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The transcriptome of newly hatched larvae as a window into the exploration of parental legacy and early life performance in Eurasian perch

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Abstract

Early developmental stages in teleost fish are characterized by profound morphological, physiological, and molecular changes that determine survival and long-term performance. In aquaculture, the larval phase remains a critical bottleneck, with mortality and variability in growth often linked to both intrinsic (genetic and non-genetic inheritance) and extrinsic (environmental) factors. This thesis investigates the transcriptomic profile of Eurasian perch (*Perca fluviatilis*) larvae at the mouth-opening stage - a pivotal developmental point that coincides with hatching and marks the transition to functional independence - to evaluate its potential as a source of information on inherited traits and developmental potential.

A stepwise, "zoom-in" experimental framework was applied across four complementary studies. In the larval transcriptome experiment (Chapter 3), newly hatched larvae from 16 different families were profiled transcriptomically to assess the larval transcriptome as a molecular glimpse of both parental legacy and early developmental status. In the Dom-Wild experiment (Chapter 4), the influence of parental origin was examined by comparing offspring from wild and domesticated broodstock, disentangling maternal and paternal contributions. Building on these findings, the egg quality experiment (Chapter 5) investigated maternal effects by examining how variation in egg quality influences offspring performance. Finally, intra-batch variation was investigated in the heart oedema case study (Chapter 6), comparing transcriptomes of deformed larvae with those of normal siblings to identify gene expression patterns associated with developmental anomalies. Across all the study, larvae were reared under standardized recirculating aquaculture system conditions, and zootechnical traits, including growth, survival, swim bladder inflation, and deformity rates, were recorded at multiple developmental checkpoints. Integration of these phenotypic data with transcriptomic analyses provided a multi-level view of early ontogeny.

Results obtained during realization of this thesis indicate that the larval transcriptome at mouth opening ("the present") contains molecular signatures reflecting both parental contributions ("the past") and factors influencing future developmental trajectory ("the future"). Specifically, maternal effects emerged as particularly prominent, especially during the earliest stages of embryogenesis, yet a dynamic and compensatory interplay between maternal and paternal factors was also evident during development. These findings challenge the traditional view of strictly coordinated parental contributions, instead revealing a flexible interaction in which paternal inputs may modulate or buffer maternal influences in a context-dependent manner. The "present" view of the larval transcriptome reveals it as a dynamic snapshot of physiology at the mouth-opening stage, marked by the activation of metabolic pathways supporting, among others, the transition

from endogenous to exogenous feeding, the maturation of sensory systems for prey detection and environmental interaction. This stage-specific molecular profile not only reflects the coordinated events required for immediate survival and functional independence but also harbors predictive information linking the present state to future performance potential. In this way, the transcriptome at mouth-opening bridges inherited molecular legacies with the developmental trajectory ahead.

Overall, this work demonstrates that the larval transcriptome at the mouth-opening stage captures both - inherited molecular legacies and indicators of prospective developmental outcomes - providing a framework for deeper investigation of early-life processes in fish. This is the first study in finfish to combine standardized zootechnical phenotyping with transcriptomic profiling at such an early stage. Beyond its contribution to fundamental knowledge, the approach offers a conceptual and methodological framework that could inform selective breeding, broodstock management, and early-life quality assessment in aquaculture, while also supporting broader ecological and evolutionary studies of early vertebrate development.

Streszczenie

Wczesne stadia rozwojowe ryb kostnoszkieletowych charakteryzują się gwałtownymi zmianami morfologicznymi, fizjologicznymi i molekularnymi, które determinują zarówno przetrwanie, jak i zdolność organizmu do dalszego życia. W akwakulturze etap larwalny pozostaje wciąż krytycznym, „wąskim gardłem", a wysoka śmiertelność i zróżnicowanie tempa wzrostu w tym okresie, wynikają zarówno z czynników wewnętrznych (dziedziczenie genetyczne i niegenetyczne), jak i zewnętrznych (środowisko) działających na larwy.
W niniejszej rozprawie przeanalizowano profil transkryptomiczny larw okonia europejskiego (*Perca fluviatilis*) w momencie otwarcia pyska – kluczowym etapie rozwoju, który następuje tuż po wykluciu i oznacza przejście do samodzielnego funkcjonowania. Celem badania była ocena przydatności tego profilu jako źródła informacji o dziedziczeniu rodzicielskim oraz potencjale rozwojowym larw.

W czterech komplementarnych doświadczeniach zastosowano stopniowe, „przybliżające" podejście eksperymentalne, w którym wyniki jednego eksperymentu stawały się „punktem wyjściowym" do kolejnego, powodując tym samym coraz głębsze „zanurzenie się" w problem badawczy. Dlatego też, w eksperymencie pierwszym, dotyczącym transkryptomu larw (Rozdział 3) poddano profilowaniu transkryptomicznemu nowo wyklute osobniki z 16 różnych rodzin, aby określić molekularne ślady dziedziczenia rodzicielskiego oraz ich wczesny status rozwojowy. W kolejnym eksperymencie Dom-Wild (Rozdział 4) zbadano natomiast wpływ pochodzenia rodziców, porównując potomstwo pochodzące od rodziców dzikich i udomowionych, co umożliwiło rozdzielenie efektów matczynych i ojcowskich wpływających na larwy. Wreszcie, opierając się na tych wynikach, w eksperymencie dotyczącym jakości jaj (Rozdział 5) oceniono wpływ czynników matczynych, analizując, w jaki sposób zmienność jakości jaj determinuje przeżywalność potomstwa. Ponadto, w badaniu dotyczącym odmy sercowej (Rozdział 6) skoncentrowano się na zmienności wewnątrz poszczególnych rodzin, porównując transkryptomy larw zdeformowanych (posiadających odmę sercową) z ich zdrowym rodzeństwem, w celu identyfikacji genów powiązanych z anomaliami rozwojowymi. We wszystkich doświadczeniach larwy hodowano w standardowych warunkach recyrkulowanych obiegów zamkniętych (RAS), monitorując ich cechy zootechniczne, takie jak: wzrost, przeżywalność, napełnienie pęcherza pławnego oraz częstość deformacji na najbardziej znaczących etapach rozwoju. Integracja uzyskanych danych fenotypowych z wynikami analiz transkryptomicznych pozwoliła uzyskać wielopoziomowy obraz wczesnej ontogenezy.

Wyniki uzyskane podczas realizacji niniejszej rozprawy wskazują, że transkryptom larw w momencie otwarcia pyska (czyli „teraźniejszość”) zawiera wzorce molekularne odzwierciedlające zarówno wkład rodzicielski („przeszłość”), jak i czynniki wpływające na przyszłą trajektorię rozwoju („przyszłość”). Szczególnie silny okazał się wpływ matczyny obserwowany we wczesnych etapach embriogenezy, jednak w trakcie rozwoju widoczna była również dynamiczna i kompensacyjna interakcja między czynnikami matczynymi i ojcowskimi. Uzyskane wyniki kwestionują tradycyjny pogląd o sztywnym podziale wpływów rodzicielskich, wskazując raczej na „elastyczną interakcję”, w której wkład ojcowski może modulować efekt matczyny w zależności od kontekstu. „Teraźniejszy” obraz transkryptomu larwy ukazuje go więc jako dynamiczny zapis fizjologii w momencie otwarcia pyska, charakteryzujący się aktywacją szlaków metabolicznych, wspierających między innymi przejście z odżywienia endogennego na egzogenne, rozwój układów sensorycznych, odpowiedzialnych za detekcję pokarmu oraz pierwsze interakcje ze środowiskiem. Profil molekularny charakterystyczny dla tego etapu nie tylko pokazuje skoordynowane procesy potrzebne larwom do przeżycia i szybkiego uniezależnienia się, ale też zawiera wskazówki pozwalające przewidywać ich dalszy rozwój. W ten sposób transkryptom larw w momencie otwarcia pyska łączy odziedziczone sygnatury molekularne z przyszłą trajektorią rozwojową.

Podsumowując, niniejsza praca wskazuje, że transkryptom larw w momencie otwarcia pyska rejestruje zarówno odziedziczone sygnatury molekularne, jak i wskaźniki dalszych możliwości rozwojowych, tworząc ramy dla głębszych badań procesów wczesnej ontogenezy u ryb. Jest to pierwsze badanie dotyczące ryb kostnoszkieletowych, w którym połączono wystandaryzowane fenotypowanie zootechniczne wraz z profilowaniem transkryptomicznym na tak wczesnym etapie życia ryby. Oprócz wkładu w podstawową wiedzę biologiczną, podejście to oferuje nowe ramy koncepcyjne i metodologiczne, które mogą stać się przydatne w hodowli selektywnej, zarządzaniu stadami rozrodczymi i ocenie jakości larw na najwcześniejszym etapie ich życia w warunkach akwakulturowych. Przedstawione podejście wspiera także szersze badania ekologiczne i ewolucyjne dotyczące szeroko rozumianego wczesnego rozwoju kręgowców.

Abbreviations

ALH	Amplitude of Lateral Head Displacement
ANOVA	Analysis of Variance
$CaCl_2$	Calcium Chloride
CASA	Computer-Assisted Sperm Analysis
cDNA	Complementary DNA
CV	Coefficient of variation
D	Deformed
DD	Domesticated♀ × Domesticated♂
DW	Domesticated♀ × Wild ♂
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
DPH	Days post hatching
DR	Deformity rate
EDR	Embryonic developmental rate
ELH	Early life history
END	End of the experiment/larval period
FDR	False Discovery Rate
FF	First feeding
FL	Fork length
FPKM	Fragments per kilobase of transcript per million mapped reads
FULTON	Fulton's condition factor
FULTON_END	Fulton's condition factor at the end of the experiment
GM	Glucose-methanol
GO	Gene Ontology
GS	Gene significance

HPF	Hours post fertilization
HR	Hatching rate
KCl	Potassium Chloride
KTA	Key traits for aquaculture
L:D	Photoperiod notation indicating hours of light (L) followed by
LIN	Linearity
log_2FC	Logarithm (base 2) Fold Change
MBT	Mid blastula transition
MCC	Maximal Clique Centrality
ME	Module eigengene
$MgSO_4$	Magnesium sulfate
miRNA	Micro RNA
MO	Mouth opening
MOT	Motility
mRNA	Messenger ribonucleic acid
MS-222	Tricaine Methanesulfonate
MZT	Maternal-to-zigotic transition
N	Normal
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NGI	Non-genetic inheritance
NGS	Next generation sequencing
OD	Oil droplet
p	p-value
padj	Adjusted p-value
PBS	Phosphate-buffered saline

PCA	Principal component analysis
piRNA	Piwi-interacting RNA
PPI	Protein-protein interaction
RAS	Recirculating aquculture system
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SBIE	Swim bladder inflation effectiveness
SD	Standard deviation
SG	Onset of somitogenesis
sGnRHa	Salmon gonadoliberin releasing hormone analogue
SGR	Specific growth rate
SGRL_TOT	Specific growth rate for length data for the entire larviculture
SGRW	Specific growth rate for weight
SGRW_TOT	Specific growth rate for weight data for the entire larviculture
SYBR	SYBR Green I
sncRNAs	Small non-coding RNAs
τ	tau index
TD	Tail detachment stage
TL	Total length
TPM	Transcripts Per Million
UFE	Unfertilized eggs
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-Line Velocity

W	Weaning
W	Wild
WBW	Wet body weight
WD	Wild♀ × Domesticated♂
WGCNA	Weighted gene co-expression network analysis
WW	Wild♀ × Wild♂
YS	Yolk sac
ZGA	Zygotic-genome activation

Chapter 1: General Introduction

"We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time."

(T.S. Eliot, Little Gidding, Four Quartets -1942-)

1.1 Fish in the world

Fishes are among the most widespread and yet most mysterious and astonishing life forms on Earth. Over the course of approximately 500 million years of evolutionary history, fishes have colonized nearly every aquatic habitat in the world, from temporary desert springs and high-altitude lakes to the icy polar seas and the abyssal zones of the deep ocean (Stepien & Haponski, 2015). With over 36,000 recognized species, they are the most diverse and abundant group of vertebrates, exhibiting unmatched phenotypic and ecological variation (Nelson et al., 2016).

Within this vast diversity of fish species, the order *Perciformes* stands out as one of the largest and most ecologically diverse groups of teleosts (Nelson, 2006). Encompassing a wide range of forms and habitats, perciforms have diversified extensively across both marine and freshwater systems. Among them, the family *Percidae* comprises a group of freshwater fishes that have successfully radiated throughout the Northern Hemisphere. This family, which includes over 200 species, such as perches (*Perca spp.*), darters (*Etheostoma spp.*), and pike-perches (*Sander spp.*), is well known for its ecological adaptability and key roles in freshwater ecosystems (Stepien & Haponski, 2015). In addition, some species, like the Eurasian perch (*Perca fluviatilis*), hold significant commercial and recreational value, leading to growing interest in its aquaculture potential and effective management of wild stocks. Their evolutionary success and ecological plasticity make them valuable models for studies in adaptive physiology, population genomics, and species diversification.

1.1.1 Eurasian perch as a valuable model species

The Eurasian perch stands out as a prominent member of percids family, showcasing remarkable ecological adaptability and a wide geographic distribution (Ning et al., 2025). As an euryvalent species, it exhibits a high tolerance to environmental fluctuations, thriving across diverse aquatic habitats, ranging from freshwater lakes, rivers, and ponds to brackish estuarine systems (Ning et al., 2025; Vasemägi et al., 2023). This physiological and behavioral flexibility has made *P. fluviatilis* a key model organism in ecological, evolutionary, and fisheries research (Ning et al., 2025).

In the last three decades, the Eurasian perch became a viable candidate for expanding European freshwater aquaculture, particularly within recirculating aquaculture systems (RAS), which is a sustainable and technologically advanced method of intensive fish farming (Fontaine & Teletchea, 2019). Its compatibility with RAS stems from its moderate growth rate, robust environmental

tolerance, and strong market appeal. While the foundational aspects of closed lifecycle aquaculture and domestication are well established for this species, several practical challenges remain unresolved (Teletchea & Fontaine, 2014), especially in achieving consistent larval survival, optimizing early growth performance, and improving reproductive efficiency. These limitations underscore the need for comprehensive, multidisciplinary research focused on elucidating the biological and environmental determinants of early-life-stage performance, ultimately to improve production consistency and support selective breeding efforts.

From among all the percids, Eurasian perch has been pointed out to be the most robust model for various areas of research, including domestication (Chen et al., 2017; De Almeida et al., 2019; Fontaine & Teletchea, 2019), reproductive physiology (Żarski et al., 2012; 2017a; 2019), chronobiology (Migaud et al., 2006; Strand et al., 2007; Żarski et al., 2021a), embryonic and larval biology (Alix et al., 2015, 2017; Henrotte et al., 2010; Palińska-Żarska et al., 2020,2021), as well as growth and nutrition (Kestemont et al., 2001; Król et al., 2019; Kupren et al., 2019), among others. This widespread research interest in Eurasian perch has been supported by the development of reliable, standardized experimental protocols for controlled reproduction, *in vitro* fertilization (Żarski et al., 2017b, 2019), as well as for larval rearing (Palińska-Żarska et al., 2019, 2020, 2021). Together, these advances have reinforced the species' value as a model for experimental studies on early life stages, enabling reproducible and biologically meaningful investigations into larval development, physiology, and performance. Furthermore, the standardized and reliable methodologies developed in Eurasian perch can be translated to other species of ecological and aquaculture importance, supporting the refinement of hatchery technologies, broodstock management, and larval rearing protocols across diverse taxa.

1.2 “Once upon a larva” (Pepin et al., 2014)

The life cycle of most teleost fish, including Eurasian perch, unfolds through four distinct stages: egg, larva, juvenile, and adult. Fertilization marks the beginning of the egg stage, which continues until hatching. At this point, the fish enters the larval stage, a critical period of rapid morphological and physiological development. This ends with metamorphosis into the juvenile phase, during which the fish gradually acquires adult-like features. The final transition occurs as the fish reaches sexual maturity, marking the adult phase (Urho, 2002).

Among these, the larval stage is arguably the most biologically complex and developmentally dynamic. It marks the transition from embryonic dependency to functional autonomy and this is the time where essential biological processes, such as organogenesis, feeding, sensory

development, and behavioural patterns, begin to unfold (Osse et al., 1997). This is where the foundation of individual performance and survival is laid, and thus where the future trajectory of each individual is being determined by interplay of intrinsic and extrinsic factors.

The scientific interest in larval fish biology, or ichthyoplankton research, began in the late 19th century. A foundational milestone came in 1865, when G.O. Sars identified floating cod eggs and described their pelagic nature, highlighting the ecological significance of early life stages in marine teleosts (Hempel, 1979). By the early 20th century, research had progressed toward larval rearing and ontogeny, driven by both scientific curiosity and concerns over declining fish stocks. Pioneering work by Hjort (1914), Blaxter (1969), and Hempel (1979) laid the groundwork for experiments towards a better understanding of the early life stages in fish.

In aquaculture, the larval stage remains a critical bottleneck due to its high mortality rates, which impact the production success (Pepin et al., 2014). This stage is characterized by substantial variability in individual growth and survival, driven primarily by environmental factors such as photoperiod, temperature, food and oxygen availability, as well as genetic, parental, and epigenetic influences (Jonsson & Jonsson, 2014). These factors collectively influence the larvae's later performance and phenotype (Jonsson & Jonsson, 2014). This complexity makes the larval phase both a constraint and a strategic point of intervention. By improving our understanding of early developmental processes, aquaculture can more precisely manage larval rearing to enhance survival, robustness, and growth.

1.2.1 What's a larva? – Morphological characteristics

At hatching, Eurasian perch larvae are extremely small, measuring between 5-6 mm and transparent. They lack fully formed fins, scales, and other adult features. Instead, they are characterized by a continuous median fin fold, which runs along the dorsal, caudal, and ventral surfaces and functions as a primitive locomotor structure. They have a yolk sac (YS), serving as an internal energy source for the first few days post-hatching (DPH) (Urho, 2002) (**Fig. 1.1**). At this early stage, their digestive system is rudimentary, and they lack a functional stomach, making them incapable of digesting complex feeds. The head is relatively large in proportion to the body and houses prominent sensory organs. Notably, the eyes are the first sensory organs to develop during embryogenesis and, by hatching, are fully pigmented and functional (Kamaszewski & Ostaszewska, 2015). Together, these features depict larvae as morphologically simple yet functionally specialized organisms, firstly entirely dependent on their yolk reserves, basic locomotor and sensory structures for survival. This early stage marks the beginning of a series of

rapid developmental transitions, during which even minor disruptions can have profound effects on growth, survival, and future performance.

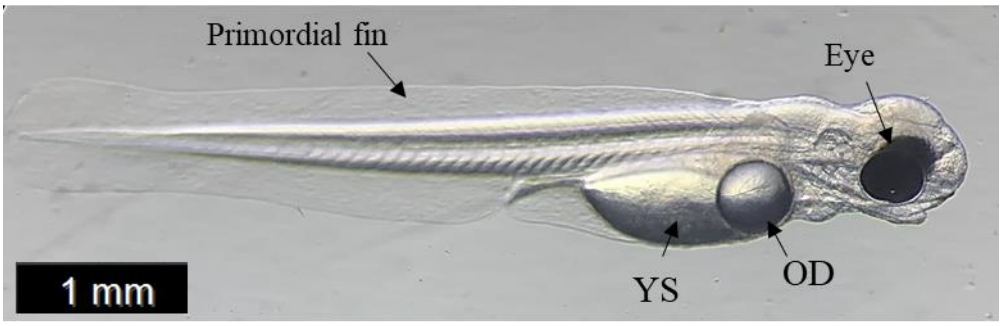

Figure 1.1: *Eurasian perch larva displaying a continuous median fin fold for primitive locomotion, a prominent yolk sac (YS) with oil droplet (OD) for early energy supply, and fully developed pigmented eyes as key sensory organs*

1.2.2 Challenges of the larvae

The larval period is a critical bottleneck in fish ontogeny, where small differences in condition have outsized consequences for survival (Pepin et al., 2014). This vulnerability is often described using the concept of "larval quality" - an integrative assessment made at hatching that combines morphological, physiological, and behavioural traits to estimate a larva's survival potential in both wild and aquaculture environments (Koumoundouros et al., 2017). High-quality larvae typically exhibit normal development, functional organs, and normal swimming behavior. In contrast, poor-quality larvae may show developmental abnormalities, such as spinal curvature, jaw malformations, or yolk and heart oedema, which will impair essential functions like feeding and locomotion and significantly reduce survival rates (Alix et al., 2017) contributing to increased early-stage mortality. While the assessment of larval quality at hatching provides a valuable early indicator of developmental success and a tool for predicting survival outcomes, it is also closely tied to performance during a series of early developmental challenges, including hatching itself, the transition from endogenous to exogenous feeding, swim bladder inflation, yolk sac absorption, cannibalism and the functional maturation of key organ systems such as the nervous, digestive, and immune systems (Osse et al., 1997). Successfully navigating these milestones is critical for future growth, survival, and overall viability.

One of the most crucial transitions in early development is the switch from endogenous (yolk-based) to exogenous (external) feeding. During the first few DPH, larvae rely on yolk reserves. As

these deplete, they must begin foraging to survive. Hjort (1914) introduced the "critical period" concept to describe this vulnerable window, during which many larvae fail to initiate feeding. The causes of this phenomenon, which affects many aquatic species, are still not fully understood. One explanatory framework is the "hydrodynamic starvation" hypothesis proposed by China & Holzman (2014), which suggests that body length and swimming performance are determinants of larval feeding success. Failure to complete this transition successfully represents one of the primary causes of high mortality during early ontogeny (Yúfera & Darias, 2007), and can greatly influence overall larval quality.

Another critical event during the larval stage is the inflation of the swim bladder, which is essential for buoyancy regulation. This process typically occurs when larvae rise to the surface to gulp air, which is then transported via the pneumatic duct from the digestive tract to the swim bladder lumen (Palińska-Żarska et al., 2019). Importantly, for Eurasian perch larvae this window of opportunity is highly time-sensitive, as the pneumatic duct remains functional for only a limited period after hatching - typically between 6- and 12-DPH (Summerfelt, 2013). Additionally, several environmental and biological factors influence the success of swim bladder inflation, including: photoperiod (light conditions), water temperature, salinity, larval body size (Woolley & Qin, 2010). Depending on the species, failure to inflate can lead either to early death or to significant physiological and ecological challenges, such as poor buoyancy control, diminished swimming ability, and stunted growth. For example, in Eurasian perch, failure in inflated swim bladder influences growth and future developmental success of the individual, though it does not cause mortality (Kupren et al., 2019). Also, in yellow perch (*Perca flavescens*), larvae with inflated swim bladders exhibit significantly higher growth rates than those that fail to inflate (Czesny et al., 2005). Similarly, in Japanese medaka (*Oryzias latipes*), larvae with inflated swim bladders display higher oxygen consumption, likely reflecting the increased metabolic demand associated with maintaining a horizontal swimming posture. Such physiological impairments have cascading effects on larval fitness. Reduced swimming efficiency and smaller body size increase susceptibility to predation, hinder prey capture, and lower foraging efficiency, collectively leading to elevated mortality during this vulnerable life stage.

In percid species, cannibalism also significantly impacts larval survival, accounting for up to 50% of mortality within the first two weeks after hatching (Król et al., 2015). This behavior is frequently observed in both freshwater and marine commercially cultivated perciforms, such as Eurasian perch, pikeperch (*Sander lucioperca*) and European seabass (*Dicentachus labrax*) (Kestemont et al., 2003). Cannibalism is often associated with growth heterogeneity within larval

cohorts, where larger individuals tend to prey on their smaller conspecifics, leading to increased early-stage mortality (Kestemont et al., 2003; Król et al., 2019). The origin of this behavior may be linked to alternative feeding strategies or genetic predispositions (Król et al., 2015). Although the precise drivers of cannibalistic selection remain under debate, understanding its underlying causes is essential for developing rearing protocols that reduce mortality and improve larval survival in aquaculture.

Overall, larval survival depends on successfully passing several key developmental checkpoints, such as normal hatching morphology, the onset of independent feeding, and effective swim bladder inflation. These events are crucial indicators of early-life performance and are commonly assessed through measurable zootechnical traits. However, while such observations offer important insights into larval phenotype, they provide limited understanding of the underlying biological mechanisms. To fully grasp why some larvae thrive while others fail, it is essential to look beyond external traits and explore the deeper processes that shape early development.

1.3 Beneath morphological traits - larvae are made of molecules

Although larval morphology offers valuable insights into developmental progression, it captures only the surface of the underlying biological complexity. Beneath these visible traits lies a dynamic molecular network involving RNA transcripts, proteins, hormones, and metabolites that collectively regulate processes such as growth, tissue differentiation, and physiological maturation (Huerlimann et al., 2024). Consequently, the larval phenotype emerges not solely from genetic blueprints but from a complex interplay of molecular inheritance, parental contributions, and environmental cues (Day & Bonduriansky, 2011; de Vienne, 2022). Yet, despite their biological importance, the molecular mechanisms shaping early development in fish remain surprisingly poorly understood, particularly in non-model and aquaculture species (Bobe & Labbé, 2010). A deeper understanding of these mechanisms is essential not only for advancing developmental biology and ecology but also for enhancing selective breeding programs through earlier, more accurate prediction of larval performance and robustness.

1.3.1 Genetic and non-genetic inheritance

Genetic inheritance has long been recognized as a cornerstone of biology, determining how traits are transmitted from one generation to the next (Danchin et al., 2011). Our understanding of inheritance has evolved significantly over the past two centuries. Early theories - ranging from

Lamarck's concept of acquired characteristics to Darwin's natural selection and Mendel's laws of inheritance - laid the groundwork for modern genetics. The discovery of DNA in the 20th century further solidified the genome as the central repository of hereditary information, with genes viewed as the primary units controlling development and function (Uller, 2013).

During fertilization, embryos receive genetic material from both the mother and the father. This DNA contains the instructions required to build, regulate, and maintain the organism. However, it is now clear that inheritance is not limited to DNA alone as this does not explain the vast diversity of phenotypes. This has led to a broader framework of heredity that includes non-genetic inheritance (NGI), which refers to the transmission of phenotypic traits across generations without changes to the DNA sequence (Adrian-Kalchhauser et al., 2020). NGI mechanisms, such as epigenetic modifications (e.g., DNA methylation, histone modification) and environmentally influenced parental molecules deposited in gametes (Uller, 2013), can shape gene expression and phenotype, with effects that in some cases persist across multiple generations (Adrian-Kalchhauser et al., 2020). Rather than serving as a substitute for genetic inheritance, NGI complements it by adding a regulatory layer that reflects environmental context.

Fish are particularly well-suited models for studying NGI due to their external fertilization and high fecundity**,** which enable controlled manipulation and observation of developmental processes from the very earliest stages (Sutton & Wilson, 2019). In species exposed to high environmental variability, such as many fish, understanding both genetic and non-genetic contributions is critical, especially during early life stages when developmental trajectories are established and phenotypic variation emerges. A comprehensive view of complex traits therefore requires integrating all shades of inheritance, both genetic and non-genetic.

1.3.2 Mechanisms of non-genetic inheritance

Non-genetic inheritance encompasses a variety of mechanisms that influence offspring development beyond the DNA sequence itself. These include epigenetic modifications such as DNA methylation, histone acetylation, and non-coding RNAs as well as cytoplasmic factors involving maternally derived mRNAs, proteins, and organelles (Feng et al., 2010; Venney et al., 2020). Many of these molecular vectors are transmitted to gametes during oogenesis or spermatogenesis, acting as biochemical imprints that carry environmental and physiological information (Adrian-Kalchhauser et al., 2020). Although many questions remain, these inherited effects are of particular interest to researchers, as they may enable rapid phenotypic adjustments to environmental change, often within just a single generation (Adrian-Kalchhauser et al., 2020).

1.3.2.1 Epigenetic modifications

Epigenetic modifications play a fundamental role in regulating gene expression and cellular functions during early development. These heritable changes occur without altering the underlying DNA sequence, yet they facilitate developmental processes and enable organisms to respond to environmental cues in ways that can be inherited across generations, potentially influencing evolutionary trajectories (Labbé et al., 2017; Macartney et al., 2018).

DNA methylation typically involves the addition of a methyl group to cytosine residues within CpG dinucleotides, often leading to gene silencing by restricting access of transcriptional machinery to DNA (Labbé et al., 2017). In fish, environmental conditions can trigger methylation changes that are stably inherited and result in phenotypic variation in the offspring. For instance, in brook charr (*Salvelinus fontinalis*), exposure of parents to elevated temperatures during sexual maturation caused heritable shifts in offspring DNA methylation patterns (Venney et al., 2022). Similarly, in shortfin molly (*Poecilia mexicana*), exposure to hydrogen sulfide induced DNA methylation alterations that persisted across generations, even after the environmental stressor was removed (Kelley et al., 2021). While, in European sea bass, a 2 °C increase in water temperature during early larval development induced genome-wide methylation changes and altered gene expression profiles (Anastasiadi et al., 2017). These findings highlight the potential of DNA methylation as a mechanism of NGI through which environmental factors can influence offspring phenotypes and potentially contribute to adaptive responses.

Histone modifications - such as acetylation and methylation of histone tails - regulate gene activity by altering chromatin structure and thereby modulating the accessibility of DNA to transcription factors (Herráez et al., 2021). In zebrafish (*Danio rerio*), these histone marks orchestrate transcriptional activation during the maternal-to-zygotic transition (MZT), a critical developmental milestone (Zhu et al., 2019a). In medaka, histone modifications such as H3K27me3 (trimethylated lysine 27 of histone H3) persist through early embryonic stages, playing a key role in controlling gene expression patterns critical for normal development (Fukushima et al., 2023). Such histone marks underscore their role in controlling transcriptional processes during fish early development, with potential implications for heritable regulation of phenotype.

1.3.2.2 Small non-coding RNAs

Small non-coding RNAs (sncRNAs) - including microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and others - are key post-transcriptional

regulators involved in processes such as gene silencing, mRNA degradation, and transposon suppression (Cheung et al., 2018; Lubzens et al., 2010). In fish, maternal deposition of miRNAs and other sncRNAs has been shown to influence tissue differentiation, growth regulation, and immune responses. For instance, miR-430 is critical for the clearance of maternal mRNAs, thereby facilitating zygotic genome activation (ZGA) and enabling proper cell lineage specification and differentiation (Romney & Podrabsky, 2018). This transition from maternal to zygotic control represents a crucial developmental checkpoint, ensuring that embryonic gene expression is correctly initiated (Pauli et al., 2011). Additionally, paternal stress in zebrafish seems to modify the small RNA composition of sperm which resulted in reduced stress responsiveness in offspring (Ord et al., 2020). Therefore, by acting as molecular messengers of parental experience, sncRNAs contribute to non-genetic inheritance, potentially encoding a form of adaptive plasticity across generations.

1.4 Parental effects

Modern biology increasingly recognizes that the phenotype of an organism is shaped not only by its own genome evolving over generations, but also - in the short term, even within a single generation - by the inherited biological legacy of its parents. Therefore, the parental legacy (encompassing both genetic material and a diverse array of non-genetic contributions) is collectively referred to as parental effects (Badyaev & Uller, 2009). In this context, parental effects are not a distinct mechanism, but rather the integrated outcome of multiple modes of inheritance (Day & Bonduriansky, 2011; Uller, 2008). These contributions influence early development long before the offspring interacts with its external environment, and they are often crucial in setting developmental trajectories (Burton & Metcalfe, 2014). In fish, parental effects can manifest in a wide range of early-life traits - from morphology and metabolism to stress responsiveness and survival - and are already evident during the first stages of embryogenesis and larval life (Bang et al., 2006; Benini et al., 2018; Palińska-Żarska et al., 2020, 2021). These effects can originate from both maternal and paternal sources and are strongly shaped by each parent's physiological condition and environmental history. This underscores the importance of considering both maternal and paternal contributions when aiming to understand the full set of factors driving larval development and performance. However, a central challenge remains: disentangling the specific influences of each parent on offspring phenotype (Bonzi et al., 2024). Understanding parental effects is therefore essential not only for explaining phenotypic variation during early ontogeny,

but also for improving selective breeding strategies and early-life management in aquaculture species.

1.4.1 Maternal effects

Historically, parental effects in fish, as in many other animal taxa, were primarily associated with maternal contributions (Bonzi et al., 2024). Maternal effects refer to the transfer of nutritional resources to the oocyte, particularly lipids and proteins which dominate the composition of the egg yolk and serve key developmental functions (Lubzens et al., 2017). Lipids, especially polyunsaturated fatty acids (PUFAs) such as DHA, EPA, and AA, are essential for embryonic and larval development. They provide structural components for cell membranes and serve as a major energy source before exogenous feeding begins (Rainuzzo, 2020). PUFA composition directly affects traits like growth, immune function, and organ formation and maternal diet is a major determinant of egg lipid quality. For instance, in Eurasian perch, an optimal DHA/EPA/AA ratio of 3:2:2 is critical for producing viable, high-quality larvae (Henrotte et al., 2010; Hou & Fuiman, 2022; Turkmen et al., 2019). While the importance of lipid sources for embryonic development is well established, the mechanisms governing fatty acid transfer and storage in oocytes remain poorly understood. Further research is needed to uncover how maternal experience (i.e., diet, temperature, photoperiod) influences egg lipid composition and how these maternal inputs shape embryonic and larval development over time.

Proteins, alongside lipids, are major components of the fish egg yolk (Reading et al., 2018). Yolk proteins such as vitellogenin (Vtg) not only supply amino acids and energy but also participate in key cellular processes like division and differentiation (Lubzens et al., 2010). Vtg and other maternally derived immune molecules (e.g., immunoglobulins, complement C3, lectins) provide crucial early immune protection, supporting embryonic survival before the immune system becomes active (Sullivan & Yilmaz, 2018; Swain et al., 2006; Wang et al., 2009). Similar to lipids, maternal diet can influence protein composition in eggs, which can affect key reproductive traits such as spawning timing and offspring viability (Reading et al., 2018). While yolk proteins are recognized as key NGI factors, the link between maternal experience, protein composition, and egg quality remains unclear.

More recently, maternal contributions have been shown to include also coding (mRNAs) and non-coding RNAs, which are deposited into the oocyte during oogenesis (Reading et al., 2018). Specifically, maternal mRNAs play regulatory roles during early development, particularly before ZGA, when the embryo is still reliant on maternally derived transcripts for gene expression control

(Reading et al., 2018). While many maternal mRNAs degrade after the MZT, some persist and continue influencing development (Bobe & Labbé, 2010). Notably, transcriptomic studies have shown that maternal mRNA profiles vary between individuals and are shaped by both genetics and environmental conditions, such as temperature (Colson et al., 2019), stress (Andersson et al., 2011), and diet (Hou et al., 2022). Differences in mRNA composition have been linked to egg quality and offspring viability in several species, including Eurasian perch, pikeperch, rainbow trout (*Oncorhynchus mykiss*) (De Almeida et al., 2019; Sullivan et al., 2015; Weber et al., 2021; Żarski et al., 2020a). Together, these findings highlight maternal mRNAs as critical NGI factors, however much remains unknown. The key questions now lie in identifying what types of information are transmitted, how they are encoded, and what functional consequences they have for offspring development and performance. A better understanding of NGI could enable targeted strategies to enhance egg quality, improve larval outcomes, and optimize production efficiency.

1.4.2 Paternal effects

Paternal effects were traditionally viewed as purely genetic, with the male's contribution to offspring development considered limited to the transfer of nuclear DNA through the sperm (Bang et al., 2006). This perspective led to the widespread assumption that fathers played a minimal role in shaping offspring phenotype. However, growing evidence now shows that sperm carries much more than just genetic material, and that paternal contributions extend into the realm of non-genetic inheritance (Immler, 2018). In addition to DNA, sperm also delivers a range of epigenetic and molecular components to the zygote at fertilization, including chromatin modifications, regulatory RNAs (e.g., miRNAs and piRNAs), and proteins (Immler, 2018). These elements can modulate gene expression and developmental processes during the early stages of life (Bonzi et al., 2024). Over the past few decades, a growing body of research has highlighted the importance of paternal effects in shaping early life history (ELH) traits, particularly during the period from fertilization to the end of larval development (Ragsdale et al., 2022; Rideout et al., 2004; Siddique et al., 2017).

Among the most studied mechanisms are sperm-derived DNA methylation patterns, which have been shown to influence gene regulation in the embryo and potentially contribute to transgenerational effects (Jiang et al., 2013; Macartney et al., 2018). More recently, Panda et al. (2024) identified specific paternal-effect genes that appear to influence the development of the Eurasian perch larval visual system, providing further evidence of targeted paternal influence on organogenesis and sensory development. Together, these findings underscore those paternal

contributions extend well beyond the genetic code, playing an active and diverse role in shaping offspring development and early-life performance.

1.5 Larval phenotypic assessment

Phenotype, in its classical sense, refers to the set of observable traits of an organism, such as size, shape, color, or growth rate. Over time, however, this concept has expanded to include any other feature across multiple biological levels, from molecular (e.g., transcript, protein, metabolite levels) and cellular, to physiological and behavioral traits (de Vienne, 2022). In larvae, as in other developmental stages, phenotypic assessment may involve recording morphological and developmental characteristics alongside molecular analyses, such as transcriptomics, to reveal gene expression patterns. This integrative approach captures phenotypic variation arising from both genetic and NGI factors, as well as environmental influences, enabling a comprehensive, multilevel characterization of larval development and performance.

1.5.1 Advanced zootechnics

Accurate phenotypic assessment is the foundation of fish biology, particularly in aquaculture, where understanding growth, survival, and robustness is essential for both basic research and breeding strategies. For species like Eurasian perch, zootechnical methods have been developed to standardize the measurement of key traits such as body length, weight, and survival rate (Debernardis et al., 2025; Panda et al., 2024). Before molecular tools became standard also in aquaculture, these observable traits served as the primary basis for evaluating fish quality and guiding selection. For example, larger individuals were often preferred, under the assumption that size reflected superior fitness or, in females, better reproductive potential (Jorgensen et al., 2011; Marshall et al., 2010). As the field advanced, researchers selected key traits for aquaculture (KTAs) - quantifiable indicators of developmental success and performance across life stages (Toomey et al., 2021). Among these, early-life KTAs, such as body size, swim bladder inflation, feeding activity, cannibalism and survival remain especially important during the larval stage. While seemingly basic, these indicators offer a practical window into individual variability and form the basis for deeper biological investigation. Additionally, since these parameters are routinely used by both researchers and aquaculture farmers, they enable quick and straightforward comparisons of fish performance not only between species, but also among different populations or cultured stocks of the same species.

Today, these phenotypic assessments are increasingly integrated with molecular approaches, including transcriptomics, proteomics, and epigenetic profiling (Sundaray et al., 2022). This combination enables researchers to connect observable traits with gene expression patterns and regulatory mechanisms, offering a more comprehensive view of how and what larval phenotype is shaped. Such an integrative framework is not only valuable for developmental biology but also holds direct application in aquaculture. Understanding the genotype–phenotype–environment relationship allows for earlier and more accurate selection of high-quality individuals, ultimately supporting more efficient and resilient breeding programs.

1.5.2 Transcriptomics

The advent of next-generation sequencing (NGS) technologies has introduced transcriptomics**,** the study of all RNA molecules expressed in a cell or tissue, including messenger RNAs (mRNAs), and various non-coding RNAs. Unlike the stable genome, the transcriptome is dynamic, reflecting the cellular responses to developmental stage, physiological status, and environmental conditions (Lowe et al., 2017). This makes transcriptomic profiling a powerful tool for understanding how genes are regulated in context-specific ways.

Transcriptomics has become a central tool in aquaculture research, offering valuable insights into both fundamental biology and practical applications. Through RNA sequencing (RNA-seq), researchers can quantify transcript abundance, identify differentially expressed genes (DEGs), and explore molecular pathways involved in key traits such as growth, immune function, stress response, and neural development (Heras, 2021). This approach has been applied across all life stages, ranging from fertilized eggs and larvae to juveniles and adults, to reveal stage-specific gene expression profiles and developmental transitions (Chapman et al., 2014; Harry & Zakas, 2023; Huerlimann et al., 2024; Mazurais et al., 2011).

In studies at the level of larvae, RNA-seq has enabled novel insights into the mechanisms and biological processes driving it. For example, in marine species transcriptomics has shed light on the molecular mechanisms underlying methamorphosis in larvae, offering new perspectives on processes that are otherwise difficult to observe (Ferraresso et al., 2013; Louro et al., 2020). Moreover, transcriptomic profiling has been instrumental in comparing wild and domesticated strains, revealing gene expression differences related to domestication traits such as metabolic efficiency, stress resilience, and immune competence (Bicskei et al., 2020; De Almeida et al., 2019; Lanes et al., 2013). Also, it can be applied in studies where effects of environmental variables, including temperature shifts**,** photoperiod changes**,** and salinity fluctuations**,** can be

explored, since it's known that diverse environmental conditions can modulate gene expression patterns that influence phenotype and survival (Oomen & Hutchings, 2017). Nonetheless, a critical application of transcriptomics in aquaculture is the investigation of parental effects, how the physiological condition, diet, or stress exposure of broodstock affects offspring gene expression (Bougas et al., 2013; Colson et al., 2019; Panda et al., 2024). For example, stress-exposed or nutritionally deficient parents can transmit altered transcriptomic signatures to larvae, impacting early developmental success (Adrian-Kalchhauser et al., 2020). These insights are invaluable not only for improving broodstock management and selective breeding programs, but also for optimizing larval rearing protocols, refining welfare standards. Linkage of phenotypic traits with molecular mechanisms offers a comprehensive understanding of fish condition and supports the development of targeted strategies to improve performance, efficiency, and overall industry outcomes.

1.6 Larvae at mouth opening stage – a glimpse into the past and future

The mouth opening (MO) stage represents a critical developmental milestone in fish larvae, marking the transition to active feeding and greater interaction with the environment (**Fig. 1.2**). In controlled rearing conditions, it also signifies the point at which larvae become functionally independent, requiring no major human intervention yet (Debernardis et al., 2025), and in Eurasian perch, mouth opening typically coincides with hatching. Transcriptomic profiling at this stage provides valuable insights into the molecular mechanisms underlying key developmental processes such as metabolism, sensory maturation, and immune system activation. Importantly, gene expression patterns during this period reflect not only inherited genetic factors but also non-genetic influences shaped by parental environments and early embryonic conditions. Overall, the MO stage bridges the legacy of parental and embryonic development ("the past") with the foundation for future growth and survival ("the future"), making it a vital focus for transcriptomic research.

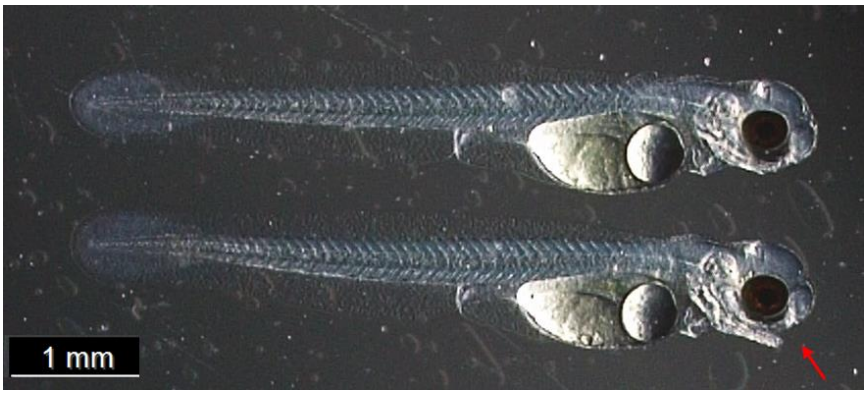

Figure 1.2: *Two larvae of Eurasian perch (Perca fluviatilis), with the lower larva displaying an open mouth (indicated by the red arrow) and the upper larva with a closed mouth.*

Chapter 2: Objectives and Hypothesis

2.1 Objectives

The central objective of this thesis is to determine what the larval transcriptome at mouth-opening stage can reveal about both parental legacy and subsequent performance in Eurasian perch. Specifically, the study (i) partitions larval gene-expression variance into maternal and paternal contributions - encompassing genetic and non-genetic inheritance - and (ii) tests the predictive value of transcriptomic signatures for early-life performance and aquaculture-relevant traits.

To explore the biological complexity encoded in the transcriptome during this stage, a progressive, zoom-in approach, moving from broad to highly specific research questions, has been adopted (**Fig. 2.1**).

This strategy involved four lines of investigations:

1. Transcriptomic characterization of early-stage larvae (Chapter 3; Larval transcriptome) - the gene expression profiles of newly hatched larvae from 16 different families were analyzed to identify the core developmental pathways and regulatory mechanisms active during the earliest stages of life.

2. Parental origin and its influence on offspring phenotype (Chapter 4; Dom-Wild) - larvae derived from wild versus domesticated broodstock were compared to disentangle the contributions of maternal and paternal origin to early developmental outcomes.

3. Maternal effects via egg quality (Chapter 5, Egg quality) - variation in egg quality was investigated using refined morphological indicators, and its effects on larval gene expression and phenotype were assessed to provide insights into the maternal legacy on offspring performance.

4. Intra-batch variation and developmental deformities (Chapter 6; Heart oedema) - transcriptomic differences between morphologically normal larvae and those displaying heart oedema deformities were examined to identify potential molecular drivers of developmental anomalies.

This approach aims to elucidate how larval phenotypes are shaped by decoding the ‘messages’ embedded in their transcriptome and identifying the factors that influence these molecular signatures.

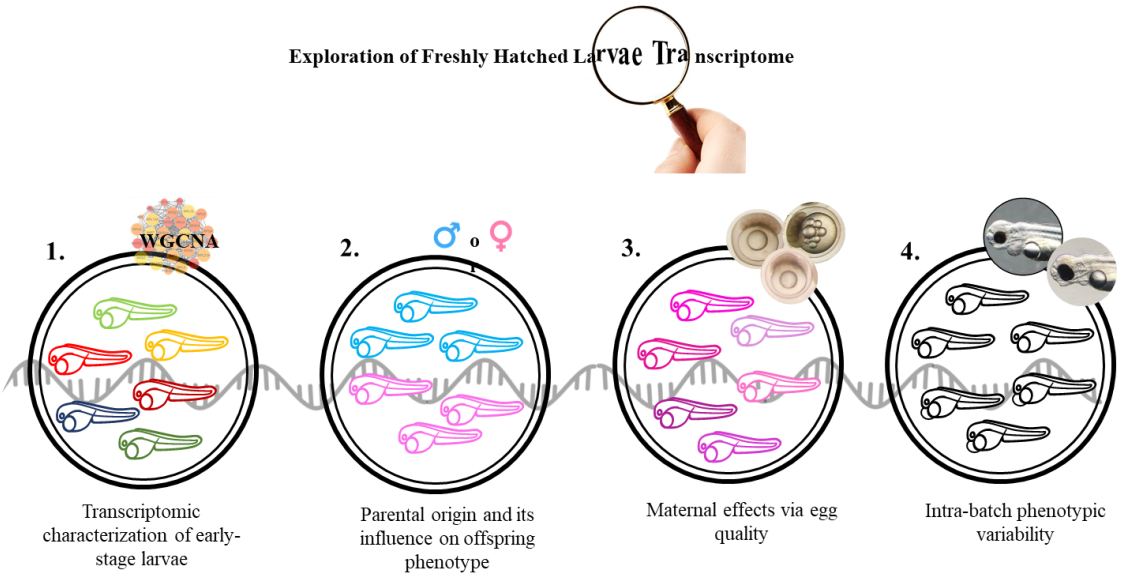

Figure 2.1: *Schematic illustration of the stepwise "zoom-in" strategy applied to investigate the information contained in the freshly hatched transcriptome of Eurasian perch larvae. The approach progressively narrows from different larval populations to specific comparisons within family. 1. Different colours represent distinct families. 2. Blue indicates paternal effect, while pink denotes maternal effect. 3. Different shades of pink correspond to different mothers. 4. Larvae in the same black colour originate from the same parents. WGCNA: Weighted Gene Co-expression Network Analysis.*

2.2 Research hypothesis

The general hypothesis of this thesis is that the transcriptome of Eurasian perch freshly hatched larvae (i.e., at mouth opening stage) contains molecular signatures that reflect intrinsic biological factors - stemming from parental effects - which together influence developmental outcomes and larval performance.

Hypothesis 1: It is proposed that transcriptomic profiles of larvae can offer predictive insight into future developmental trajectories and allow retrospective inference of parental contributions.

Hypothesis 2: Given the well-documented phenotypic differences between wild and domesticated broodstock, and the known influence of domestication on offspring from the earliest life stages, it is hypothesized that comparing larvae derived from these two parental origins will enable the disentanglement of maternal and paternal contributions - encompassing both genetic and non-genetic influences - to early phenotypic variation.

Hypothesis 3: Furthermore, it was hypothesized that variability in egg quality, assessed through zootechnical traits (i.e., fertilization rate, embryonic developmental rate, etc.), is associated with distinct gene expression patterns that mediate maternal effects on larval development.

Hypothesis 4: Finally, it is expected that transcriptomic comparisons between morphologically normal larvae and those exhibiting developmental anomalies, such as heart oedema, within the same families will reveal key molecular pathways involved in abnormal development.

Chapter 3: Does transcriptome of freshly hatched fish larvae describe past or predict future developmental trajectory?

Also reffered as: Larval transcriptome

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Contribution: writing – original draft, visualization, methodology, investigation, formal analysis, data curation, conceptualization.

3.1 Introduction

The early life history of most teleost fishes (both marine and freshwater) embraces a crucial stage known as larval period (McMenamin & Parichy, 2013). This transitional phase involves changes across morphological, behavioral, and physiological levels which lead to individual's progress towards adulthood (Urho, 2002). The dynamics and vulnerability of this phase are what make larvae important to study in order to facilitate a better understanding of the developmental journey from the egg stage to subsequent juvenile and adult stages.

Larval phase is characterized by significant events encompassing the initiation of the exogenous feeding, yolk-sac reduction, and the inflation of the swim bladder, as well as the development and functional maturation of different organs, tissues, and systems (e.g.: nervous, visual, digestive and immune systems) (Osse et al., 1997). These changes are regulated by a cascade of events as well as intrinsic (e.g., genetics, physiology) and extrinsic factors (e.g., temperature, density, nutrition - Sarropoulou et al., 2016 -) which have been a longstanding focus for the scientific community. Therefore, due to their significant influence on later developmental phases, investigations on the factors and intrinsic processes influencing larval performance are of high importance. This could also directly impact the modification of breeding and hatchery protocols by aquaculturists.

Sustainable development of aquaculture industry requires species diversification and the intensification of production to meet the rising global demand for aquatic products (Subasinghe et al., 2009). Among cultured species, Eurasian perch (*Perca fluviatilis*) is considered as a valuable model due to its commercial relevance and practise in intensive farming using recirculating aquaculture systems (RAS). Although, its production is already well-established (Fontaine & Teletchea, 2019; Palińska-Żarska et al., 2020; Policar et al., 2019), variable reproductive and larviculture performances remain still an obstacle to the improvement of its breeding efficiency. Within this framework, the production of high-quality larvae becomes paramount, as they possess a significant capacity to adapt to the aquaculture environment (Koumoundouros et al., 2017; Valente et al., 2013). Despite substantial progress in comprehending larvae biology and establishing larviculture protocols for many fish species, achieving consistently high survival rates and optimal growth potential remains a challenge (Valente et al., 2013). To overcome this, a deeper understanding of intrinsic and extrinsic factors influencing larval environmental adaptability is required. This is crucial for establishing a clear definition or, at the very least, identifying descriptors or predictors of larval quality, addressing an urgent need in the field.

Currently, the assessment of fish larvae quality is predominantly tied to morphological traits, such as body shape (i.e., absence of skeletal deformities), yolk sac morphology, inflated swim bladder, pigmentation (i.e., Boglione et al., 2013a, 2013b; Koumoundouros et al., 2010, 2017). Despite their significance, these indicators have limitations. Assessing these traits requires laborious and long-term breeding operations and their accuracy may be compromised by potential biases resulting from the rearing environment and human interventions. For this reason, there is a growing emphasis on incorporating new molecular quality indicators (e.g., gene markers) for larvae to supplement existing methods. The integration of molecular approach with conventional morphological examination will improve the efficiency of larval rearing protocols by offering a comprehensive understanding of biological processes. These molecular signatures should be found considering larvae at the earliest stage (preferably after hatching), where individuals are fully autonomous but major human intervention which could cause some stress and thus affect the overall regulation of biological processes of the fish is not yet in place (Valente et al., 2013). For Eurasian perch larvae, the mouth opening stage represents this time point (Palińska-Żarska et al., 2021). However, despite multiple evidences suggesting the potential of larval molecular profiles in predicting performance, there is still a lack of specific molecular indicators or fingerprints as reliable indicators. This stems from the lack of studies that directly align the characterisation of molecular profiles against larval traits.

Molecular profile of the larvae is influenced by a combination of genetic and non-genetic factors provided by both parents. These factors encompass various molecules (mRNAs, small RNAs, proteins, metabolites, etc.) or modifications of the genome (such as epigenetic modifications) which play a pivotal role as modulators of gene expression during the early development, influencing the phenotype of the progeny (Adrian-Kalchhauser et al., 2020). For instance, previous studies have demonstrated that transcriptomic profiling of pre-ovulatory oocytes and eggs serves as a robust predictor of their developmental competence (Chapman et al., 2014; Cheung et al., 2019). This clearly shows, that cascade-like transmission of molecular information from parents is influencing future embryonic fate. Furthermore, this transmission has been hypothesized to affect larval performance at the earliest stage (Adrian-Kalchhauser et al., 2018, 2020), suggesting that molecular profile of eggs can serve also as a predictor of future fate of fish, as tackled in several studies. For example, Colson et al. (2019) describe how the maternal molecular cargo transmitted into the eggs of rainbow trout (*Oncorhynchus mykiss*) significantly predicts the behaviour of their offspring. Despite the growing body of evidence on the crucial role of molecular parental cargo on embryonic development, there is still lack of information on

whether the transcriptome of larvae at the earliest stage is only a result of parental contributions or if there is a continuum of transmission of parental information beyond embryogenesis. Therefore, it remains to be elucidated whether the molecular portrait of larvae can also predict its future performance.

Transcriptomics has been already successfully applied to fish larvae biology research, offering valuable insights into the molecular mechanisms underlying various biological processes (Chandhini & Rejish Kumar, 2019; Ferraresso et al., 2013; Mazurais et al., 2011; Żarski et al., 2017c). While it underscores the significance of larval transcripts as a proxy to understand organisms' phenotypes, it remains unclear if such profiles can describe/predict larval outcomes. Therefore, the current study on Eurasian perch aims to explore whether the transcriptomic profile of 16 different families of larvae, each coming from different pairs of parents and collected at the mouth-opening stage, could be an indicative tool for describing parental contribution and/or predicting the future fate of the larvae. This research question has been addressed by examining detailed zootechnical features from fertilization until the end of the larval stage for each family separately, and by identifying molecular information that could serve as indicators of larval performance-related traits.

3.2 Material and methods

Sixteen diverse families of Eurasian perch larvae were obtained by controlled reproduction of wild spawners from different water bodies (**Supplementary file S3.1**). A simple mating design was carried out by crossing a single female with an individual male.

3.2.1 Broodstock origin, management and reproduction

3.2.1.1 Males' origin and management

The Eurasian perch males (average weight 190.5 ± 58 g), used for controlled reproduction, were from earthen pond systems located in Central (Rytwiany and Łyszkowice Fish Farm) and North of Poland (Iława Fish Farm). Spawners were harvested during late autumn (end of October and early November) and overwintered in the flow-through system of the Salmonid Research station of the National Inland Fisheries Research Institute (IRS-PIB) in Rutki (North Poland) under the natural photoperiod. Male individuals were captured in autumn since it is very difficult to catch them during the breeding season, and moreover, often completed or already contributed to the spawning act before being caught, which may affect sperm quality. Then, during the spawning

period, males were transferred in plastic bags with oxygen (Żarski et al., 2017d) to the 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 with a controlled photoperiod (14 L:10 D) and temperature (12 °C) until spermiation took place. The sexually mature fish were hormonally stimulated (with an intraperitoneal injection at the base of the left ventral fin) using a salmon gonadoliberin analog (sGnRHa, Bachem Chemicals, Switzerland) at a dose of 50 μg kg^{-1} (Żarski et al., 2020b). Before any manipulation, individuals were anesthetized in MS-222 (Argent, USA) at a concentration of 150 mg l^{-1}. Total length (TL), fork length (FL) and body weight (before stripping) were measured for each individual (**Supplementary file S3.1**).

3.2.1.2 Sperm sampling and cryopreservation protocols

Five days after hormonal injection, semen was collected using a catheter (to avoid contamination of the urine; Sarosiek et al., 2016) and with gentle abdominal pressure (i.e., stripping). After collection, each sample was kept on ice, and sperm motility parameters were evaluated with CASA system **(Supplementary file S3.2**), (using the CEROS II system -Hamilton-Thorne, USA; as described by Judycka et al. (2022), while the sperm concentration of fresh semen was measured using a NucleoCounter SP-100 computer aided fluorescence microscope (Chemometec, Allerød, Denmark; Nynca & Ciereszko, 2009). For this purpose, the semen was first diluted 100 times with PBS and then 51 times with Reagent S100 and loaded into the kit cassette containing propidium iodide. SemenView software (Chemometec, Denmark) was used to determine the final concentration of spermatozoa in each sample (Judycka et al., 2019).

Thereafter, sperm cryopreservation was carried out following the procedure described by Judycka et al. (2022). Briefly, the semen was diluted with a glucose-methanol (GM) extender supplemented with potassium chloride (consisting of a final concentration of 0.30 M glucose, 7.5% methanol and 25 mM KCl at 3.0×10^9/ml spermatozoa). Semen mixed with cryoprotectants was filled into 0.5 ml plastic straws and then placed on a floating rack and cryopreserved in liquid nitrogen vapor for 5 min. Next, the straws were submerged into liquid nitrogen, which ended the process. After cryopreservation, the sperm motility was re-evaluated by thawing the straws in a water bath for 10s at 40 °C. Finally, the straws were placed in liquid nitrogen storage tanks until being used for fertilization. For this operation, cryopreserved semen was used to ensure feasibility of the entire operation (in case of delayed reproduction of females) and to maintain comparable sperm quality in case the females did not ovulate simultaneously (collection of eggs from wild

females during the spawning season can take several days; Żarski et al., 2017e) and it is currently used as a standard procedure in many selective breeding programs (Judycka et al., 2022)

3.2.1.3 Females' origin and management

For this experiment, 16 wild females (average weight 493 ± 213 g) were utilized. Some individuals came from the Żurawia and Iława pond systems, which were overwintered together with males (as mentioned before). In addition, in April, wild females from Szymon and Umląg Lakes were captured using gill nets during the spawning season. In this way, we ensured significant phenotypic variability, expected to provide appropriate heterogeneity in the performance of the larvae, which was needed to fulfil the purpose of this research project. Prior to spawning, all fish were transported in plastic bags with oxygen to the CAEE-UWM where they were placed in the RAS with a controlled photoperiod (14 L:10 D) and temperature (12 °C) until ovulation. Prior to hormonal stimulation, females were first catheterized, and the oocyte maturation stage was determined following the classification proposed by Zarski et al. (2011). All the fish at the time of injection were at stage III and IV, being the most appropriate for reproduction in Eurasian perch (Zarski et al., 2011). After that, the fish were hormonally stimulated (as with males) using a salmon gonadoliberin analog (sGnRHa, Bachem Chemicals, Switzerland) at a dose of 50 μg kg^{-1} (Żarski, et al., 2020b). Before any manipulation, individuals were anesthetized in MS-222 (Argent, USA) at a concentration of 150 mg l^{-1}. During the experiment, for each fish, the total length (TL), fork length (FL), body weight and weight of the ribbon were taken (measures are provided in **Supplementary file S3.1**).

3.2.2 Egg collection and fertilization protocols

At ovulation, the eggs (ribbon) were collected through hand stripping (gentle massage of the abdomen part of the fish body as described by Żarski et al. (2011). After stripping, the number of dry eggs in 1 g was evaluated by first counting the eggs in 3 small portions (~0.2 g each) of the ribbon (Żarski et al., 2017e). In this way, the correct number of spermatozoa to be used for fertilization of each ribbon was estimated (Żarski et al., 2017b). A ribbon with an average weight of 80 ± 12 g was used to carry out the *in vitro* fertilization as described by Judycka et al. (2019). Briefly, the eggs were first activated with modified Lahnsteiner 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; Judycka et al., 2022). Hereafter, just before fertilization, straws with cryopreserved semen were thawed in a water bath at 40 °C for 10 s and placed in an Eppendorf tube. Thirty seconds after eggs activation, sperm was

added at a sperm:egg ratio of 200,000:1. The eggs were then stirred for 40 s and washed with hatchery water after ~10 min to remove excess sperm and any debris. The same procedure was followed for each female separately.

3.2.3 Egg incubation and hatching – evaluation of pre-hatching zootechnical traits

The fertilized eggs were incubated in 15 L black-walled tanks (eggs from each female separately) operating in the same RAS and placed on nets with mesh diameter of 3 mm at a temperature of 14 °C. After 12 h post fertilization (HPF), ~100 eggs from each batch were randomly sampled (in duplicate) to evaluate the fertilization rate before the maternal-to-zygotic transition (MZT), which occurs at around 13 HPF (Güralp et al., 2016), and then the embryonic development at the neurula stage (when the body of the embryo can already be easily seen, at around 3 days post fertilization). During eggs incubation, the temperature was raised to 15 °C when the embryos reached the eyed-egg stage and then to 16 °C as soon as the first spontaneously hatched larvae were observed. In Eurasian perch, hatching can last for 5 days, even for the same batch of eggs (Żarski et al., 2017f). Therefore, to ensure almost synchronous hatching, manual hatching was induced. Briefly, the eggs were moved to a bowl (each batch separately) and stirred gently. The hatched larvae were then moved back into the 15 L tanks. This operation was repeated several times until most of the larvae hatched. This moment was considered the end of hatching (0 days post hatching - DPH).

After hatching, the larvae were left for 24 h without any human interaction, and next, all the larvae within the families were volumetrically counted and stocked back to the rearing tanks with the same stocking density for each tank and each family (~ 2500 larvae per tank). Larvae from each batch were stocked into 3 separate tanks constituting separate replicates.

3.2.4 Larviculture protocol

Every family of Eurasian perch larvae was reared in triplicate in the same RAS conditions, following a set of validated and standardized larval rearing methods (i.e., advanced zootechnics) as described by Palińska-Żarska et al. (2020). The larvae were exposed to a specific thermal regime, photoperiod, and feeding schedule (**Fig. 3.1A**). The water temperature was automatically controlled throughout the rearing period. After hatching, at 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, 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; Palińska-Żarska et al., 2020). Starting from 4 DPH, the larvae began 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]) until weaning. From 17 DPH, the larvae were sharply weaned and then fed exclusively with dry feed (Perla Larva Proactive, Skretting, Norway) six times a day, pouring it into each tank in small doses, with intervals of 3 min, for approximately 15 min. At ~30 DPH, the experiment was completed, as with the temperature regime used, and the larval period was considered finished. During the rearing trial, the photoperiod was 24 L:0 D, and the light intensity measured at the water surface was 1500 lx. In addition, the oxygen level and ammonia concentration in the tanks were analyzed every two days. No oxygen level below 80% was ever observed, and the ammonia level was always lower than 0.02 mg L^{-1}. The tanks were cleaned twice a day (in the morning, 1 h after feeding, and in the evening just prior feeding), and the dead larvae were collected and counted under a microscope to assess the survival rate (%) throughout the experimental trial. In addition, from 14 DPH, dead larvae were observed under the microscope to assess the cannibalism rate by recording the number of larvae with damage to the body, especially the tail.

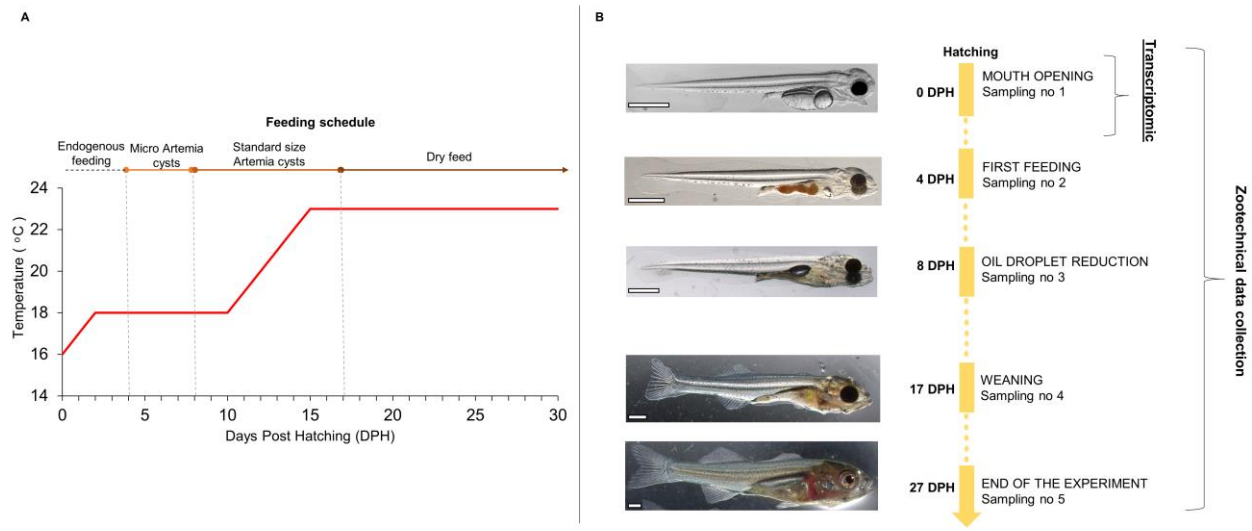

Figure 3.1: A. *Temperature regime (red curve) and the feeding schedule followed during Eurasian perch larvae rearing.* ***B.*** *Scheme of Eurasian perch larvae samplings at specific developmental stages. DPH – Days post hatching, sampling no 1 – moment of mouth opening in at least 50% of larvae, sampling no 2 – moment of exogenous feeding starting in at least 50% of perch larvae, sampling no 3 – oil droplet reduction in at least 50% of larvae, sampling no 4 – time of weaning with dry feed diet, sampling no 5 – end of experiment. Only larvae collected during sampling no 1 were used for transcriptomic analysis. At each sampling point 30 larvae per family were collected to measure total length and wet body weight.*

3.2.5 Sampling activities and evaluation of zootechnical traits

Zootechnical traits of larvae were evaluated based on data obtained during five precisely chosen developmental stages (following Palińska-Żarska et al., 2021) (**Fig. 3.1B**):

1. At the mouth opening stage (0 DPH)
2. At 4 DPH, when at least 50% of larvae started exogenous feeding,
3. At 8 DPH, in at least 50% of the larvae, almost complete reduction of oil droplets was observed,
4. At the weaning stage (17 DPH),
5. At 27 DPH, considered the end of the experiment, when at least 50% of larvae had undergone the removal of the fin fold.

