A journey to understanding larval quality in fish by integrating transcriptomics and zootechnics

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Abstract

Background: The larval stage plays a crucial role in the ontogeny of many fish species, leading to profound changes in morphology, physiology and behavior. How larvae face metamorphoses can be associated with their quality, which can vary significantly among the same family or even cohort. This variability depends on the interaction of genetic and nongenetic factors, which are key phenotypic determinants that further affect the overall survival of wild fish and adaptability of cultured stocks. Although researchers and aquaculturists often refer to 'larvae quality' as an important parameter discriminating high- and low-performing stocks, there is currently no universally accepted definition or critically verified set of quality indicators. Therefore,

in this study, we generated unique families of Eurasian perch (*Perca fluviatilis*) larvae to follow their performance during the larval period. Next, we integrated the zootechnical performance data with transcriptomics data from freshly hatched larvae to identify specific molecular quality markers.

Results: Zootechnical data revealed statistically significant differences ($p < 0.05$) in performance between 16 families reared. The RNA-seq data were processed using weighted gene correlation network analysis (WGCNA) and showed particularly significant interactions between gene modules and the length of larvae at the mouth-opening stage, emerging as an important quality-related trait. Next, by comparing the transcriptomic profiles of larvae with high and low lengths at mouth opening, we identified several differentially expressed genes, here chosen as putative larvae quality markers, involved in metabolic processes and antioxidant responses, such as fatty acid desaturase 2 (*fads2*) and glutathione peroxidase 1b (*gpx1b*).

Conclusions: The findings highlight the length of the larvae at hatching as a valid parameter of larvae quality. Initial length is not just a numerical measurement; it serves as a specific indicator that strongly influences future larval development and survival. 'Longer' larvae appear to benefit from a higher metabolic rate, enhanced antioxidant defenses and a more advanced nervous system. As a result of these insights, we propose genes such as *fads2*, *ctsl1*, and *gpx1b* as potential biomarkers for evaluating larval quality. Our study represents a significant step toward a clear understanding of the factors that determine the quality of fish larvae, with implications for improving aquaculture practices.

Keywords

Larvae-quality, Eurasian perch, growth heterogeneity, length at mouth opening, family-based design, nongenetic inheritance, WGCNA, RNA-seq

Background

The life cycle of most teleost fishes (both marine and freshwater) embraces a crucial phase known as the larval period [1]. This is described as the most dynamic stage of fish ontogeny during which individuals undergo many changes at multiple levels (e.g., morphological, behavioral, physiological) while developing into adults [2]. More precisely, larvae experience several primary events, e.g., the start of exogenous feeding, yolk-sac reduction, and inflation of the swim bladder [3], which are considered key milestones in their growth and survival [2] and, ultimately, in the success of further fish production. Larvae that successfully face these challenges are more likely to become valuable future spawners. It has been observed that larvae of many cultured species, even from the same batch, are characterized by different performances [4], which are often used to rate their quality. However, there is currently no definition of larval quality or criteria to delineate it, and thus, studies on larvae that aim at outlining quality traits are very much needed. Importantly, a clear characterization of the quality of a larva could not only broaden knowledge on the biological factors and mechanisms influencing the robustness of the progeny but also – by providing a specific “phenotypic marker” – be used to decipher mechanisms affecting gamete quality. Furthermore, it could shed light on the role of spawners in the progeny phenotype and help in developing effective selective breeding programmes, which would be useful for the improvement of the aquaculture industry [5].

For a long time, zootechnical characteristics have been used to describe and/or predict larval quality. Until now, it has been arbitrarily associated with individuals characterized by a normal shape (i.e., without any type of deformity), filled swim bladder, a nourishing yolk-sac [6], high growth rate, stress resistance [7] and certain behavioral features (e.g., good feed acquisition or high swimming speed) [2]. These traits might be, theoretically, indicative of quality in fish larvae, but there are no studies that critically challenge their applicability in a broader context. Moreover, the phenotype of fish larvae is not solely determined by genetic factors but also by the interplay between environmental conditions and parental contributions through nongenetic inheritance mechanisms (here referred to as “nongenetic”, although they are closely intertwined with DNA sequences). Jointly, they influence gene expression and shape the phenotype of an organism [8]). In summary, the quality of a larva is a complex characteristic that is influenced by the interaction of various intricate factors, and therefore, it is necessary to take them into account to obtain a broader and more complete picture.

During the larval stage, fish experience significant development, which is regulated by a cascade of events and factors. Next-generation sequencing (NGS) and gene expression analysis allowed the identification of genes involved in the immune system and stress response, as well as in the nervous system or in the mechanisms of digestion, which control and influence the fate of larvae and their performance since the earliest developmental stages [9–11]. Additionally, the growth and survival of the larvae appeared to be directly influenced by maternally derived molecules stored in the eggs during oogenesis [12]. This is mainly manifested prior to the maternal-to-zygotic transition (MZT) event, during which embryonic development is entirely under the control of the maternal genome (later, the zygotic genome

activates, and the embryo starts to produce its own molecules) [13]. However, maternal effects have also been observed beyond the MZT since, for example, lipids and proteins contained in the yolk sac are fundamental sources of energy and nutrients for hatched larvae before the onset of exogenous feeding. The composition of these molecules seems to vary depending on the nutritional status of the mother [14–16], and this variability may affect egg quality and, consequently, larval growth [17]. Moreover, maternally derived messenger RNAs (mRNAs) and proteomic profiles have been reported to be important determinants of neurodevelopment and immune functions, respectively, in offspring [18–20]. Over the past few years, the contribution of parents (especially the maternal parent) has been investigated mainly at the oocyte level, which has led to a clear definition of egg quality [21]. Nevertheless, it is not yet known how and whether gamete quality parameters can be used to predict progeny quality due to, among others, the lack of clear and objective zootechnical and molecular profiles of high-quality larvae.

In recent years, transcriptomics has been successfully applied to fish biology research [22–25]. In the current study, the transcriptome of larvae at the mouth-opening stage was analyzed, which is considered to be a stage in which embryonic development is complete, the individual is already fully autonomous and major human intervention (which could cause some stress and thus affect the overall physiological state of the fish) is not yet in place. From this perspective, the transcriptome of the whole individual provides a broad overview of gene expression and regulation, which may help in understanding biological processes related to larval performance.

In this study, we attempted to assess larval quality using Eurasian perch (*Perca fluviatilis*) as an ideal model among commercially relevant freshwater fish species

dedicated to intensive farming using recirculating aquaculture system (RAS). The production of this species is already well established [10, 26, 27], although variable reproductive and larviculture performances remain an obstacle to the expansion of its production. For this investigation, we developed a specific experimental and analytical strategy based on studying phenotypic variability among larvae obtained from heterogeneous families. This unique approach aimed at identifying, for the first time, possible “phenotypic markers” predicting the performance/quality of the larvae by combining zootechnics and transcriptomics.

Results

Zootechnical performance of larvae

The fertilization rate was similar for all 16 families (marked as F1 to F16) (Fig. 1). However, F9 was found to have a significantly lower ($p > 0.05$) embryo developmental rate (after the MZT) than F1 and F6 (Fig. 1). In addition, the within-family *t test*, which compared the pre- and post-MZT periods, revealed considerable post-MZT mortality in F4 and F12.

For the other zootechnical traits, the analysis revealed statistically significant differences ($p < 0.05$) between families for cannibalism rate, mortality, length and weight of larvae throughout the entire rearing period and specific growth rate (SGR) (for both length and weight). However, there was no significant difference in the swim bladder inflation effectiveness (SBIE) or weight of larvae at the first feeding stage. The graphs resulting from the statistical analysis, which show this heterogeneity, are shown in Additional file 1.

During the entire rearing trial, the mortality rate of each family was also recorded daily. The cumulative mortality graph (Fig. 2) shows a peak of mortality in almost all

families at the oil droplet reduction stage. In addition, a statistical analysis was performed on cumulative daily mortality, and the results of the between-family comparison are reported in Additional file 2.

To detect potential relationships between various zootechnical parameters, for both measurable and derivative (see “Data analysis and statistics” in Material and Methods), a Pearson's correlation (*r*) matrix was generated (Fig. 3). The most robust correlations ($r > 0.7$ or $r > -0.7$) were observed for the growth-related traits. For instance, the interaction between the length of the larvae at hatching and at the first feeding stage ($r = 0.72$) or the negative correlation between specific growth rate of weight for the entire larviculture period (SGRW_TOT) and larval weight at hatching ($r = - 0.77$). Alternatively, there was a negative relationship between the Fulton condition factor at weaning (Fulton_W) and the specific growth rate for weight data from weaning to the end of the larval period (SGRW_2) ($r= - 0.84$).

<u>Transcriptomic data and WGCNA</u>

Analysis of the transcriptome allowed the identification of 30,744 genes, which were further analyzed using weighted gene coexpression network analysis (WGCNA). First, a gene cluster dendrogram (see Additional file 3) was built by the correlation coefficients of each gene, which resulted in 24 different gene modules. Then, the correlation matrix between the gene modules and the measurable zootechnical traits identified significant modules ($p<0.05$) related to weight at mouth opening, cannibalism and mortality but mostly to the length at mouth opening (Fig. 4a). Specifically, 5 modules were positively correlated with length at mouth opening, and 3 modules were negatively associated (Fig. 4b). The genes were extracted from each module and grouped according to the positive and negative correlation to the length

trait. The GO analysis revealed that in positively length-related modules, ribosome biogenesis, translation processes, and rRNA processing were the most enriched biological processes (Fig. 5a-b). In contrast, in modules negatively correlated with length, the most enriched processes included neurogenesis, neuron projection development, cell junction and cell projection organization (Fig. 6 a-b). Note that the same GO analysis was also carried out for significant modules related to cannibalism, weight at mouth opening and mortality but did not show enriched GO terms (FDR<0.05). Therefore, length at mouth opening will be considered a trait of interest in this study.

<u>Differentially expressed gene (DEG) analysis and qPCR validation</u>

The analysis revealed 54 DEGs (adjusted p<0.05) (Fig. 7a). Among those, 17 were downregulated and 37 were upregulated in high-length larvae (see Additional file 4). The gene ontology enrichment analysis showed that most of these genes were related to metabolic processes, which play an essential role in growth and development in fish (Fig. 7b). After carefully reviewing all the genes and on the basis of their significant role in the survival and growth of fish larvae during their early life stages, cathepsin l1 (*ctsl1),* glutathione peroxidase 1b *(gpx1b),* fatty acid binding protein 2 *(fabp2),* fatty acid desaturase 2 *(fads2),* cytochrome c oxidase subunit 6b1 *(cox6b1),* complement component 3 *(c3)* and troponin l type 1 *(tnni1)* were chosen to be validated with real-time qPCR. Among those chosen, 5 genes involved in metabolic processes and immune resistance confirmed a significant difference (*t test*, p<0.05) (Fig. 8).

