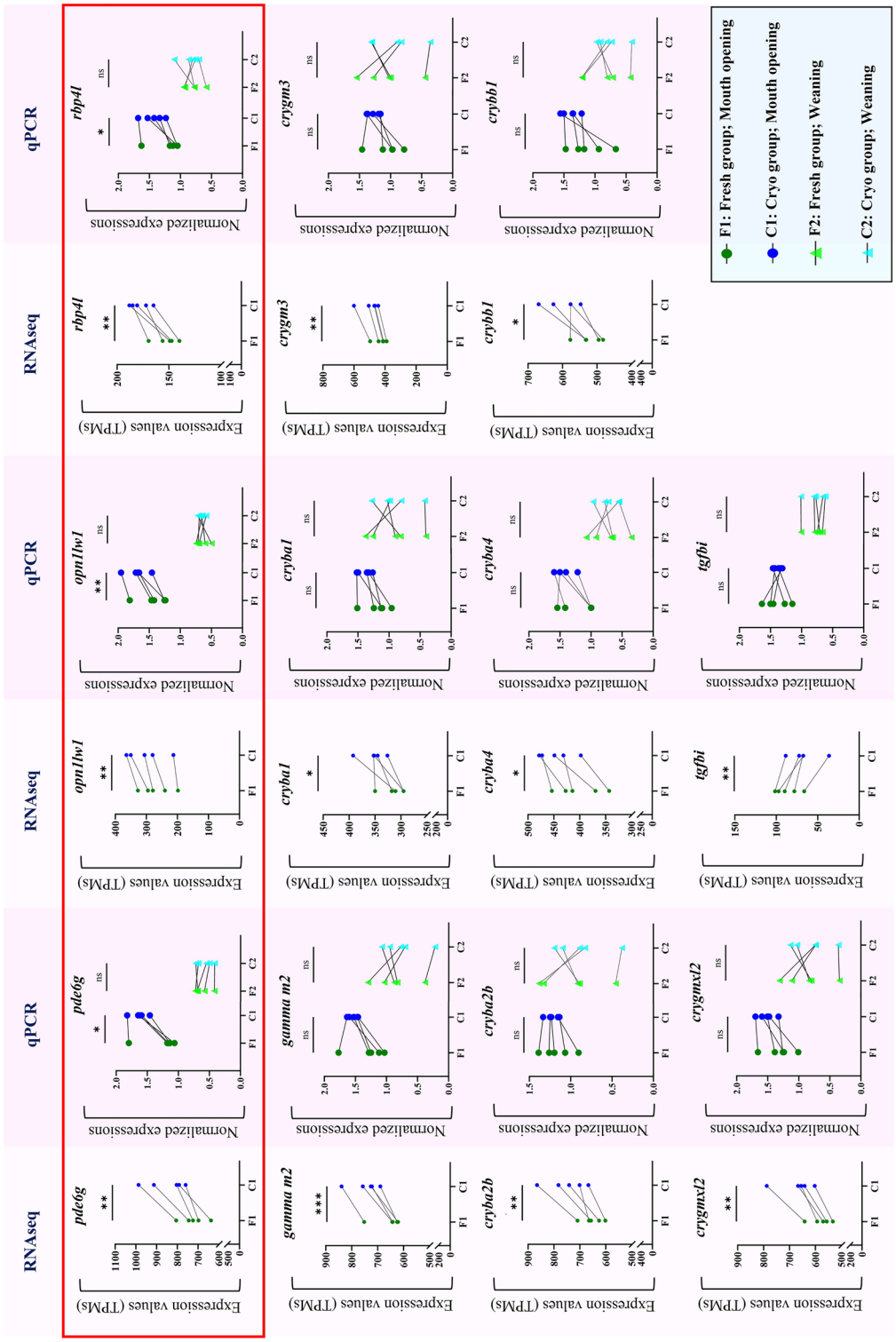
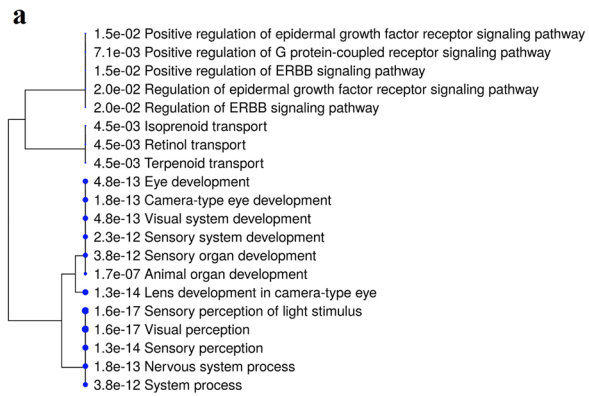
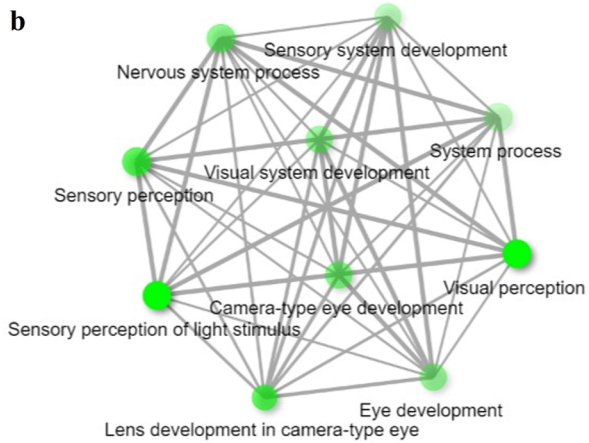
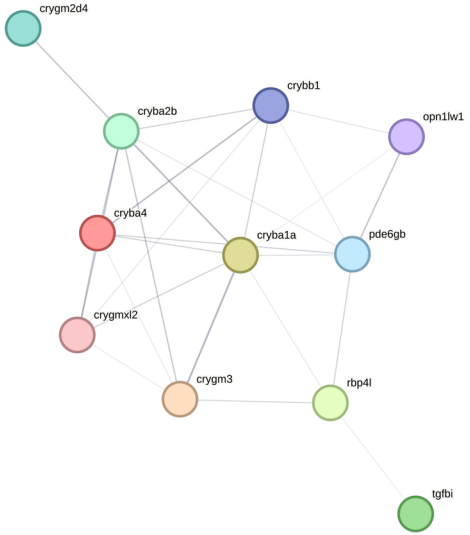