At each sampling point, 30 larvae per family were sampled to record total length (TL, ±0.01 mm) and wet body weight (WBW, ±0.1 mg). The individuals were first anesthetized (with MS-222, at a concentration of 150 mg L^{-1}) and then photographed under a stereoscopic microscope (Leica, Germany) to measure the TL. The WBW was determined with a precision laboratory scale by placing the anesthetized larvae on a nylon net (with a mesh size of approx. 200 µm) and draining the excess water with filter paper (Krejszeff et al., 2013). Additionally, two days after oil droplet reduction (10 DPH), the swim bladder inflation effectiveness (SBIE, %) was evaluated on approximately 100 larvae per family by triple counting (as described by Palińska-Żarska et al. (2020). Briefly, the individuals were first captured randomly from each tank, placed on a Petri dish, anesthetized and then counted under a stereoscopic microscope (individuals with and without a filled swim bladder).

3.2.6 Transcriptomics

3.2.6.1 Sampling and RNA extraction for molecular analysis

For transcriptomic analysis, larvae were sampled at the mouth opening stage. From each family, ten freshly hatched larvae were randomly selected, anesthetized, and preserved in RNAlater (Sigma-Aldrich, Germany) according to the manufacturer's instructions. RNA extraction was performed on these pooled samples (n = 10 per pool, one pool per family) using a TotalRNA mini-kit (A&A Biotechnology, Poland), as described by Palińska-Żarska et al. (2020). The quantity and purity of the extracted RNA were assessed using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA), ensuring absorbance ratios of A260/280–2.0 and A260/230–2.2.

Additionally, RNA quality was confirmed with an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with all samples showing RIN values > 9.0. In total, 16 RNA samples, representing each larval family, were prepared and submitted for RNA sequencing.

3.2.6.2 RNA-sequencing library preparation

Sixteen different libraries were created. RNA-seq analysis was performed by Macrogen (Amsterdam, Netherlands) using the TruSeq Stranded Total RNA kit (Illumina) with a NovaSeq600 platform, and 40 M 150 bp paired-end reads per sample were generated. The raw reads were quality controlled using FastQC software ver. 0.11.9 (Simon Andrews, 2020). Adapters and low-quality fragments of raw reads (average QPhred score < 20) were trimmed, 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* (GENO_Pfluv_1.0) obtained from the NCBI database (Sayers et al., 2022) using STAR software ver. 2.7.10a (Dobin et al., 2013) with ENCODE default options.

The annotation and estimation of the expression levels were performed using the StringTie tool ver. 2.2.0 (Pertea et al., 2015), with interpreting strand-specific sequencing the 'fr—firststrand' parameter was activated. Counts per transcript and gene were calculated using the prepDE Python script (https://github.com/gpertea/stringtie/blob/master/prepDE.py), and TPM and FPKM values were read from StringTie output data using the lncRna R library (Jastrzebski et al., 2023).

Functional annotation was performed using eggNOG-mapper (version emapper-2.1.9) (Cantalapiedra et al., 2021) based on eggNOG orthology data (Huerta-Cepas et al., 2019). Sequence searches were performed using DIAMOND (Buchfink et al., 2021).

3.2.6.3 Transcriptomic and Construction of the weighted gene co-expression network analysis (WGCNA)

To visualize the variability in transcriptomic data, a heatmap was constructed for the top 500 most variable genes using the pheatmap package (Kolde, 2022). Additionally, principal component analysis (PCA) was performed using the "prcomp" (package *stats* version 4.2.2; Team, 2021) to analyse the overall data variance. The gene counts were employed to perform weighted gene co-expression network analysis (WGCNA) with the R package, following the authors' recommendations (Langfelder & Horvath, 2008). Briefly, the hierarchical clustering of samples with Euclidean distance was used to check the presence of outliers (**Fig. 3.2**). After that, the total

gene counts were first filtered by removing all genes with <10 counts in each sample. Subsequently, variance-stabilizing transformation was performed, using the DESeq2 package (Love et al., 2014). Then, to proceed with automatic blockwise network construction, the adjacency matrix was calculated, and a soft-power threshold ($\beta = 16$, leading to signed $R^2 = 0.85$, which was the best scale-free indicators for the current analysis) was chosen based on a scale-free topology model (**Supplementary file S3.3, Fig. A**). Then, a block network signed was constructed and gene modules were identified by hierarchical clustering dendrogram **(Supplementary file S3.3, Fig. B**). The relationships among modules are visualized by a hierarchical clustering dendrogram of their eigengenes (**Supplementary file S3.3, Fig. C, D**). After, the module eigengene (ME) distances were calculated in order to detect potential relationships of modules with the zootechnical traits collected during the experiment. This will result in gene significance (GS) values and the corresponding *p*-value for all the modules and traits. To visualize the associations between modules and traits, a module-trait heatmap is generated, offering a graphical representation of the correlation patterns. We focused on the significant modules with a $p < 0.05$ and the absolute value of the correlation coefficient $|cor| \geq 0.6$. Genes embedded in the significant modules of interest were then extracted to proceed with the GO Enrichment analysis.

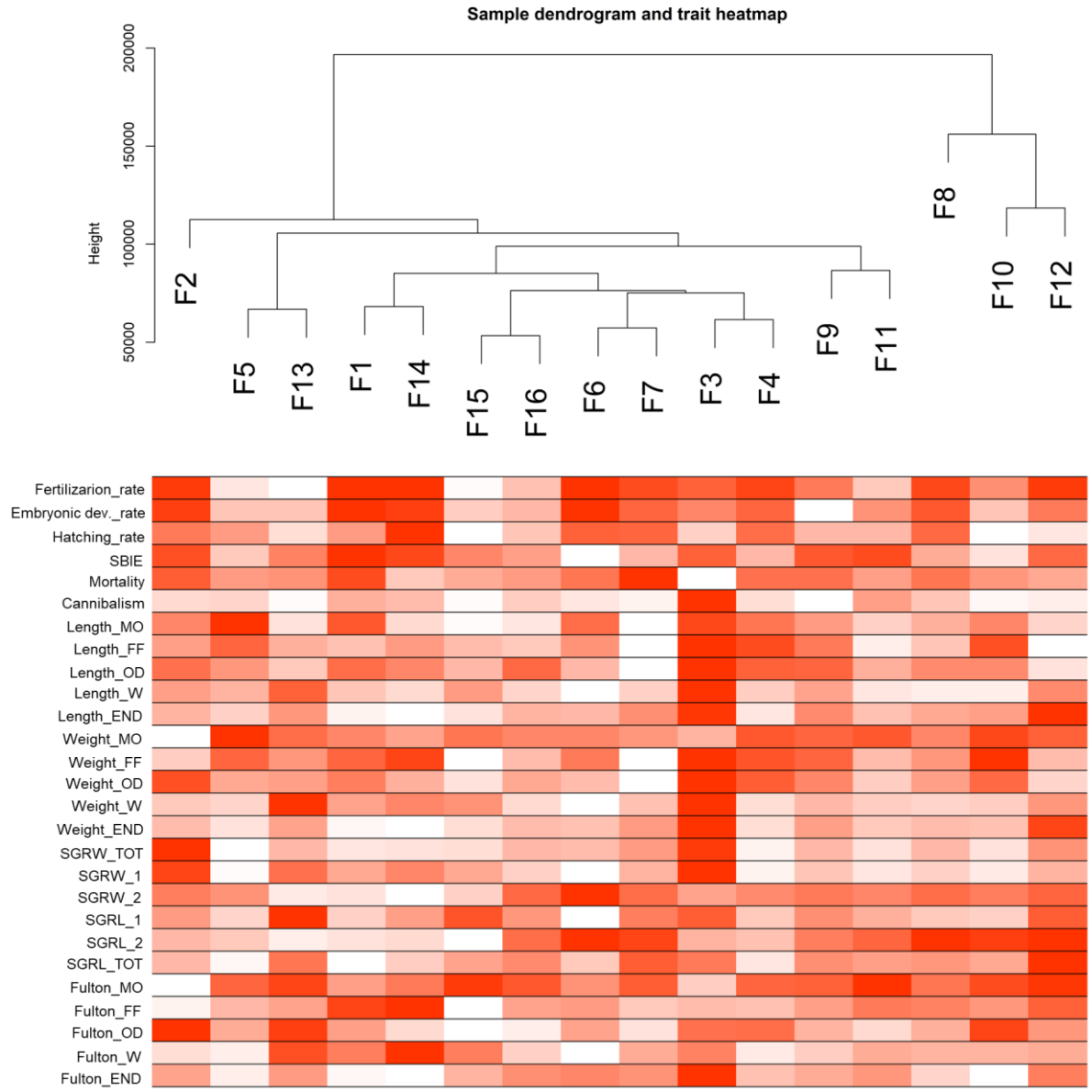

Figure 3.2: *Sample dendrogram based on their Euclidean distance and traits heatmap. The dendrogram plotted by hierarchical clustering (based on gene expression data) for the 16 families. The heatmap presented below the dendrogram represents an overview of the zootechnical traits for the corresponding families. Red color denotes higher values, while white signifies lower values of traits.*

3.2.6.4 Gene Ontology (GO) analysis

GO analysis was performed by following the approach described by Żarski et al. (2021a). Briefly, for each transcript, a protein RefSeq accession number was obtained. Next, the RefSeq identifiers were used to align the sequences against human protein in Swiss-Prot with BLASTP. After the alignment, only the best match for each protein was retained, which allowed us to retrieve

gene names and UniProt accession numbers for successfully aligned proteins, which were further used to perform GO analysis. GO analysis was performed using the ShinyGO online platform (Ge et al., 2020). First, GO analysis was performed for the genes incorporated in the significant modules obtained from the WGCNA, separately for the positively and negatively correlated modules, and the 10 most enriched biological processes were identified (FDR < 0.05). This allowed us to identify clusters that highlighted the most relevant biological processes. Next, the GO terms found in pre- and post-hatching were compared to identify specific traits unique to each period as well as those shared between them and the results were visualized using a Venn diagram (Bardou et al., 2014). Following the categorization of gene modules based on these two groups of zootechnical traits, we performed a comprehensive GO analysis, resulting in the identification of 100 enriched terms (FDR < 0.05). Subsequently, these terms underwent network analysis through hierarchical clustering. This approach facilitated the identification of distinct clusters, which were further characterized by conducting additional enrichment analysis on the gene list associated with each cluster. Through this process, we discerned the biological processes characteristic of each cluster.

3.2.6.5 Identification of hub genes

After identifying the most significant modules, we decided to examine the gene network within each module to identify hub genes, which could provide insights into the biological processes that the genes within each module may be involved in. The genes obtained from each module previously were mapped into the online search tool STRING database (Szklarczyk et al., 2017) (STRING, V12.0; https://string-db.org/), which could play a critical role in the protein-protein network (PPI). Then Cystoscape (Shannon et al., 2003) was used to visualize the networks and CytoHubba (a plugin of Cytoscape) was used to search for hub genes (Chin et al., 2014). The top nodes ranked by the Maximal Clique Centrality (MCC) algorithm in CytoHubba were seen as hub genes. Ten hub genes were selected for each significant module.

3.2.6.6 Key traits for aquaculture (KTA)

For WGCNA, we utilized all the collected larval traits to investigate their relationships with gene networks. However, to identify specific biomarkers related to particular traits, we focused on 7 key larval traits critical for the success of the aquaculture sector: mortality, cannibalism, SBIE, SGRL_TOT, SGRW_TOT, K, and the final weight of larvae at the end of rearing (Toomey et al., 2020). For this purpose, the GS values obtained from the WGCNA were used. The GS is the

correlation of gene expression profile with an external trait. It quantifies the biological importance of genes, higher absolute GS values indicate greater significance, and these values can be either positive or negative. For the current analysis, we selected the most correlated genes (|cor| > 0.7; |cor| > - 0.7) for further validation using Real-Time quantitative PCR (qPCR) (**Supplementary file S3.4**).

3.2.6.7 Reverse transcription and real-time qPCR

Real-Time qPCR validation is widely used for the identification and validation of molecular biomarkers associated with specific traits (Akbarzadeh et al., 2020; Becker et al., 2013; VanGuilder et al., 2008). For this purpose, the top positively and negatively correlated genes for each chosen trait were validated through real-time qPCR. The results were then associated to the specific zootechnical data to assess their correlation coefficient (a value between −1 and + 1). For this purpose, total RNA was reverse transcribed using a 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 5× 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.

Real-time qPCR was performed using RT–PCR Mix SYBR (A&A Biotechnology, Poland). For each qPCR (20 µl), 10 ng cDNA template was used along with 10 µl of RT PCR Mix SYBR, 0.5 µM forward and reverse primers (designed with the Primer3Plus online platform (Untergasser et al., 2007) – **Supplementary file S3.5**), 0.4 µl of HiRox and sterile water. The reactions were conducted using ViiA7 real-time PCR systems (Applied Biosystems) with the following conditions: incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. 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 analyzed using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen & Livak, 2001) as a reference for the geometric mean of four reference genes, namely, acyl-CoA dehydrogenase long chain (*acadl*), thioredoxin 2 (*txn2*), glutathione s-transferase alpha 1 (*gsta1*), and wd repeat domain 83 opposite strand (*wdr83os*), which exhibited the most stable expression level (revealed based on the transcriptomic data obtained; (Żarski et al., 2021b).

3.2.7 Data analysis and statistics

In addition to the measurable zootechnical characteristics (i.e., mortality, length, weight, cannibalism, SBIE), the specific growth rate (SGR) and the Fulton condition factor (K) were calculated. Fulton's condition factor (*K*) was calculated based on the obtained measurements according to the formula: $K = 100\ (W/TL^3)$, where W = Weight and TL = total length. In addition, SGR (% day^{-1}) was calculated according to the formula: $SGR = 100\ ((\ln Wt - \ln W0)\ \Delta t^{-1})$, where W0 = mean initial weight of the fish (g), Wt = mean final weight of the fish (g), and Δt = number of days between measurements. The SGR was calculated considering 3 different conditions/approaches: SGRW_TOT, which refers to the entire rearing trial (0 DPH to 27 DPH), SGRW_1 relative to the weight of larvae from 0 DPH to 17 DPH and SGRW_2 for larvae from 17 DPH to 27 DPH. The same calculations were made for the length data of the larvae (referred to as SGRL_TOT, SGRL_1, SGRL_2).

After assessing the normal distribution and homogeneity of variance, one-way, two-way analysis of variance (ANOVA) and Tukey's post hoc test was conducted; additionally, Kruskal–Wallis' test and Dunn-Bonferroni post hoc correction were used for analysis of data that were not normally distributed. The samples were considered significantly different when $p < 0.05$. The statistical analysis was performed using R studio software ver. 4.2.3 (Team, 2021).

Spearman's correlation matrix was used in order to evaluate the relationships between the zootechnical traits collected for all the families. The matrix was constructed in R studio using the "corrplot" package v. 0.92 (Wei et al., 2017).

Additionally, a principal component analysis (PCA) for all the zootechnical traits was first performed in R studio using FactoMineR package (Lê et al., 2008), to possibly select key traits that could serve as indicators of overall larvae performance (**Supplementary file S3.6**). However, no specific trait emerged as predominant and all collected zootechnical traits were used to conduct WGCNA.

3.3 Results

All the supplementary figures are gathered in the **Supplementary file S3.7** and are referenced hereinafter as **Fig. S3.1-S3.15**.

3.3.1 Zootechnical performance of larvae

Fertilization rate in all fish larval families varied between 63% and 97%. Surprisingly, these data didn't show any significant difference between all the 16 families (marked as F1 to F16 in **Fig.3.3**). Nevertheless, F9 was found to have significantly lower ($p < 0.05$) embryonic developmental rate (after maternal-to-zygotic transition -MZT-) compared to F1 and F6. In addition, two-way ANOVA, which compared the pre- and post-MZT period for each family of larvae, revealed considerable post-MZT mortality in only two families of larvae (F9 and F10; **Fig. 3.3**), indicating relatively high consistency between the fertilization rate and post-MZT embryonic survival.

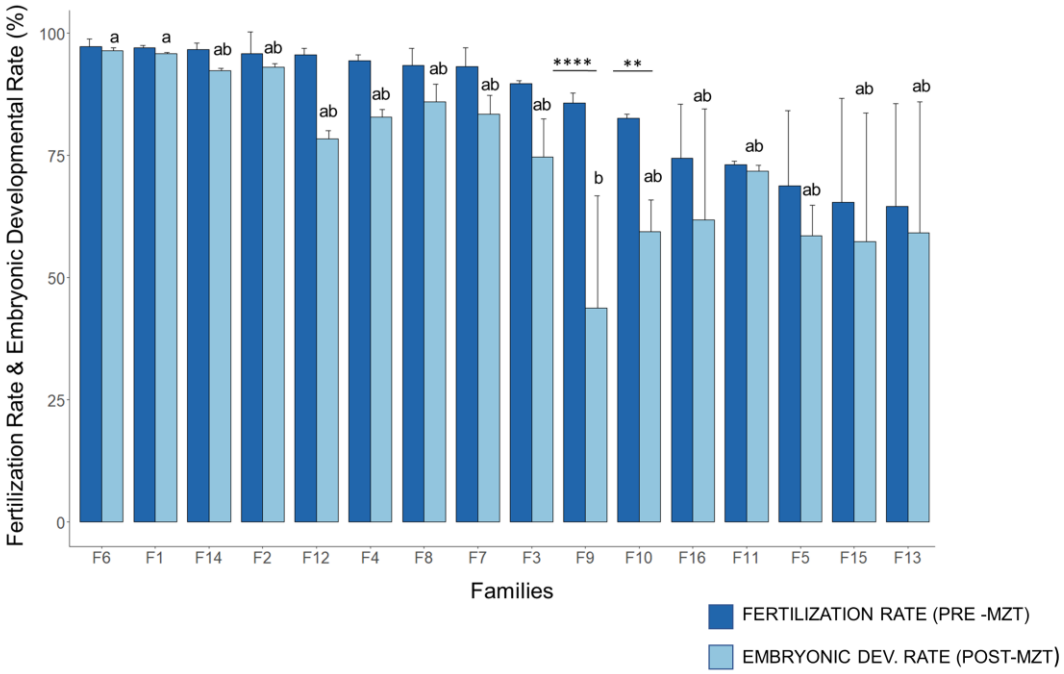

Figure 3.3**: Fertilization rate and embryonic developmental rate of 16 families of E. perch. The data are arranged in descending order according to the mean values of the fertilization rate between which no significant differences ($p > 0.05$) were recorded. Letters indicate significant differences ($p < 0.05$) between the families for the embryonic development rate. The asterisks (** – $p < 0.01$, * – $p < 0.0001$) indicate significant differences within the families and between fertilization and embryonic development rate.*

Among the remaining zootechnical traits, the analysis revealed significant differences ($p < 0.05$) between families for mortality, cannibalism (**Fig. S3.1A,B**), length and weight of larvae throughout almost the entire rearing period and SGR (for both length and weight) (**Fig. S3.2-S3.5**). However, there were no significant differences for weight of larvae at mouth opening and first

feeding stage and also for the SBIE (**Fig. S3.1C; S3.3A,B**). Also, no statistical differences ($p > 0.05$) were observed for K across different developmental stages (**Fig. S3.6**), except for larvae at the end of the experiment.

During the entire rearing trial, the mortality rate of each family was also recorded daily. The cumulative mortality graph (**Fig. 3.4**) shows a peak of mortality in mostly all larval families at oil droplet reduction stage.

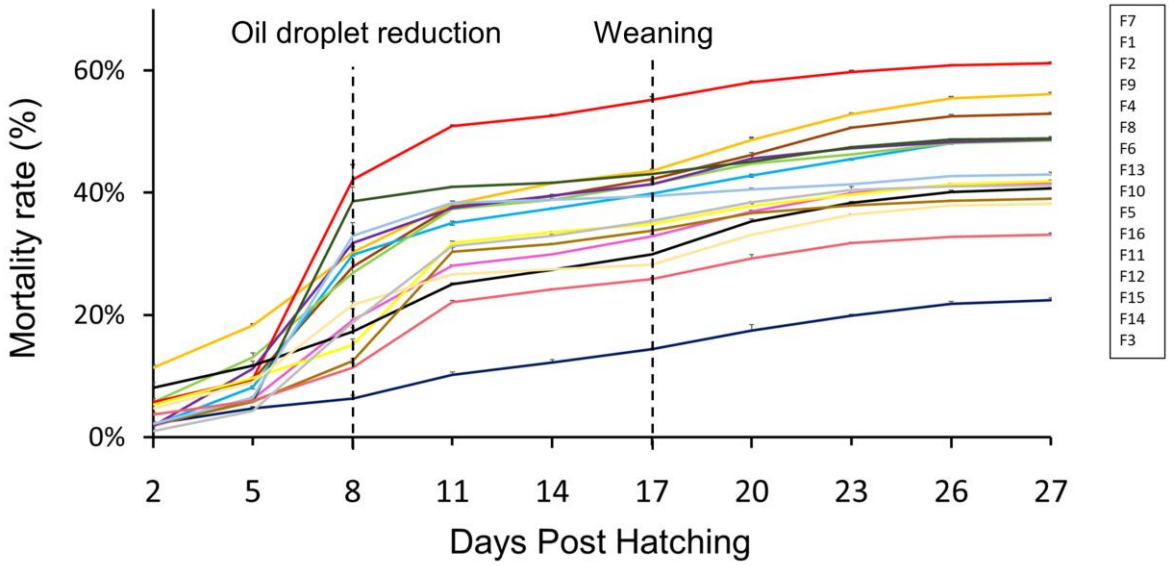

Figure 3.4: *Cumulative mortality (mean ± SD) of 16 families of E. perch larvae. Different colors stand for different families. The latter are sorted in descending order of final mortality rate recorded (they are listed in the box at the top right of the graph).*

To identify relationships among zootechnical parameters, a Spearman's correlation (r_s) matrix was constructed (**Fig. 3.5**). Interestingly positive correlations between the embryonic developmental rate parameter and fertilization/hatching rates (respectively $r_s = 0.89$ and $r_s = 0.7$) are shown. Noteworthy, robust correlations ($r_s \geq 0.6$ or $r_s \geq -0.6$) are observed for the growth-related traits. For instance, length of the larvae at mouth opening correlates positively with length at the first feeding ($r_s = 0.67$), weight at first feeding ($r_s = 0.66$) and at oil droplet reduction ($r_s = 0.63$). Specific growth rate of weight for the entire larviculture period (SGRW_TOT) is negatively correlated with larval weight at mouth opening stage ($r_s = -0.61$), while it is positively related to weight and length of larvae at the end of the experiment (respectively, $r_s = 0.86$, $r_s = 0.73$). Alternatively, the negative relationship between *K* at weaning (Fulton_W) and the

specific growth rate for weight data from weaning to the end of the larval period (SGRW_2) ($r_s = -0.65$). Also, the matrix shows negative correlations between *K* at mouth opening (Fulton_MO) and fertilization and embryonic developmental rate ($r_s = -0.65$, $r_s = -0.63$) (**Fig. 3.5**). This can be interpreted as the larvae obtained from lower quality eggs appear to be much more robust, resulting in a better *K*.

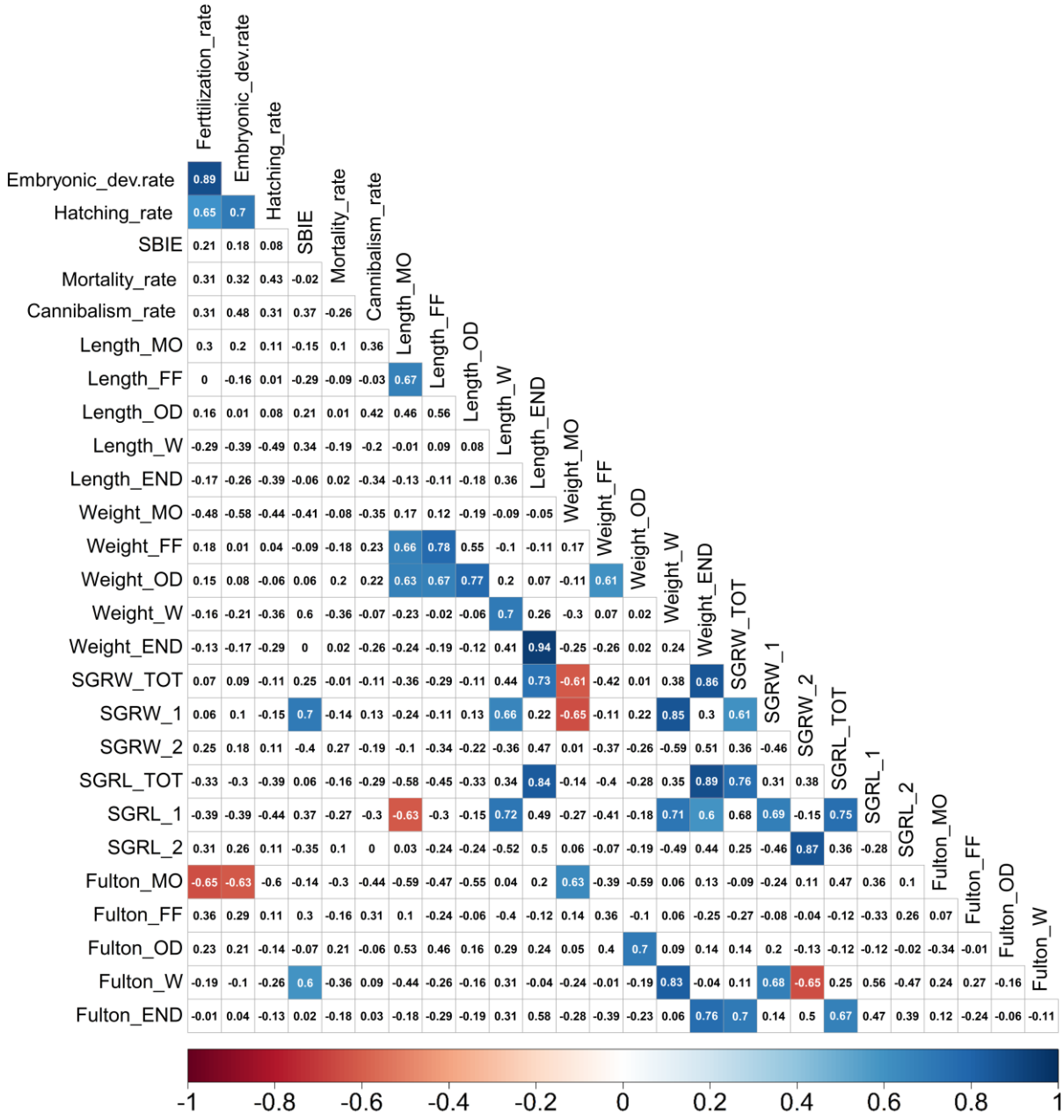

Figure 3.5: *Spearman's correlation matrix of all the zootechnical traits and for all the families together. Correlation coefficients (r_s) are shown in the squares, with significant correlations ($r_s \geq 0.6$, $p < 0.05$) indicated by colored boxes. Red colors show significant negative correlations, blue shows significant positive correlations, and white shows insignificant correlations. SBIE: swim bladder inflation effectiveness, MO: mouth opening, FF: first feeding, OD: oil droplet reduction, W: weaning, END: end of the experiment, SGRW: specific growth rate for weight, SGRL: specific*

growth rate for length data, SGR_TOT: specific growth rate for the entire larviculture period, SGR_1: specific growth rate from hatching until weaning stage, SGR_2: specific growth rate from weaning stage until the end of the experiment, Fulton: Fulton's condition factor.

Principal component analysis has been employed to identify key traits that could serve as indicators of overall larvae performance, and initially only these indicators were intended to be used for WGCNA analysis. Based on the results (**Supplementary file S3.6**), however, growth-related traits (i.e., weight and length at different developmental stages), specific growth rates and development, and survival traits (i.e., fertilization, embryonic developmental rate, and hatching rate) collectively provided a comprehensive assessment of larvae phenotypic profile, but none of them could be used separately. Therefore, as no specific trait emerged as predominant, we opted to conduct WGCNA using all the collected zootechnical traits.

3.3.2 Transcriptomic data and WGCNA analysis

After RNA-sequencing, 30,744 genes were initially identified. Following filtering procedures (explained in paragraph 3.2.6.3 in Material and Methods), 19,656 unique genes has been identified and further used for WGCNA. Visualization of 500 most variable genes (**Fig. 3.6A**) and PCA plot (**Fig. 3.6B**) illustrate significant transcriptomic diversity among the 16 families of Eurasian perch. These differences in gene expression profiles suggest unique genetic and phenotypic characteristics of each family studied. This variability observed led us to undertake identification of promising genetic markers associated with desirable traits for aquaculture using WGCNA. For this purpose, a gene cluster dendrogram was initially constructed, yielding 28 distinct gene modules (**Supplementary file S3.3, Fig. B**). Next, correlation analysis between these modules and zootechnical traits are shown in the module-traits heatmap (**Fig. 3.7**). Notably a total of 13 modules with $p < 0.05$ and correlation coefficient $|\text{cor}| \geq 0.6$ were prioritized for further scrutiny. Specifically, 7 modules exhibited notably robust correlations ($r \geq 0.6$) with embryonic developmental rate and hatching rate, indicating their association with pre-hatching traits. Conversely, 9 modules displayed significant correlations with post-hatching traits, specifically the weight of larvae at mouth opening, length at first feeding, K at mouth opening and oil droplet reduction stage. Notably, modules black, pink, and turquoise demonstrated shared correlations with both pre-hatching and post-hatching traits, suggesting their importance across different developmental stages.

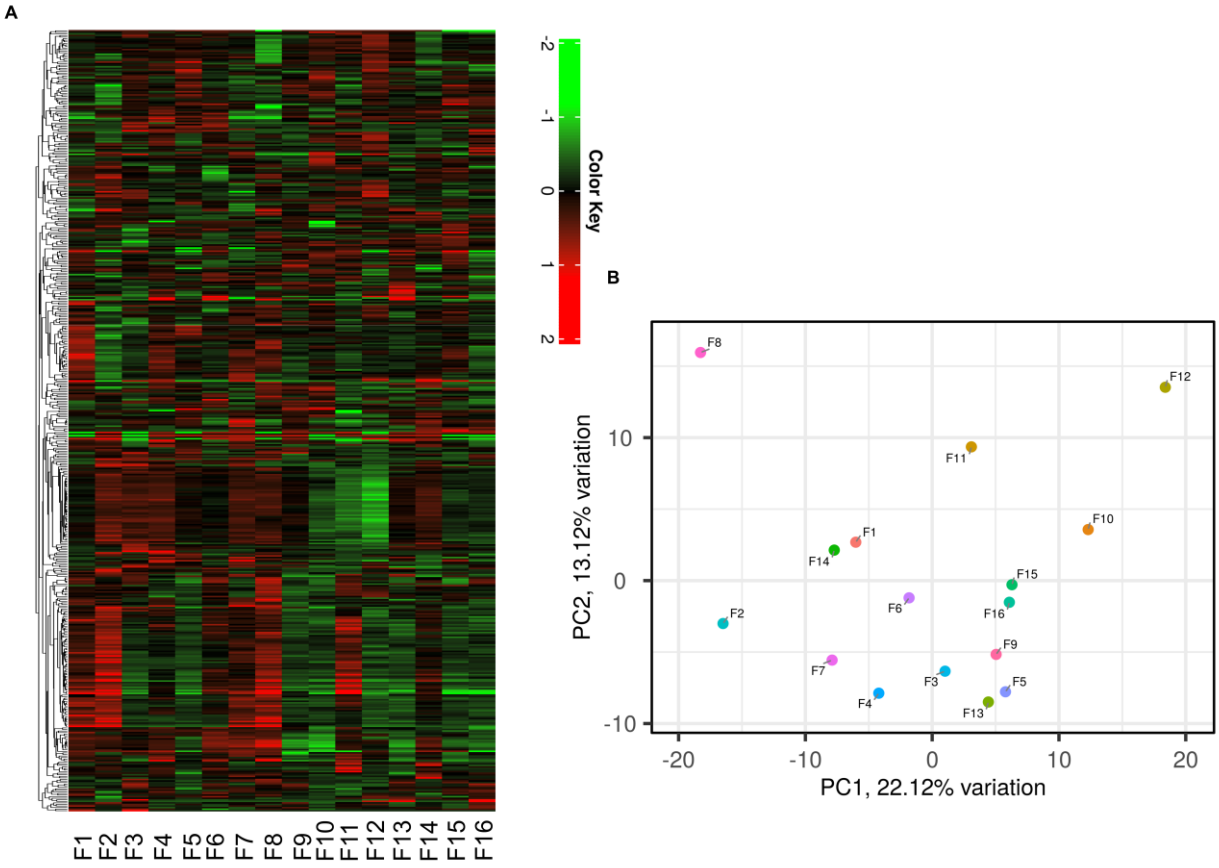

***Figure 3.6: A**. Heatmap of top 500 genes between 16 families of Eurasian perch larvae at mouth opening stage. Each row represents a gene and each column represents a sample. The color represents changes of gene expression where red shows increased expression and green decreased expression. **B**. Principal component analysis showing samples distribution.*

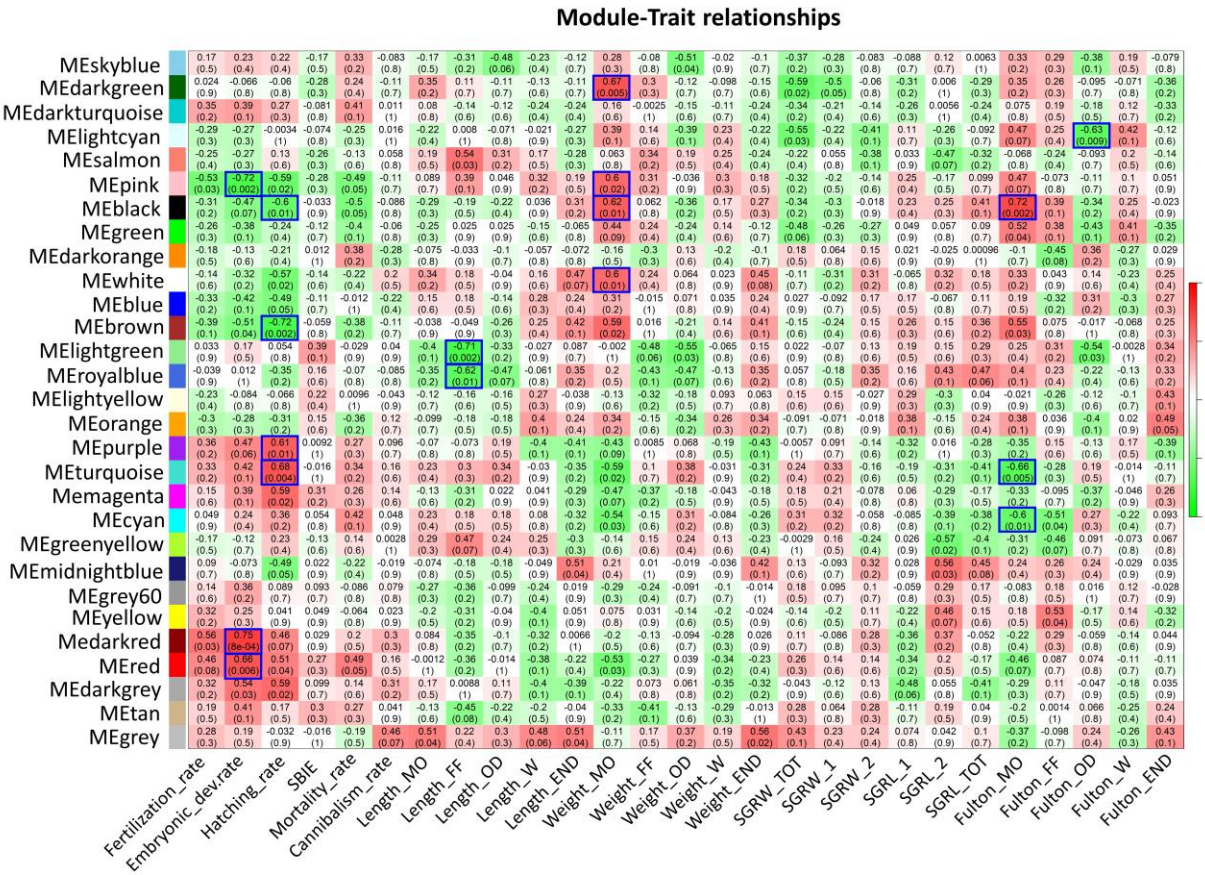

Figure 3.7: *Module-traits relationship. The module eigengene (ME) is shown in each row. Genes not assigned to any of the other modules are included in the grey module. The columns represent the zootechnical traits. The modules with high correlation values and $p < 0.05$ were identified as significant trait-related modules. The colors indicate the positive (red) and negative (green) correlations between gene modules and traits. SBIE: swim bladder inflation effectiveness, MO: mouth opening, FF: first feeding, OD: oil droplet reduction, W: weaning, END: end of the experiment, SGRW: specific growth rate for weight, SGRL: specific growth rate for length data, SGR_TOT: specific growth rate for the entire larviculture period, SGR_1: specific growth rate from hatching until weaning stage, SGR_2: specific growth rate from weaning stage until the end of the experiment, Fulton: Fulton's condition factor.*

The analysis of hub genes within significant WGCNA modules reveals key biological processes underpinning the transcriptomic diversity among Eurasian perch families (**Fig. 3.8**). The black module is associated with mitochondrial function, highlighted by ribosomal protein genes, while the brown and royalblue modules emphasize extracellular matrix organization through collagen and fibronectin genes. Chromatin modification and transcription regulation are central in the cyan module, featuring genes like *kmt2a* and *gata3*. Calcium signalling is prominent in the turquoise module, with multiple calcium channel genes. Transport and detoxification processes are represented in the darkgreen and white modules, involving solute carriers and cytochrome P450 pathways. The red and purple modules are linked to transcription regulation and cellular stress response, and lysosomal function, respectively, while the lightgreen and lightcyan modules focus

on synaptic function and cell division. Finally, the pink and darkred modules highlight ribosome biogenesis and energy metabolism.

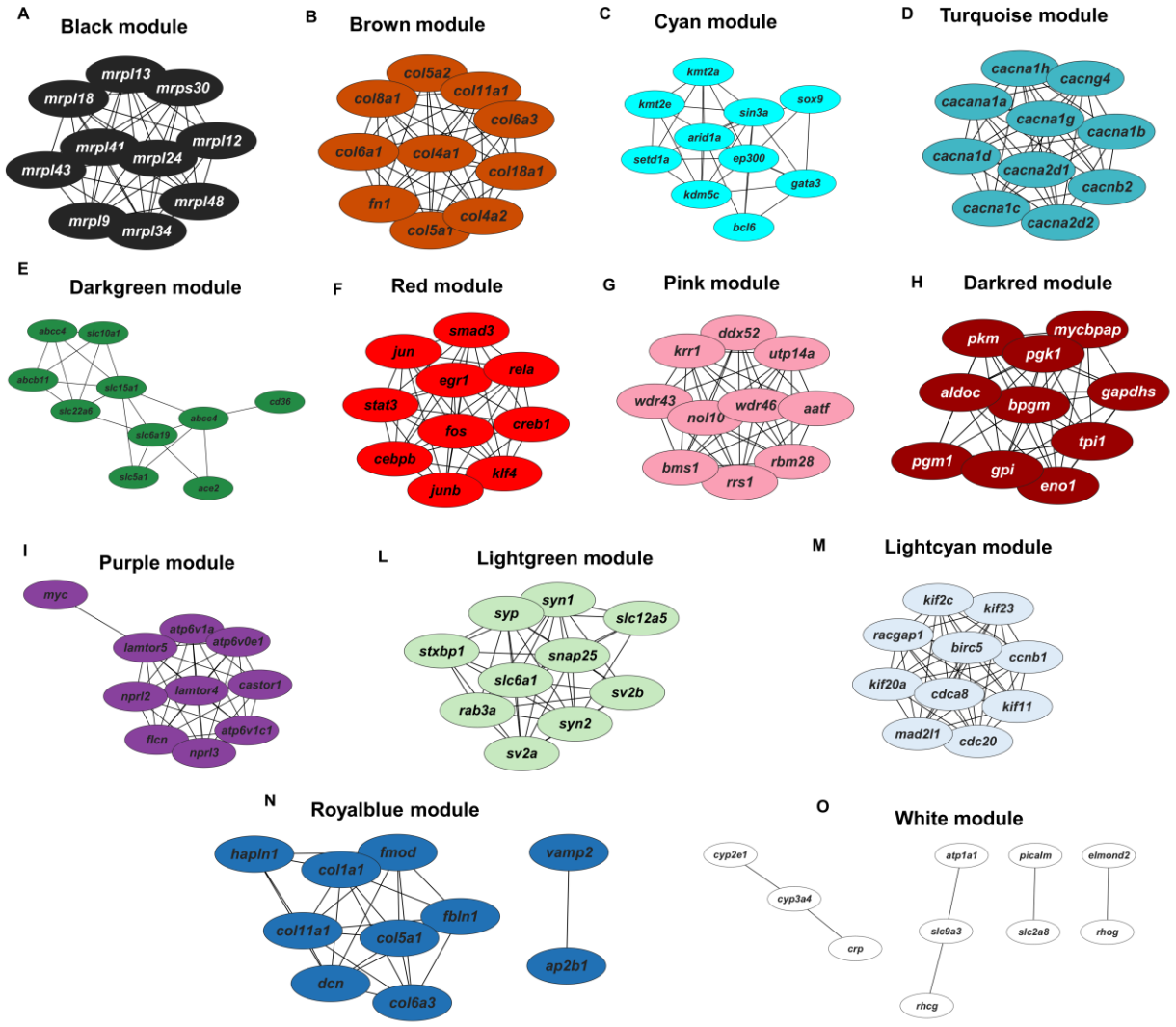

Figure 3.8: *Visualization of modules of interest and hub genes. Color of the nodes is associated with the color of the modules identified with the WGCNA analysis. The top 10 genes with the highest levels of intramodular connectivity in all the significant modules.*

Subsequent Gene Ontology (GO) Enrichment analysis was performed for those 13 modules. This analysis revealed enriched biological processes associated with specific traits. Regarding the embryonic developmental rate, negatively correlated modules were associated with terms linked to ribosome biogenesis, RNA processing, and modification (**Fig. S3.7A**). Conversely, positively correlated modules were enriched in terms related to morphogenesis, circulatory system development, and response to endogenous stimuli (**Fig. S3.7B**). For hatching rate, GO analysis indicated a positive correlation with processes primarily involved in neurogenesis, while

negatively correlated processes included those related to protein transport and metabolic processes such as organic acid and carboxylic acid metabolism (**Fig. S3.8A,B**). Modules significantly correlated with larval weight at mouth opening were exclusively positively associated, with GO analysis implicating mechanisms related to RNA processing and ribosome biogenesis (**Fig. S3.9**). In contrast, length at first feeding showed negative correlations with two modules, revealing processes associated with neurotransmitter transport, transmembrane transport, ion transport, and chemical synaptic transport (**Fig. S3.10**). The analysis of negative modules for Fulton's condition coefficient at mouth opening were associated with genes related to neurogenesis, neuron generation, and differentiation (**Fig. S3.11A**), while positive modules for Fulton's condition coefficient at mouth opening indicated involvement in translation, ribosome biogenesis, and metabolic processes (**Fig. S3.11B**). Furthermore, significant modules were identified for K at mouth opening and the oil droplet reduction phase. The latter negatively correlated with processes involved in the mitotic cell cycle, chromosome segregation, and nuclear division (**Fig. S3.12**).

In summary, it is crucial to note that the transcriptomic profile observed at the larvae's mouth opening stage is shaped by genes that correlate with traits seen before and after hatching, encompassing a variety of functions. Among the enriched biological processes, those related to regulation of transcription processes, cell differentiation and signal transduction are linked to events occurring before hatching. In contrast, processes associated with mitotic cell cycle are specific of traits manifesting after hatching (**Fig. S3.13B**). Intriguingly, there are common biological processes identified for both pre- and post-hatching traits, particularly those related to neurodevelopment (**Fig. S3.13C**). This suggests that neurodevelopment plays a key role in driving both embryonic and larval development stages.

3.3.3 Key traits for aquaculture (KTA)

WGCNA employs significant calculations to pinpoint genes intricately associated with targeted traits. These findings facilitate the identification of most correlated genes for traits such as mortality, cannibalism, SBIE, SGR for total weight and length, K and weight of larvae at the end of the experimental period. The results unveil a comprehensive table showcasing genes with high correlations to each examined parameter (**Supplementary file S3.4**), offering valuable insights into the molecular underpinnings of these traits. The qPCR validation confirmed the association of specific genes, such as selenoprotein O *(selenoo),* tripartite motif-containing protein 16 *(trim16),* solute carrier family 15 member 1 *(slc15a1),* clock-interacting pacemaker *(cipc)*, with the traits under investigation, as outlined in **Table 3.1** (see also **Fig. S3.14**). These genes are

constituting candidate markers which could serve to predict the traits they are significantly correlated with. Additionally, **Fig. S3.15** illustrates the trendlines for candidate genes that were not successfully validated using qPCR.

Table 3.1: *Most correlated genes (both positive and negative) selected for each chosen commercially relevant traits, further validated with qPCR. The resulting table includes the gene significance (GS) values obtained from weighted gene co-expression network analysis (WGCNA), correlation values from qPCR. SBIE: swim bladder inflation effectiveness, SGRW_TOT: specific growth rate for weight for the entire larviculture period, SGRL_TOT: specific growth rate for length for the entire larviculture period, Fulton_END: Fulton's condition factor at the end of the experiment.*

	CANNIBALISM		MORTALITY		SBIE		WEIGHT END		SGRW_TOT		SGRL_TOT		FULTON_END	
	selenoo	*nudt12*	*mkx*	*crcp*	*si:dkey-117m1.4*	*pycard*	*aoc1*	*cipc*	*prdm1*	*txnl4a*	*atp8a2*	*slc15a1*	*sec14l2*	*trim16*
GS	0.82	-0.85	0.77	-0.81	0.79	-0.78	0.81	-0.82	0.84	-0.84	0.87	-0.80	0.81	-0.80
qPCR	0.78	-0.31	-0.02	-0.18	-0.32	-0.33	-0.02	-0.70	0.01	-0.24	0.13	-0.72	0.04	-0.55

3.4 Discussion

Studies conducted on the early developmental stages of larvae can provide valuable insights into the factors that influence, and consequently predict their future performance. Despite advancements, our comprehension of the mechanisms responsible for successful development and survival remains limited. By combining traditional zootechnical assessments with transcriptomic analysis, our research yielded significant insights into larval biology paving the way for understanding intricate growth changes and molecular mechanisms that contribute to the performance of fish larvae. Examining 16 distinct larval families, originating from diverse parental pairs, facilitated an in-depth exploration of larval transcriptomes. The data presented draw our attention to the fact that the molecular profile of freshly hatched larvae (at mouth-opening stage) is highly indicative of pre-hatching events. This emphasizes crucial role of parental contribution in shaping the larval transcriptomic landscape. However, our findings shed also light on the importance of the larvae's transcriptomic profile in determining their future performance, being a solid foundation for further investigations.

3.4.1 Zootechnical traits

Overall, the observed growth patterns (in terms of weight and length) align with previous studies on Eurasian perch (Kupren et al., 2019; Palińska-Żarska et al., 2020). Additionally, there were no major differences observed in cannibalism intensity and SBIE when compared to previous experiments that exposed the larvae to similar experimental conditions (Kupren et al., 2019; Palińska-Żarska et al., 2020). In addition, larval mortality increased especially at the oil droplet reduction phase, reflecting the challenges encountered by the larvae when they switch entirely to exogenous feeding (Kestemont et al., 2003; Król et al., 2019; Palińska-Żarska et al., 2020). This phenomenon could be linked to the non-feeding behaviour, commonly observed in fish larval species (Yúfera & Darias, 2007).

Upon closer observation of the data obtained, although all families were reared under the same controlled conditions, and within-family genetic variability has been limited by creating them each time from a single pair of spawners, considerable variation between families in terms of zootechnical traits was observed, which reflected different performances. Growth-related differences likely influenced cannibalistic tendencies and overall fish development. Such growth heterogeneity has been commonly detected in many other fish species and has been always considered as a relevant determinant and predictor of fish survival, since it can lead to aggressiveness and mortality (Baras & Dalmeida, 2001; Carvalho et al., 2018; Kestemont et al., 2003).

The correlation analysis between various zootechnical traits provides a comprehensive overview of the interrelationships among qualitative traits, elucidating the overall growth trajectory of the offspring. Notably, a positive correlation between embryonic developmental rate and hatching rate suggests that embryos which passed the MZT mostly hatched successfully, supporting the significance of embryonic developmental rate as a reliable indicator of egg developmental competence (Bobe, 2015). Additionally, the analysis illustrates a positive correlation between the length and weight of fry at each sampling time point, indicating that larvae tend to become more robust as their length increases. Conversely, negative correlations, such as those observed between the weight of larvae at mouth opening and SGR from hatching until weaning stage and at the experiment's end, may signify a sort of compensatory mechanism in growth between early and later developmental stages. This phenomenon is commonly observed and has been documented for various fish species (Ali et al., 2003).

The observed correlations between embryonic developmental rates, fertilization and hatching rates, and between growth-related traits, provide insights into larval development dynamics and confirms that these parameters as valuable predictors of future fish performance and overall fitness (Brooks et al., 1997; Koumoundouros et al., 2017). Also, the variability observed in zootechnical traits despite identical rearing conditions implies the involvement of inherited parental factors shaping larvae features. This underscores the need to investigate the molecular background of larvae to elucidate this phenomenon.

3.4.2 Transcriptome data analysis - between past and future

The molecular profile of fish larvae can be influenced by environmental factors (e.g., temperature) as well as genetic and non-genetic inherited factors (Adrian-Kalchhauser et al., 2020). These factors collectively affect transcription, thereby shaping the final transcriptome of hatched larvae. In our current study, we maintained identical incubation conditions for the eggs, ensuring that the analyzed larval transcriptomic profiles primarily reflect solely parental contributions. Thus, in this study we show that certain portion of genes (the ones correlated with pre-hatching traits) are well reflecting parentally-derived genes variability shedding light on parental contribution to transcriptomic portrait of freshly hatched larvae. However, there is also another portion of genes, the one correlated with post-hatching traits, indicating that this transcriptomic variability is also related with future performance of the larvae.

WGCNA identifies gene modules linked to larval traits, aiming to pinpoint specific zootechnical indicators and their dependency with the larval transcriptome as well as to reveal underlying molecular mechanisms crucial in the early phases of fish growth. The results underscored significant correlation between gene networks and pre-hatching parameters, as embryonic developmental and hatching rates, as well as post-hatching traits, i.e., weight at mouth opening, length at first feeding, K at mouth opening and at oil droplet reduction stage. Overall, the GO enrichment analysis of the genes identified within modules exhibits diverse functional processes, including cell cycle, RNA processing, ribosome biogenesis, protein trafficking, apoptosis, circulatory system regulation, and neurogenesis. Notably, the functions of the identified hub genes within significant modules (**Fig. 3.8**) mostly align with the biological processes highlighted through GO analysis of all the genes in the significant modules. This underscores their importance in regulating key biological functions, indicating their potential as critical targets or biomarkers in specific biological contexts. The consistency of these findings across different fish

species (Bougas et al., 2013; Mazurais et al., 2011) further emphasizes the significance of these biological processes in shaping larval performance and underscores also its complexity in fish.

Embryonic developmental rate and hatching rate, were negatively correlated to genes involved in RNA processing, translation, ribosome biogenesis and protein transports. These processes collectively govern gene expression, cell signalling, and tissue differentiation, profoundly impacting fish larval growth and survival. Particularly, ribosomes produced abundantly during oogenesis and presumably deposited in the eggs, play a crucial role in synthesizing proteins vital for various developmental processes (Leesch et al., 2023; Qi et al., 2016; Shen et al., 2017). These maternally provided ribosomes are paramount during embryogenesis specifically until Zygotic Genome Activation (ZGA), which marks the transition of developmental control from maternal to zygotic factors (Leesch et al., 2023). Since the pre-hatching traits were negatively linked to these biological processes, our speculation is that post-ZGA, embryos likely redirect their energy towards processes -positively correlated with pre-hatching traits – like neurogenesis, sensory organ development, and morphogenesis of tubes and blood vessels, which are equally necessary for their future developmental success. These results indicate that embryos may prioritize these processes closer to hatching. The embryos, as well as post-hatching larvae, are constantly subjected to morphological and physiological modifications. Among others, the maturation of the nervous system is one of the most important events. This process is crucial for enhancing sensory perception, motor coordination, and cognitive functions in larvae (Nelson & Granato, 2022). Previous studies discussed the nervous systems' development could potentially be pre-programmed by the molecular content inherited maternally (Żarski et al., 2020a, 2021a). This, along with environmental factors may influence the trajectory of nervous system development and impact future behaviour and adaptive responses (Colson et al., 2019). In summary, right after fertilization more general but crucial functions are prioritized (i.e., ribosome biogenesis), while after ZGA processes essential for successful accomplishment of embryonic development take precedence. These subsequent events will most likely define the success of the hatching.

Molecular profile of the larvae is determined by the interplay between environmental factors, genetic background and so called non-genetic inheritance mechanisms. The latter encompass, among others, mRNAs which play a pivotal role as modulators of gene expression during the early development and consequently influencing the phenotype of the progeny (Adrian-Kalchhauser et al., 2020). It has been hypothesized that such cascade-like transmission of information from parent to progeny affects the performance of larvae and juveniles (Adrian-Kalchhauser et al., 2018; Colson et al., 2019). In this context, results of our study highlight the importance of parental

contribution in shaping the transcriptomic profile of the larvae (Harvey et al., 2013), reflecting their past. Analogically, the significant correlations with the post hatching traits are predictive of the larvae's future. Notably, those traits fall within the initial growth phases until yolk sac absorption. Up to this stage, larvae rely primarily on the nutrients stored within the yolk sac, which they inherited from the female (Callet et al., 2022). These nutrients play a crucial role in sustaining the larvae's early development by determining energetic reservoirs and indirectly controlling their growth and performance (Bachan et al., 2012; Migaud et al., 2013). The GO enrichment analysis of gene modules linked to post-hatching traits aligns with molecular processes identified in pre-hatching traits. However, contrarily to pre-hatching traits, genes associated with ribosome biogenesis exhibit a positive correlation with post-hatching traits, while those linked to neurogenesis show a negative correlation. This inversion may suggest a shift in biological process prioritization as larvae transition, depending on their developmental stage (Mathavan et al., 2005). For example, after hatching larvae may prioritize protein production to overcome crucial metamorphosis events (e.g., onset of exogenous feeding, swim bladder inflation, etc). We can hypothesize that bigger larvae at mouth opening invest more in protein translation, potentially leading to enhanced physiological development and growth rates. Also, upon first feeding larvae that exhibit larger sizes, may have already better-developed senses and a more advanced nervous system, which can explain the inverse correlation with neurogenesis pathways at this stage.

Post-hatching traits exhibit a specific association with processes linked to the cell cycle and mitosis. As larvae undergo metamorphosis and confront various challenges related to the interaction with the external environment, significant restructuring of organs and tissues occurs, with the cell cycle serving as a central mechanism (González-Quirós et al., 2007). This transition likely initiates a cascade of molecular events, indicating a profound shift in cellular activities towards enhancing the growth and development of various organs to support their functions. For instance, after the oil droplet reduction stage, larvae become entirely dependent on external feed for energy, necessitating the digestive system to efficiently process and utilize food resources. Rapid cell division and proliferation may play critical roles in optimizing system functions, enabling larvae to better adapt and respond to environmental cues.

Overall, the contrasting correlations between ribosome biogenesis and neurogenesis well reflect the dynamic nature of larval development. As larvae progress through various stages, their biological priorities shift, leading to fluctuations in gene expression patterns and molecular processes. Understanding these intricate relationships between molecular processes and larval development provides valuable insights into the adaptive strategies of organisms and the

mechanisms underlying developmental plasticity. Nonetheless, the environmental factors still play a significant role in shaping these developmental trajectories. Changes in temperature, light exposure, nutrient availability, and other environmental cues can modulate gene expression and influence the balance between different biological processes (Mazurais et al., 2011; Urho, 2002). Our results indicates that transcriptomic signature of larvae at the mouth opening stage offers significant insights into parental contributions and their influence on embryogenesis. However, its ability to predict future larval performance appears to be somewhat limited. It should be emphasized, that future larval fate is determined by the interaction of their molecular cargo with the external factors playing a major role right after hatching. It's essential to acknowledge that in our 'common garden' experiment all the environmental factors were controlled and equal for all larvae families. This may limit the detection of certain traits typically observed in wild conditions, such as exposure to pathogens or stressors. For instance, immune system traits may not be evident in our current setup, but exposing organisms to bacterial or temperature challenges could reveal the transcriptome's predictive capacity for stress or immune system functioning, which is important in fish adaptability (Elabd et al., 2017; Kammer et al., 2011; Palińska-Żarska et al., 2021). Therefore, future research should consider subjecting organisms to specific challenges to uncover additional predictive traits.

Transcriptomics is an important tool for phenotypic profiling, as it reveals gene expression patterns and their correlation with observable traits (Chandhini & Rejish Kumar, 2019). However, transcriptomics alone provides only part of the picture. To gain a more comprehensive understanding of biological processes and accurately predict phenotypic outcomes, it is crucial to integrate other omics technologies such as proteomics and metabolomics. Proteomics analyses proteins, the direct effectors of cellular functions, while metabolomics examines metabolites, the end products of cellular processes. Combining these approaches with transcriptomics offers a more holistic view of the phenotype, reflecting both genetic information and its functional outcomes (Natnan et al., 2021; Subramanian et al., 2020). Therefore, we propose that future research should adopt a multi-omics approach to improve the precision and reliability of phenotypic predictions.

3.4.3 Key traits for aquaculture (KTA) - molecular signatures

Aquaculture relies on important traits like cannibalism, survival rate, swim bladder inflation, weight, and specific growth rate for successful management and production (Toomey et al., 2021). The transcriptome of newly hatched larvae may offer predictive insights into future development, supporting aquaculture research and its production. The WGCNA has facilitated the identification

of genes strongly associated with these commercially relevant traits, potentially serving as specific gene markers.

The validation with qPCR highlighted 4 genes: *selenoo, trim16, slc15a1, cipc*; linked respectively to cannibalism, SGR for length, K and weight of larvae at the end of the experiment. Notably, *cipc* appears pivotal in regulating various physiological processes in fish, influencing behaviour, metabolism, and also clock genes seem to be implicated in thermal resistance (Hung et al., 2016); while *slc15a1* affects nutrient absorption, transport and fish growth (Romano et al., 2014; Vacca et al., 2019). *Trim16*, a member of the teleost-specific *fintrim* family (van der Aa et al., 2009), is involved in regulation of innate immunity, but it seems also involved in cell proliferation, differentiation, and metabolism (Cho et al., 2022). While its specific functions in fish larvae, particularly regarding traits like Fulton's condition factor, may require further investigation, its involvement in cellular processes suggests potential roles in larval development and physiology. Also, selenoproteins, including *selenoo*, contribute to cellular antioxidant defence mechanisms (Han et al., 2014; Sumana et al., 2023), and indirectly, they can play a role in fish growth.

These genes collectively play crucial roles in fish larval development and physiology, impacting growth, behaviour, immunity, and metabolic processes. However, out of the 14 chosen genes, only 4 were confirmed through qPCR validation. This suggests that our approach (i.e., finding gene markers based on correlation of their expression with traits) may not be entirely reliable for detecting gene markers associated with zootechnical traits. Future studies should use more relevant methods, such as conducting specific experiments that assess each trait and individual larva. This approach will help to gain a deeper and more accurate understanding of the variability in the traits expressed within larval populations. This would provide a clearer understanding of the mechanisms and interactions underlying larval traits and overall fish performance.

3.5 Conclusion

Considering all the above mentioned, larval transcriptomic profile represents a bridge between the past, stemming from the transcripts provided by parents within gametes and continuing through embryonic development, and the future, serving as a forthcoming instruction for the larval period and potentially beyond. Taking all of this into account, by analysing transcriptome of freshly hatched larvae we gather information on parentally derived molecular cargo but also on larvae adaptability, paving the way to comprehend the intricate developmental trajectories that lead to adulthood.

3.6 Data availability

Raw data from the analysis of different families of freshly hatched larvae can be accessed via the NCBI BioProject database under the PRJNA1032718 accession number.

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CRediT authorship contribution statement

Rossella Debernardis: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Katarzyna Palińska-Żarska:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Sylwia Judycka:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Abhipsa Panda:** Writing – review & editing, Investigation. **Sylwia Jarmołowicz:** Investigation. **Jan P. Jastrzębski:** Data curation. **Tainá Rocha de Almeida:** Writing – review & editing, Data curation. **Maciej Błażejewski:** Resources. **Piotr Hliwa:** Resources. **Sławomir Krejszeff:** Resources. **Daniel Żarski:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Chapter 4: 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: writing the original draft, visualization, methodology, data analysis, investigation, data curation, conceptualization.

4.1 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. 2020a) 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.

4.2 Materials and methods

4.2.1 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.

4.2.2 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. 2017a) 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 ♂) (**Fig. 4.1A**). 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. DW; WW vs. WD), with shared male or female partners across comparisons (**Fig. 4.1B**).

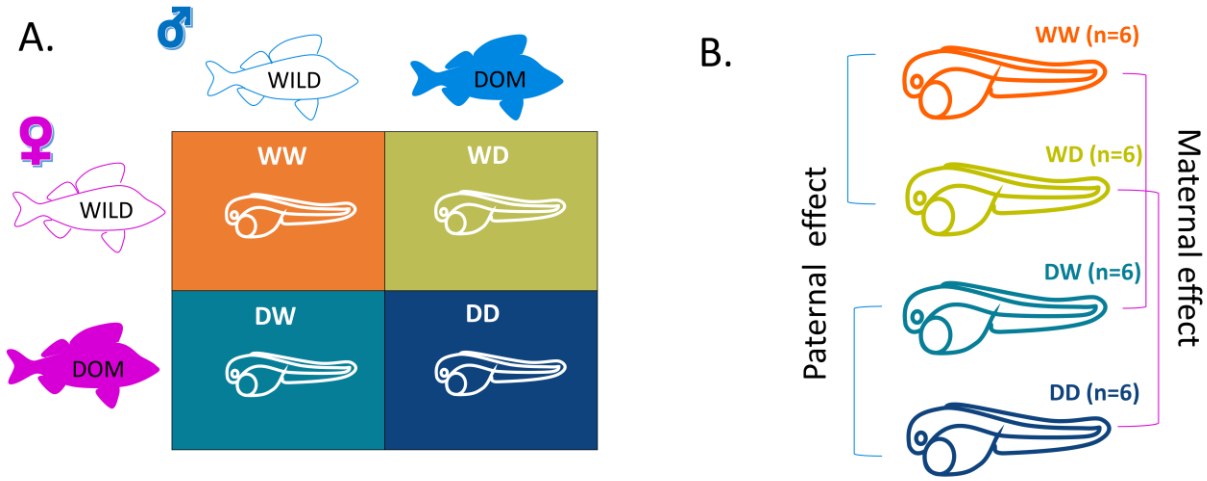

Figure 4.1: *Experimental design and Groups comparisons scheme.* ***A.*** *Crossing matrix of Wild (W) and Domesticated (D) fish, resulting in four offspring experimental groups: wild females and wild males (WW), wild females and domesticated males (WD), domesticated males (WD), domesticated females 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.*

4.2.3 Broodstock management and collection of gametes from domesticated and wild spawners

The broodstock characteristics for both wild and domesticated individuals are described in **Supplementary table S4.1**.

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. 2017g). Finally, eggs of domesticated fish were collected on day 9 following priming injection, whereas eggs of wild females on day 4 after injection (Zarski 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.

4.2.4 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., 2021b). 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. (2022) with a final concentration of 0.3 M glucose, 7.5% methanol and 25 mM KCl at 3×10^9/ml spermatozoa.

4.2.5 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. (2017a) 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 (Zarski 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 4.1**), 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 (**Fig. 4.2**), 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.

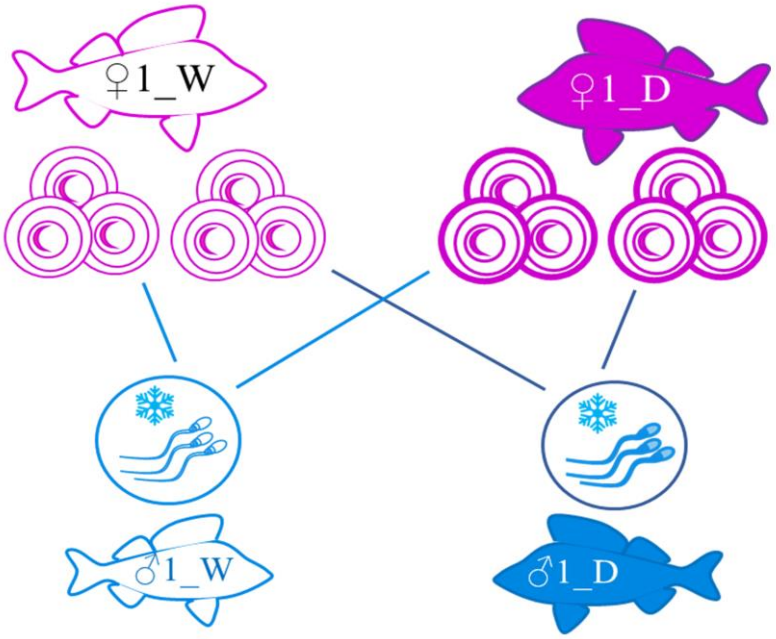

Figure 4.2: *The diagram illustrates a paired fertilization method, where eggs from a specific domesticated female (♀1_D) were fertilized in two ways: half with milt from a domesticated (♂1_D) male and the other half with wild male milt (♂1_W). Same combination of domesticated and wild males was used 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.*

4.2.6 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 (**Fig. 4.3A**). 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.