Discussion

In this study, a comparative 'family-based' experiment on Eurasian perch was conducted for the first time, in which we evaluated the performance of 16 different larval families. The results provide novel insights into larval biology and emphasize phenotypic heterogeneity as a bottleneck toward the improvement of the aquaculture sector [28]. Hence, we profiled the quality of larvae by combining a “multitrait” zootechnical approach [29] and a transcriptome analysis.

Heterogeneity of zootechnical performance – inter- and intrafamily features

Overall, the growth patterns (in terms of weight and length) observed in the larvae under investigation are consistent with other experiments on Eurasian perch [10, 30]. Additionally, there were no major differences observed in cannibalism behavior and swim bladder inflation rate when compared to a previous study that exposed the larvae to similar experimental conditions [10, 30]. Additionally, larval mortality was mostly determined by problems related to nonfeeding behavior after the oil droplet reduction stage, cannibalism and problems with swim bladder inflation [10, 28, 31]. However, upon closer observation of the experiment, although all groups were reared under the same controlled conditions, high variation between families in terms of phenotypic traits was observed, which reflected different performances. These differences can be affected by both genetic and nongenetic factors, with the latter being of maternal or paternal origin or a combination of the two [32]. Larvae from different families diverged mainly in growth-related traits, which probably influenced their cannibalistic behavior and development. Growth depensation has been commonly observed in many other fish species [28, 33, 34] and has always been considered a relevant determinant and predictor of fish survival since it can lead to aggressiveness and mortality.

Furthermore, within a cohort of fish larvae, while aspects such as feeding behavior and swim bladder inflation rate tend to show a relatively homogenous pattern, differences become apparent in regard to growth quality between siblings, leading to variations in size. This heterogeneity may be explained by individual “personality”, which influences larval efficiency in foraging food, escaping from predators or interacting with conspecifics [35]. Personality can be influenced by a complex interplay of genetics and environmental factors, as well as maternal investment in the molecular cargo of fish eggs, which can impact the neural and physiological systems influencing larval behaviors [18, 36, 37]. The interplay of these elements results in variability in individuals of the same cohort, and understanding this complexity might be crucial for defining larvae quality.

This family-based experiment allowed us to observe clear inter- and intrafamily variability. With this approach, it can be easier to understand a wide range of characteristics, including early life history characteristics and physiological responses. Moreover, since these variations are heritable, meaning they can be passed on from one generation to the next, this experimental design may represent a pivotal approach for better understanding evolutionary changes [38]. This will lead to improved selective breeding programmes and, in turn, aquaculture practices and conservation procedures.

<u>Transcriptomic profile of larvae</u>

For this study, WGCNA was employed as a tool to identify quality-related mRNAs by digging into the transcriptome and identifying correlations between the expression of genes and phenotypic traits [39]. Consistent with the analysis of zootechnical traits, the results highlight the relevance of larval length at the mouth opening stage as a

possible quality-related trait. Therefore, further differential expression analysis was performed, taking only this single parameter into account, which shed light on several crucial processes that distinguish larvae with higher length at mouth opening, indicating that this parameter is an important quality trait in Eurasian perch larvae.

A fish larva is a tiny, vulnerable but fully autonomous organism, and the initial length seems to have a profound impact on the individual's fate [40]. The size at hatching can, in fact, trigger a series of cascade reactions, which will define the future of each individual larva. Arguably, larger larvae could gather food more easily, grow faster, have better swimming abilities and escape predators or cannibals. Considering all this, the size of the larvae, especially immediately after hatching, can considerably influence their developmental success and therefore define their quality.

After hatching, the yolk reservoir in fish eggs and larvae serves as a source of essential nutrients and energy for their development and survival until they can actively gather food. Especially during the earliest life stages, larvae undergo significant body modifications (e.g., in the nervous, digestive and circulatory systems), and yolk nutrients are particularly valuable in supporting those primary changes. The utilization of these nutritional compounds in various biological processes is ensured, among other elements, by desaturase enzymes, which are mainly responsible for the biosynthesis of fatty acids. Differential expression analysis drew attention to *fads2* (fatty acid desaturase 2). It is a key enzyme in lipid metabolism and nutrient utilization, and it was upregulated in larvae with a "higher" length. Cunha et al. [41] observed an increase in the transcription of *fads2* in turbot larvae (*Scophthalmus maximus*) during the first days after hatching, suggesting *fads2* as an engine driving larval growth and lengthening. In fish, fatty acids are also essential to support various aspects of development, especially concerning the

nervous system, sensory organs and larval eyes [42]. Similar to *fads2*, cathepsin L (*ctsl1*) contributes to the proteolysis of yolk proteins, allowing nutrient absorption and supporting larval growth. For its central function, c*tsl* has already been proposed as a marker to assess egg quality in Eurasian perch [43]. The results showed that *ctsl1* was upregulated in families with longer lengths at hatching. Considering that larger larvae may be more efficient in utilizing nutrients than smaller larvae, we highlight *fads2* and *ctsl1* for their key functions, and we propose these genes as potential markers for larval quality.

During the early life stages, the larvae constantly evolve, and their perception of the surrounding environment affects their growth. As the larvae grow, their nervous system rapidly develops to facilitate their interaction with external stimuli and control their motor skills and overall behavioral abilities. Moreover, it has already been highlighted that neurogenesis events in the offspring can be affected by maternally inherited mRNA [20, 44], and this has also been reflected in the personality/behavior of the fish [18]. WGCNA showed that the genes involved in nervous system development were correlated with the length of larvae at hatching. Therefore, it could be hypothesized that neurogenesis mechanisms appear to be indirectly involved in the quality of fish larvae, influencing behavior, adaptability, and sensory perception, and these factors will define the body size of each individual. Therefore, ensuring proper neural development is crucial for larvae to thrive and grow into healthy adult fish.

Stress is one of the main factors threatening fish welfare. After hatching, larvae are vulnerable to environmental stressors that can significantly affect their development and survival. Fish larvae rely on their immune system and antioxidant activity as a crucial defense source [45]. Although every organism is equipped to counteract

stress, the perception and response to stress vary individually, and the way every larva deals with stress can be defined by genetic and nongenetic factors [46]. The present study showed that larger larvae exhibit higher expression of the glutathione peroxidase 1b (*gpx1b*) gene. This gene is involved in building the individuals' antioxidant defense and could therefore be supposed that larvae with higher length at hatching may be less susceptible to stress or otherwise respond better to challenging events [47]. Moreover, freshly hatched larvae are immediately exposed to pathogens, and complement component *c3* plays a pivotal role in the larvae's innate immune defense. According to the obtained results, *c3* showed no significant differentiation between the two groups, suggesting that the different sizes of the larvae do not seem to influence their early capacity for immune response. In a recent study, Palińska-Żarska et al. [11] observed that the *c3* gene was differentially expressed at mouth opening between Eurasian perch larvae obtained from wild and domesticated spawners. Specifically, *c3* was upregulated in wild larvae, which suggests that wild larvae may have a more robust immune response when exposed to pathogens compared to domesticated larvae (reared in a pathogen-free RAS). Therefore, the expression of the *c3* gene in larvae could be mainly determined by the origin of the parents, which for this experiment were wild.

High-length larvae showed significant differentiation for the *cox6b1* gene. The upregulation of this gene highlights an increase in metabolic rates and oxygen consumption. Perhaps this phenomenon may be explained by the Gill-Oxygen Limitation theory (GOLT) [48], which posits that the oxygen supply of fish is negatively correlated with their body size. In other words, larger fish require more oxygen to support their metabolic needs, and their gills may not be able to provide a sufficient supply, which could lead to stress. In newly hatched larvae, however, the

gills are not yet fully developed, and gas exchange is primarily regulated by the entire body and yolk sac [49]. Nevertheless, it could be reasonable to assume that larger larvae require more oxygen uptake. Therefore, the upregulation of *cox6b1* may be an adaptive response to cope with the increased oxygen demand. A study on zebrafish discussed the deficiency of *cytochrome c oxidase* that appears to affect fish swimming behavior, heart function and the development and functioning of various tissues and organs in zebrafish embryos [50]. Although Pauly's theory was developed on adult fish, it is very likely that it can also be extended to fish larvae, as other studies have confirmed that oxygen availability at the larval stage has an impact on their growth, development and overall metabolism [51, 52].

<u>Length of larvae at mouth opening – quality determinant</u>

In summary, the results of this study emphasized the relevance of length in freshly hatched larvae as a quality marker. Growth pattern, or length in this specific case, can be influenced by a combination of multiple factors and can vary from one individual to another even within the same species, population and cohort. Overall, different processes interplay in determining larval developmental success. Specifically, fatty acid enzymes seem to have a pivotal role in determining larval growth. Additionally, they contribute to protecting organisms against oxidative stress and influence the expression of genes related to immune system functions [53], and they can impact neurogenesis in young fish. We can see a larger larva as an individual less susceptible to starvation because it has potentially more energy reserves and may exhibit better swimming capabilities due to the higher metabolic rate. These advantages can contribute to better chances of survival and successful transition to adulthood. Although in aquaculture, fish size has been commonly used

as an indicator of their performance [32], here, transcriptome analyses have provided valuable support for considering fish length as a good quality parameter since the earliest larval life stage and have also highlighted specific genes that can be considered quality biomarkers.

Conclusion

In this experiment, the observation of multiple zootechnical traits and the use of WGCNA proved to be an effective approach to explore differences between multiple families of Eurasian perch larvae. The obtained results highlight the relevance of length at mouth opening during the early life stages and propose it as a possible parameter of larval quality. However, considering the great phenotypic heterogeneity observed within each family, we propose to further profile the quality of the larvae considering specific research on the evaluation of performance at the level of an individual larva. The observation of each individual separately may allow us to gather more precise and detailed information about high-quality phenotypes and bring us closer to the understanding of characteristics and factors responsible for larval quality in fishes. It is important to postulate that when dealing with tiny organisms such as larvae at mouth opening, which are approximately 6-7 mm in size, specifying a particular length as a predictor of larval performance can be challenging. Therefore, the initial length can be considered a quality trait, but it is probably more applicable within populations or within families rather than as a universal predictor between different stocks or groups. In conclusion, the length of larvae at mouth opening may serve as a predictive indicator for larval quality, but it is certainly just one of many factors that influence larval growth and survival. Therefore, using the genetic markers

identified in our study may be a more reliable indicator of larval quality, although more specific studies are still needed to confirm this hypothesis.

Material and methods

General study design

The objective of this study was to define the larvae-quality profile using an integrated zootechnical-transcriptomic approach. For this purpose, 16 unique families of Eurasian perch were obtained by controlled reproduction of wild spawners from different water bodies (Additional file 5). A simple mating design was carried out by crossing a single female with an individual male.

Broodstock management and origin

Males' origin and management

The Eurasian perch males, used for controlled reproduction, originated 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) 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, males often completed or already contributed to the spawning act before being caught, which may directly affect the quality of the sperm. Then, during the spawning period, males were transferred in plastic bags with oxygen [54] 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 controllable 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}[55]. Before any manipulation, individuals were anesthetized in MS-222 (Argent, USA) at a concentration of 150 mg l^{-1}. Total length (L_T), caudal length (L_C) and body weight (before stripping) were measured for each individual (see Additional file 5).