Figure 4. Differentially expressed genes (DEGs). Normalized expression levels of DEGs identified after transcriptomic analysis (RNAseq). Enshadowed lane of graphs (with pink colour) refer to normalized expression level obtained with qPCRs of larvae at MO stage (F1 and C1) and weaning (F2 and C2) between Fresh and Cryo group (n = 5), respectively. The results of statistical analysis are presented as follows: *ns* nonsignificant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).





c.1



c.2

rbp4l	Retinol binding protein 4,-like; Belongs to the calycin superfamily. Lipocalin family. (196 aa)
cryba2b	Crystallin, beta A2b; Belongs to the beta/gamma-crystallin family. (197 aa)
cryba1(cryba1a)	Crystallin, beta A1a; Belongs to the beta/gamma-crystallin family. (196 aa)
cryba4	Crystallin, beta A4; Belongs to the beta/gamma-crystallin family. (196 aa)
pde6g(pde6gb)	Phosphodiesterase 6G, cGMP-specific, rod, gamma, paralog b. (87 aa)
opn1lw1	Red sensitive opsin-1; Visual pigments are the light absorbing molecules that mediate vision. They consist of an apoprotein, opsin, covalently linked to cis-retinal; Belongs to the G-protein coupled receptor 1 family. Opsin subfamily. (357 aa)
crygmxl2	Crystallin, gamma MX,-like 2; Belongs to the beta/gamma-crystallin family. (176 aa)
gamma m2(crygm2d2)	Crystallin, gamma M2d2; Belongs to the beta/gamma crystallin family. (175 aa)
tgfbi	Transforming growth factor, beta induced. (677 aa)
crybb1	Crystallin, beta B1; Belongs to the beta/gamma-crystallin family. (253 aa)
crygm3(crygm2d4)	Crystallin gamma embryonic M2 type 3; Belongs to the beta/gamma-crystallin family. (173 aa)

Figure 5. In silico analysis of DEGs. (**a**) A hierarchical clustering tree summarizes the correlation among significant pathways based on the gene ontologies. Here 20 most enriched biological processes were clustered, bigger dots indicating more significant p-values. (**b**) Relationship between 10 most enriched biological processes. Two pathways are connected if they share 20% or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes. (**c.1**) STRING-db chart showing proteins (encoded by DEGs) interactions and; (**c.2**) name of the proteins (bracketed protein names are the isoforms of the same proteins in *D. rerio*).

