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DHA-enrichment of live and compound feeds influences the incidence of cannibalism, digestive function, and growth in the neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) during early life stages

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Abstract :

Pseudoplatystoma punctifer is a highly appreciated fish species native to the Amazon basin, whose commercial farming has been hampered by low survival during early life stages due to the high incidence of cannibalism and the low acceptability of compound diets at weaning. Dietary DHA is known to promote digestive system development and maturation and growth, whereas its deficiency induces stress. The aim of this study was to evaluate the effect of dietary DHA supplementation on the incidence of cannibalism, digestive physiology, and growth performance during the early life stages of P. punctifer to improve current feeding protocols. Four dietary treatments were generated using a commercial enrichment product with high DHA content. Fish larvae were fed non-enriched or enriched Artemia from 4 to 15 days post fertilization (dpf) and fed a non-enriched or enriched compound diet from 15 to 26 dpf, coinciding with the start of the juvenile stage. Growth, survival, incidence of cannibalism, proximate and fatty acid composition, histology of the intestine and liver, and quantitative gene expression of the main digestive enzymes (amy, try, ctr, pga, pla2, and lpl) were analyzed in the different dietary groups at the end of each feeding period. Results showed that dietary DHA supplementation influenced P. punctifer in a developmental stage-dependent manner. In particular, DHA-enriched Artemia provided during the larval stage contributed to reduce the incidence of cannibalism and improved survival at the early juvenile stage, while enriching the compound diet improved growth. The expression level of genes involved in protein and carbohydrate digestion (ctr, amy) was higher in groups fed enriched Artemia despite that both

enriched and non-enriched Artemia displayed similar proximate composition, suggesting that different dietary fatty acid profiles may modulate the expression of these digestive enzyme precursors. The group transitioning from enriched Artemia to non-enriched compound diet showed a higher expression of most genes at the early juvenile stage. At the histological level, the group fed non-enriched Artemia and compound diet showed a significant accumulation of lipids in the intestine relative to the liver, contrary to the rest of the groups that showed similar amounts of lipids in both tissues, indicating a more balanced lipid metabolism. The group fed both DHA-enriched Artemia and compound diet provided the best results in terms of growth, survival, incidence of cannibalism, and digestive physiology. In conclusion, this study showed that the nutritional history during the larval period affected fish nutrition and behavior during the early juvenile stage.

Highlights

► Cannibalism in *Pseudoplatystoma punctifer* is affected by nutrition. ► Dietary DHA supplied to larvae conditions the digestive physiology of juveniles. ► Dietary DHA during the larval phase reduces cannibalism at the juvenile stage. ► Dietary DHA during the early juvenile stage of *P. punctifer* promotes growth.

Keywords : DHA, Cannibalism, Nutrition, Catfish larvae, Digestive system

1. Introduction

The most cultivated catfish species in South America belong to the genus *Pseudoplatystoma* Bleeker, 1862, which are piscivorous migratory species native to the major river basins of South America and have total lengths of up to 140 cm (Buitrago–Suárez and Burr, 2007; Gisbert et al., 2022). Current aquaculture production mostly relies on interspecific (e.g., *Pseudoplatystoma reticulatum* x *Pseudoplatystoma corruscans*) and intergeneric hybrids with omnivorous pimelodid species (*Leiarius marmoratus* or *Phractocephalus hemioliopterus*), since the latter present less cannibalism during early life stages and more readily accept compound diets than the *Pseudoplatystoma* spp. parent species (Gisbert et al., 2022; Hashimoto et al., 2012). However, given the risks associated to the culture of hybrid species (Hashimoto et al., 2015, 2013), research efforts are being made to develop more efficient culture practices for pure *Pseudoplatystoma* species, as is the case for *Pseudoplatystoma punctifer* (Castelnau, 1855), a species native to the Amazon basin (e.g., Castro-Ruiz et al., 2021a, 2021b, 2019; Darias et al., 2015; Gisbert et al., 2014). The commercial farming of this highly appreciated species has been hampered by low survival during early life stages due to the high incidence of

cannibalism and the low acceptability of compound diets at weaning (Baras et al., 2011; Gisbert et al., 2014). However, recent studies have found a strong correlation between nutrition and the cannibalistic behavior in this species; in particular, that when using a feeding protocol adapted to digestive capacities and nutritional needs during early life stages, growth and survival are substantially improved (Castro-Ruiz et al., 2021a, 2021b, 2019; Darias et al., 2015).

During the early life stages of fish, an optimal dietary fatty acid composition, especially polyunsaturated fatty acids (PUFA), is essential to promote adequate development and growth (Lund et al., 2012; Mourente, 2003; Watanabe, 1993). Freshwater species have lower requirements for n-3 highly unsaturated fatty acids (HUFA) than marine fish larvae (Verreth et al., 1994). However, the fatty acid composition of *Artemia*, naturally lacking n-3 HUFAs (Sargent et al., 1999), may also affect growth in freshwater species (Bengtson et al., 1991). In the case of *P. punctifer*, previous studies have shown that *Artemia* nauplii did not satisfy the nutritional needs of this species from 12 days post-fertilization (dpf) onwards, coinciding with the beginning of the juvenile stage, leading to decreased growth and increased incidence of cannibalism (Castro-Ruiz et al., 2019; Darias et al., 2015; Gisbert et al., 2014). DHA is particularly important during the larval development due to its structural role in biomembranes, especially in neural tissues, such as the retina and the brain (Bell et al., 1996; Mourente, 2003; Wassall and Stillwell, 2008). Dietary DHA provided in adequate quantities promotes digestive system development and maturation, growth, survival, and normal morphogenesis (Cahu et al., 2003; Takeuchi, 2014; Villeneuve et al., 2005; Zambonino Infante and Cahu, 1999), whereas dietary DHA deficiencies induce physiological stress (Lund et al., 2012). Considering that stress can favor cannibalistic behavior (Naumowicz et al., 2017) and that inadequate nutrition has shown to influence the incidence of cannibalism in *P. punctifer* (Castro-Ruiz et al., 2021b; Darias et al., 2015), the aim of this study was to evaluate the influence of dietary DHA supplementation on the incidence of cannibalism, digestive physiology, and growth

performance during the early life stages in *P. punctifer*. A feeding trial in which *Artemia* metanauplii and compound diets were either enriched or not with DHA was used to elucidate whether the dietary requirements in DHA varied throughout development. The effects of these feeding regimes on the development and function of the digestive system of early juveniles of *P. punctifer* were analyzed at histological and molecular levels. Regarding the latter, the study focused on the expression of the main digestive enzyme precursors α-amylase (*amy*), phospholipase A2 (*pla2*), lipoprotein lipase (*lpl*), trypsinogen (*try*), chymotrypsinogen (*ctr*), and pepsinogen (*pga*). The outcomes of this study will contribute to improve the understanding of the nutritional needs of this species during early development and to ameliorate the deficiencies in the nutritional composition of feeds, as well as to optimize the feeding protocols used, in order to promote adequate growth and health in this Amazonian species.

2. Materials and methods

2.1. Rearing protocol

Spawning of a sexually mature couple of *P. punctifer* (♀: 4.15 kg; ♂: 1.15 kg) from a broodstock maintained in captivity at the Instituto de Investigaciones de la Amazonia Peruana (IIAP, Iquitos, Peru) was hormonally induced. The female and male were injected intramuscularly with carp pituitary extract (Argent Chemical Laboratories, Inc., Redmond, WA, USA) at 5 mg kg^{-1} and 1 mg kg^{-1} of body weight, respectively. Hormone injections were administered in two doses 12 h apart: the first at 10% and 50% of the total dose, and the second at 90% and 50% of the total dose for the female and the male, respectively. Stripping of the female, sperm collection, and fertilization procedure were performed according to Nuñez et al. (2008). Fertilized eggs (fertilization rate ~99.9%) were incubated at 28 °C in 60-L tanks connected to a freshwater recirculating system; hatching took place 18 ± 2 h later (hatching rate ~87%). Larvae were transferred at 4 days post-fertilization, dpf (5.1 ± 0.7 mm total length, TL),

into 40 L tanks (30-L water volume, 1000 larvae per tank) connected to a clear water recirculation system with mechanical and biological filters. Water conditions throughout the experiment were: temperature, 27.1 ± 0.6 °C; pH, 7.0 ± 0.6; dissolved oxygen, 8.9 ± 1.5 mg L^{-1}; NO_2^-, 0.04 ± 0.02 mg L^{-1}, NH_4^+, 0.14 ± 0.05 mg L^{-1}. Water temperature, pH, and dissolved oxygen were measured daily and NO_2^- and NH_4^+ weekly in each tank. Water supply was adjusted in each tank to assure a water flow rate of 0.2 L min^{-1}. Fish were reared from 4 to 36 dpf under a photoperiod of 0L:24D.

2.2. Experimental design and feeding protocol

Larvae were fed 5 times a day from 4 to 14 dpf *Artemia* spp. metanauplii in slight excess (0.6-12 nauplii mL^{-1}) considering larval density, weight increase, and the daily food ration. As previous studies have shown that *P. punctifer* early juveniles prefer moist over dry feeds at weaning (Fernández-Méndez et al., 2015), individuals were weaned from 15 dpf onto a compound moist diet within 4 days. Juveniles were solely fed the compound moist diet from 19 dpf onwards 5 times a day (5% fish wet weight, WW) until 27 dpf, when the nutritional trial ended. The moist diet was elaborated using a commercial compound diet (Purina®, Cargill Incorporated, Lima, Peru) containing 45% protein, 10% lipids, 2% fiber, 12% moisture and 12% ash, to which water and neutral gelatin were added (Fernández-Méndez et al., 2015). Four experimental feeding protocols were tested in triplicate: 1) the control group C fed non-enriched *Artemia* and non-enriched compound diet; 2) the group T1 fed enriched *Artemia* and non-enriched compound diet; 3) the group T2 fed non-enriched *Artemia* and enriched compound diet; and 4) the group T3 fed enriched *Artemia* and enriched compound diet (Figure 1). In the T1 and T3 groups, one-day-old *Artemia* nauplii (100 nauplii mL^{-1}) were enriched with a commercial enriching product containing high levels of DHA (Algamac 3050, Pacific Trading Aquaculture Ltd., Dublin, Ireland) for 16 h at 28 °C with 0.2 g L^{-1} of Algamac 3050, following

manufacturer's instructions. After enrichment, *Artemia* metanauplii were washed with sterile, filtered, and slightly salted water to reduce the bacterial load and remove residues of the enrichment emulsions, and kept at 4 °C in sterile, filtered, and slightly salted water with aeration until administered to the larvae. In the T2 and T3 groups, the compound moist diet was enriched with Algamac 3050 at 10%. From 27 dpf, all groups were adapted within 3 days to the commercial compound diet (Purina®) and fed solely this feed 3 times a day (5% fish WW) from 30 dpf until an additional control of survival was made at 36 dpf.