Sperm sampling and cryopreservation protocols

Five days after hormonal injection, semen was collected (average weight of male - 190.5 ± 58 g) using a catheter (to avoid contamination of the urine; [56]) and with gentle abdominal pressure (i.e., stripping). After collection, each sample was kept on ice, and sperm motility was evaluated with CASA system (using the CEROS II system -Hamilton-Thorne, USA-; as described by Judycka et al. [57], while the sperm concentration of fresh semen was measured using a NucleoCounter SP-100 computer aided fluorescence microscope (Chemometec, Allerød, Denmark; [58]). 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 [59].

Thereafter, sperm cryopreservation was carried out following the procedure described by Judycka et al. [57]. 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, and 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; [60]) and is currently used as a standard procedure in many selective breeding programs [57].

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 in section 2.3.1). 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 controllable 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. [61]. 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}[55]. 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 (L_T), caudal length (L_C), body weight and weight of the ribbon were taken (measures are provided in Additional file 5).

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. [61]). 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 [62]. In this way, the correct number of spermatozoa to be used for fertilization of each ribbon was estimated. 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. [59]. 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 [57]. 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 egg activation, sperm was added at a sperm:egg ratio of 200 000:1. The eggs were then stirred for 40 seconds and washed with hatchery water after ~10 minutes to remove excess sperm and any debris. The same procedure was followed for each female separately.

Egg incubation and hatching

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 hours post fertilization (HPF), ~100 eggs from each batch were randomly sampled (in duplicate) to evaluate the fertilization rate

before the MZT and then the embryonic development at the neurula stage (when the body of the embryo can already be easily viewed). During egg 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. 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 into the larval tanks (same from where eggs were taken). 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 family were stocked into 3 separate tanks constituting separate replicates.

Larviculture and advanced zootechnics

Every family of Eurasian perch larvae was reared in triplicate and in the same RAS conditions, following a set of validated and standardized animal husbandry methods (i.e., advanced zootechnics) as described by Palińska-Żarska et al. [10]. The larvae were exposed to a specific thermal regime, photoperiod, and feeding schedule (Fig. 9 a). 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 [10, 28]. 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 dry feed (Perla Larva Proactive, Skretting, Norway) six times a day, pouring it into each tank in small doses, with intervals of 3 minutes, for approximately 15 minutes. At ~ 30 DPH, the experiment was completed, as with the temperature regime used, and the larval period was already finished. During the rearing trial, the photoperiod was 24 L:0 D, and the light intensity measured at the water surface was 1500 lux. 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 and in the evening, 1 h after 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.

<u>Larval sampling</u>

Larval samplings were carried out at five precise developmental stages [10, 11] (Fig. 9b):

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 larval period

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 mgL^{-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 [63]. 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. [10]). 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). Additionally, to perform molecular analysis, 40 freshly hatched larvae per family were randomly sampled, anesthetized and preserved in RNAlater (Sigma Aldrich, Germany) following the manufacturer's recommendations.

RNA extraction

RNA extraction was performed for each family separately. Using a TotalRNA mini-kit (A&A Biotechnology, Poland), total RNA was extracted from a pool of 10 larvae (per family), as described by Palińska-Żarska et al. [10]. Then, the quantity and purity of the extracted RNA were evaluated using a NanoDrop 8000 spectrophotometer

(Thermo Fisher Scientific, USA). Samples showed absorbance ratios A260/280 ~2.0 and A260/230 ~2.2. The quality of the extracted total RNA was also evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), and all the samples presented RIN > 9.0.

RNA-sequencing library preparation and bioinformatic expression analysis

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 [64]. Adapters and low-quality fragments of raw reads (average *Q*Phred score < 20) were trimmed, and reads were clipped to equal lengths of 100 nt using the Trimmomatic tool ver. 0.40 [65]. The resulting read sets of the analyzed samples were mapped to a reference genome *P. fluviatilis* ver. 11.1.104 obtained from the NCBI database [66] using STAR software ver. 2.7.10a [67] with ENCODE default options.

The annotation and estimation of the expression levels were performed using the StringTie tool ver. 2.2.0 [68], with interpreting strand-specific sequencing the 'fr—firststrand' parameter was activated. Counts per transcript and gene were calculated using the *prepDE* Python script[69], and TPM and FPKM values were read from StringTie output data using the lncRna R library [70].

Functional annotation was performed using eggNOG-mapper (version emapper-2.1.9) [71] based on eggNOG orthology data [72]. Sequence searches were performed using DIAMOND [73].

Construction of the gene coexpression network analysis

The counts per transcript were employed to perform weighted gene coexpression network analysis (WGCNA) with the R package, following the authors' recommendations [74]. Briefly, the hierarchical clustering of samples with Euclidean distance was used to check the presence of outliers. Then, to proceed with automatic blockwise network construction, the adjacency matrix was calculated, and a soft-power threshold (β=18, leading to signed R^2 = 0.9) was chosen based on a scale-free topology model. Then, the adjacency matrix was transformed into a topological overlap matrix, and gene modules were identified by hierarchical clustering dendrogram. After the gene modules with comparable expression patterns were defined, the module eigengene (ME) distances were calculated to detect potential relationships of modules with the zootechnical traits collected during the experiment. Hence, we focused on the significant modules with a $p < 0.05$ and the absolute value of the correlation coefficient |cor| >0.5. Genes embedded in the significant modules of interest were then extracted to proceed with the Gene Ontology Enrichment analysis.

<u>Differential expression analysis</u>

Taking into account the results of WGCNA, the length of the larvae at mouth opening was used as a parameter to divide the families into "high" and "low" length groups. First, 6 families with the highest length and 6 families with the lowest length were chosen. Then, the coefficient of variation (CV=standard deviation/sample mean) was calculated for each family. After that, 4 families from each group with the lowest coefficient of variation were chosen (which allowed us to follow a typical commercially desired trait of low intragroup variability) to carry out the differentially expressed gene analysis. Analysis of differentially expressed genes (DEGs) was performed with the DESeq2 package in R studio [75, 76].

Gene Ontology (GO) analysis

GO analysis was performed by following the approach described by Żarski et al. [20]. 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 [77], and the most enriched biological processes (FDR<0.05) were identified. 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. The same was done for the genes resulting from the DeSeq2 analysis, and the 20 most enriched biological processes were selected. This allowed us to identify clusters that highlighted the most relevant biological processes.

Reverse Transcription and Real-Time qPCR

After examining each DEG individually, candidate genes were chosen according to their function and validated by real-time qPCR. 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 5X reaction buffer, 0.5 µl of RNAse inhibitor, 2 µl of dNTP mix and 4 µl of TranScriba reverse transcriptase. The reaction was conducted for 60 min at 42°C and then completed by heating at 70°C for 5 min.

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 [78] – see Additional file 6), 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, a melting curve was made to check the amplification specificity. Relative expression was normalized using the geometric mean of expression values recorded for 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)*, exhibiting the most stable expression level (revealed based on the transcriptomic data obtained) [20]. Finally, the data between the groups were analyzed with the *t test* (GraphPad Prism 9), and significant differences were considered at $p < 0.05$.

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, which will be referred to in the manuscript as derived zootechnical parameters. Fulton's condition factor (*K*) was calculated based on the obtained measurements according to the formula: $K = 100\ (W\ Lt^{-3})$. In addition, the specific fish growth rate (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 (0DPH to 27DPH), SGRW_1 relative to the weight of larvae from 0DPH to 17DPH and SGRW_2 for larvae from 17DPH to 27 DPH. The same calculations were made for the length data of the larvae (referred to as SGRL_TOT, SGRL_1, SGRL_2).

The statistical analyses were carried out with a one-way analysis of variance (ANOVA) and Tukey's *post hoc* test; additionally, Kruskal Wallis' test and Bonferroni *post hoc* correction were used for analysis 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 [75].

Abbreviations

c3: complement component 3

cox6b1: cytochrome c oxidase subunit 6b1

ctsl1*:* cathepsin l1

CV: Coefficient of Variation

DEGs: Differentially expressed genes

DPH: Days Post Hatching

END: End of the experiment

fabp2: fatty acid binding protein 2

fads2*:* fatty acid desaturase 2

FF: First feeding stage

FULTON_END: Fulton's condition factor at the end of the experiment

FULTON_FF: Fulton's condition factor at the first feeding stage

FULTON_MO: Fulton's condition factor at the mouth opening stage

FULTON_OD: Fulton's condition factor at oil droplet reduction

FULTON_W: Fulton's condition factor at weaning

GO: Gene Ontology

gpx1b: glutathione peroxidase 1b

MO: Mouth opening stage

MZT: maternal-to-zygotic transition

NGS: Next-generation sequencing

OD: Oil droplet reduction stage

RAS: Recirculating Aquaculture Systems

SBIE: Swim Bladder Inflation Effectiveness

SGR: Specific growth factor

SGRL: Specific growth rate for length data

SGRL_1: Specific growth rate of length from hatching until weaning stage

SGRL_2: Specific growth rate of length from weaning stage until the end of the experiment

SGRL_TOT: Specific growth rate of length for the entire larvicultural period

SGRW: Specific growth rate for weight data

SGRW_1: Specific growth rate of weight from hatching until weaning stage

SGRW_2: Specific growth rate of weight from weaning stage until the end of the experiment

SGRW_TOT: Specific growth rate of weight for the entire larviculture period

tnni1: troponin I type 1

W: Weaning stage

WGCNA: Weighted Gene Coexpression Network Analysis

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Ethic statement

All the activities conducted during the experimentation were performed following the Polish law (Act of January 15, 2015 on the protection of animals used for scientific or educational purposes) and in accordance with the Local Ethical Committee for Animal Experiments (Olsztyn, Poland) (Resolution No. 5/2023).

Availability of data and materials

Raw data from the analysis of different families of freshly hatched larvae can be accessed via the NCBI SRA database under the PRJNA1032718 accession number (restricted availability prior publication).

Competing interests

The authors declare that they have no competing interests.

Authors' information

Authors' Contribution

R.D. has contributed to the conceptualization of the experiment, performed the experiment, collected the samples, analysed data and wrote the manuscript; **K.P.-Ż.** has made substantial contributions to the conception and to the management and coordination responsibility for the research activity, conducted the experiment, collected the samples, reviewed the manuscript; **D.Ż.** contributed to the conceptualization of the experiment, funding acquisition, methodology, validation, supervision, project administration and manuscript revision; **S. Judycka** conceptualization, investigation, **A.P., M.B**. and **S. Jarmołowicz** were involved in the investigation; **P.H.** and **S.K**. provided resources for the experiment; **T.R.D.A**. and **J.P.J.** contributed to the data curation. All authors read and approved the final manuscript.

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Figure captions:

Fig. 1: 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 groups for the embryonic development rate. The asterisks (* – $p < 0.05$, ** – $p < 0.01$) indicate significant differences within the family and between fertilization and embryonic development rate.