In silico verification of DEGs as PEGs

To further investigate if the DEGs are of paternal origin, we checked for their presence in unfertilized eggs (UFE) of fish species for which data on the transcriptomic profile of UFE are available (Fig. 6). If any genes would have been abundant in UFE, we would have to reject the hypothesis that the DEGs are PEGs. As a result, we did not observe any pattern in the expression of DEGs in UFE across different species analyzed. For example, from among DEGs identified in our study we did not detect their expression in any percids' UFE (i.e. *P. fluviatilis* and *S. lucioperca*). However, 2 out of 11 DEGs were found to be present in *D. rerio,* 4 out of 11 in *O. mykiss* and 1 out of 6 in *M. anguillicaudatus*, and the ones present in different species were always different genes. Next, to additionally confirm their possible paternal influence their expression profile along the embryonic development was examined. This was done in order to check whether their expression starts after ZGA, which could strenghten our assumption that these genes play a role as PEGs since their expression starts after the paternal genome is already playing a role in the embryonic development. The results of this analysis (Fig. 7) allowed us to confirm that these genes are expressed long after ZGA, which additionally supports the hypothesis that their expression could be under the influence of males.

Tissue distribution of the DEGs

Given the literature background and the results of our experiment, where such important genes are differentially expressed, we wondered whether they are finite to just visual-sensory metabolic pathways or whether they have more to contribute to organism development. In this pursuit, we conducted in silico analysis to determine the tissue distribution of these genes across the evolutionarily distinct taxa (Fig. s3). We found that the expression of these genes was not limited to the eyes and/or brain, which could be expected for the genes directly linked with the visual system. These genes in the evolutionarily blind species cave Mexican tetra (*Astyanax mexicanus*), which has adapted to the cave environment, were also found to express these genes in various tissues[64,65].

Discussion

In fish reproductive biology research, it is commonly assumed that most of the variation in ELH in fish is attributable to parental genome and the environment provided to the progeny. However, over the years research is adding up to this fact that both the mother and the father contribute to progeny quality also via the non-genetic mechanisms[9,11]. The present study was aimed towards examining paternal effects on ELH traits in Eurasian perch by fertilizing eggs of individual females with either fresh or cryopreserved milt from the same male. Using cryopreservation as a "selection pressure" for sperm 'populations' derived from individual males, we found this had a modulatory effect on progeny's transcriptomic profile and their performance in aquaculture conditions. Consequently, we show, for the first time that paternally inherited factors may have a significant influence on the visual system via targetted gene expression modulation in the progeny.

Results of our study indcate that only the cryo-resistant cells that remained motile after thawing became carriers of genetic and non-genetic information to pass on to the next generation. It has been demonstrated in rainbow trout that sperm cryopreservation did not affect fertilization rates[66], and has no effects on development and survival during the embryo stage. However, fertilization of eggs using cryopreserved sperm led to significantly reduced larval growth after hatching[27]. This is in contrast to our study, where after using cryopreserved milt for fertilization of eggs it resulted in increased WBW of the larvae compared to larvae obtained with fresh milt at the end of our experiment. This allows us to hypothesize, that in Eurasian perch cyopreservation-induced changes are causing permanent alterations to the cryosensitive subpopulation of sperm cells which then become non-functional and are not participating in fertilization. In other words, the effects of cryopreservation observed in our experiment were mediated by changes to the composition of the sperm population, rather than changes to individual sperm.

In the last decades, non-genetic inheritance and transgenerational inheritance is being studied profoundly[67,68]. Until now the paternal non-genetic inheritance mechanisms have been associated with the methylation pattern of the genome, which is then transferred to the progeny[69]. From this perspective the overexpression of the 11

	cryba2b	*cryba4*	*crybb1*	*crygmxl2*	*gamma m2*	*crygm3*	*cryba1*	*tgfbi*	*pde6g*	*opn1lw1*	*rbp4l*
Perca fluviatilis	X	X	X	X	X	X	X	X	X	X	X
Danio rerio	X	X	✓	X	X	X	✓	X	X	X	X
Oncorhynchus mykiss	X	✓	X	✓	X	X	X	✓	✓	X	X
Sander lucioperca	X	X	X	X	X	X	X	X	X	X	X
***Misgurnus anguillicaudatus*	N/A	N/A	X	N/A	X	X	X	X	✓	N/A	N/A

Figure 6. A pictograph for the presence of our candidate genes and genes validated by qPCR (written in pink and shaded in purple, respectively) in UFE in *P. fluviatilis*, *D. rerio*, *O. mykiss*, *S. lucioperca*, *M. anguillicaudatus*. (Reference UFE sequencing done as described earlier for *P. fluviatilis*, from PhyloFish and Expression atlas (TPMs > 0.5 considered); **Results based on TMM counts[62]; N/A stands for data not available) ✓ represents presence while X represents absence of a particular gene in that species.