2.3. Fish performance

Individuals of *P. punctifer* were sampled from each tank at 14 and 26 dpf and euthanized with an overdose of Eugenol (0.05 µL mL^{-1}; Moyco®, Moyco, Lima, Peru). In order to monitor growth, 15 individuals per tank were placed in a Petri dish, photographed using a scale bar and TL was measured on the digital images (300 dpi) using ImageJ software (Schneider et al., 2012). At 14 and 26 dpf, WW was determined using an analytic microbalance (Sartorius BP 211 D, Data Weighing Systems, Inc., Elk Grove, IL, USA, ± 0.01 mg). The number of cannibals was counted in each tank twice a day (08:00 h and 17:00 h) and the incidence of cannibalism expressed as the percentage of fish displaying cannibalistic behavior at each feeding period. Two types of cannibalism were recorded: type I, when larvae were partially damaged (pectoral fins and/or stomach bitten), and type II, when individuals were completely ingested by their siblings. Survival was evaluated by counting the individuals surviving at 14, 26, and 36 dpf with respect to the number of individuals at the beginning of each feeding period and calculated considering the number of individuals sampled at each sampling point.

In the absence of an *ad hoc* ethical committee at the IIAP where this trial was conducted, the animal experimental procedures were conducted in compliance with the Guidelines of the

European Union Council (2010/63/EU) on the protection of animals used for scientific purposes.

2.4. Proximate composition and fatty acid analyses

The experimental feeds (enriched and non-enriched *Artemia* and compound diets) were sampled in triplicate (*ca*. 1 g per replicate) and stored at −80 °C until analysis. Sampled *P. punctifer* individuals at 4, 14, and 26 dpf (*ca*. 1 g per tank) were washed with distilled water and kept at −80 °C after removing the excess of water. Total lipids of the feeds and fish specimens were extracted in chloroform:methanol (2:1, v/v) using the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by overnight vacuum desiccation. Total lipids were stored in chloroform:methanol (2:1, 20 mg mL^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at −20 °C until analysis. Acid-catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane:diethyl ether (1:1, v/v), purified on thin-layer chromatography plates (Silica gel 60, VWR, Lutterworth, UK), and analyzed by gas–liquid chromatography on a Thermo Electron Trace GC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 × 0.25 mm id; SGE, Milton Keynes, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}, helium (1.2 mL min^{-1} constant flow rate) as the carrier gas and on-column injection, and flame ionization detection at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Bellefonte, PA, USA) and a well characterized fish oil and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-Card for Windows (Trace GC, Thermo Finnigan, Milan, Italy). Results of fatty acid content were expressed as a percentage of total fatty acids (TFA). Protein and carbohydrate contents were

determined following the Lowry et al. (1951) and the DuBois et al. (1956) methods, respectively.

2.5. Histological analyses

Individuals of *P. punctifer* (n = 10) were sampled at 14 and 26 dpf from each tank and fixed in buffered formaldehyde (pH = 7.2) at 4 °C overnight. The day after, individuals were dehydrated with graded series of ethanol and stored in 70% ethanol at 4 °C until further processing. After the dehydration process, individuals were embedded in paraffin with an automatic tissue processor Histolab ZX-60 Myr (Especialidades Médicas MYR SL, Tarragona, Spain). Then, paraffin blocks were prepared in an AP280-2Myr station and cut into serial sagittal sections (3 μm thick) with an automatic microtome Microm HM (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Paraffin cuts were kept at 40 °C overnight. After that, samples were deparaffinized with a graded series of xylene substitute and stained by means of hematoxylin and eosin for general micromorphological observations. Histological preparations were observed under a Leica DM2000 LED microscope equipped with a camera Leica MC170 HD (Leica Microsystems Nussloch GmbH, Nussloch, Germany) as described in Gisbert et al. (2008).

2.6. RNA extraction and gene expression analyses

Fifteen individuals per tank were used for total RNA extraction. RNA from 100 mg of whole fish homogenates at 14 and 26 dpf was extracted using TRIzol™ (Invitrogen, San Diego, CA, USA) according to manufacturer's protocol. RNA concentration and quality were determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at λ = 260 and 280 nm and by denaturing electrophoresis in TAE agarose gel (1.5 %), respectively. Total RNA was treated with DNase I Amplification Grade (Invitrogen,

San Diego, CA, USA) according to manufacturer's protocol and then reverse transcribed in 10 µL reaction volume containing 3 µg total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA, USA) with oligo (dT) (12-18) (0.5 µg/ul) and random hexamers primers (50 ng μL^{-1}), 10X RT buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCL), 25 mM MgCl2, 0.1 M DTT, 10 mM dNTP mix, SuperScript™ II RT (50 U μL^{-1}), RNaseOUT™ (40 U μL^{-1}), followed by RNase H (2 U μL^{-1}) (Invitrogen, San Diego, CA, USA) treatment. Reverse transcription reactions were carried out in a thermocycler (Mastercycle R nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer's protocol. The samples were diluted 1:20 in molecular biology grade water and stored at −20 °C until further analyses. The expression of *amy* (AC MT006358), *ctr* (AC MT006344), *try* (AC MT006359), *lpl* (AC MT006346), *pla2* (AC MT006345), and *pga* (AC MT006343) (Castro-Ruiz et al., 2021a) was analyzed in individuals from the four experimental groups at 14 and 26 dpf. Quantitative PCR analyses were carried out in triplicate in a 7300 Real-Time PCR System (Applied Biosystems, Roche, Barcelona, Spain). The amplification mix contained 1 µL cDNA, 0.5 µL primers (20 µM), and 10 µL SYBR Green Supermix (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 µL. A negative control was included (no template control) for each set of reactions on each 96-well plate. The amplification conditions were as follows: 10 min at 95 °C, 40 cycles of 20 s at 95 °C, and 1 min at 65 °C, followed by 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, and finally, 15 s at 60 °C. A standard curve was obtained by amplification of a dilution series of cDNA for calculation of the efficiency (E) for each set of primers. Real-time PCR efficiencies were determined for each gene from the slopes obtained with Applied Biosystems software, applying the equation E=10[−1/slope], where E is PCR efficiency. The relative gene expression ratio (R) for each gene was calculated according to Pfaffl's (2001) formula: $R = (E_{\text{target gene}})^{\Delta Cq \text{ target gene (mean sample} - \text{mean reference sample)}} / (E_{\text{reference gene}})^{\Delta Cq \text{ reference gene (mean sample} - \text{mean reference sample)}}$, where ΔCq is the deviation of the target sample minus

the reference sample. The relative expression of the genes was normalized using glyceraldehyde-3-phosphate dehydrogenase (*gapdh*, AC MT006341) (Castro-Ruiz et al., 2021a) as the reference gene, since it did not exhibit any significant variation in expression between samples.

2.7. Statistics

Results were expressed as mean ± standard deviation (n = 3). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). One-way ANOVA was performed to analyze differences in growth, proximate and fatty acid composition, and gene expression during development and/or among dietary treatments. All pairwise multiple comparisons were performed using the Holm-Sidak method if significant differences were found at $P < 0.05$ to discriminate the significant differences. Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, VA, USA).

3. Results

3.1. Growth performance

Growth in terms of WW and TL is shown in Figure 2. At 14 dpf, individuals presented similar WW among dietary groups, whereas individuals from T2 and T3 groups displayed higher WW than those from C and T1 groups at 26 dpf (Figure 2; $P < 0.05$). There were no differences in TL among the different dietary treatments at both sampling times (14 and 26 dpf) ($P > 0.5$).

3.2. Incidence and temporal occurrence of cannibalism

The incidence of cannibalism was reduced by half in all the enriched groups (T1, T2, and T3) and, as a consequence, survival increased 3-fold in these groups (Figure 3).

Figure 4 shows the incidence of cannibalism for each feeding period (*Artemia*, weaning, and compound diet). In general, the total incidence of cannibalism was lowest during the *Artemia* feeding period (ca. 0.5 %), increased at weaning (ca. 2 %), and peaked during the compound diet feeding period (ca. 8 %). The incidence of total cannibalism was higher in the C and T2 groups during the *Artemia* feeding period, in the C and T1 groups at weaning, and in the C group during the compound diet feeding period ($P < 0.05$, Figure 4A). In regards to the type of cannibalism (Figure 4B), the incidence of type I cannibalism was higher than type II during the *Artemia* feeding phase, being highest in the C group and lowest in the T3 group ($P < 0.05$); however, the incidence of type II cannibalism was similar in all dietary groups ($P > 0.05$, Figure 4B). During the weaning period, the T3 group presented a higher incidence of type I cannibalism ($P < 0.05$), whereas the incidence of both types of cannibalism was similar in the rest of the dietary groups (Figure 4B). Among dietary groups, the C, T1, and T3 groups presented a similar incidence of type I cannibalism, which was higher than that of the T2 group ($P < 0.05$, Figure 4B). In contrast, type II cannibalism was lowest in the T3 group ($P < 0.05$, Figure 4B). During the compound diet feeding period, the C group presented a higher incidence of type I cannibalism than of type II and also showed the highest incidence of both types of cannibalism ($P < 0.05$) among the different groups (Figure 4B).

When comparing the temporal incidence of cannibalism at morning and evening time (Figure 4C, D), cannibalism was not detected in the evening during the *Artemia* feeding phase (Figure 4C). A similar level of type I cannibalism incidence was detected in the morning in all dietary groups with ($P > 0.05$, Figure 4C), whereas type II cannibalism was only detected in similar levels ($P > 0.05$) in the morning in groups C and T1 (Figure 4D). At weaning, type I cannibalism in the morning was higher in groups T1 and T3 ($P < 0.05$), whereas it was similar at evening in all dietary groups ($P > 0.05$, Figure 4C). In groups T1 and T3, type I cannibalism was higher in the morning than at evening, whereas the opposite was found in the T2 group ($P < 0.05$, Figurer

4C). The highest and lowest incidence of type II cannibalism in the morning were detected in the C and T3 groups, respectively ($P < 0.05$), but no differences were found in the evening among dietary groups ($P > 0.05$, Figure 4D). Within dietary groups, only the C group showed a higher incidence of type II cannibalism in the morning than in the evening ($P < 0.05$, Figure 4D). Finally, during the compound diet feeding period, the incidence of type I cannibalism was similar in all dietary groups in the morning ($P > 0.05$), whereas it was higher in the C group at evening ($P < 0.05$, Figure 4C); and the C group presented the highest incidence of type II cannibalism both in the morning and in the evening (Figure 4D. Within dietary groups, only the T2 group showed a higher incidence of both types of cannibalism in the morning than in the evening ($P < 0.05$, Figure 4C, D).

