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Abstract

Environmental factors present cyclic variations during the day. Of them, high temperatures occur in the light phase, whereas low temperatures take place at night, which is known as thermocycle. Although applying thermocycles to fish larvae improves growth in some species, nothing is known about the impact during the early development of the digestive system. The aim of the present research was to investigate the effect of different temperature regimes, cycling versus constant, on the daily rhythms of digestive factors and growth of Nile tilapia (Oreochromis niloticus) larvae. For this purpose, fertilized eggs were divided into two groups: one under a thermocycle (TC) of 31ºC:25ºC day:night and other group at constant temperature of 28ºC (CTE). Photoperiod was set at 12:12h light/dark cycle. Larvae length was measured on 4, 8 and 13 days post-fertilization (dpf). Larvae samples were collected every 4 h during a 24-hour cycle on 4, 8 and 13 dpf. The expression levels of pepsinogen, chymotrypsinogen, trypsinogen, lipase, maltase, isomaltase, npy and cck were analyzed by qPCR. The results showed that larval growth was greater when larvae were reared at TC than at CTE. Moreover on 13 dpf, most analyzed genes (chymotrypsinogen, lipase, maltase, isomaltase, npy and cck) displayed daily rhythms in the TC group, but not in CTE, with most acrophases located around mealtime. These rhythms could explain the higher growth rate observed in the TC larvae due to improved feed digestion and utilization. The results can be useful for improving the rearing protocols used in larviculture and to enhance production performance.

Keywords	biological clock; larviculture; fish development; digestive function; endocrine system
Taxonomy	Fish Development, Chronobiology, Circadian Rhythm, Digestive System Physiology
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Aquaculture
Section Editor
Prof. A. Takemura
University of the Ryukyus
Nakagami-gun, Japan

Murcia 14/02/20

Dear Prof. Takemura,

I enclose the paper entitled "Effects of temperature regime (daily thermocycles *versus* constant) on growth and the ontogeny of the daily rhythms of expression of digestive factors of Nile tilapia (*Oreochromis niloticus*)" by do Espirito Santo *et al.* for possible publication in Aquaculture.

The attached paper has not been and is not intended to be published anywhere except this journal. The authors agree that if and when it is accepted by the Editors for publication in consideration of such acceptance the entire copyright in this paper pass thereupon to the Publisher, Elsevier.

Thanking you in advance,

Yours sincerely

Dr. J.F. López-Olmeda

HIGHLIGHTS

- Tilapia larvae reared under thermocycles presented a higher growth than larvae reared at constant temperature.
- In the thermocycles, larvae showed a higher number of digestive factors that displayed daily rhythms of expression.
- Most digestive factors that were rhythmic correlated with mealtime, among them proteases (*trypsinogen* and *chymotrypsinogen*), *lipase* and hormones (*npy* and *cck*).
- The use of rearing conditions similar to the natural environment, i.e. thermocycles, in tilapia larviculture is recommended.

Effects of temperature regime (daily thermocycles *versus* constant) on growth and the ontogeny of the daily rhythms of expression of digestive factors of Nile tilapia (*Oreochromis niloticus*)

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Running Title: ***Temperature affecting tilapia growth and ontogeny***

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ABSTRACT

Environmental factors present cyclic variations during the day. Of them, high temperatures occur in the light phase, whereas low temperatures take place at night, which is known as thermocycle. Although applying thermocycles to fish larvae improves growth in some species, nothing is known about the impact during the early development of the digestive system. The aim of the present research was to investigate the effect of different temperature regimes, cycling *versus* constant, on the daily rhythms of digestive factors and growth of Nile tilapia (*Oreochromis niloticus*) larvae. For this purpose, fertilized eggs were divided into two groups: one under a thermocycle (TC) of 31ºC:25ºC day:night and other group at constant temperature of 28ºC (CTE). Photoperiod was set at 12:12h light/dark cycle. Larvae length was measured on 4, 8 and 13 days post-fertilization (dpf). Larvae samples were collected every 4 h during a 24-hour cycle on 4, 8 and 13 dpf. The expression levels of *pepsinogen*, *chymotrypsinogen*, *trypsinogen*, *lipase*, *maltase*, *isomaltase*, *npy* and *cck* were analyzed by qPCR. The results showed that larval growth was greater when larvae were reared at TC than at CTE. Moreover on 13 dpf, most analyzed genes (*chymotrypsinogen*, *lipase*, *maltase*, *isomaltase*, *npy* and *cck*) displayed daily rhythms in the TC group, but not in CTE, with most acrophases located around mealtime. These rhythms could explain the higher growth rate observed in the TC larvae due to improved feed digestion and utilization. The results can be useful for improving the rearing protocols used in larviculture and to enhance production performance.

Keywords: biological clock, larviculture, fish development, digestive function, endocrine system.

INTRODUCTION

The natural environment presents cyclic and predictable variations in geophysical variables, such as light and temperature. These cyclic variations have influenced evolution and selected the occurrence of biological clocks in most living organisms. Biological rhythms confer adaptive advantages as organisms can time their physiological processes to occur at specific moments when effectiveness is greater (DeCoursey, 2004). Among the environmental variables that synchronize biological rhythms, light has been commonly regarded as the most important, but other factors, such as temperature and feeding, play an important role, especially in ectothermic animals like fish (López-Olmeda, 2017).

Temperature has a strong influence on fish behavior and physiology (Brett, 1971) as it affects processes like development, locomotion, sex differentiation, reproduction and survival (Bennett and Beitinger, 1997; Ospina-Álvarez and Piferrer, 2008; Pankhurst and King, 2010; López-Olmeda and Sánchez-Vázquez, 2011). In the natural environment, daily temperature oscillations (thermocycles) are generated in water due to the presence or absence of solar radiation (Villamizar et al., 2011). Hence temperature displays cycles alongside the light/dark cycle, with the higher temperature phase (thermophase) coinciding with day and the lower temperature phase (cryophase) coinciding with night (López-Olmeda and Sánchez-Vázquez, 2011). However, while most fish undergo thermocycles in the wild, their effects on fish biology, especially in developmental stages, have received little attention to date (Schaefer and Ryan, 2006; Villamizar et al., 2012). Fluctuating temperatures can change gene expression patterns compared to constant temperature conditions (Podrabsky and Somero, 2004). During fish development, thermocycles have been reported to increase survival and growth, lower the incidence of malformations and modify the sex ratio (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012; Sánchez-Vázquez and López-Olmeda, 2018). In tilapia (*Oreochromis*

niloticus) juveniles, the effects of thermocycles on growth were tested (Azaza et al., 2010). Small juveniles presented higher growth rates under thermocycles than constant temperatures, although this effect seemed to be lost in bigger juveniles (Azaza et al., 2010). Nevertheless, further research in tilapia and other fish species is required to confirm the positive effects of thermocycles, especially during early development.

One of the most important factors to affect the efficiency of food conversion into nutrients is the availability of the enzymes involved in this process (Jobling, 1995; Perez-Casanova et al, 2006). The maximum fish growth rate, especially during early development, strongly depends on digestive capacity (Blier et al., 1997). Thus studies on the ontogeny of digestive enzymes in fish can elucidate certain aspects of their nutritional physiology and help to solve nutritional problems challenged in larviculture (Furné et al., 2005). In fish, studies on the ontogeny of the digestive function at the molecular level and on the existence of daily rhythms of digestive factors during development and how the environment modulates these rhythms, are lacking (Rønnestad et al., 2013; Yúfera et al., 2018). To date, the existence and ontogeny of these rhythms in fish larvae have only been reported in two marine species: gilthead seabream (*Sparus aurata*) and Senegalese sole (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016).

The larviculture of Nile tilapia offers some advantages, such as using formulated diets from the start of exogenous feeding (Luz et al., 2012). However, for this species, more in-depth knowledge about the digestive physiology in the larval stage is necessary (de Moura Pereira et al., 2019). To date, research into the ontogeny of digestive factors during larval development is scarce, and only a few reports have addressed this topic (Tengjaroenkul et al., 2002; Qiang et al., 2017; de Moura Pereira et al., 2019; Silva et al., 2019). Daily rhythms in clock gene expression and factors from the endocrine axis have been reported in adult Nile tilapia (Costa

et al., 2016a, 2016b; de Alba et al., 2019), but still nothing is known about the existence of the daily rhythms in digestive factors for tilapia larvae.

Therefore, the aim of the present study was to evaluate the effects of two different temperature regimes, a daily thermocycle *versus* constant temperature, applied on the first days of Nile tilapia development, on the growth and daily rhythms of different digestive physiology parameters. These parameters involved the expression of genes that encode enzymes for protein (*pepsinogen*, *chymotrypsinogen* and *trypsinogen*), lipid (*lipase*) and carbohydrate digestion (*maltase* and *isomaltase*), and the endocrine control of food intake and digestion (*neuropeptide Y*, *npy*, and *cholecystokinin*, *cck*).

MATERIALS AND METHODS

The present research was conducted at the facilities of the Department of Physiology of the University of Murcia (Spain). Fish were reared following the Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 1201/2005 and Law 32/2007) for the use of laboratory animals. In addition, the protocols were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

Animals and experimental design

Male and female Nile tilapia (*Oreochromis niloticus*) adults were obtained from a local fish farm (Tilamur S.A., Murcia, Spain). Animals were kept in 300-liter tanks connected to a recirculation system, equipped with biological and mechanical filters. The photoperiod was set at 12:12 light/dark (LD) cycle with lights on at 09:00h. The water temperature was maintained

and controlled at 28±0.5ºC. Adult tilapias were fed a commercial diet (D-4 AlternaBasic 2P, Skretting, Spain) with 36% crude protein (CP) 3 times a day until apparent satiety.