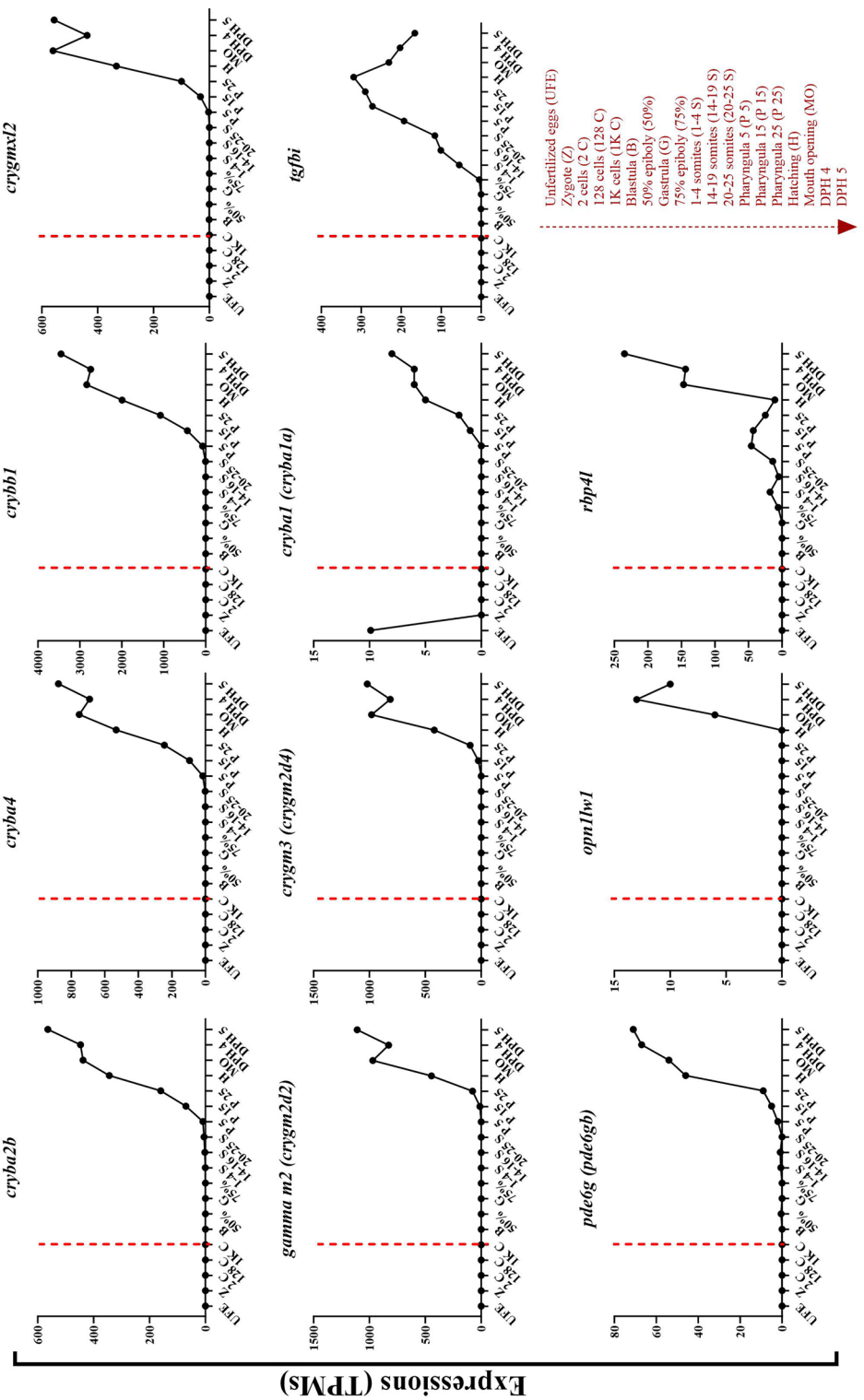


Figure 7. Expression level of identified PEGs along the zebrafish early development (data extracted from White et al.[84]). Abbreviations used on the x axes are explained on the right-bottom with the broken arrow indicating time course. Broken lines on each graph indicate the moment of zygotic genome activation.