3.3. Proximate composition, lipid classes, and total fatty acids composition of live prey and experimental diets

Proximate composition and lipid classes of the experimental diets are presented in Table 1 and 2, respectively. Protein content was similar in all experimental diets (*ca*. 37 %). Lipid content was similar (*ca*. 14 %) in all diets, except for the non-enriched compound diet, which contained a lower amount of dietary lipids (10 %). Carbohydrate content was higher in compound diets than in *Artemia* (25 % *vs* 10 %, respectively). Concerning lipid classes, total phospholipid content was higher in *Artemia* (18 %) than in compound diets (*ca*. 9 %), in which phosphatidylcholine (PC) and phosphatidylethanolamine (PE) accounted for such differences. On the contrary, total neutral lipids, were higher in compound diets (91 %) than in *Artemia* (82 %), with triglycerides (TAG) accounting for such differences (Table 2).

Total lipid and total fatty acid contents and fatty acid composition of the experimental diets are presented in Table 3. Total lipid and total fatty acid contents were similar between the experimental diets (*ca*. 141 mg g^{-1} total lipid and *ca*. 97 mg g^{-1} TFA, DW), with the exception

of the non-enriched compound diet, which presented lower values. In terms of TFA, the compound diets presented higher contents of saturated fatty acids than *Artemia.* Total monounsaturated fatty acids were more abundant in non-enriched *Artemia* and in the non-enriched compound diet compared to the respective enriched forms. Total n-6 PUFAs were higher in compound diets than in *Artemia*; LA (18:2n-6) and docosapentaenoic acid (DPA, 22:5n-6) accounted for such differences in non-enriched and enriched compound diets, respectively. Total n-3 PUFA content was higher in *Artemia* than in compound diets, with ALA accounting for such difference. Among compound diets, the enriched one contained higher total n-3 PUFA levels, as expected, due to the DHA content of the enriching product. With the exception of the non-enriched compound diet, which showed lower total PUFA content, the rest of the experimental diets presented similar total PUFA levels.

All the experimental diets had a similar ratio of total n-3/n-6 PUFA. The DHA/EPA ratio was higher in the enriched compound diet, followed by enriched *Artemia*. Enriched *Artemia* and the enriched compound diet had the highest and the lowest ARA/DHA ratio, respectively. The LA/PUFA and OA/PUFA ratios were lower in enriched than in non-enriched compound diets. The PUFA/saturated ratio was higher in *Artemia* than in the compound diets.

3.4. Proximate and total fatty acids content and composition of *P. punctifer*

Proximate composition and total lipids and fatty acids of *P. punctifer* individuals fed the different experimental diets are presented in Tables 4 and 5, respectively. The protein content of *P. punctifer* remained constant during development, and regardless of the experimental feeding protocol used ($P > 0.05$). Lipid content was similar between dietary treatments at 14 dpf (end of the *Artemia* feeding phase) ($P > 0.05$), whereas differences were found at 26 dpf (end of the compound feeding phase) ($P < 0.05$), in which the group T3 presented a higher lipid content than the rest of the dietary groups. In addition, the lipid content of the T1 group was

lower at 26 dpf than at 14 dpf ($P < 0.05$). Carbohydrate content was also similar between dietary treatments at 14 dpf ($P > 0.05$), whereas it was higher in the T3 group than in the C group at 26 dpf. Additionally, carbohydrate content of the individuals from the T3 group was higher at 26 dpf than at 14 dpf ($P < 0.05$).

The total fatty acid content of *P. punctifer* specimens generally reflected that of the experimental diets. Individuals from groups C and T2 presented higher content of total monounsaturated fatty acids ($P < 0.05$), whereas no differences among groups were observed at 26 dpf ($P > 0.05$). Further, the content of total monounsaturated fatty acids decreased from 14 to 26 dpf in all dietary groups ($P < 0.05$). The total n-6 PUFAs content was higher in groups T1 and T3 at 14 dpf, and in T2 and T3 at 26 dpf ($P < 0.05$). In addition, the total n-6 PUFAs content increased between 14 and 26 dpf in groups T2 and T3 ($P < 0.05$). The total n-3 PUFA content was similar in all dietary groups both at 14 and 26 dpf ($P > 0.05$); however, differences were found among several n-3 PUFAs. For instance, EPA content was higher in the C group and lower in the T3 group at 26 dpf; higher DPA and lower DHA contents were detected in the groups C and T2 at 14 dpf ($P < 0.05$), whereas at 26 dpf, DHA was similar in all dietary groups ($P > 0.05$). Total PUFA was higher in groups T1 and T3 at 14 dpf ($P < 0.05$), a difference that was not maintained at 26 dpf ($P > 0.05$). At 14 dpf, the groups C and T2 presented a higher ratio of total n-3/n-6 PUFA than the groups T1 and T3, whereas at 26 dpf, the groups C and T1 showed the highest ratio, and T2 and T3 the lowest ($P < 0.05$). At 14 dpf, the DHA/EPA, ARA/EPA, and PUFA/saturated ratios were lower in the groups C and T2 than in the groups T1 and T3, whereas the opposite was found for the ratios LA/PUFA and OA/PUFA ($P < 0.05$). At 26 dpf, however, the DHA/EPA and PUFA/saturated ratios were higher and the ARA/DHA and OA/PUFA ratios were lower in the T2 and T3 groups than in the C and T1 groups ($P < 0.05$).

3.5. Histological analyses

The main histological changes in lipid deposition in the liver and intestine of *P. punctifer* individuals reared under different dietary treatments are shown in Figure 5. Histological analyses at 14 dpf revealed the presence of fattier livers in groups fed non-enriched *Artemia* (C, T2) in comparison to those fed enriched *Artemia* (T1, T3). Lipid deposits in the intestine were similar in all dietary treatments, mainly corresponding to very low-density lipoproteins (Figure 5). At the end of the nutritional trial (26 dpf), groups fed non-enriched compound diets (C, T1) showed low-moderate lipid deposits in the hepatic parenchyma, whereas groups fed enriched compound diets (T2, T3) presented a moderate-high lipid accumulation in the liver. Hepatic diverticula were visible and spaced in groups with low lipid accumulation, whereas this space disappeared in fish displaying significant lipid accumulation. Lipid deposits in the intestine in fish from the C and T1 groups where located throughout the folds of the posterior part of the intestine. The totality of the enterocytes in group C contained lipid deposits and the size (diameter) and surface of the lipid inclusions varied from 7 to 14 μm and from 210 to 450 μm^2, respectively. In the T1 group, the size of lipid inclusions varied between 7 and 10 μm in diameter and between 140 and 400 μm^2 in surface, and the level of accumulation was low. The group T2 presented a low-moderate lipid accumulation in the intestine, with a diameter and surface of lipid inclusions that ranged from 3 to 13 μm, and from 70 to 140 μm^2, respectively. The lipid accumulation did not affect the shape or the organization of the intestinal folds, nor were there observed signs of epithelial damage. The group T3 presented moderate levels of intestinal lipid deposits with sizes and surfaces ranging from 3 to 14 μm and from 250 to 350 μm^2, respectively. Contrary to groups C and T1, most lipids were observed in the apical zone of the villi in the group T3 (Figure 5). The C group showed a significant accumulation of lipids in the posterior intestine (steatosis) compared to the liver, contrary to the other groups that presented similar amounts of lipids in both tissues, indicating a more balanced lipid metabolism.

3.6. Gene expression of digestive enzymes

Gene expression of the analyzed digestive enzymes was differentially modulated between both dietary treatments and feeding periods (Figure 6). At the end of the *Artemia* feeding period (14 dpf), *amy* expression was higher in the T3 group than in the C group ($P < 0.05$). The expression of *try* and *pga* was not influenced by the dietary treatments ($P > 0.05$), whereas the expression of *ctr* was higher in the enriched groups (T1 and T3) than in the C group ($P < 0.05$). The expression of *pla2* was highest in the T2 and lowest in the T3 groups ($P < 0.05$). The *lpl* expression was highest in the T2 group and lowest in the T1 group ($P > 0.05$). At the end of the nutritional trial (26 dpf), the expression of all analyzed genes, except for *lpl*, was highest in the T1 group ($P < 0.05$). Unexpectedly, the expression of these genes decreased in the T3 group to the levels of the rest of the dietary treatments ($P < 0.05$), despite having also been fed enriched *Artemia* as in the T1 group. The expression of *amy* increased in the groups C, T1, and T2 between 14 and 26 dpf ($P < 0.05$) and remained stable in the T3 group ($P > 0.05$). The expression of *try* increased only in the T1 and T2 groups ($P < 0.05$); whereas *ctr* expression increased in the C and T1 groups, decreased in the T3 group ($P < 0.05$), and remained constant in the T2 group ($P > 0.05$). The expression of *pla2* increased during development in the groups T1, T2, and T3 ($P < 0.05$) and was invariable in the C group ($P > 0.05$). The expression of *lpl* and *pga* decreased in the C and T2 groups ($P < 0.05$) and remained stable in the groups T1 and T3 for *lpl* ($P > 0.05$), whereas for *pga*, the gene expression decreased in T1 ($P < 0.05$) and remained constant in T3 ($P > 0.05$).

4. Discussion

Present results showed that the DHA enrichment during the *Artemia* feeding period (larval period) did not have any effect on growth performance. This could be related to the fact that

freshwater species are able to synthesize DHA de novo from 18:3 precursors and thus often have lower n-3 HUFA requirements than marine fish larvae (Bell and Sargent, 2003). Similarly, a nutritional study performed in *Clarias gariepinus* showed that changes in dietary fatty acid composition did not affect larval growth (Verreth et al., 1994). However, under current experimental condition we have shown that DHA-enriched *Artemia* metanauplii reduced the incidence of cannibalism and likely contributed to favor the survival observed at the end of the experimental period. Nutritional deficiencies can favor size dispersion and promote cannibalism (Baras and Jobling, 2002). In this study, however, size dispersion was similar between all dietary treatments throughout the trial. The incidence of cannibalism in *P. punctifer* increased at weaning, peaked two days after the end of the weaning period, and disappeared five days later. Therefore, the cannibalistic behavior at weaning seemed to rather be a response to the change of the diet than to size dispersion.