After acclimation, tilapia breeders were induced with human Chorionic Gonadotropin hormone (hCG, Sigma Aldrich, St. Louis, USA) as described elsewhere (Fernandes et al., 2013). Briefly, females were injected with 1500 IU/kg of body weight and males with 500 IU/kg. The administration for females was divided into two injections: the first consisted of 500 IU/kg injected at the end of the light phase and the second one (1,000 IU/kg) was applied 12 h after the first dose (at the beginning of the light phase on the next day). Males received a single injection at the same time as the second dose for females. After hCG administration, animals were placed together. After 24 h, sperm and eggs were collected by stripping and *in vitro* fertilization was performed (Fernandes et al., 2013). This procedure allowed us to immediately obtain fertilized eggs, which were used in the experiments.

Fertilized eggs were obtained from five different groups of tilapia breeders. Each group consisted in 3 females and 5-6 males. Then fertilized eggs were pooled together and distributed in incubators for Cichlid eggs (Alimar SA, Murcia, Spain) (200 eggs per incubator) in two distinct systems with different temperature regimes: one system with a daily thermocycle (TC) of 31ºC:25ºC, and another with a constant temperature (CTE) of 28ºC. Both systems were recirculating water systems that were connected to mechanical and biological filters. The photoperiod was set at 12:12 LD, with lights on at 09:00h (set as *Zeitgeber* time 0 h, ZT0 h). In the TC system, the thermophase (high temperature phase) of 31ºC coincided with the light phase, whereas the cryophase (low temperature) of 25ºC coincided with the dark phase (Suppl. Fig. 1). The average water temperature in the TC system throughout the day was 28.02ºC. Thus the larvae reared in this system were subjected to the same degree days as the animals reared in the CTE group, at an average water temperature of 28.03±0.33ºC (mean±S.D.). In the TC system, water temperature was modified by water heaters (Askoll, Povolaro, Italy) and coolers

(AquaMedic 1500, Titan GmbH, Bissendorf, Germany), controlled by electronic timers (Bachmann GmbH & Co, Stuttgart, Germany). Water temperature was recorded continuously throughout the experiment by an underwater data recorder (HOBO pendant, Onset Computer Corporation, Massachusetts, USA). Larvae were reared in incubators until 7 days post fertilization (dpf), when they were transferred to 9-liter tanks connected to the same temperature system. Exogenous feeding began on 7 dpf. Larvae were fed a semi-purified diet containing 42% CP and 4100 Kcal/kg, formulated as described elsewhere (Silva et al., 2019). Larvae were fed in excess 4 times a day in the first half of the light phase (ZT1, ZT3, ZT5 and ZT7 h). Tilapia embryo/larvae were maintained under the experimental conditions from 0 to 13 dpf.

A total of 924 tilapia larvae were used in the experiments (462 larvae per group). On 4, 8 and 13 dpf, whole larvae samples were collected every 4 h during a 24-hour cycle at the following time points: ZT 2, ZT 6, ZT 10, ZT 14, ZT 18 and ZT 22 h. Larvae were pooled for each replicate and six replicates (n=6) were collected for each group, time point and day. Larvae were stored in 1.5 ml tubes and immediately frozen and stored at -80ºC until analyzed. The number of larvae used in the pool differed depending on the sampling day: 5 larvae/pool on 4 dpf; 4 larvae/pool on 8 dpf; 3 larvae/pool on 13 dpf. The larvae collected on 8 and 13 dpf were fed on the sampling day.

Larval growth

The growth of the tilapia larvae in both temperature groups (TC and CTE) was evaluated on 4, 8 and 13 dpf. For this purpose, 30 larvae were used for each treatment, and 10 larvae were used for each measurement day. Larvae were removed from the aquarium and transferred to a Petri dish. Then each larvae was photographed using a binocular (Leica EZ4 HD, Leica Microsystems GmbH, Wetzlar, Germany) with an incorporated digital camera. Photos were stored in a computer and analyzed later with the ImageJ image processing software (Abramoff

et al., 2004). Larvae were measured longitudinally by considering standard length (Suppl. Fig. 2).

Real-time RT-PCR analysis

Larvae pools were transferred to sterile tubes and homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions, using a tissue homogenizer for mechanical homogenization (TissueLyser LT, Qiagen, Hilden, Germany). RNA was dissolved in DEPC water (Invitrogen, CA, USA) and RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was first treated with 1U of DNase (Dnase I, Thermo Fisher), followed by retrotranscription using a commercial kit (qScript cDNA Synthesis Kit, Quantabio, Beverly, USA). The quantitative PCR (qPCR) reactions were performed by the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All the samples were run in duplicate and the qPCR reactions were performed in a final volume of 20 µl. The quantitative PCR analyses were performed in a light thermocycler (7500 RT-PCR system, Applied Biosystems) following this protocol: 15 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Melting curves were run after amplification to ensure that only one DNA species was amplified. All the primer sequences (Table 1) were designed with the Primer 3 plus software (Untergasser et al., 2012). The relative amplification efficiencies of all the genes were analyzed by cDNA dilution curves and were found to be similar for all the genes. Primer concentrations were determined by means of a primer dilution curve. The primers for *chymotrypsinogen*, *pepsinogen*, *lipase*, *npy*, *maltase* and *βactin* were added at a reaction concentration of 200 nM. The primers for *trypsinogen*, *isomaltase* and *cck* were added at a final concentration of 400 nM. The relative expression of all the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The reference gene, *βactin*, was selected after verifying that its coefficient of variation (CV) was lower than 5%.

Data analysis

All the results are expressed as mean±SEM. The SPSS software (v. 19.0, IBM, Armonk, USA) was used to detect any statistically significant differences between groups and time points. Normality of the data was previously assessed by the Kolmogorov-Smirnov test and homogeneity of variance was also verified using Levene's test. The data from each gene and day were subjected to a two-way ANOVA, followed by Duncan's *post hoc* test, to check for statistically significant differences in gene expressions between groups (TC *versus* CTE) and sampling points (ZTs). The existence of significant rhythmicity was tested for all the genes by the Cosinor analysis, performed with the "EL TEMPS" software (v.1.179, Prof. Dıez-Noguera, University of Barcelona, Spain). The Cosinor analysis is based on the least squares approach of time series data with a cosine function of a known period of type Y = Mesor + Amplitude * cos (2π(t-Acrophase)/Period). The Cosinor analysis also provides the statistical significance of the rhythm by an F-test of the variance, accounted for by the waveform *versus* a straight line of zero-amplitude (null hypothesis). Larval growth among groups was compared on each sampling day by a Student's t-test. The significance threshold was set at $p<0.05$ for all the tests.

RESULTS

Larval growth

The temperature regime in which tilapia embryo and larvae were raised had significant effects on growth, with TC inducing a greater growth than CTE (Figure 1). On 4 dpf, the larvae subjected to the TC treatment had a significantly longer length (5.61±0.03 mm) than those under CTE of the same age (5.47±0.03 mm) (t-test, $p<0.05$). On 8 dpf, the CTE larvae had caught up with the TC larvae (7.01±0.02 and 7.05±0.07 mm for CTE and TC, respectively) and no

significant differences between treatments were detected (t-test, $p>0.05$). By 13 dpf, the TC treatment larvae once again obtained significantly higher length values (mean 8.09±0.07 mm) compared to those in the CTE treatment (average 7.77±0.09 mm) (t-test, $p<0.05$).

Pepsinogen expression

No daily rhythms (Cosinor, $p>0.05$) were found for *pepsinogen* relative expression in the 4, 8 or 13 dpf tilapia larvae in either of the tested temperature regimes (TC or CTE) (Fig. 2). Significant differences were observed depending on the group and time of day on both 4 and 13 dpf (two-way ANOVA, $p<0.05$) (Fig. 2A and 2C). On 4 dpf, a significant peak of *pepsinogen* expression was observed in the TC group at ZT 2 h, whereas the significant peak was observed in the CTE group at ZT 2 h on 13 dpf. In addition, statistical differences were observed on 13 dpf between the overall expression of both groups, which were higher for CTE than for TC (two-way ANOVA, $p<0.05$). No significant differences were observed in *pepsinogen* expression on 8 dpf (two-way ANOVA, $p>0.05$).

Chymotrypsinogen expression

The relative expression of *chymotrypsinogen* displayed significant daily rhythms (Cosinor, $p <0.05$) for the 4 dpf larvae maintained in the CTE group, on 8dpf in both treatments (TC and CTE) and for the 13 dpf larvae maintained in TC (Fig. 3). In addition, the acrophases varied between the significant detected rhythms (Table 2). In the CTE group, acrophases were located in the dark phase at ZT 18:06h and ZT 21:35h for 4 and 8 dpf, respectively. In the TC group, the acrophase was located at the beginning of the dark phase on 8 dpf (ZT 14:02h) and shifted to the beginning of the light phase on 13 dpf (ZT 02:36h).

Moreover, *chymotrypsinogen* expression in the 8 dpf larvae showed significant differences depending on the groups and time of day, with a higher expression in the TC group

at both ZT 10 and 14h (two-way ANOVA, $p>0.05$) (Fig. 3B). Differences on 8 dpf were also observed between the overall expression of both groups, which were higher for TC than for CTE (two-way ANOVA, $p<0.05$). No statistically significant differences were observed in *chymotrypsinogen* on 4 and 13 dpf (two-way ANOVA, $p>0.05$).