Fig. 2: Cumulative mortality (mean ± SD) of 16 families of E. perch larvae. Different colors stand for different families. Families are sorted in descending order of final mortality rate recorded (they are listed in the box at the top right of the graph).

Fig. 3: Pearson's correlation matrix of the measurable and derivative zootechnical 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 numbers in the squares correspond to Pearson's correlation coefficient values (*r*).

Fig. 4: (a) Module-trait relationship. The module eigengene (ME) is shown in each row. 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. (b). Five significant modules correlated with the length at mouth opening trait.

SBIE: swim bladder inflation effectiveness, MO: mouth opening, FF: first feeding, OD: oil droplet reduction, W: weaning, END: end of the experiment

Fig. 5: Tree plot (a) of clustering analysis and (b) network of the 10 most enriched ontological terms for genes embedded in modules positively related to length at mouth opening (darker nodes are more significantly enriched gene sets; larger nodes represent larger gene sets; thicker edges represent more overlapping genes). For the network graph, a cutoff of 0.3 (default) was set, meaning that the two nodes were connected whenever at least 20% of genes were shared by the visualized GO term.

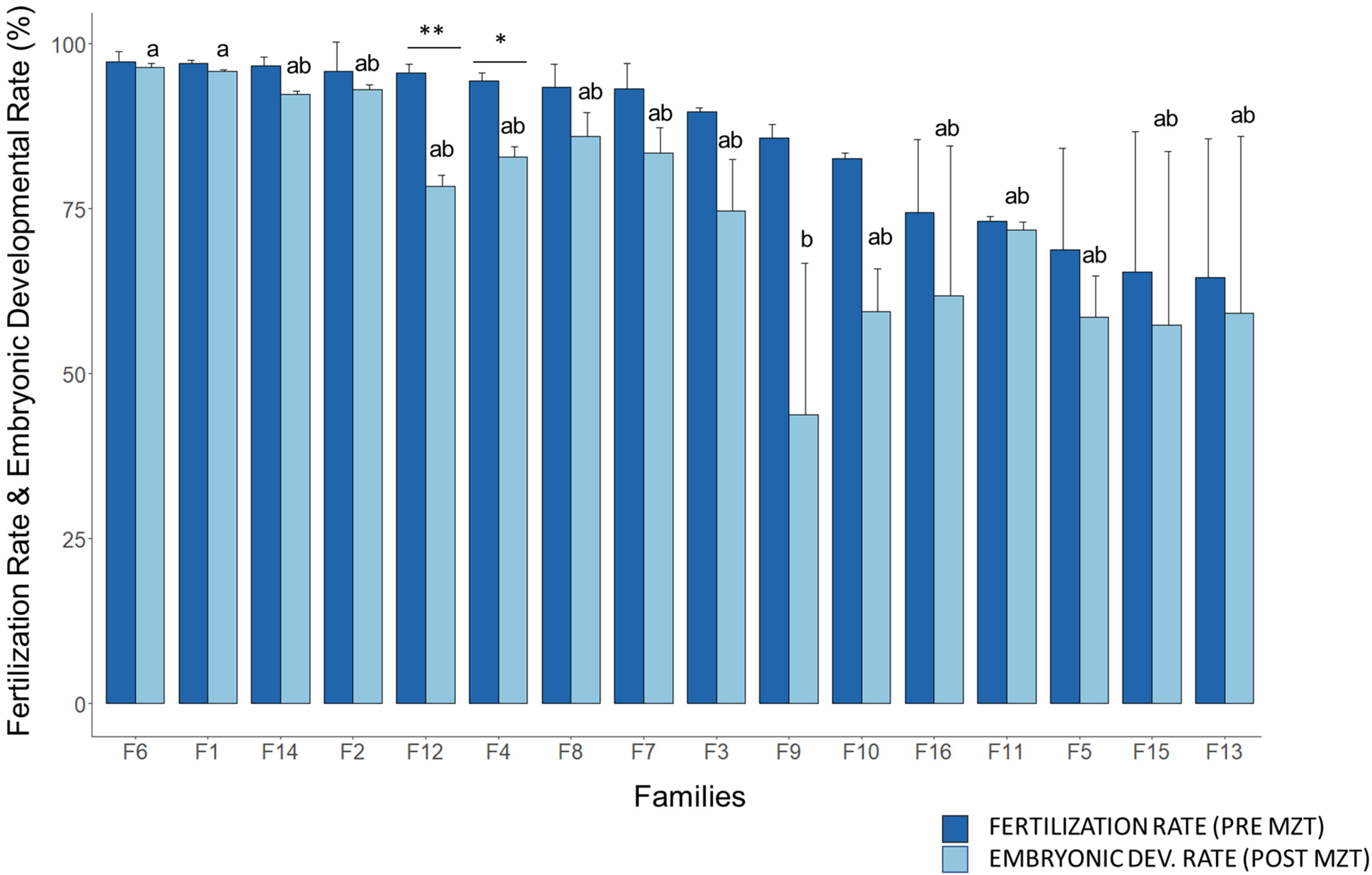
Fig. 6: Tree (a) plot of clustering analysis and network (b) of the 10 most enriched ontological terms for genes embedded in modules negatively correlated with length at mouth opening (darker nodes are more significantly enriched gene sets; larger nodes represent larger gene sets; thicker edges represent more overlapping genes). For the network graph, the default cutoff at 0.3 was set, meaning that the two nodes are connected whenever at least 20% of genes are shared by the visualized GO term.

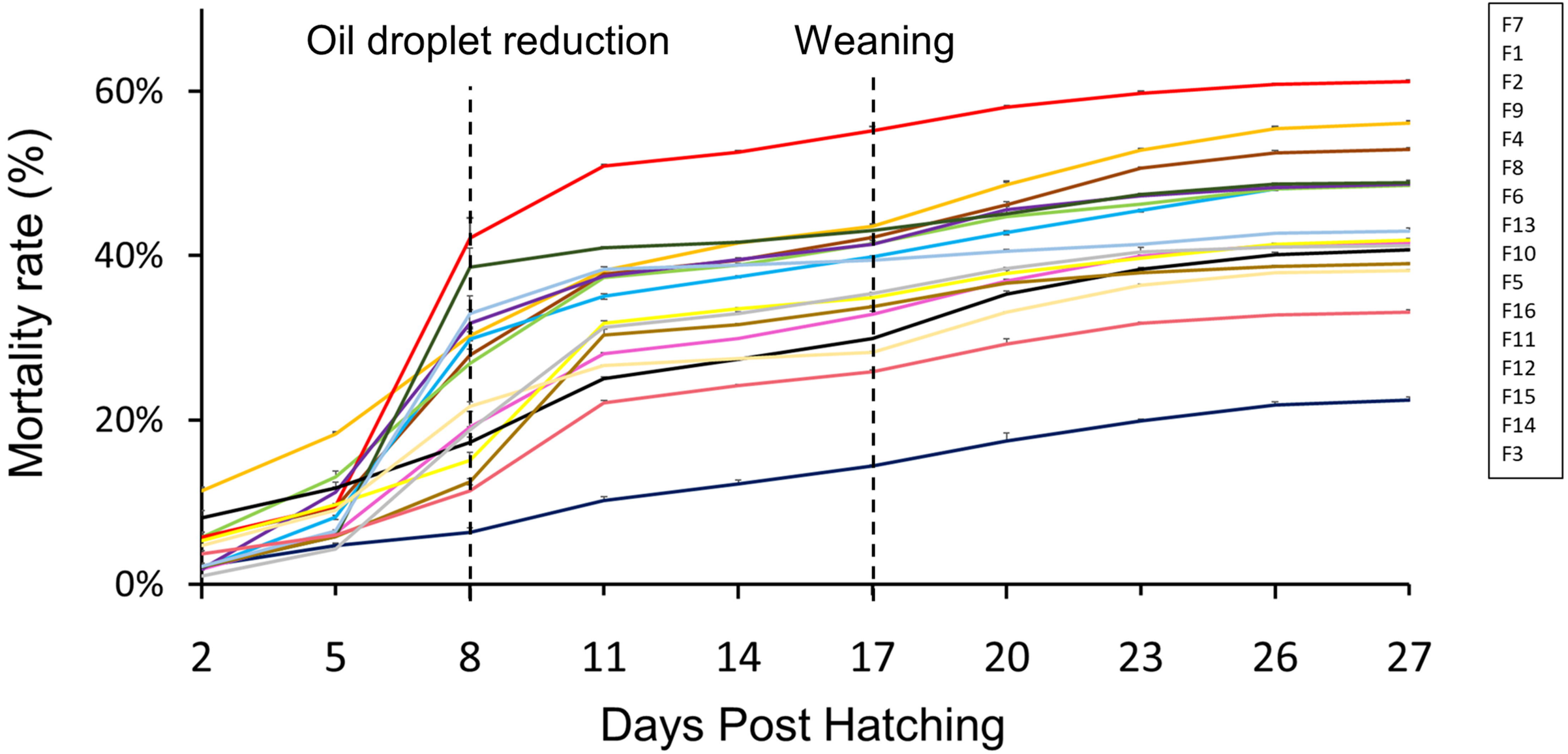
Fig. 7: (a) Heatmap of 54 differentially expressed genes (DEGs) between high- and low-length larvae (n = 4 for each group). The expression level for each gene is presented using a color intensity scale, where blue and red indicate over- and underexpression, respectively. (b) Tree plot of clustering analysis of the 20 most enriched ontological terms

Fig. 8: The relative gene expression in larvae characterized by low and high length at the mouth opening stage obtained after RNA-seq (white-shaded bars) and real-time qPCR (blue-shaded bars). The results of the statistical analysis are presented as follows: ns – nonsignificant, * – $p < 0.05$, ** – $p < 0.01$). ***c3***: complement component 3, ***cox6b1****:* cytochrome c oxidase subunit 6b1, ***ctsl1****:* cathepsin l1, ***fabp2****:* fatty acid binding protein 2,

fads2*:* fatty acid desaturase 2, ***gpx1b***: glutathione peroxidase 1b, ***tnni1****:* troponin I type 1. Descriptions and ontologies of all the genes are presented in Additional file 4.

Fig. 9: (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 and end of larval period.