4.2.7 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) (**Fig. 4.3A**). The deformity rate was counted at 2 DPH (**Fig. 4.3B**). 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.

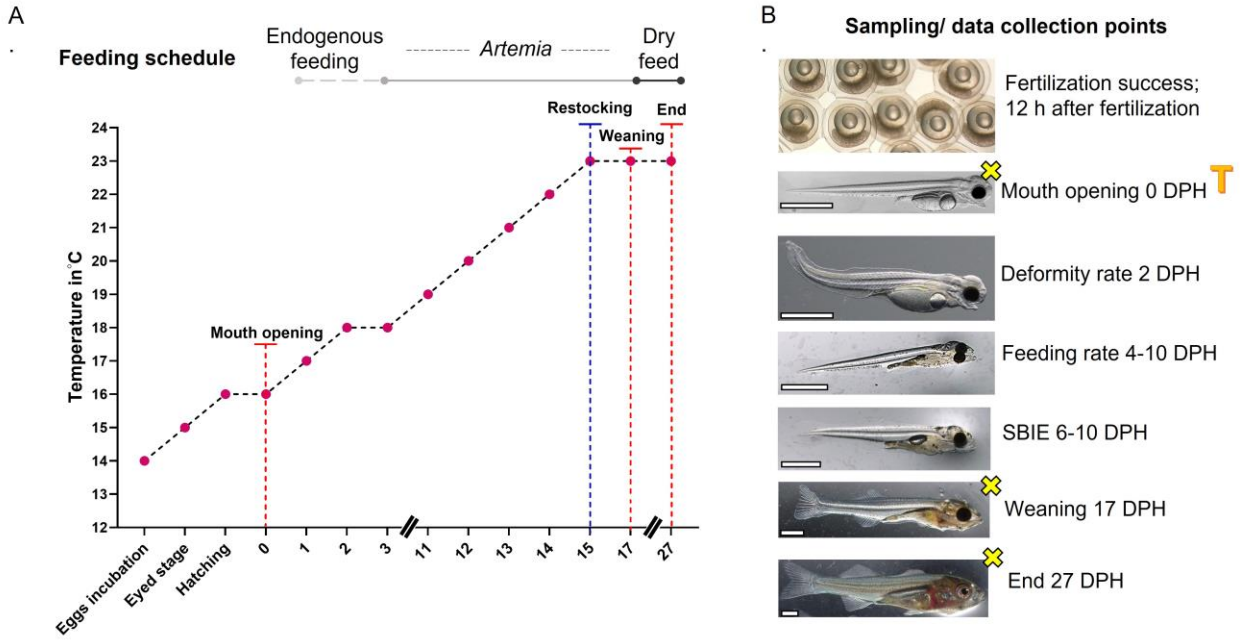

Figure 4.3: *Protocol of eggs incubation and larvae rearing followed for the experiment.* ***A.*** *Rearing schedule and temperature regimen used for embryos and larvae.* ***B.*** *Sampling points for zootechnical data collection. Marked with yellow crosses are the sampling points for both zootechnical data collection and molecular analysis. T= describe the moment when collected larvae were used for transcriptomic analysis. DPH: Days post hatch*

4.2.8 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.

4.2.9 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.

4.2.10 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 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., 2013) and quantified with RSEM (Li & Dewey, 2011). A summary table with general statistics of the RNA-seq data is provided in **Supplementary file S4.2**.

4.2.11 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 (α = 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).

4.2.12 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.

4.2.13 *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) (**Fig. 4.4**). 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 (**Fig. 4.4, Supplementary File S4.3**). 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.

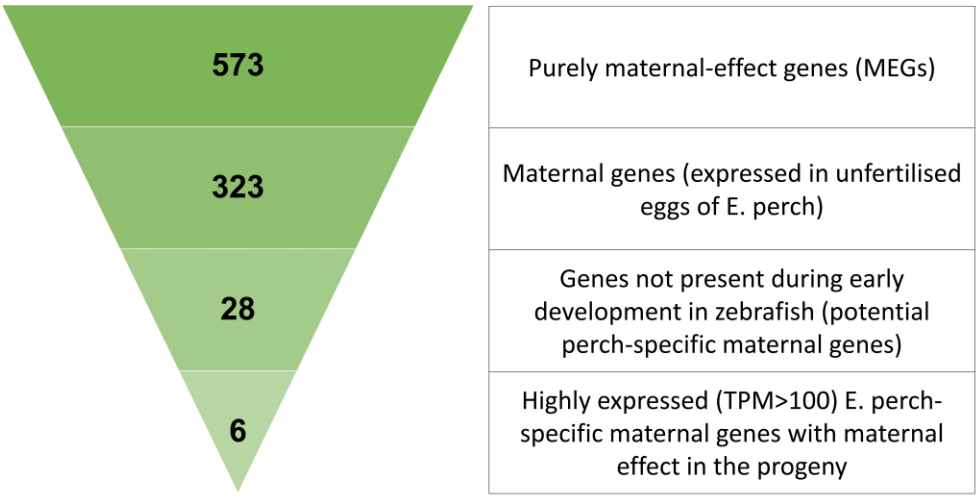

***Figure 4.4**: Graphic representation of the filtering cascade followed used to select the six Eurasian-perch-specific maternal genes with documented maternal-effect candidates.*

4.2.14 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 5× 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. 2021a), using TPMs calculated for all the biological replicates. The sequences of the designed primers are presented in **Supplementary file S4.4**.

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 (Livak & Schmittgen, 2001). Data were normalized using geometric mean of reference genes.

4.2.15 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).

4.3 Results

4.3.1 Zootechnical data

No significant differences in fertilization rates were observed when comparing the influence of both paternal- and maternal-effects across groups (**Fig. 4.5**). 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 (**Fig. 4.6**). Also, no differences were observed for deformity rate (**Fig. S4.5A, in Supplementary file S4.5**). 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 (**Fig. S4.5A, in Supplementary file S4.5**).

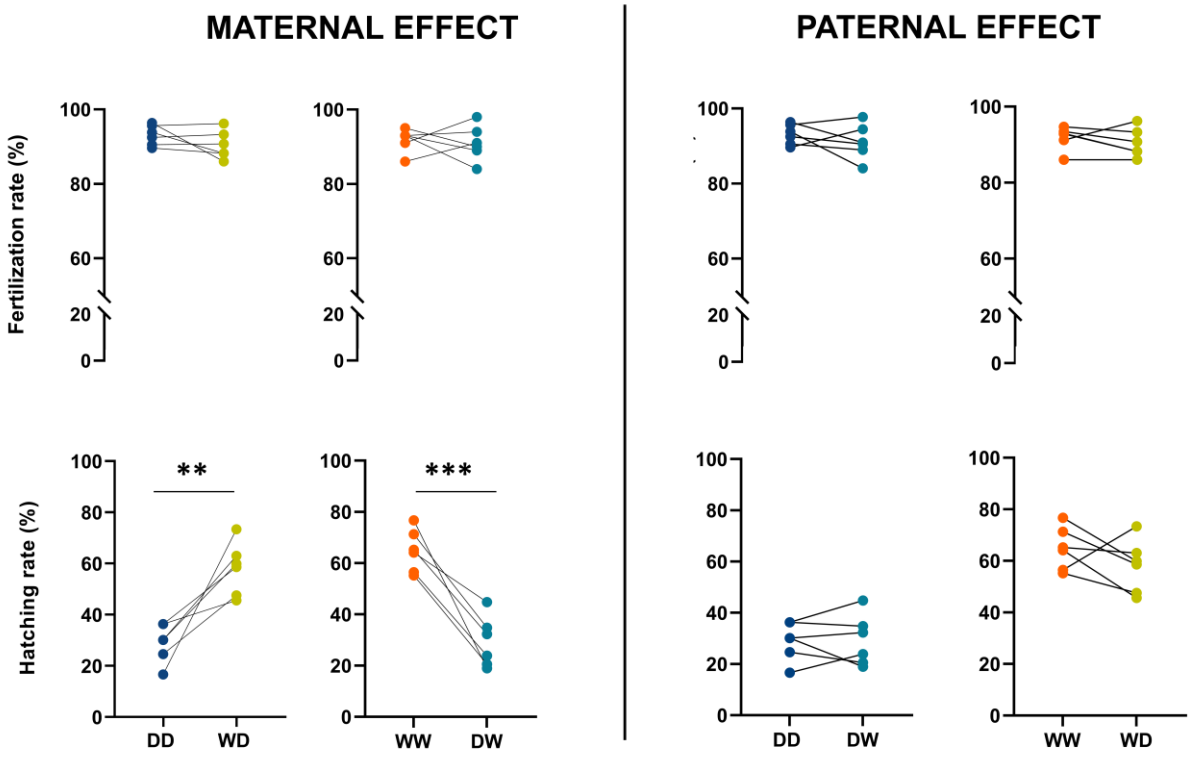

Figure 4.5: *Fertilization rate and hatching rate for all the families of Eurasian perch. The asterisks (**p < 0.01, ***p < 0.001) show significant differences between the groups. D: Domesticated; W: Wild*

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 (**Fig. 4.6**). 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 (**Fig. S4.5B, in Supplementary file S4.5**).

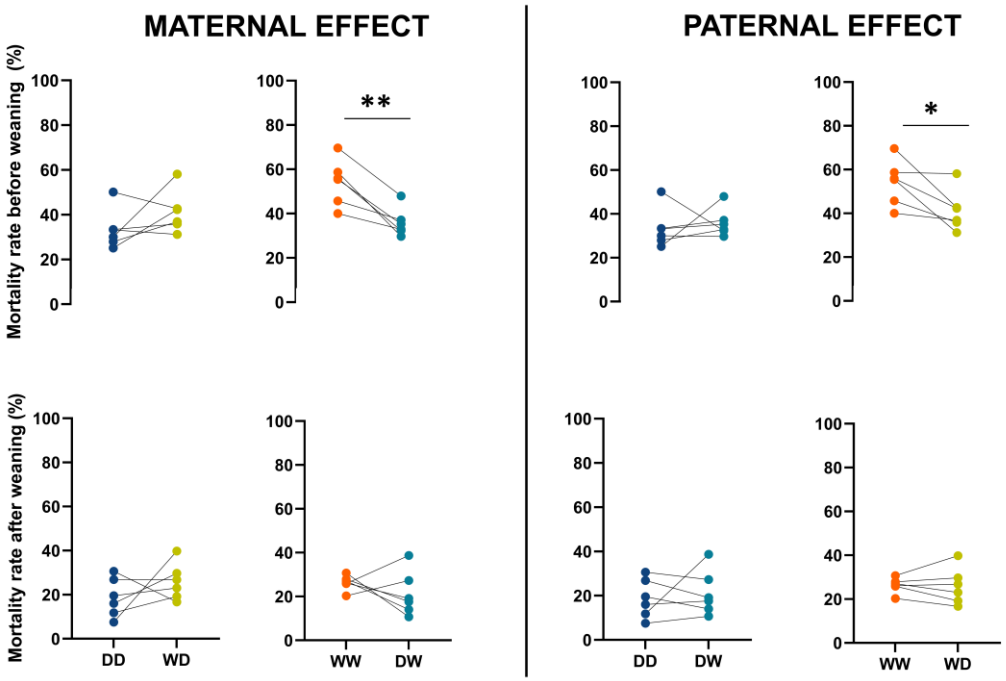

Figure 4.6: *Mortality rate before and after weaning for all the families of Eurasian perch. The asterisks (*p< 0.05, **p < 0.01) show significant differences between the groups. D: Domesticated; W: Wild*

The feeding rate was evaluated over a 7-days period (from 4 DPH to 10 DPH) to study kinetics of feeding onset (**Fig. 4.7 and 4.8**). Significant differences were mainly observed when males were crossed with domesticated females (**Fig. 4.8**). 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 (**Fig. 4.7**). Moreover, the larvae coming from domesticated females consistently showed a higher SBIE, regardless of the phenotype of the male they were paired with.

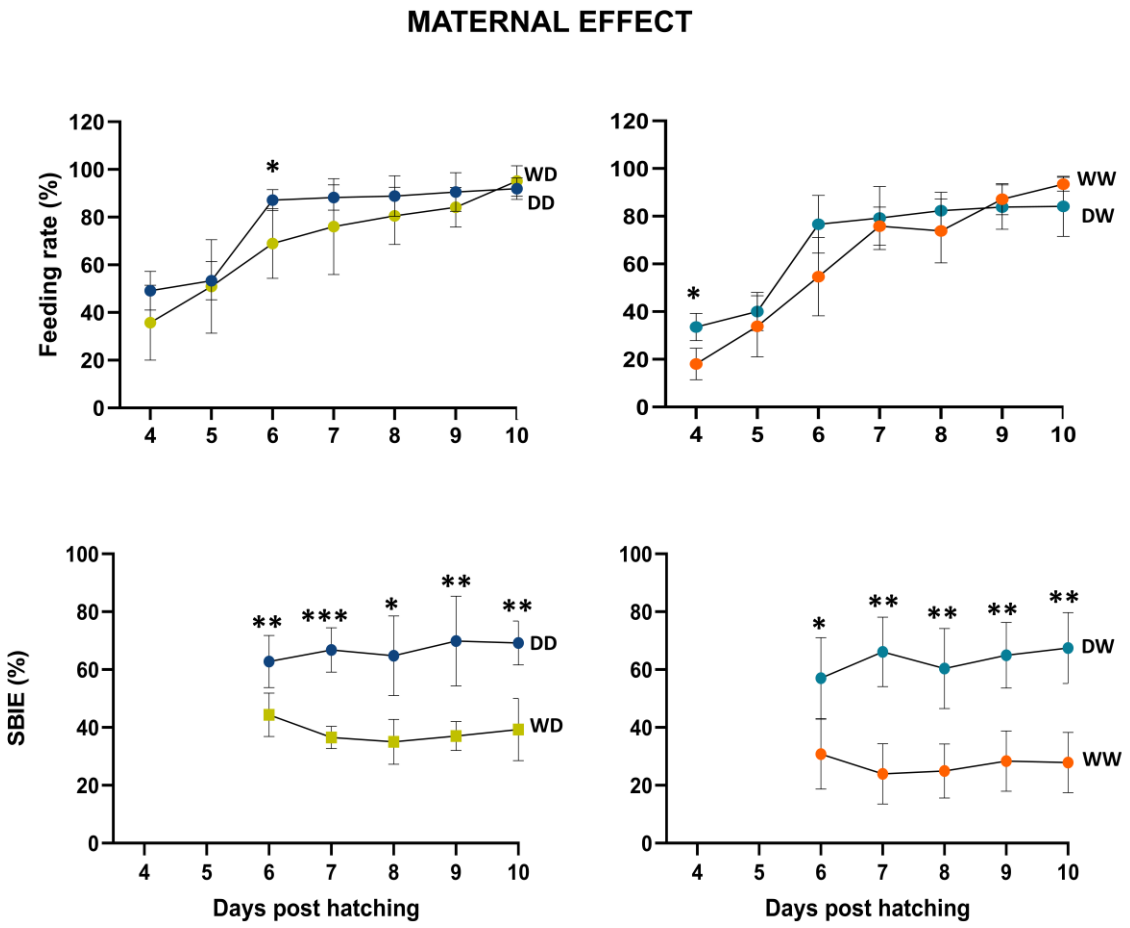

Figure 4.7: *Feeding rate and SBIE kinetics (%) for all Eurasian perch families analyzed for maternal-effect. The asterisks (*p< 0.05, **p < 0.01, ***p < 0.001) show significant differences between the groups over time. SBIE – swim bladder inflation effectiveness; D: Domesticated; W: Wild*

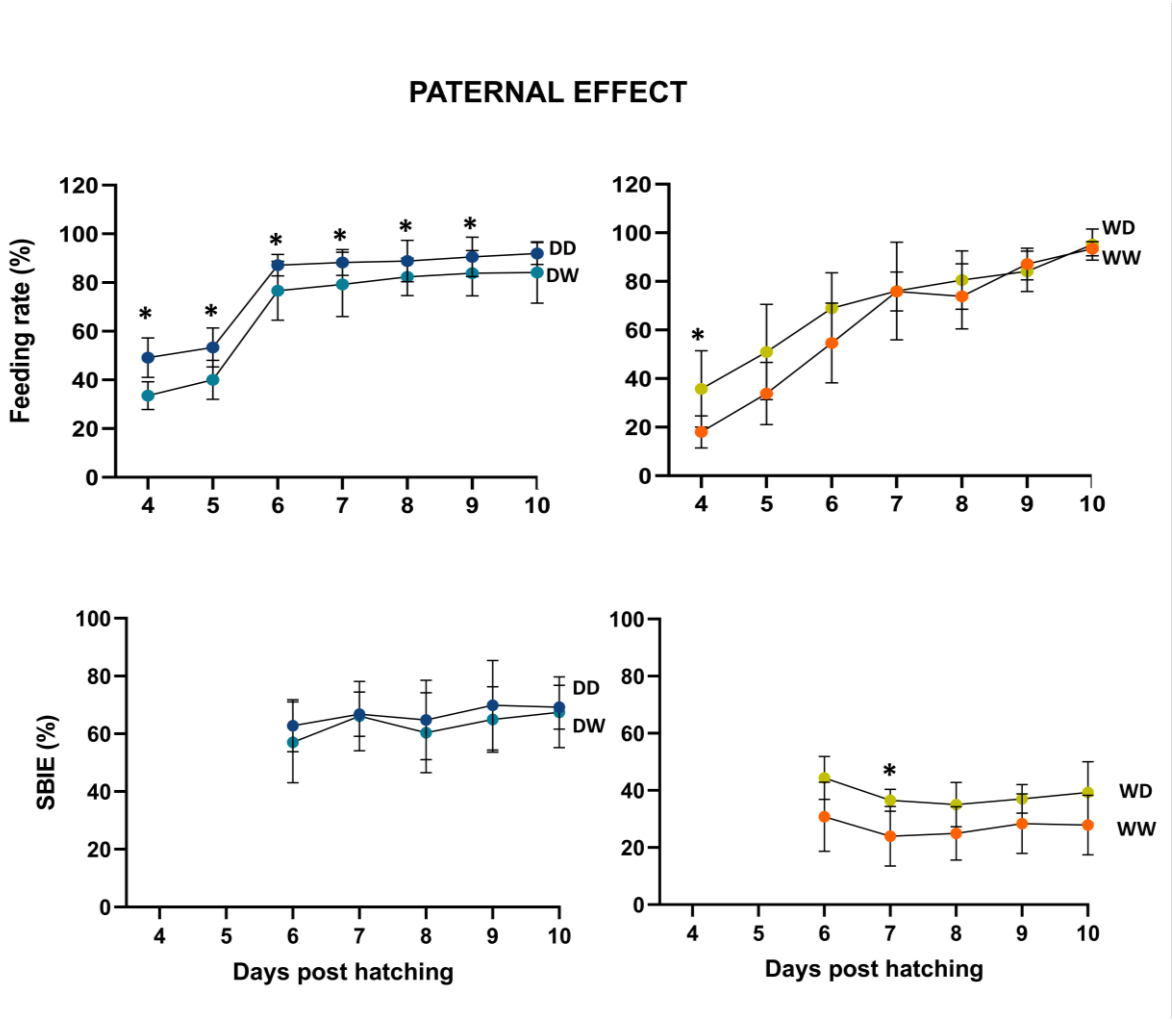

Figure 4.8: *Feeding rate and SBIE kinetics (%) for all Eurasian perch families analyzed for paternal-effect. The asterisks (*p< 0.05) show significant differences between the groups over time. SBIE – swim bladder inflation effectiveness; D: Domesticated; W: Wild*

Significant differences in total length (TL) (**Fig. 4.9**) 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.

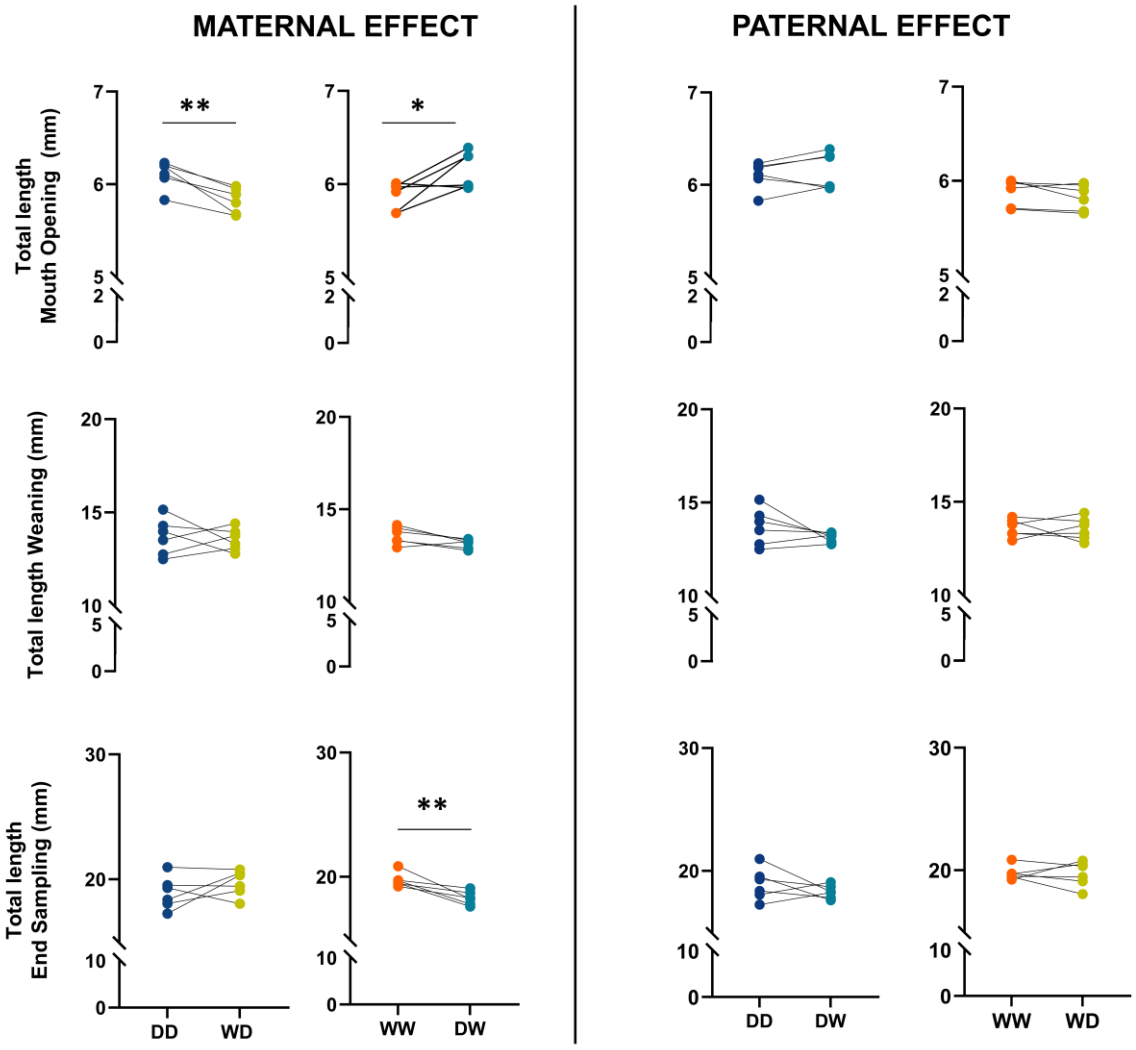

Figure 4.9: *Total length (TL; mm) of larvae measured at different developmental stages for all Eurasian perch families. The asterisks (*p< 0.05, **p < 0.01) show significant differences between the groups over time. D: Domesticated; W: Wild*

For wet body weight (WBW) (**Fig. 4.10**), 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.

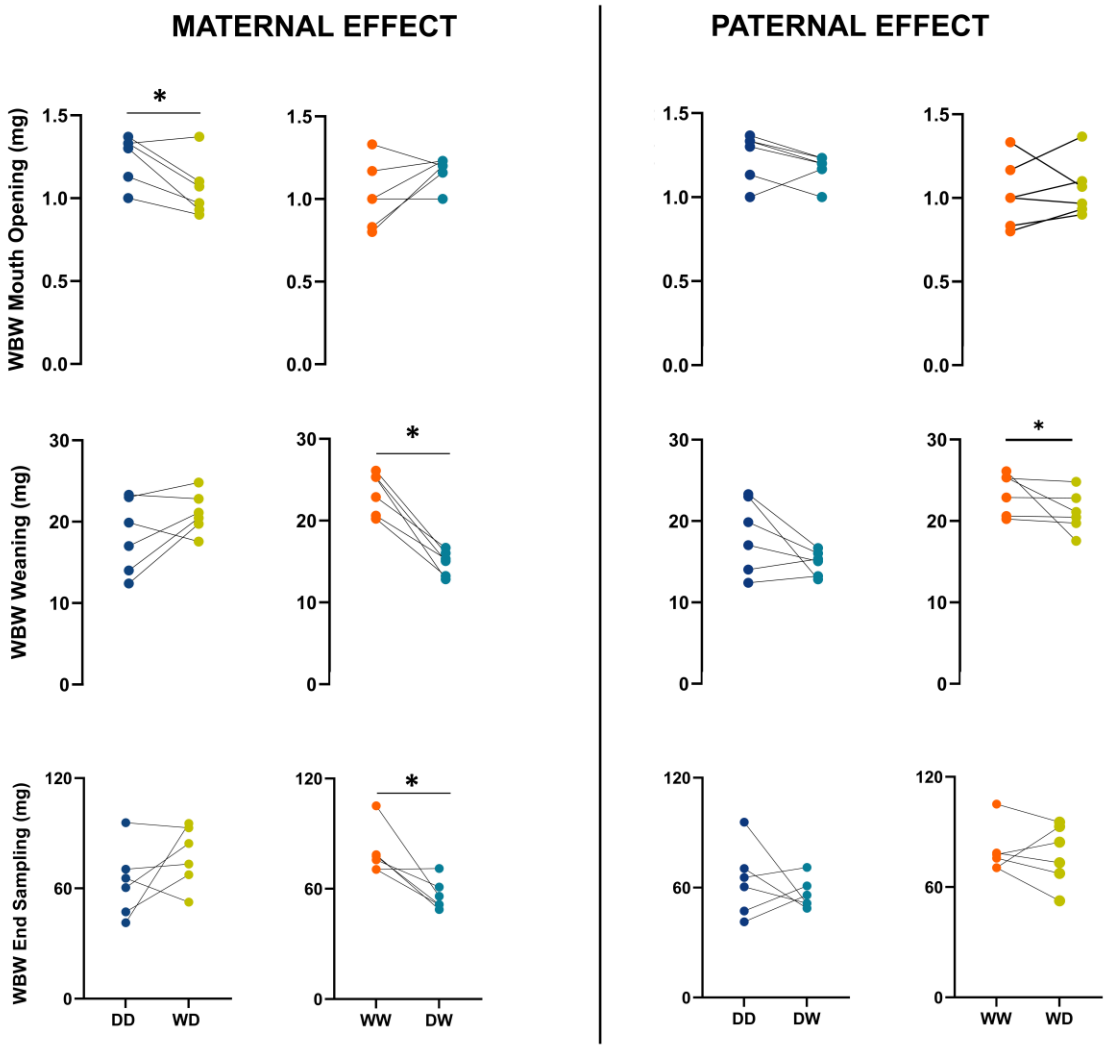

Figure 4.10: *Wet body weight (WBW; mg) of larvae measured at different developmental stages for all Eurasian perch families. The asterisks (*p< 0.05) show significant differences between the groups over time. D: Domesticated; W: Wild*

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.

4.3.2 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 S4.3**). Visualization of 100 most variable genes (**Fig. 4.11A**) shows significant transcriptomic diversity among the experimental groups and the PCA (**Fig. 4.11B**) reveals that the family distribution is clearly influenced by the female origin.

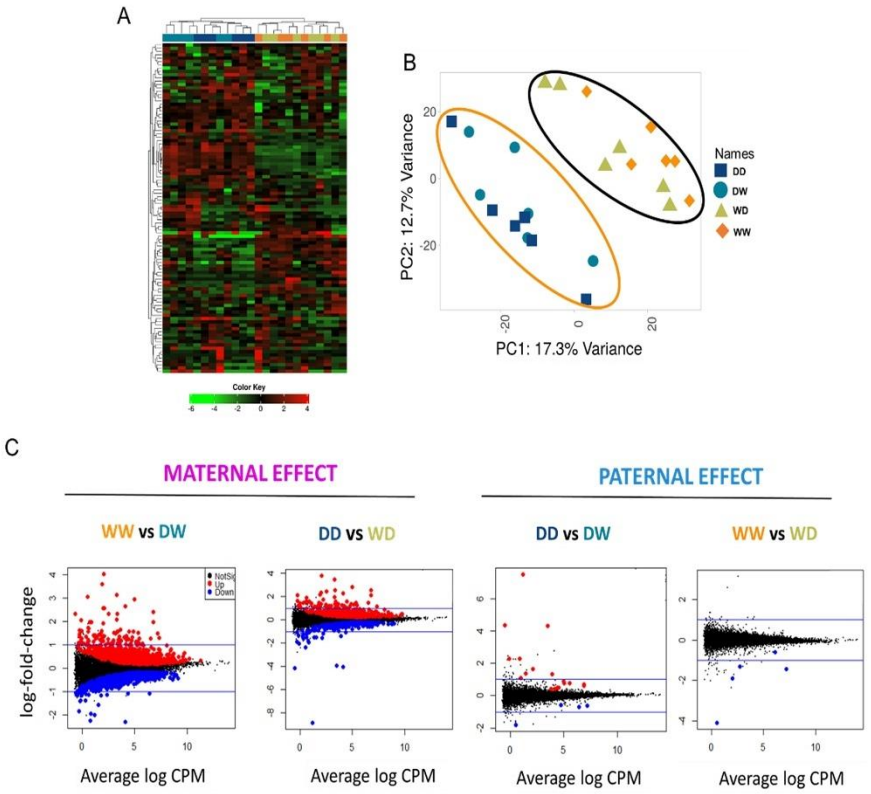

Figure 4.11: A. *Heatmap showing hierarchical non-supervised clustering of 100 most variable genes in the transcriptome of freshly hatched Eurasian perch larvae.* ***B.*** *Principal component analysis for all the Eurasian perch families created. The analyses were done on the basis of female origin - 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; D: Domesticated; W: Wild*

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 (**Fig. S4.5D, see Supplementary file S4.5**).

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 (**Fig. 4.11C**). 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 (**Fig. 4.11C**). To check for any overlap between groups, a Venn intersection analysis was performed (**Fig. 4.12**). 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 (**Fig. S4.5E, see Supplementary file S4.5**). 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 (**Fig. S4.5F in Supplementary file S4.5**).

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).

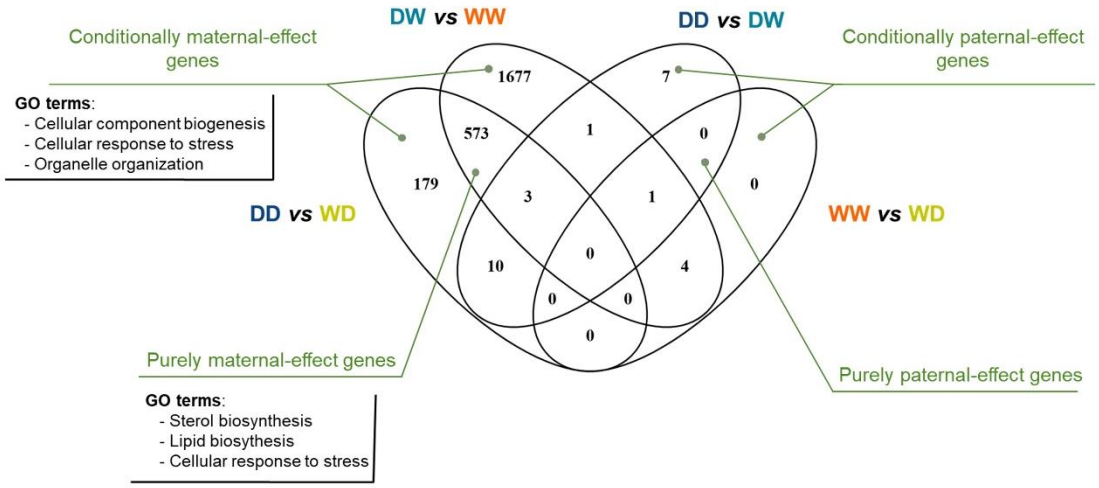

***Figure 4.12**: Venn diagram illustrating the overlapping genes classified as purely maternal-effect and paternal-effect genes, as well as those exclusive to conditionally maternal- and paternal-effect genes across different comparisons. Additionally, the figure lists the top three most enriched Gene Ontology (GO) terms associated with purely maternal-effect genes (n=573) and all conditionally maternal-effect genes (n=1856). D: Domesticated, W: Wild*

4.3.3 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 4.1, Fig. 4.13**).

Table 4.1: *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.

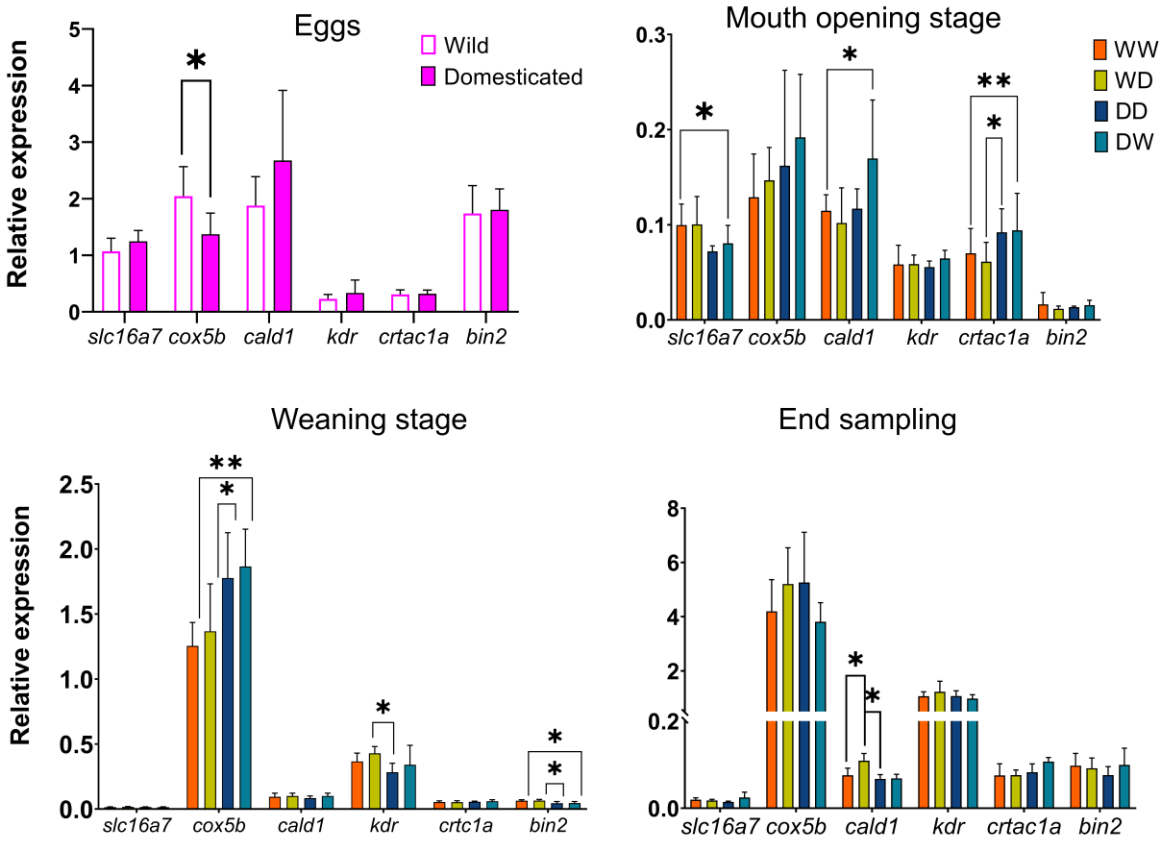

Figure 4.13: *RT-qPCR for the 6 genes selected across the egg stage and various larval stages (from mouth opening to the end of the larval period, here referred as end sampling). The results of statistical analysis are presented as follows: *p < 0.05, **p < 0.01. D: Domesticated; W: Wild*

4.4 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.

4.4.1 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.

4.4.2 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.

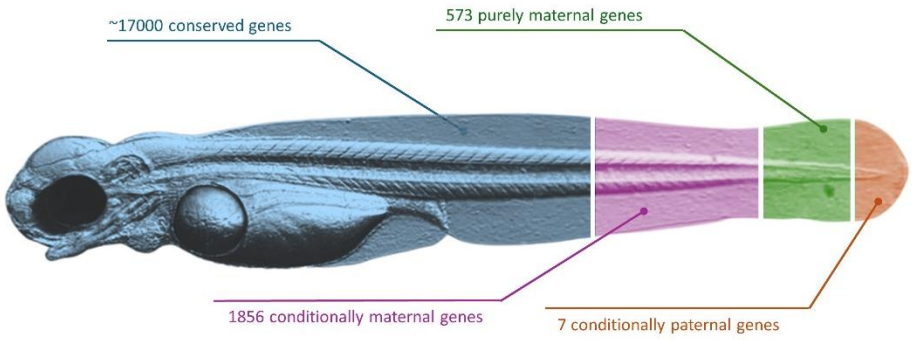

Figure 4.14: *Presumed transcriptomic portrait of a freshly hatched Eurasian perch larva considering parental contributions.*

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 (**Fig. 4.14**). 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. (2021a) 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.

4.4.2.1 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.

4.5 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.

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 PRJNA1196822 accession number.

4.7 Acknowledgments

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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

***RD** has been responsible for experiment; laboratory works and data analysis related to maternal effect

****AP** has been responsible for experiment; laboratory works and data analysis related to paternal effect

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Chapter 5: Transcriptomic profile of fish eggs reflects embryonic survival potential, not larval fate

Also referred as: Egg quality

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Status: In preparation

Contribution: writing the original draft, visualization, methodology, data analysis, investigation, data curation, conceptualization.

5.1 Introduction

Prediction of larval quality at the egg stage has been pointed out as a major challenge in aquaculture. Early prediction would not only enable the selection of high-quality egg batches but also higher larval survival and growth, thereby improving hatchery efficiency, identification of superior spawners, production costs, and ultimately selective breeding programs (Ienaga et al., 2021; Schaefer et al., 2018). This expectation stems from the common assumption that high-quality eggs invariably produce healthy, robust larvae, whereas low-quality eggs yield weak ones (Fernández Míguez et al., 2024; Kjørsvik et al., 1990). However, discussions on egg quality often conflate two separate aspects: the ability of an egg to complete embryogenesis and the quality of larvae that result from it. In practice, egg quality is usually assessed at the batch level using measures such as fertilization rate, cleavage success, or embryonic survival (Bobe & Labbé, 2010). Since low-quality batches produce very few viable larvae, it makes difficult to directly evaluate larval performance. As a result, the common assumption that low egg quality leads to poor larval quality remains largely untested. This gap limits the predictive power of current egg quality indicators, most of which are often species-specific and not easily generalized (Schaerlinger & Żarski 2015).

Egg quality, defined as the capacity of an egg to achieve fertilization and develop into a viable larva (Bobe & Labbé, 2010), is a result of a complex interplay between genetic, environmental, and physiological factors (Kjørsvik et al., 2003; Reading et al., 2018). Traditional egg quality metrics such as egg size, lipid droplet morphology, cleavage patterns (Brooks et al., 1997; Żarski et al., 2011), as well as biochemical indicators like fatty acid profiles and amino acids content (Henrotte et al., 2010; Reading et al., 2018) often fail to predict larval success due to species- or even population-specific variability and context-dependence (Migaud et al., 2013; Żarski et al., 2017c). Even fertilization rates, commonly used as an indicator of egg quality (Bobe & Labbé, 2010), can be misleading. In fact, eggs with identical fertilization success may show drastic differences in embryo development and hatching outcomes (Kjørsvik et al., 2003). Notably, developmental failures frequently arise even after fertilization, when the zygotic genome activation (ZGA) takes place. Before ZGA, embryo development is primarily directed by maternal RNA and proteins deposited in the egg. However, after ZGA, maternal transcripts are replaced by the embryo's own genetic material (Tadros & Lipshitz, 2009). When disruptions occur after this transition, it means that not only maternal contributions but also zygotic factors play a critical role in determining developmental success, and it becomes difficult to pinpoint the exact cause of

failure (Bobe, 2015; Cheung et al., 2019). This complexity makes it difficult to reliably predict developmental outcomes based solely on early-stage egg quality indicators.

To address these limitations, transcriptomic profiling of gametes has emerged as a promising tool for identifying molecular pathways and gene markers linked to egg quality (Żarski et al. 2017d; Cheung et al. 2019), which could help in monitoring and further understanding causes underlying lowered egg quality. Furthermore, despite ongoing progress in this field, a consistent molecular fingerprint predictive of larval quality remains elusive (Migaud et al., 2013). This stems partly from methodical reasons. Usually, for molecular analysis a randomly pooled eggs are being used following their classification to either "high" or "low" quality groups without excluding eggs characterized by apparent deterioration (such as those with severe lipid droplet fragmentation, advanced overripening, or intraovarian aging heralded by lack of intracellular integrity; for details see - Żarski et al. 2021a) which are essentially incapable of successful fertilization. Including these egg samples can distort molecular analyses by introducing signals unrelated to true developmental competence (Migaud et al. 2013; Żarski et al. 2021a). To overcome this limitation, Żarski et al. (2021a) conducted a study on pikeperch (*Sander lucioperca*) using a specific preselection strategy: only eggs with confirmed fertilization success were included in the transcriptomic analysis, and these were subsequently classified according to their embryonic developmental outcomes. This refined approach enabled to identify novel maternal transcripts being predictive for developmental competence. However, as in many other studies in this field, the relationship between altered molecular cargo in eggs and the resulting larval phenotype, and its implications for larval fate, has received little attention. Bridging this knowledge gap is essential to understand how maternal contributions shape offspring performance and to evaluate whether transcriptomic variation in eggs truly determines larval fate.

Eurasian perch (*Perca fluviatilis*) is a freshwater species with growing commercial value (Fontaine and Teletchea, 2019) and it serves as a unique model for studying reproductive biology and development in fish subjected to extreme, intensive rearing conditions (Ning et al., 2025). Although the reproductive cycle and production methods of Eurasian perch are well established (Fontaine and Teletchea, 2019), studies exploring intrinsic factors that influence egg quality - and how these, in turn, affect larval performance - remain limited. By linking egg phenotype with larval outcomes, this study seeks to challenge long-held assumptions and offer a more nuanced understanding of the extent to which variations in egg quality shape larval performance. Specifically, we address the question: Can eggs of lower quality still produce viable and robust larvae? Furthermore, by analysing both unfertilized eggs (UFE) and larvae at the transcriptomic

level, we aim to explore the relationship between maternal transcriptomic profiles and offspring phenotype. Additionally, one common assumption is that high-quality eggs in fish lead to improved larval survival and growth (Bobe, 2015). However, it remains unclear whether "high quality" larvae, those not exhibiting any visible developmental abnormalities, perform differently when originating from eggs of varying quality, especially when reared together. Therefore, in the present study, we additionally compared the rearing efficiency of Eurasian perch larvae derived from various quality eggs, assessing their performance both in separate (group-wise) and mixed culture conditions simulating typical aquaculture practice. As a result, we aimed at refining current interpretations of egg quality, assessing its true predictive value for larval performance, and providing a framework for improving selection strategies in commercial aquaculture.

5.2 Materials and Methods

5.2.1 Ethics statement

The study was conducted in accordance with European and national regulations on fish welfare and was approved by the Local Animal Research Ethics Committee (resolution no. 5/2023). The animal research procedures were documented following the ARRIVE guidelines (https://arriveguidelines.org) for reporting animal studies.

5.2.2 Broodstock management

For this experiment, domesticated Eurasian perch spawners were used. Notably, using eggs obtained through commercial-scale fertilization allowed us to closely reflect industry practices, ensuring that the results would be directly reflect the real-world aquaculture conditions. The spawners belonged to the eighth domesticated generation (F8) and were reared in a recirculating aquaculture system (RAS) at the Percitech fish farm in Switzerland. The experiment was conducted in November, being a natural reproductive period for this particular broodstock, which was specifically programmed over generations to spawn at that time with the application of a well-established company photo-thermal protocol. In accordance with Żarski et al. (2019), the fish were subjected to a standardized hormonal induction procedure prior to spawning. Briefly, the broodstock was kept at photoperiod of 14 hours of light and 10 hours of darkness (14L:10D) and water temperature of 12°C (Żarski et al., 2019). To induce and synchronize ovulation, females received two injections of a salmon gonadoliberin analogue (sGnRHa, BACHEM, Switzerland) at doses of 10 µg kg^{-1} and 25 µg kg^{-1}, administered seven days apart. Meanwhile, males received a

single injection of 25 µg kg^{-1} sGnRHa, and milt was collected seven days later, coinciding with the optimal sperm collection window for this species (Żarski, et al. 2017g). Egg collection took place on day nine after the first injection. Prior to handling, all fish were anesthetized with MS-222 (Argent, USA) at a concentration of 150 mg L^{-1}.

5.2.3 Experimental design

A simple experimental design was used in which each egg batch was fertilized with the same pooled semen, collected by mixing sperm from 10 males (Fig.1). This commonly used strategy minimized potential biases from individual males (Bobe, 2015). This approach helps to avoid confounding effects due to specific male–female compatibility, ensuring that any observed differences more accurately reflect maternal contributions (Siddique et al., 2017), what has also been reported for Eurasian perch (Kucharczyk et al., 2001).

Thirty egg ribbons, each from a separate female, were fertilized. After assessing fertilization rates, only egg batches with fertilization success above 75% (a threshold considered high in commercial set-up; Bernáth et al. 2016) were selected, resulting in 17 distinct batches (**Fig. 5.1**). Fertilization success was evaluated separately for each batch (in triplicate), by counting approximately 100 embryos under the stereomicroscope before they reached the mid-blastula transition (MBT) stage.

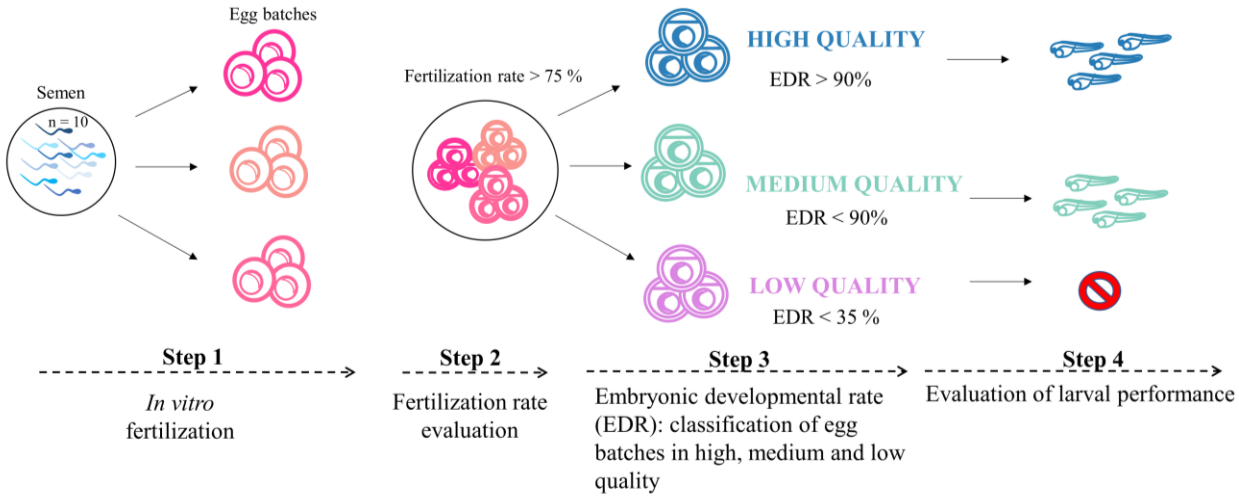

Figure 5.1*: Experimental design and classification of egg quality in Eurasian perch.*
Step 1: In vitro fertilization was performed using pooled semen to reduce paternal effects.
Step 2: Egg batches were initially evaluated for fertilization success, with only those achieving fertilization rates above 75% selected for further study.
Step 3: Pre-selected egg batches were then classified into high, medium, or low quality based on their embryonic developmental rate (EDR).
Step 4: Larval rearing of larvae originated from high- and medium-quality eggs.

5.2.4 Eggs classification into high, medium and low quality

Following MBT, the embryonic developmental success was evaluated by direct counting under the stereomicroscope (Leica, Germany) embryos at the onset of somitogenesis (SG), when the embryonic axes are formed, and tail detachment stage (TD). These stages represent critical developmental checkpoints at which embryo viability can be reliably assessed. On these same samples, we further evaluated hatching rate (HR) and deformity rate (DR). Such precise monitoring of the developmental competence of all the 17 egg batches allowed us to categorize them into three quality classes: 6 batches were classified as high-quality, 6 as medium-quality, and 5 as low-quality (**Table 5.1**).

Table 5.1: *Classification criteria for egg batches based on the onset of somitogenesis (SG), and tail detachment (TD) stage. Threshold values define high-, medium-, and low-quality eggs categories.*

Quality Category	**SG (%)**	**TD (%)**
High quality	> 90	90-97
Medium quality	< 90	53-83
Low quality	< 35	—

5.2.5 Gamete's collection and Fertilization

5.2.5.1 Milt collection

Milt was collected from 10 males by gentle abdominal pressure using a catheter (Galmed, Poland) to prevent contamination with urine or blood. After collection, the milt samples were pooled and diluted with an isotonic medium (FISH STOP, Noah Genetics, Switzerland) designed for short-term fish semen preservation, according to the manufacturer's protocol. The diluted pooled semen was stored at 4 °C until fertilization.

5.2.5.2 Egg collection and *in vitro* fertilization

The *in vitro* fertilization procedures were carried out as a routine hatchery procedure. Eggs were obtained by gently applying pressure to the abdomen. Each egg ribbon (average weight 169.5 ± 65.5 g) was collected separately into a clean, dry beaker. Fertilization has been performed according to the standardized protocol (see Żarski et al. 2012), with modification involving using distilled water for gametes activation. Briefly, eggs underwent first a 20-second pre-activation

treatment by adding 500 ml of distilled water in two portions. Following activation, 20 ml of diluted semen was added to each ribbon in two steps: an initial 10 ml, followed by an additional 10 ml after 20 seconds. After fertilization, excess milt and residual debris were removed by rinsing the eggs with hatchery water. The eggs were then incubated at 11 °C in the facility's round incubators. Prior to fertilization, unfertilized egg samples (0.3 gr) were collected in triplicate into cryotubes and promptly snap-frozen in liquid nitrogen for further molecular analysis.

5.2.6 Eggs incubation

Fertilized eggs were transported from Switzerland to Olsztyn, Poland, in individual plastic bags containing a mixture of 70% water and 30% oxygen. To maintain a stable temperature during the approximately 16-hour journey, each bag was placed inside a hermetic Styrofoam container with 500 g of ice, following the procedure described by Palińska-Żarska et al. (2020). Temperature was regularly monitored during transit and remained stable at 11 °C, comparable to conditions typically used for wild-caught embryos. Handling was kept to a minimum, and the protocol followed procedures previously validated for Eurasian perch transport (e.g., Palińska-Żarska et al. 2020; 2021). Although a slight effect of transport on early development cannot be entirely ruled out, it was considered negligible due to stable and the same conditions for all the egg batches, and the consistency observed between embryonic development rates measured prior to transport and the subsequent phenotypic outcomes.

Upon arrival, water temperature in the transport bags measured still 11 °C, that is why the eggs were first acclimated, then transferred to 15 L tanks with black walls and a top water inflow system, operating within the same RAS at 13 °C. Next day, eggs were spread across mesh screens placed inside the tanks, with a mesh diameter of approximately 3 mm, and the temperature was gradually increased to 14 °C. Once embryos reached the eyed-egg stage, the water temperature was raised again to 15 °C. Throughout embryo incubation and subsequent larval rearing, a continuous photoperiod 24L:0D, (1500 lux at the water surface) was maintained. When the first larvae began to hatch, the temperature was further increased to 16 °C. To promote synchronous hatching, manual assistance was applied by placing egg ribbons into bowls filled with water from the system and gently stirring them and this process was repeated until the majority of larvae had hatched. The day of hatching was defined as day 0 post-hatching (0 DPH). Following hatching, the larvae were left undisturbed for 24 hours. At 2 DPH, they were volumetrically counted and then stocked into triplicates at a density of 1,500 larvae per tank (Debernardis et al., 2025).

5.2.7 Larvae rearing

Larvae originating from high- and medium-quality eggs were reared under controlled conditions following the protocol described by Palińska-Żarska et al. (2020; 2021). In brief, at 1 DPH the water temperature was increased to 17°C, then further raised to 18°C at 2 DPH, maintaining this temperature steadily until 10 DPH. From 11 DPH onward, the temperature was gradually elevated by 1°C per day until reaching 23°C, which is considered optimal for Eurasian perch larval growth (Kestemont et al. 2003; Debernardis et al. 2025).

Larval mortality counts began at 3 DPH, conducted twice daily immediately following tank cleaning. Starting from 4 DPH, larvae were fed Artemia sp. nauplii *ad libitum* three times daily - initially with micro Artemia cysts (from San Francisco Bay, California, USA) for the first four days, then switching to standard size Artemia cysts (260,000 nauplii per gram, GSL origin) (Santos et al., 2021). From 4 DPH foraging rates and swim bladder inflation efficiency (SBIE %) were monitored by randomly sampling approximately 100 larvae per tank and examining them under a stereomicroscope (Leica, Germany). Both foraging and SBIE assessments continued until 10 DPH, by which time larvae had fully absorbed their yolk sacs. Beyond this point, no further increase in SBIE was observed, and larvae that had not initiated foraging by then did not survive. From 12 DPH, dead larvae were examined microscopically to evaluate occurrences of type I cannibalism, characterized by partial ingestion of prey (Kestemont et al., 2003). Two days before weaning (15 DPH), larvae from each batch were manually counted and restocked at a standardized density of 350 larvae per tank. Weaning occurred at 17 DPH, and from this time larvae were fed *ad libitum* exclusively with dry feed (Perla Larva Proactive, Skretting, Norway) three times daily. Small amounts of feed were sprinkled into each tank over approximately 15 minutes per feeding.

Throughout the experiment, dissolved oxygen levels in tanks were monitored daily using an Aquaculture Oximeter Polaris (Oxyguard, Belgium) and consistently remained above 80% saturation. Ammonia and nitrite concentrations were measured every two days with a DR1900 Portable Spectrophotometer and remained below 0.02 mg L^{-1}. The study ended at 27 DPH, when under the applied rearing protocol more than 50% of the larvae completed the larval stage (Palińska-Żarska et al. 2020, Debernardis et al. 2025).

5.2.8 Larval sampling and Zootechnical measurements

Larvae were anesthetized with MS-222 (150 mg L^{-1}) prior to every manipulation. Larval zootechnical traits (i.e., length and weight) were collected at three key developmental stages, based on protocols by Palińska-Żarska et al. (2020) and Debernardis et al. (2025):

1. mouth opening stage (0-1 DPH): At this stage, at least 50% of the larvae had their mouths open, indicating they were ready to feed independently while still experiencing minimal human intervention.
2. weaning stage (17 DPH): The point at which larvae transition to compound diets.
3. end of larval period (27 DPH): Defined as when at least 50% of larvae have completed the larval stage, marking the experiment's end.

At each stage, 30 larvae per family (10 per tank) were collected to measure total length (TL, ±0.01 mm) and wet body weight (WBW, ±0.1 mg). For TL, larvae were photographed under a stereomicroscope (Leica, Germany), while WBW was measured by placing anesthetized larvae on a nylon net (approximate mesh size 200 μm) and gently removing excess water with filter paper (Krejszeff et al., 2013). In addition, yolk sac and/or oil droplet volumes were measured from the photographs of larvae at mouth opening stage. Additionally, at mouth opening, another 30 larvae per batch were preserved in RNAlater (Sigma-Aldrich, Germany) for further transcriptomic analysis.

5.2.9 RNA extraction

Total RNA was extracted individually from unfertilized eggs of each female, as well as from larvae (pool of n=10) originating from the same female. After extraction the concentration and purity of the RNA were evaluated using DS-11 spectrophotometer (Denovix), showing absorbance ratios of A260/280 ≥ 2.0 and A260/230 ≥ 2.2. The quality of RNA extracted from larvae was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with all exhibiting RIN values ≥ 9.0. RNA from eggs was evaluated using the TapeStation system (Agilent Technologies, USA), also showing RIN values ≥ 9.0. All were subsequently shipped for transcriptomic analysis.

5.2.10 RNA sequencing and library preparation

29 different libraries were created (12 from larvae and 17 from UFE). RNA-seq analysis was outsourced to weSEQ.IT using the TruSeq Stranded mRNA kit (Illumina) with a NovaSeq X, PE150 platform, and 40 M 150 bp paired-end reads per sample were generated.

Raw reads were subjected to quality control using FastQC software version 0.11.9 (Simon Andrews, 2020). Adapters and low-quality fragments of raw reads (average QPhred score < 20) were trimmed, and reads were trimmed to equal lengths of 140 nt 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., 2013) with default ENCODE options.

5.2.11 Differential expression genes (DEGs) analysis

RNA-seq data from larvae and egg samples were analysed using the DESeq2 package in RStudio (version 4.2.2), following the authors' recommendations (Love et al., 2014), and *apeglm* for log fold-change shrinkage (Zhu et al., 2019b). Gene count data for the samples were filtered in order to have at least half of libraries in which there were at least 10 reads. Differences were considered significant when p-adjusted value was inferior to α (α = 0.05) and log2fold change above 1 or less than -1, and specific DEGs were found. To explore the variability in transcriptomic data, Principal Component Analysis (PCA) plot was created for visualization using RStudio.

DEGs identified from the various egg quality comparisons were then cross-compared to determine shared genes, and the overlaps were visualized using a Venn diagram (Bardou et al., 2014).

5.2.12 Gene Ontology (GO) Analysis

GO analysis was performed using the approach outlined by Żarski et al. (2021). The Eurasian perch transcriptome was initially mapped to the human proteome in the Swiss-Prot database. Sequence alignment was then performed using BLASTX, with the top match for each protein selected to provide gene names and UniProt accession numbers. These gene names were subsequently input into the ShinyGO platform (Ge et al., 2020) to carry out the GO analysis. The analysis focused on the DEGs found and the 10 most enriched biological processes were identified, based on an p-adjusted value threshold of < 0.05.

5.2.13 Zootechnical data analysis and statistics

After assessing normality and homogeneity of variance, appropriate statistical tests were performed for the zootechnical traits collected: one-way ANOVA for normally distributed data with equal variances, Kruskal-Wallis test for non-parametric comparisons among multiple groups, unpaired t-test for normally distributed two-group comparisons, and Mann-Whitney U test for non-parametric two-group comparisons. The samples were considered significantly different when $p < 0.05$. The statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, USA).

5.2.14 Mixed Larval Rearing Trial

To investigate whether larvae originating from different egg quality groups might interact differently when reared together, we performed an additional experiment. Larvae from four high-quality egg batches were pooled proportionally and stocked at a density of 1,500 larvae per tank (in triplicate). The same procedure was applied for larvae from four medium-quality eggs. Finally, larvae from both high- and medium-quality eggs were mixed proportionally and stocked at the same density and in triplicates (**Fig. 5.2**). These larvae were reared under the same conditions as in the main experiment, and their zootechnical parameters were recorded following the same methodology.

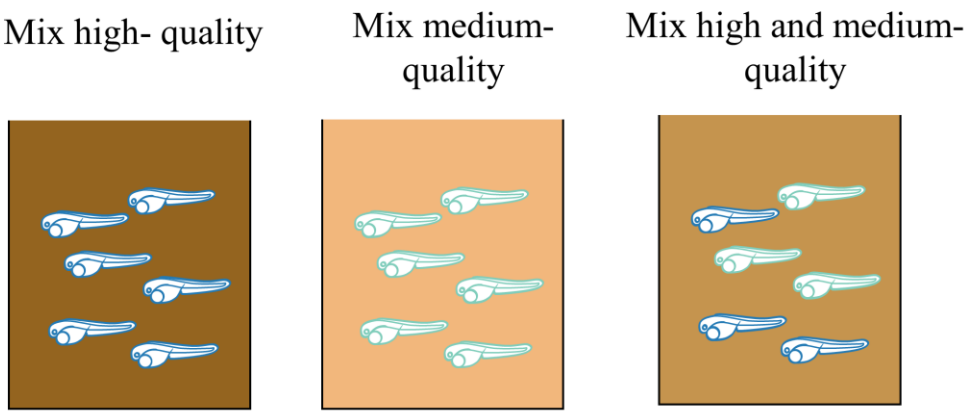

***Figure 5.2**: Schematic representation of experimental groups based on larval quality classification. Groups include: (1) mix high-quality larvae, (2) mix medium-quality larvae, and (3) mixed group containing equal proportions of high- and medium-quality larvae. In blue high-quality larvae; green represents medium-quality larvae.*

5.3 Results

5.3.1 Zootechnical parameters

Egg quality

At the fertilization stage, statistically significant difference was observed only between high- and low-quality egg batches (**Fig. 5.3A**). In contrast, embryonic developmental success differed significantly between low-quality eggs and both the high- and medium-quality groups, with no significant difference detected between high- and medium-quality eggs in the overall three-group comparison (**Fig. 5.3A**). This apparent discrepancy arises because the global analysis considered variability across all groups simultaneously, reducing the apparent difference between high and medium qualities, whereas the pairwise comparison shown in **Figure 5.4** revealed a significant difference in embryonic developmental rate between these two groups, which were further considered in the larval rearing trials. In the low-quality group, developmental success declined sharply during embryonic development, resulting in an insufficient number of viable embryos for successful larval rearing, that is why the low-quality group was removed from the larval rearing experiment. In medium-quality batches, developmental success began to decline after the MBT. Conversely, high-quality eggs maintained embryonic success rates comparable to their initial fertilization rates (**Fig. 5.3B**).

Furthermore, statistically significant differences were detected between medium- and high-quality groups in: embryonic developmental rate, late embryonic developmental rate (at tail detachment stage), and hatching rate (**Fig. 5.4**). No difference for deformity rate were observed.

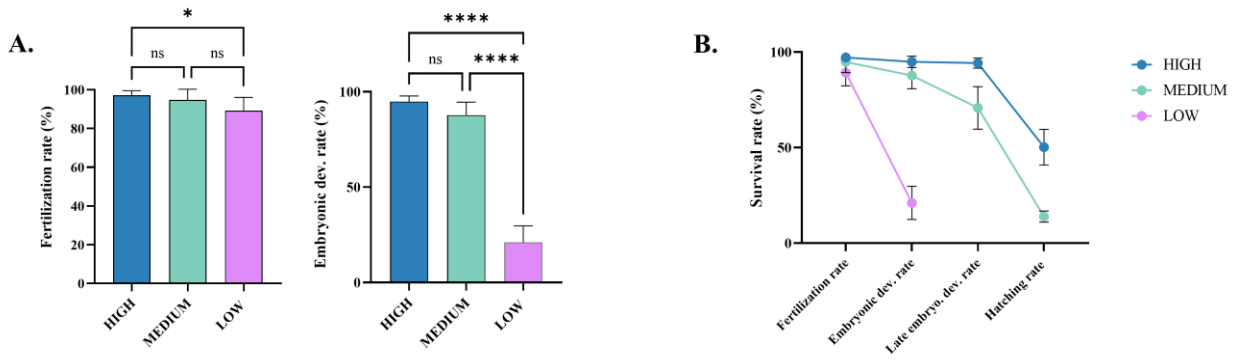

Figure 5.3: ***A.*** *Statistical comparison for fertilization rates and embryonic developmental rates of high-, medium-, and low-quality egg batches in Eurasian perch.* ***B.*** *Survival dynamics of embryos from different egg quality groups across different time points. Asterisks indicate significant differences between groups (*p < 0.05, ****p < 0.0001). ns: not significant.*

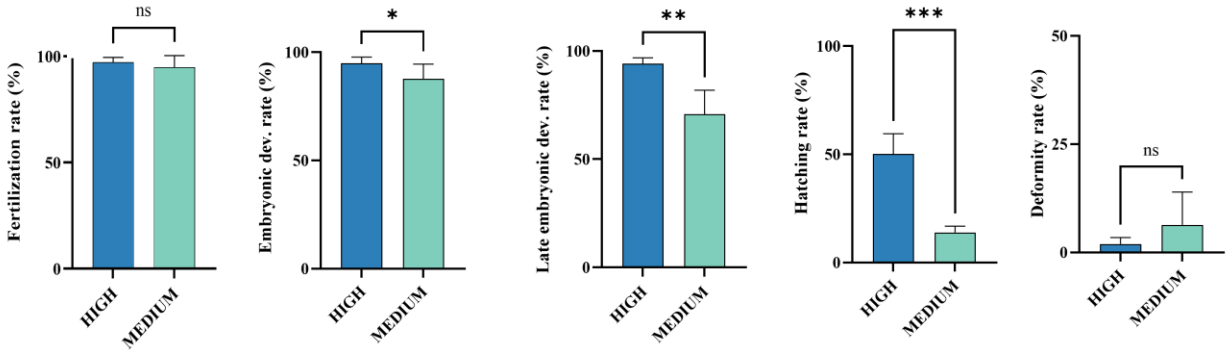

Figure 5.4: *Comparison of fertilization rate, embryonic development rate, late embryonic development rate, hatching rate, and deformity rate between only the high- and medium-quality egg groups, as these were the only groups yielding viable larvae for downstream phenotyping (*p < 0.05, **p < 0.01, ***p < 0.001). ns: not significant.*

Larval rearing

No significant differences were found in larval TL and WBW at any of the measured time points (mouth opening, weaning, and end of the larval period) (**Fig. 5.5**). Similarly, no differences were observed in yolk sac or oil droplet volumes (**Fig. 5.6B**). Foraging rate analysis revealed a difference at 5 DPH, while the SBIE showed a significant difference between the high- and medium-quality groups only at 6 DPH (**Fig. 5.6A, C**).