Trypsinogen expression

The relative expression of *trypsinogen* in tilapia larvae showed significant daily rhythms (Cosinor, $p<0.05$) in the larvae raised in TC (on both 4 and 8 dpf), but not for the larvae raised at CTE (Cosinor, $p>0.05$) (Fig. 4). The acrophase of *trypsinogen* for the 4 dpf TC was located at the beginning of the light phase (ZT 03:10 h) and shifted to the end of the light phase in the 8 dpf larvae (ZT 10:11 h) (Table 2). In addition, *trypsinogen* in the 4 dpf larvae displayed significant differences (two-way ANOVA $p <0.05$), with a significantly higher expression at ZT 2 h in the TC group (Figure 4A). Differences were also observed on 4 dpf between the overall expression of both groups, which was higher for TC than for CTE (two-way ANOVA, $p<0.05$). No statistically significant differences were observed in *trypsinogen* expression on 8 and 13 dpf (two-way ANOVA, $p>0.05$).

Lipase expression

Lipase expression only displayed significant daily rhythms in the tilapia maintained under the TC conditions on both 4 and 13 dpf (Cosinor, $p<0.05$) (Fig. 5). The acrophases in these two stages occurred at similar times, in the first half of the light phase (ZT 03:54h and 02:47h on 4 and 13 dpf, respectively) (Table 2). No significant daily rhythms were detected on any of the days analyzed for the CTE group, nor for the 8 dpf larvae from the TC group (Cosinor, $p>0.05$).

Both the 4 and 8 dpf larvae showed significant differences between groups and time points in *lipase* expression (two-way ANOVA, $p<0.05$) (Fig. 5A and 5B). On 4 dpf, expression peaked at ZT 02:00h for both groups. On 8 dpf, *lipase* expression in the CTE animals also peaked at ZT 02:00h. However in this larval stage, this peak was lost in the TC larvae compared to the 4 dpf larvae. Furthermore, differences in overall expression were observed on both 4 and 8 dpf (two-way ANOVA, $p<0.05$), with a higher *lipase* expression throughout the day in the CTE group *versus* the TC group. No statistically significant differences were observed in *lipase* expression on 13 dpf (two-way ANOVA, $p>0.05$).

Maltase expression

The only significant daily rhythm in *maltase* expression was detected in the 13 dpf larvae from the TC group (Cosinor, $p<0.05$) (Figure 6). In these animals, *maltase* expression peaked around the middle of the dark phase, at ZT 17:27 h (Table 2). No significant daily rhythms were detected in the other days and groups (Cosinor, $p>0.05$). Moreover, no statistically significant differences between groups and time of day were found in *maltase* expression on any of the analyzed days (two-way ANOVA, $p>0.05$).

Isomaltase expression

On 13 dpf, significant daily rhythms of *isomaltase* were found in both groups (Cosinor, $p<0.05$) (Fig. 7). These rhythms presented opposite acrophases, with *isomaltase* expression peaking around the middle of the dark phase in the TC group (ZT 19:33h) and about the middle of the light phase in the CTE group (ZT 05:28h) (Table 2). In the 13 dpf larvae, differences in *isomaltase* were observed depending on the group and time of day, with a higher significant expression at ZT 18:00h and ZT 22:00h h in the TC larvae (two-way ANOVA, $p<0.05$) (Fig. 7C). Differences in the overall expression were observed on 13 dpf, with a higher *isomaltase*

expression in the TC than in the CTE group (two-way ANOVA, $p<0.05$). Neither significant daily rhythms nor differences between groups and time points were found for *isomaltase* expression in 4 and 8 dpf larvae (Cosinor, $p>0.05$) (two-way ANOVA, $p>0.05$).

Npy expression

Significant daily rhythms (Cosinor, $p<0.05$) were found for *npy* expression in 4 dpf larvae maintained in CTE and the 13 dpf larvae from the TC group (Fig. 8). Acrophases were located in the middle of the dark phase (ZT 18:38h) in the 4 dpf CTE larvae and in the first half of the light phase (ZT 03:00h) in the 13 dpf TC larvae (Fig. 8A and 8C) (Table 2). No significant daily rhythms were detected for the other days and groups (Cosinor, $p>0.05$). The 13 dpf larvae presented significant differences in *npy* expression between groups and time points (two-way ANOVA, $p<0.05$) (Fig. 8C). The tilapia larvae from the CTE group showed the highest expression at ZT 22:00h, which was higher than for the TC animals at ZT 02:00h and 06:00h, and was also a higher overall expression compared to the larvae from the TC group (two-way ANOVA, $p<0.05$). No statistically significant differences were observed in *npy* expression on 4 and 8 dpf (two-way ANOVA, $p>0.05$).

Cck expression

The only significant daily rhythm in *cck* expression was detected in the 13 dpf larvae from the TC group (Cosinor, $p<0.05$) (Fig. 9). The acrophase of this rhythm was located in the first half of the light phase, close to the middle of the day (ZT 04:19h) (Fig. 9C) (Table 2). No significant daily rhythms were detected for the other days and groups (Cosinor, $p>0.05$), and no statistically significant differences between groups and time of day appeared in *cck* expression on any analyzed day (two-way ANOVA, $p>0.05$).

DISCUSSION

In the present research, the existence of daily rhythms in the expression of several genes encoding different digestive enzymes and hormones were analyzed in tilapia larvae, as were the effects of temperature regime on the ontogeny of these rhythms. In general, rearing tilapia larvae under a thermocycle improved the occurrence of daily rhythms in these factors as most displayed significant rhythms on 13 dpf, which was not observed in the larvae maintained at constant temperature (Fig. 10). The acrophases (time of maximum value) of the factors involved in protein and lipids digestion, as well as hormones were located in the morning, at around mealtime, on 13 dpf in the TC group (Figure 10). The acrophases of the factors involved in carbohydrate digestion were located at night (Fig. 10). This greater number of significant rhythms correlated also with growth as the TC larvae length was longer than that of the CTE group larvae.

The existence of daily rhythms in digestive enzymes in fish was first described in adults (Vera et al., 2007; Montoya et al., 2010; López-Olmeda et al., 2012). In adult tilapia, daily rhythms have been reported in total protease activity, both acid and alkaline (Guerra-Santos et al., 2017). Here we showed daily rhythms in the expression of genes that encode digestive enzymes in tilapia. Currently, it is necessary to examine this topic in-depth because studying gene expression will allow us to know how enzymatic digestion is programmed, activated and modulated (Rønnestad et al., 2013; Yúfera et al., 2018). Studying the existence of daily rhythms in larval physiology is also crucial because they are present in many processes, although studies usually focus only on one single daily sample (Yúfera et al., 2018).

In recent years, the ontogeny of rhythms in digestive factors has been reported in the larvae of two marine species: gilthead seabream and Senegalese sole (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016; Zeytin et al., 2016). In both these species, most of the larval

stages analyzed had already started the exogenous feeding and, under these conditions, daily rhythms in digestive enzymes correlated with feeding (Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016). This is similar to the result herein found for the TC larvae on 13 dpf, where proteases (*chymotrypsinogen* and *trypsinogen*) and *lipase* presented the highest values around feeding time. The food digestion process depends, among other factors, on the presence and availability of adequate digestive enzymes throughout the gastrointestinal tract (Gisbert et al., 2013). The enzymes observed to display rhythms in tilapia larvae are amongst the most important for protein (trypsin and chymotrypsin) and lipid digestion (lipase acting to release fatty acids and glycerol) (Almeida et al., 2018; Durigon et al., 2019). Besides, hormones *npy* and *cck* also presented rhythms with the highest values at around mealtime in the 13 dpf larvae reared at TC. Npy is a growth hormone (Gh) regulator (Peng and Peter, 1997) that stimulates food consumption in fish (Narnaware et al., 2000). In addition, Cck is one of the most important regulators of food intake and digestion in fish and is largely responsible for the secretion of pancreatic enzymes (Koven et al., 2002; Volkoff et al., 2005; Zhang et al., 2018). The rhythms displayed by these endocrine factors, which were synchronized with mealtime in the TC larvae, could have improved the digestive processes and nutrient utilization in these larvae compared to the CTE larvae. Taken together, the rhythms in both digestive enzymes and hormones could have increased the efficiency of physiological processes, leading to improved efficiency of food intake, digestion and growth. This, in turn, would have led to the enhanced growth rates observed in the TC larvae compared with the CTE group.

On the other hand, *maltase* and *isomaltase* presented shifted acrophases and peaked at nighttime. These genes encode for enzymes that act in the final steps of the digestion of dietary carbohydrates (Tengjaroenkul et al., 2002). The peaks of maltase and isomaltase could have occurred later than other enzymes given the process phase in which they are involved. In addition, carbohydrate digestibility is generally poor in fish, although omnivorous fish like

tilapia have better digestion rates (Moon, 2001; Kamalam et al., 2017). Thus one possibility could be that carbohydrates remained for longer in the gut and, hence, the expression of these enzymes was delayed. On the other hand, gene expression does not necessarily correlate with the activity of the encoded enzyme (Yúfera et al., 2018), which could be the case for *maltase* and *isomaltase* and would explain why their rhythms are shifted compared to mealtime and to the other analyzed factors.