DHA is essential for the development of the brain and the ontogeny of behavior in fish (Benítez-Santana et al., 2012, 2007; Ishizaki et al., 2001; Masuda et al., 1999). Dietary reduction in n-3 PUFAs, and hence in fish tissues, has been shown to reduce larval escaping behavior (Benítez-Santana et al., 2012). In mammals, inadequate intake of DHA is associated with elevated behavioral indices of anxiety, aggression, and depression (Fedorova and Salem, 2006) and, both in mammals and fish, DHA is known to reduce stress (Lund et al., 2012; Lund and Steenfeldt, 2011; Takeuchi et al., 2003; Xu et al., 2016). Considering that groups fed enriched *Artemia* contained higher DHA levels, as well as higher PUFA/saturated and lower LA/PUFA and OA/PUFA ratios in their tissues than groups fed non-enriched *Artemia*, one could speculate that the reduced incidence of cannibalism in the former groups could be associated with a better developed nervous system and escaping behavior, as well as reduced stress in those specimens. This hypothesis is based on the results of cannibalism observed in the juveniles from the group T1 (fed enriched *Artemia*, but non-enriched compound diet), where the incidence of

cannibalism was reduced to the levels of the groups T2 and T3 after weaning despite being fed a non-enriched compound diet. Further research will be necessary to confirm this hypothesis.

At weaning, a transition period could be observed in the cannibalistic behavior, in which the introduction of the enriched compound diet reduced the incidence of cannibalism (groups T2 and T3), whereas the transition from enriched *Artemia* to a non-enriched compound diet temporarily increased the cannibalistic behavior (T1 group). The fact that the cannibalistic behavior of the T1 group at weaning (from 15 dpf) did not last and decreased at 26 dpf to similar levels of cannibalism as those of groups T2 and T3 suggested that the change of diet induced transitory stress; and that the dietary DHA provided during the larval phase had a conditioning effect leading to a reduced cannibalistic behavior in juveniles. In line with this, the long-term effect on anxiolytic behavior has been observed in fish with an early history of DHA and EPA nutritional deficiencies (Lund et al., 2012). In particular, first feeding pikeperch (*Sander lucioperca*) larvae fed a DHA-deficient diet increased stress sensitivity in the future juveniles, whereas the opposite was found with a DHA-rich diet (Lund et al., 2012). In *P. punctifer*, the hypothesis that a low dietary DHA content early in development promoted stress in juveniles was further supported by the fact that total cannibalism was higher in the C group at the end of the trial, and that C group displayed higher incidence of type II cannibalism, both in the morning and in the evening, than the T1 group. Another indicator of dietary conditioning during the larval stage was the higher levels of expression of the majority of the digestive genes analyzed in the T1 group compared to the C group, as a similar gene expression profile at 26 dpf would have been expected in these groups fed a non-enriched compound diet. These higher levels of gene expression in the T1 group at 26 dpf were not reflected in a better performance in terms of growth, as this group presented a similar wet weight as the C group, which was lower than that of the groups fed the enriched compound diet.

The biosynthesis and metabolism of fatty acids differed in fish larvae fed non-enriched and enriched *Artemia.* Despite having offered them the same amount of dietary LA, the larvae fed enriched *Artemia* showed higher ARA content than the larvae fed non-enriched *Artemia*. Fish fed enriched *Artemia* biosynthesized DPA and ARA from LA, resulting in a lower LA tissue accumulation than in the fish fed non-enriched *Artemia*. On the contrary, groups fed non-enriched *Artemia* showed some ARA synthesis and lipid accumulation in tissues, but not DPA. As a consequence, the contents of ARA and LA were lower and higher, respectively, in the C and T2 groups than in those fed enriched *Artemia* (T1, T3). The differences in the DPA n-6 biosynthesis between both dietary groups could be due to a desaturation process (delta 4 desaturase) from 22:4n-6 contained in enriched *Artemia*, which was absent in non-enriched *Artemia*. This would indicate that delta 4 desaturase also exists in this species (Monroig et al., 2018). The levels of OA were higher in non-enriched than in enriched *Artemia* and this same pattern was reflected in the fish tissue. The contents of 20:4n-3 and 22:5n-3, both substrates for the elongase Elovl2 (Monroig et al., 2018), were similar in both enriched and non-enriched *Artemia.* However, groups fed enriched *Artemia* presented lower contents of 20:4n-3 and 22:5n-3 than those fed non-enriched *Artemia*, suggesting that elongation of these PUFAs was promoted in these dietary groups to produce DHA. The higher EPA content in enriched (3% TFA) versus non-enriched (1% TFA) *Artemia* accounted for this biosynthetic pathway, as all dietary groups presented similar EPA content in their tissues (3% TFA). While fish larvae fed non-enriched *Artemia* had to synthesize EPA *de novo* to accumulate 3% TFA in their tissues, the larvae fed enriched *Artemia* had covered their EPA requirements through the diet.

During the *Artemia* feeding phase, groups fed with non-enriched *Artemia* showed higher lipid deposition in the liver than individuals fed enriched *Artemia,* which could be associated with the lower PUFA/saturated and higher OA/PUFA ratios observed in these groups compared to those fed enriched *Artemia* (Boglino et al., 2012). The higher *lpl* expression observed in fish

fed non-enriched *Artemia* might have helped to prevent hepatic steatosis, as LPL is essential in buffering the circulatory TAG load, which protects against ectopic TAG accumulation (Frayn, 2002). The similar lipid accumulation in the intestine in all dietary groups indicated a differential regulation in the lipid uptake by the liver when comparing the enriched and non-enriched dietary groups. Indeed, differential regulation in lipid-related gene expression was observed between them, where *pla2* and *lpl* expression was higher in non-enriched than in enriched groups. This could be associated to the higher OA, LA, and ALA contents of the larvae fed non-enriched *Artemia* (C, T2). Therefore, despite that dietary DHA did not have a significant effect on growth during the larval phase of *P. punctifer*, it seemed to be important for preserving larval quality and tissue health. Fatty acids have a role in controlling LPL activity to assure that fatty acids are not formed more rapidly than they can be taken up by the peripheral tissue (Saxena et al., 1989). In the present study, there was an inverse correlation between lipid accumulation in the liver and *lpl* gene expression in all dietary groups. For instance, fish fed non-enriched *Artemia* showed higher levels of *lpl* expression together with lower liver lipid accumulation than their congeners fed enriched *Artemia*. In the absence of differences in lipid classes (including PL and TAG) between enriched and non-enriched *Artemia*, the most important difference in fatty acid composition between them was their DHA content. These results may indicate that DHA decreased *lpl* gene expression, which likely controlled the lipid uptake in the liver and this might explain the lower levels of lipid accumulation in the liver observed in the enriched groups (T1, T3). A similar trend was also observed for *pla2* expression among dietary groups. However, at the early juvenile stage, the C group showed a significant accumulation of lipids in the posterior intestine (steatosis) compared to the liver, contrary to the other groups that presented similar amounts of lipids in both tissues. The accumulation of large lipid droplets (mainly consisting of TAG) in the intestine of fish is generally due to a reduced lipid export from the intestinal mucosa to the circulatory system (Fontagné et al., 1998). In this

sense, although the differences were not statistically significant between the dietary groups, *lpl* expression in the C group tended to be lower, which might be indicating its action in modulating TAG deposition in the liver in these individuals.

The dietary switch from enriched *Artemia* to the non-enriched compound diet (T1 group) had a marked impact on the expression of the digestive enzymes analyzed. Despite having a similar carbohydrate content in both enriched and non-enriched compound diets, which was higher than in enriched *Artemia,* an increase in *amy* expression was only observed in the T1 group, but not in juveniles from the T3 group. In addition, even though all the enriched and non-enriched diets had similar protein contents, *pga*, *try*, and *ctr* gene expression was up-regulated in the T1 group. These results suggest a clear interaction between dietary fatty acids and genes involved in carbohydrate and protein digestion, as seen previously (Castro-Ruiz et al., 2021b; Darias et al., 2015). When comparing the fatty acid composition of enriched *Artemia* and the non-enriched compound diet, LA and EPA were found in higher amounts in the non-enriched compound diet as compared to the enriched *Artemia*. Therefore, these two fatty acids likely accounted for the increased gene expression of *amy*, *pga*, *try*, *pla2*, and *ctr* observed in the T1 group. In fact, dietary LA has been shown to induce *amy*, *try*, and *ctr* gene expression in the hepatopancreas of juvenile grass carp (*Ctenopharyngodon idellus*) (Zeng et al., 2016). These authors also demonstrated that an optimal dietary LA:ALA ratio of 1.03 was necessary to promote growth in this species, which they considered was partly attributed to the enhancement of trypsin, chymotrypsin, lipase, and amylase activities. In the present study, the T1 group presented, together with the C group, the lowest growth at 26 dpf. This suggests either a post-transcriptional negative regulation of these enzymes and/or the influence of other fatty acids in the nutrient metabolism in the T1 group, leading to a lower weight gain. For instance, similar to the C group, the n-3/n-6 PUFA ratio was higher in juveniles from the T1 group than from the T2 and T3 groups. These results suggest that a diet rich in n-6 PUFA promoted weight gain in

P. punctifer. Although growth at the end of the experiment was similar in T2 and T3, when considering all the variables analyzed, the feeding protocol used in the T3 group favored a more balanced digestive physiology compared to the other dietary treatments, including a more balanced lipid metabolism and fat storage in the hepatic parenchyma and intestinal epithelium. In addition, enriched *Artemia* in the T3 group contributed to reduce the incidence of cannibalism, which led to a better survival rate at the end of the trial.