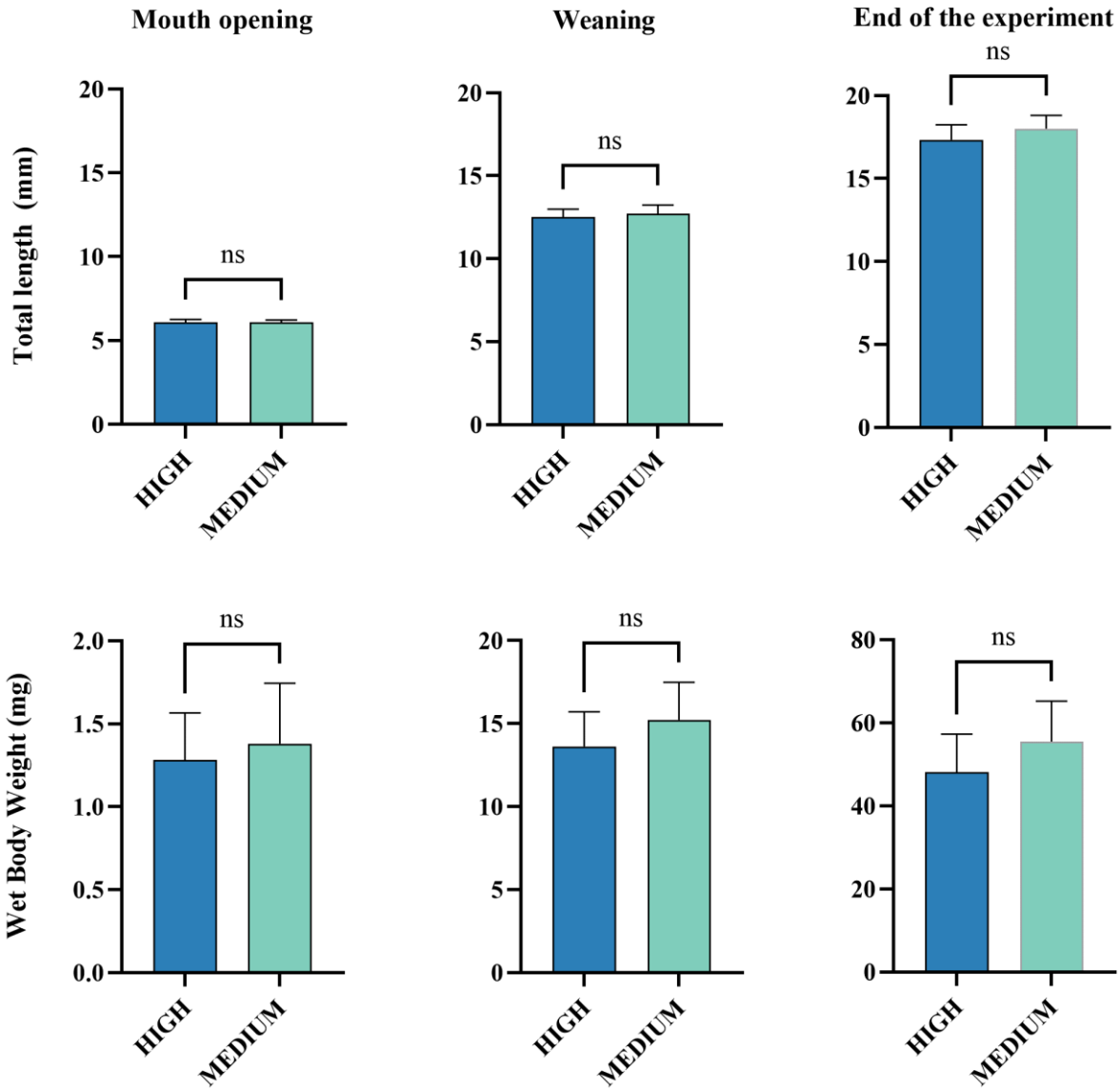

Figure 5.5: *Total length (mm) and wet body weight (mg) of Eurasian perch larvae originating from high- and medium-quality eggs, measured at different developmental stages. Differences between groups were not significant (ns).*

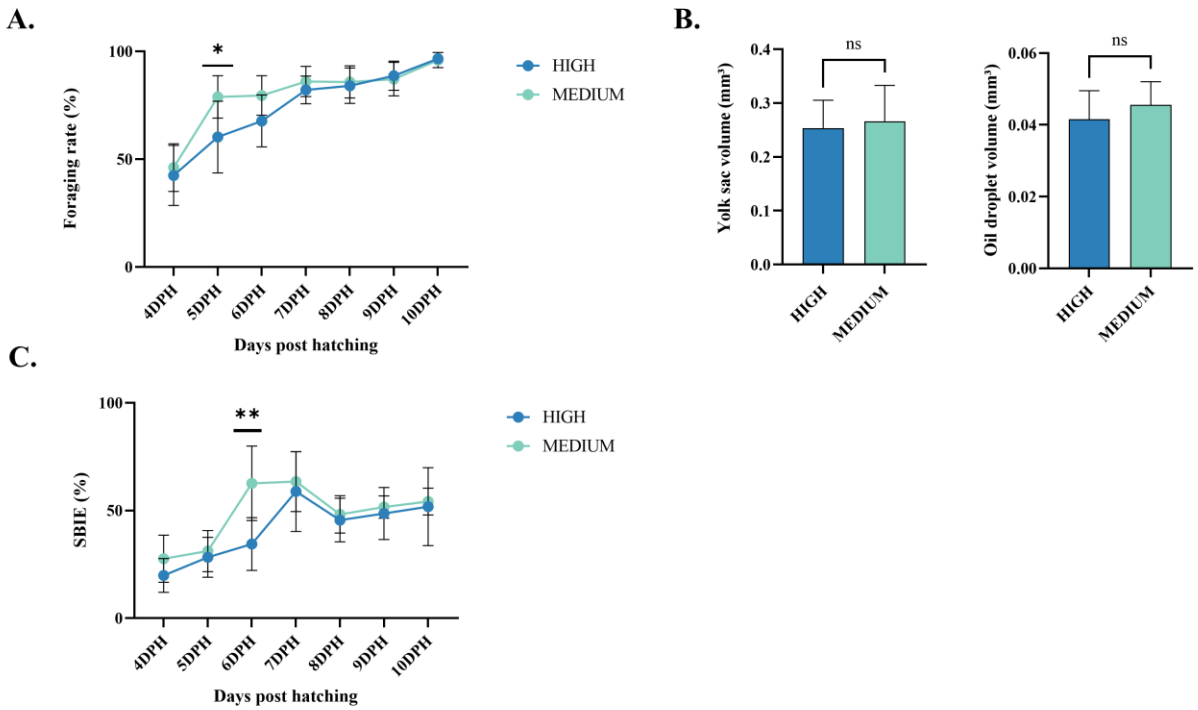

Figure 5.6: A. *Foraging rate* ***B.*** *Yolk sac and oil droplet volume and* ***C.*** *SBIE kinetics (%) for Eurasian perch larvae coming from high and medium-quality groups. The asterisks (*p<0.05, **p< 0.01) show significant differences between the groups at a specific time point. ns: not significant.*

Analysis of total mortality and cannibalism did not reveal any differences between groups (**Fig. 5.7A-B**). However, when considering only mortality before weaning, a significant difference ($p < 0.05$) emerged between the high- and medium-quality groups, with the high-quality group exhibiting higher mortality rate (**Fig. 5.7A**).

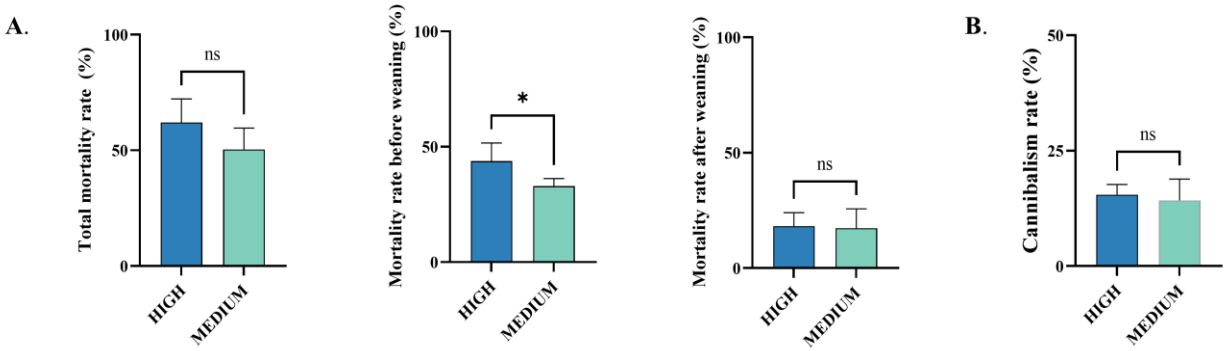

Figure 5.7: A. *Total mortality at the end of the experiment (%), as well as mortality before and after weaning (%), of Eurasian perch larvae originating from high- and medium-quality egg batches.* ***B.*** *Cannibalism rates among larvae from high- and medium-quality eggs. Asterisk (*p < 0.05) indicate statistically significant differences between groups. ns: not significant.*

5.3.2 Transcriptomic data

Egg quality

RNA-seq analysis of UFE of high, medium, and low quality initially identified 30,744 genes. After filtering, 12,632 protein-coding genes remained and were used for differential gene expression (DEG) analysis. The unsupervised heatmap of the top variable genes (**Fig. 5.8A**) reveals partial clustering by egg quality. High-quality eggs tend to exhibit more homogeneous gene expression patterns, forming a tighter cluster, whereas medium-quality samples are more heterogeneous. Low-quality samples show moderate clustering with some variability. These patterns are in line with the PCA graph (**Fig. 5.8B**) and highlight transcriptomic distinctions associated with egg quality. The analysis revealed distinct DEGs (*padj* < 0.05, |log2 FC| > 1). Specifically, 34 DEGs were identified between high- and medium-quality eggs, 40 DEGs between high- and low-quality eggs, and 95 DEGs between medium- and low-quality egg batches (**Supplementary file S5.1**).

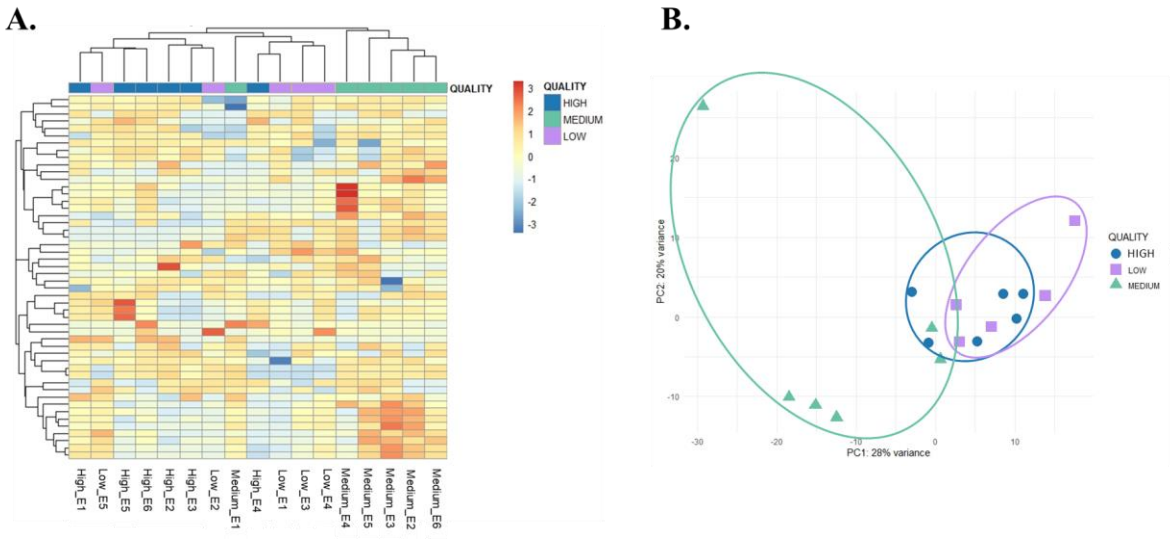

Figure 5.8: A. *Unsupervised hierarchical clustering heatmap of the 50 most variable genes in Eurasian perch egg samples (high, medium and low-quality). Each row corresponds to a gene, and each column represents an individual sample. The colour scale indicates relative gene expression (z-score), with red denoting higher expression and blue denoting lower expression.* ***B.*** *Principal Component Analysis (PCA) plot showing the distribution of high-, medium- and low-quality unfertilised egg samples based on gene expression profiles. Each quality group is represented by a distinct colour and symbol.*

Gene Ontology (GO) enrichment analysis of DEGs from the comparison between medium- and high-quality eggs showed genes involved in immune system regulation and immune responses (**Fig. 5.9A**). In contrast, DEGs distinguishing low- from medium-quality eggs were enriched for

GO terms associated with the cell cycle, organelle organization, and cell adhesion (**Fig. 5.9B**). Finally, the comparison between low- and high-quality eggs revealed significant enrichment for GO categories related to chromosome organization, histone modification, and DNA replication mechanisms (**Fig. 5.9C**).

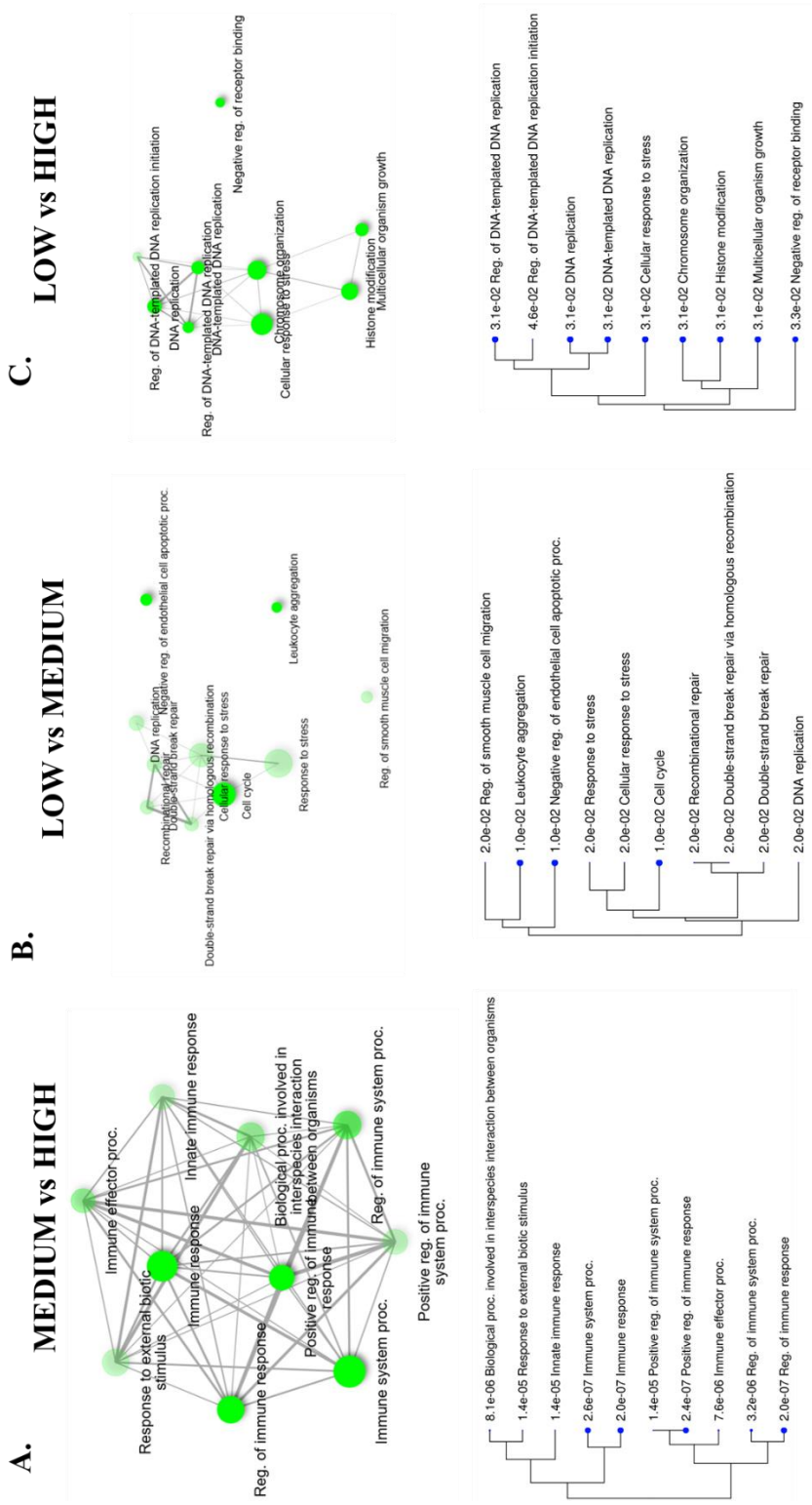

Figure 5.9: *Tree view and network visualization of the top 10 most significantly enriched Gene Ontology (biological process) terms associated with differentially expressed genes between* ***A****. medium and high-quality eggs,* ***B****. low and medium-quality eggs, and* ***C****. low and high-quality eggs.*

A Venn diagram was used to determine the presence of shared DEGs across pairwise comparisons, aiming to identify genes consistently associated with differences in egg quality (**Fig. 5.10, Supplementary file S5.2**). This analysis revealed 11 DEGs common to both the low vs. medium and medium vs. high comparisons, mainly linked to immune system processes. Additionally, 19 DEGs were common to the low vs. medium and low vs. high comparisons, with functional enrichment suggesting involvement in DNA replication, metabolic pathways, and cell cycle regulation. This common DEGs from each of those comparisons can be interpreted as being specific to the quality group that is constant in both comparisons.

A closer look at the 19 DEGs shared between the low vs medium and low vs high comparisons, therefore specific for the low group, revealed genes involved in DNA repair pathways, such as *atm* (serine-protein kinase ATM), as well as genes linked to zygotic genome activation, including *ep400* (E1A–binding protein p400) and *otulina* (OTU deubiquitinase with linear linkage specificity A), which plays a specific role during fertilization. Those genes were consistently up-regulated in low-quality eggs.

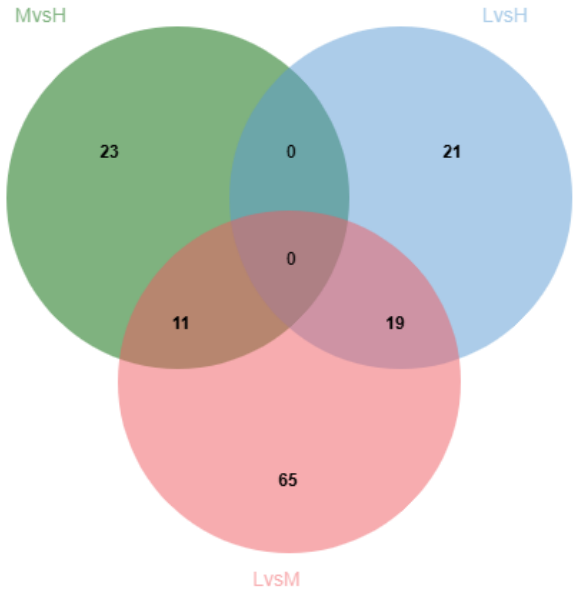

***Figure 5.9**: Venn diagram illustrating the overlap of differentially expressed genes (DEGs) identified across three pairwise comparisons of egg quality groups: medium vs. high (MvsH), low vs. high (LvsH), and low vs. medium (LvsM). This visualization highlights shared and unique DEGs among the comparisons, providing insight into the molecular differences associated with varying egg quality.*

Larval rearing

Following RNA-seq analysis of larval samples, a total of 30,744 genes were initially detected. After filtering based on expression levels, 20,081 protein-coding genes remained and were used for differential expression (DEG) analysis to investigate potential differences among larval groups originating from eggs of high and medium-quality. A heatmap of the 50 most variable genes (**Fig. 5.11A**) shows an overall consistent expression pattern across groups. This is consistent with the PCA plot (**Fig. 5.11B**), which does not reveal a strong separation between high and medium-quality groups. However, larvae from the high-quality egg group exhibit a more compact clustering pattern compared to the medium-quality group, which appeared more dispersed. The analysis of the larval transcriptome at the mouth opening stage did not identify any differentially expressed genes (DEGs) meeting the criteria of *padj* < 0.05 and |log2 FC| > 1.

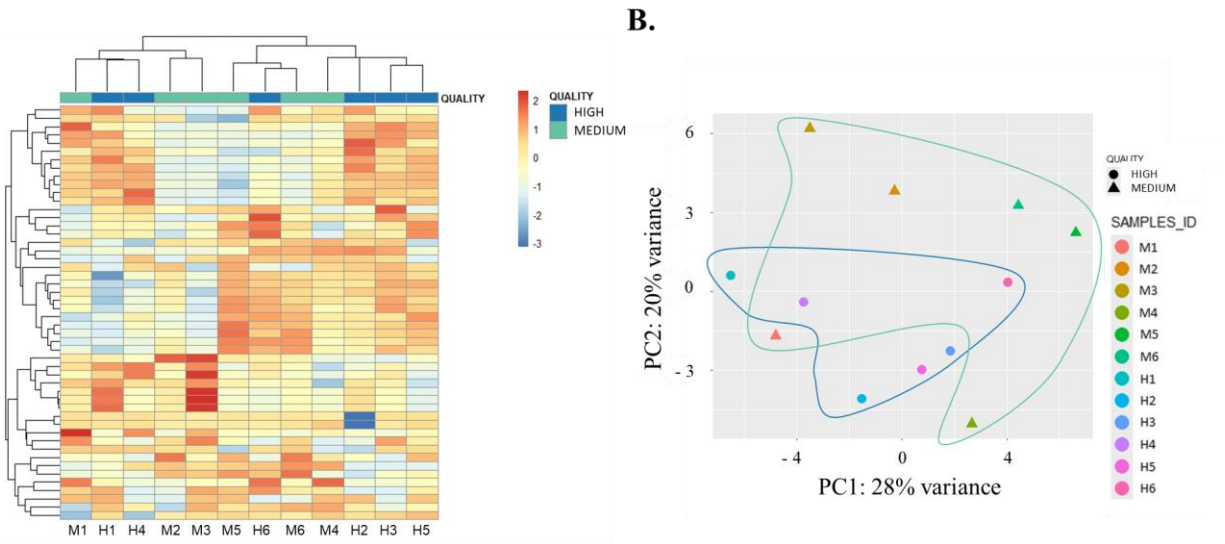

Figure 5.10: A. *Unsupervised hierarchical clustering heatmap of the 50 most variable genes in E. perch larval samples. Each row corresponds to a gene, and each column represents an individual sample. The colour scale indicates relative gene expression (z-score), with red denoting higher expression and blue denoting lower expression.* ***B.*** *Principal Component Analysis (PCA) plot showing the distribution of high- and medium-quality larval samples based on gene expression profiles. Circles represent high-quality samples, and triangles represent medium-quality samples. Each sample is coloured uniquely to distinguish individual identities.*

5.3.3 Mixed Larval Rearing Trial

The analysis revealed no significant differences among the groups across all measured parameters, including body weight and length at both weaning and the end of larval period, as well

as foraging rate and mortality (**Fig. 5.12 A-C**). A significant difference in SBIE was observed at 3 DPH, with the highest value recorded in the mixed group (**Fig. 5.12 D**).

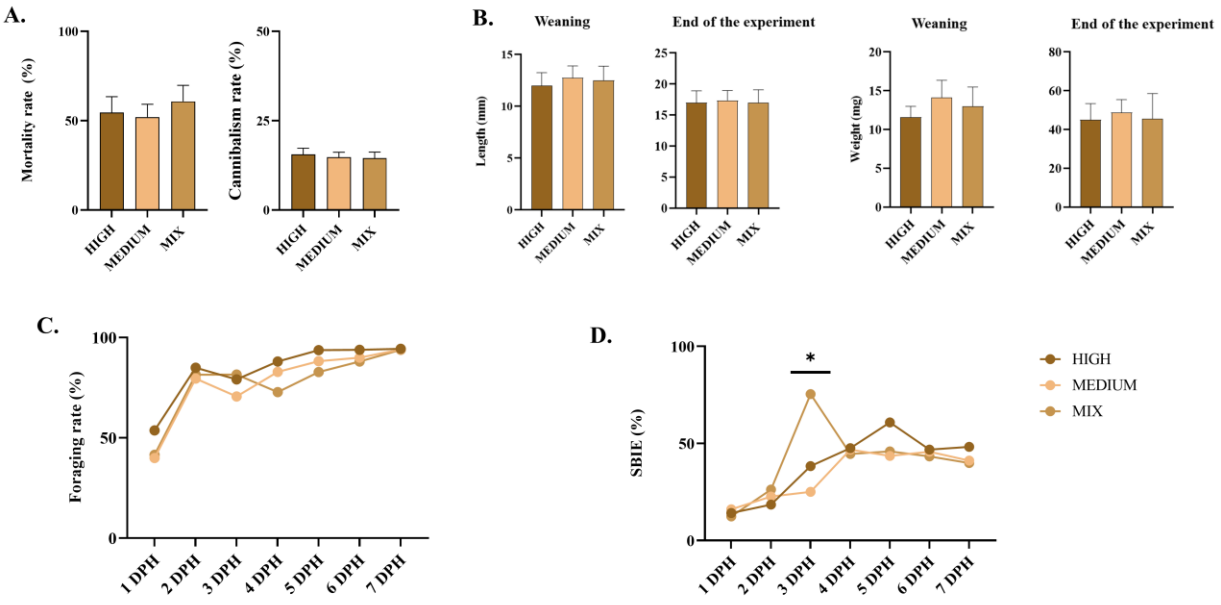

Figure 5.11: ***A.*** *Total mortality and cannibalism rates (%),* ***B.*** *Larval length and weight at both weaning and the end of the experiment,* ***C.*** *Foraging rate kinetics and* ***D.*** *Swim bladder inflation effectiveness (SBIE) kinetics evaluated in three larval groups of Eurasian perch a group composed of high-quality larvae, a group of medium-quality larvae, and a mixed group combining high- and medium-quality individuals. Asterisks (*p < 0.05) indicate statistically significant differences between groups.*

5.4 Discussion

In this study, we shed light on the concept of late egg developmental competence, defined as the capacity of an egg to sustain normal development beyond fertilization and through critical embryonic checkpoints such as the mid-blastula transition (MBT), onset of somitogenesis (SG) and tail detachment (TD). Our results provide the first evidence for molecular and phenotypic indicators of late egg developmental competence in Eurasian perch. Consequently, and contrary to traditional assumptions, egg quality groups, from which we were able to obtain a suitable number of larvae for further phenotyping, did not reliably predict larval performance in this species. While high-quality eggs exhibited greater embryonic survival and higher hatching rates than medium-quality eggs, the resulting larvae performed similarly across groups in both zootechnical traits and transcriptomic profiles. These findings challenge the common reliance on fertilization rate as a proxy for developmental potential (Bobe & Labbé, 2010; Fernández Míguez et al., 2024) and instead highlight that, in Eurasian perch, embryonic developmental success and

larval viability are governed by more complex regulatory mechanisms that cannot be captured by egg or early embryonic developmental assessments alone (Bobe, 2015).

A key strength of this study is the use of a commercial-scale fertilization protocol, closely mirroring standard hatchery practices. Notably, to minimize paternal genetic effects and focus mainly on maternal contributions, fertilizations were performed using pooled sperm from multiple males, a common strategy in gamete quality studies (Bobe, 2015; Siddique et al., 2017) and in the commercial hatcheries. This approach reduces the influence of individual male variability and specific male-female compatibility, thereby allowing a more reliable assessment of intrinsic egg quality (Bobe, 2015). Although it cannot completely eliminate all paternal contributions, this approach significantly strengthens the conclusion that differences in developmental trajectories were primarily driven by maternal factors. All egg batches were selected based on high fertilization rates (>75%), yet they exhibited marked differences in later embryonic developmental outcomes. This aligns with the definition of egg quality as the capacity to be fertilized and support further development (Bobe & Labbé, 2010). In our study, low-quality eggs mostly failed to progress beyond the MBT. Importantly, while differences in embryonic developmental rate between medium- and high-quality eggs were already statistically detectable at MBT, these differences could still be considered relatively subtle. However, the most profound differences between these two groups were recorded at hatching. The hatching process represents a critical challenge test for the embryo, requiring the coordination of multiple developmental systems to enable the larva to successfully emerge from the chorion (Ługowska & Sarnowski, 2011). Therefore, the presented data demonstrate that, despite embryogenesis being completed successfully, only those embryos that developed into a sufficiently competent phenotype were capable of hatching. This shows that fertilization rate remains useful to exclude very poor batches, but it is not sufficient to predict late embryonic competence or hatching success. This does not contradict earlier definitions in the literature, but rather challenges the common practice of treating fertilization success alone as a proxy for overall egg quality. These findings advocate for a more refined approach, also in the commercial hatcheries, suggesting that successful development through later embryonic stages serves as a more reliable indicator of egg competence, whenever the paternal factor is minimized.

Transcriptomic profiling of unfertilized eggs revealed both between-group and within-group variability, particularly in medium- and low-quality batches. This variability provides insight into how maternal molecular inputs may influence developmental potential. The PCA showed that high-quality egg batches clustered more tightly, indicating more consistent transcriptomic profiles and suggesting of a well-coordinated maternal RNA program. In contrast,

medium-quality batches displayed broader dispersion and weaker clustering, reflecting greater heterogeneity in maternal provisioning, potentially due to the presence of compromised oocytes within the same batch. Such internal variability complicates group-level comparisons and supports previous findings that the inclusion of compromised, deteriorated or overripe eggs can mask meaningful transcriptomic signals (Migaud et al., 2013; Schaefer et al., 2018; Żarski et al., 2021b). Because these differences were detected in unfertilized eggs, prior to zygotic genome activation, they reflect purely maternal RNA and protein content (Weber et al., 2021). The pronounced developmental failures around MBT, a phase governed by maternal control of cell cycle and mRNA turnover (Bobe, 2015; Tadros & Lipshitz, 2009), together with the use of pooled sperm to minimize paternal variation and male–female compatibility effects (Kucharczyk et al., 2001; Siddique et al., 2017), further support the conclusion that phenotypic discrepancies in late egg developmental competence is most likely rooted in maternal origin.

The transcriptome of the unfertilized egg provides a valuable resource for identifying markers that can predict late egg developmental competence and explain post-MBT developmental anomalies (Cheung et al., 2019). Among the DEGs identified across egg quality groups in previous studies, several are associated with essential cellular processes such as DNA repair and zygotic genome activation (ZGA). For instance, *atm* (serine-protein kinase ATM) is involved in DNA repair pathways in oocytes (Stringer et al., 2018) , while *ep400* (E1A-binding protein p400) has been shown in mice to regulate ZGA (Tian et al., 2024). In zebrafish (*Danio rerio*), *otulina* was reported as dysregulated in low-quality eggs, and CRISPR/Cas9-mediated disruption in females produced largely unfertilizable eggs, resulting in marked subfertility through altered Wnt signaling, which is critical for early developmental processes (Cheung et al., 2019). Notably, in our current study *atm, otulina* and *ep400* emerged as consistently differentially expressed and up-regulated in low-quality egg groups. This pattern suggests that these genes form part of the molecular signature associated with reduced egg quality and may help explain developmental abnormalities observed after MBT; possibly via impaired DNA repair (*atm*), disrupted zygotic genome activation (*ep400*), and altered fertility pathways (*otulina*). These genes represent promising candidate biomarkers for assessing late egg developmental competence in Eurasian perch. Future research may further investigate these candidate genes to verify their functional importance in early embryonic development.

Despite significant transcriptomic variation in unfertilized eggs, newly hatched larvae exhibited uniformity in both gene expression and zootechnical traits. No DEGs were detected between larvae originating from high- and medium-quality egg batches, and both groups exhibited

similar phenotypic outcomes. This outcome may reflect a developmental filtering effect ("filtering" hypothesis), where only embryos with sufficient robustness - those able to overcome early developmental checkpoints such as ZGA - successfully hatch and develop further. While maternal provisioning provides the initial molecular toolkit for development, our results suggest that post-fertilization regulatory mechanisms play a decisive role in shaping embryos outcomes. Another possible interpretation is rooted in the concept of canalization ("buffering" hypothesis), in which early molecular differences are buffered during development, resulting in stable and standardized phenotypes (Horta-Lacueva et al., 2023). Within this framework, ZGA acts as a reset point, minimizing the impact of initial within-batch molecular variability and allowing surviving embryos to follow similar developmental trajectories. Alternatively, the observed uniformity may stem from selective survival within heterogeneous batches (Rauwerda et al., 2016). In this scenario, medium-quality batches likely contained a spectrum of eggs, from well-provisioned to impaired. Those with inadequate molecular machinery may have failed at early checkpoints and were lost, leaving only the most robust embryos - comparable in quality to those from high-quality batches - to survive until hatching, and hatch. Both hypotheses are consistent with the transcriptomic and phenotypic similarity observed at the larval stage. The key difference lies in whether these similarities result from development actively correcting early differences ("buffering" hypothesis) or from the selective survival of only those embryos with sufficient developmental potential ("filtering" hypothesis). Importantly, due to extremely low survival, larvae from low-quality eggs were not included in downstream analysis. As a result, it was not possible to determine whether their transcriptomic profiles would have aligned with those of other groups. Prior studies indicate that disrupted maternal provisioning can have altered or delayed effects, so it's plausible that had these larvae survived, they may have followed distinct developmental trajectories (Żarski et al. 2021a). Nonetheless, our findings underscore that late egg developmental competence, not initial egg classification, is a more meaningful predictor of larval quality. This supports a dynamic approach to egg quality assessment in aquaculture, where monitoring embryonic progression through key checkpoints provides better predictive value than static early-stage quality parameter (Fernández Míguez et al., 2024).

An additional highlight of this study is the consistency of results across two rearing strategies: one where the larvae were kept in separate, batch-specific groups and another where larvae from different egg quality groups were raised together in a mixed larval rearing trial. Interestingly, both approaches led to similar zootechnical outcomes, with larvae from both high- and medium-quality eggs performing comparably. This supports the common-garden experimental

principle, where a uniform environment reduces external variation, making genetic or parental influences easier to detect (de Villemereuil et al., 2016). The lack of differences between families in mixed larval rearing groups further strengthens the conclusion that maternal egg quality, not the rearing conditions or random family differences, is the main factor influencing early development. In other words, even when larvae share the same rearing environment with siblings from other batches, only those embryos that successfully pass early developmental checkpoints, which depend heavily on the mother's contribution (Lubzens et al., 2017), survive and develop with similar gene expression patterns. Therefore, although incubating low-quality eggs typically results in fewer hatched larvae (due to high mortality during development), the few survivors can still be considered high-quality individuals. Therefore, the huge variability in larval performance often seen within egg batches or even families, especially in Eurasian perch (Palińska-Żarska et al. 2020), likely comes from other, still unknown factors, which need further attention through more targeted experiments.

In summary, this study highlights the complex and non-linear relationship between egg quality, embryonic development, and larval phenotype. Our findings reframe the concept of egg quality and demonstrate that fertilization rate alone is not a reliable predictor of egg quality and, importantly, that reduced egg quality does not necessarily translate into poor larval performance. In this context, late egg developmental competence emerges as a more robust predictor of egg quality than traditional metrics such as fertilization rate. While maternal provisioning provides the initial molecular framework for development, our results suggest that maternal factors still constitute important regulatory mechanisms also post-fertilization, particularly after the MBT, playing a decisive role in determining larval viability and phenotype. Although, this has already been evidenced earlier (Pelegri, 2003; Vastenhouw et al., 2019) our results additionally strengthen the need for reframing how we assess egg quality, shifting the focus from static maternal traits to the dynamic capacity of embryos to regulate and stabilize development. Future studies could build on these results by incorporating targeted validation of key genes (e.g., *atm, ep400*); or focusing on single-egg analyses may help uncover key mechanisms underlying early developmental success, contributing to select specific markers of quality and improving egg quality assessment protocols and more targeted selection strategies in hatchery operations.

5.5 Data availability

Raw RNAseq data of different groups of larvae at mouth opening and unfertilized eggs can be accessed via the NCBI BioProject database under the accession numbers PRJNA1321851. Note that the data will become publicly available upon manuscript acceptance.
Reviewers can access the data using the following link https://dataview.ncbi.nlm.nih.gov/object/PRJNA1321851?reviewer=hcg5t27og00u60lu3otvhp3m28 .

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CRediT authorship contribution statement

Rossella Debernardis: Writing-original draft, Methodology, Visualization, Investigation, Data analysis, Data curation, Conceptualization. **Katarzyna Palińska-Żarska**: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Jan P. Jastrzębski**: Data curation. **Abhipsa Panda**: Investigation. Joanna Nynca: Writing-review & editing. **Daniel Żarski**: Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Chapter 6: Heart oedema in freshly hatched larvae of Eurasian perch is associated with multi-tissue gene dysregulation

Also referred as: Heart oedema

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Status: Submitted to *Genomics*

Contribution: writing the original draft, visualization, methodology, data analysis, investigation, data curation, conceptualization.

6.1 Introduction

Aquaculture's rapid growth is hindered, among others, by challenges in producing high-quality larvae, typically defined as those with normal morphology capable of developing into healthy juveniles and adults (Koumoundouros et al., 2017). A major issue in larval rearing is the occurrence of deformities, which can lead to impaired growth, high mortality rates, and ultimately compromise stock quality and production efficiency (Boglione et al., 2013a; Chandra et al., 2024). Types and frequencies of these deformities can vary widely not only between species and populations but also among individuals within the same family (Aydın et al., 2024; Boglione et al., 2013b; Puvanendran et al., 2015; Tong et al., 2012). This variability complicates efforts to identify causative factors and to develop effective prevention strategies. Nevertheless, targeted studies focusing on specific deformity types could help explore their underlying causes, providing valuable insights into the factors influencing larval phenotypic quality and contributing to aquaculture industry sustainability.

Deformities in fish can result from a wide range of factors, including biotic factors such as pathogens and rearing conditions (Boglione et al., 2013; Chandra et al., 2024), and abiotic factors such as temperature (Kupren et al., 2011; Réalis-Doyelle et al., 2016), water quality (Naz et al., 2023), photoperiod (Villamizar et al., 2011) or nutrition (Cahu et al., 2003). Additionally, genetic predispositions may also play a role in the manifestation of specific malformations (Chandra et al., 2024; Lorenzo-Felipe et al., 2021). Early-onset deformities (visible already at hatching), in particular, may as well result from exposure to environmental pollutant during embryogenesis (Sfakianakis et al., 2015), inherited genetic factors (Lorenzo-Felipe et al., 2021), or non-genetic parental influences, where environmental conditions experienced by the parents can induce molecular changes in gametes and affect embryonic development through epigenetic mechanisms (Adrian-Kalchhauser et al., 2020). Despite their critical impact on larval survival and performance, deformities present at hatching remain largely understudied, as most research has focused on deformities occurring at juvenile or adult stages (Chandra et al., 2024).

Newly hatched larvae can already exhibit a range of deformities, including skeletal malformations such as kyphosis, lordosis, and scoliosis, as well as abnormalities affecting yolk, heart, eyes, jaw, and mouth (Alix et al., 2017; Chandra et al., 2024). Skeletal deformities are the most common type of malformation in fish larvae, regardless of their underlying cause, with incidence rates varying notably across species. In Atlantic cod (*Gadus morhua*), 4–12% of larvae are affected (Puvanendran et al., 2015), while in European flounder (*Platichthys flesus*) the rate

rises to approximately 23% (Aydın et al., 2024). In Senegalese sole (*Solea senegalensis*), skeletal deformities in early juveniles are particularly prevalent, ranging from 40% to 80% (de Azevedo et al., 2017). Although common, skeletal deformities are not necessarily lethal (depending on their severity), they can significantly impact survival when they co-occur with other malformations or impair essential functions such as swimming and feeding, ultimately increasing the risk of mortality (Boglione et al., 2013). In contrast to skeletal abnormalities, cardiovascular deformities, particularly heart oedema, are severe and usually lethal, substantially compromising larval stock quality (Brette et al., 2014; Incardona et al., 2004). Despite their impact, studies focusing specifically on heart oedema in newly hatched larvae remain limited. Most available research investigates heart oedema as a result of embryonic exposure to environmental toxicants such as crude oil or polycyclic aromatic hydrocarbons (PAHs) (Incardona et al., 2004), rather than its spontaneous occurrence in aquaculture settings.

Heart oedema, characterized by fluid accumulation around the cardiac region, is often present at hatching and frequently results in death within a few days due to its severe physiological effects (Alix et al., 2017; Perrichon et al., 2017). This condition markedly reduces survival rates and causes significant economic losses in aquaculture (Alix et al., 2017; Aydın et al., 2024). In Eurasian perch (*Perca fluviatilis*), Alix et al. (2017) highlight that heart oedema, along with yolk sac oedema, are the most common deformities in larvae derived from domesticated broodstock reared in recirculating aquaculture systems (RAS). Specifically, cardiac oedema was observed in 27% of deformed larvae, and while co-occurring with yolk oedema, these accounted for 85% of all malformations (Alix et al., 2017). Despite its prevalence and crucial function in fish survival, mechanisms underlying the formation of heart oedema remain poorly understood.

To address this gap, the present study explores the heart oedema phenotype in newly hatched Eurasian perch larvae using a transcriptomic approach, analyzing gene expression differences between larvae exhibiting the heart oedema phenotype and their morphologically normal siblings from the same family. The study uses offspring from an 8th-generation domesticated broodstock, offering a controlled context for exploring potential molecular signatures associated with cardiac malformations. By examining this critical early developmental stage within the same family, we aimed to investigate whether deformities may be driven by inherited non-genetic factors rather than direct environmental exposure or genetic background.

6.2 Materials and Methods

6.2.1 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.

6.2.2 Broodstock management

This study utilized domesticated Eurasian perch spawners, specifically five males and five females. A simple experimental design was employed, in which each female was paired with a single male, resulting in the creation of five unique families.

The spawners belonged to the 8th domesticated generation, reared in RAS at the Percitech fish farm in Chavornay (Switzerland). Fish underwent a standardized hormonal induction protocol, as outlined by Żarski et al. (2019). Briefly, before spawning the broodstock was kept at photoperiod of 14 hours of light and 10 hours of darkness (14L:10D) and a stable water temperature of 12°C. To induce and synchronize spawning, females received two injections of a salmon gonadoliberin analogue (sGnRHa, BACHEM, Switzerland), administered seven days apart at doses of 10 µg kg^{-1} and 25 µg kg^{-1}, respectively (Żarski et al., 2019). Meanwhile, milt was collected seven days after the males received a single 25 µg kg^{-1} hormonal dose, aligning with the optimal sperm collection window for this species (Żarski et al., 2017g).

Egg collection took place on day nine after the first injection. Prior to handling, all fish were anesthetized with MS-222 (Argent, USA) at a concentration of 150 mg L^{-1}.

6.2.3 Milt collection and cryopreservation

Milt was collected from 5 males (average weight 540 ± 90 g) by gently applying pressure to the abdomen. To prevent contamination with urine or blood, a catheter (Galmed, Poland) was used during collection.

Sperm motility was assessed using a Computer-Assisted Sperm Analysis (CASA) system (CEROS II, Hamilton Thorne) through two-step activation protocol. Fresh milt samples were initially diluted at a 1:50 ratio, while frozen/thawed samples were diluted 1:5 in an immobilization solution containing 150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, and 20 mM Tris (pH 8.0). Subsequently, all samples underwent further dilution at 1:20 in an activation

medium composed of 75 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 1 mM $CaCl_2 \times 2H_2O$, and 20 mM Tris (pH 8.0), with the addition of 0.5% bovine serum albumin to enhance motility (Judycka et al., 2022).

Key parameters measured included motility percentage (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}). These metrics were used to verify that the samples were of consistently high and comparable quality. Additionally, fresh milt concentration was quantified using the NucleoCounter SP-100 (Chemometec, Allerød, Denmark) following the methodology outlined by (Judycka et al., 2019).

Cryopreservation was performed according to the protocol established by (Judycka et al., 2022). The final cryoprotectant composition included 0.3 M glucose, 7.5% methanol and 25 mM KCl, with a sperm concentration of 3×10^9/ml.

6.2.4 Egg collection and *in vitro* fertilization

Eggs were obtained from 5 selected domesticated females (average weight 456 ± 82 g), by gently applying pressure to the abdomen. Each ribbon (average weight 120 ± 29 g) was collected into a clean and dry beaker.

Prior to *in vitro* fertilization, 0.5 ml frozen milt straws were thawed by submerging them in a 40°C water bath for 10 seconds before being transferred into an Eppendorf tube. Meanwhile, the eggs underwent a 30-second pre-activation treatment using a diluted Woynarovich solution at a 1:5 ratio, as described by (Zarski et al., 2012).

Fertilization was then carried out by adding milt to the eggs at an optimized sperm-to-egg ratio of 100,000:1 (Judycka et al., 2019). After adding the thawed milt, the eggs were gently mixed for 30 seconds to ensure uniform fertilization. After approximately 10 minutes, excess milt and any residual debris were removed by rinsing the eggs with hatchery water.

6.2.5 Eggs incubation

Eggs were transported from Switzerland to Olsztyn, (NE Poland), in individual plastic bags containing a mixture of 70% water and 30% oxygen. To maintain a stable temperature during transit, each bag was placed inside a styrofoam container with 500 g of ice (Palińska-Żarska et al., 2020). Transport lasted approximately 16 hours, spanning the developmental period from mid-blastula to ~80% epiboly. Water temperature was monitored regularly and remained stable at 10 °C throughout. Handling was minimised in accordance with protocols previously validated for

perch (e.g., Palińska-Żarska et al. 2020, 2021). Upon arrival, fertilized eggs were acclimatized before transfer to the RAS system. Incubation took place in 15 L tanks with black walls and an upper water inflow, all connected to the same RAS. Eggs were evenly spread over mesh screens (~3 mm diameter) and maintained in water at 14 °C.

Fertilization success was assessed 12 hours for fertilization (12 HPF), separately for each family (in duplicate) by microscopically counting approximately 100 embryos before they reached the mid-blastula transition stage.

Throughout embryos incubation, the photoperiod was set to continuous illumination (24 hours light: 0 hours dark; 1500 lux measured at the water surface). When embryos developed eyed-egg stage (8 days post-fertilization), the water temperature was increased to 15°C and as soon as the first larvae began hatching (10 days post-fertilization), the temperature was further raised to 16°C. To ensure synchronized hatching, larvae were hatched manually by transferring the ribbons into separate bowls filled with water from the rearing tanks and gently stirring. This process was repeated few times until the majority of larvae had emerged from egg shells (Debernardis et al., 2025). This moment was recorded as 0 days post-hatching (DPH).

6.2.6 Larvae sampling and rearing

At hatching, larvae were carefully examined under a stereomicroscope, and those showing heart oedema deformities were meticulously selected (**Fig. 6.1**).

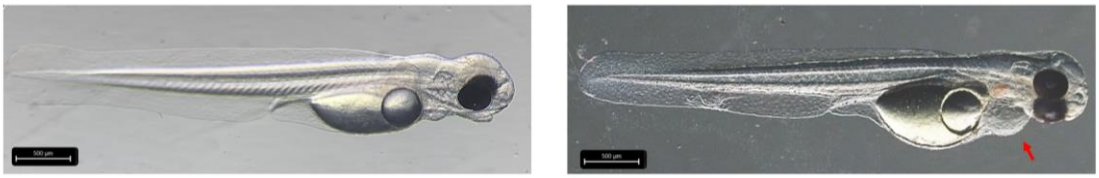

Figure 6.1*: Comparative exemplary images of Eurasian perch (Perca fluviatilis) larvae. Left: Normally-shaped larva without visible signs of oedema. Right: Individual displaying heart oedema (indicated by red arrow).*

Each deformed larva was photographed individually and preserved in 0.2 mL tubes containing FixRNA (Euryx, Poland), ensuring proper selection of larvae with consistent phenotypes for subsequent molecular analysis (same procedure was followed for each family). To facilitate a direct comparison, normal-shaped larvae from each family were also collected, enabling a paired analysis. Importantly, sampling was conducted on 0 DPH. Also, only larvae exhibiting heart oedema as the sole deformity were selected for analysis, in order to minimize potential transcriptomic noise from additional malformations. Heart oedema was identified through visual

assessment by an experienced researcher familiar with the specific phenotype, which was adequate for the objectives of this study.

To confirm that heart oedema constitutes a lethal phenotype, approximately 30 deformed and 30 normally shaped larvae from each family were maintained separately in dedicated incubators with continuous water flow. From 4 DPH, the larvae were fed *Artemia* sp. nauplii ad libitum twice daily. Larvae were monitored daily from hatching following the standardized rearing protocol for Eurasian perch (Debernardis et al., 2025).

6.2.7 RNA extraction

Total RNA was extracted separately from two pools of larvae per family: one pool consisting of larvae with heart oedema (n=5) and another pool of normally shaped larvae (n=10). This was done for each family separately. After extraction the concentration and purity of the RNA were evaluated using DS-11 spectrophotometer (Denovix), showing absorbance ratios of A260/280 ≥ 2.0 and A260/230 ≥ 2.2. Quality of the RNA was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with all samples exhibiting RIN values ≥ 9.0. RNA samples were then sent for transcriptomic analysis. Due to limited availability of larvae exhibiting solely heart oedema, each biological replicate for the oedema group consisted of pooled RNA from 5 individual larvae, while each replicate for the morphologically normal group consisted of RNA pooled from 10 individuals. A total of 5 independent biological replicates were generated per group. While the unequal pool sizes may affect the variance structure between groups, this approach was necessary given the low incidence of the pure oedema phenotype under our experimental conditions. All pools were processed independently to maintain biological replication. Appropriate normalization methods and dispersion estimation, implemented in DESeq2 package, were applied to account for potential differences arising from this design.

Additionally, RNA was extracted (using the protocol previously described) from Eurasian perch embryos at key developmental stages for Real-time qPCR analysis: the 128-cell division stage (12 HPF), which occurs before the mid-blastula transition (MBT) when embryonic development is solely under maternal control. This is followed by the half-epiboly stage (36 HPF), occurring after MBT, where paternal influence emerges, and zygotic genome activation takes place. Subsequent stages include eye cup formation (60 HPF), tail detachment (100 HPF), the eyed-egg stage (180 HPF), and hatching (0 DPH). These stages were chosen to enable the assessment of candidate gene expression dynamics across major developmental milestones of early development.

6.2.8 RNA sequencing and library preparation

Ten different libraries were created. RNA-Seq libraries were prepared and sequenced by Macrogen (Amsterdam, Netherlands) using the TruSeq Stranded mRNA kit (Illumina) with a NovaSeq6000 platform, and 40 M 150 bp paired-end reads per sample were generated. Read files have been processed with nf-core/rnaseq v3.12.0 (Patel, 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., 2013) and quantified with RSEM (B. Li & Dewey, 2011).

6.2.9 Differential expression genes (DEGs) analysis

RNA-seq data were analysed by performing pair-wise comparisons using the DESeq2 package in RStudio (version 4.2.2), following the authors' recommendations (Love et al., 2014), and *apeglm* for log fold-change shrinkage (Zhu et al., 2019b). Gene count data for the larval samples were filtered in order to have at least 6 libraries in which there were at least 10 reads. Groups from deformed and normal-shaped larvae were compared using the design: ~ *pairs* + *condition;* pairs standing for the 10 groups of larvae and condition referring to deformed (with heart-oedema) and normal-shaped larvae. Differences were considered significant when false discovery rate (FDR) was inferior to α (α = 0.05) and specific DEGs were found. To explore the variability in transcriptomic data, a heatmap and a Principal Component Analysis (PCA) plot was created for visualization using RStudio.

6.2.10 Gene Ontology (GO) Analysis

GO analysis was performed using the approach outlined by (Żarski et al., 2021b). The Eurasian perch transcriptome was initially mapped to the human proteome in the Swiss-Prot database. Sequence alignment was then performed using BLASTX, with the top match (based on the lowest e-value) for each protein selected to provide gene names and UniProt accession numbers. These gene names were subsequently input into the ShinyGO platform (Ge et al., 2020) to carry out the GO analysis. The analysis focused on the DEGs found and the 20 most enriched biological processes were identified, based on an FDR threshold of < 0.05.

6.2.11 *In silico* analysis

DEGs meeting the thresholds of $p < 0.05$ and $|\log_2 FC| > 1$ were further analyzed for tissue specificity. Specifically, we utilized the PhyloFish database (Pasquier et al., 2016), which provides *de novo* assembled transcriptome and gene expression data from various tissues and organs of different ray-finned fishes. The specificity of genes related to specific tissues in Eurasian perch was evaluated by calculating the tau (τ) index (Mank et al., 2008; Yanai et al., 2005). Genes were considered tissue-specific when $\tau > 0.85$ (Nguinkal et al., 2021). From this subset, genes specific to heart tissue were identified and selected as candidate markers for heart oedema. These candidate genes were subsequently validated using qPCR.

6.2.12 Reverse transcription, primer designing and Real-time (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 for the larval samples and 2 µg of total RNA for the embryos, was mixed with 4 µl of 5× 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 selected genes along with 7 reference genes for RT- qPCR were designed using Primer3Plus software version 3.3.0 (Untergasser et al., 2007). The reference genes were selected based on their lowest coefficient of variation (CV) recorded in our transcriptomic data (Żarski et al., 2021b), using TPMs calculated for all the biological replicates. The genes and sequences of the designed primers are shown in Supplementary file 1. For egg samples, four common reference genes were used: beta actin (*b-actin*), ribosomal protein S20 (*rps20*), ribosomal protein 4X (*rps4x*), elongation factor 1 (*elof1*) (designed primers are shown in **Supplementary file S6.1**). Unlike larvae, for which reference genes were selected from their RNA-seq data, for egg samples reference genes were selected based on previous studies demonstrating their stable expression in fish eggs and early developmental stages, in order to improve normalization accuracy (Deloffre et al., 2012; Gao et al., 2020).

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 cycles parameters involved 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. For each gene, a standard curve was generated using a series of six two-fold dilutions to determine the specific reaction efficiency. Efficiencies between 85% and 110% were considered acceptable. Gene expression changes were then analyzed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$) as described by (Livak & Schmittgen, 2001). Data were normalized using geometric mean of reference genes.

Finally, statistical analyses between groups (deformed vs. normal larvae) were performed using either a paired t-test or the Mann–Whitney U test, depending on data assumptions, in GraphPad Prism (version 9.4.1). Differences were considered statistically significant at $p < 0.05$.

6.3 Results

By 6 DPH, all heart-oedematose larvae had died. Although mortality occurred also in normal-shaped larvae was significantly lower and within expectations for this stage (**Fig. 6.2**).

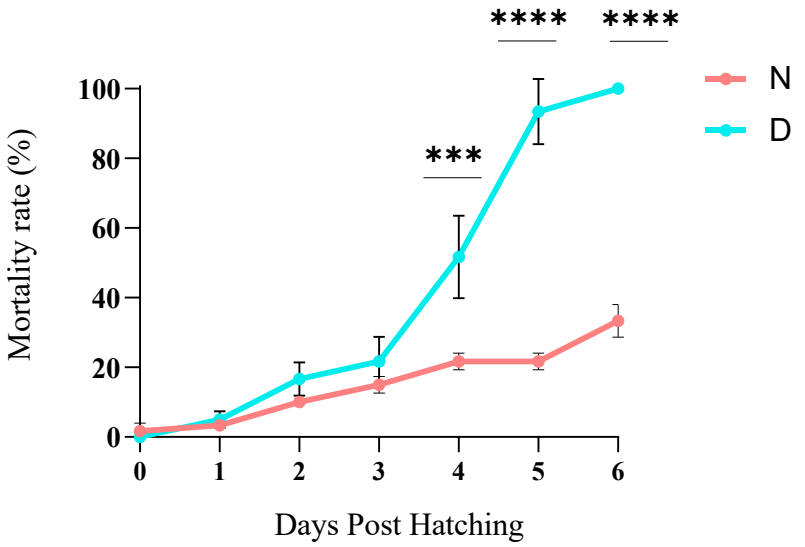

Figure 6.2: *Cumulative mortality (mean ± SD) from 0–6 days post-hatching (DPH) in deformed (D) and normal (N) larvae.*

RNA-seq data from heart oedema and normal-shaped larvae were analyzed using differential gene expression analysis. Following RNA-seq analysis, a total of 30,744 genes were initially found. After the filtering process for expression level, 20,227 protein-coding genes were identified, which were then used for identifying differentially expressed genes (DEGs).

The PCA graph (**Fig. 6.3A**) shows a strong distinction between the 2 groups of larvae. In fact, samples with same phenotype tend to cluster together, indicating consistency in gene expression

within groups. The sample distance heatmap (**Fig. 6.3B**) further supports the PCA plot, showing that the two groups have distinct gene expression patterns.

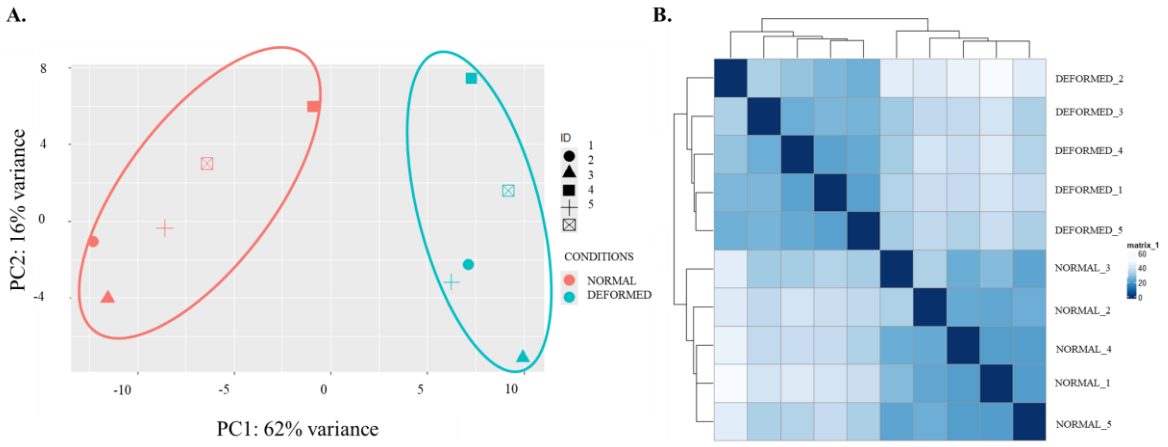

***Figure 6.3: A**. Principal component analysis (PCA) of RNA-seq data from Eurasian perch larvae illustrating sample distribution. Different colors indicate distinct experimental conditions, while symbols represent paired groups. Samples cluster according to condition, with the red ellipse encompassing normal larvae and the blue ellipse representing larvae exhibiting heart oedema (deformed). **B**. Heat map of sample-to-sample distances generated with DeSeq2 software package showing Euclidean distances between samples.*

The DESeq2 analysis identified 5,964 differentially expressed genes (DEGs) with $p < 0.05$ and $|\log2FC| > 0$ (**Supplementary file S6.2**). Visualization of the top 100 differentially expressed genes (**Fig. 6.4**) shows significant transcriptomic diversity among the groups (i.e., normal and heart-oedema larvae).

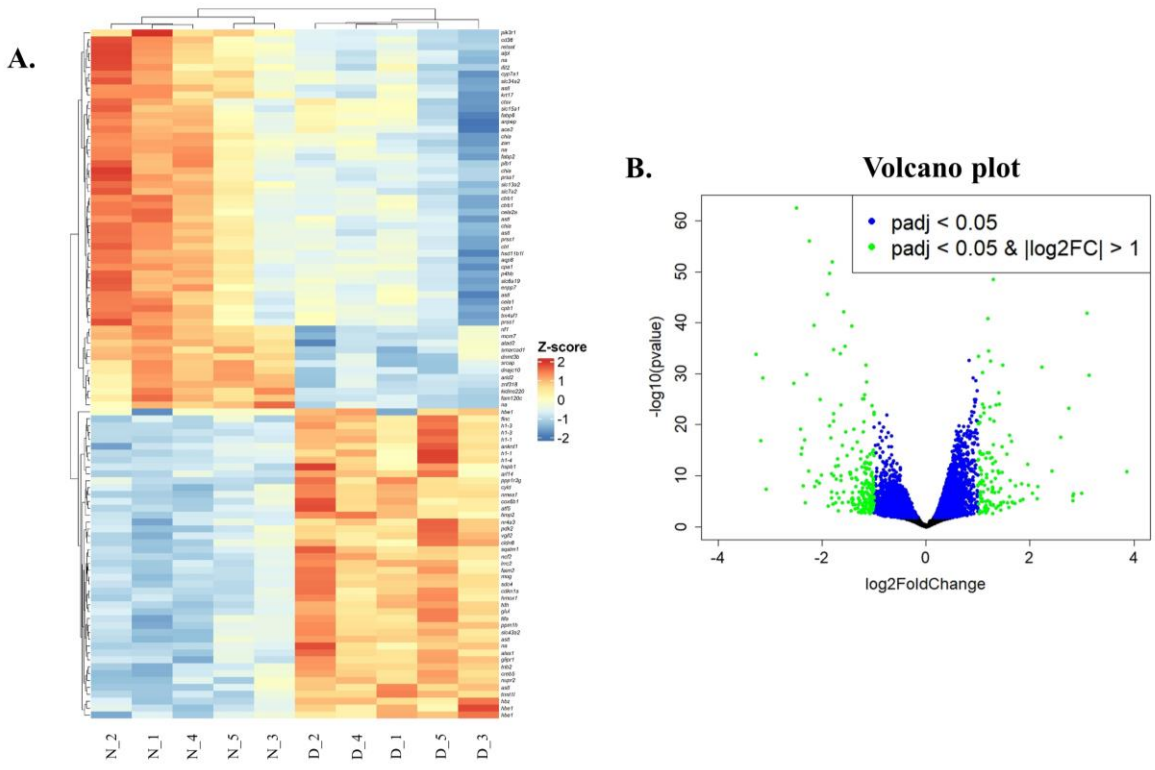

Figure 6.4: ***A****. Heatmap showing the unsupervised hierarchical clustering of 100 differentially expressed genes between normal (N) and deformed (D) larvae of Eurasian perch.* ***B****. Volcano plot of DEGs (genes with padj < 0.05 in blue, genes with padj < 0.05 and log₂ fold change greater than 1 or less than -1 in green).*

Gene Ontology enrichment revealed that these DEGs are predominantly involved in biological processes such as cellular structure organization, metabolic and catabolic pathways, cell cycle regulation, signaling, and development (**Fig. 6.5A**).

Of the total DEGs, 507 DEGs met the criteria of $p < 0.05$ and a $\log_2$ fold change greater than 1 or less than -1. These genes were predominantly associated with DNA replication, metabolic processes, and responses to stress (**Fig. 6.5B**).

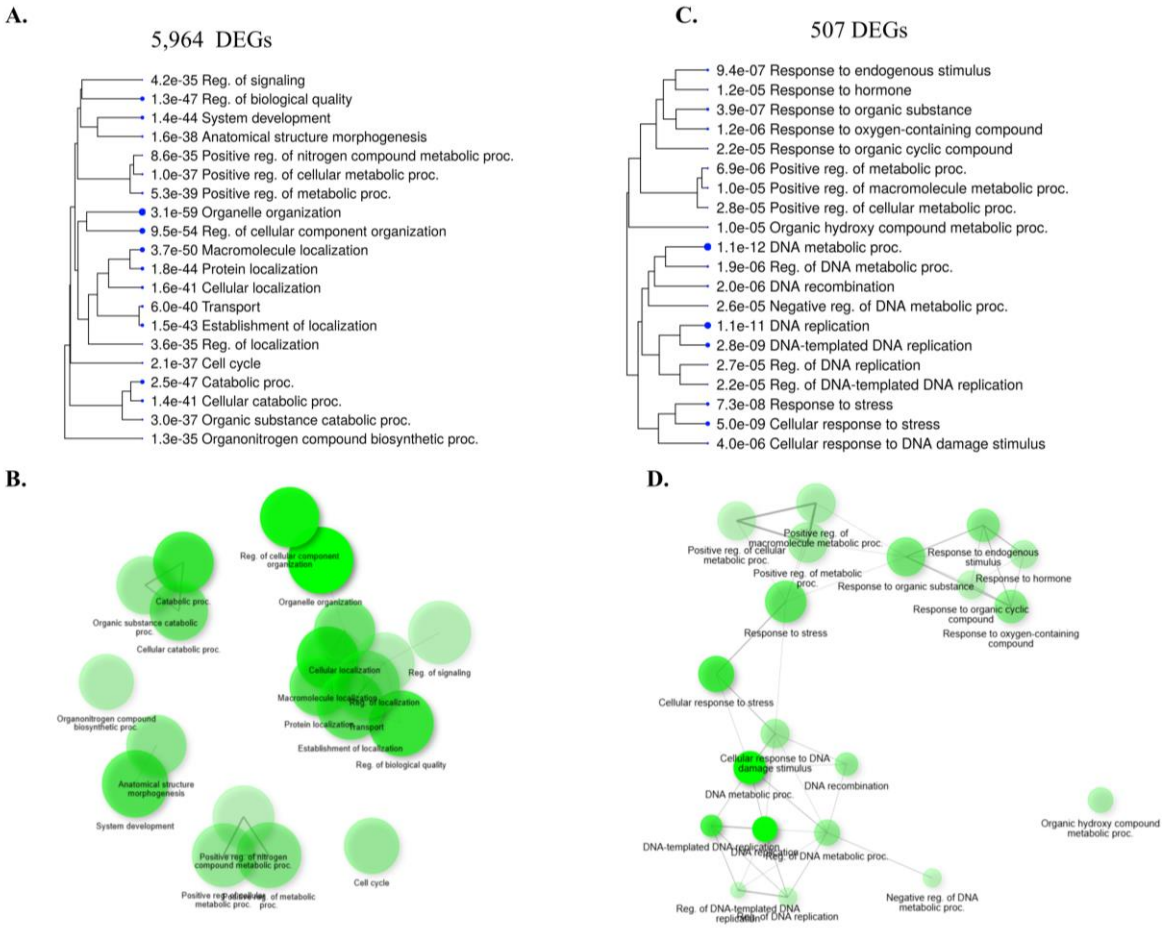

Figure 6.5: ***A****. Tree view and* ***B.*** *Network visualization showing the 20 most significantly enriched GO (biological process) for the 5,964 DEGs (padj < 0.05) found between normal and deformed (with heart-oedema) Eurasian perch larvae.* ***C.*** *Tree view and* ***D.*** *network visualization showing the 20 most significantly enriched GO (biological process) for the 507 DEGs (padj<0.05 and |log2FC| > 1) found between normal and deformed (with heart-oedema) Eurasian perch larvae.The tree views show hierarchical clustering of enriched GO terms based on semantic similarity, with larger blue dots indicating higher statistical significance (lower adjusted p-values). In the network visualizations, node size reflects the number of associated DEGs, while darker green color indicates stronger enrichment. Edges represent functional or semantic similarity between GO terms, with shorter connections indicating greater overlap.*

DEGs with *p*adj < 0.05 and $|\log_2 FC| > 1$ were analyzed for tissue specificity. As shown Among them, 180 DEGs did not exhibit tissue-specific expression ($\tau < 0.85$), while the majority of the remaining genes showed high specificity ($\tau > 0.85$), predominantly for the testis and intestine, while only six DEGs were found to be specific to the kidney. The GO analysis (**Fig. 6.6**) revealed that genes without tissue specificity are primarily involved in fundamental biological processes such as chromosome and chromatin organization, as well as transcription regulation. In contrast, genes specific to the testis and ovary are enriched in pathways related to DNA replication, repair, and the cell cycle. Liver-specific genes are associated with lipid and fatty acid metabolism, while those in the intestine are linked to digestive processes, intestinal absorption, and vascular transport.