Previous studies have highlighted the importance of maintaining embryos/larvae under rearing conditions that are similar to the natural conditions that animals experience in the wild. This mainly involves using environmental cycles instead of constant conditions. For instance, it has been reported that LD cycles are required for correct circadian clock maturation in fish, and constant lighting conditions (either darkness or light) can delay or even suppress the appearance of daily rhythms (Ziv and Gothlif, 2006; Martín-Robles et al., 2013; Cuesta et al., 2014; Di Rosa et al., 2015). The temperature factor has been less studied than light. In zebrafish, thermocycles are able to synchronize the circadian rhythms of embryos/larvae during the first days of development in the absence of lighting cues, which indicates that thermocycles are a strong environmental signal for the ontogeny of the clock in fish (Lahiri et al., 2014). Likewise in tilapia, thermocycles enhance the ontogeny of digestive circadian rhythms, which highlights the importance of this signal. In addition, the results from both zebrafish (Lahiri et al., 2014) and our study could indicate an important role of thermocycles on fish circadian clock development, especially for freshwater species.

Furthermore, when fish develop under similar cyclic environmental variations to those they experience in the natural environment, they show beneficial effects on parameters other than the ontogeny of the circadian system. Tilapia juveniles presented higher growth rates under thermocycles than at constant temperatures, although this effect was lost as fish were becoming bigger and size dispersal was greater (Azaza et al., 2010). In addition, Senegalese sole and

zebrafish present higher growth rates and a lower incidence of malformations when they are kept, during the early development, in thermocycles compared to constant temperatures (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012; Sánchez-Vázquez and López-Olmeda, 2018). This agrees with the results obtained in tilapia growth with thermocycles. In these previous studies, however, the only analyzed molecular or physiological markers to explain the effects on development induced by thermocycles were some genes involved in sex differentiation as thermocycles also affect sex ratios (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012). Thus, the present results obtained in tilapia are the first to shed light on the molecular mechanisms used by thermocycles to improve growth, which indicates that at least one of the factors may be faster maturation and better synchronization of the rhythms of digestive physiology.

CONCLUSIONS

In summary, our research shows that applying daily thermocycles during early development improves the ontogeny and maturation of the daily rhythms of digestive enzymes in tilapia. This turns into a higher growth in the larvae reared under thermocycles, which may be due to higher digestion rates and better food efficiency. Therefore, employing similar conditions to natural environmental conditions (e.g. thermocycles instead of constant temperatures) is suggested when designing protocols for tilapia larviculture.

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FIGURE LEGENDS

Table 1. Primer sequences used for the quantitative analysis by qPCR.

Table 2. Acrophase (in *Zeitgeber* Time, h) and statistical significance values of the genes subjected to Cosinor analysis.

Figure 1. Standard length (mm) of tilapia larvae on 4, 8 and 13 days post fertilization (dpf) at a 12:12LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (black bars) or constant temperature (CTE) of 28ºC (white bars). Asterisks indicate statistically significant differences between groups at the same age (t-test, $p<0.05$).

Figure 2. Daily variations in the relative expression of *pepsinogen* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature(CTE) of 28ºC (represented by white squares, □). No statistically significant daily rhythms were detected in any group (Cosinor, $p>0.05$). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, $p<0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT0 h corresponds to light onset.

Figure 3. Daily variations in the relative expression of *chymotrypsinogen* analyzed in tilapia larvae on 4 (A) and 13 (B) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented

by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, $p<0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

Figure 4. Daily variations in the relative expression of *trypsinogen* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, $p<0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

Figure 5. Daily variations in the relative expression of *lipase* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, $p<0.05$). The white and black bars

above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

Figure 6. Daily variations in the relative expression of *maltase* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). No significant differences were observed between groups and time points (two-way ANOVA, $p>0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

Figure 7. Daily variations in the relative expression of *isomaltase* analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, $p<0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

Figure 8. Daily variations in the relative expression of *neuropeptide Y* (*npy*) analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were

subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). Different letters indicate statistically significant differences between time points on the same graph (two-way ANOVA, $p<0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

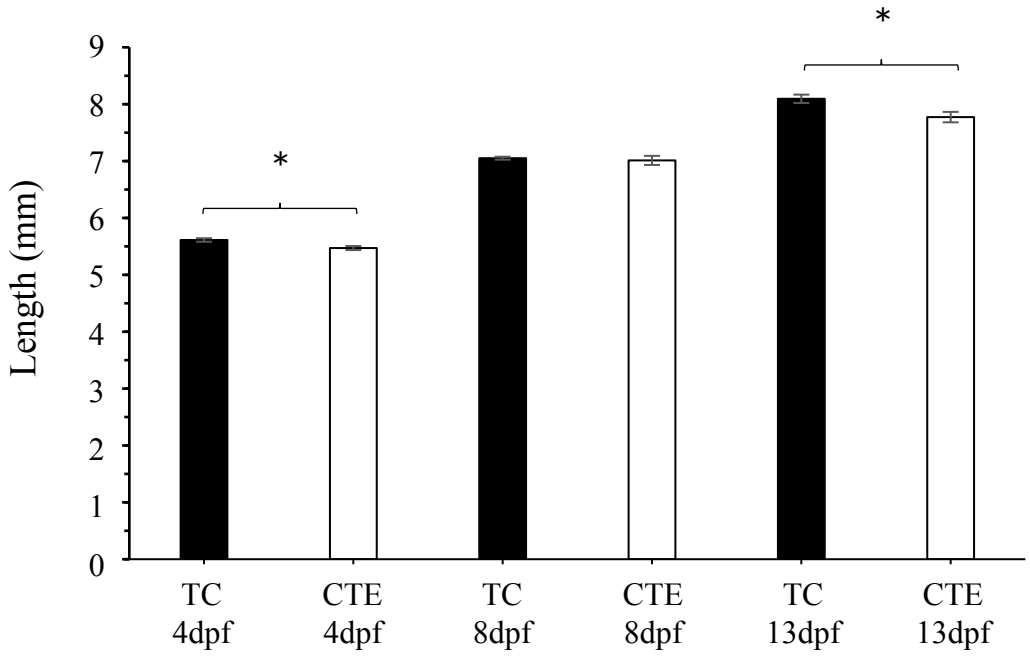
Figure 9. Daily variations in the relative expression of *cholecystokinin* (*cck*) analyzed in tilapia larvae on 4 (A), 8 (B) and 13 (C) days post fertilization (dpf) at a 12:12 LD cycle. Larvae were subjected to two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC (represented by black circles, ●) or constant temperature (CTE) of 28ºC (represented by white squares, □). The sinusoidal dashed line represents the adjustment to a sinusoidal rhythm whenever the Cosinor test was significant ($p<0.05$). No significant differences were observed between groups and time points (two-way ANOVA, $p>0.05$). The white and black bars above each graph represent the light phase and dark phase, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), where ZT 0 h corresponds to light onset.

Figure 10. Map of the acrophases of the digestive factors and hormones analyzed in tilapia larvae from 4, 8 and 13 days post fertilization (dpf) at a 12:12 LD cycle and by two different temperature regimes: a thermocycle (TC) of 31ºC:25ºC or constant temperature (CTE) of 28ºC. The acrophases from the larvae reared in the TC group on 4, 8 and 13 dpf are represented by black squares, triangles and circles, respectively. The acrophases from the larvae reared in the CTE group on 4, 8 and 13 dpf are represented by white squares, triangles and circles, respectively. The acrophase is indicated only for the statistically significant rhythms

(Cosinorp<0.05). The name of each represented gene is indicated on the left. The white and black bars above the graph represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset.

Supplementary Figure 1. Daily average water temperature throughout the experiments in the two temperature regimes herein tested: a thermocycle (TC) of 31ºC:25ºC (dashed line) or constant temperature (CTE) of 28ºC (continuous line). The presented data are expressed as mean±S.D. The white and black bars above the graph represent the light and dark phases, respectively. Time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset.

Supplementary Figure 2. Standard tilapia larvae length measurement.