5. Conclusions

The present results showed that dietary DHA levels influenced the physiology and cannibalistic behavior of *P. punctifer* in a stage-dependent manner. In particular, providing DHA-enriched *Artemia* during the larval stage contributed to reducing the incidence of cannibalism and improved survival, whereas the DHA-enriched compound diet during the early juvenile stage improved growth. Altogether, the group fed both DHA-enriched *Artemia* and compound diet (T3) provided the best results in terms of growth, survival, incidence of cannibalism, and digestive physiology. This study also showed that the nutritional history during the larval period affected fish nutrition and behavior during the early juvenile stage. The shift observed in the incidence of cannibalism at weaning between dietary treatments demonstrated that cannibalism is strongly affected by nutrition in *P. punctifer*. Moreover, the incidence of both types of cannibalism was modulated by the nutritional composition of the diet. More research is needed to better understand the effects of early dietary fatty acid composition on the digestive metabolism at the early juvenile stage and the mechanisms linking the anti-stress effects of DHA and the cannibalistic behavior in this species.

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Table 1. Proximate composition (in % of dry matter) of the different experimental diets. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant among the experimental diets (one-way ANOVA, $P < 0.05$).

Proximate Composition (%)	**Non-enriched *Artemia***	**Enriched *Artemia***	**Non-enriched compound diet**	**Enriched compound diet**
Proteins	36.77±0.67	35.20±0.46	38.79±6.22	37.74±0.81
Lipids	13.56±0.03^{a}	14.61±0.64^{a}	9.92±0.29^{b}	13.67±0.92^{a}
Carbohydrates	10.73±0.48^{b}	9.01±0.25^{b}	24.76±0.91^{a}	24.95±0.98^{a}
Moisture	79.52±0.45^{a}	81.03±1.37^{a}	43.99±0.26^{b}	33.06±0.42^{c}

Table 2. Lipid classes (in % of dry matter) analyzed in the experimental diets. Data expressed as mean ± SD (n = 3). Different superscript letters denote differences statistically significant among diets (one-way ANOVA, $P < 0.05$).

	Non-enriched Artemia	**Enriched Artemia**	**Non-enriched compound diet**	**Enriched compound diet**
SM	0.261±0.09^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
Lyso PC	1.08±0.10^{ab}	0.86±0.09^{b}	1.39±0.09^{a}	1.03±0.17^{ab}
PC	7.85±0.57^{a}	7.57±1.02^{a}	5.75±0.34^{ab}	4.90±0.20^{b}
PS	0.98±0.45	1.07±0.49	0.91±0.10	0.88±0.12
PI	1.60±0.59	1.51±0.66	0.00±0.00	0.00±0.00
Lyso PE	1.14±0.50	1.14±0.31	0.00±0.00	0.00±0.00
PE	5.78±0.75^{a}	5.83±0.76^{a}	1.63±0.22^{b}	1.19±0.02^{b}
Total PL	*18.55±2.09^{a}*	*17.98±1.92^{a}*	*9.68±0.75^{b}*	*8.00±0.24^{b}*
MAG	5.67±1.25^{a}	3.79±0.65^{a}	0.00±0.00^{b}	0.00±0.00^{b}
CHOL	4.66±1.55	6.14±2.05	7.44±0.70	5.39±0.36
FFA	14.63±1.38^{a}	12.08±2.27^{ab}	9.27±1.41^{ab}	6.98±1.21^{b}
TAG	48.35±3.69^{b}	53.43±3.75^{b}	69.71±0.56^{a}	75.77±1.62^{a}
SE+W	8.14±0.90^{a}	6.59±1.08^{ab}	3.90±0.61^{b}	3.87±0.29^{b}
Total NL	*81.45*±2.09^{b}	*82.02*±1.92^{b}	*90.32*±0.75^{a}	*92.00*±0.24^{a}

CHOL, cholesterol; FFA, free fatty acids; Lyso PE, lysophosphatidylethanolamine; Lyso PC, lysophosphatidylcholine; MAG, monoacylglycerols; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; SE, sterolesters; SM, sphingomieline; TAG, triacylglycerides; W, wax.

Table 3. Total lipid and total fatty acids contents and fatty acid composition (in % TFA) analyzed in the experimental diets. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant among diets (one-way ANOVA, $P < 0.05$). DW, dry weight.

	Non enriched Artemia	**Enriched Artemia**	**Non enriched compound diet**	**Enriched compound diet**
Total lipid (mg g^{-1} DW)	138.77±2.78^{a}	146.87±5.54^{a}	99.16±2.90^{b}	136.68±0.92^{a}
Total fatty acid (mg g^{-1} DW)	93.22±8.59^{a}	101.31±2.97^{a}	63.15±6.96^{b}	96.55±0.73^{a}
14:0	0.53±0.05^{c}	0.69±0.20^{c}	2.65±0.04^{b}	4.77±0.61^{a}
16:0	11.82±1.06^{b}	12.25±1.88^{b}	23.33±1.88^{a}	24.27±1.67^{a}
18:0	3.79±0.58	4.65±0.95	5.05±0.28	3.43±0.29
Total saturated	16.14±0.63^{b}	17.59±1.13^{b}	31.04±1.64^{a}	32.47±1.99^{a}
16:1	2.49±0.90	1.54±0.88	5.55±2.68	2.54±0.72
18:1n-9 (OA)	30.24±0.45^{a}	27.72±0.42^{b}	21.63±0.96^{c}	14.01±0.53^{d}
20:1	0.25±0.14^{b}	0.20±0.28^{b}	1.21±0.21^{a}	0.90±0.10^{a}
Total monounsaturated	32.98±0.39^{a}	29.47±0.18^{b}	28.39±1.51^{b}	17.45±0.29^{c}
18:2n-6 (LA)	6.72±0.11^{b}	6.44±0.11^{b}	9.20±0.72^{a}	6.48±0.00^{b}
18:3n-6	0.00±0.00	0.35±0.50	0.08±0.11	0.23±0.22
20:4n-6 (ARA)	0.06±0.11^{b}	0.71±0.16^{ab}	1.21±0.31^{a}	0.57±0.21^{ab}
22:4n-6	0.00±0.00^{b}	1.39±0.14^{a}	0.32±0.28^{b}	0.00±0.00^{b}
22:5n-6 (DPA)	0.00±0.00^{b}	0.00±0.00^{b}	0.18±0.25^{b}	6.20±0.16^{a}
Total n-6 PUFA	6.78±0.21^{b}	8.89±0.89^{b}	10.99±1.45^{a}	13.49±0.15^{a}
18:3n-3 (ALA)	35.66±0.56^{a}	30.56±0.30^{b}	1.57±0.04^{c}	1.04±0.02^{c}
18:4n-3	5.08±0.32^{a}	3.88±0.07^{b}	1.31±0.05^{c}	1.18±0.23^{c}
20:4n-3	1.13±0.14^{a}	1.05±0.13^{a}	0.44±0.04^{b}	0.65±0.07^{b}
20:5n-3 (EPA)	1.19±0.15^{d}	3.06±0.20^{c}	10.88±0.30^{a}	7.34±0.40^{b}
21:5n-3	0.00±0.00	0.00±0.00	0.13±0.18	0.06±0.08
22:5n-3 (DPA)	0.00±0.00^{b}	0.03±0.04^{b}	1.59±0.54^{a}	0.97±0.26^{a}
22:6n-3 (DHA)	0.00±0.00^{d}	4.39±0.06^{c}	12.95±0.68^{b}	25.05±1.28^{a}
Total n-3 PUFA	43.05±0.72^{a}	42.97±0.12^{a}	28.87±1.39^{c}	36.30±1.71^{b}
Total PUFA	49.83±0.92^{a}	51.85±1.02^{a}	39.87±2.84^{b}	49.78±1.86^{a}
(n-3)/(n-6)	6.35±0.10	4.86±0.47	2.64±0.22	2.69±0.10
DHA/EPA	0.00±0.00^{d}	1.44±0.07^{b}	1.19±0.03^{c}	3.41±0.01^{a}
ARA/DHA	0.00±0.00^{c}	0.16±0.03^{a}	0.09±0.02^{b}	0.02±0.01^{c}
ARA/EPA	0.06±0.10	0.23±0.04	0.11±0.03	0.08±0.02
LA/PUFA	0.13±0.00^{b}	0.12±0.00^{b}	0.23±0.00^{a}	0.13±0.00^{b}
OA/PUFA	0.61±0.01^{a}	0.53±0.00^{b}	0.54±0.01^{b}	0.28±0.00^{c}
PUFA/Saturated	3.09±0.18^{a}	2.96±0.25^{a}	1.29±0.16^{b}	1.54±0.15^{b}

Table 4. Proximate composition (in % of dry matter) of *P. punctifer* individuals at 4 dpf (yolk sac larvae) and at 14 dpf (end of the *Artemia* feeding phase) and 26 dpf (end of the moist compound feeding phase) fed the different dietary treatments. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant among dietary treatments (one-way ANOVA, $P < 0.05$). Different asterisks denote differences statistically significant among ages within a dietary treatment. Dietary treatment codes: C, non-enriched *Artemia* and non-enriched compound diet; T1, enriched *Artemia* and non-enriched compound diet; T2, non-enriched *Artemia* and enriched compound diet; T3, enriched *Artemia* and enriched compound diet.

	4 dpf	14 dpf				26 dpf			
		C	T1	T2	T3	C	T1	T2	T3
Proteins	53.63±4.62	57.09±1.26	48.89±3.28	54.92±4.65	51.25±1.45	52.66±2.39	55.32±4.81	45.86±1.48	51.22±0.90
Lipids	22.98±0.65	13.05±2.68	12.08±0.86*	14.09±1.79	12.24±1.82	9.08±1.08^{b}	9.92±0.28^{b**}	11.22±0.13^{b}	13.67±0.13^{a}
Carbohydrates	2.90±0.44	3.55±0.40	3.57±0.25	3.70±0.28	3.29±0.32**	3.14±0.13^{b}	3.61±0.24^{ab}	3.67±0.25^{ab}	5.49±0.76^{a*}
Moisture	0.95±0.05	1.32±0.25	1.42±0.07**	1.51±0.09**	1.43±0.03	1.84±0.08	1.76±0.09*	1.82±0.14*	1.58±0.09

Table 5. Total lipid and total fatty acids contents and fatty acid composition (in % TFA) analyzed in *P. punctifer* individuals at 4 dpf (yolk sac larvae) and at 14 dpf (end of the *Artemia* feeding phase) and 26 dpf (end of the moist compound feeding phase) fed the different dietary treatments. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant among dietary treatments (one-way ANOVA, P < 0.05). Different asterisks denote differences statistically significant between ages within a dietary treatment (one-way ANOVA, *P* < 0.05). Dietary treatment codes: C, non-enriched *Artemia* and non-enriched compound diet; T1, enriched *Artemia* and non-enriched compound diet; T2, non-enriched *Artemia* and enriched compound diet; T3, enriched *Artemia* and enriched compound diet; DW, dry weight.

	4 dpf	14 dpf				26 dpf			
		C	T1	T2	T3	C	T1	T2	T3
Total lipid (mg g^{-1} DW)	229.76±6.54	130.54±26.83	120.77±8.59	140.91±17.94	122.41±18.23	90.75±3.52b	99.22±10.78b	112.18±1.30ab	136.75±1.31a
Total fatty acid (mg g^{-1} DW)	130.93±7.86	72.60±17.75	64.77±6.58	78.29±8.23	68.72±13.28	40.49±3.04d	50.20±4.26c	63.68±1.13b	77.70±2.83a
14:0	0.28±0.03	$0.24±0.03^{a**}$	$0.29±0.02^{a**}$	$0.20±0.01^{b**}$	$0.30±0.03^{a**}$	$0.42±0.03^{d*}$	$0.76±0.04^{c*}$	$1.37±0.06^{b*}$	$1.73±0.11^{a*}$
16:0	22.50±0.53	$15.46±0.26^{ab**}$	$15.86±0.60^{a**}$	$14.85±0.02^{b**}$	$15.63±0.21^{ab**}$	$20.55±0.76^{c*}$	$21.99±0.30^{b*}$	$23.96±0.52^{a*}$	$25.64±0.20^{a*}$
18:0	21.09±0.78	$14.02±0.22^{**}$	14.76±0.46	$13.95±0.49^{*}$	$14.40±0.34^{*}$	$15.88±0.45^{a*}$	$14.29±0.08^{b}$	$10.80±0.15^{c**}$	$9.87±0.24^{d**}$
Total saturated	43.99±1.28	$29.82±0.34^{ab**}$	$31.02±1.02^{a**}$	$29.08±0.49^{b**}$	$30.43±0.18^{ab**}$	$37.09±1.17^{*}$	$37.33±0.32^{*}$	$36.43±0.73^{*}$	$37.56±0.13^{*}$
16:1	1.03±0.08	$1.06±0.01^{a}$	$0.85±0.09^{b**}$	$1.07±0.08^{a**}$	$0.85±0.05^{b**}$	$1.23±0.13^{c}$	$1.76±0.15^{b*}$	$1.98±0.00^{ab*}$	$2.34±0.06^{a*}$
18:1n-9 (OA)	16.43±0.29	$17.70±0.26^{a*}$	$15.38±0.25^{b*}$	$17.79±0.23^{a*}$	$15.49±0.47^{b*}$	$14.16±0.30^{ab**}$	$14.57±0.00^{a**}$	$12.66±0.16^{c**}$	$13.59±0.12^{b**}$
20:1	0.60±0.03	$0.45±0.01^{**}$	$0.44±0.02^{**}$	0.52±0.09	0.45±0.01	$0.54±0.03^{*}$	$0.61±0.01^{*}$	0.62±0.05	0.33±0.47
Total monounsaturated	20.28±0.40	$26.48±0.68^{a*}$	$23.49±0.53^{b*}$	$26.88±0.67^{a*}$	$23.78±0.79^{b*}$	$19.64±0.75^{**}$	$20.46±0.15^{**}$	$18.62±0.33^{**}$	$19.24±0.41^{**}$
18:2n-6 (LA)	1.91±0.05	$4.67±0.03^{a*}$	$3.56±0.26^{b}$	$4.63±0.08^{a*}$	$3.59±0.18^{b**}$	$3.24±0.27^{b**}$	$4.14±0.04^{a}$	$4.32±0.10^{a**}$	$4.79±0.22^{a*}$
18:3n-6	0.35±0.08	0.91±0.13	$0.65±0.16^{*}$	0.76±0.20	$0.86±0.06^{*}$	$0.66±0.07^{a}$	$0.00±0.00^{b**}$	$0.53±0.02^{ab}$	$0.51±0.14^{ab**}$
20:4n-6 (ARA)	3.12±0.12	$1.26±0.10^{b**}$	$2.69±0.09^{a}$	$1.21±0.08^{b**}$	$2.78±0.06^{a*}$	$2.81±0.03^{a}$	$2.86±0.11^{a*}$	$1.94±0.15^{b*}$	$1.71±0.04^{b**}$
22:4n-6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
22:5n-6 (DPA)	0.35±0.01	$0.05±0.08^{b**}$	$2.23±0.14^{a}$	$0.00±0.00^{b**}$	$2.29±0.17^{a**}$	$1.21±0.04^{d*}$	$2.09±0.02^{c}$	$4.66±0.03^{b*}$	$4.76±0.04^{a*}$
Total n-6 PUFA	7.12±0.15	$7.73±0.37^{b}$	$9.80±0.41^{a}$	$7.49±0.44^{b**}$	$10.17±0.12^{a**}$	$8.67±0.34^{c}$	$9.61±0.11^{b}$	$11.93±0.17^{a*}$	$12.16±0.11^{a*}$
18:3n-3 (ALA)	0.45±0.15	$14.69±0.41^{a*}$	$11.42±1.01^{b*}$	$15.27±0.68^{a*}$	$11.59±0.27^{b*}$	$1.38±1.21^{**}$	$0.85±0.10^{**}$	$1.05±0.03^{**}$	$0.89±0.05^{**}$
18:4n-3	0.00±0.00	$3.23±0.14^{a*}$	$2.26±0.09^{b*}$	$3.32±0.08^{a*}$	$2.23±0.02^{b*}$	$0.70±0.10^{**}$	$0.64±0.04^{**}$	$0.78±0.02^{**}$	$0.84±0.04^{**}$
20:4n-3	0.34±0.01	$3.06±0.19^{a*}$	$2.33±0.09^{b*}$	$3.14±0.12^{a*}$	$2.36±0.11^{b*}$	$1.04±0.02^{**}$	$0.75±0.11^{**}$	$0.78±0.13^{**}$	$0.67±0.08^{**}$
20:5n-3 (EPA)	4.08±0.07	$3.08±0.08^{**}$	$3.49±0.21^{**}$	$3.03±0.37^{**}$	$3.26±0.11^{**}$	$5.12±0.24^{a*}$	$5.02±0.18^{ab*}$	$4.11±0.36^{ab*}$	$3.97±0.03^{b*}$
21:5n-3	0.25±0.02	$0.16±0.02^{**}$	$0.19±0.05^{**}$	0.30±0.15	$0.15±0.01^{**}$	$0.36±0.06^{*}$	$0.30±0.02^{*}$	0.31±0.00	$0.35±0.02^{*}$
22:5n-3 (DPA)	1.84±0.08	$2.11±0.09^{a**}$	$1.55±0.09^{b**}$	$1.98±0.06^{a}$	$1.67±0.10^{b}$	$2.67±0.10^{a*}$	$2.09±0.02^{b*}$	$1.85±0.10^{c}$	$1.55±0.03^{d}$
22:6n-3 (DHA)	17.13±1.28	$5.49±0.39^{b**}$	$10.83±0.67^{a**}$	$5.57±0.44^{b**}$	$11.00±0.71^{a**}$	$20.19±0.89^{*}$	$20.54±0.59^{*}$	$22.21±0.53^{*}$	$21.65±0.35^{*}$
Total n-3 PUFA	24.08±0.98	31.82±0.52	32.07±0.38*	32.61±0.17*	32.27±0.48*	31.47±0.03	30.19±0.82**	31.10±0.86**	29.92±0.24**
Total PUFA	31.20±1.13	39.55±0.75b	41.86±0.76a	40.09±0.27b**	42.45±0.60a	40.14±0.37ab	39.80±0.71b	43.03±1.03a*	42.08±0.13ab
(n-3)/(n-6)	3.38±0.07	4.12±0.18a*	3.28±0.11b	4.37±0.29a*	3.17±0.01b*	3.63±0.11a**	3.15±0.14b	2.61±0.03c**	2.46±0.04c**
DHA/EPA	4.20±0.38	1.78±0.13b**	3.10±0.17a**	1.85±0.14b**	3.38±0.27a**	3.95±0.03b*	4.09±0.02b*	5.42±0.35a*	5.45±0.12a*
ARA/DHA	0.18±0.01	0.23±0.01ab*	0.25±0.02a*	0.22±0.00b*	0.25±0.01a*	0.14±0.02a**	0.14±0.00a**	0.09±0.00b**	0.08±0.00b**
ARA/EPA	0.76±0.04	0.41±0.04b	0.77±0.07a*	0.40±0.03b**	0.85±0.03a*	0.55±0.06	0.57±0.00**	0.47±0.01*	0.43±0.01**
LA/PUFA	0.06±0.00	0.12±0.00a*	0.08±0.00b**	0.12±0.00a*	0.08±0.01b**	0.08±0.01b**	0.10±0.00a*	0.10±0.00a**	0.11±0.01a*
OA/PUFA	0.53±0.03	0.45±0.01a*	0.37±0.00b	0.44±0.01a*	0.36±0.02b*	0.35±0.00a**	0.37±0.01a	0.29±0.01c**	0.32±0.00b**
PUFA/Saturated	75.19±0.14	69.37±0.44b**	72.89±0.58a**	69.17±0.33b**	72.88±0.72a**	77.23±0.80b*	77.13±0.39b*	79.45±0.30a*	79.63±0.26a*