Fertilization Rate & Embryonic Developmental Rate (%)
100
75
50
25
0
a
a
ab
ab
**
ab
*
ab
ab
ab
ab
b
ab
ab
ab
ab
ab
ab
F6
F1
F14
F2
F12
F4
F8
F7
F3
F9
F10
F16
F11
F5
F15
F13
Families
FERTILIZATION RATE (PRE MZT)
EMBRYONIC DEV. RATE (POST MZT)


Oil droplet reduction
Weaning
Mortality rate (%)
60%
40%
20%
0%
2
5
8
11
14
17
20
23
26
27
Days Post Hatching
F7
F1
F2
F9
F4
F8
F6
F13
F10
F5
F16
F11
F12
F15
F14
F3

	Hatching.rate	Fertilization.rate	Embryonic.dev.rate	SBIE	Mortality	Cannibalism	Length_MO	Length_FF	Length_OD	Length_W	Length_END	Weight_MO	Weight_FF	Weight_OD	Weight_W	Weight_END	SGRW_TOT	SGRW_1	SGRW_2	SGRL_1	SGRL_2	SGRL_TOT	Fulton_MO	Fulton_FF	Fulton_OD	Fulton_W	Fulton_END
Hatching.rate	1																										
Fertilization.rate	0.61	1																									
Embryonic.dev.rate	0.7	0.77	1																								
SBIE	0	0.12	0.11	1																							
Mortality	0.38	0.29	0.28	-0.07	1																						
Cannibalism	0.06	0.18	0.28	0.34	-0.55	1																					
Length_MO	0.1	0.25	0.15	-0.22	-0.06	0.39	1																				
Length_FF	-0.04	0.02	-0.2	-0.33	-0.37	0.29	0.72	1																			
Length_OD	0.03	0.16	-0.03	0.13	-0.33	0.56	0.59	0.7	1																		
Length_W	-0.46	-0.23	-0.3	0.42	-0.47	0.34	0.1	0.23	0.18	1																	
Length_END	-0.43	0.11	-0.21	0.05	-0.28	0.2	-0.02	-0.03	-0.11	0.56	1																
Weight_MO	-0.37	-0.46	-0.54	-0.42	-0.07	-0.32	0.05	0.06	-0.24	-0.22	-0.06	1															
Weight_FF	0.09	0.25	0.01	-0.15	-0.37	0.37	0.68	0.78	0.64	0.04	-0.04	0.2	1														
Weight_OD	-0.05	0.22	0.05	0.03	-0.2	0.45	0.68	0.77	0.84	0.32	0.09	-0.29	0.65	1													
Weight_W	-0.37	-0.21	-0.18	0.52	-0.54	0.37	-0.12	0.11	0.08	0.85	0.38	-0.2	0.13	0.19	1												
Weight_END	-0.41	0.12	-0.15	0.12	-0.35	0.31	-0.02	-0.01	-0.05	0.64	0.98	-0.14	-0.05	0.11	0.45	1											
SGRW_TOT	-0.02	0.35	0.24	0.32	-0.08	0.33	-0.02	-0.05	0.11	0.52	0.66	-0.77	-0.21	0.3	0.34	0.7	1										
SGRW_1	-0.06	0.12	0.18	0.66	-0.29	0.41	-0.09	0.02	0.21	0.73	0.31	-0.75	-0.06	0.34	0.78	0.39	0.72	1									
SGRW_2	0.06	0.26	0.05	-0.53	0.28	-0.14	0.12	-0.08	-0.14	-0.35	0.41	0.07	-0.18	-0.09	-0.66	0.35	0.25	-0.49	1								
SGRL_1	-0.48	-0.36	-0.37	0.49	-0.36	0.02	-0.53	-0.24	-0.21	0.78	0.49	-0.2	-0.38	-0.14	0.8	0.54	0.43	0.67	-0.39	1							
SGRL_2	0.06	0.35	0.11	-0.39	0.22	-0.13	-0.13	-0.3	-0.31	-0.47	0.47	0.14	-0.11	-0.26	-0.51	0.37	0.17	-0.45	0.83	-0.32	1						
SGRL_TOT	-0.41	-0.08	-0.27	0.16	-0.17	-0.08	-0.6	-0.45	-0.44	0.38	0.81	-0.08	-0.44	-0.33	0.36	0.79	0.54	0.29	0.26	0.7	0.45	1					
Fulton_MO	-0.4	-0.54	-0.54	-0.19	-0.07	-0.46	-0.54	-0.37	-0.54	-0.2	-0.02	0.81	-0.22	-0.63	-0.06	-0.08	-0.63	-0.55	-0.03	0.18	0.18	0.31	1				
Fulton_FF	0.22	0.35	0.31	0.27	-0.1	0.16	-0.02	-0.23	0	-0.28	-0.09	0.2	0.42	-0.11	0.07	-0.12	-0.29	-0.1	-0.24	-0.23	0.18	-0.08	0.19	1			
Fulton_OD	-0.14	0.18	0.09	-0.11	0.02	0.02	0.47	0.49	0.25	0.34	0.29	-0.18	0.41	0.73	0.23	0.24	0.36	0.32	0	0.01	-0.08	-0.05	-0.42	-0.13	1		
Fulton_W	-0.12	-0.13	0.02	0.57	-0.39	0.22	-0.4	-0.16	-0.07	0.36	-0.05	-0.16	0.07	-0.1	0.78	-0.01	-0.01	0.59	-0.84	0.57	-0.47	0.18	0.13	0.39	-0.09	1	
Fulton_END	-0.23	0	-0.03	0.12	-0.29	0.38	-0.11	-0.09	-0.04	0.55	0.7	-0.32	-0.28	0.01	0.34	0.8	0.69	0.37	0.36	0.5	0.2	0.62	-0.17	-0.35	0.03	-0.1	1

-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1

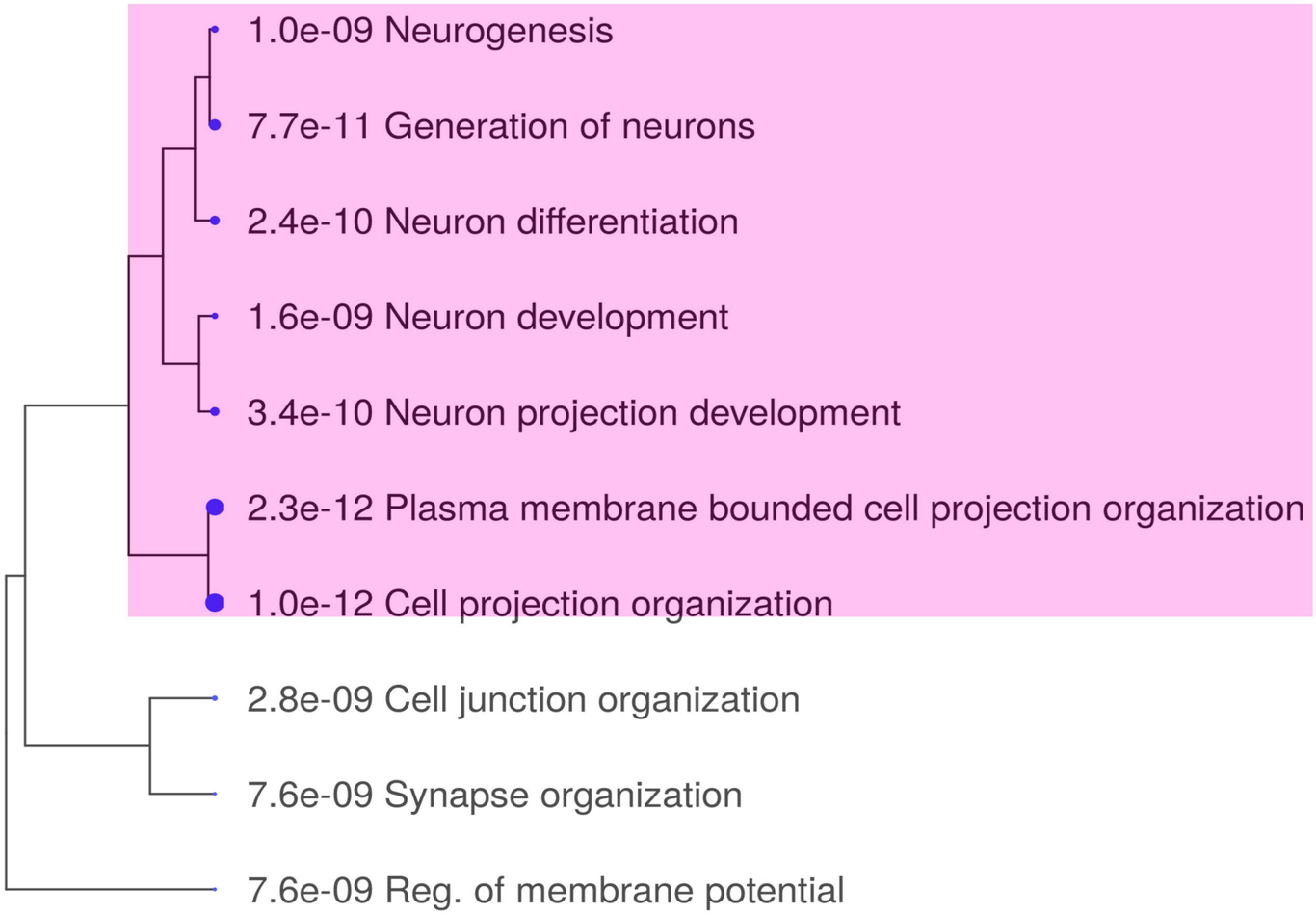
a

	Fertilization.rate	Embryonic.dev..rate	SBIE	Mortality.rate	Cannibalism.rate	LengthMO	LengthFF	LengthOD	LengthW	LengthEND	WeightMO	WeightFF	WeightOD	WeightW	WeightEND
MEblue	0.18 (0.5)	-0.15 (0.6)	-0.24 (0.4)	-0.3 (0.3)	0.009 (1)	0.52 (0.04)	0.66 (0.006)	0.31 (0.2)	0.29 (0.3)	0.24 (0.4)	-0.058 (0.8)	0.5 (0.05)	0.38 (0.2)	0.089 (0.7)	0.21 (0.4)
MEmagenta	-0.044 (0.9)	-0.3 (0.3)	-0.43 (0.1)	-0.068 (0.8)	-0.53 (0.03)	0.071 (0.8)	0.36 (0.2)	-0.082 (0.8)	-0.061 (0.8)	0.03 (0.9)	0.15 (0.6)	0.14 (0.6)	0.0055 (1)	-0.22 (0.4)	-0.044 (0.9)
MEpink	-0.28 (0.3)	-0.31 (0.2)	-0.24 (0.4)	-0.27 (0.3)	0.11 (0.7)	0.57 (0.02)	0.61 (0.01)	0.22 (0.4)	0.21 (0.4)	-0.048 (0.9)	0.41 (0.1)	0.37 (0.2)	0.25 (0.4)	-0.056 (0.8)	-0.029 (0.9)
MEyellow	-0.26 (0.3)	-0.25 (0.3)	-0.21 (0.4)	-0.47 (0.06)	0.05 (0.9)	0.22 (0.4)	0.53 (0.03)	0.079 (0.8)	0.24 (0.4)	0.079 (0.8)	0.11 (0.7)	0.22 (0.4)	0.16 (0.6)	0.05 (0.9)	0.11 (0.7)
MEgreen	0.33 (0.2)	0.03 (0.9)	-0.29 (0.3)	0.17 (0.5)	0.0061 (1)	0.65 (0.007)	0.51 (0.04)	0.4 (0.1)	0.082 (0.8)	0.024 (0.9)	-0.0089 (1)	0.47 (0.07)	0.44 (0.09)	-0.067 (0.8)	-0.021 (0.9)
MEtan	0.48 (0.06)	0.16 (0.6)	0.024 (0.9)	-0.2 (0.5)	0.41 (0.1)	0.53 (0.04)	0.47 (0.07)	0.52 (0.04)	0.39 (0.1)	0.36 (0.2)	-0.39 (0.1)	0.46 (0.07)	0.53 (0.03)	0.3 (0.3)	0.38 (0.2)
MEdarkred	0.45 (0.08)	0.2 (0.5)	0.28 (0.3)	-0.13 (0.6)	0.15 (0.6)	0.11 (0.7)	0.051 (0.9)	0.25 (0.4)	0.2 (0.5)	0.25 (0.4)	-0.56 (0.02)	0.25 (0.3)	0.25 (0.3)	0.3 (0.3)	0.2 (0.5)
MEgreenyellow	0.28 (0.3)	-0.054 (0.8)	-0.0083 (1)	-0.39 (0.1)	-0.029 (0.9)	0.056 (0.8)	0.29 (0.3)	0.11 (0.7)	0.33 (0.2)	0.42 (0.1)	-0.36 (0.2)	0.25 (0.4)	0.16 (0.6)	0.3 (0.3)	0.4 (0.1)
MEpurple	0.33 (0.2)	0.016 (1)	-0.29 (0.3)	0.1 (0.7)	-0.31 (0.2)	0.13 (0.6)	0.25 (0.3)	0.099 (0.7)	0.11 (0.7)	0.16 (0.5)	-0.31 (0.2)	0.14 (0.6)	0.16 (0.5)	0.04 (0.9)	0.11 (0.7)
MEbrown	0.14 (0.6)	0.13 (0.6)	0.072 (0.8)	0.24 (0.4)	0.3 (0.3)	0.61 (0.01)	0.23 (0.4)	0.33 (0.2)	0.076 (0.8)	-0.19 (0.5)	0.17 (0.5)	0.33 (0.2)	0.3 (0.3)	0.017 (0.9)	-0.17 (0.5)
MEdarkturquoise	0.43 (0.1)	0.33 (0.2)	0.067 (0.8)	0.36 (0.2)	0.4 (0.1)	0.45 (0.08)	0.098 (0.7)	0.49 (0.05)	-0.21 (0.4)	-0.14 (0.6)	-0.044 (0.9)	0.32 (0.2)	0.42 (0.1)	-0.22 (0.4)	-0.12 (0.7)
MElightyellow	-0.27 (0.3)	-0.18 (0.5)	-0.22 (0.4)	0.5 (0.05)	-0.45 (0.08)	-0.081 (0.8)	-0.26 (0.3)	-0.31 (0.2)	-0.43 (0.1)	-0.41 (0.1)	0.65 (0.006)	-0.15 (0.6)	-0.32 (0.2)	-0.34 (0.2)	-0.48 (0.06)
MEblack	-0.13 (0.6)	0.14 (0.6)	0.16 (0.6)	0.33 (0.2)	0.12 (0.7)	-0.23 (0.4)	-0.37 (0.2)	-0.076 (0.8)	-0.29 (0.3)	-0.36 (0.2)	0.18 (0.5)	-0.32 (0.2)	-0.18 (0.5)	-0.23 (0.4)	-0.29 (0.3)
MEred	-0.03 (0.9)	0.23 (0.4)	0.27 (0.3)	0.43 (0.1)	0.043 (0.9)	-0.23 (0.4)	-0.56 (0.02)	-0.13 (0.6)	-0.28 (0.3)	-0.29 (0.3)	0.05 (0.9)	-0.3 (0.3)	-0.21 (0.4)	-0.082 (0.8)	-0.29 (0.3)
MElightgreen	-0.21 (0.4)	-0.23 (0.4)	-0.43 (0.09)	0.045 (0.9)	-0.2 (0.5)	-0.36 (0.2)	-0.12 (0.7)	-0.089 (0.7)	-0.068 (0.8)	0.032 (0.9)	-0.023 (0.9)	-0.28 (0.3)	-0.26 (0.3)	-0.084 (0.8)	0.045 (0.9)
MEcyan	0.057 (0.8)	0.067 (0.8)	0.088 (0.7)	0.49 (0.05)	-0.13 (0.6)	-0.25 (0.4)	-0.39 (0.1)	-0.21 (0.4)	0.042 (0.9)	-0.086 (0.8)	-0.12 (0.7)	-0.27 (0.3)	-0.28 (0.3)	0.15 (0.6)	-0.068 (0.8)
MEroyalblue	0.16 (0.6)	0.2 (0.5)	0.068 (0.8)	0.26 (0.3)	-0.25 (0.3)	-0.53 (0.04)	-0.34 (0.2)	-0.4 (0.1)	0.013 (1)	-0.14 (0.6)	-0.29 (0.3)	-0.3 (0.3)	-0.39 (0.1)	0.24 (0.4)	-0.12 (0.7)
MEdarkgreen	-0.23 (0.4)	-0.22 (0.4)	-0.16 (0.5)	0.34 (0.2)	-0.82 (1e-04)	-0.66 (0.005)	-0.51 (0.04)	-0.58 (0.02)	-0.51 (0.04)	-0.29 (0.3)	0.25 (0.3)	-0.37 (0.2)	-0.58 (0.02)	-0.26 (0.3)	-0.41 (0.1)
MEsalmon	-0.31 (0.2)	-0.27 (0.3)	-0.45 (0.08)	0.47 (0.07)	-0.68 (0.004)	-0.26 (0.3)	-0.16 (0.6)	-0.26 (0.3)	-0.7 (0.002)	-0.52 (0.04)	0.43 (0.1)	-0.25 (0.3)	-0.28 (0.3)	-0.69 (0.003)	-0.62 (0.01)
MEgrey60	0.24 (0.4)	0.094 (0.7)	-0.19 (0.5)	0.58 (0.02)	-0.39 (0.1)	-0.23 (0.4)	-0.27 (0.3)	-0.036 (0.9)	-0.25 (0.3)	-0.11 (0.7)	-0.17 (0.5)	-0.18 (0.5)	-0.045 (0.9)	-0.13 (0.6)	-0.19 (0.5)
MEturquoise	-0.044 (0.9)	0.17 (0.5)	0.28 (0.3)	0.2 (0.5)	-0.16 (0.5)	-0.67 (0.004)	-0.76 (6e-04)	-0.42 (0.1)	-0.26 (0.3)	0.003 (1)	-0.16 (0.6)	-0.49 (0.05)	-0.42 (0.1)	0.014 (1)	-0.032 (0.9)
MElightcyan	0.42 (0.1)	0.22 (0.4)	-0.022 (0.9)	-0.014 (1)	-0.22 (0.4)	-0.3 (0.3)	-0.19 (0.5)	-0.081 (0.8)	-0.036 (0.9)	0.33 (0.2)	-0.49 (0.05)	-0.073 (0.8)	-0.022 (0.9)	0.056 (0.8)	0.28 (0.3)
MEmidnightblue	0.35 (0.2)	0.29 (0.3)	0.29 (0.3)	0.2 (0.5)	-0.16 (0.6)	-0.43 (0.1)	-0.5 (0.05)	-0.2 (0.4)	-0.016 (1)	0.17 (0.5)	-0.43 (0.1)	-0.24 (0.4)	-0.17 (0.5)	0.23 (0.4)	0.12 (0.6)
MEgrey	-0.16 (0.6)	-0.29 (0.3)	-0.27 (0.3)	-0.43 (0.1)	-0.012 (1)	0.31 (0.2)	0.61 (0.01)	0.14 (0.6)	0.27 (0.3)	0.15 (0.6)	0.12 (0.7)	0.3 (0.3)	0.22 (0.4)	0.033 (0.9)	0.16 (0.5)

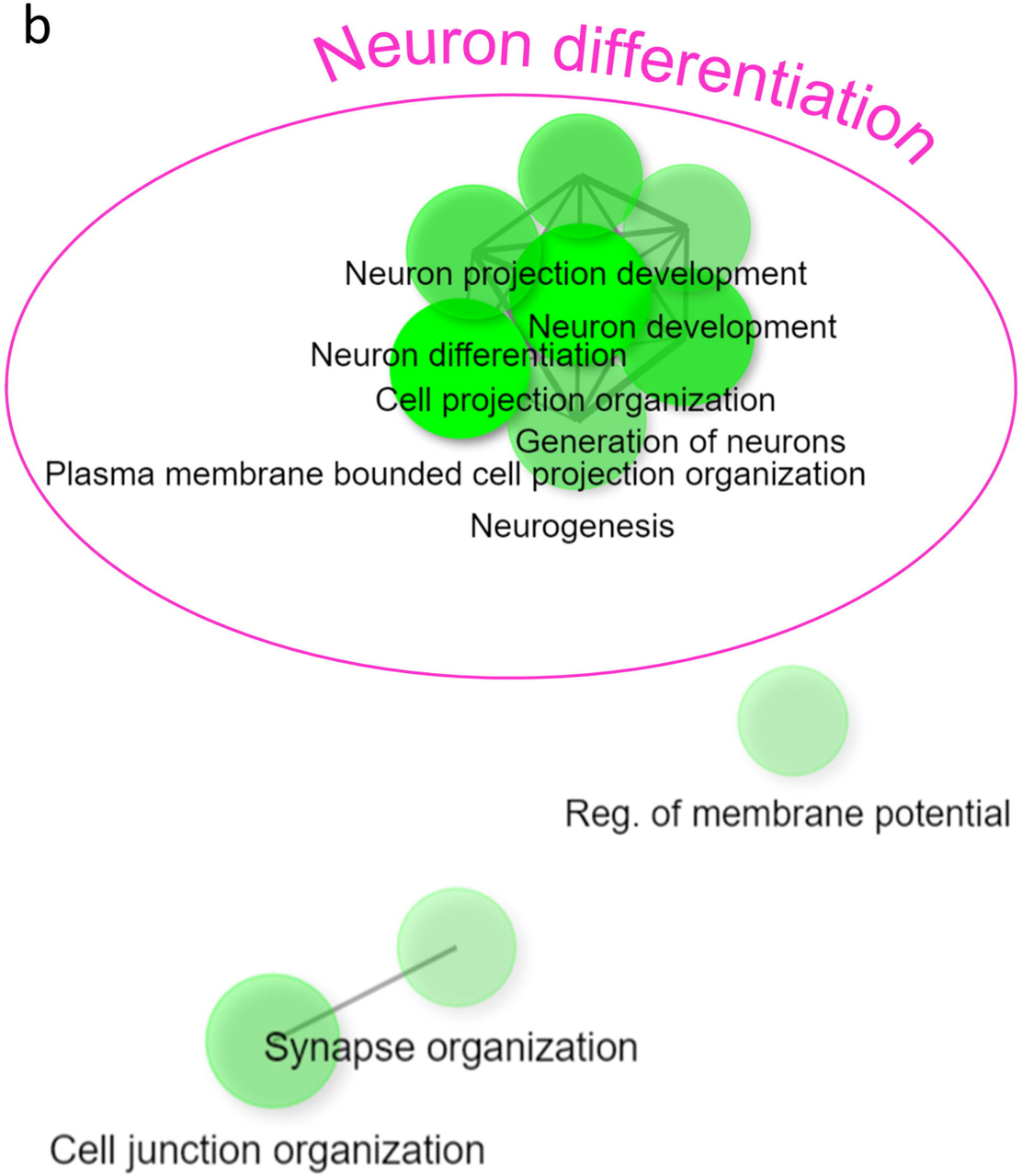
b

	LengthMO
MEblue	0.52 (0.04)
MEpink	0.57 (0.02)
MEgreen	0.65 (0.007)
MEtan	0.53 (0.04)
MEbrown	0.61 (0.01)
MEroyalblue	-0.53 (0.04)
MEdarkgreen	-0.66 (0.005)
MEturquoise	-0.67 (0.004)

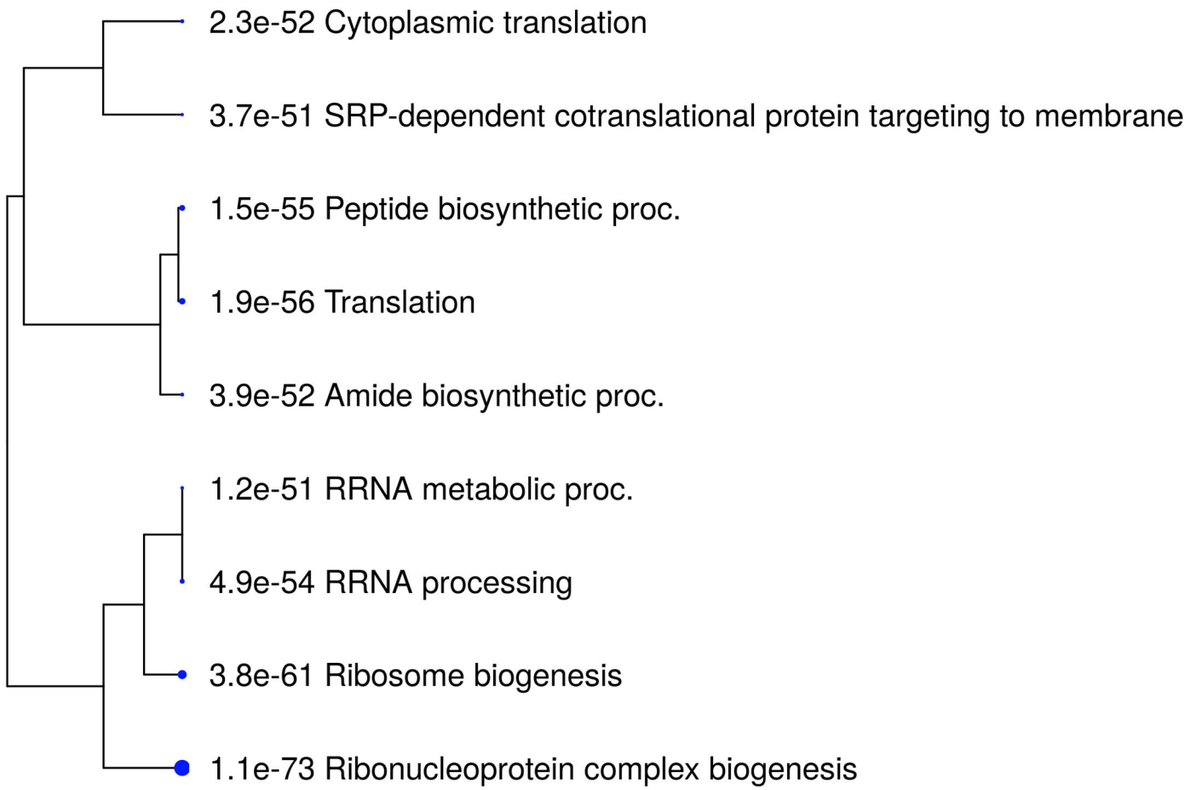
a


1.0e-09 Neurogenesis
7.7e-11 Generation of neurons
2.4e-10 Neuron differentiation
1.6e-09 Neuron development
3.4e-10 Neuron projection development
2.3e-12 Plasma membrane bounded cell projection organization
1.0e-12 Cell projection organization
2.8e-09 Cell junction organization
7.6e-09 Synapse organization
7.6e-09 Reg. of membrane potential

b

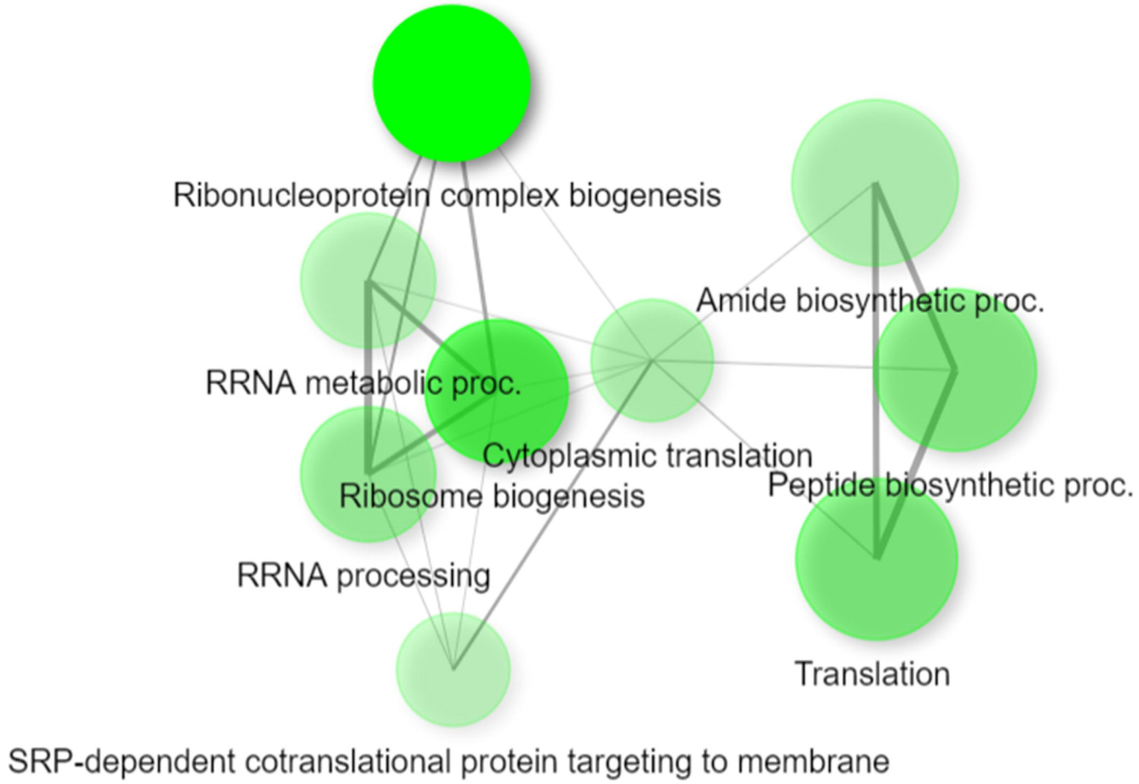

Neuron differentiation
Neuron projection development
Neuron development
Neuron differentiation
Cell projection organization
Generation of neurons
Plasma membrane bounded cell projection organization
Neurogenesis
Reg. of membrane potential
Synapse organization
Cell junction organization

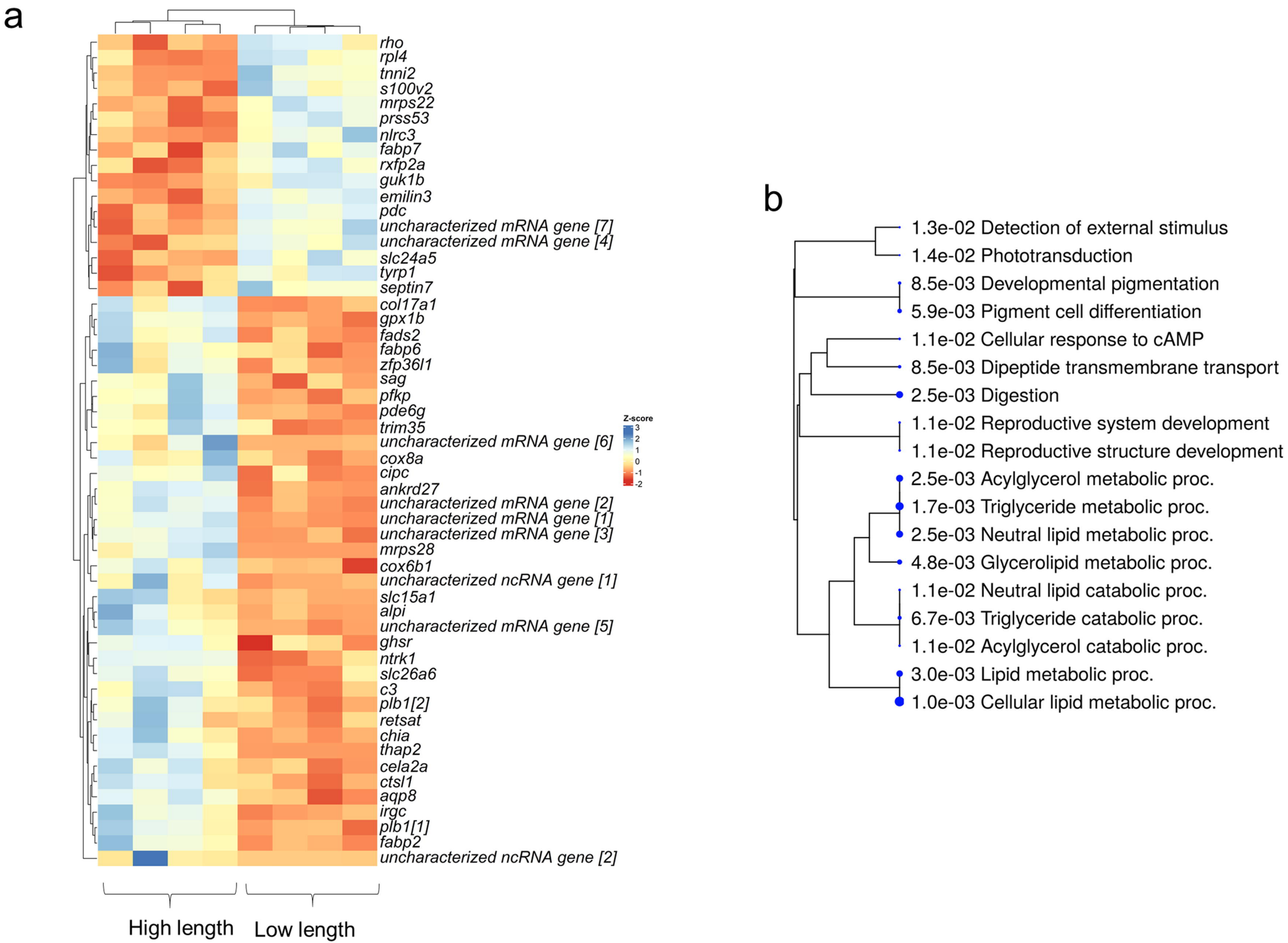
a

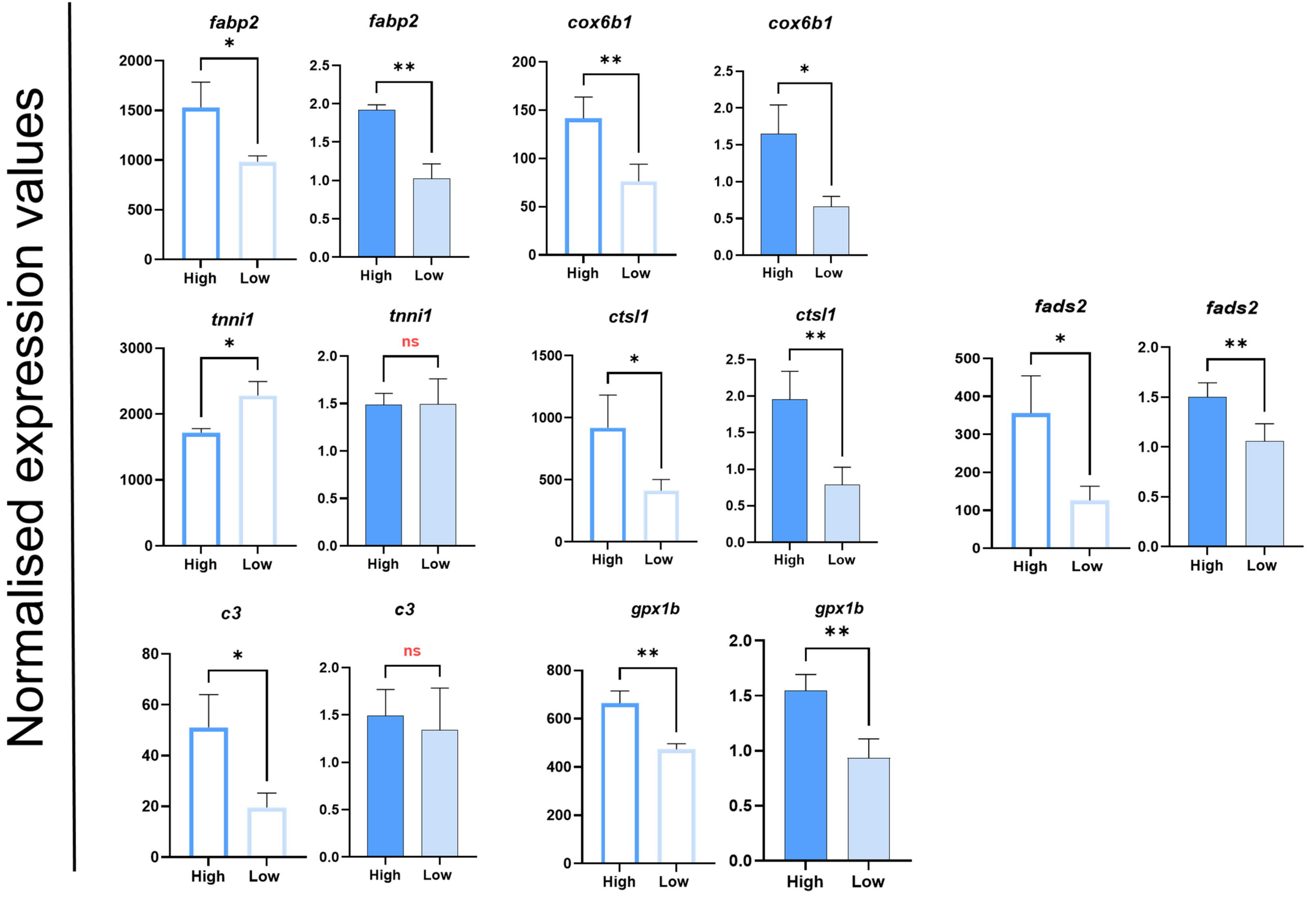


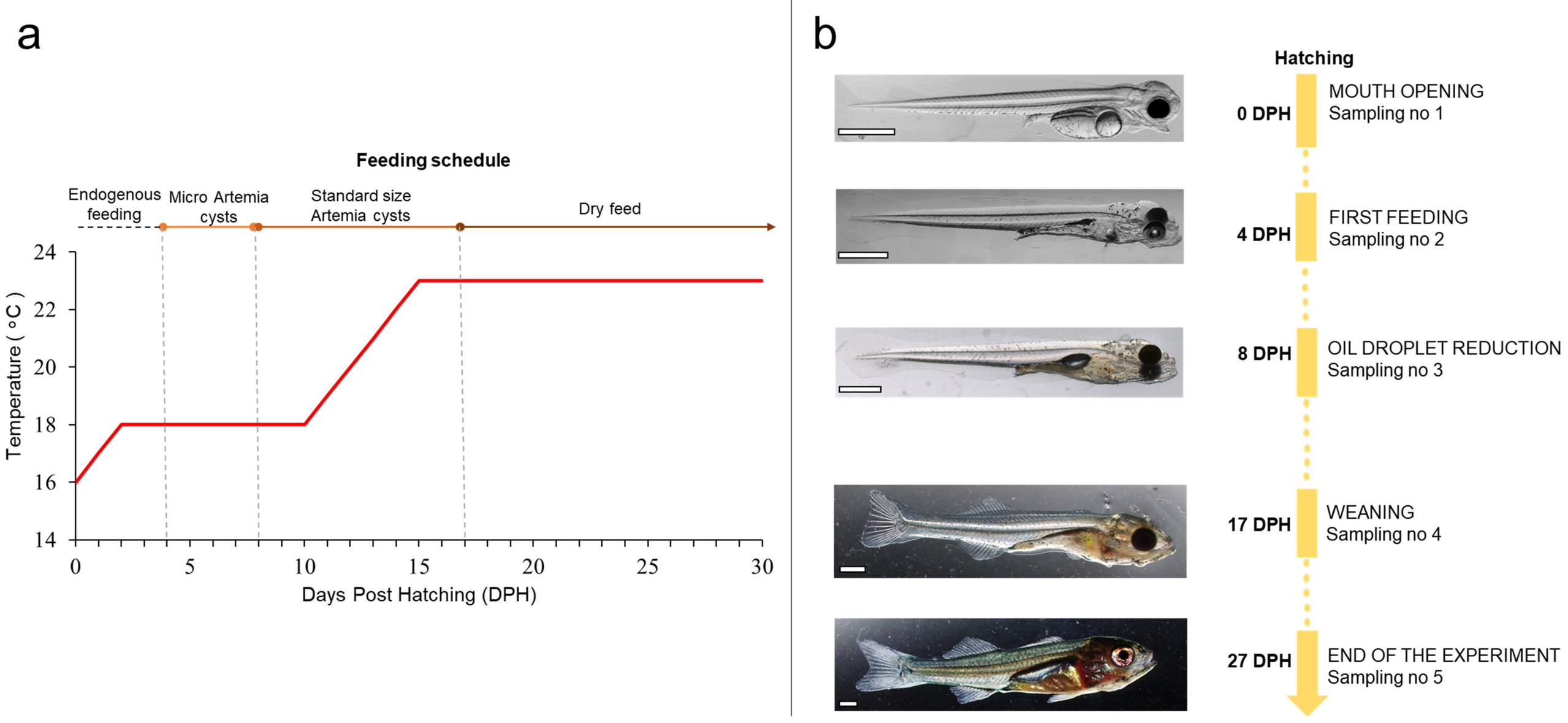
b

Translation




a
rho
rpl4
tnni2
s100v2
mrps22
prss53
nlrc3
fabp7
rxfp2a
guk1b
emilin3
pdc
uncharacterized mRNA gene [7]
uncharacterized mRNA gene [4]
slc24a5
tyrp1
septin7
col17a1
gpx1b
fads2
fabp6
zfp36l1
sag
pfkp
pde6g
trim35
uncharacterized mRNA gene [6]
cox8a
cipc
ankrd27
uncharacterized mRNA gene [2]
uncharacterized mRNA gene [1]
uncharacterized mRNA gene [3]
mrps28
cox6b1
uncharacterized ncRNA gene [1]
slc15a1
alpi
uncharacterized mRNA gene [5]
ghsr
ntrk1
slc26a6
c3
plb1[2]
retsat
chia
thap2
cela2a
ctsl1
aqp8
irgc
plb1[1]
fabp2
uncharacterized ncRNA gene [2]
Z-score
3
2
1
0
-1
-2
High length
Low length
b
1.3e-02 Detection of external stimulus
1.4e-02 Phototransduction
8.5e-03 Developmental pigmentation
5.9e-03 Pigment cell differentiation
1.1e-02 Cellular response to cAMP
8.5e-03 Dipeptide transmembrane transport
2.5e-03 Digestion
1.1e-02 Reproductive system development
1.1e-02 Reproductive structure development
2.5e-03 Acylglycerol metabolic proc.
1.7e-03 Triglyceride metabolic proc.
2.5e-03 Neutral lipid metabolic proc.
4.8e-03 Glycerolipid metabolic proc.
1.1e-02 Neutral lipid catabolic proc.
6.7e-03 Triglyceride catabolic proc.
1.1e-02 Acylglycerol catabolic proc.
3.0e-03 Lipid metabolic proc.
1.0e-03 Cellular lipid metabolic proc.


Normalised expression values
fabp2
*
2000
1500
1000
500
0
High
Low
fabp2
**
2.5
2.0
1.5
1.0
0.5
0.0
High
Low
cox6b1
**
200
150
100
50
0
High
Low
cox6b1
*
2.5
2.0
1.5
1.0
0.5
0.0
High
Low
tnni1
*
3000
2000
1000
0
High
Low
tnni1
ns
2.0
1.5
1.0
0.5
0.0
High
Low
ctsl1
*
1500
1000
500
0
High
Low
ctsl1
**
2.5
2.0
1.5
1.0
0.5
0.0
High
Low
fads2
*
500
400
300
200
100
0
High
Low
fads2
**
2.0
1.5
1.0
0.5
0.0
High
Low
c3
*
80
60
40
20
0
High
Low
c3
ns
2.0
1.5
1.0
0.5
0.0
High
Low
gpx1b
**
800
600
400
200
0
High
Low
gpx1b
**
2.0
1.5
1.0
0.5
0.0
High
Low


a
Feeding schedule
Endogenous feeding
Micro Artemia cysts
Standard size Artemia cysts
Dry feed
Temperature (°C)
24
22
20
18
16
14
0
5
10
15
20
25
30
Days Post Hatching (DPH)
b
Hatching
0 DPH
MOUTH OPENING
Sampling no 1
4 DPH
FIRST FEEDING
Sampling no 2
8 DPH
OIL DROPLET REDUCTION
Sampling no 3
17 DPH
WEANING
Sampling no 4
27 DPH
END OF THE EXPERIMENT
Sampling no 5