Length (mm)
9
8
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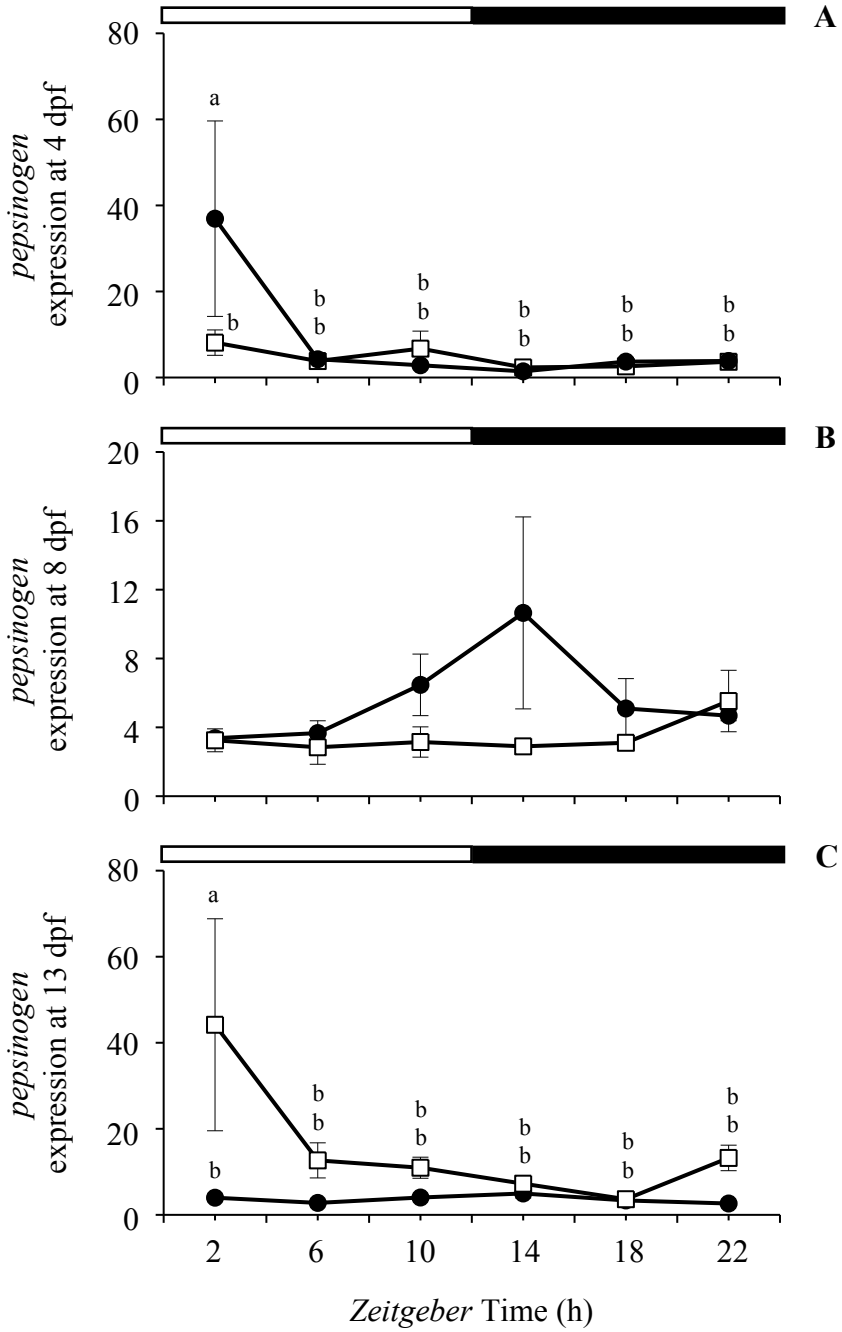
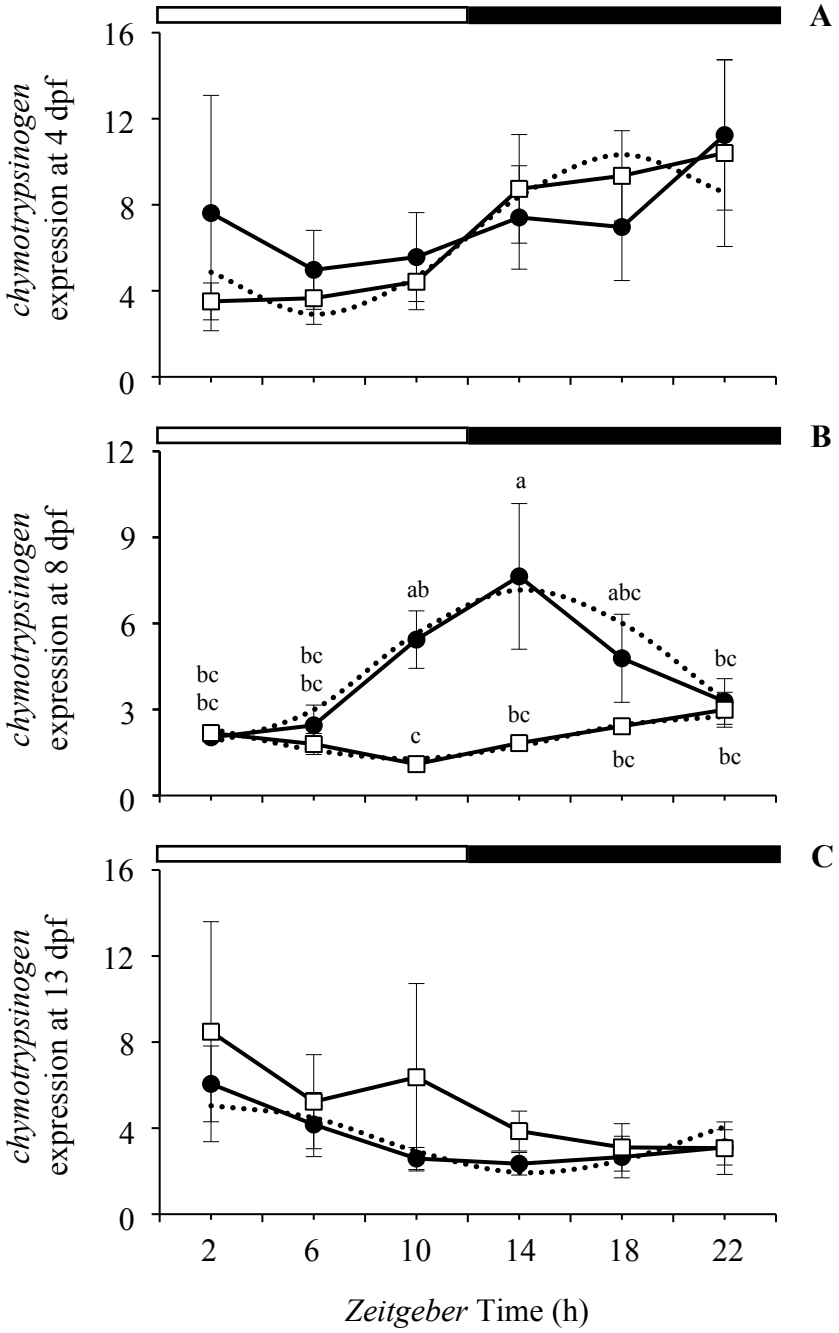
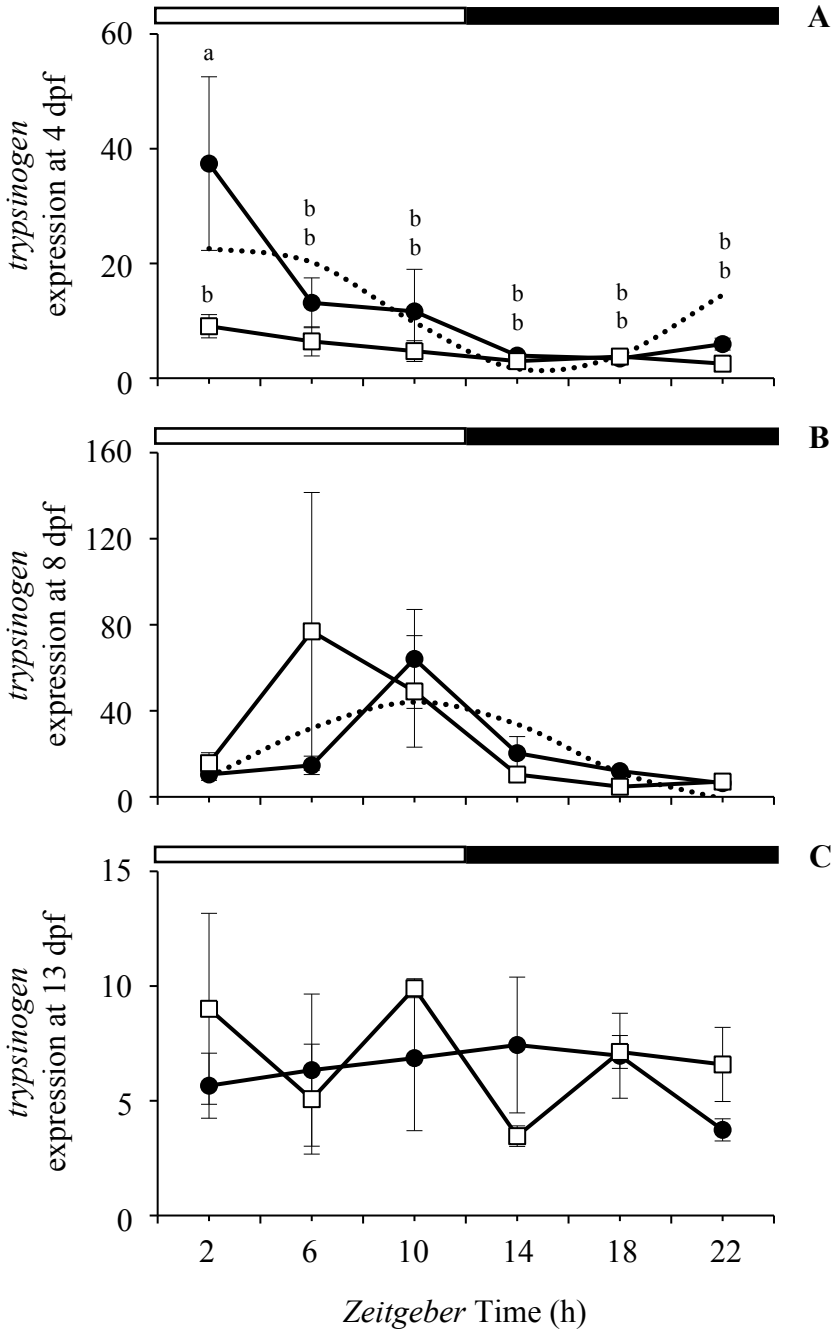
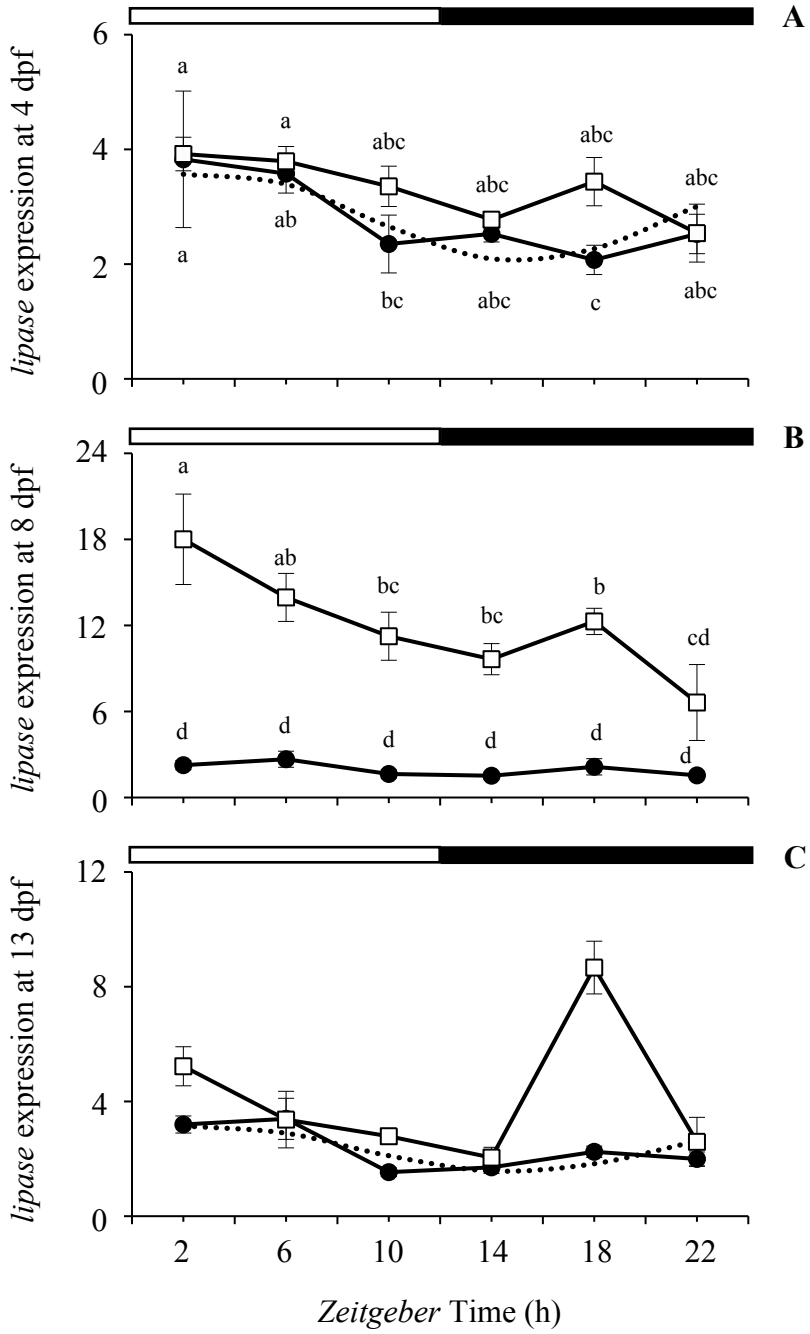
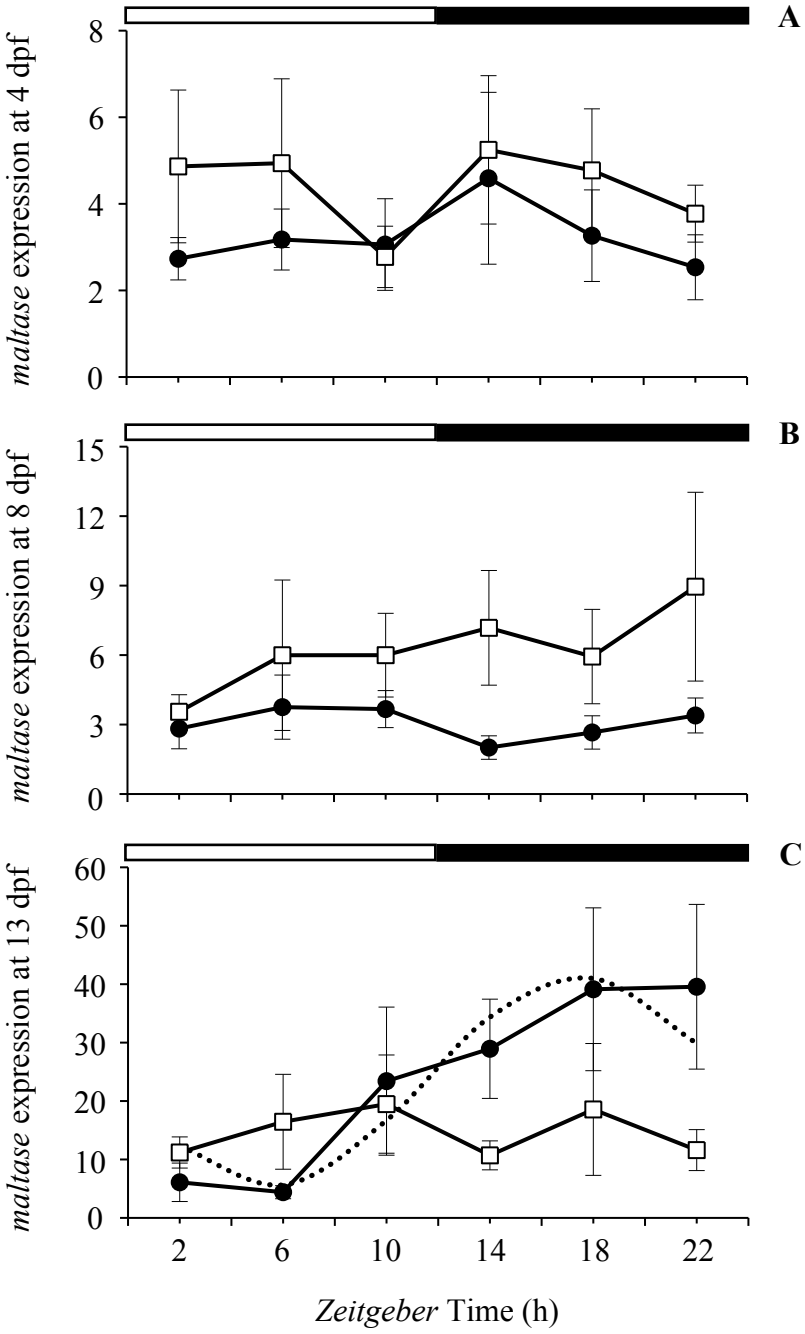
TC 8dpf
CTE 8dpf
TC 13dpf
CTE 13dpf