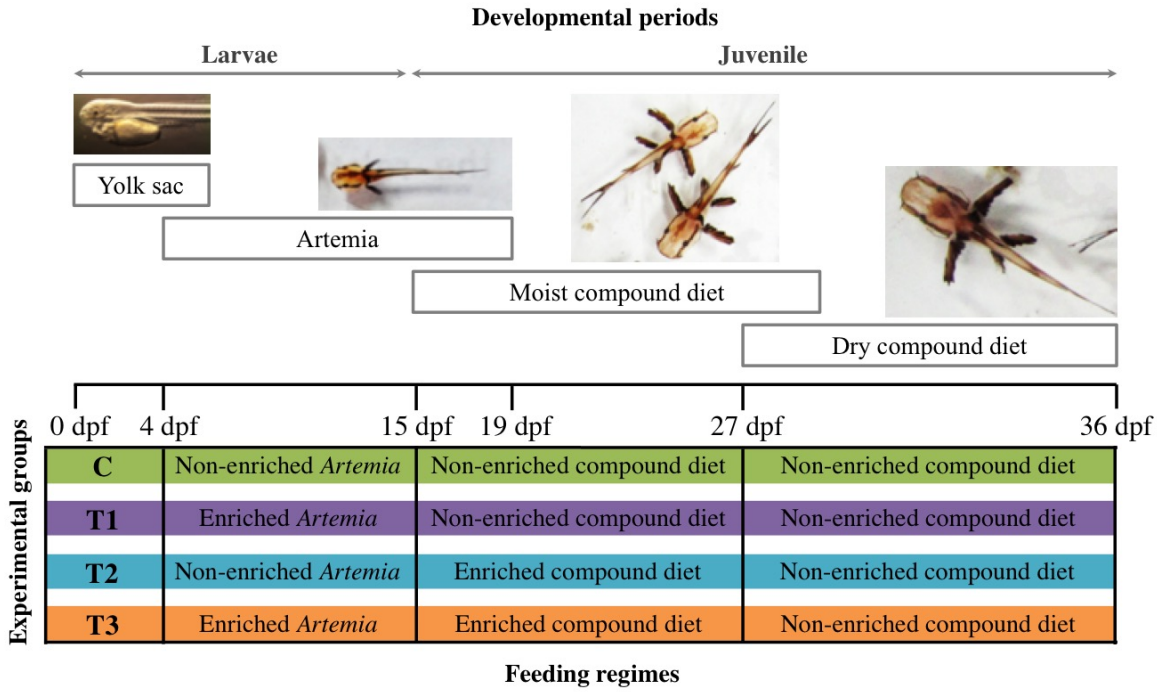

Figure 1. Experimental design of the nutritional trial. Different feeding windows coinciding with different developmental stages were used to create four experimental groups: the C (control) group was fed non-enriched *Artemia* during the larval period (4-14 dpf) and non-enriched compound diet during the early juvenile stage (15-26 dpf); the group T1 was fed enriched *Artemia* but non-enriched compound diet; the group T2 was fed non-enriched *Artemia* but enriched compound diet; and the group T3 was fed both enriched *Artemia* and compound diet. All groups were exclusively fed a non-enriched compound diet from 27 to 36 dpf. Enrichment was done with a reference commercial product, Algamac 3050. dpf, days post-fertilization.

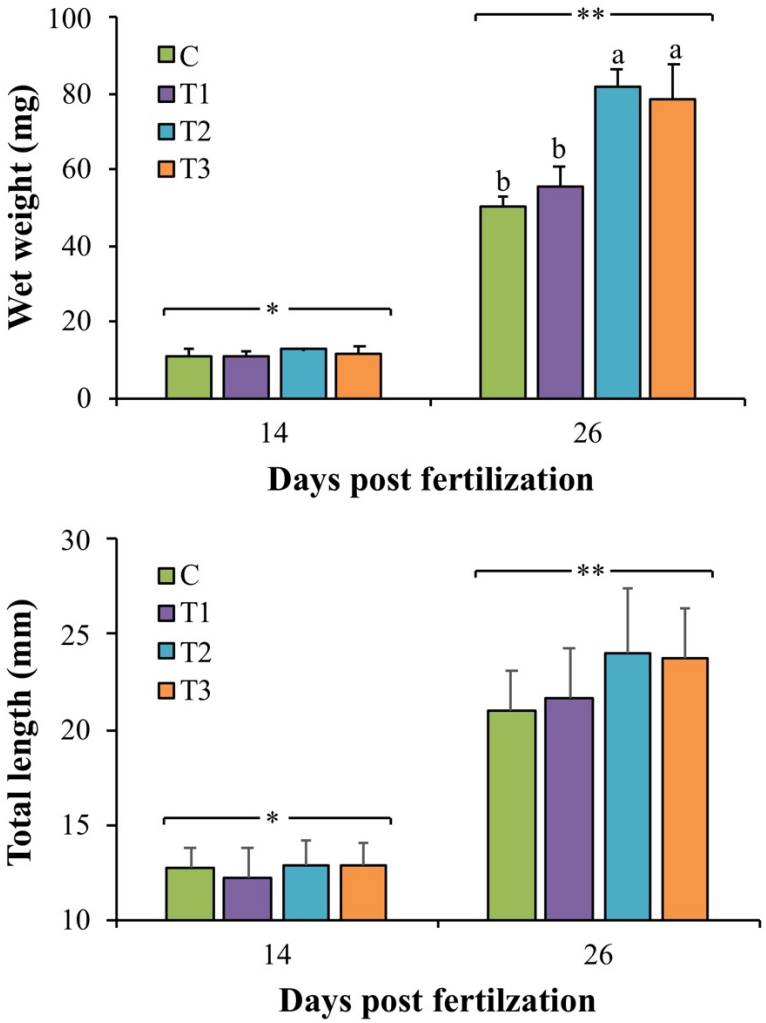

Figure 2. Growth in terms of wet weight (mg) and total length (mm) of *P. punctifer* at 14 dpf (end of the *Artemia* feeding phase, larval stage) and 26 dpf (end of the experimental compound diet feeding phase, juvenile stage). Data are presented as means ± S.D. (n = 45). Values with a different letter denote significant differences among dietary treatments within a developmental stage and asterisks denote significant differences during development within a dietary treatment (one-way ANOVA, $P < 0.05$).

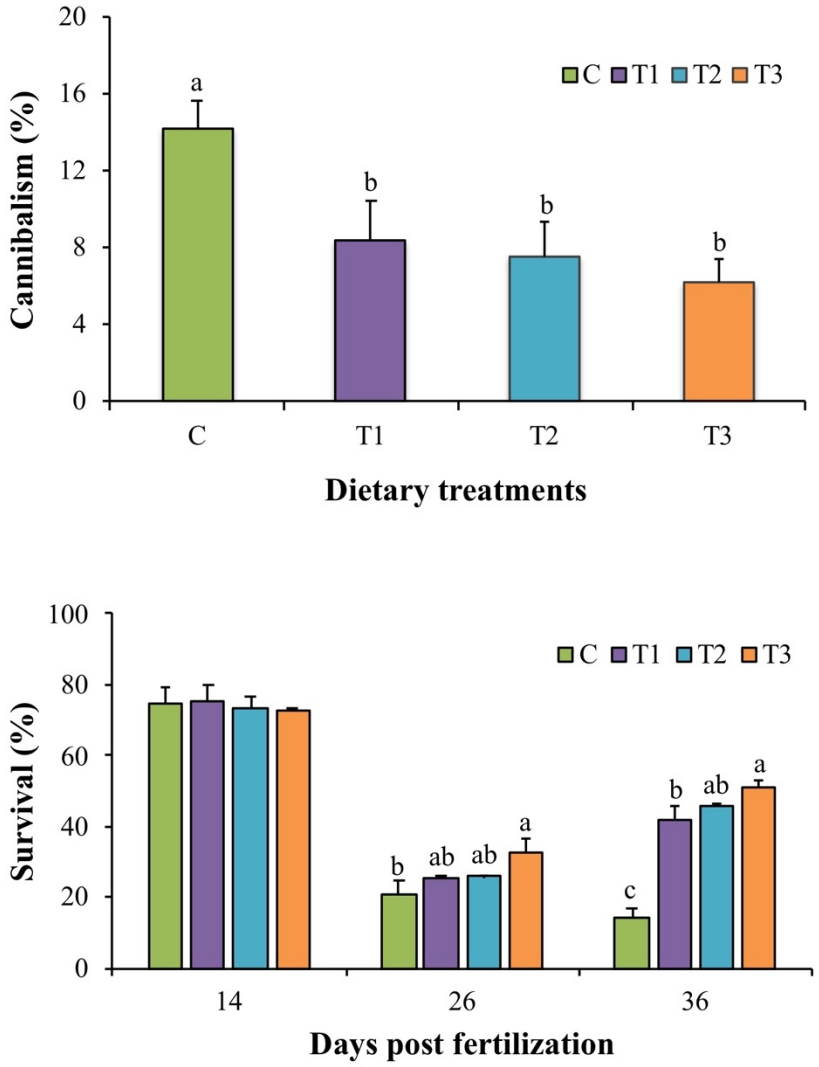

Figure 3. Incidence of total cannibalism at 26 dpf (end of the experimental compound diet feeding phase, juvenile stage), and survival at 14 dpf (end of the *Artemia* feeding phase, larval stage), 26 dpf (end of the nutritional trial, juvenile stage) and 36 dpf (end of the experimental trial, juvenile stage) of *P. punctifer* fed the different experimental feeding protocols. Data are presented as means ± S.D. (n = 45). Values with a different letter denote significant differences among dietary treatments (one-way ANOVA, $P < 0.05$).