identified PEGs (in Cryo group) in our study suggest heterogeneity of the sperm cells within the same sperm sample representing distinct populations in terms of cryo-resistance and possibly epigenetic status. It is important to note, that in the same individual fish various subpopulations of spermatozoa with different effect on the progeny's phenotype can be identified[70,71]. Additionally, it has already been reported that the cryopreservation of fish sperm is a selective process indicating existence of various subpopulations of sperm, with different cryo-resistance, within the same sperm sample[72]. Therefore, we can suppose that cryo-resistant, and thus functional spermatozoa (the ones which can actually reach the micropyle and contribute to the development of the embryo) from the Cryo group may have different methylation state when compared to functional spermatozoa in the fresh sperm (where generally more spermatozoa are capable of contributing to fertilization, also the ones with modified epigenetic status), allowing us to observe deregulated expression of these genes in the progeny. Considering our results, this brings us closer to the hypothesis that the sperm yielding hypermethylation of these genes are the ones possibly being cryo-sensitive leading to losing their fertilizing capacity. Of course, the confirmation and possible understanding of the exact mechanisms are to be elucidated in the future, but at this point this seems to be among plausible explanation of overexpression of the PEGs identified in our study. However, we cannot dismiss the possibility that also other non-genetic factors, either independently or in combination with the cell's methylation state, that might contribute to the observed differences. Having in mind that the proteomic profile of cryo-resistant spermatozoa before and after cryopreservation is really minor[72], the possible alternative mechanisms could include the role of small non-coding RNAs, which have been reported to be sperm-derived carriers of important heritable information, as potential influencers[73].

We observed no significant differences in larval performances at their early life stages from the phenotypical point of view except for one important trait of the offspring, being the WBW, recorded to be higher at the end of the growth trial in Cryo group. This is contradictory to other studies, where fish obtained using cryopreserved sperm for fertilization were characterized by lower phenotypical performance[27]. It should be highlighted, that from among all the DEGs identified in our study, 10 genes were related to the visual system development, and all of them were upregulated in the Cryo group. This allows us to hypothesize that upregulation of these genes aids the development of visual organs in Cryo group and, consequently, facilitating catching their prey faster and more efficiently. As compelling as this result may sound, we want to emphasize that it is only a supposition, and observed phenotypic consequences could also possibly stemming from interplay of these genes with the remaining transcriptomic repertoire or other molecules. Therefore, this hypothesis should be critically tested during a specifically designed future study.

In this study, we have demonstrated that fertilization of eggs with cryopreserved milt resulted in overexpression of genes related with the eye development. Most of these genes (*crygmxl2*, *cryba2b*, *cryba4*, *crybb1*, *cryba1* and *crygm3)* belong to the crystallin superfamily of genes with highly abundant proteins in vertebrate lineages. They are anciently identified in vertebrates and nonchordates, as α-, β-, and γ-crystallins as main sub-families[74]. Underwater, the lens alone provides almost all the focusing power in fish, while in terrestrial species, the cornea provides most focusing power and the lens is mainly used for fine control of image formation[75]. Certain orthologs of *cryba2b* and *crygmxl2* are directly involved in lens formation. This was demonstrated by Krall et al.[76], when they used zebrafish model and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology to lose the function of lens developing gene regulatory networks (GRNs) in *foxe3* mutant. They observed smaller eyes and defective lens formation 72 hours post fertilization, after the zygotic genome activation has already begun. The Crystallin family genes which have turned out to be DEGs in our experiment does not limit only to fishes but expands to higher vertebrates like human cataract lens[77]. It should be highlighted, that our positively validated candidate genes, *pde6g, opn1lw1* and *rbp4l* are found as orthologs in Atlantic salmon where they were found to be responsible for ocular cataract disorders along with other genes, increasing the prevelance of vertebral deformities[78]. The homologs of *pde6g* are been vigorously studied in lower vertebrate model species, as it is one important gene for many retinal degeneration diseases[79]. However, the functionality of these genes has not been evaluated basing on the food intake efficiency. Our results, for the first time indicate direct linkage between DEGs responsible for eyes development with weight of larvae which indicates male influence. Moreover, our in silico analysis of expressions kinetics along the embryonic development in zebrafish (as shown on Fig. 7) confirmed that all the 11 genes are being extensively expressed long after 1K cell stage i.e. after the zygotic genome activation[80]. Lack of considerable expression before that event provides direction that paternally-derived molecular cargo is important in shaping the expression of these genes. Our study provides for the first time, an indirect evidence that this important group of genes, could have role in development of eyes in fishes and other taxa, and are under paternal influence.

As mentioned, major function of the DEGs identified in our study is clearly related to the development of the visual system in fishes. However, the analysis of tissue distribution (see Fig. s3) indicates that their function is somehow more complex than only to development of the eyes. This is especially evident when comparing two forms of the same species – eye-less cave Mexican tetra (*Astyanax mexicanus* - cave) and surface Mexcan tetra (*Astyanax mexicanus* – surface), where the cave form is having multi-tissue expression of these genes despite not developing eyes at all. This indicates, that paternal effect over the expression of the genes identified in our study may have much more wider consequences, not limited to the formation of the eyes. This also partially explains the compensation of expression of the genes at the whole organism level observed at the end of the study. However, a very important point to note here is that since we checked the gene expression differences in larvae at the weaning stage, subjecting the whole oragnism to qPCR, this might have masked the differential expression of our DEGs. In the future, it would be more accurate to study gene expression patterns in tissues-specific manner in developing progeny. It has been reported that the eyes in Eurasian perch at hatching are constituting significant component of the entire body, as the visual system is crucial for survival of the larvae[81]. Later the eyes are not growing anymore so rapidly as the rest of the body, especially the organs reponsible for the digestion, stating one more reason to maintain tissues-specificity during future analysis. Having in mind, that all the DEGs were

found to be expressed in at least some of the digestive-system-related organs in various species (See Fig. s3), we may suggest that the differences in expression of these genes in eyes were simply blurred by the expression of those genes in much more rapidly developing organs. This brings our attention to the fact, that our approach (i.e. studying transcriptome of the whole larvae right after hatching – at mouth opening stage) has been suitable for identification of the novel PEGs, but also has a certain limitations stemming from biases coming from allometric development of various organs during the larval stages. Therefore, a more combined approach, with various research techniques, is recommended in the future studies to explore the paternal contribution in a more holistic way. Nevertheless, thanks to the approach employed in our study we were able to identify novel PEGs and draw prospects for future works focusing on the visual development as a paternally-contributed process.

Conclusions and future aspects

In the present study, our results clearly demonstrate the robustness of sperm cryopreservation to explore paternal contribution to the progeny in Eurasian perch. Cryopreservation being used as a challenge test here, exhibited the "survival of the fittest" trait in sperm, and we could identify PEGs. Using phenotypical and transcriptomic approach we observed that the larvae by the end of the rearing period were higher in weights possibly because of the higher expression of genes responsible for the development of eyes in the Cryo group. Here, we refer to DEGs identified, mostly responsible for the visual perception and lens formation that helped the larvae from Cryo group to feed on their prey more efficiently. We also confirmed the absence of expressions of these genes in UFE which means that their expression is not from a maternal genome, but is under paternal effect. Furthermore, we learnt that the role of these genes is not just confined to the development of the eyes but also several other tissues of fish species varying on the phylogenetic tree, including blind Mexican tetra. With this study, we identified novel PEGs and a future direction to learn more about how does the father determine gene expression patterns in the progeny. With our findings, and from a fundamental scientific angle, we also show that sperm-selection-mediated phenotypic consequences are clearly an overlooked type of paternal effect which warrants further study about its mechanisms, consequences, and evolutionary importance in different taxa. Additional studies on the basis of current findings would include the behavioural changes in larvae obtained after fertilizng eggs with either fresh or cryopreserved sperm, what would allow to verify the hypothesized here phenotypic consequences in progeny. Additionally, it would be highly interesting to develop specific scientific approach to test whether cryopreservation-induced alteration of intrasperm variability can be selective for certain sperm genotypes in fishes[82,83]. This study underscores the significance of our understanding of paternal contributions and encourages to undertake even more challenging endeavors that may bring significant advancements in the fields of fish developmental biology and aquaculture.

Data availability

Raw data of RNAseq used for analysis in the present study can be accessed via the NCBI SRA database (PRJNA1073695).

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Competing interests

The authors declare no competing interests.

Additional information

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