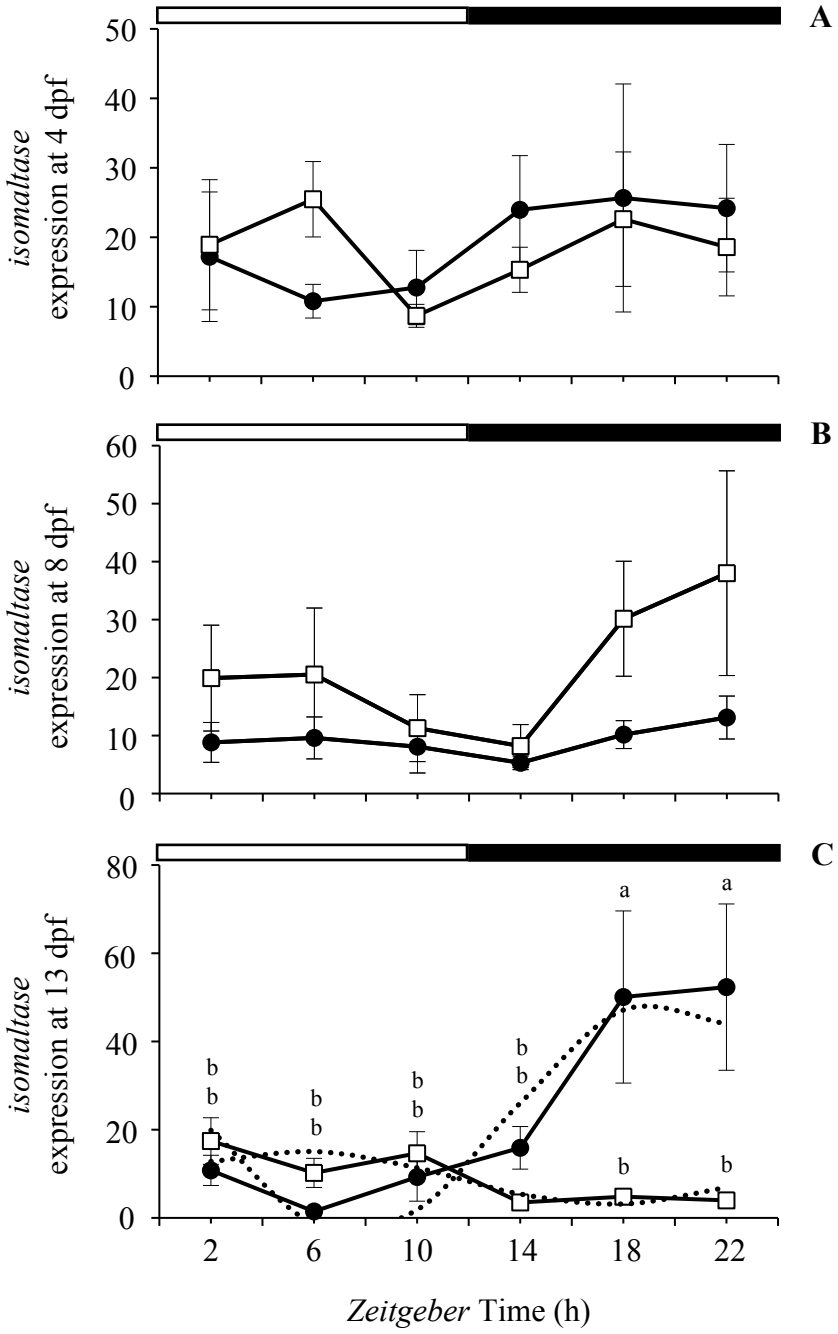

A
B
C
pepsinogen expression at 4 dpf
pepsinogen expression at 8 dpf
pepsinogen expression at 13 dpf
Zeitgeber Time (h)
0
20
40
60
80
4
8
12
16
2
6
10
14
18
22
a
b