Genes expressed in muscle tissue are connected to skeletal muscle development and apoptosis. In the brain, the predominant functions involve neurogenesis and neuronal development, whereas in bones, the focus is on ion and inorganic compound transport. Gills show enrichment for genes involved in cell migration, protein metabolism regulation, and locomotion. Interestingly, genes specific for embryo and kidney tissues did not significantly cluster into any specific GO terms.

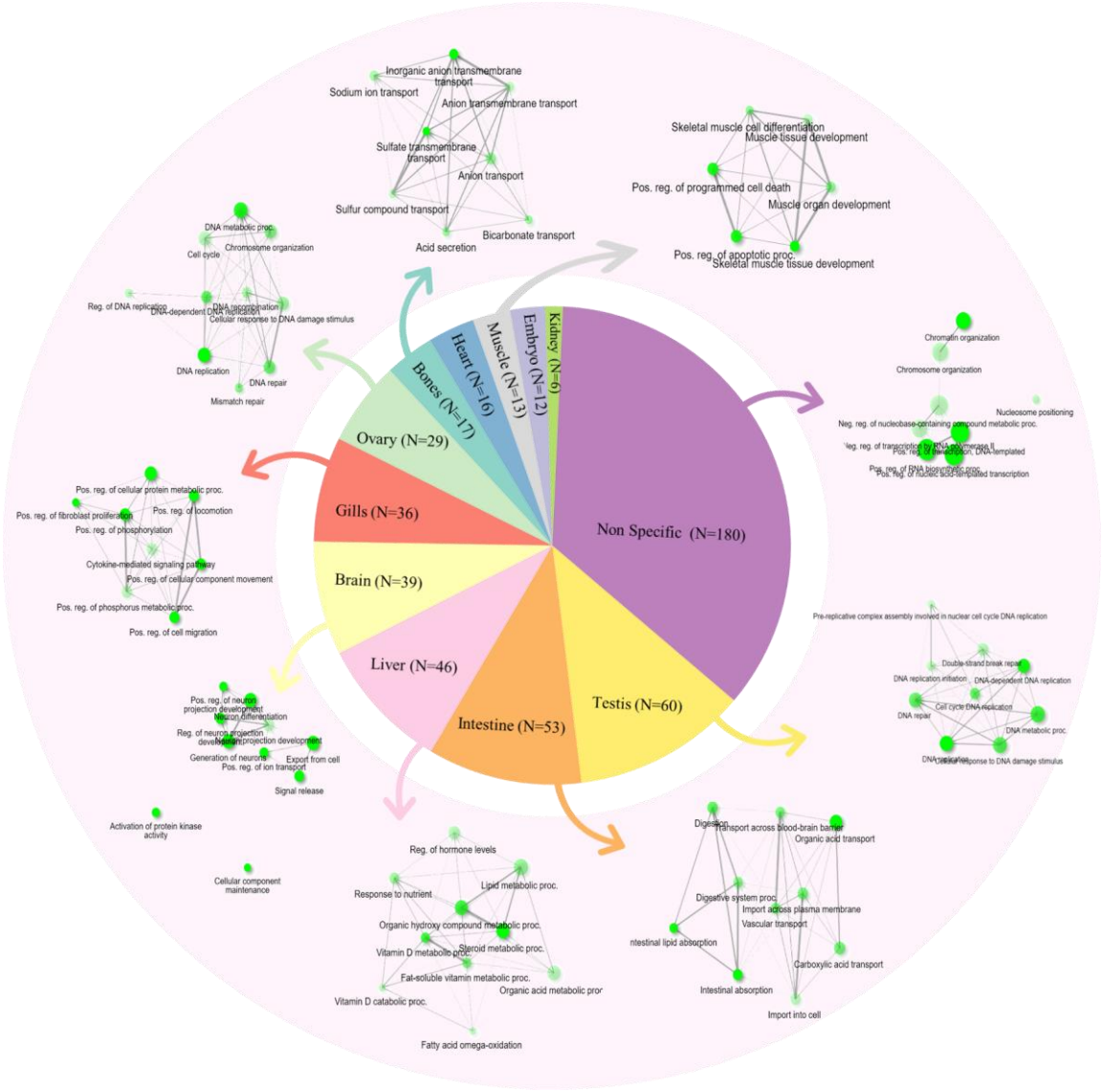

Figure 6.6: *Pie chart and Networks of Differentially Expressed Genes (DEGs) Grouped by Tissue and Specificity. It visualizes DEGs (padj < 0.05, $|log_2FC| > 1$), grouped by tissue and classified as either tissue-specific or non-specific. Each slice represents a tissue, sized according to the number of genes (indicated in brackets), and colored by tissue type. Gene specificity was determined based on expression patterns using the Tau index ($\tau > 0.85$). Network visualization shows the 10 most significantly enriched GO (biological process) for the genes specific for each tissue (no enriched GO terms were identified for embryo or kidney samples). For heart tissue, Gene Ontology analysis is shown in Figure 6.7B-C.*

Further screening revealed that only 16 of the 507 DEGs were heart-specific ($\tau > 0.85$) in Eurasian perch (see **Table 6.1** and **Supplementary file S6.2**). These 16 heart-specific genes exhibited differential expression patterns associated with heart oedema in larvae (**Fig. 6.7A**), with functional enrichment analyses highlighting their involvement in glucose metabolism and oxygen transport (**Fig. 6.7B-C**).

Table 6.1: *List of 16 candidate heart-specific genes identified in Eurasian perch in this study.*

Gene_id	***Transcript accession number***	***Gene name***	***Human ortholog***	***log2Fold Change***	***padj***
nppb	XM_039799442.1	Natriuretic peptides A	*NPPA*	1.84	2.50E-07
mb	XM_039816669.1	Myoglobin	*MB*	1.96	0.0012
corin	XM_039821935.1	Atrial natriuretic peptide-converting enzyme	*CORIN*	-1.36	0.005
gck	XM_039803535.1	Glucokinase	*GCK*	-1.97	2.86E-06
f2r	XM_039803294.1	Proteinase-activated receptor 1	*F2R*	1.02	0.001
LOC120575701	XM_039826528.1	Heat shock protein beta-1	*HSPB1*	1.06	3.24E-13
LOC120574977	XM_039825524.1	ADP-ribosylation factor-like protein 14	*ARL14*	1.04	7.38E-12
LOC120555608	XM_039794442.1	Hexokinase-1	*HK1*	1.06	1.57E-06
LOC120574104	XM_039824188.1	Hemoglobin subunit epsilon	*HBE1*	1.26	3.78E-30
LOC120564151	XM_039808898.1	Filamin-C	*FLNC*	1.13	1.68E-21
hbae5	XM_039789004.1	Hemoglobin subunit zeta	*HBZ*	1.30	1.21E-45
LOC120550815	XM_039787679.1	Cytochrome c oxidase subunit 6B1	*COX6B1*	1.33	3.32E-11
timp2b	XM_039825831.1	Metalloproteinase inhibitor 2	*TIMP2*	1.07	1.86E-13
LOC120562001	XM_039805394.1	GTPase IMAP family member 7	*GIMAP7*	-1.33	0.038
c13h18orf21	XM_039821127.1	UPF0711 protein C18orf21	*C18ORF21*	1.35	0.0007
LOC120551738	XM_039789276.1	Hemoglobin subunit epsilon	*HBE1*	1.49	2.22E-29

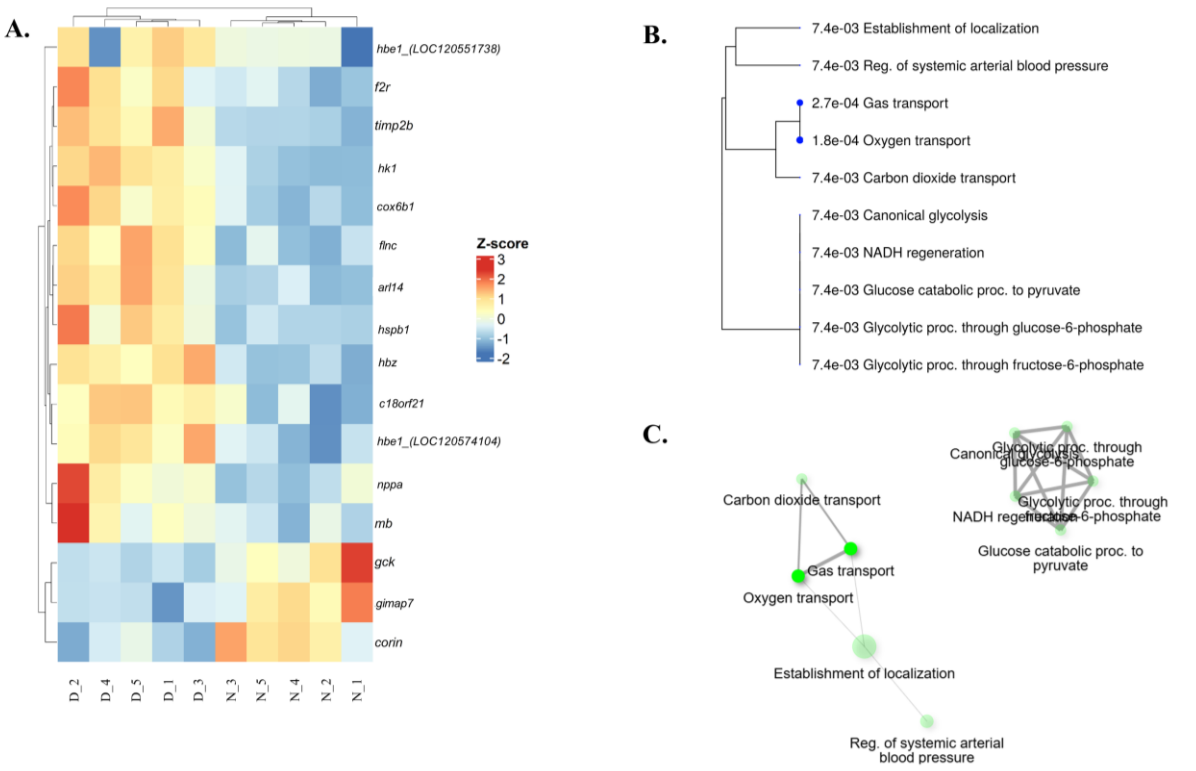

Figure 6.7: A. *The unsupervised hierarchical clustering of 16 heart-specific differentially expressed genes between normal (N) and deformed (D) larvae of Eurasian perch.* ***B.*** *Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for the 16 DEGs found between normal and deformed (with heart-oedema) Eurasian perch larvae. The tree views show hierarchical clustering of enriched GO terms based on semantic similarity, with larger blue dots indicating higher statistical significance (lower adjusted p-values).* ***C.*** *In the network visualizations, node size reflects the number of associated DEGs, while darker green color indicates stronger enrichment. Edges represent functional or semantic similarity between GO terms, with shorter connections indicating greater overlap.*

These 16 heart-specific genes were selected for qPCR validation. Of the 16 candidates, 7 were positively validated: *gck* (glucokinase), *arl14* (ADP-ribosylation factor-like protein 14), *hbz* (hemoglobin subunit zeta) and *c18orf21* (UPF0711 protein C18orf21), *nppa* (natriuretic peptides A), *hbe1* (hemoglobin subunit epsilon) and *flnc* (filamin-C) (**Fig. 6.8**). Note that, validation of the gene *corin* (atrial natriuretic peptide-converting enzyme) was not possible due to unsuccessful primer design. Consequently, for this gene (shown in the supplementary **Figure S6.1**), only the RNA-seq expression data are presented.

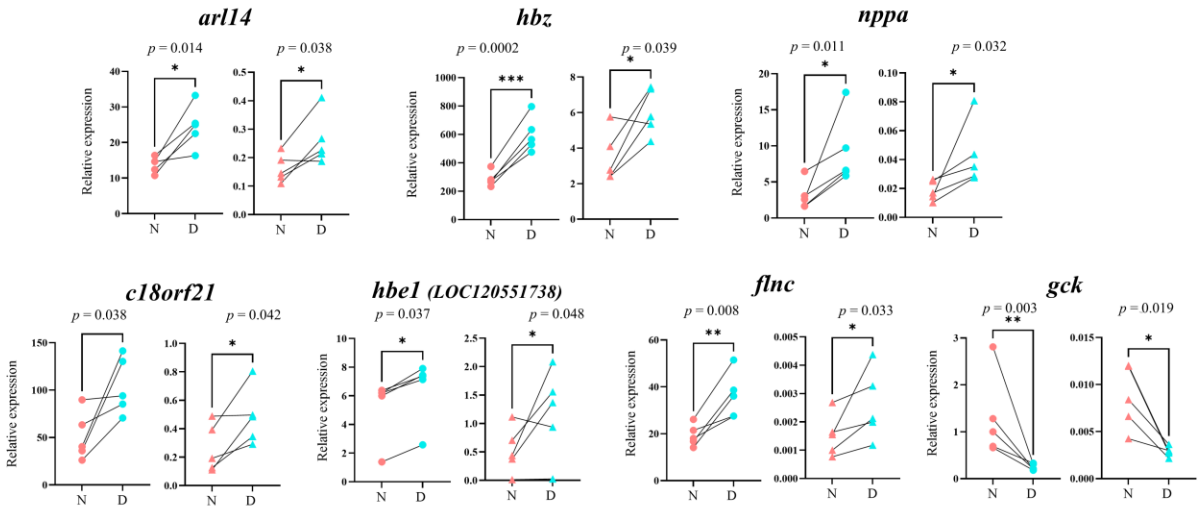

Figure 6.8: *The relative gene expression level of successfully validated genes in deformed (D) and normal (N) larval groups obtained after RNA-seq (circles) and real-time qPCR analysis (triangles). Data marked with an asterisk were statistically different (*p < 0.05, **p < 0.01, ***p < 0.01). Specific p-value is reported for each comparison.*

For the genes that were positively validated, we examined their expression profiles across key developmental stages, ranging from early embryogenesis to hatching (**Fig. 6.9**). The results revealed a broadly conserved temporal pattern among the seven genes, characterized by minimal expression during early stages (12–100 HPF), followed by an upregulation at later stages, particularly from 180 HPF to 0 DPH. Specifically, *arl14, hbz, gck, nppa*, and *flnc* exhibited low expression from 12 HPF through 100 HPF, followed by a pronounced increase from 180 HPF (eyed-egg stage), peaking at hatching (0 DPH). *hbe1* differed slightly, showing an earlier onset of upregulation beginning at 100 HPF (tail detachment stage), with continued increase through to hatching. Interestingly, *c18orf21* showed a distinct expression pattern, with relatively high expression at 12 HPF, a sharp decline by 36 HPF, and a gradual growth in expression from 180 HPF onwards.

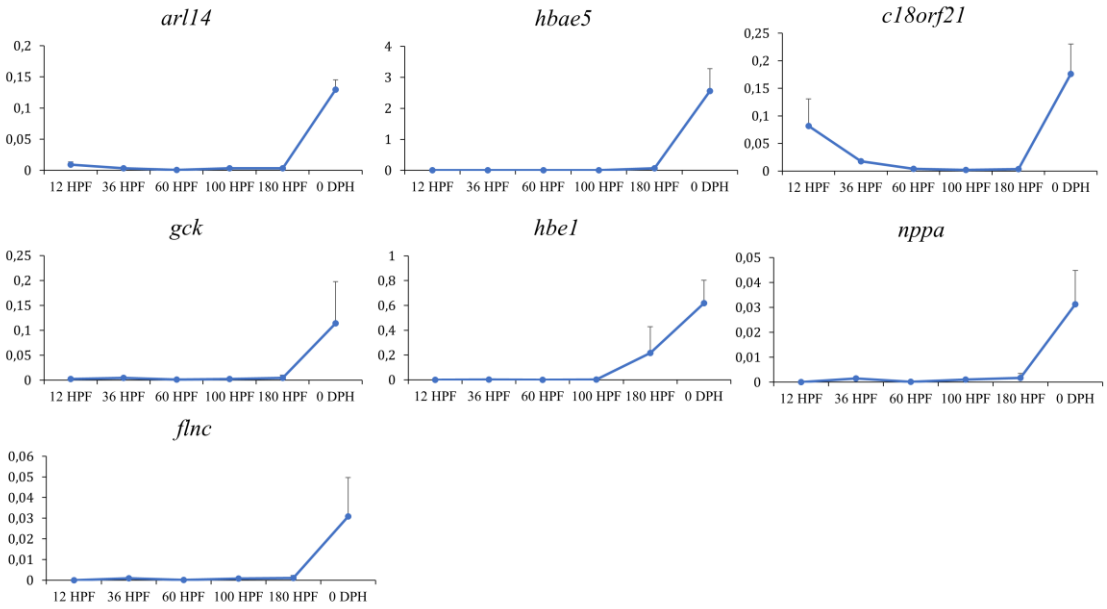

***Figure 6.9**: Kinetics of the expression of the 7 positively validated genes by qPCR, at specific key stages critical for the development and survival of the Eurasian perch embryo 128-cell division stage (12 hours after fertilization, HPF), half epiboly stage (36 HPF), eye cup formation (60 HPF), tail detachment (100 HPF), eyed-egg stage (180 HPF) and hatching (0 days post hatching, DPH).*

While 7 out of 16 candidate genes were successfully validated by qPCR, this partial validation is not uncommon due to differences in sensitivity and methodology between RNA-seq and qPCR. Nevertheless, the validated genes provide strong evidence for different expression of heart specific genes associated with the heart oedema phenotype and constituting solid set of heart-oedema-related markers.

6.4 Discussion

This study provides novel insights into the heart oedema phenotype in Eurasian perch progeny. Our findings deepen the understanding of the molecular landscape underlying early cardiac deformities and reveal specific transcriptomic changes in freshly hatched larvae. The results also highlight that heterogeneity among gametes from the same individual - arising either from genetic mutations or from non-genetic influences - contributes to phenotypic diversity within families, of which heart oedema represents a specific manifestation.

The heart is one of the first organs to develop and function in fish, and its proper development is crucial for the regulation of other physiological processes (Incardona & Scholz, 2016). At

embryonic and larval stages, considered highly vulnerable and sensitive phases in the fish life cycle, even the impaired development of a single organ can critically compromise survival and long-term viability (Perrichon et al., 2017). Larvae exhibiting heart oedema usually show impaired swimming ability, which likely limits their capacity to actively forage and ultimately reduced their chances of survival (Incardona and Scholz, 2016). In this study, deformed individuals died around 6 DPH. Mortality also occurred among morphologically normal larvae, but at rates typical for this developmental window near the oil-droplet absorption stage, when mortality usually peaks (Debernardis et al., 2025). This highlights the critical importance of proper cardiac function during early development and the need for in-depth molecular investigations to elucidate the mechanisms of this phenotype.

Transcriptomic comparisons between larvae with heart oedema and their normally developed siblings revealed a distinct molecular signature associated with the oedema phenotype. The PCA further supported this distinction, showing clear segregation between the two groups and highlighting the robustness of the transcriptomic differences. These results suggest that heart oedema is not just a morphological defect but is strongly associated with alterations in gene expression. The identified set of genes constitute valuable resource which may potentially lead to a deeper understanding of the mechanisms involved in or associated with this alteration. Importantly, the 16 identified heart-specific genes are predominantly involved in oxygen transport and glucose metabolism. Their dysregulation may play a critical role in cardiac performance and contribute to the development of heart oedema.

Glucokinase (*gck*), a key regulator of glucose metabolism, was found to be downregulated in deformed larvae, suggesting impaired energy transformation. Since glucose metabolism fuels heart rate and muscle activity critical for hatching success (Gao et al., 2022), reduced *gck* expression in oedematose larvae may compromise energy supply, leading to diminished cardiac output and impaired circulatory function. In contrast, the embryonic hemoglobin genes *hbz* and *hbe1* were upregulated in larvae exhibiting heart oedema. These hemoglobins, essential for oxygen transport, have been previously associated with hypoxic responses in zebrafish (*Danio rerio*), where paternal hypoxic exposure led to elevated embryonic hemoglobin expression in offspring (Ragsdale et al., 2022). Their upregulation here may reflect a compensatory mechanism in response to impaired oxygen delivery caused by reduced cardiac efficiency. Supporting the critical role of heart development genes, *nppa*, involved in early cardiac formation, osmoregulation, and blood pressure regulation, also showed altered expression. Its functional importance is emphasized by studies showing that zebrafish mutants lacking *nppa/nppb* develop cardiac malformations and oedema

(Grassini et al., 2018), underscoring its critical role in heart development. Likewise, *flnc*, which encodes filamin C, a structural protein that anchors membrane proteins to the cytoskeleton and ensures muscle integrity, has been linked to cardiomyopathies across species (Song et al., 2022). underscoring its role in maintaining cardiac structure and function. Lastly, the gene *arl14*, although not previously associated directly with heart malformations, is involved in intracellular trafficking, and related ADP-ribosylation factors have been implicated in angiogenesis and vascular development in zebrafish (Chen et al., 2012), suggesting its potential indirect involvement in cardiovascular integrity. Collectively, these expression patterns highlight a coordinated molecular response to compromised cardiac function in deformed larvae, involving genes critical for energy metabolism, oxygen transport, structural integrity, and vascular development.

Together, these findings highlight how heart deformities in fish larvae arise from a multifactorial disruption of essential physiological pathways, including glucose metabolism, oxygen transport, and the regulation of genes critical for cardiac structure and development. Notably, genes, such as *gck*, *hbz*, *nppa*, *flnc*, and *arl14*, show higher expression during the eyed-egg stage, a key period for organogenesis and tissue maturation. Their altered expression during this critical developmental window likely contributes directly to impaired cardiac morphogenesis and compromised circulation, highlighting the eyed-egg stage as a particularly sensitive period during which disruptions can lead to heart oedema. Interestingly, *c18orf21* also emerged as significantly differentially expressed between deformed and normal larvae. Although this gene has not previously been associated with cardiac development or function, it showed specific expression patterns during embryogenesis. The gene shows high expression early on, drops sharply, and then gradually increases again after 180 HPF. This timing hints that it could be involved in early developmental processes and later in tissue maturation. Its specific expression profile highlights the need for further research to understand its potential role in embryonic development and heart defects.

Beyond heart-specific DEGs, other genes showed specificity to other tissues such as gonads, intestine, brain, and gills. This suggests that in heart-oedema larvae, the observed gene dysregulation is not limited to the heart itself, but reflects a broader, multi-tissue response, even though these changes are not detectable morphologically and heart oedema remains the only visible sign. The changes in gene expression may be driven not only by the oedema but also by other concurrent biological processes occurring across different tissues. Notably, many of the DEGs identified in the larval transcriptome were specific to the gonads. While this may seem unexpected since larvae are not yet sexually mature, it reflects the concept that gene functions can

shift during development (Seifert, 2015). For example, testis or ovary-specific genes in larvae seem initially to fulfill broad biological functions (i.e., DNA replication and cell cycle regulation) before taking on more specialized functions that support sexual maturation later in life. Similarly, genes without current tissue specificity may assume more specialized functions as development progresses. Together, these findings reinforce the idea that gene expression during early development is highly dynamic and context-dependent.

The study suggests that early developmental outcome, even under similar genetic and environmental background, can be shaped by factors inherited through gametes, including both subtle genetic variation (i.e., gene mutations) and non-genetic mechanisms. Despite sharing the same parental origin, offspring displayed marked phenotypic differences within the same batch. As proposed by Vogt et al. (2008) such "developmental variation" or "developmental noise" may arise from random or intangible processes during oogenesis and embryogenesis. Differences in maternal provisioning of mRNAs and proteins (Bobe & Labbé, 2010; Yilmaz et al., 2017), as well as epigenetic contributions such as sperm DNA methylation (Jiang et al., 2013; Panda et al., 2024) could contribute to these early-life differences, despite uniform genetics and rearing conditions. This underscores that both maternal and paternal influences on offspring phenotype may be unequal and unpredictable, adding complexity to our understanding of developmental biology (Garfield et al., 2013). Importantly, previous work has shown that deformities in fish larvae can also have genetic basis, with mutations or chromosomal abnormalities (e.g., triploidy, mosaicism) leading to morphological malformations (mostly skeletal) in several species (Babcock et al., 2014; Berillis, 2017; Fopp-Bayat et al., 2021; Fraser et al., 2021). Such genetic changes can be inherited through gametes and may interact with environmental factors to influence deformity incidence (Chandra et al., 2024). Therefore, by exploring the transcriptome of heart oedematose larvae, this study provides a framework for understanding how molecular disruptions during early development can have profound consequences for survival. At the same time, it encourages future integrative approaches, where genetic, proteomic and epigenetic analyses of both, gametes and embryos along the early development will enable to explore how gene expression is controlled and influenced by parental factors in genetically similar individuals raised under controlled conditions.

While this study offers valuable insights into the molecular signatures linked to cardiac deformities in Eurasian perch larvae, some limitations need to be acknowledgments. Due to the small size of the larvae, whole-body transcriptomic analysis was necessary, which limits tissue-specific resolution. Future studies could employ whole-mount in situ hybridization to validate heart-specific expression of key candidate genes. Also, functional validation using techniques such

as morpholino knockdown or CRISPR-Cas9 gene editing would further clarify roles of these genes in cardiac structure and function.

In summary, this study provides a novel perspective on heart oedema arising spontaneously during embryogenesis in Eurasian perch larvae, occurring in the absence of identifiable external stressors such as environmental toxins or pollutants. Although the experiment did not establish definitive underlying mechanisms, it identified several candidate genes that are likely central to heart development and function. The findings suggest that the heart-oedema anomaly reflects more than a simple morphological defect. Instead, it indicates widespread dysregulation across multiple tissues. This underscores the complexity of embryonic development and the important role of inherited molecular signals in driving phenotypic variability. Identifying early molecular markers of developmental anomalies may support more targeted strategies, such as screening broodstock to select parents less likely to produce offspring with deformities, ultimately reducing mortality and enhancing aquaculture outcomes.

6.5 Data availability

Raw RNAseq data of different groups of freshly hatched larvae can be accessed via the NCBI BioProject database under the PRJNA1313406 and PRJNA1196822 accession numbers. Note that the data will become publicly available upon manuscript acceptance. Please access the data using the following links:
https://dataview.ncbi.nlm.nih.gov/object/PRJNA1313406?reviewer=jaevspti5a77qfq5tt2i5jj8me
https://dataview.ncbi.nlm.nih.gov/object/PRJNA1196822?reviewer=48q6c09n1m664426ui07sk58nv

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CRediT authorship contribution statement

Rossella Debernardis: Writing-original draft, Methodology, Visualization, Investigation, Data analysis, Data curation, Conceptualization. **Katarzyna Palińska-Żarska**: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Sylwia Judycka**: Writing – review & editing, Methodology, Investigation, Conceptualization. **Abhipsa Panda**: Investigation. **Christophe Klopp** & **Tainá Rocha de Almeida**: Writing – review & editing, Data curation.

Daniel Żarski: Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Chapter 7: General discussion

"What we call the beginning is often the end. And to make an end is to make a beginning"

(T.S. Eliot, Little Gidding, Four Quartets -1942-)

7.1 What does the transcriptome say?

This thesis presents a comprehensive transcriptomic exploration of newly hatched Eurasian perch larvae, a model freshwater species of growing aquaculture importance. By integrating large-scale gene expression profiling with assessments of key zootechnical traits, such as growth performance, deformity incidence, and survival, across four complementary experimental frameworks, this work sought to unravel the molecular and zootechnical determinants of early developmental success. The approach functioned as consequential zoom-in, moving from broad family-level comparisons to the disentangling of parental effects, then narrowing to maternal influence through egg-quality analyses, and finally zooming in on fine-scale within-batch transcriptomic differences between normal and abnormal (heart oedema) larvae. Collectively, these studies shed light on the dynamic processes that shape fish development at its earliest stages and emphasise the importance of both genetic and non-genetic parental contributions on offspring phenotype. Ultimately, the transcriptome of newly hatched larvae emerges as a molecular bridge linking the parental legacy (the past), the molecular phenotype at hatching (the present), and the developmental trajectories that follow (the future), offering new insights into early developmental processes in fish.

7.2 Past – parental effects and molecular legacy

The "past" of a larva is encoded in the molecular materials which inherits at fertilization, a complex blend of genetic sequences and non-genetic factors from both parents (Adrian-Kalchhauser et al., 2020; Uller, 2008). In Eurasian perch, the results of the current thesis show that this parental legacy is deeply embedded in the larval transcriptome, shaping developmental potential well before environmental influences act. As discussed in Chapter 3 (larval transcriptome study), the transcriptome of larvae at mouth opening serves as a molecular archive of gamete-derived RNA transcripts provided by both parents, with maternal contributions often predominating, in agreement with previous studies (Domínguez-Petit et al., 2022; Wolf & Wade, 2009). This inherited molecular landscape not only sets the starting conditions for embryos and larvae development but also influences their responses to post-hatch environmental challenges.

Traditionally, larval phenotype formation has been viewed as a static and straightforward process, in which each parent's equal genetic contribution translates into equal phenotypic influence (Wells, 2014; Zhao et al., 2020). However, the findings presented in Dom-Wild experiment (Chapter 4), which disentangled maternal and paternal influences, challenge this

assumption. It revealed that larval phenotype emerges from a complex and dynamic interplay between maternal and paternal effects. Within this broader framework, the present thesis found that maternal influences predominated, particularly in zootechnical traits such as hatching rate, growth, swim bladder inflation, and survival. Consequently, these results provide evidence, that maternal influence is not only restricted to provision the oocyte with molecular resources that drive early embryogenesis before zygotic genome activation (ZGA), as it is commonly assumed (Vastenhouw et al., 2019), but also beyond. While the maternal transcriptome is generally thought to be largely cleared at ZGA, evidence from zebrafish and mammals indicates that this process is selective, with subsets of maternal RNAs escaping immediate degradation. These transcripts can remain transiently active after ZGA and may contribute to downstream molecular cascades influencing subsequent developmental stages (Giraldez et al., 2006; Sha et al., 2020). On the other hand, paternal effects, although less pronounced, were nonetheless important, fine-tuning the regulation of expression of specific genes in the progeny. The results presented in Dom-Wild suggest that paternal contributions may 'come to voice' in particular developmental or environmental contexts, or when maternal regulation allows, thereby modulating gene expression and shaping offspring phenotype. This perspective aligns with findings in other taxa. In mice, Mashoodh et al. (2018) showed that mothers can actively modulate the expression of paternal influences on offspring development, whereas in the large white butterfly (*Pieris brassicae*), Ducatez et al. (2012) similarly found that parental effects rarely act independently, but instead result from interactions between maternal and paternal traits influenced by environmental context. These findings reinforce our assessment that parental effects are not equal but complementary, with maternal provisioning setting the baseline and paternal contributions providing context-dependent regulatory refinement.

The significance of maternal provisioning was further demonstrated in Chapter 5 (egg quality), where transcriptomic analysis of unfertilised eggs of differing quality revealed dysregulation of key genes - *atm*, *ep400*, and *otulina* - in low-quality eggs, which exhibited sharp developmental failure after the mid-blastula transition. This suggests that successful embryogenesis could depend on the oocyte's DNA repair capacity and fertilisation competence. Notably, the gene *otulina* has previously been linked to poor egg quality in zebrafish (Cheung et al., 2019), reinforcing its potential role as a molecular marker. The dysregulation of these molecular factors likely underpins developmental robustness, while their absence or dysregulation predisposes embryos to early developmental failure. These findings also suggest that variation in egg quality may originate from heterogeneous deposition of molecular factors during oogenesis, leading to differences in

developmental potential even within the same batch of eggs already before fertilisation. Overall, this emphasises that the egg's molecular endowment decisively shapes embryonic potential. Such variation occurs both between females, reflecting differences in their capacity to cope with environmental influences, and among oocytes within the same ovary, reflecting intrinsic heterogeneity in resource allocation during egg formation. Also, the within-batch comparison in Chapter 6 (heart oedema case study), comparing larvae with normal morphology to siblings exhibiting heart oedema, revealed that even larvae from the same parents, reared under identical conditions, can diverge sharply in their morphology and transcriptomic profiles. This within-batch variability, detectable as early as hatching, strongly supports the idea proposed in the egg quality study, that heterogeneous deposition of molecular factors during oogenesis or variation in their regulation can lead to developmental differences. Similar variation in maternal provisioning has been documented in two sibling species of ascidian (*Ciona intestinalis* and *Ciona robusta*), where differences in the quantity or composition of deposited factors can shift across generations, suggesting that this heterogeneity may be both dynamic and heritable (Sato et al., 2024). In zebrafish, Rauwerda et al. (2016) likewise found that, although most variation in maternal mRNA composition occurs between clutches from different mothers, a subtler but measurable level of within-clutch variability still exists. This fine-scale variability, layered on top of broader maternal and paternal effects, underscores the dynamic and non-uniform nature of early fish development. Ultimately, the "past" provides an important foundation upon which the present molecular phenotype is built and from which future developmental pathways emerge.

In sum, this thesis shows that early developmental potential is largely pre-figured by the molecular legacy inherited through gametes. Maternal inputs establish the baseline, paternal inputs provide context-dependent regulatory refinement, and intra-batch heterogeneity introduces additional variation, even among siblings reared under identical conditions. This integrated, multi-scale evidence turns "parental effects" from a conceptual framework into a concrete, measurable component of the mouth-opening-stage larval transcriptome in Eurasian perch.

7.3 Present – transcriptome of freshly hatched larvae

If the "past" of a larva is embedded in the molecular legacy inherited from its parents, the "present" is captured in the transcriptome at hatching, a real-time molecular snapshot of the organism at the moment it transitions from embryonic development to independent functional larva. This stage is pivotal because it represents the larva's intrinsic molecular state, established before external environmental influences take hold. Understanding this present moment is crucial:

it reveals the developmental programs already completed during embryogenesis, identifies the biological processes actively sustaining early life, and defines the molecular foundation upon which future growth, performance, and survival will depend.

From the experiment described in Dom-Wild (Chapter 4), we identified a subset of genes that were conserved across larval groups irrespective of the parental backgrounds. These consistently expressed genes, representing a molecular "reference state," were strongly enriched for functions related to nervous system development and intracellular transport. The importance of neurogenesis-related processes at hatching is consistent with the rapid functional maturation of sensory and motor systems needed for example for first feeding, as reported for Eurasian perch by Kupren et al. (2019). Similarly, enrichment for intracellular transport terms reflects the high metabolic and biosynthetic activity required to support cell growth, tissue differentiation, and morphogenesis. These conserved pathways are likely essential components of the baseline molecular machinery for early larval development in Eurasian perch, and are consistent with observations in other teleost larvae, where nervous system development and cellular organisation processes dominate the transcriptome at hatching (e.g., Ferraresso et al., 2013; Marisaldi et al., 2021). This conclusion was further reinforced by an additional comprehensive comparison of all RNA-seq datasets generated in this thesis (performed for the purpose of the present chapter), which revealed that over 87% of expressed genes were shared across experiments, regardless of experimental design or parental origin (**Fig. 7.1**). These conserved genes were consistently associated with fundamental biological processes ensuring proper cellular organisation, efficient macromolecule synthesis and transport, and coordinated system-level development - particularly of the nervous system (**Fig. 7.2A**). Notably, the nervous system has previously been proposed as a key target of non-genetic inheritance at the egg stage (as shown by Colson et al., 2019 and Żarski et al., 2021b) and this view is now strengthened by the present whole-larva transcriptome data. These functions likely constitute the essential molecular reference line sustaining early larval viability.

Alongside this conserved core, each experimental context also revealed a distinct set of context-specific genes (so called because their expression is specific to a given scenario or parental background and does not persist across other conditions), highlighting biological priorities shaped by the specific scenario or parental background (**Fig. 7.2B**). In larval transcriptome research (Chapter 3), these genes were predominantly enriched in processes related to regulation of immune system and ions transport. In dom-wild and heart oedema studies (Chapter 4 and 6), the non-overlapping gene set showed enrichment in pathways regulating signal transduction, cell

differentiation, and structural development. In contrast, the genes highlighted in egg quality experiment (Chapter 5) were involved in negative regulation of intracellular signals, amine and catecholamine metabolism, and a smaller subset linked to immune regulation (**Fig. 7.2B**). While these context-specific pathways are equally important for larval functioning and survival, they appear more sensitive to parental influences, indicating that certain components of early molecular programming are not universally conserved but instead shaped by their environmental history or genetic background. In other words, early development seems to include a robust core of conserved pathways, complemented by a layer of context-specific genes that can be adaptively fine-tuned in response to parental and environmental contexts. This dual structure highlights how parental effects can modulate specific aspects of early molecular programming without disrupting its fundamental framework.

Taken together, these findings establish the transcriptome as far more than a static list of expressed genes. It comprises two functional layers. The first is a large, conserved core of genes consistently active across all datasets, providing a reliable molecular baseline for the mouth opening stage - useful for cross-study comparisons or selecting stable normalisers -. The second is a smaller, context-specific set of genes expressed only in certain families or experimental scenarios, reflecting biological processes that vary with parental background or experimental conditions and different sampling or analysis. This framework has practical implications for functional studies, including gene editing approaches such as CRISPR/Cas9. Editing any of the conserved genes at this stage is likely to impact multiple systems and may cause early lethality, underscoring the need for caution, which should be taken under consideration at the designing stage of the experiment. By contrast, targeting context-specific genes may reveal effects only in the backgrounds where these genes are active. Therefore, the transcriptomic profile at hatching becomes more than just a catalogue of genes, but a functional map distinguishing what is consistently active from what is context-dependent. This map not only informs experimental design, but also points toward the “future” of larval development, where molecular indicators at hatching could be used to guide strategies for optimising growth and survival.

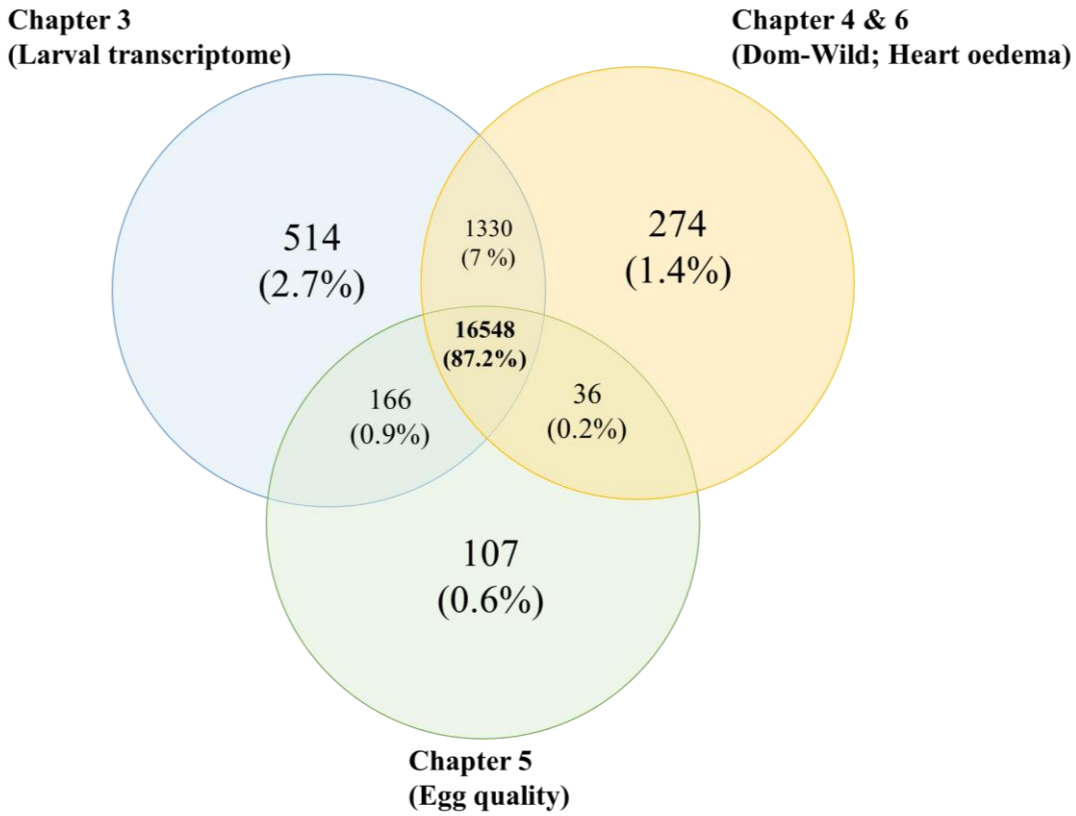

Figure 7.1*: Venn diagram showing the overlap of expressed genes among the three experimental datasets in this thesis. Genes were included if they were expressed in at least 75% of samples within each experiment and had a TPM > 1. A total of 16,548 genes (87.2%) were common to all the experiments, representing the conserved core transcriptome of newly hatched Eurasian perch larvae. The remaining genes were either shared between two experiments or unique to a single experiment, reflecting context-specific transcriptomic signatures. Note: the "normal larvae" group in the heart oedema experiment (Chapter 6) is identical to the Dom–Wild dataset (Chapter 4); therefore, only three circles are shown.*

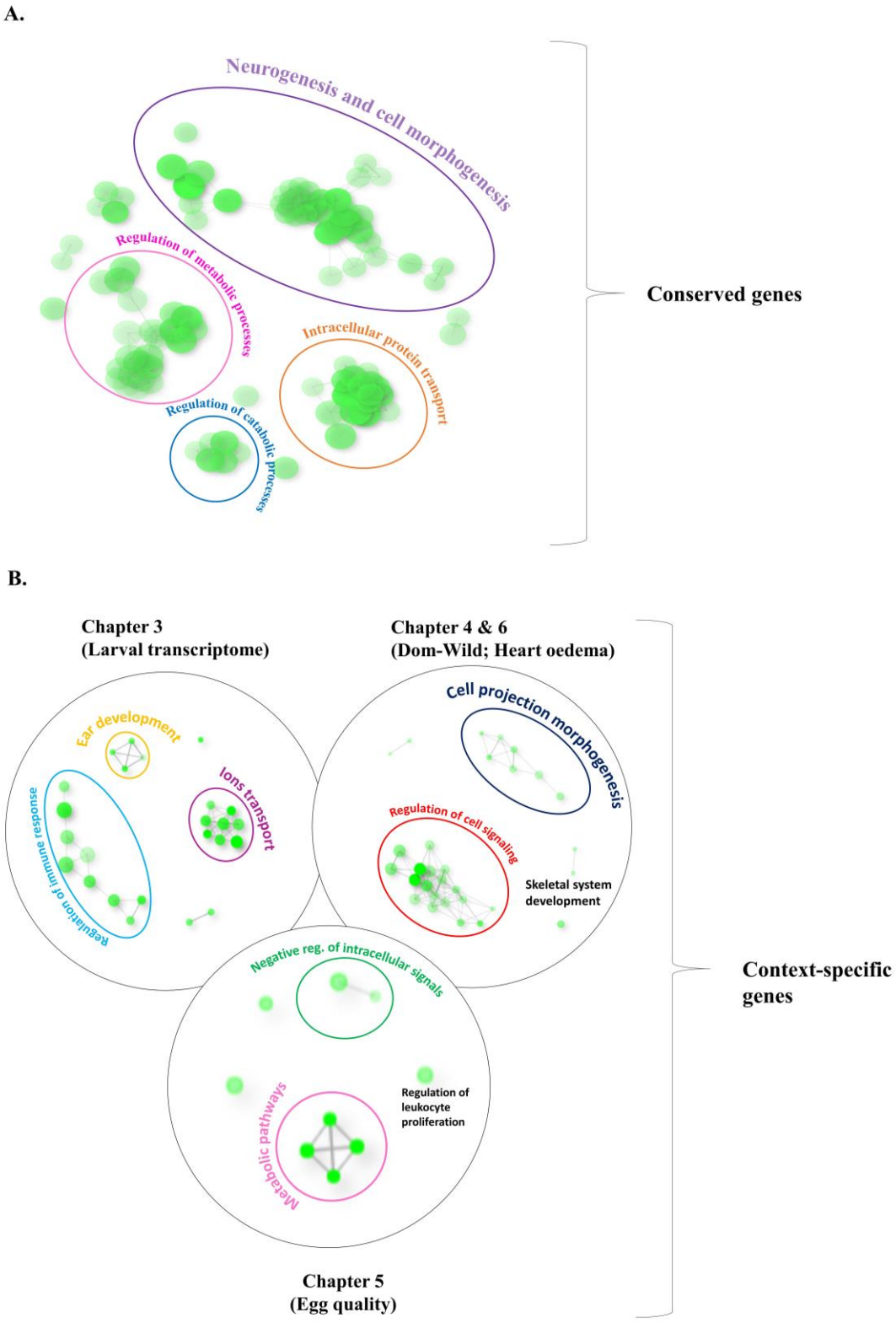

Figure 7.2: *Overview of networks highlighting clusters of enriched biological processes for* ***A.*** *conserved and* ***B.*** *context-specific genes. Larger clusters denote broader functional categories, including neurogenesis, regulation of metabolic processes, intracellular protein transport, immune response, and signaling regulation. Note: the "normal larvae" group in the heart oedema experiment (Chapter 6) is identical to the Dom–Wild dataset (Chapter 4); therefore, only three circles are shown.*

7.4 Future – From molecular insight to developmental outcomes

Among the key findings of this thesis is that transcriptome at hatching can describe not only a larva's origins and mirror its current state, but can also provide valuable clues about its developmental trajectory (Debernardis et al., 2025). The correlations observed across the experiments, between transcriptome profiles at hatching and later zootechnical traits suggest that transcriptomics could serve as a valuable early-stage predictive tool for larval performance in finfishes. This potential is clear in larval transcriptome study, where WGCNA enabled identification of gene modules whose expression at hatching was strongly correlated with post-hatch growth related traits. Most of these genes were involved in cell cycle and mitosis, supporting the organ and tissue remodelling that occurs after hatching as larvae adapt to their environment. It suggests that key aspects of larval performance can be already encoded in the transcriptome at hatching, and that the molecular program at this stage underpins subsequent growth and survival. Within this framework, several candidate genes emerged as potential early molecular markers for larval performance: s*elenoo* (antioxidant defence), *trim16* (immune regulation and stress resilience), *slc15a1* (nutrient uptake and growth), and *cipc* (circadian regulation) were each strongly correlated with post-hatching traits. While not previously validated as larval performance predictors in aquaculture species, their known biological roles align closely with the physiological demands of early development, making them promising targets for future studies.

Only a few studies to date have evaluated the predictive value of transcriptomic profiles in early-life stages of aquatic animals. In European eel (*Anguilla anguilla*) early-stage larval transcriptome profiling has identified gene expression patterns associated with developmental failures and early mortality, offering a potential tool to enhance larval survival (Jéhannet et al., 2021). Similarly, in webfoot octopus (*Amphioctopus fangsiao*), transcriptomic analyses at multiple time points after hatching have provided important insights into larval growth and development (Li et al., 2023). While these studies highlight the potential for using early-stage molecular data to forecast later outcomes, they focus on single contexts. Here, for the first time in finfish, a broader, multi-scenario framework has been built, integrating transcriptome data with rich phenotyping across parental contributions, egg-quality classes, and within-batch deformity status. This multi-layered approach links molecular state to performance traits with unique scope, offering new perspective into the intrinsic cues that guide larvae through their developmental journey.

Yet, the predictive power of newly hatched larvae transcriptome is limited by two key factors: environmental influences and intrinsic variability. Notably, the growth and survival of larvae after

hatching is strongly influenced by environmental factors, such as feed quality, rearing density, or water parameters (Burton & Metcalfe, 2014; Jonsson & Jonsson, 2014). Nonetheless, the way larvae respond to these conditions often depends on the molecular "toolkit" inherited from their parents. This parental legacy can prime individuals for efficient resource allocation, enhanced stress tolerance, and robust growth under favourable conditions or, if suboptimal, limit their adaptive capacity. Dom-Wild study supports this view, showing that while around 80% of a larva's transcriptome profile is conserved regardless of parental origin, approximately 20% is shaped by maternal and/or paternal contributions. Although smaller, this fraction may be decisive in determining future developmental responses. Building on this, egg quality trial explores how variability in maternal provisioning during oogenesis can influence which individuals ultimately succeed, highlighting its potential as an early selection criterion. However, larvae that successfully hatched from eggs of different quality showed remarkably consistent transcriptomic profiles and zootechnical traits, indicating that those which survived had passed key developmental checkpoints and converged towards a similar molecular and phenotypic state. This finding emphasizes the idea that the transcriptome at hatching captures a stable "conserved" profile for quality larvae, while emphasising the need to go back to the parental legacy, captured in the molecular composition of the gametes, to understand the factors that determine which individuals successfully reach this stage.

Extending this focus on intrinsic variability, heart-oedema experiment explores differences within families. Morphologically deformed (heart-oedema) individuals, despite sharing the same parental origin and rearing conditions as their normal siblings, showed a distinct transcriptomic signature at hatching. This included dysregulation of genes involved in cardiac development (*nppa, flnc*), oxygen transport (*hbz*) and glucose metabolism (*gck*). Such differences, detectable before any post-hatching environmental influences, underscore the transcriptome's sensitivity to subtle developmental deviations and its potential as a diagnostic tool for identifying at-risk individuals early. While such variability can blur predictions at the individual level, the present results show that meaningful forecasting of performance is still possible. This suggests that the transcriptome profiles of larvae at mouth opening captures robust molecular signals that persist despite individual-level noise. However, to refine predictive accuracy, future studies should focus on single-larva transcriptomic analyses and experimentally track individuals from hatching through later developmental stages. Such approaches could enhance both our fundamental understanding of early-life performance and the practical use of transcriptomics in aquaculture.

Chapter 8: Conclusions

This thesis provides a novel and comprehensive analysis of the whole-body transcriptome of Eurasian perch larvae at the mouth-opening stage, integrating it with detailed zootechnical evaluations to address the central objective: determining what the transcriptome of freshly hatched larvae reveals about both parental legacy and subsequent performance. At the broadest level, the transcriptomic characterisation of early-stage larvae identified a conserved core of highly expressed genes, enriched for nervous system development, intracellular transport, and macromolecule organisation. These pathways form a baseline molecular "reference state" for early larval viability, supporting the hypothesis 1 that the MO stage transcriptome captures essential developmental programs completed during embryogenesis and can be used to predict early larval performance. Also, by comparing larvae originated from wild and domesticated broodstock, the thesis addresses the hypothesis 2, that disentangling maternal and paternal influences reveals distinct contributions to early phenotypic variation. The results showed that parental effects are not equal but rather complementary, with maternal provisioning setting the developmental baseline and paternal inputs fine-tuning regulatory processes. The egg-quality study partially confirmed hypothesis 3, which proposed that variation in maternal provisioning could be detected at the transcriptomic level in unfertilised eggs and can halt embryonic development, however these differences are not evident in the transcriptomes of larvae that successfully hatch. This finding supports the hypothesis that egg quality leaves a molecular imprint that influences developmental potential, even if its effects are no longer visible at the larval stage in individuals that pass critical early checkpoints. Finally, the within-batch comparison of morphologically normal and heart-oedema larvae addressed hypothesis 4, anticipating that deformities would be associated with distinct transcriptomic patterns, underscoring the value of newly hatched larvae RNA profiles for identifying developmental anomalies early. Therefore, together, these results demonstrate that the transcriptome at hatching serves as a window into the parental legacy and the future developmental trajectories, while offering a detailed view of the biological processes underpinning larval survival.

The integration of transcriptomic and zootechnical data at this scale and across multiple biological contexts is, to our knowledge, unprecedented in finfish, and it proved essential for revealing both the conserved molecular framework of larval development and the context-specific pathways shaped by parental background or egg quality. Looking ahead, we recommend expanding this integrated approach by including additional molecular layers (proteomics, metabolomics, epigenomics) or tracking single-larva transcriptomic profiling. Such advances will

uncover additional molecular mechanisms, strengthen predictive capacity, and enhance both fundamental developmental biology and the practical optimisation of selective breeding and larval rearing in aquaculture. In the words of T.S. Eliot, "*What we call the beginning is often the end. And to make an end is to make a beginning".* The molecular legacy present at hatching both encapsulates the journey so far and sets the stage for what is to come.

Chapter 9: Implications and Recommendations

While this study provides novel evidences for the value of the larvae transcriptome profiles, several limitations identified should be acknowledged. First, the work examined by high-throughput transcriptomic profiling only a single developmental time point, capturing the molecular state before major environmental influences but not the dynamic transcriptomic changes that occur during later ontogeny. Future studies should therefore include multiple larval stages to track how transcriptomic profiles evolve, to characterise the kinetics of gene expression changes, and to map the gene networks that drive key developmental stages. Such temporal resolution would not only improve predictive accuracy but also provide insights into how early molecular programs shape later phenotypes. In addition, the analyses focused solely on transcriptomics, and integrating proteomics, metabolomics, and epigenetic profiling would offer a more complete understanding of the mechanisms linking early molecular states to performance outcomes. Finally, RNA-seq was conducted on pooled larval samples, which reveals average expression trends but may mask the considerable variability observed both between and within families, which were manifested at the zootechnical level, moving toward single-larva transcriptomic and zootechnical analyses would allow direct associations between individual molecular profiles and phenotypic traits, improve prediction of developmental trajectories, and potentially identify markers linked to robustness, growth, deformity risk and more. Linking these larval profiles back to the molecular composition of the gametes could clarify how parental provisioning shapes offspring outcomes. Extending such analyses to other species would help determine whether these mechanisms are consistent across aquaculture-relevant species as well as in non-cultured species. Addressing these limitations by appropriately planned and executed experiments will further expand the knowledge generated within this thesis. Moreover, future studies addressing the identified limitations may even further facilitating adoption of the findings of this thesis into a robust tool for larval quality assessment in aquaculture. Consequently, the findings of this thesis enabled to bring a novel knowledge, but also constitutes an important inspiration for a plethora of different scientific fields – from developmental biologists investigating early-life molecular mechanisms, to aquaculture-oriented practitioners willing to expand production based on knowledge and modern analytical tools.

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Chapter 14: Literature

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Chapter 15: Supplementary data

Supplementary files related to Chapter 3: "Does transcriptome of freshly hatched fish larvae describe past or predict future developmental trajectory?"

Supplementary file S3.1: Broodstock characteristics

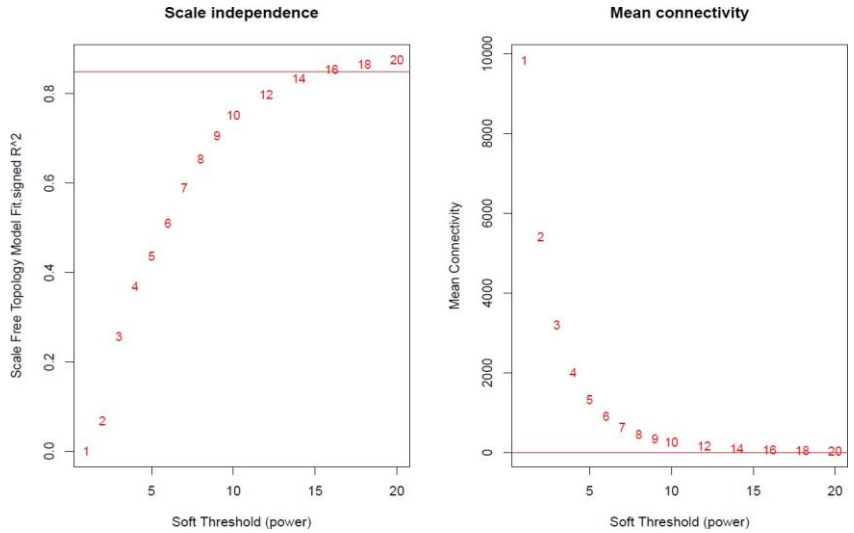
FAMILIES	BROODSTOCK	ORIGIN	WEIGHT (g)	TOTAL LENGTH (cm)	FORK LENGTH (cm)	AGE
F1	FEMALE 1	Żurawia	343	28.2	24.8	6
	MALE 1	Żurawia(Rytwiany Pond System)	203	25.7	21.3	4
F2	FEMALE 2	Szymon lake	571	32	27.5	7
	MALE 2	Żurawia(Rytwiany Pond System)	359	29.5	26	4
F3	FEMALE 3	Żurawia	236	24.6	21.8	5
	MALE 3	Żurawia(Rytwiany Pond System)	151	23.2	20	4
F4	FEMALE 4	Żurawia	299	26.8	23.8	5
	MALE 4	Żurawia(Rytwiany Pond System)	242	25.9	22.8	5
F5	FEMALE 5	Żurawia	302	25.8	24	6
	MALE 5	Żurawia(Rytwiany Pond System)	152	23.5	20.5	4+
F6	FEMALE 6	Żurawia	297	25	24	6
	MALE 6	Żurawia(Rytwiany Pond System)	218	25.9	23	5+
F7	FEMALE 7	Umląg lake	216	23.5	21.5	5
	MALE 7	Żurawia(Rytwiany Pond System)	154	23.5	20.3	?
F8	FEMALE 8	Żurawia	285	26.5	23	5
	MALE 8	Żurawia(Rytwiany Pond System)	196	24.6	21.5	5+
F9	FEMALE 9	Szymon lake	608	35.5	32	7
	MALE 9	Żurawia(Rytwiany Pond System)	196	25.5	22.5	?
F10	FEMALE 10	Szymon lake	763	35	31.4	8
	MALE 10	Żurawia(Rytwiany Pond System)	160	24.1	20.8	?
F11	FEMALE 11	Umląg lake	336	28	24.5	6
	MALE 11	Żurawia(Rytwiany Pond System)	137	22	19.2	?
F12	FEMALE 12	Umląg lake	810	36.5	33	7
	MALE 12	Żurawia(Rytwiany Pond System)	105	20.7	17.5	?
F13	FEMALE 13	Szymon lake	611	35.3	30.8	7
	MALE 13	Żurawia(Rytwiany Pond System)	142	22.5	19.8	?
F14	FEMALE 14	Szymon lake	725	37.2	32	7
	MALE 14	Iława	246	26.1	23	4
	FEMALE 15	Szymon lake	720	37.3	32	5

F15	MALE 15	Iława	223	25.8	22.2	4
F16	FEMALE 16	Szymon lake	695	34.3	30.5	6
	MALE 16	Żurawia(Rytwiany Pond System)	133	22.2	18.5	3+

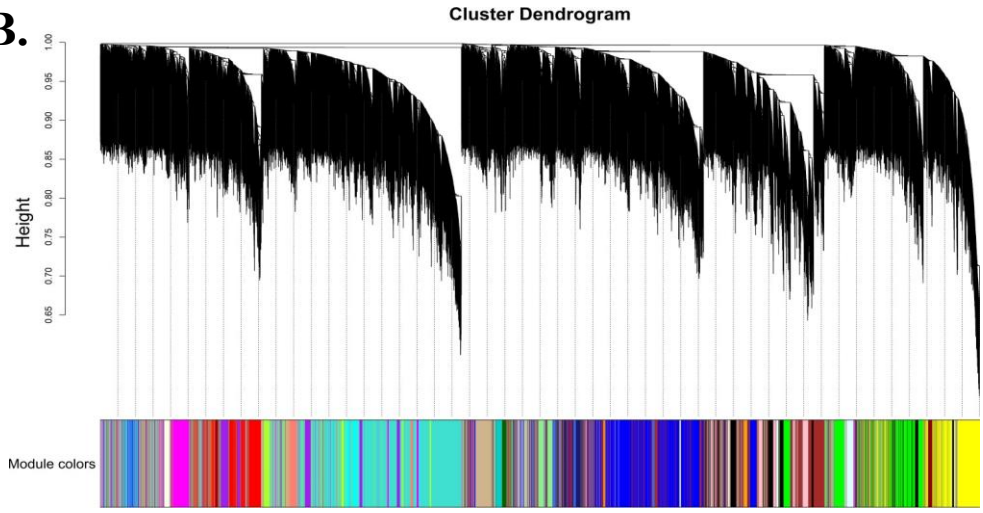
Supplementary file S3.2: Sperm evaluation with CASA before and after cryopreservation. ALH: Amplitude of Lateral Head Displacement; LIN: Linearity; VAP: Average Path Velocity; VCL: Curvilinear Velocity; VSL: Straight Line Velocity; MOT: percentage of sperm motility.

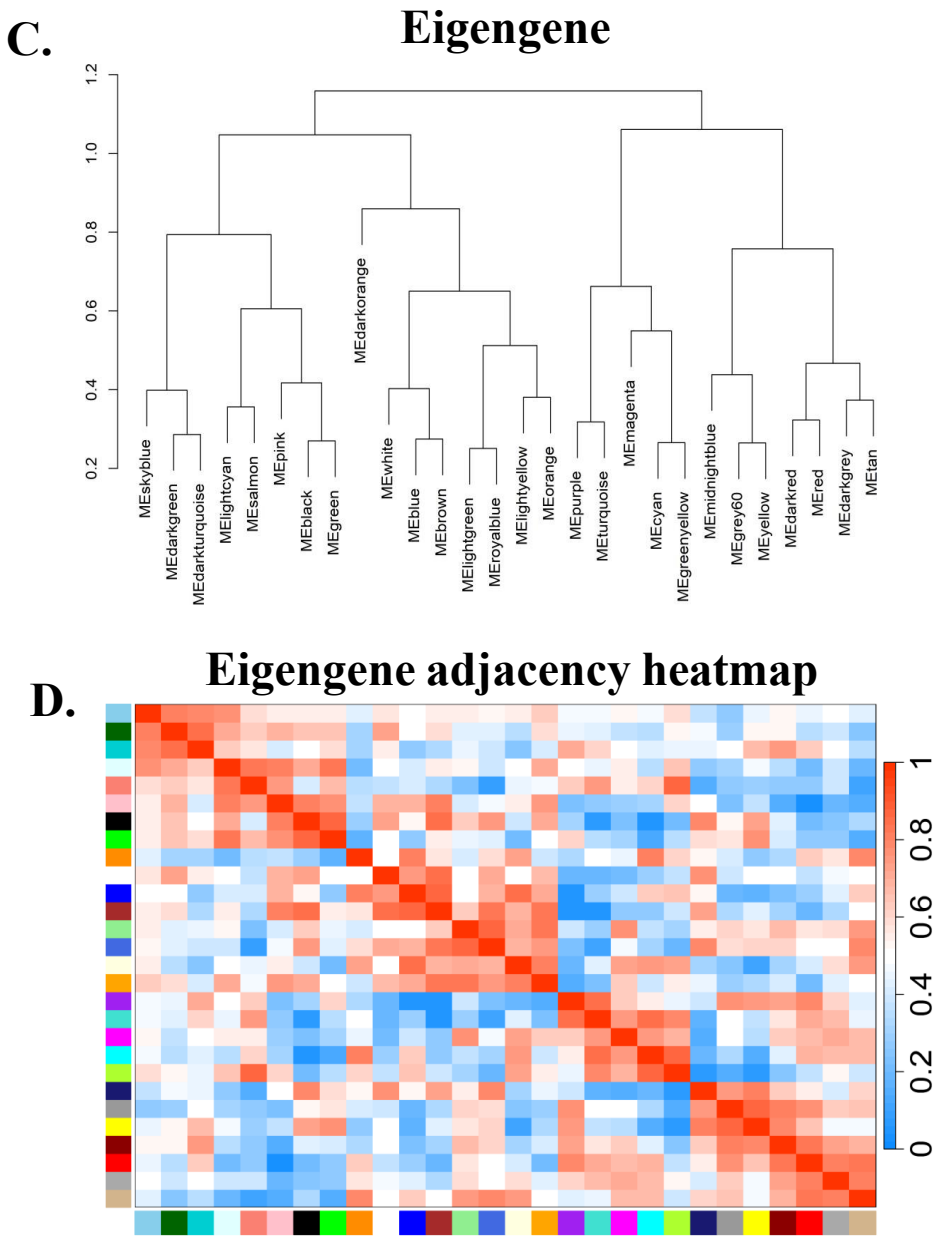
Males	ALH (µm)	LIN (%)	VAP (µm s^{-1})	VCL (µm s^{-1})	VSL (µm s^{-1})	MOT (%)
1	7.556148	72.14422	184.0915	205.7676	163.0863	75.3
2	7.933224	81.7436	227.7508	243.9181	201.3321	87.6
3	8.887574	81.3074	228.7303	245.4705	202.2623	80.4
4	10.35598	75.90818	214.9773	236.3426	182.0324	90
5	7.100678	74.39593	175.9103	198.9887	153.0129	82.7
6	6.693855	79.79078	183.4773	200.7846	164.2914	84.2
7	7.257437	80.20573	197.5499	211.8014	173.8595	80.2
8	8.525166	74.15175	191.4987	214.5812	162.5481	90.5
9	7.811111	79.93286	199.2677	216.7182	176.257	86.9
10	7.653057	75.85913	183.2043	205.2517	157.7023	89.3
11	6.946595	78.89905	190.2624	208.2974	167.1579	86.1
12	6.497107	82.37009	194.6677	209.386	176.4434	80.9
13	8.169308	77.52857	194.3661	214.7507	168.3114	89.7
14	7.514402	77.69066	189.9809	210.0948	166.0552	90.3
15	7.065888	77.29461	182.6261	203.5702	158.9418	87.9
16	8.346438	78.78947	200.7829	219.6534	174.7468	85.7
1 cryo	5.183588	67.98289	117.5497	145.9357	104.5426	61.3
2 cryo	4.540228	70.2931	120.6054	147.0256	112.78	50.8
3 cryo	5.096318	68.08394	120.8708	151.0514	110.6403	58.6
4 cryo	4.124404	64.94624	95.60346	126.8639	86.79023	63.2
5 cryo	5.029983	65.09456	118.4535	147.959	104.9009	59.8
6 cryo	4.504754	63.51034	103.8411	134.6898	94.96765	55
7 cryo	4.495647	61.96903	100.6436	128.4414	92.18875	52.1
8 cryo	4.389957	64.37805	104.8808	134.6037	96.41796	56.3
9 cryo	4.449642	62.10599	98.48766	130.6613	89.44179	59.6
10 cryo	4.273771	72.05066	119.7826	145.1478	111.0787	59.8
11 cryo	4.425791	59.93989	91.90695	127.2347	83.11011	59.6
12 cryo	4.201456	73.01287	123.2177	146.9202	114.8439	57.4
13 cryo	4.267513	63.86021	101.0722	132.3957	92.65259	54.2
14 cryo	4.409236	65.01599	108.3475	132.5867	98.41082	75
15 cryo	4.547335	69.00627	113.5295	137.3622	102.7892	74.4
16 cryo	4.926946	75.25546	144.1598	166.9635	130.6906	73.2

A.


Scale independence
Scale Free Topology Model Fit,signed R^2
Soft Threshold (power)
Mean connectivity
Mean Connectivity
Soft Threshold (power)

B.


Cluster Dendrogram
Height
Module colors



Supplementary file S3.3: **Construction of the gene co-expression network analysis (WGCNA). A.** Determination of soft-threshold power in the WGCNA. Analysis of the scale-free index for various soft-threshold powers (β) and analysis of the mean connectivity for various soft-threshold powers. **B.** Gene cluster dendrogram based on topological overlap. The colors show the module assignment determined by the Tree cut. Each colored line represents a color-coded module containing a set of highly connected genes. **C.** An eigengene dendrogram to identify groups of correlated modules. **D.** Eigengene adjacency heatmap of different gene co-expression modules. Red represents high adjacency and blue represents low adjacency.

Supplementary file S3.4: Genes with high correlations (both positive and negative) to specific key aquaculture traits. **A**. Genes related to cannibalism. **B**. Genes highly correlated to mortality **C**. Genes most correlated to SBIE. **D**. Genes highly correlated to SGR for length **E**. Genes highly correlated to SGR for weight. **F**. Genes highly correlated to weight of larvae at the end of the experiment **G.** Genes highly correlated to Fulton's condition factor. GS: Gene Significance.

A.

GENE_ID	TRANSCRIPT_ACCESSION_NUMBER	HUMAN ORTHOLOGS	GS	*p-value*
selenoo2	XM_039791163.1	*SELENOO*	0.823647	8.80E-05
pex2	XM_039790640.1	*PEX2*	0.759129	0.000649
LOC120559301	XM_039800928.1	*TMEM198*	0.748497	0.000851
LOC120558849	XM_039800144.1	*NA*	0.744127	0.000948
LOC120560490	XM_039803062.1	*ABCB9*	0.722085	0.001585
LOC120543986	XM_039777447.1	*AR*	0.711043	0.002015
nudt12	XM_039803805.1	*NUDT12*	-0.84789	3.35E-05
LOC120573883	XM_039823776.1	*NA*	-0.83261	6.27E-05
LOC120547285	XM_039782855.1	*MTUS1*	-0.78394	0.000326
LOC120556999	XM_039796989.1	*TASOR*	-0.78027	0.000363
mmp25b	XM_039788073.1	*MMP17*	-0.77709	0.000397
c8h15orf40	XM_039807462.1	*C15orf40*	-0.76682	0.000529
pds5b	XM_039776878.1	*PDS5B*	-0.76502	0.000555
rhbdl1	XM_039787716.1	*RHBDL1*	-0.76073	0.000622
sh2d3cb	XM_039802731.1	*SH2D3C*	-0.75116	0.000796
cmah	XM_039791006.1	*CMAHP*	-0.74459	0.000937
LOC120549684	XM_039786766.1	*NA*	-0.73278	0.001243
LOC120568916	XM_039816668.1	*MARCHF2*	-0.72786	0.001391
LOC120571615	XR_005641248.1	*NA*	-0.72719	0.001413
lactbl1b	XM_039798927.1	*LACTBL1*	-0.72094	0.001626
nid2a	XM_039793428.1	*NID2*	-0.7084	0.002131
rxfp3.2b	XM_039810374.1	*RXFP3*	-0.70289	0.002389

B.

GENE_ID	TRANSCRIPT_ACCESSION_NUMBER	HUMAN ORTHOLOGS	GS	*p-value*
mkxa	XM_039789540.1	*MKX*	0.773396	0.000441
LOC120557731	XM_039798307.1	*TCP11L1*	0.771872	0.00046
LOC120575215	XM_039825900.1	*SOX8*	0.757658	0.000674
LOC120554598	XM_039793599.1	*IL1RAPL1*	0.754489	0.000732
rtn4rl2a	XM_039816981.1	*RTN4RL2*	0.753633	0.000748
LOC120556829	XM_039796566.1	*CPNE1*	0.739755	0.001054
klf5a	XM_039782934.1	*KLF5*	0.739543	0.001059
fynb	XM_039782603.1	*FYN*	0.738856	0.001077
LOC120571709	XM_039820779.1	*CSPG5*	0.725929	0.001454
si:ch1073- 145m9.1	XM_039821276.1	*NA*	0.725098	0.001481
LOC120574026	XM_039824018.1	*PDZK1*	0.723493	0.001536

LOC120557984	XM_039798751.1	*BHLHE40*	0.719774	0.001668
morn4	XM_039784652.1	*MORN4*	0.719557	0.001676
rcbtb1	XM_039793148.1	*RCBTB1*	0.718855	0.001702
pdcd4a	XM_039797508.1	*PDCD4*	0.718174	0.001728
lnx2a	XM_039790511.1	*LNX2*	0.713051	0.00193
bmpr1ba	XM_039804081.1	*BMPR1B*	0.711197	0.002008
LOC120551619	XM_039789114.1	*KCNH7*	0.710976	0.002018
si:ch211- 215i13.3	XM_039819213.1	*BAALC*	0.706731	0.002206
rapgef2	XM_039813634.1	*RAPGEF2*	0.705271	0.002275
zgc:122979	XM_039778904.1	*DNAJB5*	0.704972	0.002289
LOC120570925	XM_039819623.1	*HLF*	0.703696	0.00235
fam43b	XM_039799236.1	*FAM43B*	0.70188	0.00244
LOC120575713	XM_039826547.1	*MLXIPL*	0.700779	0.002495
LOC120544023	XM_039777522.1	*CRCP*	-0.80575	0.000165
cnpy2	XM_039798701.1	*CNPY2*	-0.77553	0.000415
si:ch211-217a12.1	XM_039790223.1	*GPT2*	-0.76661	0.000532
adck2	XM_039808041.1	*ADCK2*	-0.75944	0.000644
LOC120567770	XM_039814785.1	*GTF3A*	-0.7538	0.000745
polr1f	XM_039821120.1	*POLR1F*	-0.7476	0.00087
cxcr3.2	XM_039805568.1	*CXCR2*	-0.74109	0.001021
LOC120563727	XM_039808101.1	*ERGIC2*	-0.73826	0.001092
enoph1	XM_039780775.1	*ENOPH1*	-0.73681	0.00113
gmpr2	XM_039821768.1	*GMPR2*	-0.72868	0.001366
ddx52	XM_039778580.1	*DDX52*	-0.72852	0.001371
LOC120555473	XM_039794172.1	*PLEKHF2*	-0.72695	0.001421
LOC120558123	XM_039799029.1	*DTNBP1*	-0.72347	0.001537
dnajc30b	XM_039786891.1	*DNAJC30*	-0.7209	0.001627
uts2d	XM_039812033.1	*XOXO*	-0.72076	0.001632
LOC120546050	XM_039780806.1	*STOML2*	-0.71954	0.001677
si:ch211-214j24.10	XM_039792521.1	*NA*	-0.71752	0.001753
pus3	XM_039788994.1	*PUS3*	-0.71697	0.001773
tomm20a	XM_039784734.1	*TOMM20*	-0.71547	0.001832
mpp6b	XM_039820817.1	*PALS2*	-0.71288	0.001937
LOC120545887	XM_039780516.1	*PBDC1*	-0.71155	0.001993
golt1bb	XM_039807427.1	*GOLT1B*	-0.71108	0.002013
lactb	XM_039808237.1	*LACTB*	-0.71033	0.002045
LOC120561432	XM_039804553.1	*TTF1*	-0.70951	0.002081
cacng5b	XM_039823832.1	*CACNG5*	-0.70509	0.002283
LOC120573711	XM_039823626.1	*CAAP1*	-0.70418	0.002327

C.

GENE_ID	TRANSCRIPT_ACCESSION NUMBER	HUMAN ORTHOLOGS	GS	*p-value*
si:dkey-117m1.4	XM_039803324.1	*NA*	0.791894	0.000256
arsg	XM_039788329.1	*ARSG*	0.769645	0.000489
kdrl	XM_039813298.1	*FLT1*	0.738975	0.001074
LOC120561638	XM_039804800.1	*NA*	0.737386	0.001115
malsu1	XM_039807250.1	*MALSU1*	0.725822	0.001457
npdc1b	XM_039802865.1	*NPDC1*	0.70592	0.002244
barx2	XM_039821365.1	*BARX2*	0.701486	0.002459
LOC120575397	XM_039826188.1	*PYCARD*	-0.77819	0.000385
aggf1	XM_039797527.1	*AGGF1*	-0.76316	0.000583
LOC120567195	XM_039814111.1	*PCDHGC5*	-0.75051	0.000809
cubn	XM_039789731.1	*CUBN*	-0.73351	0.001222
lrrk1	XM_039794699.1	*LRRK1*	-0.73125	0.001287
tbc1d30	XM_039792075.1	*TBC1D30*	-0.72395	0.00152
st7l	XM_039798844.1	*ST7*	-0.71834	0.001721
tmem216	XM_039813676.1	*TMEM216*	-0.71455	0.001869
LOC120556874	XM_039796687.1	*NA*	-0.70092	0.002488

D.

GENE_ID	TRANSCRIPT_ACCESSION_NUMBER	HUMAN ORTHOLOGS	GS	*p-value*
atp8a2	XM_039789439.1	*ATP8A2*	0.873817	9.72E-06
gpr179	XM_039803243.1	*GPR158*	0.764197	0.000567
LOC120571047	XM_039819747.1	*ZNF184*	0.758972	0.000651
polr2b	XM_039813488.1	*POLR2B*	0.753756	0.000745
dscama	XM_039777637.1	*DSCAM*	0.753162	0.000757
tekt1	XM_039820506.1	*TEKT1*	0.747324	0.000876
adamtsl7	XM_039801823.1	*THSD4*	0.742693	0.000982
gpr176	XM_039782698.1	*GPR176*	0.730583	0.001307
chrnb1	XM_039823570.1	*CHRNB1*	0.721703	0.001598
plxnc1	XM_039792449.1	*PLXNC1*	0.70922	0.002094
LOC120550477	XM_039787166.1	*A2ML1*	0.707241	0.002183
slc15a1a	XM_039817133.1	*SLC15A1*	-0.8001	0.000198
cln3	XM_039825647.1	*CLN3*	-0.79474	0.000235
LOC120547353	XR_005637056.1	*NA*	-0.79011	0.000271
faxca	XM_039782711.1	*FAXC*	-0.76895	0.000499
pnp5a	XM_039823439.1	*PNP*	-0.76177	0.000605
cuedc2	XM_039783959.1	*CUEDC2*	-0.74137	0.001014
LOC120549428	XM_039786345.1	*THBS1*	-0.7346	0.001191
LOC120567821	XM_039814879.1	*MYOC*	-0.73376	0.001215
loxl3b	XM_039785199.1	*LOXL3*	-0.72527	0.001476
LOC120559460	XM_039801160.1	*NKX2-3*	-0.72465	0.001496

ccbe1	XM_039780041.1	*CCBE1*	-0.72083	0.001629
LOC120565036	XM_039810342.1	*ALPI*	-0.71272	0.001944
fam221a	XM_039819369.1	*FAM221A*	-0.71005	0.002058
elna	XM_039824656.1	*NA*	-0.70996	0.002062
LOC120554227	XM_039792984.1	*ZNF84*	-0.70834	0.002133
ythdc1	XM_039779087.1	*YTHDC1*	-0.70611	0.002235
LOC120559933	XR_005639388.1	*XOXO*	-0.70561	0.002259
LOC120570652	XM_039819131.1	*PER2*	-0.70494	0.00229
si:ch211- 176g6.2	XM_039789392.1	*RADIL*	-0.70441	0.002316
LOC120549424	XM_039786332.1	*CHAC1*	-0.70326	0.002371
csnk2a2b	XM_039809336.1	*CSNK2A2*	-0.70322	0.002373
gareml	XM_039782726.1	*GAREM2*	-0.70121	0.002473

E.

GENE_ID	**TRANSCRIPT_ACCESSION_NUMBER**	**HUMAN ORTHOLOGS**	**GS**	***p-value***
prdm1b	XM_039821118.1	*PRDM1*	0.83547	5.60E-05
agpat4	XM_039783933.1	*AGPAT4*	0.780512	0.00036
apoba	XM_039785565.1	*APOB*	0.778804	0.000378
tgfbr3	XM_039810773.1	*TGFBR3*	0.766218	0.000537
LOC120546953	XM_039782234.1	*EVA1C*	0.756307	0.000698
ildr2	XM_039818909.1	*ILDR2*	0.750336	0.000813
dtx4a	XM_039823160.1	*DTX4*	0.739289	0.001066
c20h14orf180	XM_039785648.1	*CNST*	0.731303	0.001286
zgc:171971	XM_039788201.1	*POLR3D*	0.730263	0.001317
LOC120568699	XM_039816370.1	*SLC25A24*	0.729841	0.00133
neurl1aa	XM_039776668.1	*NEURL1*	0.722336	0.001576
arrb1	XM_039777112.1	*ARRB1*	0.717657	0.001747
LOC120564302	XM_039809154.1	*H1-4*	0.716955	0.001774
LOC120545456	XM_039779784.1	*CSPG4*	0.713425	0.001915
LOC120559901	XM_039801983.1	*TRIM7*	0.712669	0.001946
slc4a5b	XM_039779149.1	*SLC4A5*	0.711218	0.002007
LOC120555597	XM_039794414.1	*B3GALT2*	0.706184	0.002232
LOC120564168	XM_039808937.1	*ARL2BP*	0.704097	0.002331
tfap2a	XM_039790007.1	*TFAP2A*	0.703349	0.002367
txnl4a	XM_039821238.1	*TXNL4A*	-0.84056	4.56E-05
muc13b	XM_039789342.1	*MUC13B*	-0.80523	0.000167
mid1ip1l	XM_039813944.1	*MID1IP1*	-0.80393	0.000175
c21h10orf88	XM_039789081.1	*PAAT*	-0.75839	0.000661
si:ch211- 59o9.10	XM_039807797.1	*RNF38*	-0.75607	0.000703
nkapd1	XM_039782424.1	*NKAPD1*	-0.74988	0.000822
LOC120547184	XM_039782660.1	*KCNK2*	-0.74846	0.000852
zanl	XM_039823009.1	*ZAN*	-0.74358	0.000961
si:dkey- 57a22.11	XM_039818970.1	*SGO1*	-0.74333	0.000967
LOC120557765	XM_039798369.1	*NAT8*	-0.73551	0.001166

LOC120544654	XM_039778556.1	*RRM1*	-0.73228	0.001257
fabp2	XM_039806486.1	*FABP2*	-0.72505	0.001483
chtf8	XM_039808917.1	*CHTF8*	-0.72309	0.00155
LOC120556667	XM_039796312.1	*CHIA*	-0.72078	0.001631
LOC120544521	XM_039778329.1	*CIPC*	-0.71893	0.001699
cdc14aa	XM_039816763.1	*CDC14A*	-0.71262	0.001948
LOC120562327	XM_039806016.1	*ZNF154*	-0.70764	0.002165
cryz	XM_039815914.1	*CRYZ*	-0.70485	0.002295
mrps6	XM_039784102.1	*MRPS6*	-0.70438	0.002317
tango2	XM_039804096.1	*TANGO2*	-0.70236	0.002415
cald1b	XM_039808207.1	*CALD1*	-0.70128	0.00247

F.

GENE_ID	**TRANSCRIPT_ACCESSION_NUMBER**	**HUMAN ORTHOLOGS**	**GS**	***p-value***
LOC120552004	XM_039789614.1	*AOC1*	0.811459	0.000136
gpr176	XM_039782698.1	*GPR176*	0.778565	0.000381
pou2f3	XM_039826492.1	*POU2F3*	0.768447	0.000506
hck	XM_039800624.1	*HCK*	0.764008	0.00057
LOC120555597	XM_039794414.1	*B3GALT2*	0.75433	0.000735
LOC120550477	XM_039787166.1	*A2ML1*	0.747012	0.000883
LOC120553155	XM_039791254.1	*PAH*	0.743519	0.000962
ddc	XM_039806765.1	*DDC*	0.737704	0.001107
ccser1	XM_039802935.1	*CCSER1*	0.72681	0.001425
b3gat2	XM_039784296.1	*B3GAT2*	0.71187	0.00198
LOC120564637	XM_039809793.1	*KBTBD13*	0.70476	0.002299
fam167ab	XM_039781499.1	*FAM167A*	0.704741	0.0023
rhot1b	XM_039805836.1	*RHOT1*	0.70351	0.002359
pde6a	XM_039813735.1	*PDE6A*	0.701177	0.002475
tmem41aa	XM_039817756.1	*TMEM41A*	0.700037	0.002533
LOC120544521	XM_039778329.1	*CIPC*	-0.82163	9.48E-05
slc6a3	XM_039807297.1	*SLC6A3*	-0.78493	0.000316
arl16	XM_039823881.1	*ARL16*	-0.78248	0.00034
LOC120545153	XM_039779225.1	*MEF2C*	-0.759	0.000651
adamtsl5	XM_039794130.1	*ADAMTSL5*	-0.75741	0.000679
LOC120544064	XM_039777595.1	*TMEM164*	-0.75202	0.000779
pip4p2	XM_039821087.1	*PIP4P2*	-0.74411	0.000948
areg	XM_039802087.1	*AREG*	-0.74311	0.000972
klhl17	XM_039801927.1	*KLHL17*	-0.74149	0.001011
fam120b	XM_039795670.1	*FAM120B*	-0.73926	0.001067
dram2b	XM_039798976.1	*DRAM2*	-0.72332	0.001542
LOC120560625	XM_039803303.1	*CEP120*	-0.72328	0.001543
LOC120559933	XR_005639388.1	*NA*	-0.71929	0.001686
LOC120547353	XR_005637056.1	*NA*	-0.717	0.001772
fam126a	XM_039820770.1	*HYCC1*	-0.71461	0.001867
LOC120550569	XM_039787169.1	*TRIM16*	-0.71175	0.001985

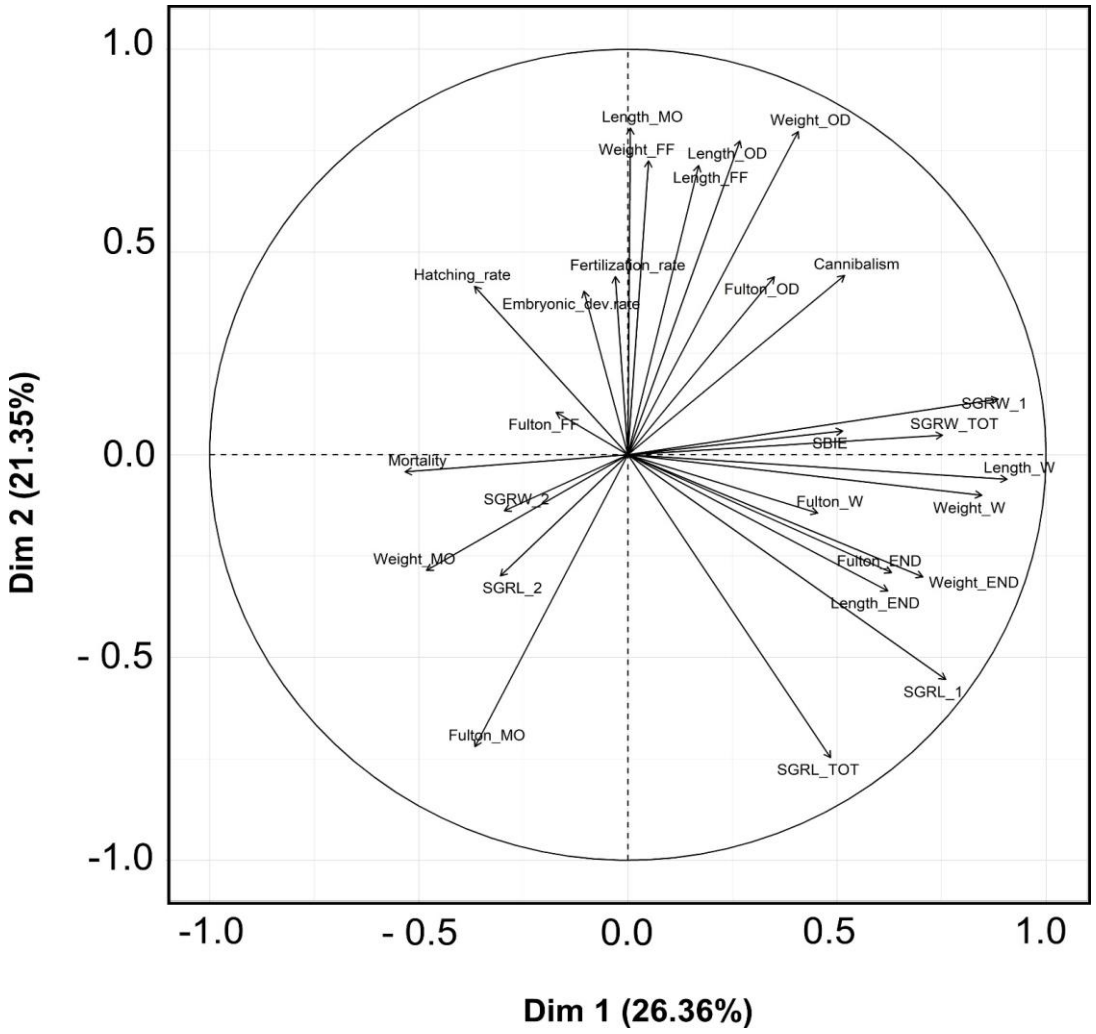
map3k14a	XM_039824035.1	*MAP3K14*	-0.71164	0.001989
cbx8b	XM_039816174.1	*CBX8*	-0.70928	0.002092
trh	XM_039800554.1	*TRH*	-0.70785	0.002156

G.

GENE_ID	TRANSCRIPT_ACCESSION_NUMBER	HUMAN ORTHOLOGS	GS	*p-value*
LOC120560720	XM_039803505.1	*SEC14L2*	0.813423	0.000127
si:dkey-6n21.13	XM_039823385.1	*P2RY2*	0.786148	0.000305
gpr176	XM_039782698.1	*GPR176*	0.78102	0.000355
esr1	XM_039780941.1	*ESR1*	0.758584	0.000658
LOC120545314	XM_039779562.1	*NA*	0.748516	0.000851
c20h14orf180	XM_039785648.1	*CNST*	0.744554	0.000938
fbln1	XM_039808895.1	*FBLN1*	0.737168	0.001121
LOC120564637	XM_039809793.1	*KBTBD13*	0.729903	0.001328
zbtb44	XM_039781008.1	*ZBTB44*	0.723127	0.001548
LOC120547035	XM_039782385.1	*ESRRG*	0.722119	0.001584
LOC120572928	XM_039822453.1	*BTNL9*	0.715178	0.001844
irx6a	XM_039794924.1	*IRX6*	0.712514	0.001953
nrros	XM_039789546.1	*NRROS*	0.709855	0.002066
rnf6	XM_039789936.1	*RNF6*	0.70552	0.002263
tacr3a	XM_039805998.1	*TACR3*	0.702018	0.002433
mapk8b	XM_039787632.1	*MAPK8*	0.700128	0.002528
LOC120550569	XM_039787169.1	*TRIM16*	-0.8027	0.000182
p2ry2.1	XM_039822179.1	*P2RY2*	-0.76879	0.000501
LOC120559933	XR_005639388.1	*NA*	-0.76254	0.000593
LOC120567767	XM_039814778.1	*CCL25*	-0.75263	0.000767
nmur3	XM_039796436.1	*NMUR2*	-0.73464	0.00119
ogfod2	XM_039802729.1	*OGFOD2*	-0.72915	0.001351
anapc10	XM_039823076.1	*ANAPC10*	-0.72902	0.001355
LOC120563179	XM_039807335.1	*NPR1*	-0.72414	0.001514
prr15lb	XM_039789397.1	*PRR15L*	-0.71812	0.00173
scn1lab	XM_039793357.1	*SCN2A*	-0.71675	0.001782
LOC120569990	XM_039818213.1	*CYBRD1*	-0.70614	0.002234
LOC120569118	XM_039816997.1	*THAP2*	-0.70318	0.002375

Supplementary file S3.5: Primers used for validation and normalization of expression level of candidate genes

	Primers	
Gene symbol	**Forward**	**Reverse**
selenoo	CGTTGGGCTCTGAACTGGAT	TCCGGCTCTTCTTTTCCCAC
nudt12	TCCTGCTTGGTTTGCCATCA	GCCTCTTCCTCGTTGAGCTT
sec14l2	CTTGGGAGGAACGTGGAGTC	CAGGAAAGAGTTTGGGGGCT
trim16	AAAGCCTGTCCTCCCTCCTG	CTCTGCTCCATGTGTTGCCT
mkx	TACAAGCACCGAGACAACCC	TGCCTCACCGTGTTCTTCAG
crcp	TTCCTGACCACCATGATGCC	TGCTCCTCTGATAACCGCTC
atp8a2	GCCAGATCCTTTTTGAGCGC	AGCATTCTGGGTGATTCGGT
slc15a1	TGACAACGATCCTCTGGTGC	GTGTTTGTCGCCGTCCTTTG
prdm1	AAACAGCACCTACCTCAGCA	ATCTGGTCGTCGGGTGTGTC
txnl4a	GGTGGACATCACAGAAGTGC	TCTCCTGCTTGTCCTCCATT
aoc1	CCAAACCAACCCCAACATCAC	GCCTTGACTCAAAGCGGACA
cipc	TAAAGAGAGGACACAGCCGC	CCTTTCCCTCATGGCTCTCC
pycard	TCGCAGTCATTTTGGATGAGC	GCTTTCAGAGGCCCAGAGTAG
si:dkey-117m1.4	TCATCACAAACTGCGGGACA	GCGGGCTTCTCTTTAGGACA
Houskeeping genes		
txn2	CGCGAGGTCTCCTTTAACGT	ATCGCCAGGTCTGTGTGATC
acadl	ATCTTCAGGCAAAGCGTCCG	ATACATTTGCTCCTCCCACG
gsta.1	AGCAAAGGACCGCTACCTTC	CCTCCAACATCAGGGTGCAT
wdr83os	TGAAGTGGTGTGCCTGGATC	GCTGTGGGTTCTGGAGGTAC



Supplementary file S3.6: Principal component analysis (PCA) plot showing the variation among 16 Eurasian perch larvae groups in terms of zootechnical traits. Vectors indicate the direction and strength of each trait to the overall distribution.

Supplementary file S3.7: Supplementary figures

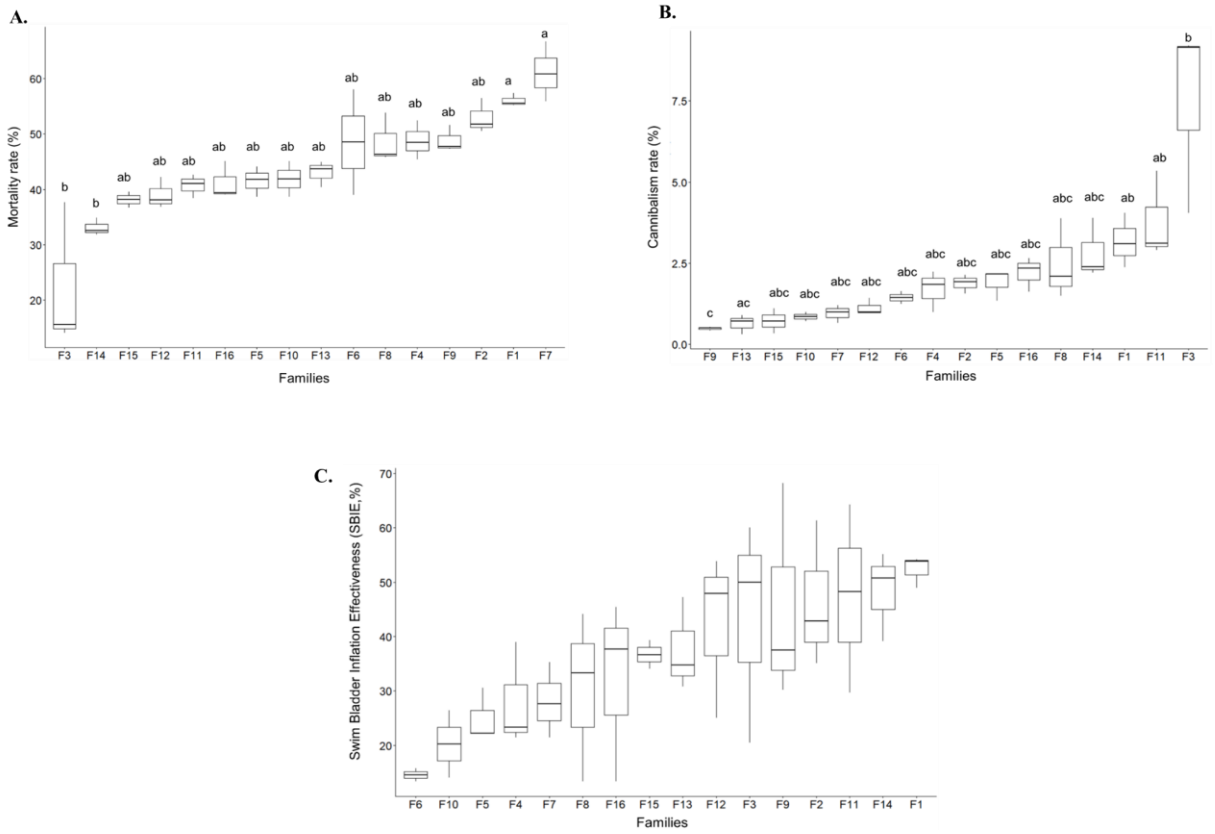


Figure S3.1: A: *Box plot diagrams of mortality rate.* ***B.*** *Box plot diagram of Cannibalism rate. C. Box plot diagram of swim bladder inflation effectiveness rate (SBIE). The graphs show statistical comparisons between the lengths of larvae from 16 E. perch families at different developmental stages. The experimental groups are arranged in an ascending order according to their mean values and the letters indicate significant differences ($p<0.05$) between the groups.*

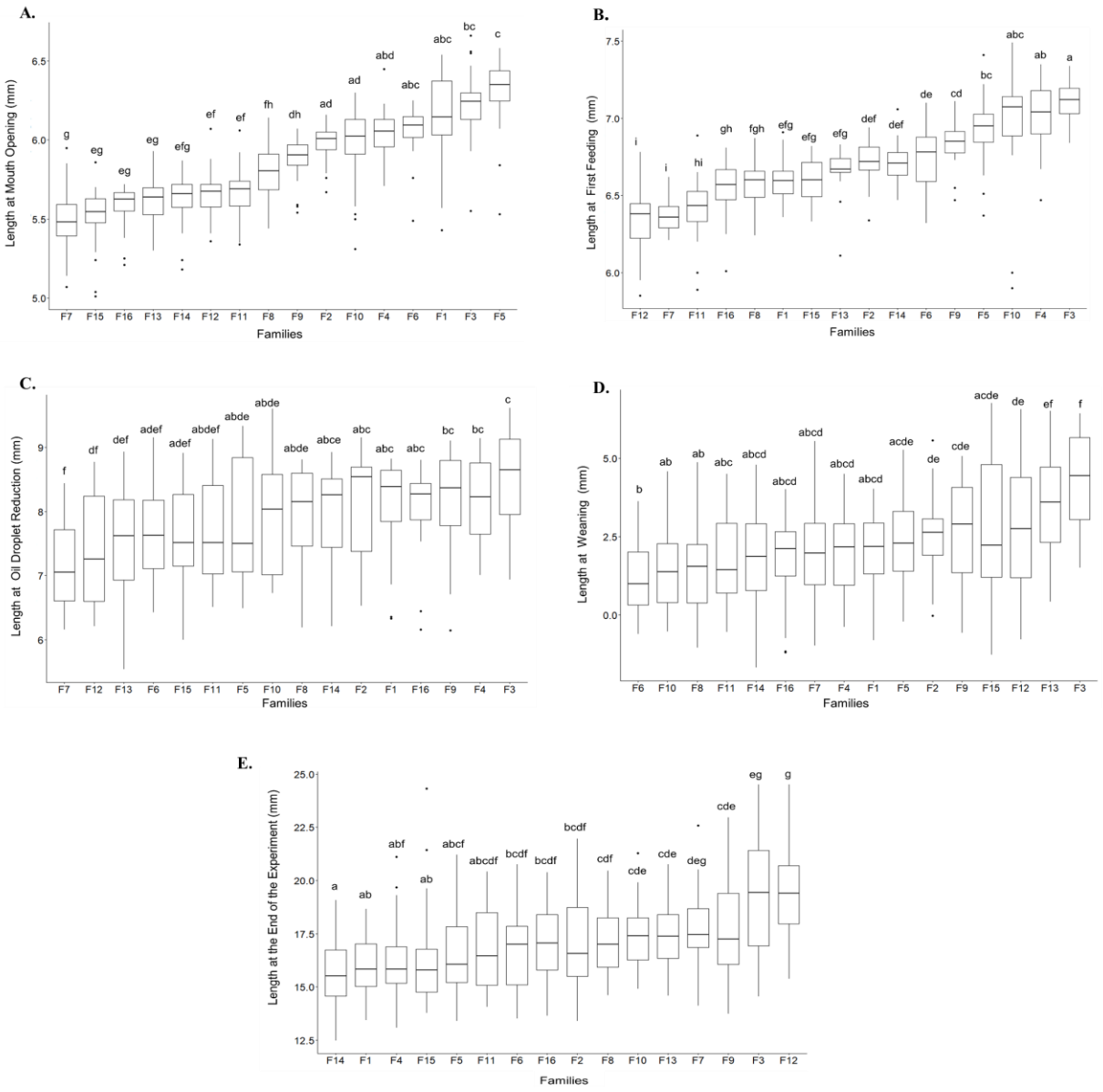


Figure S3.2*: Box plot diagrams of total length of fish larvae at* ***A.*** *mouth opening.* ***B.*** *first feeding stage.* ***C.*** *oil droplet reduction stage.* ***D.*** *weaning stage. E. the end of the experiment. The graphs show statistical comparisons between the lengths of larvae from 16 E. perch families at different developmental stages. The experimental groups are arranged in an ascending order according to their mean values and the letters indicate significant differences ($p<0.05$) between the groups.*

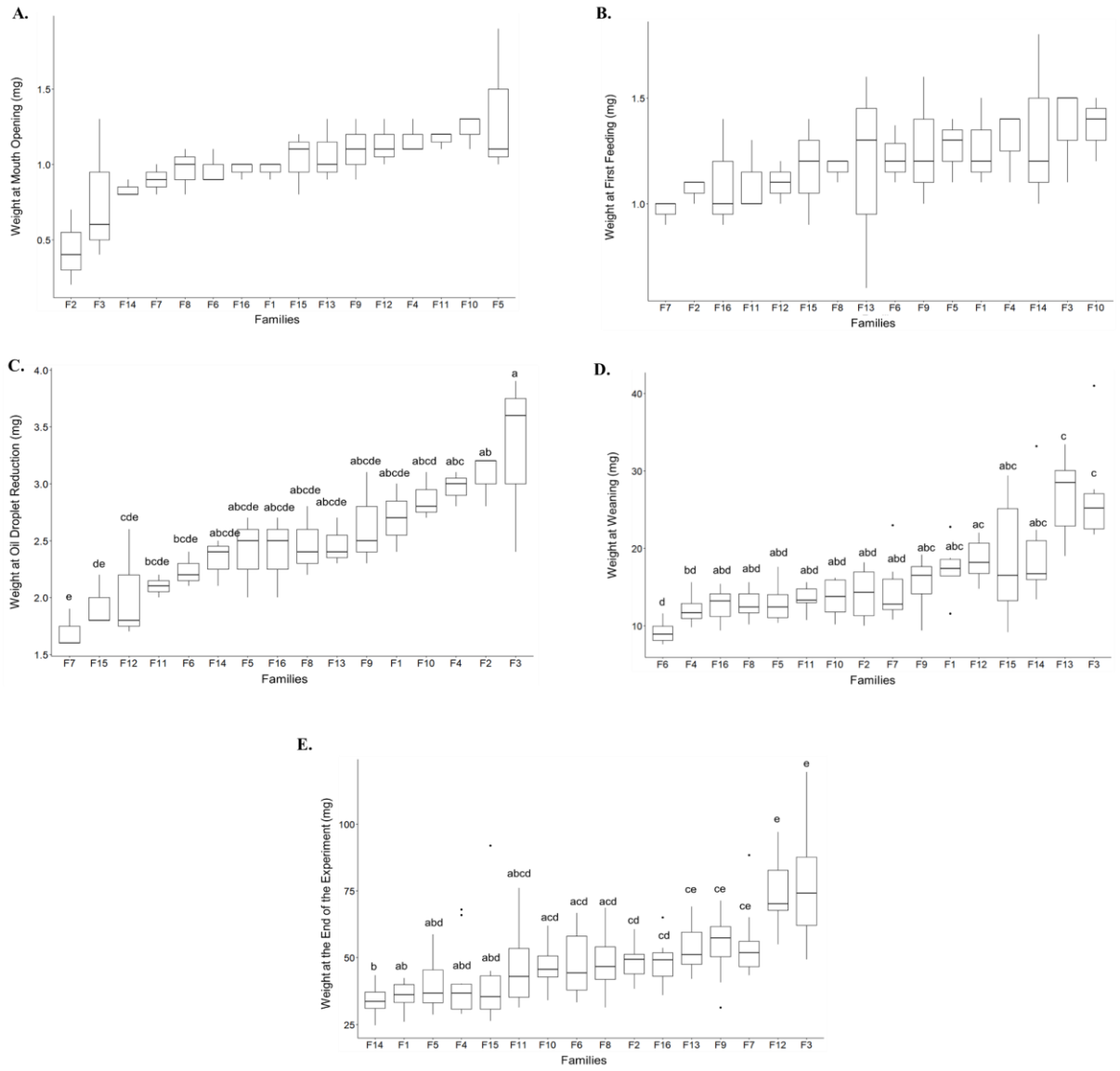


Figure S3.3: *Box plot diagrams of weight of fish larvae at* ***A.*** *mouth opening.* ***B.*** *first feeding stage.* ***C.*** *oil droplet reduction stage.* ***D.*** *weaning stage.* ***E.*** *the end of the experiment. The graphs show statistical comparisons between the weights of larvae from 16 E. perch families at different developmental stages. The experimental groups are arranged in an ascending order according to their mean values and the letters (when present) indicate significant differences ($p<0.05$) between the groups.*

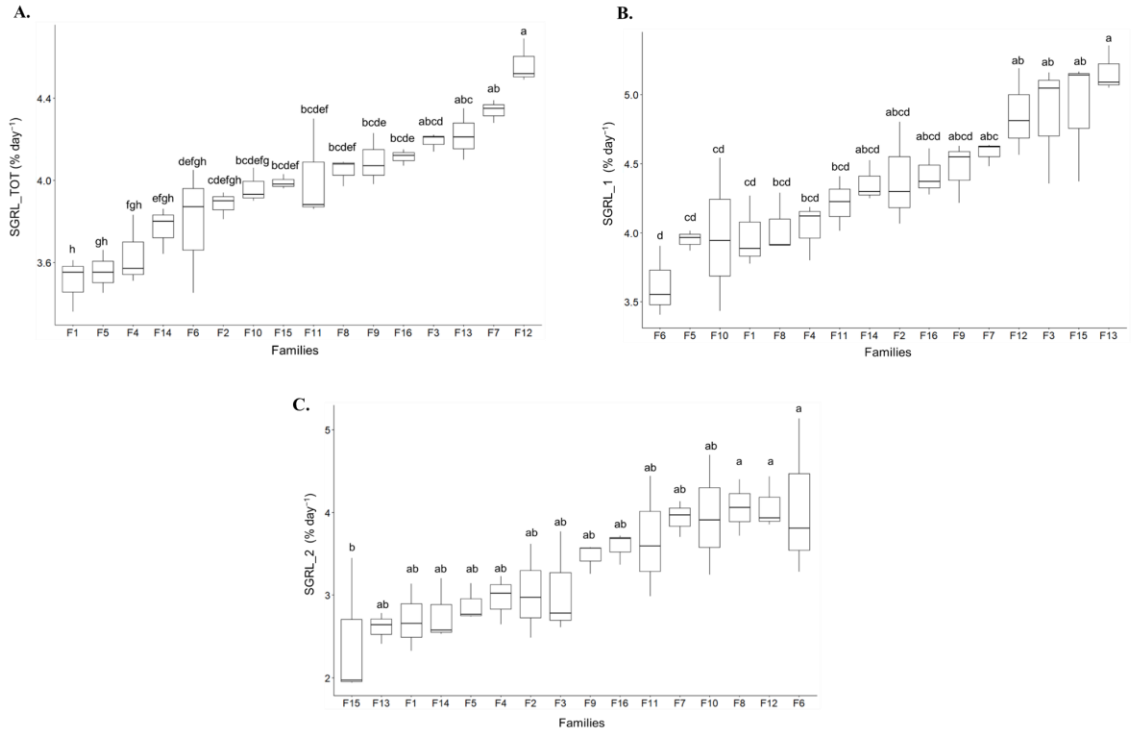


Figure S3.4: *Box plot diagram of **A.** Specific growth rate for length (SGRL_TOT) of larvae considering the entire rearing period (0 DPH-27DPH). **B**. Specific growth rate for length (SGRL_1) of larvae from hatching until the weaning stage (0 DPH-17DPH). **C.** Specific growth rate for length (SGRL_2) of larvae from weaning until the end of the experiment (17 DPH- 27 DPH). The graphs show statistical comparisons between the SGR of larvae from 16 E. perch families at different developmental stages. The experimental groups are arranged in an ascending order according to their mean values and the letters indicate significant differences (p<0.05) between the groups.*

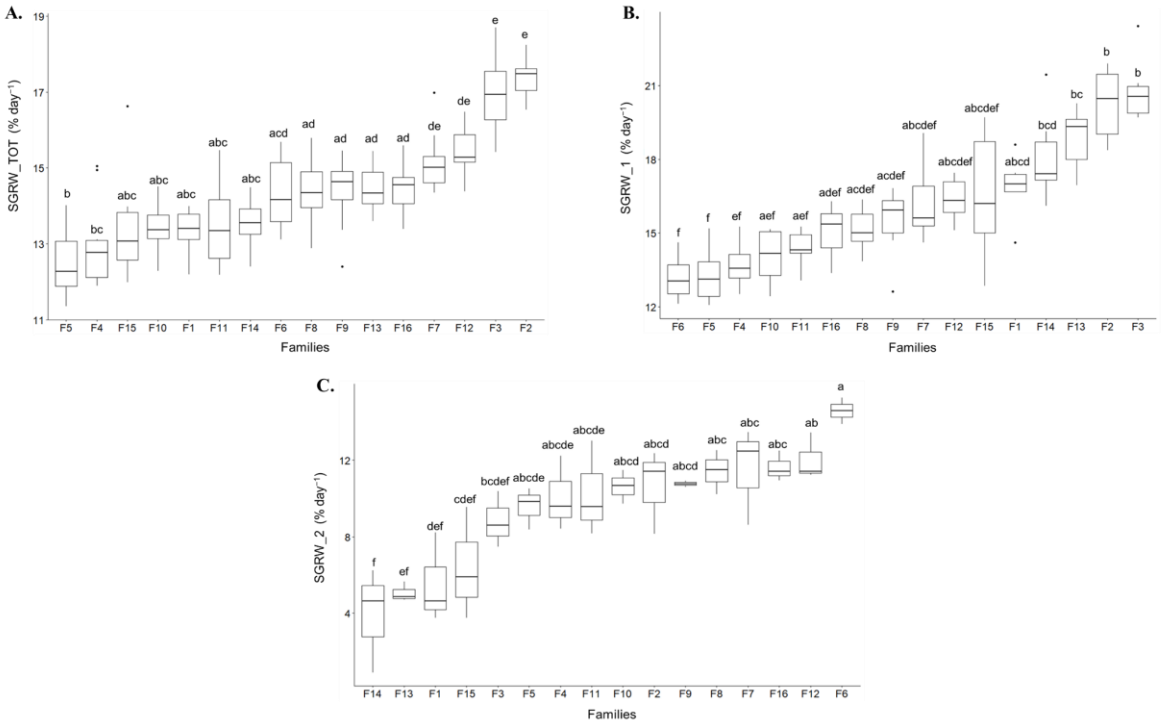


Figure S3.5: *Box plot diagram of **A.** Specific growth rate for weight (SGRW_TOT) of larvae considering the entire rearing period (0 DPH-27DPH). **B**. Specific growth rate for weight (SGRW_1) of larvae from hatching until the*

weaning stage (0 DPH-17DPH). ***C.*** *Specific growth rate for weight (SGRW_2) of larvae from weaning until the end of the experiment (17 DPH- 27 DPH). The graphs show statistical comparisons between the SGR of larvae from 16 E. perch families at different developmental stages. The experimental groups are arranged in an ascending order according to their mean values and the letters indicate significant differences ($p<0.05$) between the groups.*

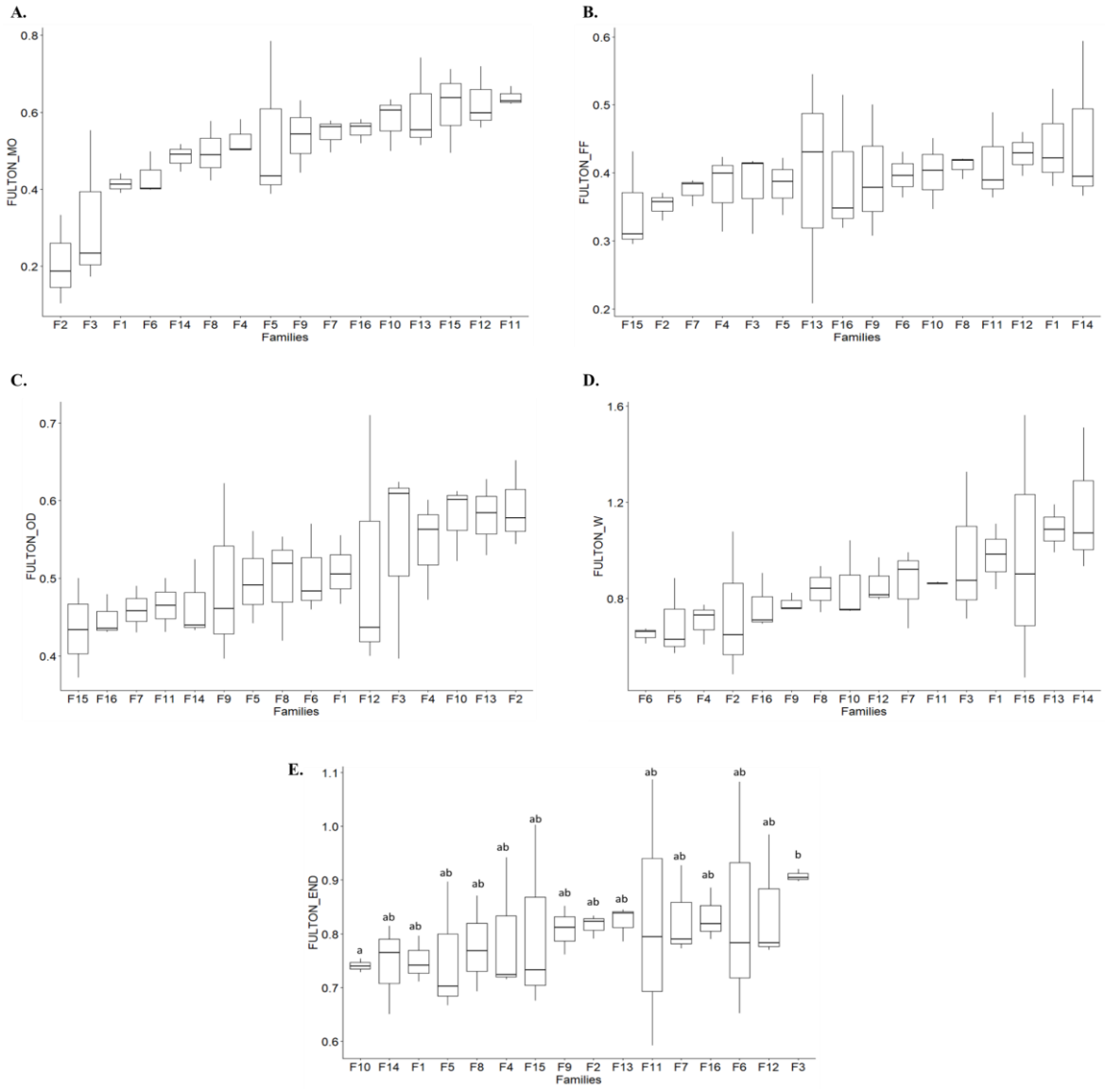


Figure S3.6: *Box plot diagram of* ***A.*** *Fulton's condition factor (K) of larvae at mouth opening.* ***B****. Fulton's condition factor (K) of larvae at first feeding stage.* ***C****. Fulton's condition factor (K) of larvae at oil droplet reduction stage.* ***D****. Fulton's condition factor (K) of larvae at Weaning stage.* ***E****. Fulton's condition factor (K) of larvae at the end of the experiment. The graphs show statistical comparisons between the K of larvae from 16 E. perch families at different developmental stages. The experimental groups are arranged in an ascending order according to their mean values and the letters (when present) indicate significant differences ($p<0.05$) between the groups.*

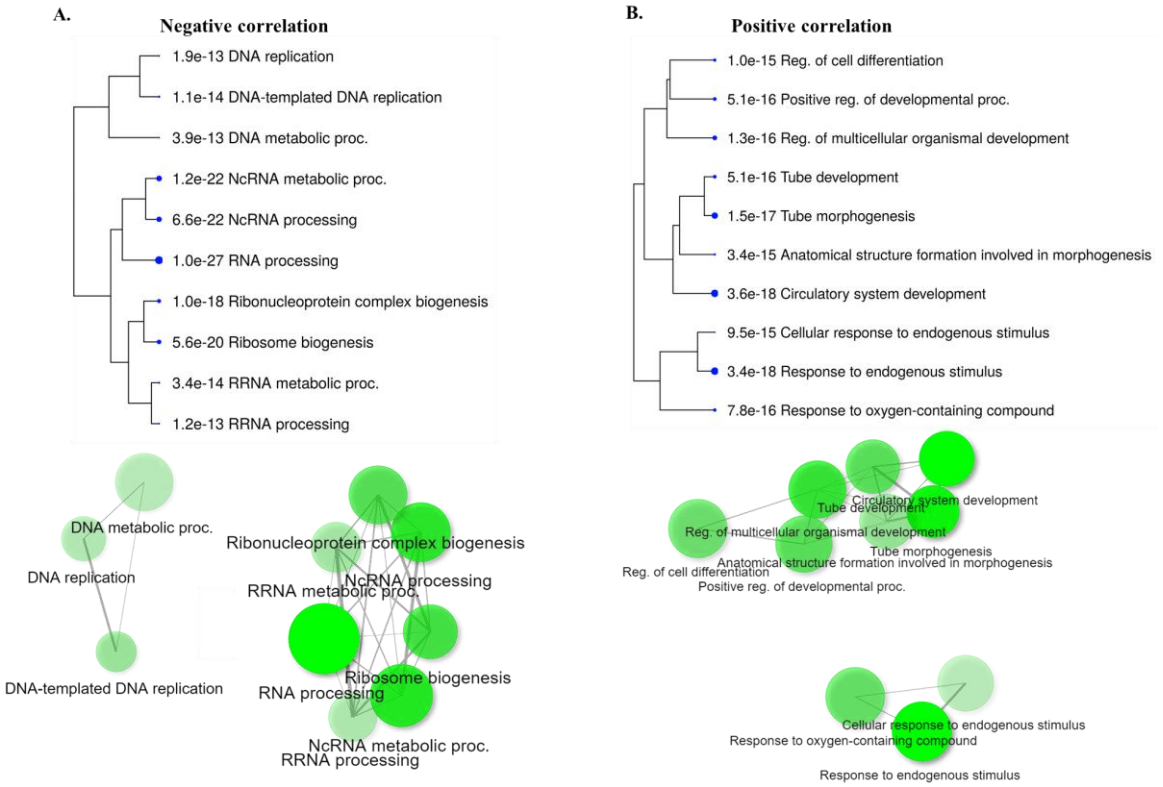


Figure S3.7: *Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for most significant gene modules related to embryonic developmental rate. A. Negative correlated modules B. Positive correlated modules*

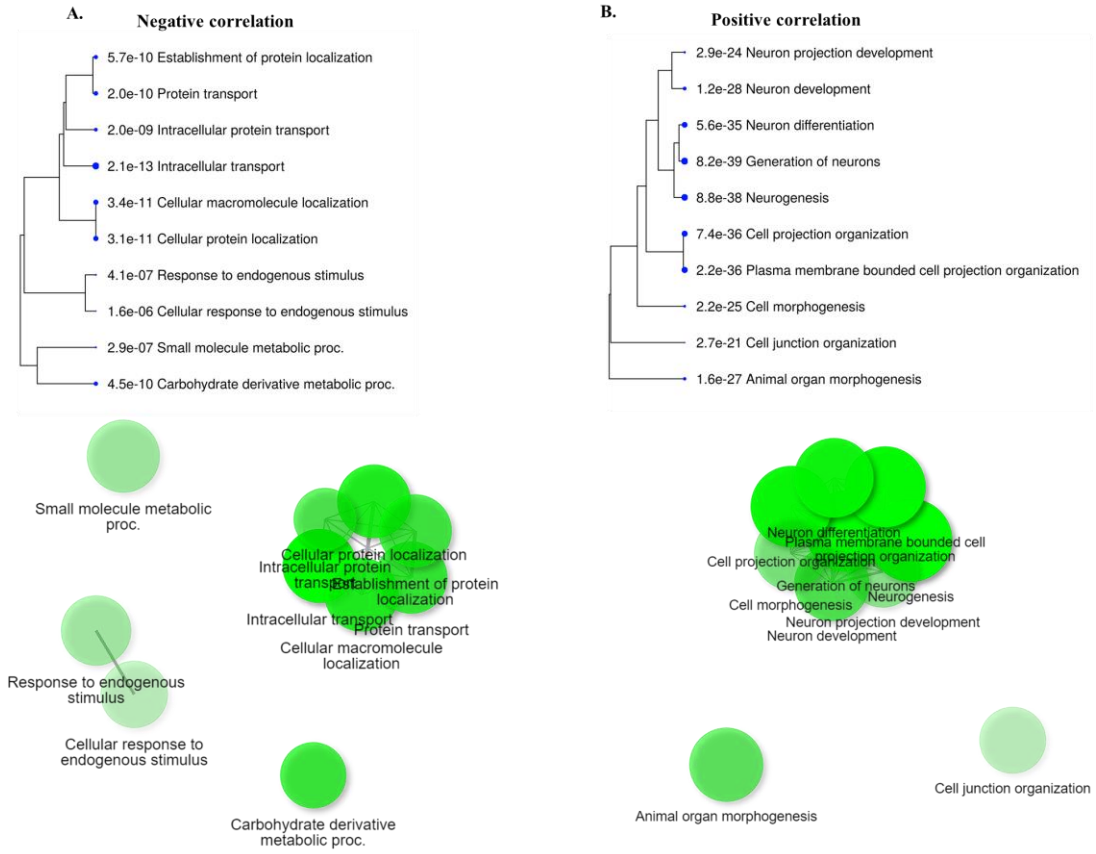


Figure S3.8*: Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for the most significant gene modules related to hatching rate. A. Negative correlated modules B. Positive correlated modules.*

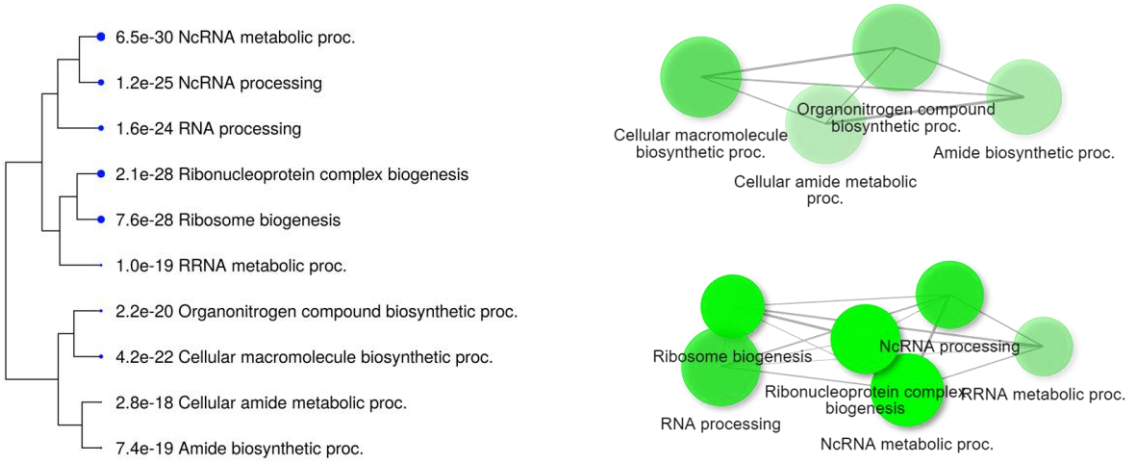


Figure S3.9: *Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for most significant gene modules positively related to weight of larvae at mouth opening.*

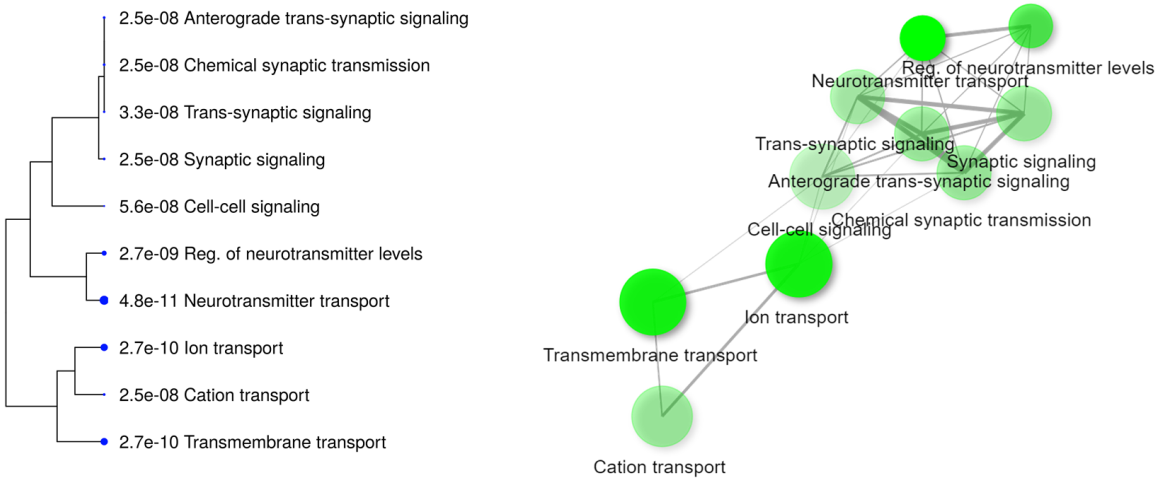


Figure S3.10: *Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for most significant gene modules negatively related to length of larvae at first feeding.*

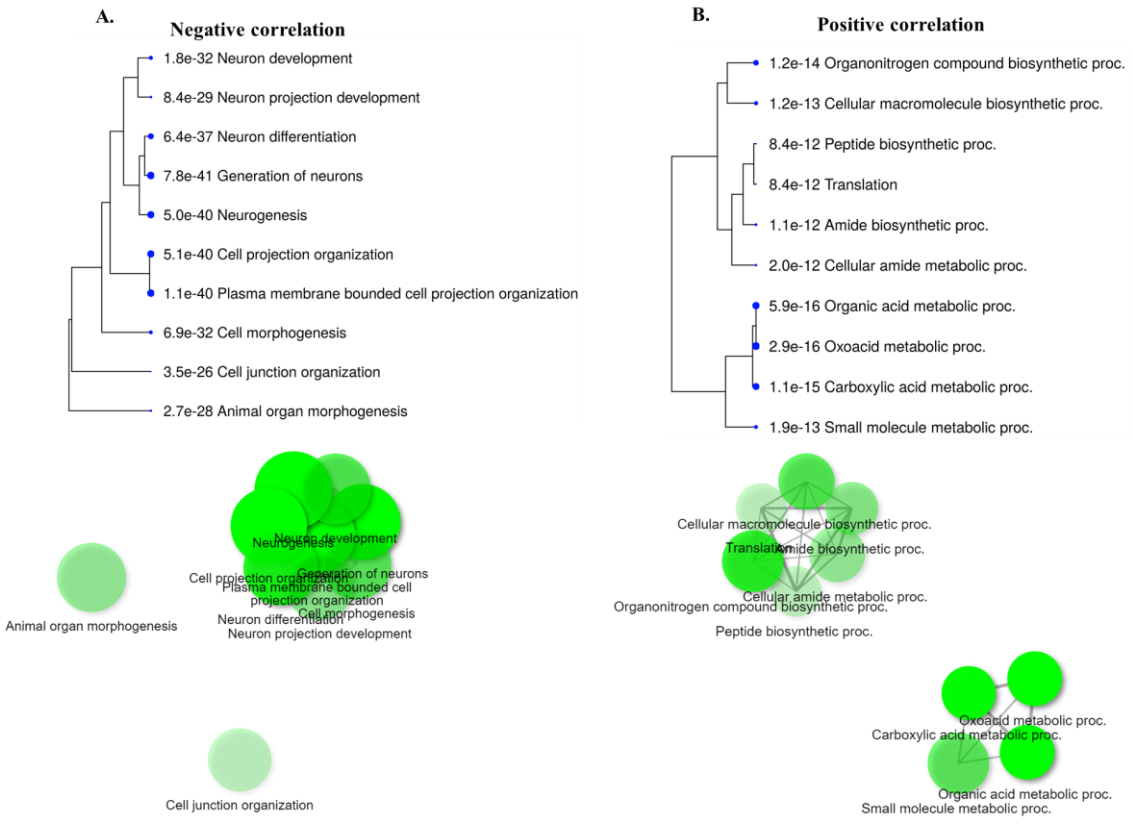


Figure S3.11: *Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for most significant gene modules related to Fulton's condition factor (K) of larvae at mouth opening.* ***A****. Negative correlated modules.* ***B****. Positive correlated modules*

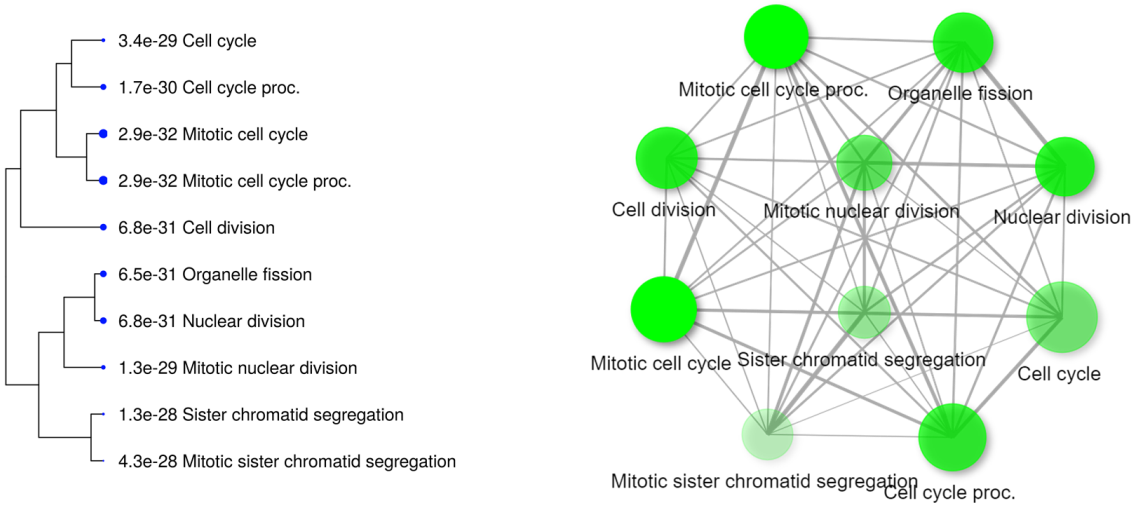


Figure S3.12: *Tree view and network visualization showing the 10 most significantly enriched GO (biological process) for most significant gene modules negatively correlated to Fulton's condition factor (K) of larvae at oil droplet reduction stage.*

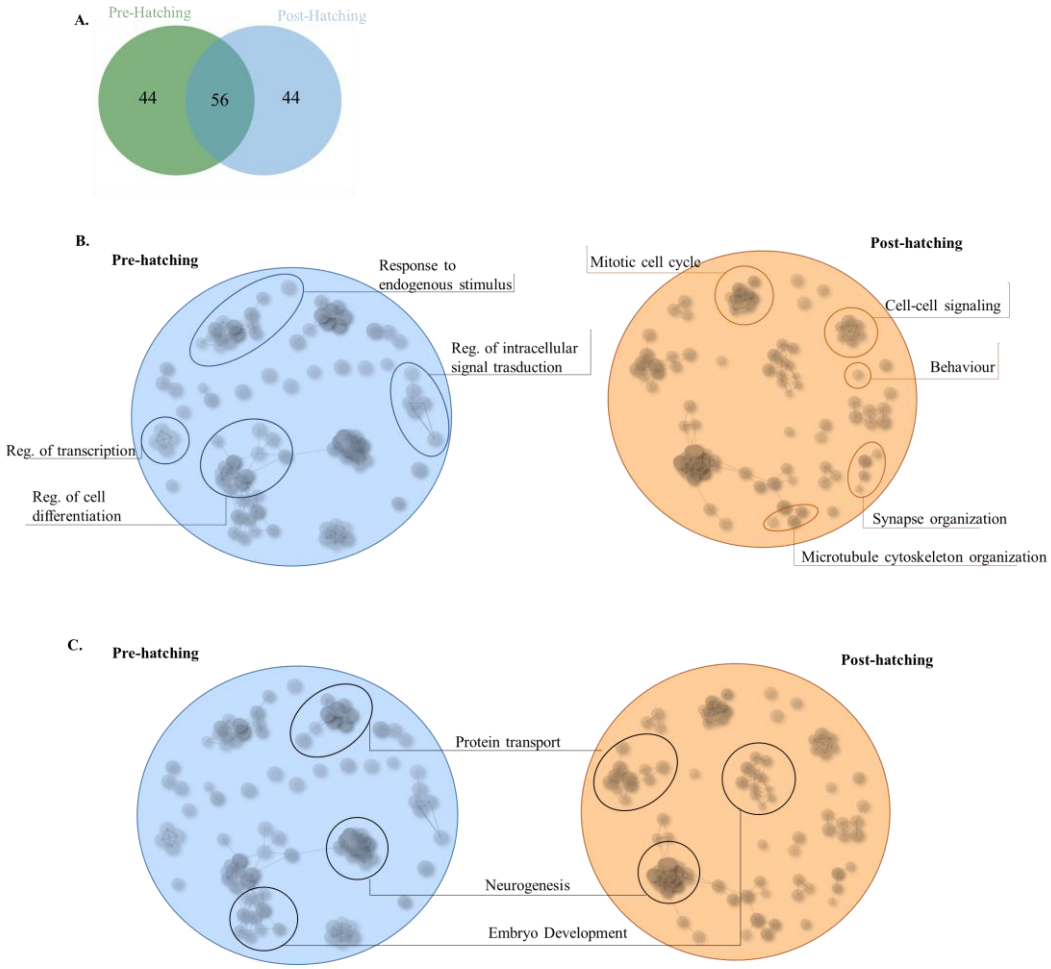


Figure S3.13: *Clustering of the 100 most enriched biological processes (BPs) obtained during the functional enrichment analysis (FDR < 0.05)* ***A.*** *Venn diagram showing the number of pre- and post-hatching specific and common GO terms.* ***B.*** *Circled clusters are those indicating pre-hatching and post-hatching traits specific cluster.* ***C.*** *Circled clusters are those indicating common clusters between pre- and post-hatching indicators.*

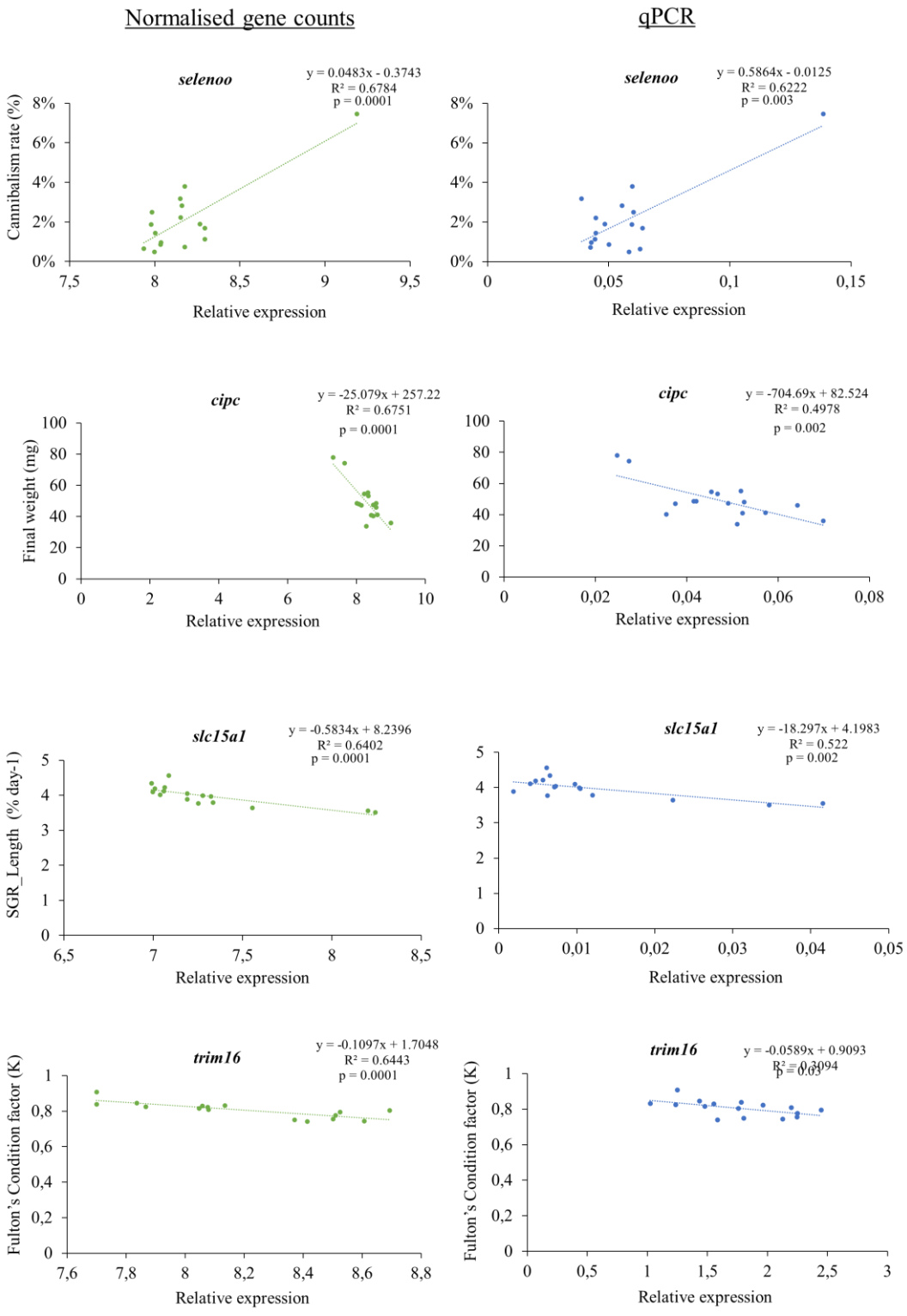
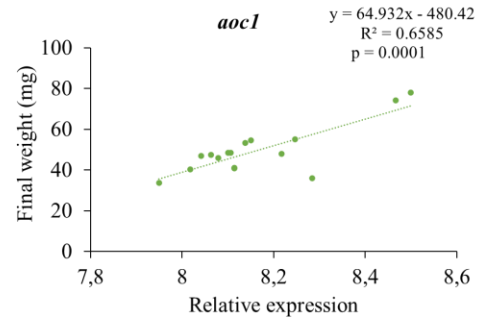
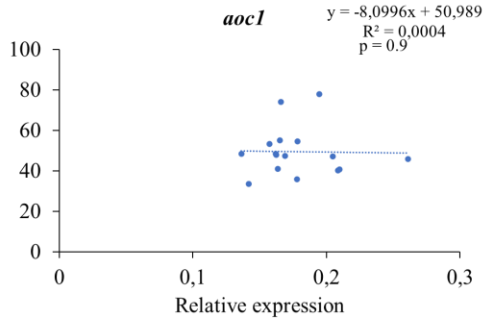
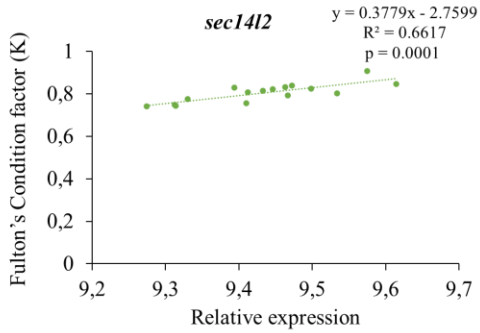
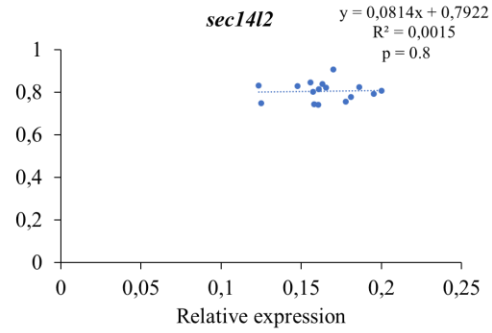


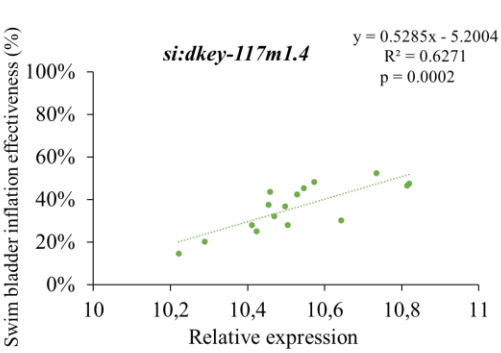
Figure S3.14*: Correlation analysis of candidate gene expression levels with associated traits. Blue trendline: Relationship between successfully qPCR-validated genes and their associated traits. Green trendline: Relationship between RNA-seq expression values of candidate genes and their associated traits.*

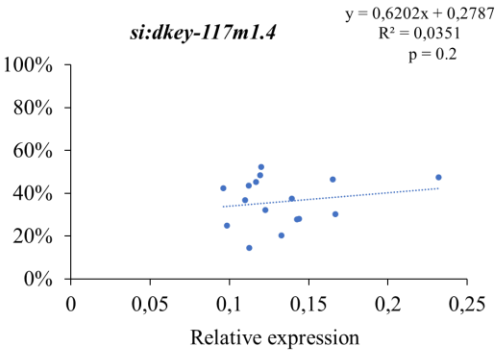

aoc1
y = 64.932x - 480.42
R² = 0.6585
p = 0.0001
Final weight (mg)
Relative expression

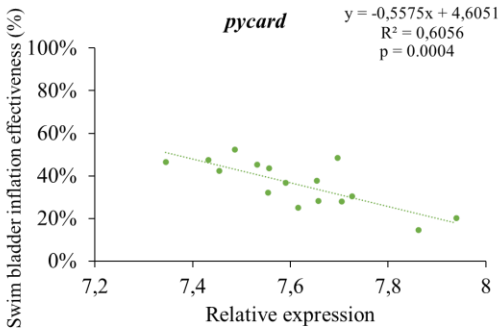

aoc1
y = -8,0996x + 50,989
R² = 0,0004
p = 0.9
Relative expression

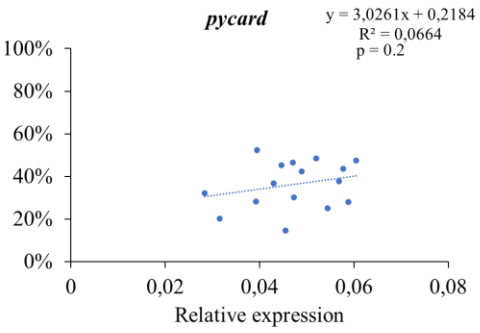

sec14l2
y = 0.3779x - 2.7599
R² = 0.6617
p = 0.0001
Fulton's Condition factor (K)
Relative expression

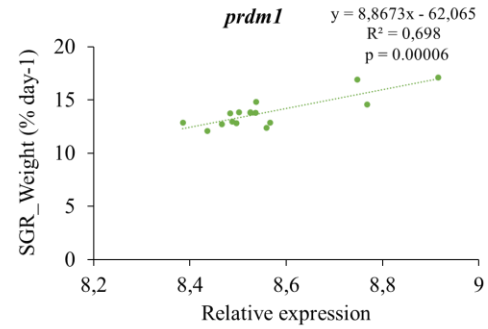

sec14l2
y = 0,0814x + 0,7922
R² = 0,0015
p = 0.8
Relative expression

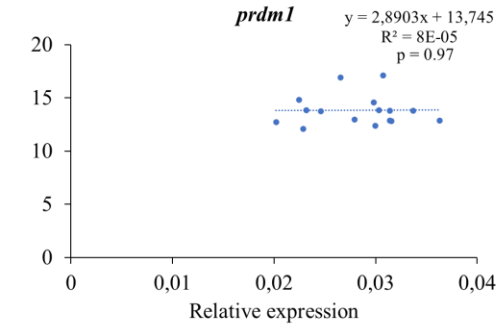

si:dkey-117m1.4
y = 0.5285x - 5.2004
R² = 0.6271
p = 0.0002
Swim bladder inflation effectiveness (%)
Relative expression


si:dkey-117m1.4
y = 0,6202x + 0,2787
R² = 0,0351
p = 0.2
Relative expression


pycard
y = -0,5575x + 4,6051
R² = 0,6056
p = 0.0004
Swim bladder inflation effectiveness (%)
Relative expression


pycard
y = 3,0261x + 0,2184
R² = 0,0664
p = 0.2
Relative expression


prdm1
y = 8,8673x - 62,065
R² = 0,698
p = 0.00006
SGR_Weight (% day-1)
Relative expression


prdm1
y = 2,8903x + 13,745
R² = 8E-05
p = 0.97
Relative expression

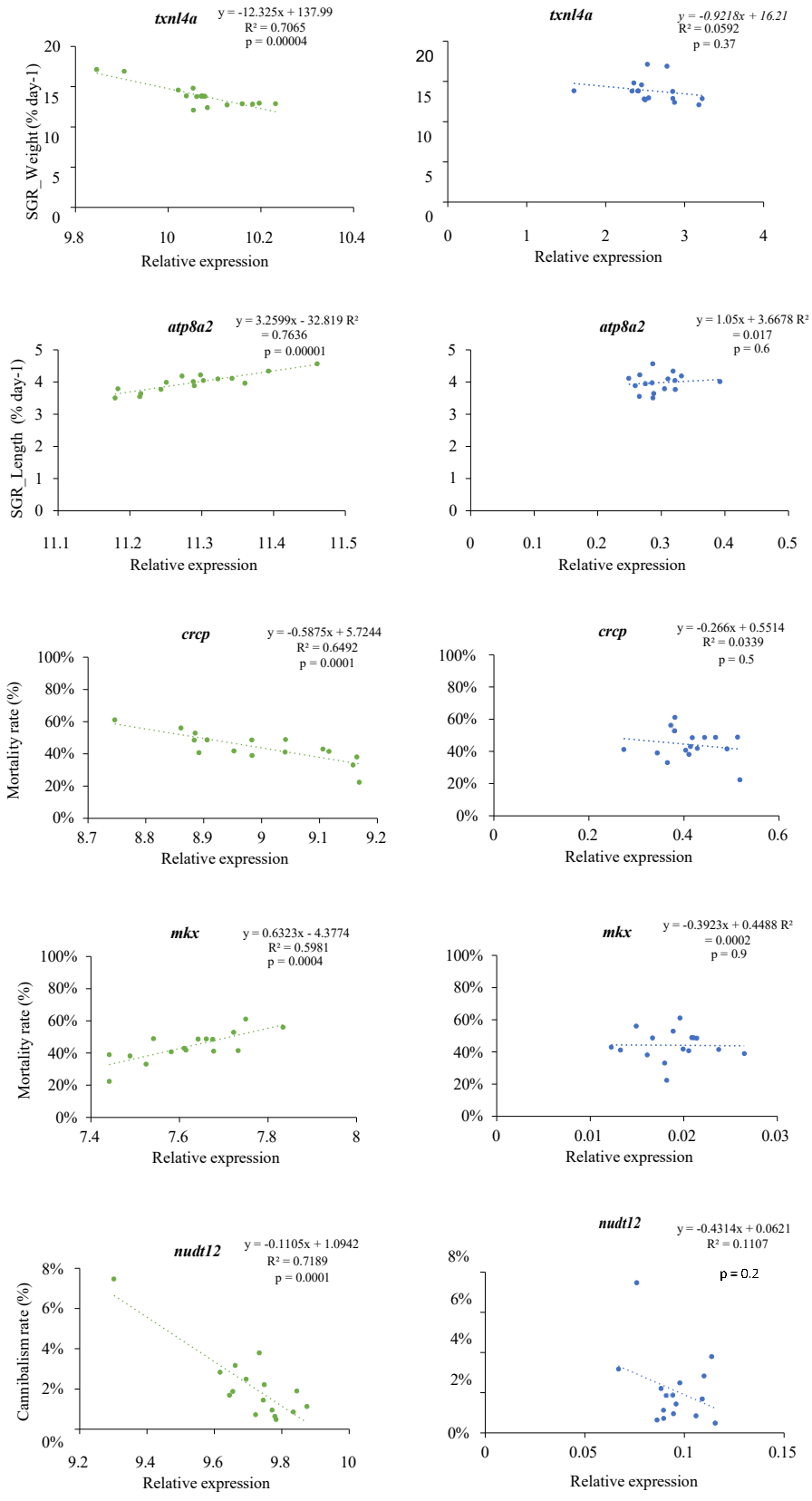


***Figure S3.15**: Correlation analysis of candidate gene expression levels with associated traits, not successfully validated using qPCR. Green trendline: Relationship between RNA-seq expression values of candidate genes and their associated traits. Blue trendline: Relationship between qPCR-validated candidate genes and their associated traits.*

Supplementary files related to chapter 4: "Dynamic interplay of maternal and paternal contributions to offspring phenotype in Eurasian perch"

Supplementary file S4.1: Broodstock characteristics for both wild and domesticated individuals and milt evaluation with CASA after cryopreservation. WBW: Wet Body Weight, LT: Total Length; W: Weight. ALH: Amplitude of Lateral Head Displacement; LIN: Linearity; VAP: Average Path Velocity; VCL: Curvilinear Velocity; VSl: Straight Velocity; MOT: percentage of sperm motility.

MALES									
WILD	**WBW (g)**	**LT (cm)**	**Sperm concentration ($x10^9$ spermatozoa ml^{-1})**	**MOT (%)**	**LIN (%)**	**ALH (µm)**	**VAP ($µms^{-1}$)**	**VCL ($µms^{-1}$)**	**VSL ($µms^{-1}$)**
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	35.5	26.4	82.4	75.9	5.7	134.1	157.9	123.2
Male 2	467	33.7	25.9	69.6	68.5	4.8	105.9	137.0	97.2
Male 3	468	34.0	34.2	73.9	72.1	4.8	115.8	144.5	105.6
Male 4	450	33.5	29.2	87.0	75.0	5.7	134.1	160.2	121.9
Male 5	615	35.8	28.3	73.5	68.2	5.0	116.2	145.1	105.1
Male 6	491	32.9	37.5	85.6	56.4	5.3	78.4	120.6	66.8

FEMALES				
WILD	**WBW(g)**	**LT (cms)**	**W ribbon (g)**	**Egg density (g^{-1})**
Female 1	470	28.7	110	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	31.1	92	404
Female 2	549	30.6	142	479
Female 3	563	33.5	159	485
Female 4	427	30.9	106	552
Female 5	447	32.2	150	601
Female 6	539	33.7	88	514

Supplementary file S4.2: 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 file S4.3: 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 the large file size, the complete tables are available at the following link:
https://cloud.pan.olsztyn.pl:18443/share.cgi?ssid=dc60cbadbd8c4ed9afb15f015bdf80a5

Supplementary file S4.4: Details of the primers used for RT-qPCR 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	

Supplementary file S4.5: Supplementary figures related to zootechnical traits results and the gene ontology (GO) analysis.

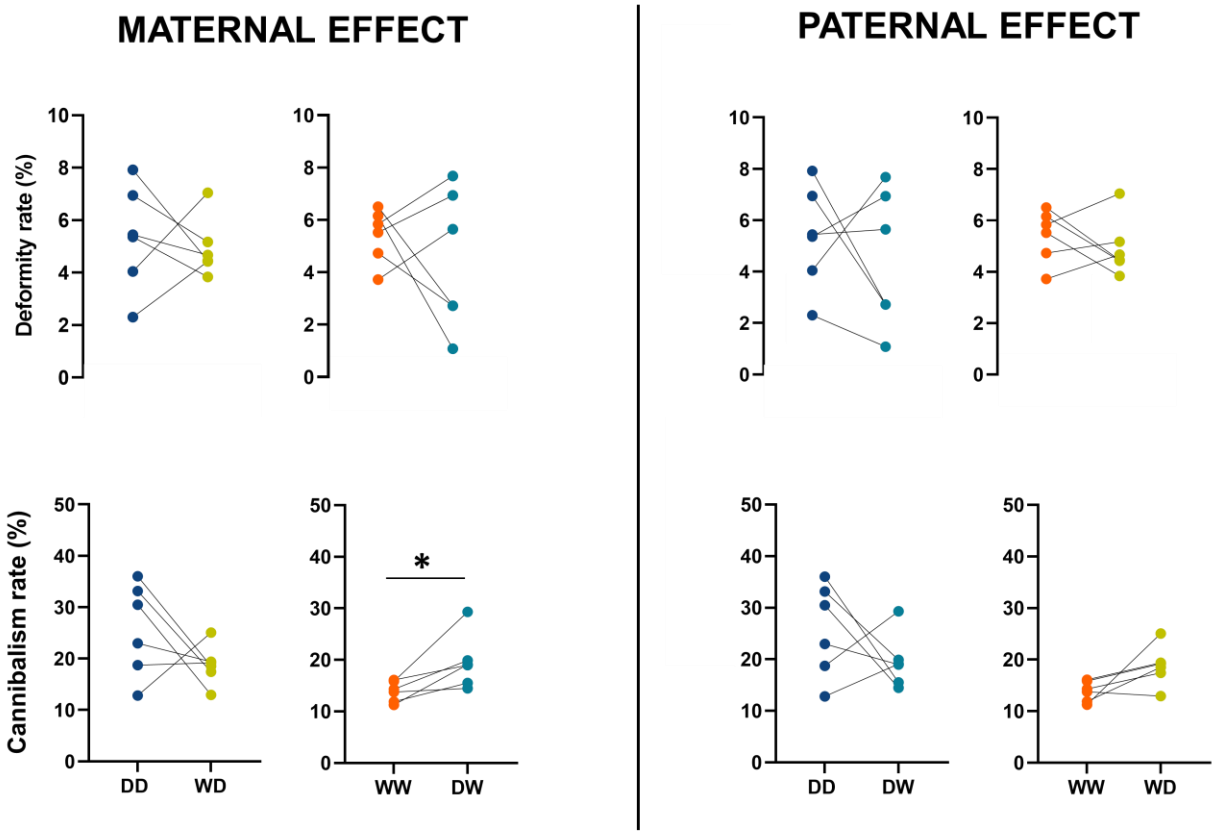


Figure S4.1: *Deformity and Cannibalism rate (%) measured for all the Eurasian perch families. Asterisk show significance difference (*p< 0.05)*

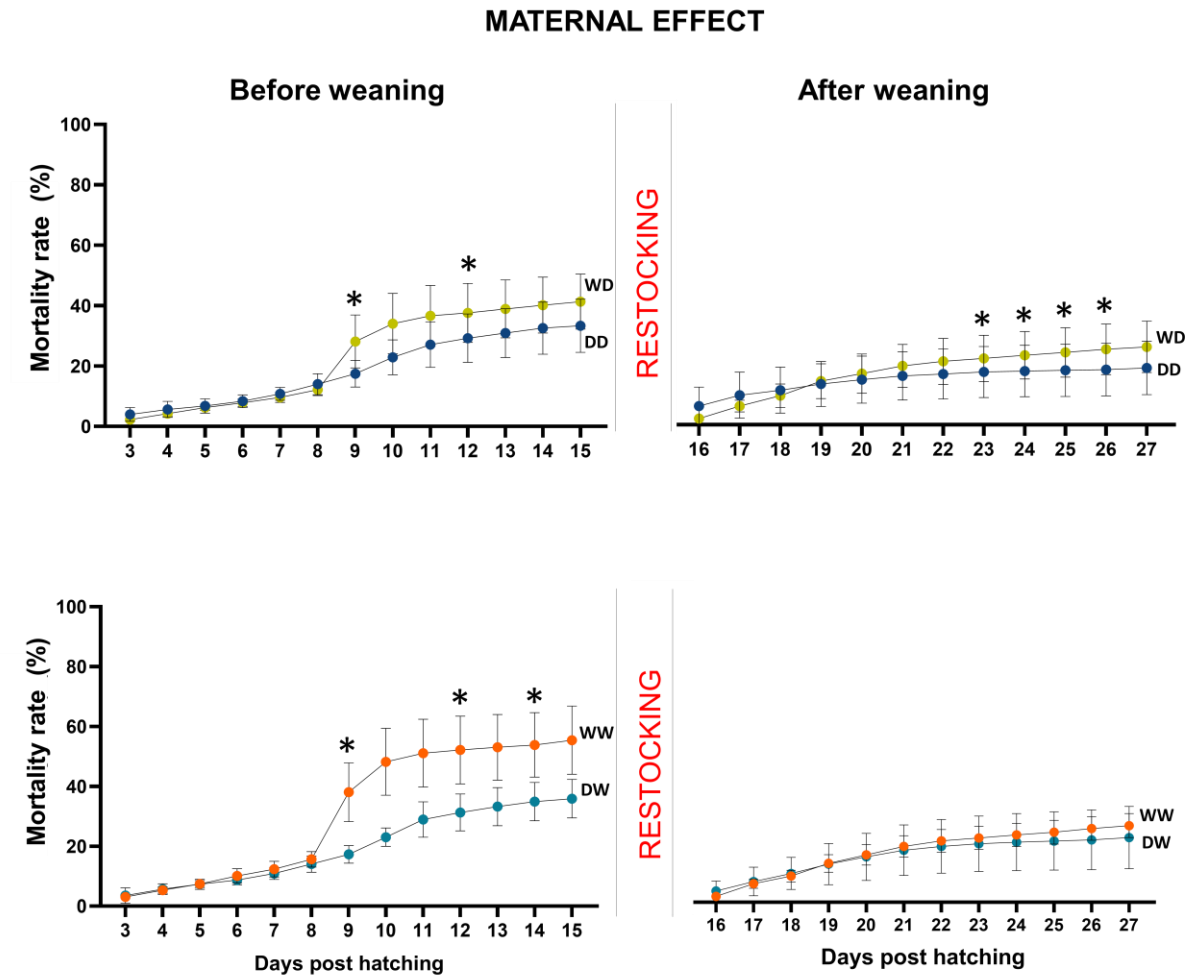


Figure S4.2: *Cumulative mortality (mean ± SD) before and after restocking for all crossings of Eurasian perch larvae analysed for maternal-effect.*

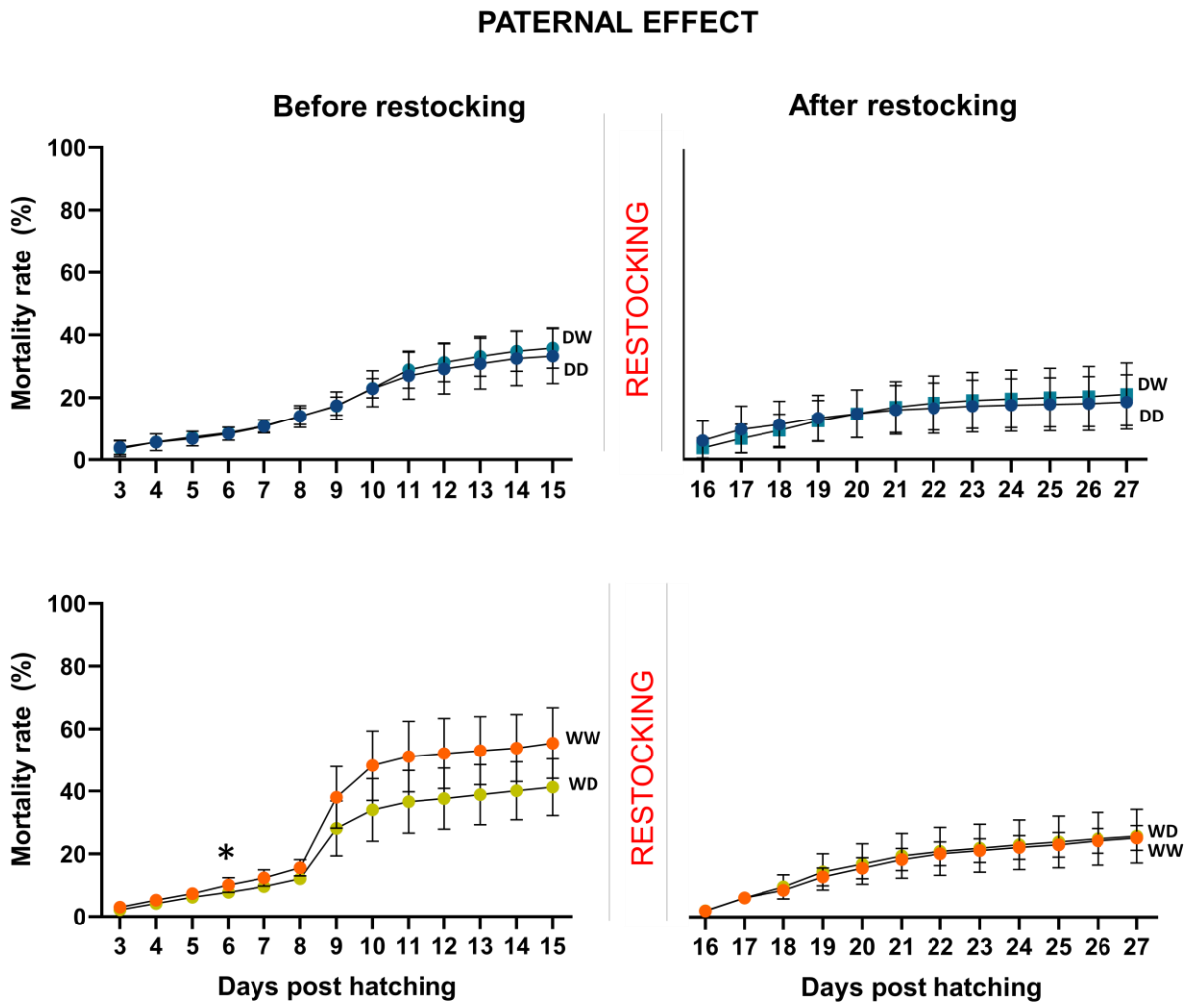


***Figure S4.3**: Cumulative mortality (mean ± SD) before and after restocking for all crossings of Eurasian perch larvae analysed for paternal-effect.*

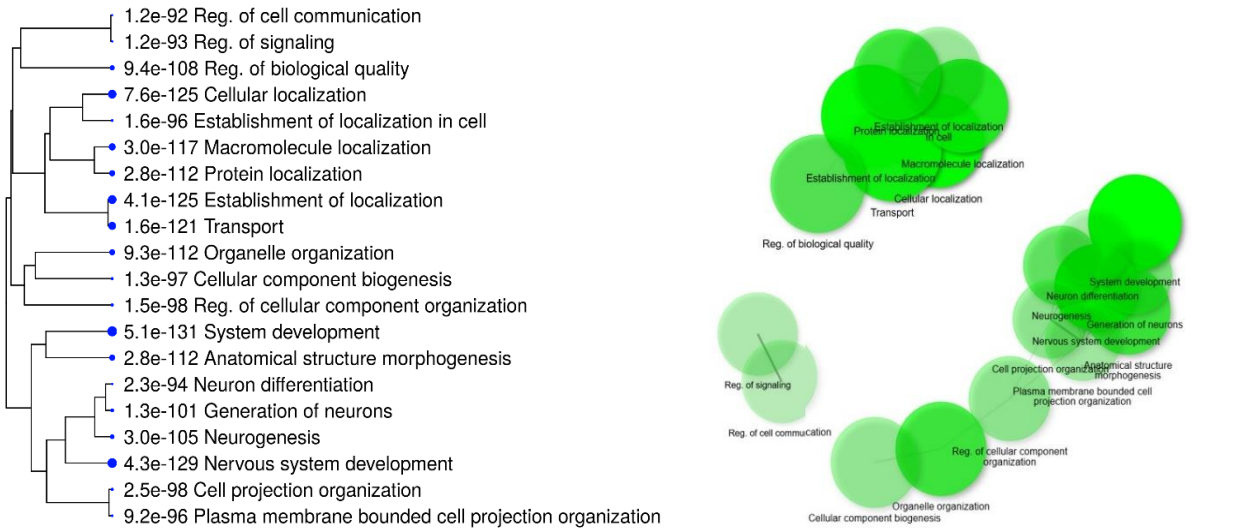


Figure S4.4: *Tree view and network visualization showing the 20 most significantly enriched GO (biological process) for non-differentially expressed genes.*

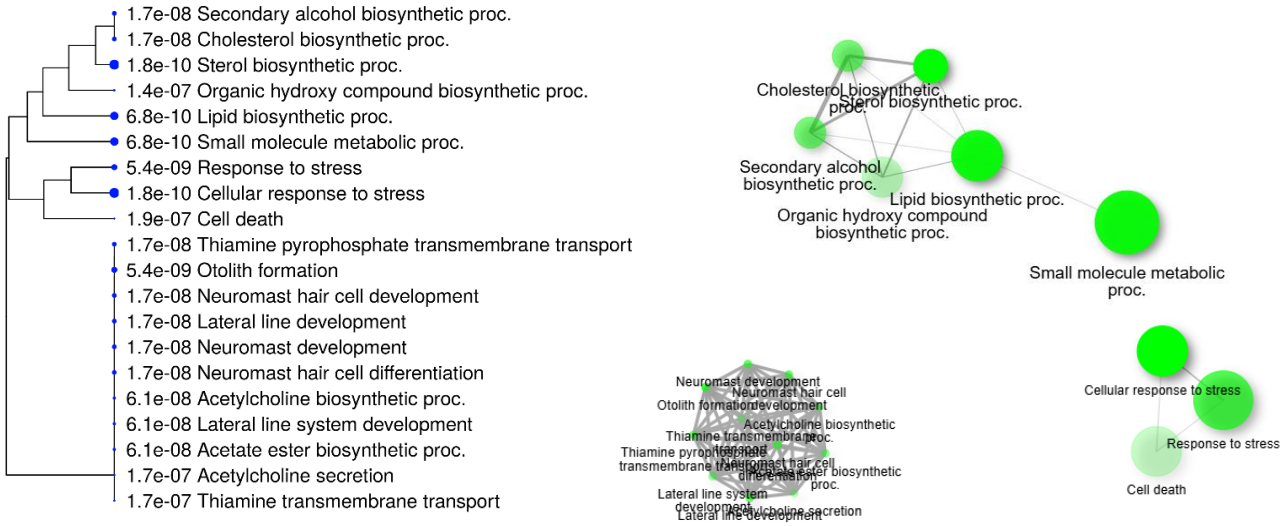


Figure S4.5: *Tree view and network visualization showing the 20 most significantly enriched GO (biological process) for purely maternal-effect genes.*

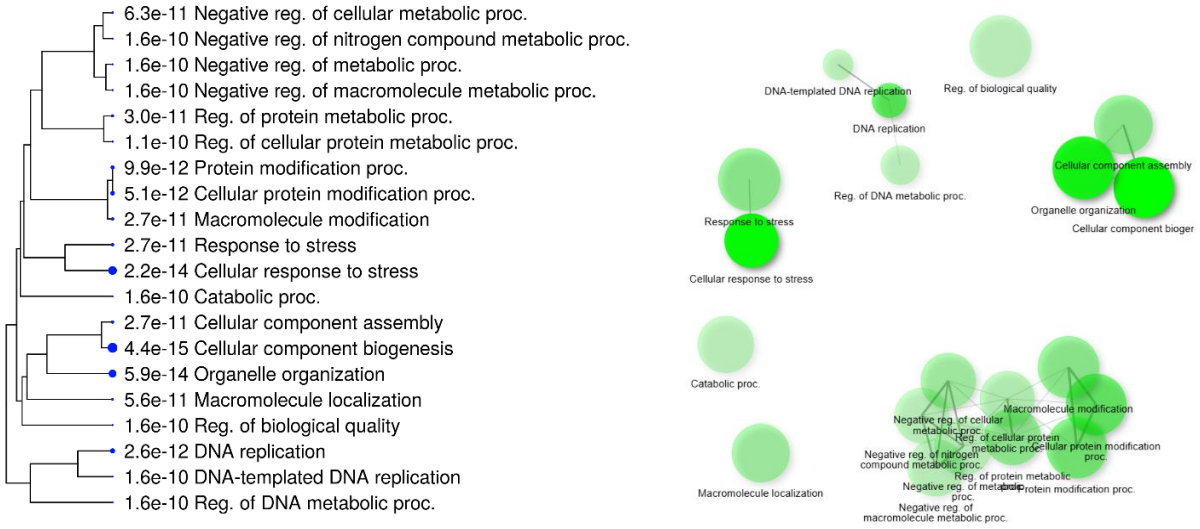


Figure S4.6: *Tree view and network visualization showing the 20 most significantly enriched GO (biological process) for all the conditionally maternal-effect genes.*

Supplementary files related to chapter 5: "Transcriptomic profile of fish eggs reflects embryonic survival potential, not larval fate"

Supplementary file S5.1: Differentially Expressed Genes (DEG) analysis of RNA-seq data from unfertilised eggs (UFE) of varying quality in Eurasian perch (*Perca fluviatilis*). This file contains: **A.** List of DEGs (*p*adj < 0.05, |log2FC| > 1) identified in the comparison between medium- and high-quality UFE. **B.** List of DEGs (*p*adj < 0.05, |log2FC| > 1) identified in the comparison between low- and high-quality UFE. **C.** List of DEGs (*p*adj < 0.05, |log2FC| > 1) identified in the comparison between low- and medium-quality UFE. Due to the large file size, the complete tables are available at the following link: https://cloud.pan.olsztyn.pl:18443/share.cgi?ssid=dc60cbadbd8c4ed9afb15f015bdf80a5

Supplementary file S5.2: List of 11 differently expressed genes (DEGs) shared between the medium vs high (MvsH) and low vs medium (LvsM) comparisons. Also, 19 DEGs common to the low vs high (LvsH) and low vs medium (LvsM) comparisons. The list provides Gene_IDs specific to Eurasian perch.

MvsH|LvsM	LvsH|LvsM
fabp4a	*atm*
LOC120553090	*ep400*
apoc1	*fbxo5*
LOC120544243	*garnl3*
LOC120570166	*im:7147486*
LOC120551416	*lmf2a*
si:ch211-212k18.7	*LOC120551300*
msna	*LOC120561483*
fcer1g	*mcm4*

LOC120566074	*mn1b*
nr4a3	*mphosph10*
	ncoa6
	notch3
	otulina
	ppm1aa
	top1l
	wdhd1
	wrnip1
	zgc:195212

Supplementary files related to Chapter 6: "Heart oedema in freshly hatched larvae of Eurasian perch is associated with multi-tissue gene dysregulation"

Supplementary file S6.1: Primers used for validation and normalization of expression level of candidate genes.

			Primers	
Gene_Id	Human orthologs	Transcript ID	**Forward**	**Reverse**
nppb	*NPPA*	XM_039799442.1	TGAGGAAGCGATCAGGGAGT	GGAGCCTATTCTGTCCATCCG
mb	*MB*	XM_039816669.1	TGCCATCCTCAAACCTCTAGC	CGGCGTCAATGTCAGCAATG
gck	*GCK*	XM_039803535.1	CAGGCTGAAGAAGGAGGAGC	TCAGGGGTGGAGCAGACATA
f2r	*F2R*	XM_039803294.1	CGGTGATCTACATGCTGAACCT	TGACAACTCTGCACATGAAGGA
LOC120575701	*HSPB1*	XM_039826528.1	GCCTTTCCTGGACATGCACT	GCAGCCTCTCCATGTACTCC
LOC120574977	*ARL14*	XM_039825524.1	CGGGTCCTGAGGTACGAGA	TTTGAGGTCCAGTGTCAGGC
LOC120555608	*HK1*	XM_039794442.1	GGCCTACTACTTCACTGAGTTGA	GAGCCCGTTCTCCATTTCCC
LOC120574104	*HBE1*	XM_039824188.1	TGGAAACCTCTACAACGCCG	GCAGTCGGACAGGAGTTTGA
LOC120564151	*FLNC*	XM_039808898.1	AGAGTCCCTTTCACATTACCGT	GAGGTCATCTTCACACCCACA
hbae5	*HBZ*	XM_039789004.1	CCGACATGAGTTTGAGCGAC	AGTAGGTCTTGGTTTGCGGG
LOC120550815	*COX6B1*	XM_039787679.1	GCCAGATTCCCCAACCAGAA	CTTGTACCAGTCACAGGGGG
timp2b	*TIMP2*	XM_039825831.1	CCCTTAACTCAACCACTGTCCA	TCACCACAGAGCACACACATT
LOC120562001	*GIMAP7*	XM_039805394.1	GCTGGGCAGATACACCGAAG	TCAAGAAGGTCCCCATGAGT
c13h18orf21	*C18ORF21*	XM_039821127.1	CCCACAGTCAACCCACAGAG	AAGAGCCTGACCCTGGAGTG
LOC120551738	*HBE1*	XM_039789276.1	TGGACCCCGACAACTTCAAG	TGGTACTGCTTTCCCAGAGC
Houskeeping genes for larvae				
	DLST	XM_039786142.1	CGGTCCAGCGTCTTCCAAAT	
dlst				CTTTCTCCCACCTCACGTCC
myl12.1	*MYL12B*	XM_039790257.1	AGGACCTTCACGACATGCTG	TGTGCCATTGAGCTTCTCCC
LOC120564404	*CAPZA2*	XM_039809324.1	TGGAAATTCACCGTCTCCCC	TGACATGGACTCCTGGACCT
taf10	*TAF10*	XM_039801277.1	GTAAAGCCTGCCCTCTCCAC	TCAGAAGCCTCAAAGCCAGC
tomm7	*TOMM7*	XM_039821187.1	GGTTTCAAACGAGGAGCCGA	GCATGGTCTCGATGAGGTGA
acaa2	*ACAA2*	XM_039804369.1	TAGCCAAACTGCCTCCTGTC	GCTTGTGTTCATTCAGCGCA
cfl1l	*DSTN*	XM_039783749.1	CGTCTGGAGTCAAAGTCGCT	GTCCAGATCTTTTGCCCGGT

Houskeeping genes for eggs				
rps20	*RPS20*	XM_039789688.1	GGCACCTGTTGAGACTGAGG	TCCTTAGCCCCACGGATCAG
rps4x	*RPS4X*	XM_039822117.1	TCAACGACACCATCCGCATC	CAAGTTAGCACCGCCAGTCA
actb	*ACT*	XM_039824642.1	ATTGCCCCACCAGAGCGTAA	ACTCATCGTACTCCTGCTTGC

Supplementary file S6.2: Differentially Expressed Genes (DEG) Analysis. This file contains gene lists from the DEG analysis: **A.** Complete list of 5,964 DEGs (*padj* < 0.05, $|\log_2 FC| > 0$). **B.** Subset of 507 DEGs meeting the criteria (*padj* < 0.05, $|\log_2 FC| > 1$), along with their tissue-specificity scores (τ). **C.** List of 16 identified heart-specific genes**.** Due to the large file size, the complete tables are available at the following link:
https://cloud.pan.olsztyn.pl:18443/share.cgi?ssid=dc60cbadbd8c4ed9afb15f015bdf80a5

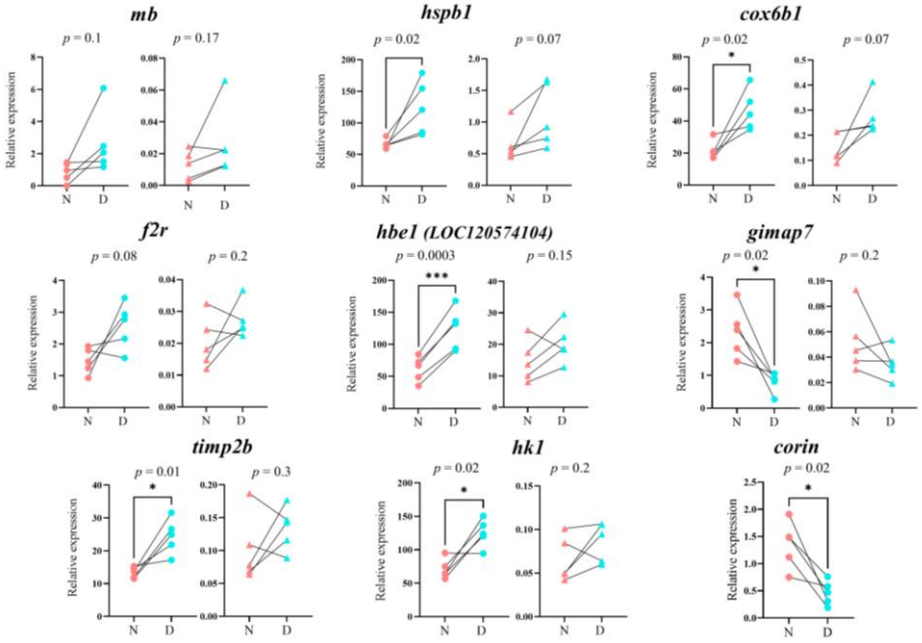


Figure S6.1: *Relative expression levels of candidate genes were not successfully validated. Circles correspond to RNA-seq analysis, while triangles represent real-time qPCR analysis. Data points marked with an asterisk indicate statistically significant differences (*p < 0.05, ***p < 0.001). Specific p-value is reported for each comparison. N: Normal, D: Deformed.*

Chapter 16: Author's 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*

Rossella Debernardis
(imię i nazwisko / *name and surname*)

Olsztyn, 01.07.2025
miejscowość, data / *city, date*

0009-0004-7691-7170
[ORCID]

Oświadczenie
Statement

Niniejszym oświadczam, że w pracy „Does transcriptome of freshly hatched fish larvae describe past or predict future developmental trajectory?", Debernardis Rossella; Palińska-Żarska Katarzyna; Judycka Sylwia; Panda Abhipsa; Jarmołowicz Sylwia; Jastrzębski Jan P.; Rocha de Almeida Tainá; Błażejewski Maciej; Hliwa Piotr; Krejszeff Sławomir; Żarski Daniel; *Aquaculture*, *595*, p.741486 (10.1016/j.aquaculture.2024.741486) mój udział polegał na redakcji i przygotowaniu pierwszej wersji manuskryptu, wizualizacji, metodologii, badaniach, analiza danych, opracowaniu danych, konceptualizacji.[7]

I hereby declare that in the scientific paper „Does transcriptome of freshly hatched fish larvae describe past or predict future developmental trajectory?", Debernardis Rossella; Palińska-Żarska Katarzyna; Judycka Sylwia; Panda Abhipsa; Jarmołowicz Sylwia; Jastrzębski Jan P.; Rocha de Almeida Tainá ; Błażejewski Maciej; Hliwa Piotr; Krejszeff Sławomir; Żarski Daniel; Aquaculture, 595, p.741486 (10.1016/j.aquaculture.2024.741486) my contribution consisted of writing – original draft, visualization, methodology, investigation, formal analysis, data curation, conceptualization.[7]

Rossella Debernardis
podpis kandydata/ *signature of the candidate*

Podpisy współautora/współautorów
Signatures of the co-author/co-authors:

Sylwia Judycka
Daniel Żarski
Abhipsa Panda
Piotr Hliwa
Jan Jastrzębski
Maciej Błażejewski
Katarzyna Palińska-Żarska
Sylwia Jarmołowicz

1

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

W celu jednoznacznego określenia wkładu kandydatów w powstanie publikacji naukowej, przyjmuje się następującą klasyfikację:

- **Konceptualizacja**: Tworzenie idei; formułowanie lub rozwijanie głównych celów, hipotez i założeń badawczych.
- **Opracowanie danych:** Zarządzanie danymi, w tym ich anotacja (tworzenie metadanych), przechowywanie danych badawczych do użytku bieżącego i/lub ponownej analizy.
- **Analiza danych:** Zastosowanie technik statystycznych, matematycznych, obliczeniowych lub innych metod do analizy danych badawczych.
- **Pozyskanie finansowania**: Zdobycie środków finansowych na realizację projektu, który doprowadził do powstania publikacji.
- **Badania:** Prowadzenie procesu badawczego, w tym przeprowadzanie eksperymentów oraz zbieranie danych.
- **Metodologia:** Opracowanie metod badawczych; tworzenie modeli badawczych.
- **Zarządzanie projektem:** Koordynacja i zarządzanie planowaniem oraz realizacją działań badawczych.
- **Oprogramowanie:** Programowanie; projektowanie programów komputerowych; implementacja kodu komputerowego i wspierających algorytmów; testowanie istniejących komponentów kodu.
- **Zasoby:** Zapewnienie materiału badawczego, odczynników, narzędzi badawczych, pacjentów, próbek laboratoryjnych, zwierząt doświadczalnych, sprzętu, zasobów obliczeniowych lub innych narzędzi analitycznych.
- **Walidacja:** Weryfikacja powtarzalności i odtwarzalności wyników badań, eksperymentów oraz innych rezultatów naukowych.
- **Wizualizacja:** Przygotowanie, opracowanie i/lub prezentacja wyników badawczych, w szczególności wizualizacja danych.
- **Redakcja i przygotowanie pierwszej wersji manuskryptu:** Opracowanie, stworzenie i/lub prezentacja pracy naukowej, w szczególności napisanie pierwszej wersji manuskryptu.
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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*

Rossella Debernardis
(imię i nazwisko / *name and surname*)

0009-0004-7691-7170
[ORCID]

Olsztyn, 22.07.2025
miejscowość, data / *city, date*

Oświadczenie
Statement

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Rossella Debernardis
podpis kandydata/ *signature of the candidate*

Podpisy współautora/współautorów
Signatures of the co-author/co-authors:

Abhipsa Panda
Sylwia Judycka
Daniel Żarski
Piotr Hliwa
Katarzyna Palińska-Żarska
Sylwia Jarmołowicz

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

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(imię i nazwisko / *name and surname*)

0009-0004-7691-7170
[ORCID]

Olsztyn, 22.07.2025
miejscowość, data / *city, date*

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Signature valid
Dokument podpisany przez Rossella Debernardis
Data: 2025.09.08 10:25:28 CEST

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podpis kandydata/ *signature of the candidate*

Podpisy współautora/współautorów
Signatures of the co-author/co-authors:

.....

.....

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

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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*

Rossella Debernardis
(imię i nazwisko / *name and surname*)

0009-0004-7691-7170
[ORCID]

Olsztyn, 22.07.2025
miejscowość, data / *city, date*

Oświadczenie
Statement

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Signature valid
Dokument podpisany przez Rossella Debernardis
Data: 2025.09.09 10:[illegible] CEST

.....
podpis kandydata/ *signature of the candidate*

Podpisy współautora/współautorów
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18.08.2025, 10:01:19
Signed on the platform Autenti

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

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Rossella Debernardis
(imię i nazwisko / *name and surname*)

0009-0004-7691-7170
[ORCID]

Olsztyn, 04.09.2025
miejscowość, data / *city, date*

Oświadczenie
Statement

Niniejszym oświadczam, że w pracy „Transcriptomic profile of fish eggs reflects embryonic survival potential, not larval fate", Debernardis Rossella, Palińska-Żarska Katarzyna, Jastrzębski Jan P., Panda Abhipsa, Nynca Joanna, Żarski Daniel; w przygotowaniu, mój udział polegał na przygotowaniu pierwszej wersji manuskryptu, wizualizacji, metodologii, analizie danych, badaniach, opracowaniu danych, konceptualizacji.[7]

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Rossella Debernardis
podpis kandydata/ *signature of the candidate*

Podpisy współautora/współautorów
Signatures of the co-author/co-authors:

Abhipsa Panda
Daniel Żarski
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Jan Jastrzębski
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Olsztyn, 09.09.2025
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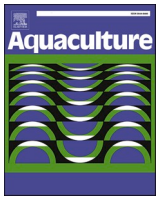

Does transcriptome of freshly hatched fish larvae describe past or predict future developmental trajectory?

Rossella Debernardis [a], Katarzyna Palińska-Żarska [b], Sylwia Judycka [a], Abhipsa Panda [a], Sylwia Jarmołowicz [b], Jan P. Jastrzębski [c], Tainá Rocha de Almeida [a], Maciej Błażejewski [d], Piotr Hliwa [d], Sławomir Krejszeff [e], Daniel Żarski [a,*]

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ABSTRACT

Transcriptomic analysis of freshly hatched fish larvae offers insights into phenotype development, yet it remains uncertain whether it reflects parental influence or predicts individual growth. This research scrutinizes the transcriptome of 16 Eurasian perch (*Perca fluviatilis*) larval families (at mouth opening stage, coinciding in this species with hatching) alongside pre- and post-hatching zootechnical traits. Despite consistent fertilization rates, significant variations in embryonic development and larval performance highlight diverse phenotypes studied. It enabled us to bring our attention to the fact that transcriptome of larvae at mouth opening stage can serve as a window into both the parental contributions and the future performance of the larvae. Our analysis shed light on ribosome biogenesis, neurogenesis, and the cell cycle, as important processes shaping early larval performance. Additionally, we propose a set of predictive, validated gene markers associated with further larval performance and key aquaculture traits, such as *selenoo* (associated with cannibalism), *trim16* (fulton's condition factor), *slc15a1* (specific growth factor), and *cipc* (final weight). This study delves into the descriptive and predictive nature of the transcriptomic portrait of newly hatched larvae, paving the way to comprehend the intricate developmental pathways from fertilization towards juvenile stage.

1. Introduction

The early life history of most teleost fishes (both marine and freshwater) embraces a crucial stage known as larval period (McMenamin and Parichy, 2013). This transitional phase involves changes across morphological, behavioral, and physiological levels which lead to individual's progress towards adulthood (Urho, 2002). The dynamics and vulnerability of this phase are what make larvae important to study in order to facilitate a better understanding of the developmental journey from the egg stage to subsequent juvenile and adult stages.

Larval phase is characterized by significant events encompassing the initiation of the exogenous feeding, yolk-sac reduction, and the inflation of the swim bladder, as well as the development and functional maturation of different organs, tissues, and systems (e.g.: nervous, visual, digestive and immune systems) (Osse et al., 1997). These changes are regulated by a cascade of events as well as intrinsic (e.g., genetics, physiology) and extrinsic factors (e.g., temperature, density, nutrition (Sarropoulou et al., 2016)) which have been a longstanding focus for the scientific community. Therefore, due to their significant influence on later developmental phases, investigations on the factors and intrinsic processes influencing larval performance are of high importance. This could also directly impact the modification of breeding and hatchery protocols by aquaculturists.

Sustainable development of aquaculture industry requires species diversification and the intensification of production to meet the rising global demand for aquatic products (Subasinghe et al., 2009). Among cultured species, Eurasian perch (*Perca fluviatilis*) is considered as a valuable model due to its commercial relevance and practise in intensive

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farming using recirculating aquaculture systems (RAS). Although, its production is already well-established (Fontaine and Teletchea, 2019; Palińska-Żarska et al., 2020; Policar et al., 2019), variable reproductive and larviculture performances remain still an obstacle to the improvement of its breeding efficiency. Within this framework, the production of high-quality larvae becomes paramount, as they possess a significant capacity to adapt to the aquaculture environment (Koumoundouros et al., 2017; Valente et al., 2013). Despite substantial progress in comprehending larvae biology and establishing larviculture protocols for many fish species, achieving consistently high survival rates and optimal growth potential remains a challenge (Valente et al., 2013). To overcome this, a deeper understanding of intrinsic and extrinsic factors influencing larval environmental adaptability is required. This is crucial for establishing a clear definition or, at the very least, identifying descriptors or predictors of larval quality, addressing an urgent need in the field.

Currently, the assessment of fish larvae quality is predominantly tied to morphological traits, such as body shape (i.e., absence of skeletal deformities), yolk sac morphology, inflated swim bladder, pigmentation (i.e., Koumoundouros, 2010; Boglione et al., 2013a, 2013b; Koumoundouros et al., 2017). Despite their significance, these indicators have limitations. Assessing these traits requires laborious and long-term breeding operations and their accuracy may be compromised by potential biases resulting from the rearing environment and human interventions. For this reason, there is a growing emphasis on incorporating new molecular quality indicators (e.g., gene markers) for larvae to supplement existing methods. The integration of molecular approach with conventional morphological examination will improve the efficiency of larval rearing protocols by offering a comprehensive understanding of biological processes. These molecular signatures should be found considering larvae at the earliest stage (preferably after hatching), where individuals are fully autonomous but major human intervention which could cause some stress and thus affect the overall regulation of biological processes of the fish is not yet in place (Valente et al., 2013). For Eurasian perch larvae, the mouth opening stage represents this time point (Palińska-Żarska et al., 2021). However, despite multiple evidences suggesting the potential of larval molecular profiles in predicting performance, there is still a lack of specific molecular indicators or fingerprints as reliable indicators. This stems from the lack of studies that directly align the characterisation of molecular profiles against larval traits.

Molecular profile of the larvae is influenced by a combination of genetic and non-genetic factors provided by both parents. These factors encompass various molecules (mRNAs, small RNAs, proteins, metabolites, etc.) or modifications of the genome (such as epigenetic modifications) which play a pivotal role as modulators of gene expression during the early development, influencing the phenotype of the progeny (Adrian-Kalchhauser et al., 2020). For instance, previous studies have demonstrated that transcriptomic profiling of pre-ovulatory oocytes and eggs serves as a robust predictor of their developmental competence (Chapman et al., 2014; Cheung et al., 2019). This clearly shows, that cascade-like transmission of molecular information from parents is influencing future embryonic fate. Furthermore, this transmission has been hypothesized to affect larval performance at the earliest stage (Adrian-Kalchhauser et al., 2018, 2020), suggesting that molecular profile of eggs can serve also as a predictor of future fate of fish, as tackled in several studies. For example, Colson et al. (2019) describe how the maternal molecular cargo transmitted into the eggs of rainbow trout (*Oncorhynchus mykiss*) significantly predicts the behaviour of their offspring. Despite the growing body of evidence on the crucial role of molecular parental cargo on embryonic development, there is still lack of information on whether the transcriptome of larvae at the earliest stage is only a result of parental contributions or if there is a continuum of transmission of parental information beyond embryogenesis. Therefore, it remains to be elucidated whether the molecular portrait of larvae can also predict its future performance.

Transcriptomics has been already successfully applied to fish larvae biology research, offering valuable insights into the molecular mechanisms underlying various biological processes (Chandhini and Rejish Kumar, 2019; Ferraresso et al., 2013; Mazurais et al., 2011; Żarski et al., 2017e). While it underscores the significance of larval transcripts as a proxy to understand organisms' phenotypes, it remains unclear if such profiles can describe/predict larval outcomes. Therefore, the current study on Eurasian perch aims to explore whether the transcriptomic profile of 16 different families of larvae, each coming from different pairs of parents and collected at the mouth-opening stage, could be an indicative tool for describing parental contribution and/or predicting the future fate of the larvae. This research question has been addressed by examining detailed zootechnical features from fertilization until the end of the larval stage for each family separately, and by identifying molecular information that could serve as indicators of larval performance-related traits.

2. Material and methods

Sixteen diverse families of Eurasian perch larvae were obtained by controlled reproduction of wild spawners from different water bodies (Supplementary file S1). A simple mating design was carried out by crossing a single female with an individual male.

2.1. Broodstock origin, management and reproduction

2.1.1. Males' origin and management

The Eurasian perch males (average weight 190.5 ± 58 g), used for controlled reproduction, were from earthen pond systems located in Central (Rytwiany and Łyszkowice Fish Farm) and North of Poland (Iława Fish Farm). Spawners were harvested during late autumn (end of October and early November) and overwintered in the flow-through system of the Salmonid Research station of the National Inland Fisheries Research Institute (IRS-PIB) in Rutki (North Poland) under the natural photoperiod. Male individuals were captured in autumn since it is very difficult to catch them during the breeding season, and moreover, often completed or already contributed to the spawning act before being caught, which may affect sperm quality. Then, during the spawning period, males were transferred in plastic bags with oxygen (Żarski et al., 2017a) to the 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 with a controlled photoperiod (14 L:10 D) and temperature (12 °C) until spermiation took place. The sexually mature fish were hormonally stimulated (with an intraperitoneal injection at the base of the left ventral fin) using a salmon gonadoliberin analog (sGnRHa, Bachem Chemicals, Switzerland) at a dose of 50 μg kg^{-1} (Żarski et al., 2020a). Before any manipulation, individuals were anesthetized in MS-222 (Argent, USA) at a concentration of 150 mg l^{-1}. Total length (TL), fork length (FL) and body weight (before stripping) were measured for each individual (Supplementary file S1).

2.1.2. Sperm sampling and cryopreservation protocols

Five days after hormonal injection, semen was collected using a catheter (to avoid contamination of the urine; Sarosiek et al., 2016) and with gentle abdominal pressure (i.e., stripping). After collection, each sample was kept on ice, and sperm motility parameters were evaluated with CASA system (Supplementary file S2), (using the CEROS II system -Hamilton-Thorne, USA; as described by Judycka et al. (2022), while the sperm concentration of fresh semen was measured using a Nucleo-Counter SP-100 computer aided fluorescence microscope (Chemometec, Allerød, Denmark; Nynca and Ciereszko, 2009). For this purpose, the semen was first diluted 100 times with PBS and then 51 times with Reagent S100 and loaded into the kit cassette containing propidium iodide. SemenView software (Chemometec, Denmark) was used to determine the final concentration of spermatozoa in each sample (Judycka et al., 2019).

Thereafter, sperm cryopreservation was carried out following the procedure described by Judycka et al. (2022). Briefly, the semen was diluted with a glucose-methanol (GM) extender supplemented with potassium chloride (consisting of a final concentration of 0.30 M glucose, 7.5% methanol and 25 mM KCl at 3.0×10^9/ml spermatozoa). Semen mixed with cryoprotectants was filled into 0.5 ml plastic straws and then placed on a floating rack and cryopreserved in liquid nitrogen vapor for 5 min. Next, the straws were submerged into liquid nitrogen, which ended the process. After cryopreservation, the sperm motility was re-evaluated by thawing the straws in a water bath for 10s at 40 °C. Finally, the straws were placed in liquid nitrogen storage tanks until being used for fertilization. For this operation, cryopreserved semen was used to ensure feasibility of the entire operation (in case of delayed reproduction of females) and to maintain comparable sperm quality in case the females did not ovulate simultaneously (collection of eggs from wild females during the spawning season can take several days; Żarski et al., 2017b) and it is currently used as a standard procedure in many selective breeding programs (Judycka et al., 2022).

2.1.3. Females' origin and management

For this experiment, 16 wild females (average weight 493 ± 213 g) were utilized. Some individuals came from the Żurawia and Iława pond systems, which were overwintered together with males (as mentioned before). In addition, in April, wild females from Szymon and Umląg Lakes were captured using gill nets during the spawning season. In this way, we ensured significant phenotypic variability, expected to provide appropriate heterogeneity in the performance of the larvae, which was needed to fulfil the purpose of this research project. Prior to spawning, all fish were transported in plastic bags with oxygen to the CAEE-UWM where they were placed in the RAS with a controlled photoperiod (14 L:10 D) and temperature (12 °C) until ovulation. Prior to hormonal stimulation, females were first catheterized, and the oocyte maturation stage was determined following the classification proposed by Żarski et al. (2011). All the fish at the time of injection were at stage III and IV, being the most appropriate for reproduction in Eurasian perch (Żarski et al., 2011). After that, the fish were hormonally stimulated (as with males) using a salmon gonadoliberin analog (sGnRHa, Bachem Chemicals, Switzerland) at a dose of 50 μg kg^{-1} (Żarski et al., 2020a). Before any manipulation, individuals were anesthetized in MS-222 (Argent, USA) at a concentration of 150 mg l^{-1}. During the experiment, for each fish, the total length (TL), fork length (FL), body weight and weight of the ribbon were taken (measures are provided in Supplementary file S1).

2.2. Egg collection and fertilization protocols

At ovulation, the eggs (ribbon) were collected through hand stripping (gentle massage of the abdomen part of the fish body as described by Żarski et al. (2011)). After stripping, the number of dry eggs in 1 g was evaluated by first counting the eggs in 3 small portions (~0.2 g each) of the ribbon (Żarski et al., 2017b). In this way, the correct number of spermatozoa to be used for fertilization of each ribbon was estimated (Żarski et al., 2017c). A ribbon with an average weight of 80 ± 12 g was used to carry out the in vitro fertilization as described by Judycka et al. (2019). Briefly, the eggs were first activated with modified Lahnsteiner 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; Judycka et al., 2022). Hereafter, just before fertilization, straws with cryopreserved semen were thawed in a water bath at 40 °C for 10 s and placed in an Eppendorf tube. Thirty seconds after eggs activation, sperm was added at a sperm:egg ratio of 200,000:1. The eggs were then stirred for 40 s and washed with hatchery water after ~10 min to remove excess sperm and any debris. The same procedure was followed for each female separately.

2.3. Egg incubation and hatching – evaluation of pre-hatching zootechnical traits

The fertilized eggs were incubated in 15 L black-walled tanks (eggs from each female separately) operating in the same RAS and placed on nets with mesh diameter of 3 mm at a temperature of 14 °C. After 12 h post fertilization (HPF), ~100 eggs from each batch were randomly sampled (in duplicate) to evaluate the fertilization rate before the maternal-to-zygotic transition (MZT), which occurs at around 13 HPF (Güralp et al., 2016), and then the embryonic development at the neurula stage (when the body of the embryo can already be easily seen, at around 3 days post fertilization). During eggs incubation, the temperature was raised to 15 °C when the embryos reached the eyed-egg stage and then to 16 °C as soon as the first spontaneously hatched larvae were observed. In Eurasian perch, hatching can last for 5 days, even for the same batch of eggs (Żarski et al., 2017d). Therefore, to ensure almost synchronous hatching, manual hatching was induced. Briefly, the eggs were moved to a bowl (each batch separately) and stirred gently. The hatched larvae were then moved back into the 15 L tanks. This operation was repeated several times until most of the larvae hatched. This moment was considered the end of hatching (0 days post hatching - DPH).

After hatching, the larvae were left for 24 h without any human interaction, and next, all the larvae within the families were volumetrically counted and stocked back to the rearing tanks with the same stocking density for each tank and each family (~ 2500 larvae per tank). Larvae from each batch were stocked into 3 separate tanks constituting separate replicates.

2.4. Larviculture protocol

Every family of Eurasian perch larvae was reared in triplicate in the same RAS conditions, following a set of validated and standardized larval rearing methods (i.e., advanced zootechnics) as described by Palińska-Żarska et al. (2020). The larvae were exposed to a specific thermal regime, photoperiod, and feeding schedule (Fig. 1A). The water temperature was automatically controlled throughout the rearing period. After hatching, at 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, 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; Palińska-Żarska et al., 2020). Starting from 4 DPH, the larvae began 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]) until weaning. From 17 DPH, the larvae were sharply weaned and then fed exclusively with dry feed (Perla Larva Proactive, Skretting, Norway) six times a day, pouring it into each tank in small doses, with intervals of 3 min, for approximately 15 min. At ~30 DPH, the experiment was completed, as with the temperature regime used, and the larval period was considered finished. During the rearing trial, the photoperiod was 24 L:0 D, and the light intensity measured at the water surface was 1500 lx. In addition, the oxygen level and ammonia concentration in the tanks were analyzed every two days. No oxygen level below 80% was ever observed, and the ammonia level was always lower than 0.02 mg L^{-1}. The tanks were cleaned twice a day (in the morning, 1 h after feeding, and in the evening just prior feeding), and the dead larvae were collected and counted under a microscope to assess the survival rate (%) throughout the experimental trial. In addition, from 14 DPH, dead larvae were observed under the microscope to assess the cannibalism rate by recording the number of larvae with damage to the body, especially the tail.

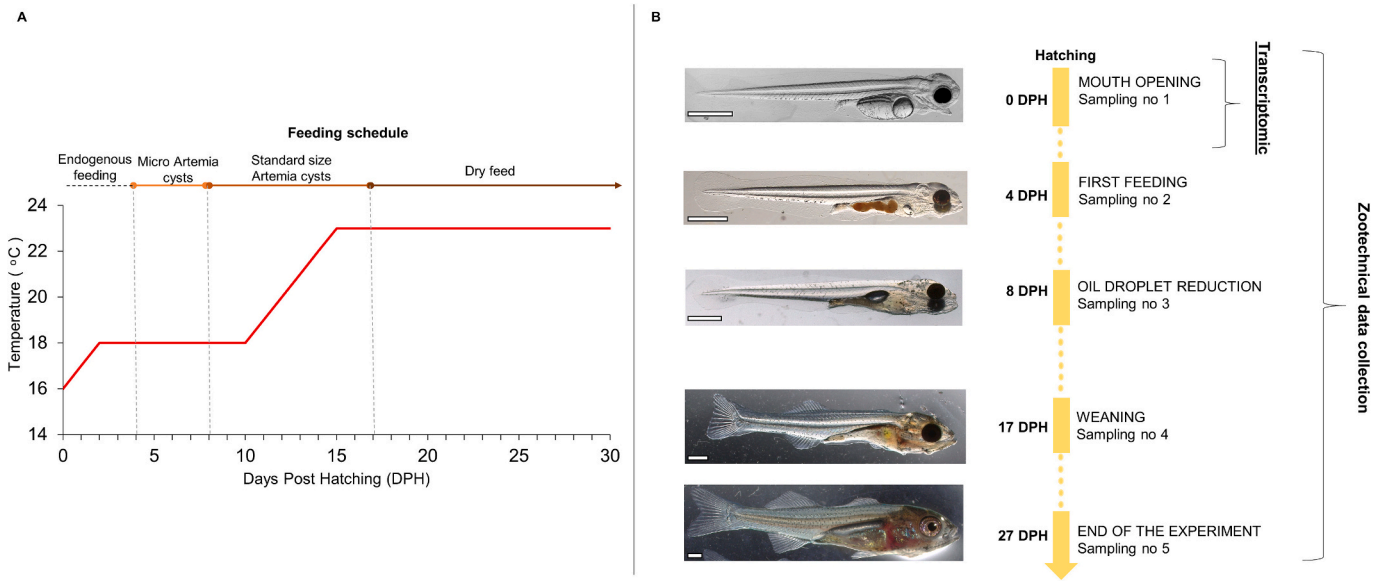

Fig. 1. (A) Temperature regime (red curve) and the feeding schedule followed during Eurasian perch larvae rearing. (B) Scheme of Eurasian perch larvae samplings at specific developmental stages. DPH – Days post hatching, sampling no 1 – moment of mouth opening in at least 50% of larvae, sampling no 2 – moment of exogenous feeding starting in at least 50% of perch larvae, sampling no 3 – oil droplet reduction in at least 50% of larvae, sampling no 4 – time of weaning with dry feed diet, sampling no 5 – end of experiment. Only larvae collected during sampling no 1 were used for transcriptomic analysis. At each sampling point 30 larvae per family were collected to measure total length and wet body weight. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Sampling activities and evaluation of zootechnical traits

Zootechnical traits of larvae were evaluated based on data obtained during five precisely chosen developmental stages (following Palińska-Żarska et al., 2021) (Fig. 1B):

1. At the mouth opening stage (0 DPH),
2. At 4 DPH, when at least 50% of larvae started exogenous feeding,
3. At 8 DPH, in at least 50% of the larvae, almost complete reduction of oil droplets was observed,
4. At the weaning stage (17 DPH),
5. At 27 DPH, considered the end of the experiment, when at least 50% of larvae had undergone the removal of the fin fold.

At each sampling point, 30 larvae per family were sampled to record total length (TL, ±0.01 mm) and wet body weight (WBW, ±0.1 mg). The individuals were first anesthetized (with MS-222, at a concentration of 150 mg L^{-1}) and then photographed under a stereoscopic microscope (Leica, Germany) to measure the TL. The WBW was determined with a precision laboratory scale by placing the anesthetized larvae on a nylon net (with a mesh size of approx. 200 μm) and draining the excess water with filter paper (Krejszeff et al., 2013). Additionally, two days after oil droplet reduction (10 DPH), the swim bladder inflation effectiveness (SBIE,%) was evaluated on approximately 100 larvae per family by triple counting (as described by Palińska-Żarska et al., 2020). Briefly, the individuals were first captured randomly from each tank, placed on a Petri dish, anesthetized and then counted under a stereoscopic microscope (individuals with and without a filled swim bladder).

2.6. Transcriptomics

2.6.1. Sampling and RNA extraction for molecular analysis

For transcriptomic analysis, larvae were sampled at the mouth opening stage. From each family, ten freshly hatched larvae were randomly selected, anesthetized, and preserved in RNAlater (Sigma-Aldrich, Germany) according to the manufacturer's instructions. RNA extraction was performed on these pooled samples ($n = 10$ per pool, one pool per family) using a TotalRNA mini-kit (A&A Biotechnology, Poland), as described by Palińska-Żarska et al. (2020). The quantity and purity of the extracted RNA were assessed using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA), ensuring absorbance ratios of A260/280–2.0 and A260/230–2.2. Additionally, RNA quality was confirmed with an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with all samples showing RIN values >9.0. In total, 16 RNA samples, representing each larval family, were prepared and submitted for RNA sequencing.

2.6.2. RNA-sequencing library preparation

Sixteen different libraries were created. RNA-seq analysis was performed by Macrogen (Amsterdam, Netherlands) using the TruSeq Stranded Total RNA kit (Illumina) with a NovaSeq600 platform, and 40 M 150 bp paired-end reads per sample were generated. The raw reads were quality controlled using FastQC software ver. 0.11.9 (Andrews, S.). Adapters and low-quality fragments of raw reads (average QPhred score < 20) were trimmed, 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* (GENO_Pfluv_1.0) obtained from the NCBI database (Sayers et al., 2022) using STAR software ver. 2.7.10a (Dobin et al., 2013) with ENCODE default options.

The annotation and estimation of the expression levels were performed using the StringTie tool ver. 2.2.0 (Pertea et al., 2015), with interpreting strand-specific sequencing the 'fr—firststrand' parameter was activated. Counts per transcript and gene were calculated using the prepDE Python script (https://github.com/gpertea/stringtie/blob/master/prepDE.py), and TPM and FPKM values were read from StringTie output data using the lncRna R library (Jastrzebski et al., 2023).

Functional annotation was performed using eggNOG-mapper (version emapper-2.1.9) (Cantalapiedra et al., 2021) based on eggNOG orthology data (Huerta-Cepas et al., 2019). Sequence searches were performed using DIAMOND (Buchfink et al., 2021).

2.6.3. Transcriptomic and Construction of the weighted gene co-expression network analysis (WGCNA)

To visualize the variability in transcriptomic data, a heatmap was constructed for the top 500 most variable genes using the pheatmap package (Kolde, 2022). Additionally, principal component analysis (PCA) was performed using the "prcomp" (package *stats* version 4.2.2; Team, R.C, 2021) to analyse the overall data variance. The gene counts were employed to perform weighted gene co-expression network analysis (WGCNA) with the R package, following the authors' recommendations (Langfelder and Horvath, 2008). Briefly, the hierarchical clustering of samples with Euclidean distance was used to check the presence of outliers (Fig. 2). After that, the total gene counts were first filtered by removing all genes with <10 counts in each sample. Subsequently, variance-stabilizing transformation was performed, using the DESeq2 package (Love et al., 2014). Then, to proceed with automatic blockwise network construction, the adjacency matrix was calculated, and a soft-power threshold ($\beta = 16$, leading to signed $R^2 = 0.85$, which was the best scale-free indicators for the current analysis) was chosen based on a scale-free topology model (Supplementary file S3, Fig. A). Then, a block network signed was constructed and gene modules were identified by hierarchical clustering dendrogram (Supplementary file S3, Fig. B). The relationships among modules are visualized by a hierarchical clustering dendrogram of their eigengenes (Supplementary file S3, Fig. C, D). After, the module eigengene (ME) distances were calculated in order to detect potential relationships of modules with the zootechnical traits collected during the experiment. This will result in gene significance (GS) values and the corresponding *p*-value for all the modules and traits. To visualize the associations between modules and traits, a module-trait heatmap is generated, offering a graphical representation of the correlation patterns. We focused on the significant modules with a $p < 0.05$ and the absolute value of the correlation coefficient $|cor| \geq 0.6$. Genes embedded in the significant modules of interest were then extracted to proceed with the GO Enrichment analysis.

2.6.4. Gene Ontology (GO) analysis

GO analysis was performed by following the approach described by Żarski et al. (2021). Briefly, for each transcript, a protein RefSeq accession number was obtained. Next, the RefSeq identifiers were used to align the sequences against human protein in Swiss-Prot with BLASTP. After the alignment, only the best match for each protein was retained, which allowed us to retrieve gene names and UniProt accession numbers for successfully aligned proteins, which were further used to perform GO analysis. GO analysis was performed using the ShinyGO online platform (Ge et al., 2020). First, GO analysis was performed for

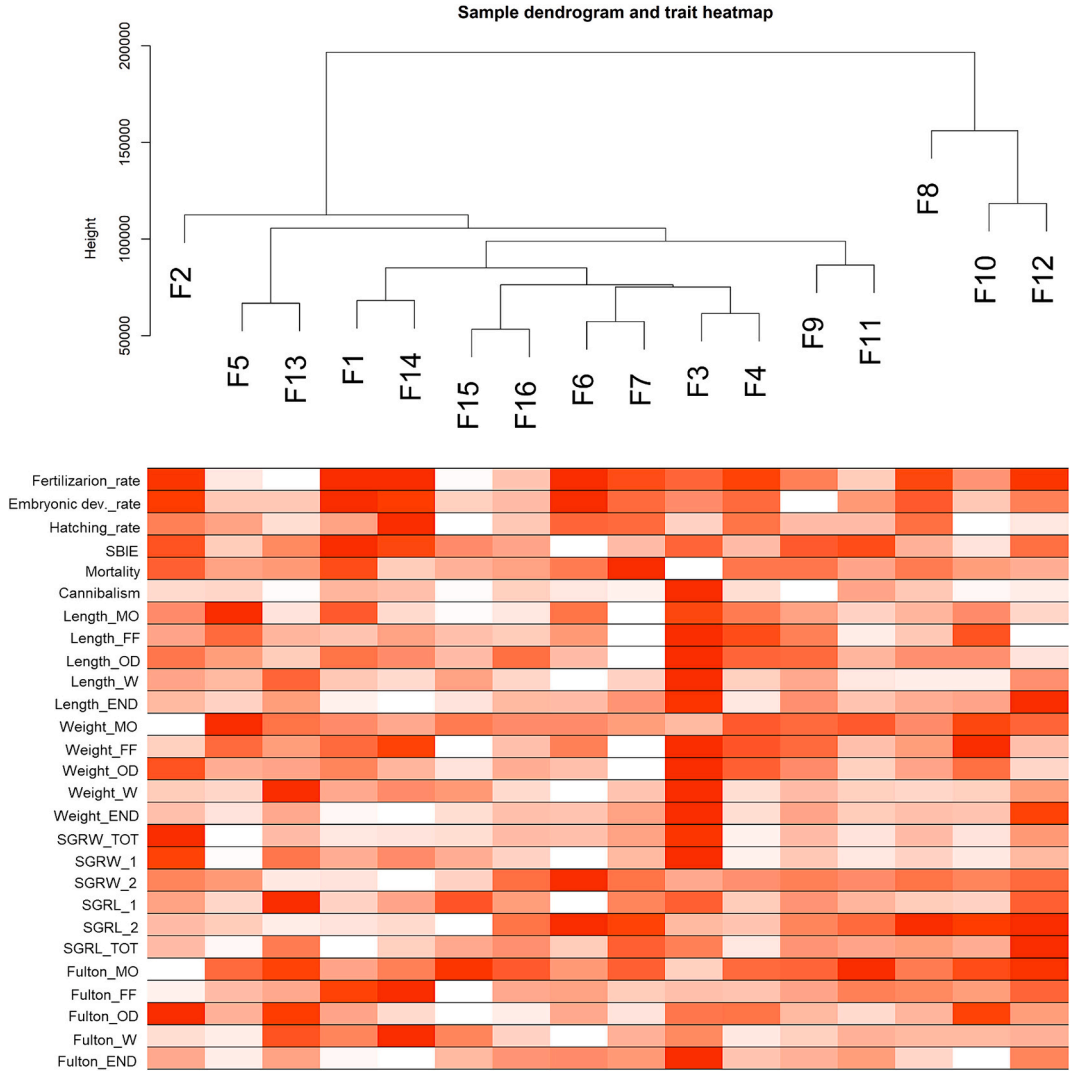

Fig. 2. Sample dendrogram based on their Euclidean distance and traits heatmap. The dendrogram plotted by hierarchical clustering (based on gene expression data) for the 16 families. The heatmap presented below the dendrogram represents an overview of the zootechnical traits for the corresponding families. Red color denotes higher values, while white signifies lower values of traits.

the genes incorporated in the significant modules obtained from the WGCNA, separately for the positively and negatively correlated modules, and the 10 most enriched biological processes were identified (FDR < 0.05). This allowed us to identify clusters that highlighted the most relevant biological processes. Next, the GO terms found in pre- and post-hatching were compared to identify specific traits unique to each period as well as those shared between them and the results were visualized using a Venn diagram (Bardou et al., 2014). Following the categorization of gene modules based on these two groups of zootechnical traits, we performed a comprehensive GO analysis, resulting in the identification of 100 enriched terms (FDR < 0.05). Subsequently, these terms underwent network analysis through hierarchical clustering. This approach facilitated the identification of distinct clusters, which were further characterized by conducting additional enrichment analysis on the gene list associated with each cluster. Through this process, we discerned the biological processes characteristic of each cluster.

2.6.5. Identification of hub genes

After identifying the most significant modules, we decided to examine the gene network within each module to identify hub genes, which could provide insights into the biological processes that the genes within each module may be involved in. The genes obtained from each module previously were mapped into the online search tool STRING database (Szklarczyk et al., 2017) (STRING, V12.0; https://string-db.org/), which could play a critical role in the protein-protein network (PPI). Then Cytoscape (Shannon et al., 2003) was used to visualize the networks and CytoHubba (a plugin of Cytoscape) was used to search for hub genes (Chin et al., 2014). The top nodes ranked by the Maximal Clique Centrality (MCC) algorithm in CytoHubba were seen as hub genes. Ten hub genes were selected for each significant module.

2.6.6. Key traits for aquaculture (KTA)

For WGCNA, we utilized all the collected larval traits to investigate their relationships with gene networks. However, to identify specific biomarkers related to particular traits, we focused on 7 key larval traits critical for the success of the aquaculture sector: mortality, cannibalism, SBIE, SGRL_TOT, SGRW_TOT, K, and the final weight of larvae at the end of rearing (Toomey et al., 2020). For this purpose, the GS values obtained from the WGCNA were used. The GS is the correlation of gene expression profile with an external trait. It quantifies the biological importance of genes, higher absolute GS values indicate greater significance, and these values can be either positive or negative. For the current analysis, we selected the most correlated genes (|cor| > 0.7; |cor| > - 0.7) for further validation using Real-Time quantitative PCR (qPCR) (Supplementary file S4).

2.6.7. Reverse transcription and real-time qPCR

Real-Time qPCR validation is widely used for the identification and validation of molecular biomarkers associated with specific traits (Akbarzadeh et al., 2020; Becker et al., 2013; VanGuilder et al., 2008). For this purpose, the top positively and negatively correlated genes for each chosen trait were validated through real-time qPCR. The results were then associated to the specific zootechnical data to assess their correlation coefficient (a value between −1 and + 1). For this purpose, total RNA was reverse transcribed using a 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 5× 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.

Real-time qPCR was performed using RT–PCR Mix SYBR (A&A Biotechnology, Poland). For each qPCR (20 μl), 10 ng cDNA template was used along with 10 μl of RT PCR Mix SYBR, 0.5 μM forward and reverse primers (designed with the Primer3Plus online platform (Untergasser et al., 2007) – Supplementary file S5), 0.4 μl of HiRox and sterile water. The reactions were conducted using ViiA7 real-time PCR systems (Applied Biosystems) with the following conditions: incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. After amplification, the efficiency of each primer was calculated using the Real-time PCR Miner program (Zhao and Fernald, 2005). Then, the changes in gene expression were analyzed using the delta delta Ct (2–ΔΔCt) method (Schmittgen and Livak, 2001) as a reference for the geometric mean of four reference genes, namely, acyl-CoA dehydrogenase long chain (*acadl*), thioredoxin 2 (*txn2*), glutathione s-transferase alpha 1 (*gsta1*), and wd repeat domain 83 opposite strand (*wdr83os*), which exhibited the most stable expression level (revealed based on the transcriptomic data obtained; Żarski et al. 2021).

2.7. Data analysis and statistics

In addition to the measurable zootechnical characteristics (i.e., mortality, length, weight, cannibalism, SBIE), the specific growth rate (SGR) and the Fulton condition factor (K) were calculated. Fulton's condition factor (*K*) was calculated based on the obtained measurements according to the formula: $K = 100\ (W/TL^3)$, where W = Weight and TL = total length. In addition, SGR (% day^{-1}) was calculated according to the formula: $SGR = 100\ ((\ln Wt - \ln W0)\ \Delta t^{-1})$, where W0 = mean initial weight of the fish (g), Wt = mean final weight of the fish (g), and Δt = number of days between measurements. The SGR was calculated considering 3 different conditions/approaches: SGRW_TOT, which refers to the entire rearing trial (0 DPH to 27 DPH), SGRW_1 relative to the weight of larvae from 0 DPH to 17 DPH and SGRW_2 for larvae from 17 DPH to 27 DPH. The same calculations were made for the length data of the larvae (referred to as SGRL_TOT, SGRL_1, SGRL_2).

After assessing the normal distribution and homogeneity of variance, one-way, two-way analysis of variance (ANOVA) and Tukey's post hoc test was conducted; additionally, Kruskal–Wallis' test and Dunn-Bonferroni post hoc correction were used for analysis of data that were not normally distributed. The samples were considered significantly different when $p < 0.05$. The statistical analysis was performed using R studio software ver. 4.2.3 (Team, R.C, 2021).

Spearman's correlation matrix was used in order to evaluate the relationships between the zootechnical traits collected for all the families. The matrix was constructed in R studio using the “corrplot” package v. 0.92 (Wei et al., 2017).

Additionally, a principal component analysis (PCA) for all the zootechnical traits was first performed in R studio using FactoMineR package (Lê et al., 2008), to possibly select key traits that could serve as indicators of overall larvae performance (Supplementary file S6). However, no specific trait emerged as predominant and all collected zootechnical traits were used to conduct WGCNA.

3. Results

All the supplementary figures are gathered in the Supplementary file S7 and are referenced hereinafter as Fig. S1-S15.

3.1. Zootechnical performance of larvae

Fertilization rate in all fish larval families varied between 63% and 97%. Surprisingly, these data didn't show any significant difference between all the 16 families (marked as F1 to F16 in Fig.3). Nevertheless, F9 was found to have significantly lower ($p < 0.05$) embryonic developmental rate (after maternal-to-zygotic transition -MZT-) compared to F1 and F6. In addition, two-way ANOVA, which compared the pre- and post-MZT period for each family of larvae, revealed considerable post-MZT mortality in only two families of larvae (F9 and F10; Fig.3), indicating relatively high consistency between the fertilization rate and post-MZT embryonic survival.

Among the remaining zootechnical traits, the analysis revealed significant differences ($p < 0.05$) between families for mortality,

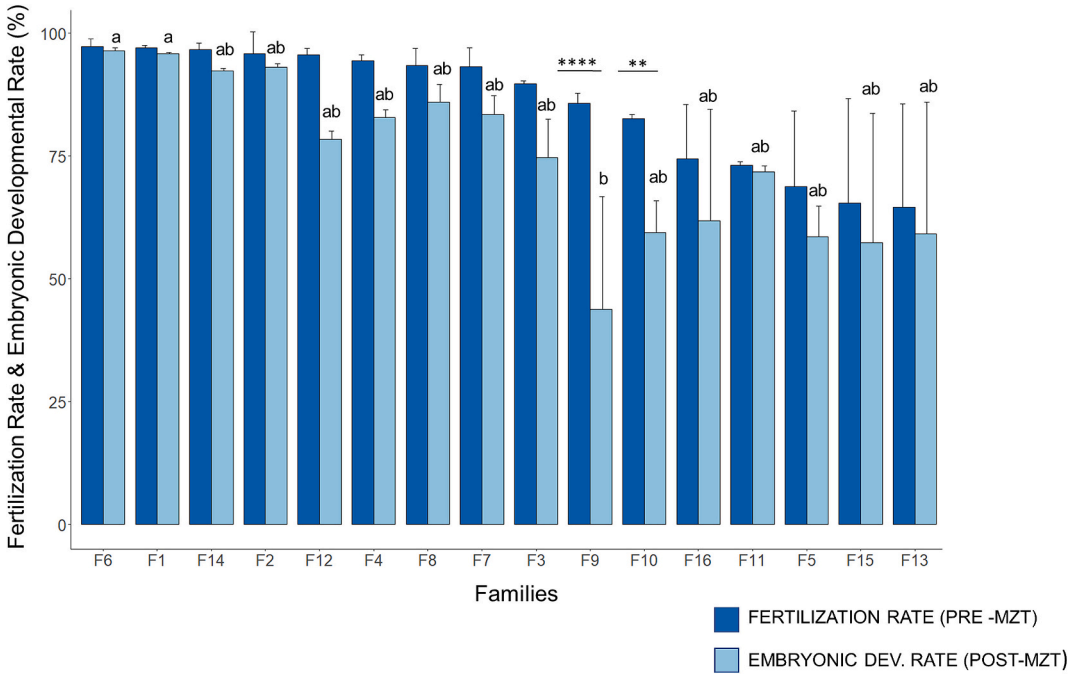

Fig. 3. Fertilization rate and embryonic developmental rate of 16 families of E. perch. The data are arranged in descending order according to the mean values of the fertilization rate between which no significant differences ($p > 0.05$) were recorded. Letters indicate significant differences ($p < 0.05$) between the families for the embryonic development rate. The asterisks (** – $p < 0.01$, ****– $p < 0.0001$) indicate significant differences within the families and between fertilization and embryonic development rate.

cannibalism (Fig. S1A,B), length and weight of larvae throughout almost the entire rearing period and SGR (for both length and weight) (Fig. S2-S5). However, there were no significant differences for weight of larvae at mouth opening and first feeding stage and also for the SBIE (Fig. S1C; S3A,B). Also, no statistical differences ($p > 0.05$) were observed for K across different developmental stages (Fig. S6), except for larvae at the end of the experiment.

During the entire rearing trial, the mortality rate of each family was also recorded daily. The cumulative mortality graph (Fig. 4) shows a peak of mortality in mostly all larval families at oil droplet reduction stage.

To identify relationships among zootechnical parameters, a Spearman's correlation (r_s) matrix was constructed (Fig.5). Interestingly positive correlations between the embryonic developmental rate parameter and fertilization/hatching rates (respectively $r_s = 0.89$ and $r_s = 0.7$) are shown. Noteworthy, robust correlations ($r_s \geq 0.6$ or $r_s \geq -0.6$) are observed for the growth-related traits. For instance, length of the larvae at mouth opening correlates positively with length at the first feeding ($r_s = 0.67$), weight at first feeding ($r_s = 0.66$) and at oil droplet reduction ($r_s = 0.63$). Specific growth rate of weight for the entire larviculture period (SGRW_TOT) is negatively correlated with larval weight at mouth opening stage ($r_s = -0.61$), while it is positively related to weight and length of larvae at the end of the experiment (respectively, $r_s = 0.86$, $r_s = 0.73$). Alternatively, the negative relationship between *K* at weaning (Fulton_W) and the specific growth rate for weight data from weaning to the end of the larval period (SGRW_2) ($r_s = -0.65$). Also, the matrix shows negative correlations between *K* at mouth opening (Fulton_MO) and fertilization and embryonic developmental rate ($r_s = -0.65$, $r_s = -0.63$) (Fig. 5). This can be interpreted as the larvae obtained from lower quality eggs appear to be much more robust, resulting in a better *K*.

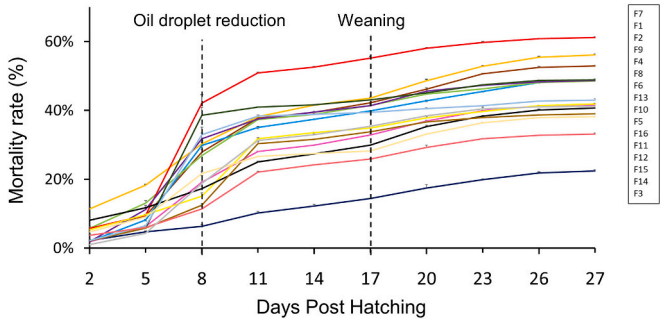

Fig. 4. Cumulative mortality (mean ± SD) of 16 families of E. perch larvae. Different colors stand for different families. The latter are sorted in descending order of final mortality rate recorded (they are listed in the box at the top right of the graph).

Principal component analysis has been employed to identify key traits that could serve as indicators of overall larvae performance, and initially only these indicators were intended to be used for WGCNA analysis. Based on the results (Supplementary file S6), however, growth-related traits (i.e., weight and length at different developmental stages), specific growth rates and development, and survival traits (i.e., fertilization, embryonic developmental rate, and hatching rate) collectively provided a comprehensive assessment of larvae phenotypic profile, but none of them could be used separately. Therefore, as no specific trait emerged as predominant, we opted to conduct WGCNA using all the collected zootechnical traits.

3.2. Transcriptomic data and WGCNA analysis

After RNA-sequencing, 30,744 genes were initially identified. Following filtering procedures (explained in paragraph 2.6.3 in Material and Methods), 19,656 unique genes has been identified and further used for WGCNA. Visualization of 500 most variable genes (Fig. 6A) and PCA plot (Fig. 6B) illustrate significant transcriptomic diversity among the 16 families of Eurasian perch. These differences in gene expression profiles suggest unique genetic and phenotypic characteristics of each family studied. This variability observed led us to undertake identification of promising genetic markers associated with desirable traits for

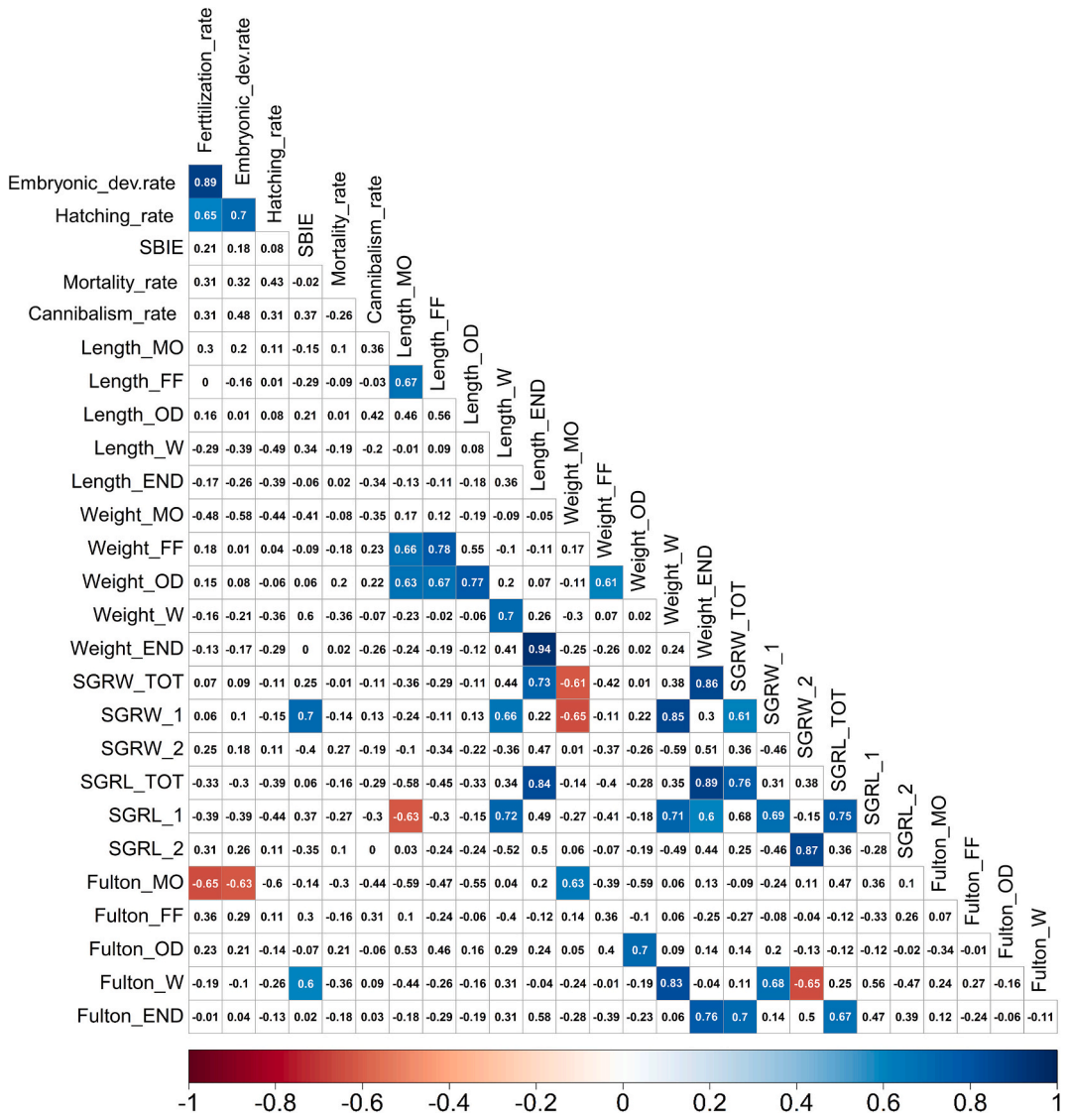

Fig. 5. Spearman's correlation matrix of all the zootechnical traits and for all the families together. Correlation coefficients (r_s) are shown in the squares, with significant correlations ($r_s \geq 0.6$, $p < 0.05$) indicated by colored boxes. Red colors show significant negative correlations, blue shows significant positive correlations, and white shows insignificant correlations. SBIE: swim bladder inflation effectiveness, MO: mouth opening, FF: first feeding, OD: oil droplet reduction, W: weaning, END: end of the experiment, SGRW: specific growth rate for weight, SGRL: specific growth rate for length data, SGR_TOT: specific growth rate for the entire larviculture period,
SGR_1: specific growth rate from hatching until weaning stage, SGR_2: specific growth rate from weaning stage until the end of the experiment, Fulton: Fulton's condition factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

aquaculture using WGCNA. For this purpose, a gene cluster dendrogram was initially constructed, yielding 28 distinct gene modules (Supplementary file S3, Fig. B). Next, correlation analysis between these modules and zootechnical traits are shown in the module-traits heatmap (Fig. 7). Notably a total of 13 modules with $p < 0.05$ and correlation coefficient $|\text{cor}| \geq 0.6$ were prioritized for further scrutiny. Specifically, 7 modules exhibited notably robust correlations ($r \geq 0.6$) with embryonic developmental rate and hatching rate, indicating their association with pre-hatching traits. Conversely, 9 modules displayed significant correlations with post-hatching traits, specifically the weight of larvae at mouth opening, length at first feeding, K at mouth opening and oil droplet reduction stage. Notably, modules black, pink, and turquoise demonstrated shared correlations with both pre-hatching and post-hatching traits, suggesting their importance across different developmental stages.

The analysis of hub genes within significant WGCNA modules reveals key biological processes underpinning the transcriptomic diversity among Eurasian perch families (Fig. 8). The black module is associated with mitochondrial function, highlighted by ribosomal protein genes, while the brown and royalblue modules emphasize extracellular matrix organization through collagen and fibronectin genes. Chromatin modification and transcription regulation are central in the cyan module, featuring genes like *kmt2a* and *gata3*. Calcium signalling is prominent in the turquoise module, with multiple calcium channel genes. Transport and detoxification processes are represented in the darkgreen and white modules, involving solute carriers and cytochrome P450 pathways. The red and purple modules are linked to transcription regulation and cellular stress response, and lysosomal function, respectively, while the lightgreen and lightcyan modules focus on synaptic function and cell division. Finally, the pink and darkred modules highlight ribosome biogenesis and energy metabolism.

Subsequent Gene Ontology (GO) Enrichment analysis was performed for those 13 modules. This analysis revealed enriched biological processes associated with specific traits. Regarding the embryonic

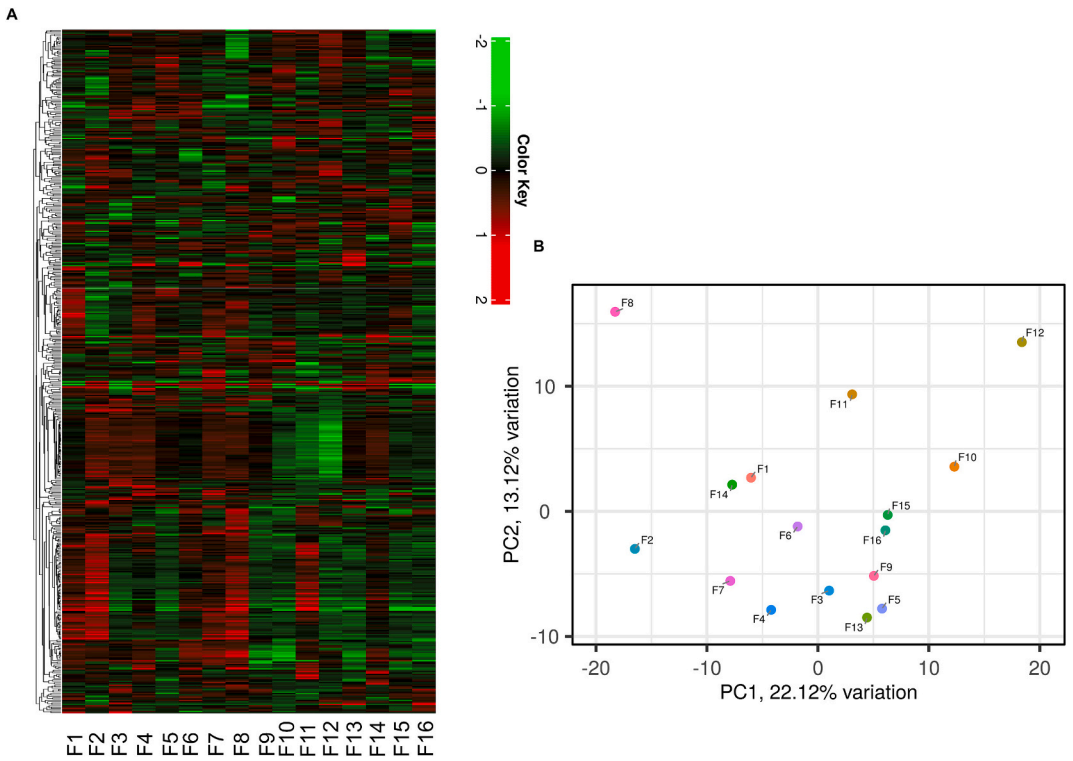

Fig. 6. (A) Heatmap of top 500 genes between 16 families of Eurasian perch larvae at mouth opening stage. Each row represents a gene and each column represents a sample. The color represents changes of gene expression where red shows increased expression and green decreased expression. (B) Principal component analysis showing samples distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

developmental rate, negatively correlated modules were associated with terms linked to ribosome biogenesis, RNA processing, and modification (Fig. S7A). Conversely, positively correlated modules were enriched in terms related to morphogenesis, circulatory system development, and response to endogenous stimuli (Fig. S7B). For hatching rate, GO analysis indicated a positive correlation with processes primarily involved in neurogenesis, while negatively correlated processes included those related to protein transport and metabolic processes such as organic acid and carboxylic acid metabolism (Fig. S8A,B). Modules significantly correlated with larval weight at mouth opening were exclusively positively associated, with GO analysis implicating mechanisms related to RNA processing and ribosome biogenesis (Fig. S9). In contrast, length at first feeding showed negative correlations with two modules, revealing processes associated with neurotransmitter transport, transmembrane transport, ion transport, and chemical synaptic transport (Fig. S10). The analysis of negative modules for Fulton's condition coefficient at mouth opening were associated with genes related to neurogenesis, neuron generation, and differentiation (Fig. S11A), while positive modules for Fulton's condition coefficient at mouth opening indicated involvement in translation, ribosome biogenesis, and metabolic processes (Fig. S11B). Furthermore, significant modules were identified for K at mouth opening and the oil droplet reduction phase. The latter negatively correlated with processes involved in the mitotic cell cycle, chromosome segregation, and nuclear division (Fig. S12).

In summary, it is crucial to note that the transcriptomic profile observed at the larvae's mouth opening stage is shaped by genes that correlate with traits seen before and after hatching, encompassing a variety of functions. Among the enriched biological processes, those related to regulation of transcription processes, cell differentiation and signal transduction are linked to events occurring before hatching. In contrast, processes associated with mitotic cell cycle are specific of traits manifesting after hatching (Fig. S13B). Intriguingly, there are common biological processes identified for both pre- and post-hatching traits, particularly those related to neurodevelopment (Fig. S13C). This suggests that neurodevelopment plays a key role in driving both embryonic and larval development stages.

3.3. Key traits for aquaculture (KTA)

WGCNA employs significant calculations to pinpoint genes intricately associated with targeted traits. These findings facilitate the identification of most correlated genes for traits such as mortality, cannibalism, SBIE, SGR for total weight and length, K and weight of larvae at the end of the experimental period. The results unveil a comprehensive table showcasing genes with high correlations to each examined parameter (Supplementary file S4), offering valuable insights into the molecular underpinnings of these traits. The qPCR validation confirmed the association of specific genes, such as *selenoprotein O (selenoo), tripartite motif-containing protein 16 (trim16), solute carrier family 15 member 1 (slc15a1), clock-interacting pacemaker (cipc)*, with the traits under investigation, as outlined in Table 1 (see also Fig. S14). These genes are constituting candidate markers which could serve to predict the traits they are significantly correlated with. Additionally, Fig. S15 illustrates the trendlines for candidate genes that were not successfully validated using qPCR.

4. Discussion

Studies conducted on the early developmental stages of larvae can provide valuable insights into the factors that influence, and consequently predict their future performance. Despite advancements, our comprehension of the mechanisms responsible for successful development and survival remains limited. By combining traditional zootechnical assessments with transcriptomic analysis, our research yielded significant insights into larval biology paving the way for understanding intricate growth changes and molecular mechanisms that contribute to the performance of fish larvae. Examining 16 distinct larval families, originating from diverse parental pairs, facilitated an in-depth

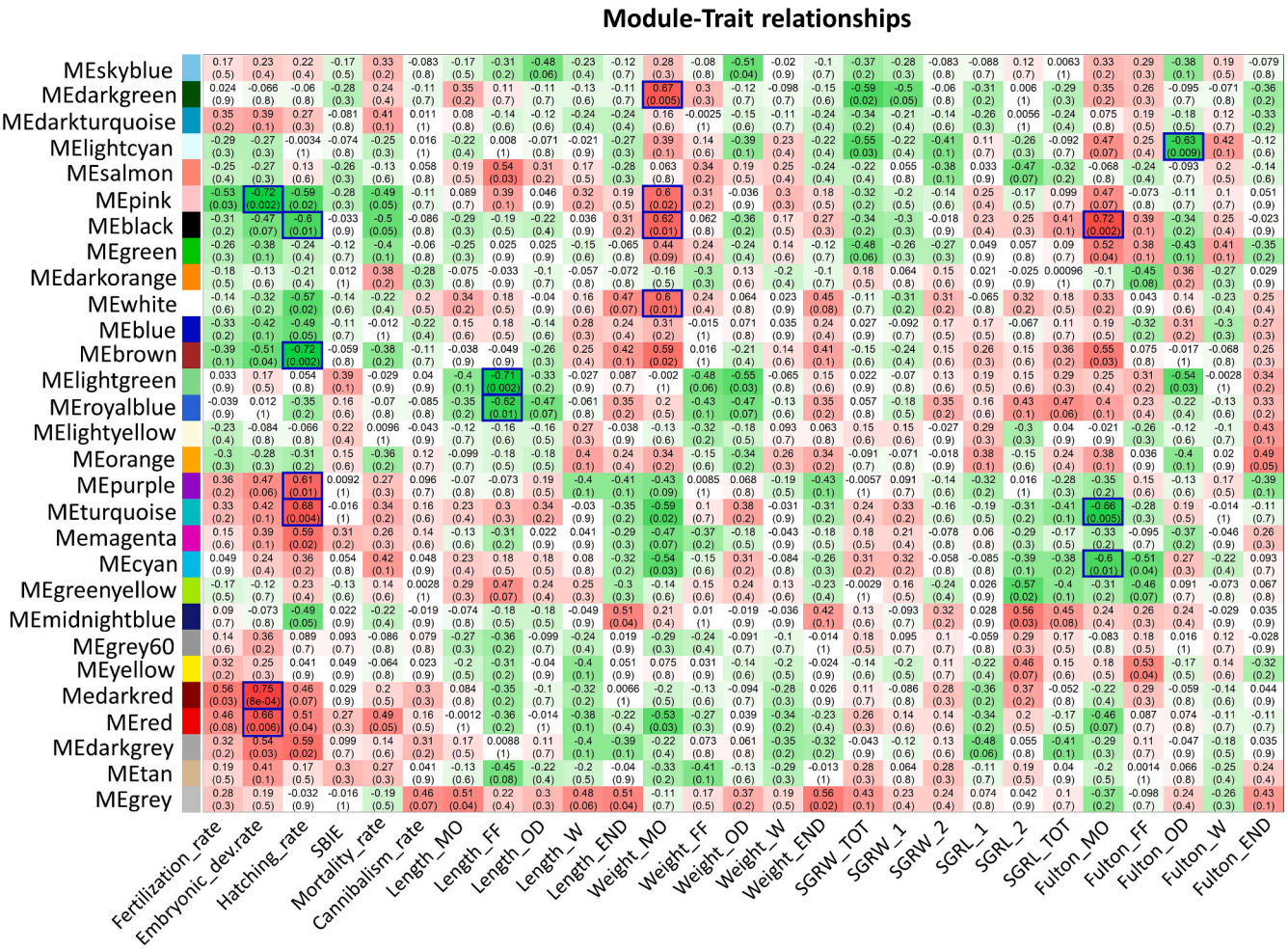

Fig. 7. Module-traits relationship. The module eigengene (ME) is shown in each row. Genes not assigned to any of the other modules are included in the grey module. The columns represent the zootechnical traits. The modules with high correlation values and $p < 0.05$ were identified as significant trait-related modules. The colors indicate the positive (red) and negative (green) correlations between gene modules and traits. SBIE: swim bladder inflation effectiveness, MO: mouth opening, FF: first feeding, OD: oil droplet reduction, W: weaning, END: end of the experiment, SGRW: specific growth rate for weight, SGRL: specific growth rate for length data, SGR_TOT: specific growth rate for the entire larviculture period, SGR_1: specific growth rate from hatching until weaning stage, SGR_2: specific growth rate from weaning stage until the end of the experiment, Fulton: Fulton's condition factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

exploration of larval transcriptomes. The data presented draw our attention to the fact that the molecular profile of freshly hatched larvae (at mouth-opening stage) is highly indicative of pre-hatching events. This emphasizes crucial role of parental contribution in shaping the larval transcriptomic landscape. However, our findings shed also light on the importance of the larvae's transcriptomic profile in determining their future performance, being a solid foundation for further investigations.

4.1. Zootechnical traits

Overall, the observed growth patterns (in terms of weight and length) align with previous studies on Eurasian perch (Kupren et al., 2019; Palińska-Żarska et al., 2020). Additionally, there were no major differences observed in cannibalism intensity and SBIE when compared to previous experiments that exposed the larvae to similar experimental conditions (Kupren et al., 2019; Palińska-Żarska et al., 2020). In addition, larval mortality increased especially at the oil droplet reduction phase, reflecting the challenges encountered by the larvae when they switch entirely to exogenous feeding (Kestemont et al., 2003; Król et al., 2019; Palińska-Żarska et al., 2020). This phenomenon could be linked to the non-feeding behaviour, commonly observed in fish larval species (Yúfera and Darias, 2007).

Upon closer observation of the data obtained, although all families were reared under the same controlled conditions, and within-family genetic variability has been limited by creating them each time from a single pair of spawners, considerable variation between families in terms of zootechnical traits was observed, which reflected different performances. Growth-related differences likely influenced cannibalistic tendencies and overall fish development. Such growth heterogeneity has been commonly detected in many other fish species and has been always considered as a relevant determinant and predictor of fish survival, since it can lead to aggressiveness and mortality (Baras and Dalmeida, 2001; Carvalho et al., 2018; Kestemont et al., 2003).

The correlation analysis between various zootechnical traits provides a comprehensive overview of the interrelationships among qualitative traits, elucidating the overall growth trajectory of the offspring. Notably, a positive correlation between embryonic developmental rate and hatching rate suggests that embryos which passed the MZT mostly hatched successfully, supporting the significance of embryonic developmental rate as a reliable indicator of egg developmental competence (Bobe, 2015). Additionally, the analysis illustrates a positive correlation between the length and weight of fry at each sampling time point, indicating that larvae tend to become more robust as their length

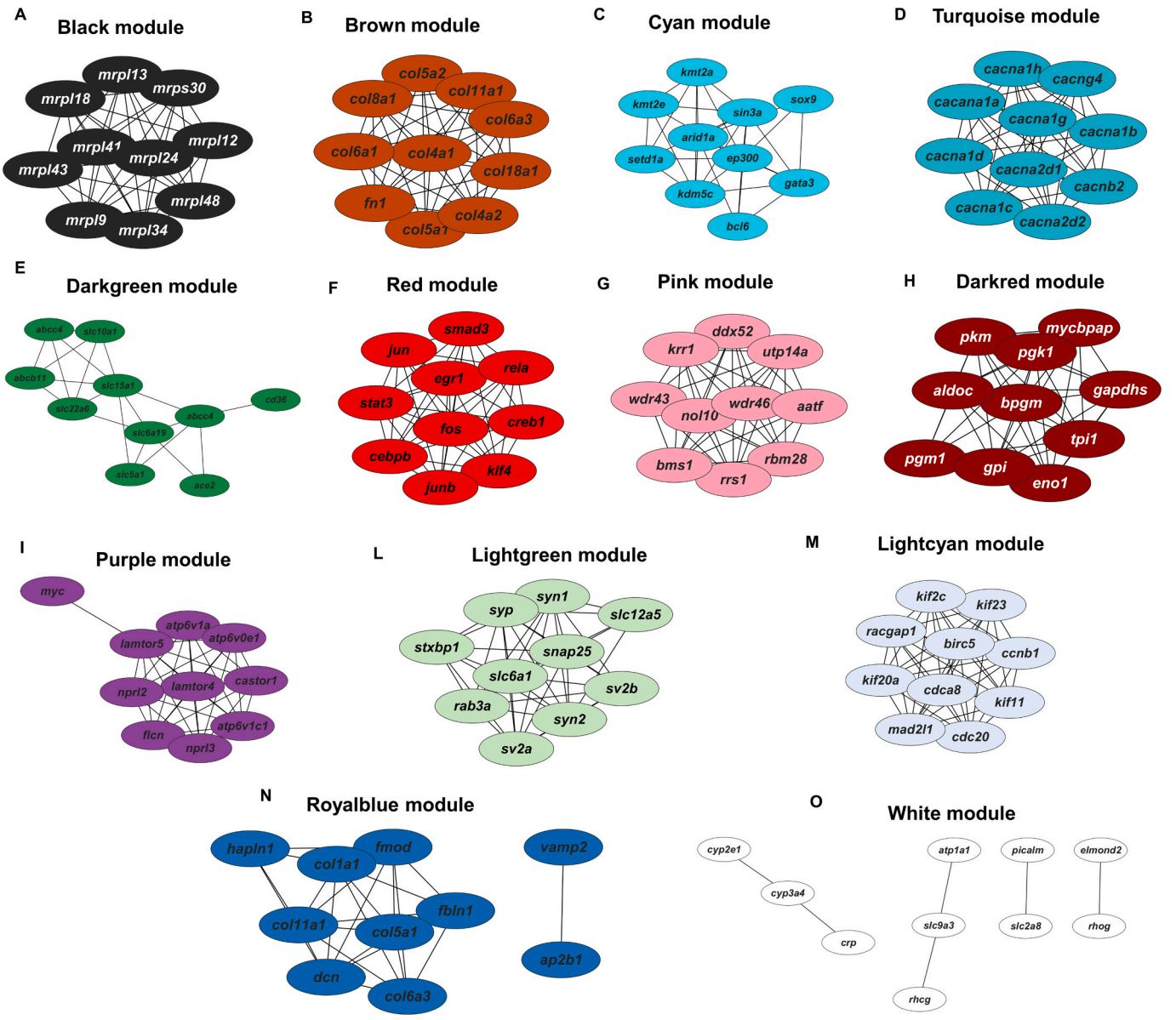

Fig. 8. Visualization of modules of interest and hub genes. Color of the nodes is associated with the color of the modules identified with the WGCNA analysis. The top 10 genes with the highest levels of intramodular connectivity in all the significant modules.

Table 1
Most correlated genes (both positive and negative) selected for each chosen commercially relevant traits, further validated with qPCR. The resulting table includes the gene significance (GS) values obtained from weighted gene co-expression network analysis (WGCNA), correlation values from qPCR. SBIE: swim bladder inflation effectiveness, SGRW_TOT: specific growth rate for weight for the entire larviculture period, SGRL_TOT: specific growth rate for length for the entire larviculture period, Fulton_END: Fulton's condition factor at the end of the experiment.

	CANNIBALISM		MORTALITY		SBIE		WEIGHT END		SGRW_TOT		SGRL_TOT		FULTON_END	
	selenoo	*nudt12*	*mkx*	*crcp*	*si:dkey-117 m1.4*	*pycard*	*aoc1*	*cipc*	*prdm1*	*txnl4a*	*atp8a2*	*slc15a1*	*sec14l2*	*trim16*
GS	0.82	−0.85	0.77	−0.81	0.79	−0.78	0.81	−0.82	0.84	−0.84	0.87	−0.80	0.81	−0.80
qPCR	0.78	−0.31	−0.02	−0.18	−0.32	−0.33	−0.02	−0.70	0.01	−0.24	0.13	−0.72	0.04	−0.55

increases. Conversely, negative correlations, such as those observed between the weight of larvae at mouth opening and SGR from hatching until weaning stage and at the experiment's end, may signify a sort of compensatory mechanism in growth between early and later developmental stages. This phenomenon is commonly observed and has been documented for various fish species (Ali et al., 2003).

The observed correlations between embryonic developmental rates, fertilization and hatching rates, and between growth-related traits, provide insights into larval development dynamics and confirms that these parameters as valuable predictors of future fish performance and overall fitness (Brooks et al., 1997; Koumoundouros et al., 2017). Also, the variability observed in zootechnical traits despite identical rearing conditions implies the involvement of inherited parental factors shaping larvae features. This underscores the need to investigate the molecular background of larvae to elucidate this phenomenon.

4.2. Transcriptome data analysis - between past and future

The molecular profile of fish larvae can be influenced by environmental factors (e.g., temperature) as well as genetic and non-genetic inherited factors (Adrian-Kalchhauser et al., 2020). These factors collectively affect transcription, thereby shaping the final transcriptome of hatched larvae. In our current study, we maintained identical incubation conditions for the eggs, ensuring that the analyzed larval transcriptomic profiles primarily reflect solely parental contributions. Thus, in this study we show that certain portion of genes (the ones correlated with pre-hatching traits) are well reflecting parentally-derived genes variability shedding light on parental contribution to transcriptomic portrait of freshly hatched larvae. However, there is also an other portion of genes, the one correlated with post-hatching traits, indicating that this transcriptomic variability is also related with future performance of the larvae.

WGCNA identifies gene modules linked to larval traits, aiming to pinpoint specific zootechnical indicators and their dependency with the larval transcriptome as well as to reveal underlying molecular mechanisms crucial in the early phases of fish growth. The results underscored significant correlation between gene networks and pre-hatching parameters, as embryonic developmental and hatching rates, as well as post-hatching traits, i.e., weight at mouth opening, length at first feeding, K at mouth opening and at oil droplet reduction stage. Overall, the GO enrichment analysis of the genes identified within modules exhibits diverse functional processes, including cell cycle, RNA processing, ribosome biogenesis, protein trafficking, apoptosis, circulatory system regulation, and neurogenesis. Notably, the functions of the identified hub genes within significant modules (Fig. 8) mostly align with the biological processes highlighted through GO analysis of all the genes in the significant modules. This underscores their importance in regulating key biological functions, indicating their potential as critical targets or biomarkers in specific biological contexts. The consistency of these findings across different fish species (Mazurais et al., 2011; Bougas et al., 2013) further emphasizes the significance of these biological processes in shaping larval performance and underscores also its complexity in fish.

Embryonic developmental rate and hatching rate, were negatively correlated to genes involved in RNA processing, translation, ribosome biogenesis and protein transports. These processes collectively govern gene expression, cell signalling, and tissue differentiation, profoundly impacting fish larval growth and survival. Particularly, ribosomes produced abundantly during oogenesis and presumably deposited in the eggs, play a crucial role in synthesizing proteins vital for various developmental processes (Leesch et al., 2023; Qi et al., 2016; Shen et al., 2017). These maternally provided ribosomes are paramount during embryogenesis specifically until Zygotic Genome Activation (ZGA), which marks the transition of developmental control from maternal to zygotic factors (Leesch et al., 2023). Since the pre-hatching traits were negatively linked to these biological processes, our speculation is that post-ZGA, embryos likely redirect their energy towards processes -positively correlated with pre-hatching traits – like neurogenesis, sensory organ development, and morphogenesis of tubes and blood vessels, which are equally necessary for their future developmental success. These results indicate that embryos may prioritize these processes closer to hatching. The embryos, as well as post-hatching larvae, are constantly subjected to morphological and physiological modifications. Among others, the maturation of the nervous system is one of the most important events. This process is crucial for enhancing sensory perception, motor coordination, and cognitive functions in larvae (Nelson and Granato, 2022). Previous studies discussed the nervous systems' development could potentially be pre-programmed by the molecular content inherited maternally (Żarski et al., 2020b, 2021). This, along with environmental factors may influence the trajectory of nervous system development and impact future behaviour and adaptive responses (Colson et al., 2019). In summary, right after fertilization more general but crucial functions are prioritized (i.e., ribosome biogenesis), while after ZGA processes essential for successful accomplishment of embryonic development take precedence. These subsequent events will most likely define the success of the hatching.

Molecular profile of the larvae is determined by the interplay between environmental factors, genetic background and so called non-genetic inheritance mechanisms. The latter encompass, among others, mRNAs which play a pivotal role as modulators of gene expression during the early development and consequently influencing the phenotype of the progeny (Adrian-Kalchhauser et al., 2020). It has been hypothesized that such cascade-like transmission of information from parent to progeny affects the performance of larvae and juveniles (Adrian-Kalchhauser et al., 2018; Colson et al., 2019). In this context, results of our study highlight the importance of parental contribution in shaping the transcriptomic profile of the larvae (Harvey et al., 2013), reflecting their past. Analogically, the significant correlations with the post hatching traits are predictive of the larvae's future. Notably, those traits fall within the initial growth phases until yolk sac absorption. Up to this stage, larvae rely primarily on the nutrients stored within the yolk sac, which they inherited from the female (Callet et al., 2022). These nutrients play a crucial role in sustaining the larvae's early development by determining energetic reservoirs and indirectly controlling their growth and performance (Bachan et al., 2012; Migaud et al., 2013). The GO enrichment analysis of gene modules linked to post-hatching traits aligns with molecular processes identified in pre-hatching traits. However, contrarily to pre-hatching traits, genes associated with ribosome biogenesis exhibit a positive correlation with post-hatching traits, while those linked to neurogenesis show a negative correlation. This inversion may suggest a shift in biological process prioritization as larvae transition, depending on their developmental stage (Mathavan et al., 2005). For example, after hatching larvae may prioritize protein production to overcome crucial metamorphosis events (e.g., onset of exogenous feeding, swim bladder inflation, etc). We can hypothesize that bigger larvae at mouth opening invest more in protein translation, potentially leading to enhanced physiological development and growth rates. Also, upon first feeding larvae that exhibit larger sizes, may have already better-developed senses and a more advanced nervous system, which can explain the inverse correlation with neurogenesis pathways at this stage.

Post-hatching traits exhibit a specific association with processes linked to the cell cycle and mitosis. As larvae undergo metamorphosis and confront various challenges related to the interaction with the external environment, significant restructuring of organs and tissues occurs, with the cell cycle serving as a central mechanism (González-Quirós et al., 2007). This transition likely initiates a cascade of molecular events, indicating a profound shift in cellular activities towards enhancing the growth and development of various organs to support their functions. For instance, after the oil droplet reduction stage, larvae become entirely dependent on external feed for energy, necessitating the digestive system to efficiently process and utilize food resources. Rapid cell division and proliferation may play critical roles in optimizing system functions, enabling larvae to better adapt and respond to environmental cues.

Overall, the contrasting correlations between ribosome biogenesis and neurogenesis well reflect the dynamic nature of larval development. As larvae progress through various stages, their biological priorities shift, leading to fluctuations in gene expression patterns and molecular processes. Understanding these intricate relationships between molecular processes and larval development provides valuable insights into the adaptive strategies of organisms and the mechanisms underlying developmental plasticity. Nonetheless, the environmental factors still play a significant role in shaping these developmental trajectories. Changes in temperature, light exposure, nutrient availability, and other environmental cues can modulate gene expression and influence the balance between different biological processes (Mazurais et al., 2011; Urho, 2002). Our results indicates that transcriptomic signature of

larvae at the mouth opening stage offers significant insights into parental contributions and their influence on embryogenesis. However, its ability to predict future larval performance appears to be somewhat limited. It should be emphasized, that future larval fate is determined by the interaction of their molecular cargo with the external factors playing a major role right after hatching. It's essential to acknowledge that in our 'common garden' experiment all the environmental factors were controlled and equal for all larvae families. This may limit the detection of certain traits typically observed in wild conditions, such as exposure to pathogens or stressors. For instance, immune system traits may not be evident in our current setup, but exposing organisms to bacterial or temperature challenges could reveal the transcriptome's predictive capacity for stress or immune system functioning, which is important in fish adaptability (Elabd et al., 2017; Kammer et al., 2011; Palińska-Żarska et al., 2021). Therefore, future research should consider subjecting organisms to specific challenges to uncover additional predictive traits.

Transcriptomics is an important tool for phenotypic profiling, as it reveals gene expression patterns and their correlation with observable traits (Chandhini and Rejish Kumar, 2019). However, transcriptomics alone provides only part of the picture. To gain a more comprehensive understanding of biological processes and accurately predict phenotypic outcomes, it is crucial to integrate other omics technologies such as proteomics and metabolomics. Proteomics analyses proteins, the direct effectors of cellular functions, while metabolomics examines metabolites, the end products of cellular processes. Combining these approaches with transcriptomics offers a more holistic view of the phenotype, reflecting both genetic information and its functional outcomes (Natnan et al., 2021; Subramanian et al., 2020). Therefore, we propose that future research should adopt a multi-omics approach to improve the precision and reliability of phenotypic predictions.

4.3. *Key traits for aquaculture (KTA) - molecular signatures -*

Aquaculture relies on important traits like cannibalism, survival rate, swim bladder inflation, weight, and specific growth rate for successful management and production (Toomey et al., 2021). The transcriptome of newly hatched larvae may offer predictive insights into future development, supporting aquaculture research and its production. The WGCNA has facilitated the identification of genes strongly associated with these commercially relevant traits, potentially serving as specific gene markers.

The validation with qPCR highlighted 4 genes: *selenoo, trim16, slc15a1, cipc*; linked respectively to cannibalism, SGR for length, K and weight of larvae at the end of the experiment. Notably, *cipc* appears pivotal in regulating various physiological processes in fish, influencing behaviour, metabolism, and also clock genes seem to be implicated in thermal resistance (Hung et al., 2016); while *slc15a1* affects nutrient absorption, transport and fish growth (Romano et al., 2014; Vacca et al., 2019). *Trim16*, a member of the teleost-specific *fintrim* family (van der Aa et al., 2009), is involved in regulation of innate immunity, but it seems also involved in cell proliferation, differentiation, and metabolism (Cho et al., 2022). While its specific functions in fish larvae, particularly regarding traits like Fulton's condition factor, may require further investigation, its involvement in cellular processes suggests potential roles in larval development and physiology. Also, selenoproteins, including *selenoo*, contribute to cellular antioxidant defence mechanisms (Han et al., 2014; Sumana et al., 2023), and indirectly, they can play a role in fish growth.

These genes collectively play crucial roles in fish larval development and physiology, impacting growth, behaviour, immunity, and metabolic processes. However, out of the 14 chosen genes, only 4 were confirmed through qPCR validation. This suggests that our approach (i.e., finding gene markers based on correlation of their expression with traits) may not be entirely reliable for detecting gene markers associated with zootechnical traits. Future studies should use more relevant methods, such as conducting specific experiments that assess each trait and individual larva. This approach will help to gain a deeper and more accurate understanding of the variability in the traits expressed within larval populations. This would provide a clearer understanding of the mechanisms and interactions underlying larval traits and overall fish performance.

5. Conclusion

Considering all the above mentioned, larval transcriptomic profile represents a bridge between the past, stemming from the transcripts provided by parents within gametes and continuing through embryonic development, and the future, serving as a forthcoming instruction for the larval period and potentially beyond. Taking all of this into account, by analysing transcriptome of freshly hatched larvae we gather information on parentally derived molecular cargo but also on larvae adaptability, paving the way to comprehend the intricate developmental trajectories that lead to adulthood.

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CRediT authorship contribution statement

Rossella Debernardis: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Katarzyna Palińska-Żarska:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Sylwia Judycka:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Abhipsa Panda:** Writing – review & editing, Investigation. **Sylwia Jarmołowicz:** Investigation. **Jan P. Jastrzębski:** Data curation. **Tainá Rocha de Almeida:** Writing – review & editing, Data curation. **Maciej Błażejewski:** Resources. **Piotr Hliwa:** Resources. **Sławomir Krejszeff:** Resources. **Daniel Żarski:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

Data availability

Raw data from the analysis of different families of freshly hatched larvae can be accessed via the NCBI BioProject database under the PRJNA1032718 accession number.

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Appendix A. Supplementary data

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