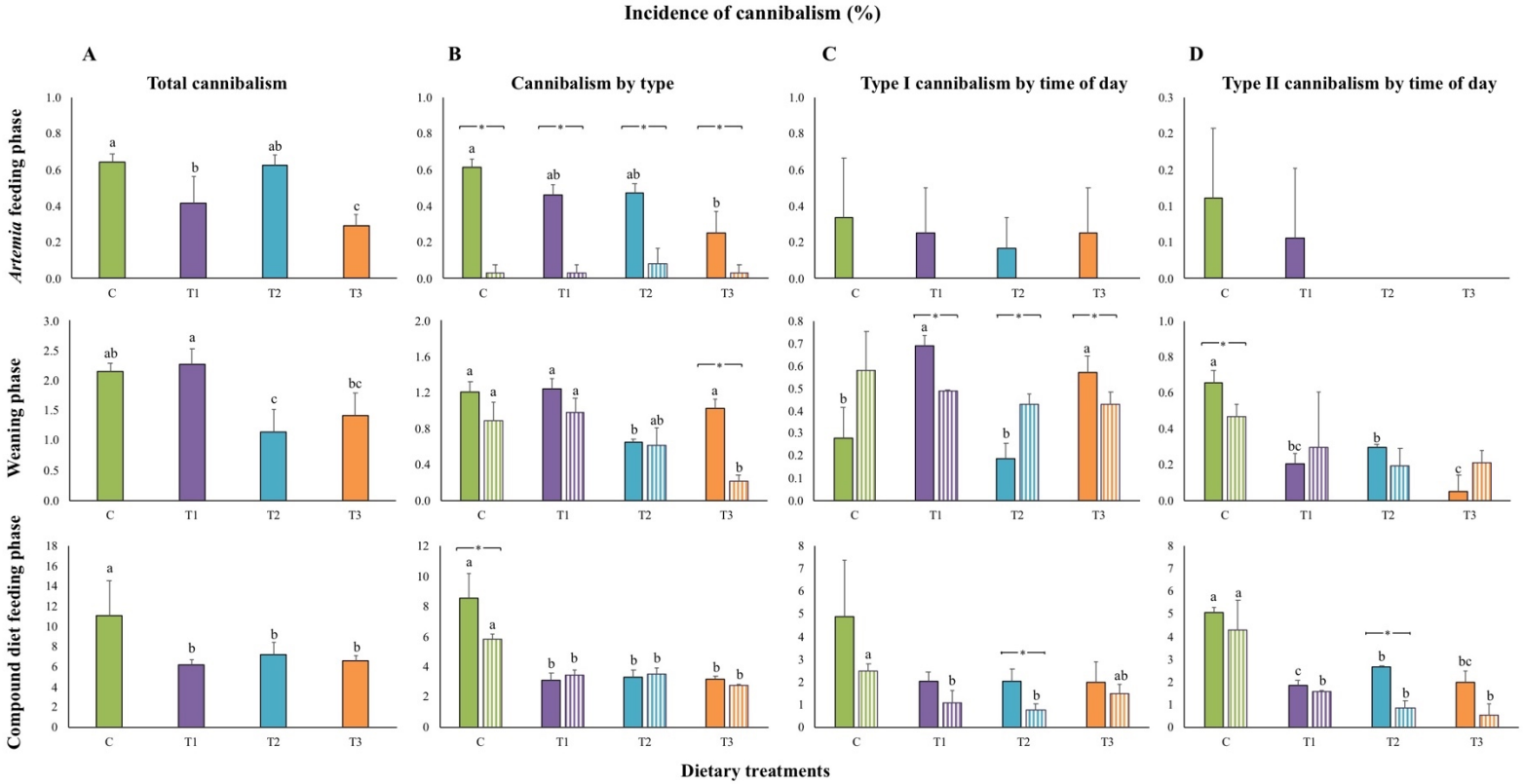

Figure 4. Incidence of cannibalism of *P. punctifer* at different developmental and feeding stages. The incidence of cannibalism is shown by A) total cannibalism, B) type of cannibalism (I or II), and C and D) time of the day for each type of cannibalism. In B, full bars indicate type I cannibalism and lined bars indicate type II cannibalism. In C and D, full bars indicate day time, while lined bars indicate night time. Data are presented as means ± S.D. (n = 45). Values with a different letter denote significant differences among dietary treatments within a developmental stage and asterisks denote significant differences between types of cannibalism within a dietary treatment or between times of the day within a dietary treatment (one-way ANOVA, $P < 0.05$).

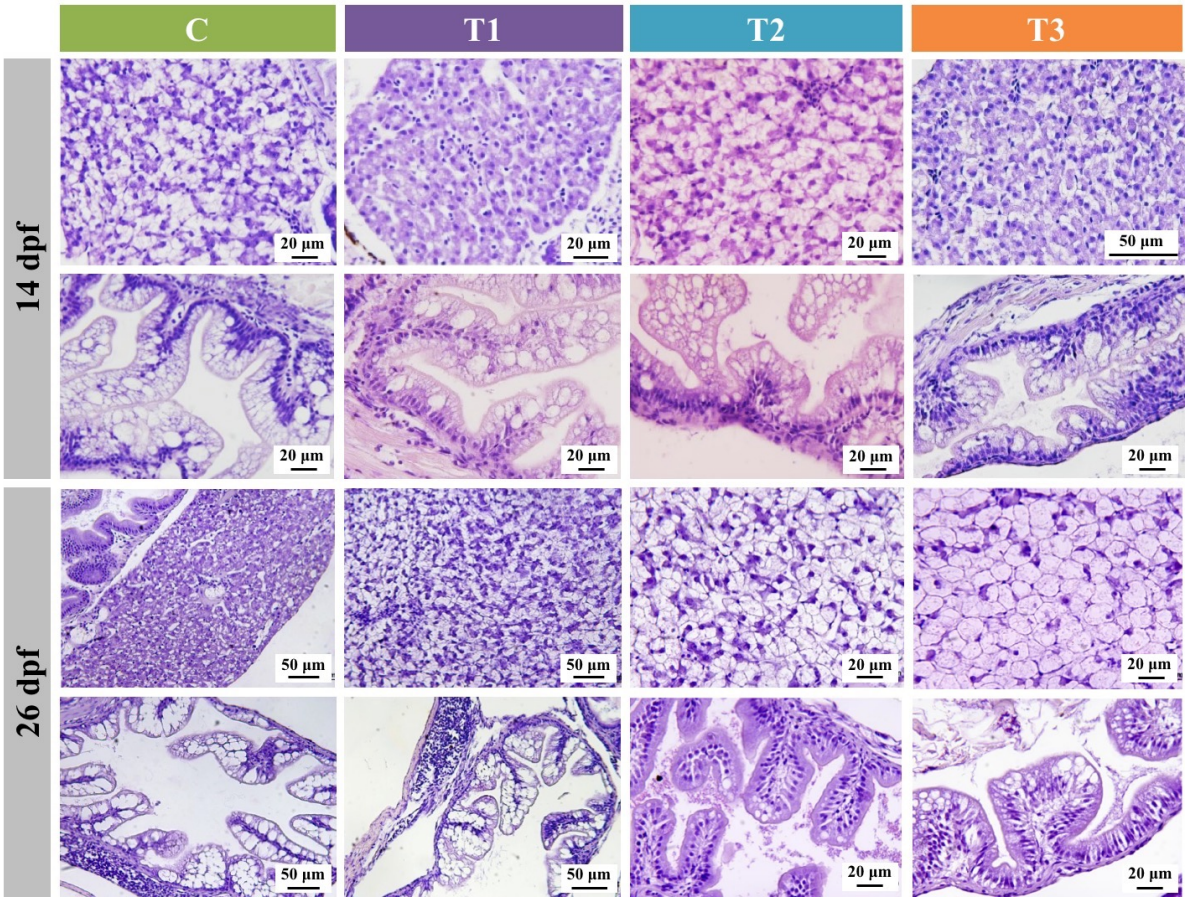

Figure 5. Longitudinal paraffin sections of the liver and anterior intestine of *P. punctifer* at 14 and 26 dpf fed the different experimental treatments. Staining, hematoxylin-eosin. While lipid deposits in the intestine are similar in all dietary groups at 14 dpf, note the fattier livers of groups C and T2. At 26 dpf, groups T2 and T3 showed the fattiest livers and lipid accumulation in the intestine was concentrated in the apical zone, whereas in groups C and T1 lipids were accumulated along the intestinal folds. Contrary to the rest of the dietary groups, note the important accumulation of lipids in the intestine (steatosis) compared to the liver in the C group.

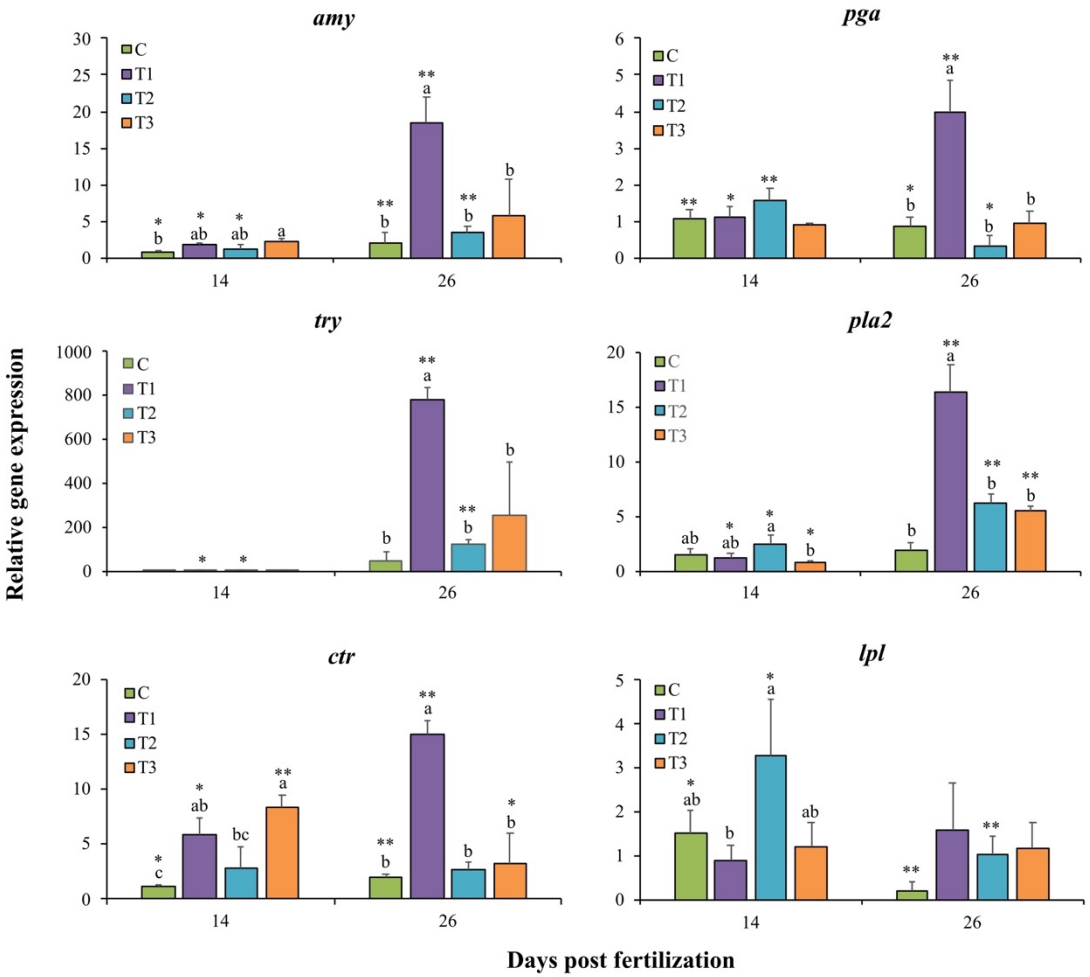

Figure 6. Relative expression of α-amylase (*amy*), trypsinogen (*try*), chymotrypsin (*ctr*), pepsinogen (*pga*), phospholipase A2 (*pla2*), and lipoprotein lipase (*lpl*) genes in *P. punctifer* at 14 and 26 dpf. Data are presented as means ± S.D. (n = 9). Values with a different letter denote significant differences among dietary treatments within a developmental stage and asterisks denote significant differences during development within a dietary treatment (one-way ANOVA, $P < 0.05$).