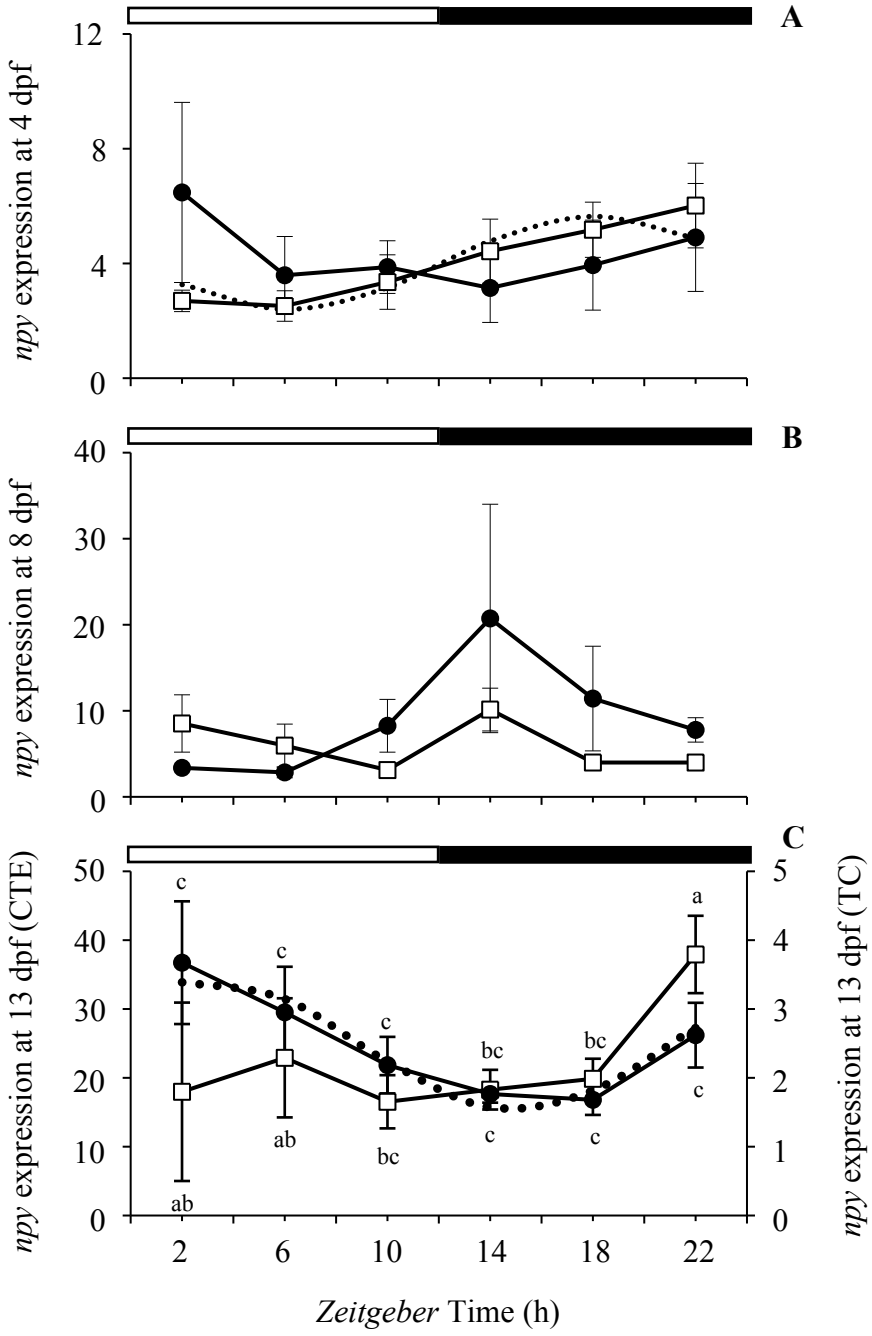

A
B
C
chymotrypsinogen expression at 4 dpf
chymotrypsinogen expression at 8 dpf
chymotrypsinogen expression at 13 dpf
0
4
8
12
16
3
6
9
a
ab
abc
bc
c
2
6
10
14
18
22
Zeitgeber Time (h)

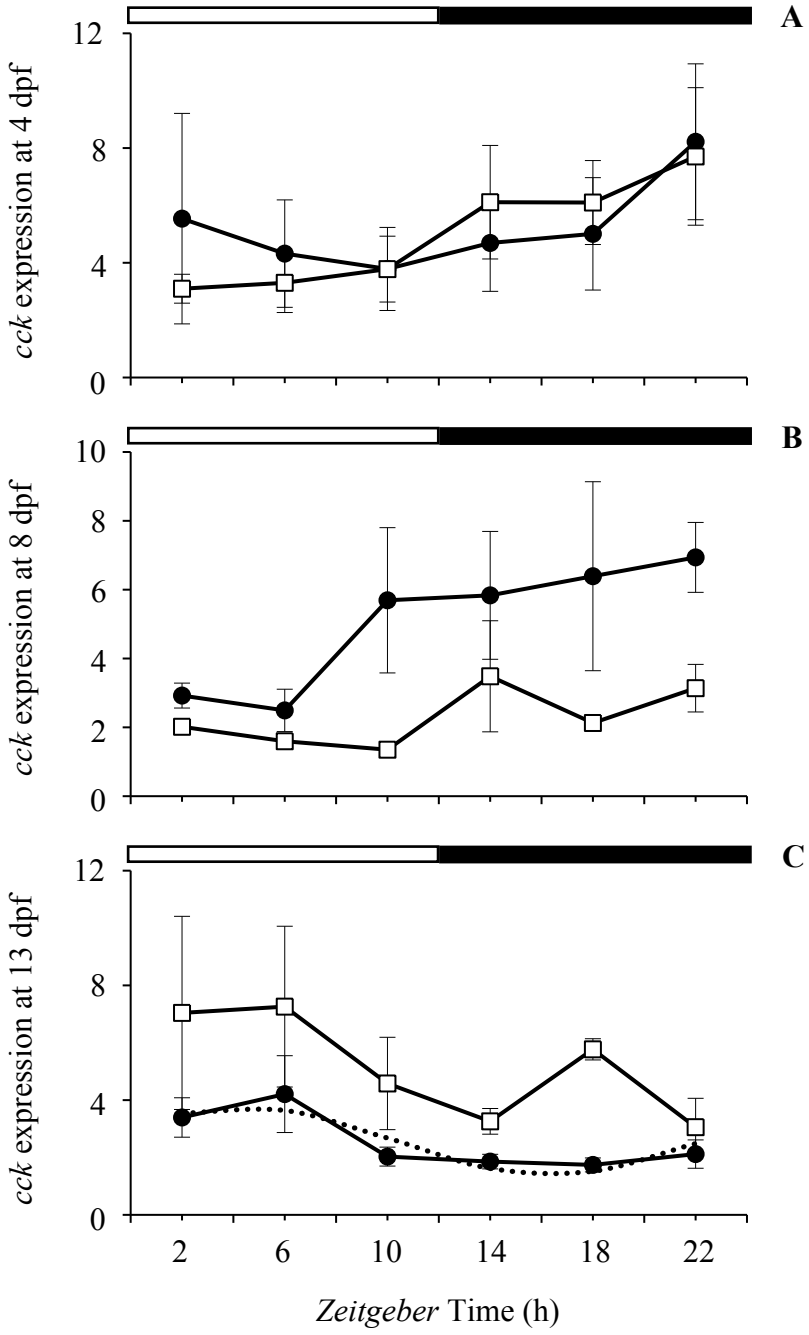

A
B
C
trypsinogen expression at 4 dpf
trypsinogen expression at 8 dpf
trypsinogen expression at 13 dpf
60
40
20
0
160
120
80
40
0
15
10
5
0
a
b
b
b
b
b
b
b
b
b
b
b
2
6
10
14
18
22
Zeitgeber Time (h)

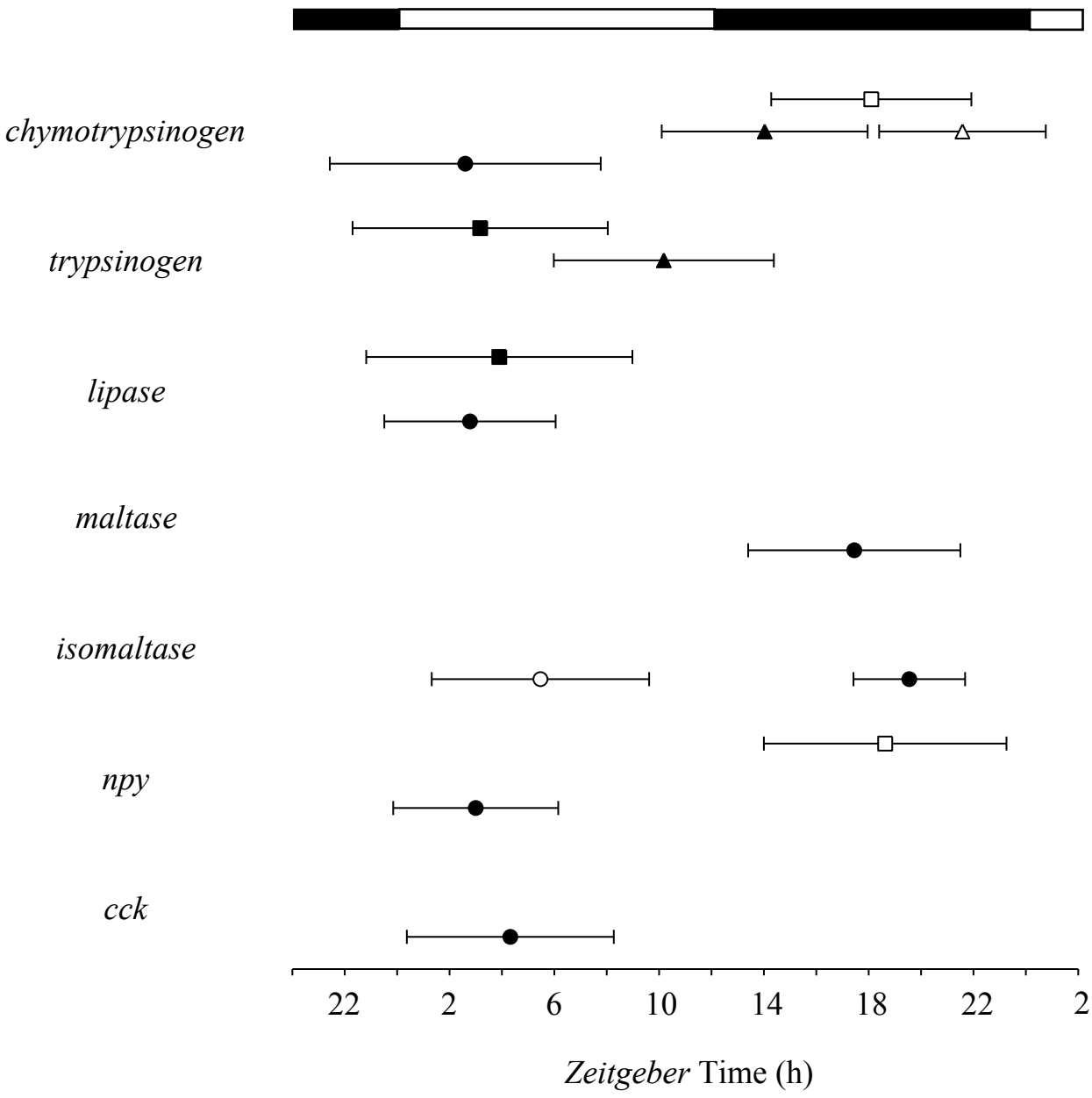

A
B
C
lipase expression at 4 dpf
lipase expression at 8 dpf
lipase expression at 13 dpf
Zeitgeber Time (h)
0
2
4
6
8
12
18
24
2
6
10
14
18
22
a
ab
abc
bc
c
d
cd

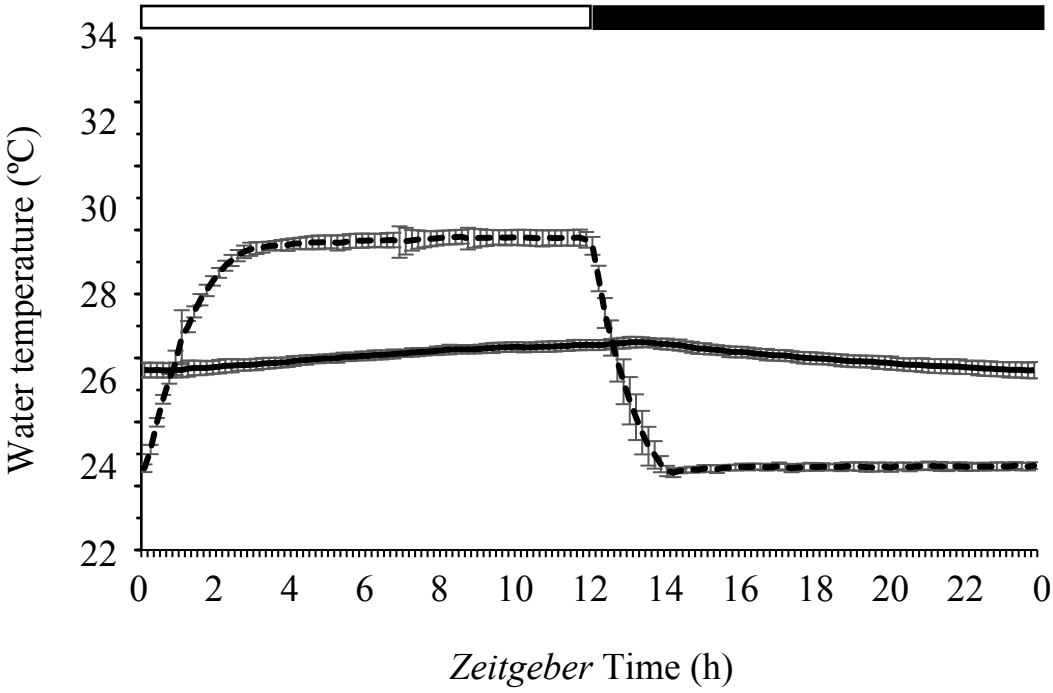

A
B
C
maltase expression at 4 dpf
maltase expression at 8 dpf
maltase expression at 13 dpf
0
2
4
6
8
3
9
12
15
10
20
30
40
50
60
2
6
10
14
18
22
Zeitgeber Time (h)

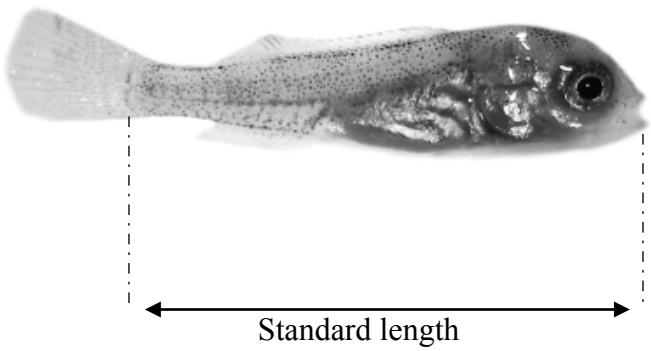

A
B
C
isomaltase expression at 4 dpf
isomaltase expression at 8 dpf
isomaltase expression at 13 dpf
0
10
20
30
40
50
60
80
2
6
10
14
18
22
a
b
Zeitgeber Time (h)


A
B
C
12
8
4
0
40
30
20
10
0
50
40
30
20
10
0
5
4
3
2
1
0
npy expression at 4 dpf
npy expression at 8 dpf
npy expression at 13 dpf (CTE)
npy expression at 13 dpf (TC)
c
c
c
bc
bc
a
c
ab
ab
bc
c
c
2
6
10
14
18
22
Zeitgeber Time (h)


A
B
C
cck expression at 4 dpf
cck expression at 8 dpf
cck expression at 13 dpf
0
4
8
12
2
6
10
14
18
22
Zeitgeber Time (h)


chymotrypsinogen
trypsinogen
lipase
maltase
isomaltase
npy
cck
22
2
6
10
14
18
22
2
Zeitgeber Time (h)


Water temperature (ºC)
34
32
30
28
26
24
22
0
2
4
6
8
10
12
14
16
18
20
22
0
Zeitgeber Time (h)


Standard length

Gene	F/R	Sequence (5′-3′)	Ensembl/GenBank Accession Number
pepsinogen	F	TGACCAATGACGCTGACTTG	JQ043215.1
	R	GGAGGAACCGGTGTCAAAAATG	
chymotrypsinogen	F	TTCTGCCTTCGCTTCTCATC	ENSONIG00000003237
	R	TTCAACGCCATCTGCTACTG	
trypsinogen	F	AGTGCGCAAAGAACTCTGTG	AY510093.1
	R	AATGTTGTGCTCACCAAGGC	
lipase	F	CTACAACTGCTCCACCAGCA	NM_001279753.1
	R	GGTGTAGTCGGTGAGCCATT	
maltase	F	ACGGTGGAATCACAGGACTC	XM_005459498.4
	R	GAAGGCTGCTGATGTGTTCA	
iomaltase	F	GGATCATTCTTCTGGGACGA	XM_003441717.4
	R	AGGTTGTGCTGTGGGGTTAG	
cck	F	AGAAACTCCACGGCAAACAG	ENSONIG00000019439.1
	R	ACTCATACTCCTCTGCACTGC	
npy	F	ACACCCAACACTGCTTGAAG	ENSONIG00000004499
	R	TGTTGCACAGATGACGACTC	
βactin	F	TGGTGGGTATGGGTCAGAAAG	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA	

Genes	dpf	Acrophase (ZT h)	
		TC	CTE
pepsinogen	4	□	□
	8	□	□
	13	□	□
chymotrypsinogen	4	□	18:06 ± 3:49*
	8	14:02 ± 3:56*	21:35 ± 3:11*
	13	2:36 ± 5:10*	□
trypsinogen	4	3:10 ± 4:52*	□
	8	10:11± 4:12*	□
	13	□	□
lipase	4	3:54 ± 5:05*	□
	8	□	□
	13	2:47 ± 3:16**	□
maltase	4	□	□
	8	□	□
	13	17:27 ± 4:03*	□
isomaltase	4	□	□
	8	□	□
	13	19:33 ± 2:08**	5:28 ± 4:09**
npy	4	□	18:38 ± 4:38 *
	8	□	□
	13	3:00 ± 3:09**	□
cck	4	□	-
	8	-	□
	13	4:19 ± 3:57*	-

Cosinor *p<0.05 **p<0.01. Acrophase is not indicated for non-significant genes (p> 0.05).

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: