Daily rhythms of the expression of genes from the somatotropic axis: The influence on tilapia (*Oreochromis niloticus*) of feeding and growth hormone administration at different times

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Running Title: ***Somatotropic axis and growth hormone response in tilapia***

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ABSTRACT

The aim of this research was to investigate the presence of daily rhythms in the somatotropic axis of tilapia fed at two times (mid-light, ML or mid-dark, MD) and the influence of the time of day of growth hormone (GH) administration on the response of this axis. Two different GH injection times were tested: ZT 3 (3 h after lights on) and ZT 15 (3 h after lights off). In both experiments, the mRNA expression levels of hypothalamic pituitary adenylate cyclase-activating polypeptide (*pacap*), pituitary growth hormone (*gh)*, liver insulin-like growth factors (*igf1* and *igf2a*), and liver and muscle growth hormone receptors (*ghr1* and *ghr2*) and IGF receptors (*igf1ra* and *igf2r*) were evaluated by means of qPCR. Daily rhythms were observed in the liver for *ghr1*, *ghr2* and *igf2r* but only in fish fed at ML, with the acrophases located in the light phase (ZT 3:30, 3:31 and 7:38 h, respectively). In the muscle, *ghr1* displayed a significant rhythm in both groups and *ghr2* in ML fed fish (acrophases at ZT 5:29, 7:14 and 9:23 h). The time of both GH administration and feeding influenced the response to GH injection: ML fed fish injected with GH at ZT 15 h showed a significant increase in liver *igf1*, *igf2a* and *ghr2*; and muscle *ghr2* expression. This is the first report that describes the existence of daily rhythms in the somatotropic axis of tilapia and its time-dependent responses of GH administration. Our results should be considered when investigating the elements of the somatotropic axis in tilapia and GH administration.

Keywords: pituitary adenylate cyclase-activating polypeptide; growth hormone; insulin-like growth factor; growth hormone receptor; IGF receptor; GH chronopharmacology; teleost fish.

INTRODUCTION

An understanding of growth physiology in fish is essential for the improvement of animal production. The somatotropic axis is the principal stimulator of growth in fish, primarily due to its effects on muscle hypertrophy and hyperplasia (Cerdá-Reverter and Canosa, 2009). The stimulatory signal is initiated in the hypothalamus, where the pituitary adenylate cyclase-activating polypeptide (PACAP) is produced (Montero et al., 2000). This factor stimulates the production of growth hormone (GH) in the pituitary gland, which in turn generates signaling pathways that involve tissues such as the liver

and the muscle and other factors such as insulin-like growth factors (IGFs) (Chang and Wong, 2009).

GH or somatotropin is a hormone from the family of cytokines that is classified in teleost fish as a single-chain polypeptide protein. GH is produced and released from the anterior pituitary and acts as an important regulator of metabolism and somatic growth in many fish species (Canosa et al., 2007). In fish, treatment with exogenous GH effectively stimulates both somatic and lineal growth (Holloway and Leatherland, 1998), sex maturation, gametogenesis and steroidogenesis (Reindl and Sheridan, 2012), and the adaptation to marine water of anadromous fish (Makino et al., 2007). GH actions are triggered by binding the hormone to GH receptors (GHRs), which are present in the cells of the target tissues. Two GHR subtypes have been described in teleost fish: GHR1 and GHR2. GHR1 is structurally the most similar to tetrapod GHR, whereas GHR2 seems to be restricted to teleosts (Fuentes et al., 2013). Nevertheless, the functions of each GHR in fish remain unclear (Di Prinzio et al., 2010).

IGFs are the primary mediators of the effects of growth induced by GH in vertebrates and can exert their actions in an autocrine, paracrine and endocrine manner (Le Roith et al., 2001). IGFs regulate numerous processes involved in growth, such as the stimulation of protein synthesis and the inhibition of proteolysis, proliferation, differentiation, cell migration and survival (Duan et al., 2010; Wood et al., 2005). Two IGFs are present in vertebrates, IGF-1 and IGF-2 (Reindl and Sheridan, 2012). In juvenile fish, *igf1* mRNA expression has been reported in all of the tissues of the organism, thus indicating the importance of this factor as one of the main stimulators of somatic growth (Biga et al., 2004; Shamblott and Chen, 1993). The presence of IGF-2 in fish was reported later than the presence of IGF-1. IGF-2 has been characterized in salmon (Palamarchuk et al., 1999), rainbow trout (Shamblott et al., 1998) and zebrafish (*Danio rerio*) (White et al., 2009), and its role in growth has been less studied than that of IGF-1. IGFs actions are driven by binding these factors to their receptors, IGF1R and IGF2R, which are present in the cell membranes of most of the organism's tissues (Caruso and Sheridan, 2011).

Biological rhythms can be defined as endogenous events that are repeated in a regular manner and are controlled by environmental factors that cycle in a regular and predictable form, such as light and temperature (Morgan, 2004). These rhythms offer an adaptive advantage because animals can time processes such as feeding and reproduction to occur during specific periods of the day and/or the year, increasing the

possibility of success and minimizing energy expenditure (López-Olmeda et al., 2012). Biological rhythms are present in many parts of the mammalian endocrine system (Haus, 2007). Most studies of the rhythms in the somatotropic axis have been performed in humans and rodents (Veldhuis and Bowers, 2003). In fish, studies on endocrine rhythms in the somatotropic axis have primarily been performed in Salmonids, such as the rainbow trout (*Oncorhynchus mykiss*) and the Atlantic salmon (*Salmo salar*), showing a great variability depending on life stage and environmental conditions (Ebbesson et al., 2008; Gélineau et al., 1996; Reddy and Leatherland, 2003). Thus, knowledge of the existence and regulation of rhythms in the fish growth system remains scarce.

Chronopharmacology is an area that links biological rhythms with pharmacology on the basis that the efficacy of a drug would vary in a rhythmic manner as many physiological variables display circadian variations (Dallmann et al., 2014). In mammals, the application of chronopharmacology primarily focuses on cancer therapy (Dallmann et al., 2014). In the case of GH treatment in humans, several studies have reported variations of GH effects depending on the time of administration, with stronger effects when the hormone is administered during nighttime (Janukonyté et al., 2013). This coincides with the endogenous daily rhythm of GH production in humans, which shows higher levels during darkness (Veldhuis & Bowers, 2003). However, the mechanism that underlies this different response to exogenous GH depending on the time of the day remains unknown. One interesting hypothesis would link the time-dependent response to GH with rhythms in GH receptors, especially in the tissues that produce IGF such as the liver. Rhythms in GH receptors have been described in rodents and they correlate with IGF-1 production (Itoh et al., 2004). However, no study has correlated to date the different effects of the time of day of GH administration with GH receptors expression, its number and/or affinity on target tissues.

Tilapia (*Oreochromis niloticus*) is an omnivorous fish species that is native to Africa; it belongs to the phylogenetic group of Cichlids (Eknath and Hulata, 2009). This fish has a high tolerance to intensive culture, the capacity to reproduce throughout the year, good market acceptance, high resistance to diseases and relative easiness for genetic manipulation related to improving production (Ng and Romano, 2013). Tilapia is bred and cultured worldwide and is the second most cultured freshwater fish after the carp (*Cyprinus carpio*) (Ng and Romano, 2013). However, despite the importance of tilapia,

very little is known about its biological rhythms and circadian system; the only studies performed have focused on daily rhythms of behavior (Fortes-Silva et al., 2010).

The objective of this paper was to describe the presence of daily rhythms of gene expression of key factors in the somatotropic axis of tilapia: two pituitary adenylate cyclase-activating polypeptide (*pacap1a* and *pacap1b*) in the hypothalamus; growth hormone (*gh*) in the pituitary; two insulin-like growth factors (*igf1* and *igf2b*), two IGF receptors (*igf1ra* and *igf2r*) and two GH receptors (*ghr1* and *ghr2*) in the liver; and GH and IGF receptors (*ghr1*, *ghr2*, *igf1ra* and *igf2r*) in the muscle. In addition, in a second experiment the effects of different times (ML vs. MD) of feeding and different times of GH administration, daytime (ZT3) vs. nighttime (ZT15), on all parameters of the somatotropic axis were tested.

MATERIALS AND METHODS

Animals and housing

The experiments were conducted using 96 fish with a mean body weight of 89.0 ± 5.77 g (mean ± S.E.M.). Fish were obtained from the Polytechnic University of Madrid (Spain) and housed at the laboratory of Chronobiology of the University of Murcia. Fish were placed in 200 L tanks that were located in a recirculation water system equipped with biological and mechanical filters. Water temperature was controlled at 28 °C. Several parameters of water quality such as pH, dissolved oxygen, ammonia, nitrate and nitrite were measured daily. Photoperiod was set at a 12:12 h light:dark (LD) cycle, with lights on at 8 h local time (Zeitgeber Time 0 h, ZT 0 h). During acclimation and experimental periods, fish were fed a commercial diet with 36% crude protein (D-4 Alterna Basic 2P, Skretting AS, Spain) at a daily rate of 1% of their body weight.

Experimental design

All of the experimental procedures complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) on the use of laboratory animals.

Experiment 1. Daily rhythms in the somatotropic axis

Fish (N=48) were divided in two groups. One group was fed at ZT 6 h (middle of the day, ML) and the other at ZT 18 h (middle of the night, MD). The fish were divided in

eight tanks (4 tanks per group, n=6 fish per tank) so that each tank could be sampled at a single sampling point, thus avoiding the stress induced by several sampling events in the same tank. Fish were fed using automatic feeders (Eheim, Germany) and the amount of food delivered was adjusted to a daily ratio of 1% of body weight. Fish were kept under the experimental conditions for 40 days, allowing them to synchronize to the feeding time. At the end of this period, fish were sampled every 6 h during a 24 h cycle, collecting samples at ZT 3, 9, 15 and 21 h. Fish were anesthetized with eugenol (clove oil essence, Guinama, Valencia, Spain) at a concentration of 50 µL/L. Blood was collected by puncture of the caudal vein using heparinized syringes (Sigma, H6278, 25,000 units/3 mL of 0.6% NaCl solution) and was kept on 1.5 ml sterile tubes containing heparin. Blood was then centrifuged at 3000 rpm for 15 minutes at 4 °C and plasma was separated and stored at -80 °C until analysis. The fish were then killed by decapitation, and samples from the hypothalamus, pituitary, liver and muscle were collected and kept in sterile Eppendorf tubes, which were immediately frozen on dry ice and then stored at -80 °C until analysis. Fish manipulation and tissue collection during the dark phase were performed under a dim red light.

Experiment 2. Influence of the time of day in the response to GH administration

This experiment was performed after analyzing the results of experiment 1, thus enabling us to establish the two different times used for GH administration. Fish (N=48) were placed in eight tanks (n=6 fish per tank) and divided in two groups (4 tanks per group) that were fed at two different times: one group fed at ZT 6 h (ML) and the other group fed at ZT 18 h (MD). As in the first experiment, fish were fed a daily ratio of 1% of their body weight using automatic feeders; they were kept under the experimental conditions for 40 days. At the end of this period, two solutions were prepared: one solution contained GH (human recombinant GH, Genotonorm Miniquick, Pfizer, New York, USA) and the control solution used a saline vehicle (VEH) (0.9% NaCl dissolved in bidistilled water). The four tanks from each feeding group were classified according to the solution injected and the time of day as follows: fish injected with GH at ZT 3 h (3 h after lights on), fish injected with VEH at ZT 3 h, fish injected with GH at ZT 15 h (3 h after lights off), and fish injected with VEH at ZT 15 h. GH solution was prepared at a concentration of 2 mg/ml, and the dose administered was 2 mg/kg of fish body weight. At the moment of the injection, fish were first slightly anesthetized using eugenol at a concentration of 10 µL/L, then fish were weighted, the dose was calculated

and the injection was performed. Both GH and VEH were administered intramuscularly, in the left side of the fish in a point midway between the base of the dorsal fin and the lateral line. The administration of mammalian GH, the dose used and time of sampling after GH injection were selected according to previously published studies in fish (Inui et al., 1985; Mancera & McCormick, 1998; Miwa & Inui, 1985; Sangiao-Alvarellos et al., 2006; Shamblott et al., 1995). Sample collection was performed 10 hours after GH administration in all groups; thus, samples from fish injected at ZT 3 h were collected at ZT 13 h and samples from fish injected at ZT 15 h were collected at ZT 1 h the following day. Samples from blood, the hypothalamus, the pituitary gland, the liver and muscle were collected as described for Experiment 1. Fish manipulation, injection and tissue collection during the dark phase were performed under a dim red light.

Plasma GH analysis

Plasma GH levels were measured by means of a commercial Salmon GH ELISA kit (Catalog No E0044s, EIAab Science Co. LTD, Wuhan, China). The homology between salmon GH and tilapia GH accounts for 62 % of identity and 78 % of similarity. The kit was validated for tilapia samples performing a parallelism test, consisting of serial dilutions of tilapia plasma containing known amounts of GH and comparing the resulting values with the standard curve, with recovery values of 92.4 ± 3.5 %.

Real time RT-PCR analysis

Samples of the hypothalamus, the pituitary gland, the liver and muscle were transferred to sterile tubes containing 0.5 ml of Trizol (Invitrogen, CA, USA). Tissue samples were mechanically homogenized and total RNA extraction was performed according to the manufacturer's instructions (Invitrogen). The RNA pellet was dissolved in sterile DEPC water. In the next step, total RNA (1 µg) was retro-transcribed using a commercial kit (QuantiTect Reverse Transcription Kit, Qiagen, Germany), which included a step involving genomic DNA elimination. The cDNA was subjected to quantitative PCR analyses using a light thermocycler (7500 Real-Time PCR system, Applied Biosystems, CA, USA) pursuant to the following protocol: 95ºC for 15 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Quantitative PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems). All of the samples were run in triplicate. The expression of *pacap1a* and *pacap1b* was analyzed in the hypothalamus; *gh* expression was measured in the pituitary; *igf1*, *igf2b*, *igf1ra*, *igf2r*,

ghr1 and *ghr2* expression was analyzed in the liver; and *igf1ra*, *igf2r*, *ghr1* and *ghr2* expression was analyzed in the muscle. Primer sequences are shown in Table 1. The primers were designed using Primer3 software (Rozen and Skaletsky, 2000). The relative amplification efficiencies of all of the genes were analyzed using cDNA dilution curves, verifying that they were similar for all genes. The PCR reaction was performed in a final volume of 20 µL. Primers for *pacap1a*, *ghr2*, *igf1*, *igf2b* and *igf2r* were added at a final concentration of 200 nM, and *pacap1b*, *gh*, *ghr1* and *igf1ra* were added at a final concentration of 400 nM. Primer concentrations were determined using a primer dilution curve.

Data analysis

The relative expression of all genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The first normalization was performed for all samples using the geometric mean of two reference genes: *elongation factor 1α* (*ef1α*) and *18s* (Vandesompele et al., 2002). Primer sequences for both genes were obtained from a previously published paper (Yang et al., 2013). Housekeeping genes were selected after checking that the coefficient of variation (C.V.) for each gene within each tissue was lower than 5%. The second normalization was performed, for data from Experiment 1, using as the reference the sample with the lowest value within each gene, tissue and feeding method. For the Experiment 2, the average value of each VEH group was calculated and then these data were used as the references for the second normalization for their respective GH groups.

Data for each variable analyzed in Experiment 1 were subjected to one-way ANOVA, followed by a Tukey's *post hoc* test, to check for significant differences between times of day within a single feeding group (ML or MD). Data were also subjected to a Cosinor analysis to check for the existence of a significant daily rhythm. Cosinor analysis is based on the least squares approximation of time series data with a cosine function of known period of the type Y = Mesor + Amplitude * cos ((2π(t-Acrophase)/Period). Cosinor analysis also identifies the statistical significance of the rhythm through an F-test of the variance accounted for by the waveform versus a straight line of zero amplitude (null hypothesis).

Data for each variable from Experiment 2 were subjected to Student's t-test to check for significant differences between GH and VEH administered at one particular time (ZT3 or ZT15) within each feeding group. In addition, data from the groups injected with GH

(normalized with the average value of their respective VEH group) were subjected to one-way ANOVA, followed by a Tukey's *post hoc* test, to check for significant differences between GH injection times and feeding times for each gene.

Statistical analyses (one-way ANOVA and t-test) were performed using SPSS software (v. 19.0, IBM, Armonk, NY, USA). Cosinor analysis was performed using El Temps (version 1.275, Prof. Díez-Noguera, University of Barcelona). The significance threshold (α) was set at 0.05 in all of the statistical tests performed.

RESULTS

Experiment 1. Daily rhythms in the somatotropic axis

Among all of the genes from the somatotropic axis analyzed, it was the GH receptors as well as liver *igf2r* that displayed statistically significant daily rhythms (Cosinor, $p<0.05$) (Table 2). Moreover, a differential effect due to feeding time was observed. Fish fed at ML showed rhythmicity in *ghr1*, *ghr2* and *igf2r* in the liver (Figure 1A-B), with the acrophases of both *ghrs* located close to ZT 3:30 h (3:30 h after lights on) and the acrophase of *igf2r* at ZT 7:38 h (Table 2), whereas fish fed at MD did not display significant rhythms in the genes analyzed in the liver. The expression of *ghr1* and *ghr2* also showed statistically significant differences depending on the time of the day (ANOVA, $p<0.05$) (Figure 1A-B). In addition, although significant daily rhythms were not revealed by Cosinor ($p>0.05$), *igf1* in fish fed at ML displayed statistically significant differences depending on the time of the day (ANOVA, $p<0.05$), with the highest values being found at ZT 3 h and the lowest values at ZT 9 h (Figure 1D). Conversely, among all of the genes analyzed in the muscle, only *ghr1* and *ghr2* showed statistically significant rhythmicity (Cosinor, $p<0.05$) (Table 2) (Figure 2A-B). Rhythms in *ghr1* expression were observed in both groups (ML and MD feeding), although the acrophase differed between feeding treatments, occurring 1:45 h earlier in the ML feeding group than in the MD group (ZT 5:29 and 7:14 h for ML and MD, respectively). Rhythms in *ghr2* expression were only observed in the ML group and showed an acrophase located at ZT 9:23 h (Table 2). In addition, *ghr2* in the ML feeding group showed differences between time points, with the highest values at ZT 9 and the lower values at ZT 21 h (ANOVA, $p<0.05$) (Figure 2B); and *igf1ra* in the MD group showed higher values at ZT 9 than at ZT 3 and 15 h (ANOVA, $p<0.05$) (Figure 2C).

That notwithstanding, the rest of the genes analyzed (hypothalamic *pacap1a* and *pacap1b*; pituitary *gh*; liver *igf2a*; and liver and muscle *igf2r*) showed neither statistically significant rhythmicity (Cosinor, $p>0.05$) nor significant differences depending on the time of the day (ANOVA, $p>0.05$). Plasma GH also showed neither significant rhythms nor significant differences between time points, displaying constant levels throughout the day in both groups, with average values of 38.5 ± 3.6 and 33.7 ± 4.3 ng/ml in ML and MD fish, respectively. Finally, no expression of the gene *igf1ra* could be detected in the liver tissue samples.

Experiment 2. Influence of the time of day in the response to GH administration

After analyzing the results of Experiment 1, only several of the factors analyzed displayed variations depending on the time of the day (liver *ghr1*, *ghr2*, *igf1* and *igf2r*; muscle *ghr1*, *ghr2* and *igf1ra*). In all of these parameters, the highest values were located during the light phase. For that reason, one GH administration time was set at ZT 3 h, thus allowing the exogenous GH to exert its actions throughout the light phase and the other time at ZT 15 h, thus allowing the exogenous GH to exert its actions throughout the dark phase.

The analysis of GH administration in tilapia revealed that both the time of injection of this hormone and the feeding time influenced the response of some of the factors from the somatotropic axis. In the pituitary, the highest *gh* expression values were observed in the animals from the ML group injected at ZT 3 h; those values were significantly higher than *gh* values in the MD group injected at ZT 15 h (ANOVA, $p<0.05$) (Figure 3A). In parallel with pituitary *gh* values, the highest values of *pacap1b* in the hypothalamus were detected in the ML group fish injected at ZT 3 h; those values were significantly higher than the *pacap1b* expression levels in the MD group injected at the same time (ANOVA, $p<0.05$) (Figure 3B). However, no significant differences of either *gh* or *pacap1b* were found between the animals injected with GH and their VEH controls (t-test, $p>0.05$).

In the liver, both the time of day of GH administration and the feeding time influenced the response in this tissue. GH injected at ZT 15 h in fish fed at ML produced a significant increase in both *igf1* and *igf2a* compared to the VEH controls (t-test, $p<0.05$) (Figure 4A-B). The increase in *igf1* and *igf2a* in this group was significantly higher than in the ML feeding group injected at ZT 3 h and in the two MD feeding groups (injected at ZT 3 and 15 h) (ANOVA, $p<0.05$) (Figure 4A-B). GH injection in the ML group had

a significant effect on *ghr1* levels depending on the time of administration, with higher values observed when GH was injected at ZT 15 h compared with the injection at ZT 3 h (ANOVA, $p<0.05$) (Figure 4C). In addition, GH injection at ZT 15 h increased *ghr2* expression levels compared with the VEH groups in both ML and MD fed fish (t-test, $p<0.05$) (Figure 4D). This increase in *ghr2* in the ML group injected at ZT 15 h was significantly higher than the values observed in the fish injected at ZT 3 h from both groups (ML and MD feeding) (ANOVA, $p<0.05$) (Figure 4D).

In muscle, only GH receptors displayed significant variations (Figure 5). With respect to *ghr1*, its expression was significantly reduced by GH injection at ZT 15 h in the ML group, compared both with its own VEH control and with those of the rest of the groups (t-test, $p<0.05$) (ANOVA, $p<0.05$) (Figure 5A). In the case of *ghr2*, GH administration at ZT 15 h in the ML group significantly increased the expression of this gene compared with both the VEH and the groups fed at MD (t-test, $p<0.05$) (ANOVA, $p<0.05$) (Figure 5B). In addition, GH injection at ZT 15 h in the MD group also increased *ghr2* expression compared to its VEH control (t-test, $p<0.05$) (Figure 5B).

Finally, hypothalamic *pacap1a*, liver *igf2r* and muscle *igf1ra* and *igf2r* showed no statistically significant differences, neither between injected groups (ANOVA, $p>0.05$) nor between each GH administered group and its VEH control (t-test, $p>0.05$).

DISCUSSION

Among all of the evaluated factors from the somatotropic axis of Nile tilapia, GH receptors (*ghr1* and *ghr2*) and one IGF receptor (*igf2r*) displayed significant daily rhythms (Cosinor), with their acrophases located during the light phase. In addition, liver *igf1* and muscle *igf1ra* displayed significant daily differences (not sinusoidal). The feeding time at which fish were acclimated influenced these factors, as the observed results were different depending on the feeding time (ML vs. MD). In addition, the response of the somatotropic axis elements to exogenous GH administration was time-dependent. The most effective time for inducing a physiological response was GH injection at ZT 15 h (3 h after lights off) in the fish fed at ML, which stimulated the expression of liver *igf1*, *igf2a* and *ghr2* and muscle *ghr2*.

The rhythmic control of fish endocrinology by the hypothalamus and the pituitary gland has been reported in previous papers, although studies have primarily focused on reproduction (Ando et al., 2014; Okuzawa and Gen, 2013; Zucchi et al., 2013) and the

stress axis (López-Olmeda et al., 2013). However, there are few studies on the rhythms in the somatotropic axis of fish and to our knowledge, none of those studies considered tilapia. Research on GH rhythms in fish has focused on GH plasma contents (Björnsson et al., 2002; Ebbesson et al., 2008; Zhang et al., 1994) but not pituitary *gh* expression. In this study, no rhythmicity was observed either in plasma GH or in *gh* expression in tilapia juveniles, although it should be noted that GH rhythms in fish may show a great variability depending on life stage, with rhythmicity lost in some stages (Ebbesson et al., 2008). However, daily rhythms were observed in the expression of GH receptors (*ghr1* and *ghr2*) in GH target tissues such as liver and muscle; to our knowledge, this is the first study to report such *ghr* expression rhythms. Thus, it could be hypothesized that although GH did not display rhythmic variations, its actions on target tissues would actually be rhythmic because they would occur driven by rhythmic changes in GH receptors. In addition, with respect to the IGF system, significant differences were observed in liver *igf1* expression depending on the time of the day in animals fed at ML, with the highest expression located at ZT 3 h, which coincides with the acrophase of *ghrs* in these animals. GH is the main regulator of IGF production in a wide variety of tissues in vertebrates (Piwien-Pilipuk et al., 2002; Wood et al., 2005); therefore, in the present study, the differences of *igf1* could be driven by daily changes in the density of GH receptors at specific points of the day.

Previous studies *in vivo* have shown that GH injections increase the expression of *igf1* and/or *igf2* in the liver of several fish species such as carp (Tse et al., 2002; Vong et al., 2003), rainbow trout (Shamblott et al., 1995) and channel catfish (*Ictalurus punctatus*) (Peterson and Small, 2005). In this research, an acute GH injection stimulated *igf1* and *igf2a* in the liver. This effect was dependent on both the time of the day in which GH was injected and feeding time, since the stimulation was only observed in fish fed at ML which were injected with GH during the dark phase (ZT 15 h). In fish, both GH and IGF-1 promote growth, although IGF-1 seems to be ultimately responsible for growth (Picha et al., 2008). Thus, a higher effect of exogenous GH for promoting growth in tilapia would be expected when it is administered at night, driven by a higher stimulation of the IGF system.

In teleost fish, two different GH receptors (*ghr1* and *ghr2*) have been identified (Di Prinzio et al., 2010; Fuentes et al., 2013). In this experiment, *ghr2* expression was stimulated in both liver and muscle, but only when GH was injected during the nighttime, as was observed with the liver *igfs*. In the case of *ghr2*, however, the effects

399 did not depend on feeding time. Conversely, muscle *ghr1* expression was decreased by
400 the GH injection at ZT 15 h in the ML-fed animals. Previous studies have reported the
401 effects of GH administration on *ghr* expression, which resulted in a variety of
402 responses. In mice, chronic GH treatment causes an increase in liver *ghr* levels, whereas
403 acute GH administration has the opposite effect, causing a reduction in *ghr* expression
404 (Baxter and Zaltsman, 1984; Maiter et al., 1988). In fish, most of the studies have been
405 performed using GH transgenic fish or chronic GH administration for long periods
406 (Kim et al., 2015; Singh and Lal, 2008). In cultured rainbow trout hepatocytes, GH
407 treatment resulted in an increase of both *ghr1* and *ghr2* expression (Very and Sheridan,
408 2007). The studies of acute GH administration to fish *in vivo* are scarce. The different
409 response of GH receptors to GH treatment seems to vary depending on the tissue
410 studied and GH delivery method (acute vs. chronic). In this study, *ghr2* showed a rapid
411 response, increasing its levels after GH administration. Thus, it is possible that *ghr2*
412 could be related to the response to acute GH administration, whereas *ghr1* could be
413 more closely related to the response to chronic GH treatments. Nevertheless, further
414 studies are required to elucidate this hypothesis.
415 PACAP is considered the primary physiological factor that stimulates the release of GH
416 in fish (Mitchell et al., 2008; Wong et al., 2005). In this group of vertebrates, two
417 PACAP isoforms are present, although it is unknown whether they have different
418 functions (Chang and Wong, 2009). In this study, both PACAP isoforms expressions,
419 *pacap1a* and *pacap1b*, were analyzed. Only *pacap1b* showed significant differences in
420 the GH administration experiment; those differences matched the differences observed
421 in pituitary *gh* in the same fish, thus pointing that *pacap1b* could be more effective than
422 *pacap1a* for inducing GH production.
423 The effects of two different feeding times (ML vs. MD) on the rhythms of the
424 somatotropic axis and the response to exogenous GH were also evaluated. In general, a
425 higher number of factors related to the somatotropic axis displayed rhythms, and the
426 effects of exogenous GH were higher in the animals fed at ML than in the animals fed at
427 MD. Although tilapia has been described to be able to feed at night under some
428 conditions (using self-feeding devices), it seems to be a mostly diurnal animal,
429 displaying its activity during the light phase (Fortes-Silva and Sánchez-Vázquez, 2012;
430 Fortes-Silva et al., 2010). One hypothesis explaining the different results obtained from
431 different feeding times would be that restricted feeding at night disrupts either the
432 circadian system or the circadian control of the somatotropic axis. Thus, daytime

feeding seems to be a better option than night feeding for tilapia, at least for maintaining the daily rhythms present in this species.

In summary, the regulation and response of the somatotropic axis of fish is a rhythmic and complex process. In tilapia, daily variations in this axis seem to occur mainly at the level of receptors. In addition, GH administration at different times of the day induced a different response, with the highest effects observed when this hormone was administered during the nighttime in animals fed during the light phase (the active phase of that species). These results should be considered for future studies on the somatotropic axis of fish because different results could be obtained depending on the time of the day in which samples are collected and depending on feeding conditions. The results also highlight the idea that the treatment of fish with exogenous hormones may vary depending of the time of the day of administration. Therefore, the time of administration of those compounds should be evaluated to maximize the treatment's efficiency.

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DECLARATION OF INTEREST

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FIGURE LEGENDS

Table 1. Primer sequences used for real-time PCR.

Table 2. Acrophase, mesor and amplitude values calculated with Cosinor analysis. Data are indicated only for parameters that showed a significant daily rhythm (Cosinor, $p<0.05$). Data are expressed as value ± fiducial limits (set at 95%). The acrophase is

indicated in ZT. Values of mesor and amplitude correspond to relative expression. * $p<0.05$; ** $p>0.005$.

Figure 1. Daily variations of relative expression of *ghr1* (A), *ghr2* (B), *igf2r* (C) and *igf1* (D) in the liver of tilapia fed in the middle of the light phase (ML). Only parameters that had statistically significant daily rhythmicity or significant differences between times of the day have been plotted. The dashed curve represents the cosine function calculated from a significant Cosinor analysis ($p<0.05$). Different letters indicate statistically significant differences between time points (ANOVA, $p<0.05$). White and black bars above the graphs indicate the light and dark periods, respectively, of the LD cycle. Feeding time is indicated by the black arrows.

Figure 2. Daily variations of relative expression of *ghr1* (A), *ghr2* (B) and *igf1ra* (C) in the muscle of tilapia fed either in the middle of the light phase (ML) (white squares) or in the middle of the dark phase (MD) (black circles). Only parameters that had statistically significant daily rhythmicity or significant differences between times of the day have been plotted. The dashed and dotted curves represent the cosine functions calculated from a significant Cosinor analysis ($p<0.05$) for the ML and MD feeding groups, respectively. Different letters indicate statistically significant differences between time points (ANOVA, $p<0.05$), upper-case and lower-case letters indicate significant differences between points of ML and MD groups, respectively. The white and black bars above the graphs indicate the light and dark periods, respectively, of the LD cycle. Feeding time is indicated by the black arrows.

Figure 3. Effects of GH administration on pituitary *gh* (A) and hypothalamic *pacap1b* (B) relative expression in tilapia. The influence of feeding time and the time of day of injection on the response to exogenous GH was evaluated. GH was injected into different animals from two groups that were fed at different times: mid-light (ML) and mid-dark (MD). GH was administered to these groups at two different time points: ZT 3 h (3 h after lights on) and ZT 15 h (3 h after lights off). Samples were collected 10 hours after GH administration. Data (mean ± S.E.M., n = 6) are represented as the variation with respect to the mean value from a control group injected with a vehicle (VEH) and sampled at the same times. Data from each variable were subjected to Student's t-test to check for differences between GH and VEH values at one time point

690 of injection and to one-way ANOVA to check for differences between the GH-injected
691 groups. Asterisks indicate significant differences between the GH and the VEH groups
692 (t-test, $p<0.05$); different letters indicate significant differences between GH-injected
693 groups (ANOVA, $p<0.05$).
694
695 **Figure 4.** Effects of GH administration on liver *igf1* (A), *igf2a* (B), *ghr1* (C) and *ghr2*
696 (D) relative expression in tilapia. The influence of feeding time and the time of day of
697 injection on the response to exogenous GH was evaluated. GH was injected into
698 different animals from two groups that were fed at different times: mid-light (ML) and
699 mid-dark (MD). GH was administered to these groups at two different time points: ZT 3
700 h (3 h after lights on) and ZT 15 h (3 h after lights off). Samples were collected 10
701 hours after GH administration. Data (mean ± S.E.M., n = 6) are represented as the
702 variation with respect to the mean value from a control group injected with a vehicle
703 (VEH) and sampled at the same times. Data from each variable were subjected to
704 Student's t-test to check for differences between GH and VEH values at one time point
705 of injection and to one-way ANOVA to check for differences between GH-injected
706 groups. Asterisks indicate significant differences between the GH and the VEH groups
707 (t-test, $p<0.05$); different letter indicate significant differences between GH-injected
708 groups (ANOVA, $p<0.05$).
709
710 **Figure 5.** Effects of GH administration on muscle *ghr1* (A) and *ghr2* (B) relative
711 expression in tilapia. The influence of feeding time and the time of day of injection on
712 the response to exogenous GH was evaluated. GH was injected into different animals
713 from two groups that were fed at different times: mid-light (ML) and mid-dark (MD).
714 GH was administered to these groups at two different time points: ZT 3 h (3 h after
715 lights on) and ZT 15 h (3 h after lights off). Samples were collected 10 hours after GH
716 administration. Data (mean ± S.E.M., n = 6) are represented as the variation with
717 respect to the mean value from a control group injected with a vehicle (VEH) and
718 sampled at the same times. Data from each variable were subjected to Student's t-test to
719 check for differences between GH and VEH values at one time point of injection and to
720 one-way ANOVA to check for differences between GH-injected groups. Asterisks
721 indicate significant differences between the GH and the VEH groups (t-test, $p<0.05$);
722 different letters indicate significant differences between GH-injected groups (ANOVA,
723 $p<0.05$).

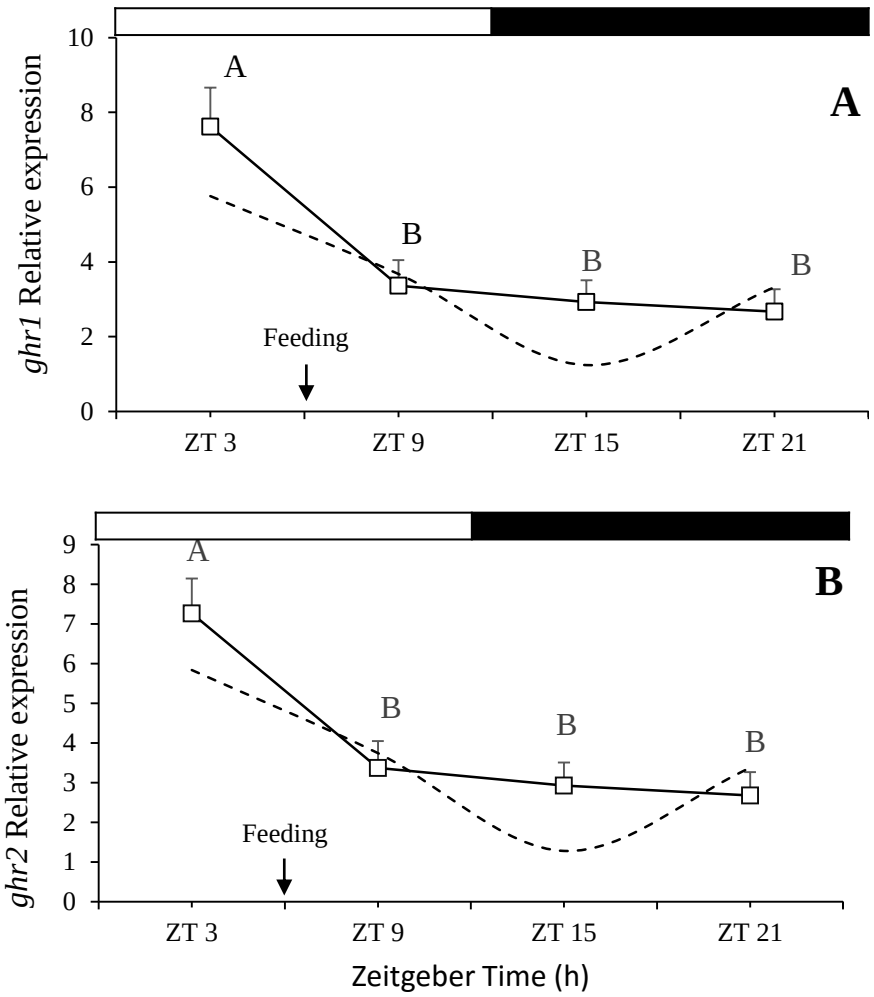
Table 1. Primer sequences used for real-time PCR.

Gene	Ensembl number	F/R	Primer Sequence (5´- 3´)
pacap1a	ENSONIG00000006092	F	TACAGCCGCTACAGAAAGCA
		R	GTTTCAGCCATTCTCCCAAA
pacap1b	ENSONIG00000009205	F	TAAACGACGACGCATACACC
		R	GTATTTCTGCACGGCCATCT
gh	ENSONIG00000009191	F	GCAACGTCAGCTCAACAAAA
		R	ACAGCCTTGGTGAAATCTGG
ghr1	ENSONIG00000015182	F	TATCAAGGGACCAGGAGACG
		R	TTGTTTTGAGTGCGAAGCTG
ghr2	ENSONIG00000012787	F	CTAGCTGTGCTTCCCCAGAC
		R	GTCCAGATCGAGGTGTGGTT
igf1	ENSONIG00000017800	F	TCCTGTAGCCACACCCTCTC
		R	ACAGCTTTGGAAGCAGCACT
igf2b	ENSONIG00000014499	F	AGTGATGCCCGCACTAAAAC
		R	TCCGCGTGCCTCTTATACTT
igf1ra	ENSONIG00000015115	F	TTTTGCCCAACGGTAATCTC
		R	CTTGGTGGGCTTTGTGTTTT
igf2r	ENSONIG00000015757	F	CGGCATCCTCCAACTAACAT
		R	AGCGGTGGAGAACTCAAAGA

Table 2. Acrophase, mesor and amplitude values calculated with Cosinor analysis. Data are indicated only for parameters that showed a significant daily rhythm (Cosinor, $p<0.05$). Data are expressed as value ± fiducial limits (set at 95 %). The acrophase is indicated in ZT. Values of mesor and amplitude correspond to relative expression.
* $p<0.05$; ** $p>0.005$

Tissue	Gene	Feeding Time	Significance	Acrophase (ZT)	Amplitude (r.e.)	Mesor (r.e.)
Liver	*ghr1*	ML	**	3:30 ± 2:58	2.27 ± 1.7	3.50 ± 0.98
	ghr2	ML	**	3:31 ± 2:56	2.19 ± 1.55	3.46 ± 0.84
	igf2r	ML	*	7:38 ± 4:40	0.64 ± 0.6	1.99 ± 0.35
Muscle	*ghr1*	ML	**	5:29 ± 2:07	2.64 ± 1.38	3.55 ± 0.77
	ghr1	MD	*	7:14 ± 4:37	6.13 ± 5.41	6.59 ± 3.15
	ghr2	ML	*	9:23 ± 4:25	14.48 ± 13.25	18.75 ± 7.37

Fig. 1


A
B
B
B
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
ghr1 Relative expression
A
B
B
B
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
ghr2 Relative expression
Zeitgeber Time (h)

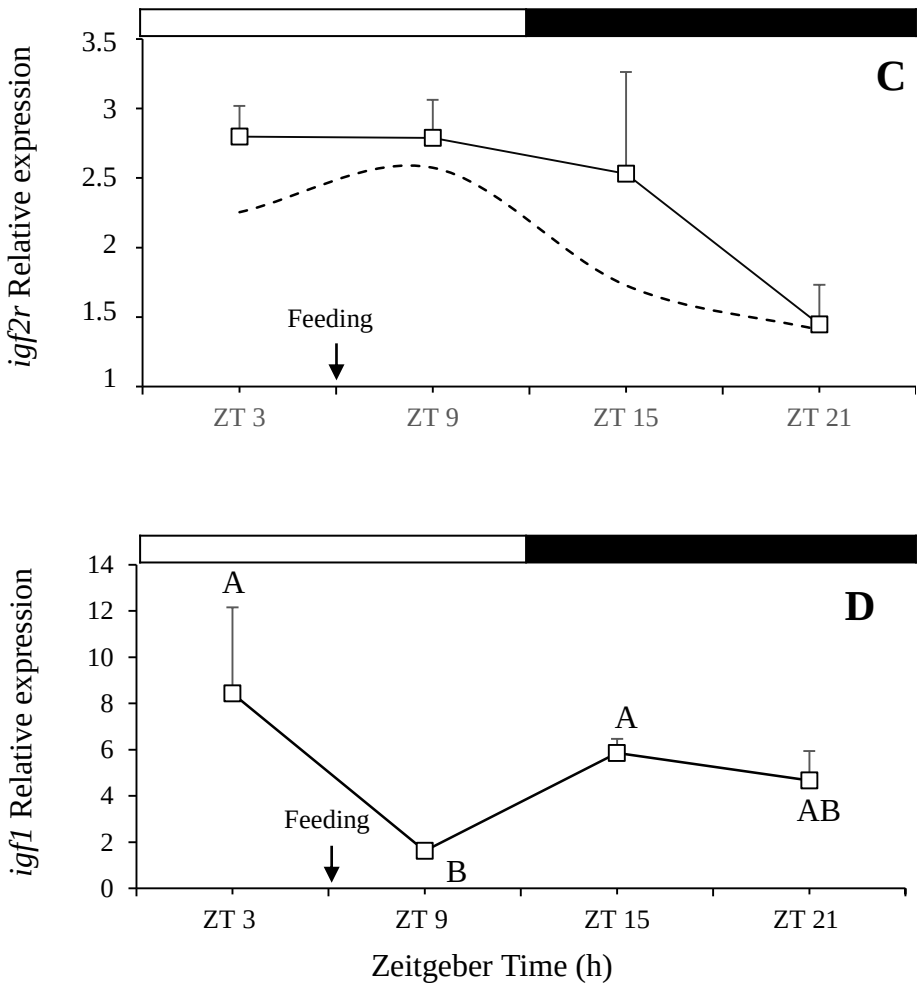

C
igf2r Relative expression
3.5
3
2.5
2
1.5
1
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
D
igf1 Relative expression
14
12
10
8
6
4
2
0
A
A
AB
B
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
Zeitgeber Time (h)

Fig. 2

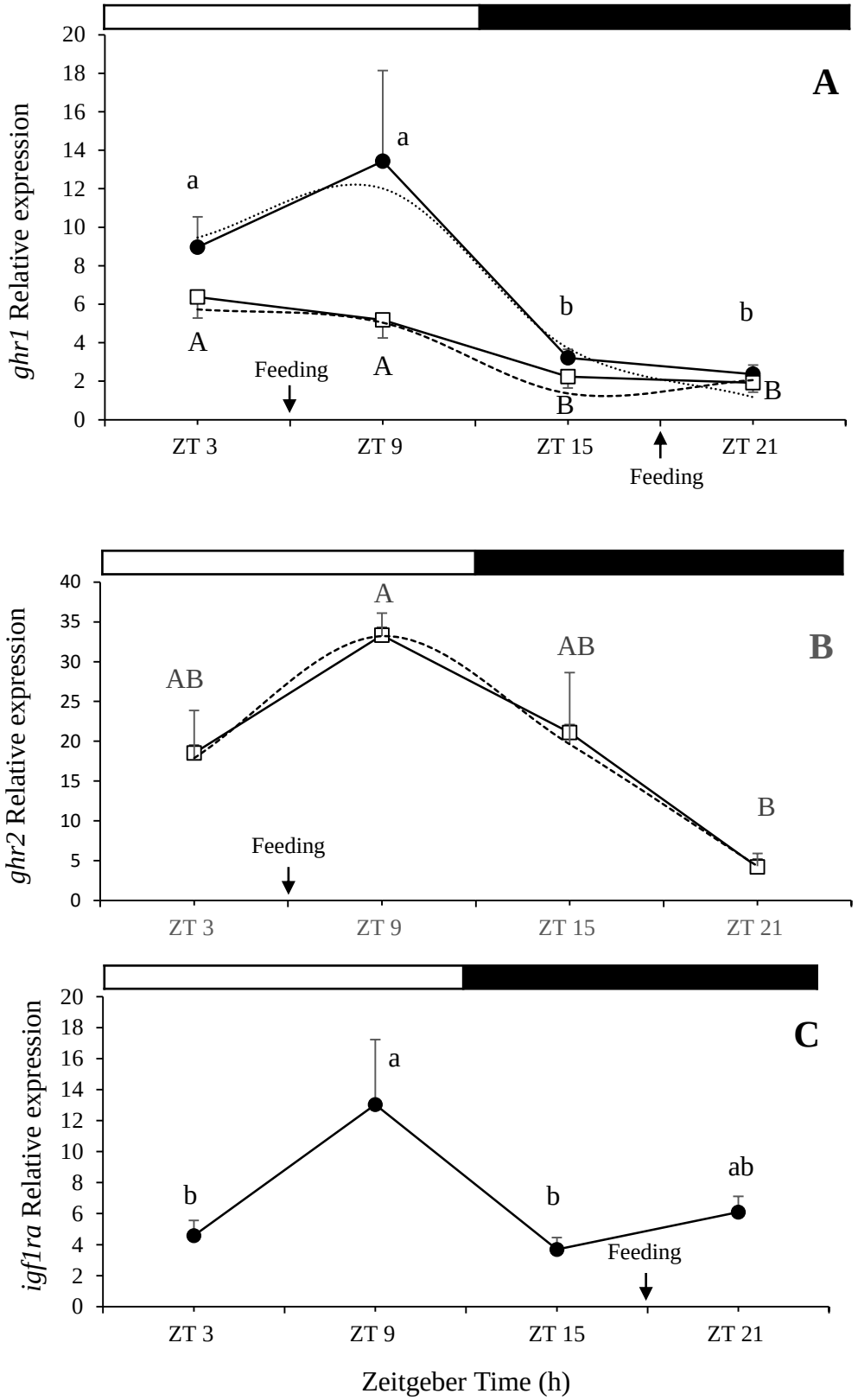

A
ghr1 Relative expression
20
18
16
14
12
10
8
6
4
2
0
a
a
b
b
A
A
B
B
Feeding
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
B
ghr2 Relative expression
40
35
30
25
20
15
10
5
0
A
AB
AB
B
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
C
igf1ra Relative expression
20
18
16
14
12
10
8
6
4
2
0
a
b
b
ab
Feeding
ZT 3
ZT 9
ZT 15
ZT 21
Zeitgeber Time (h)

Fig. 3

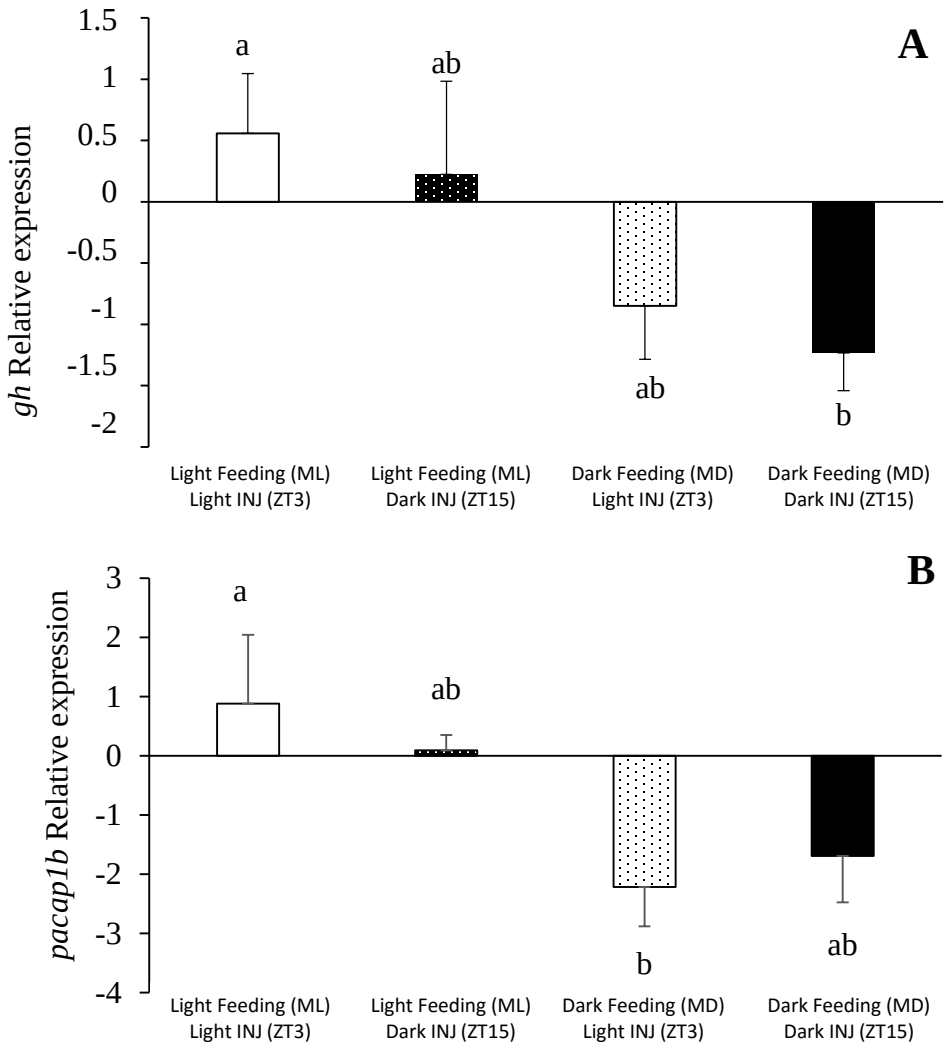
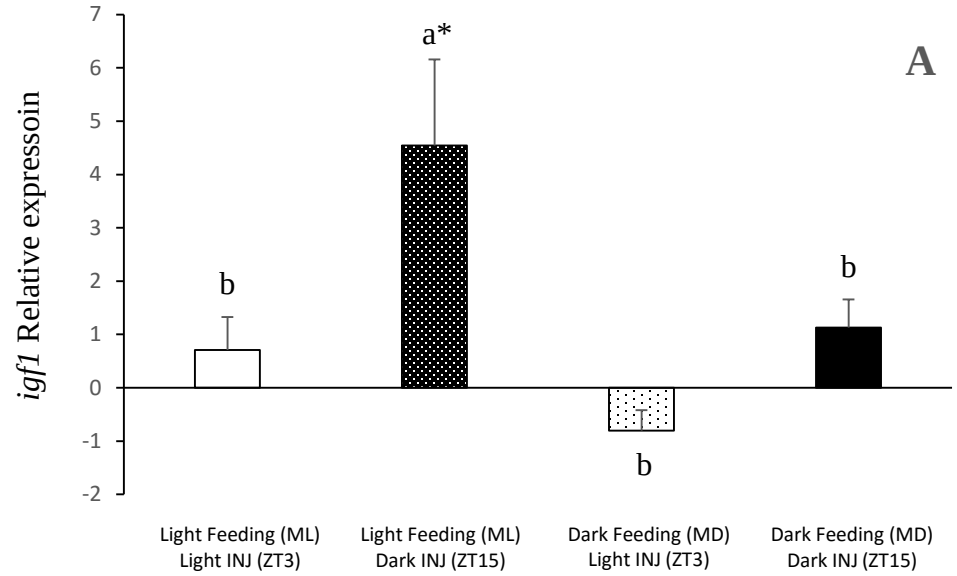
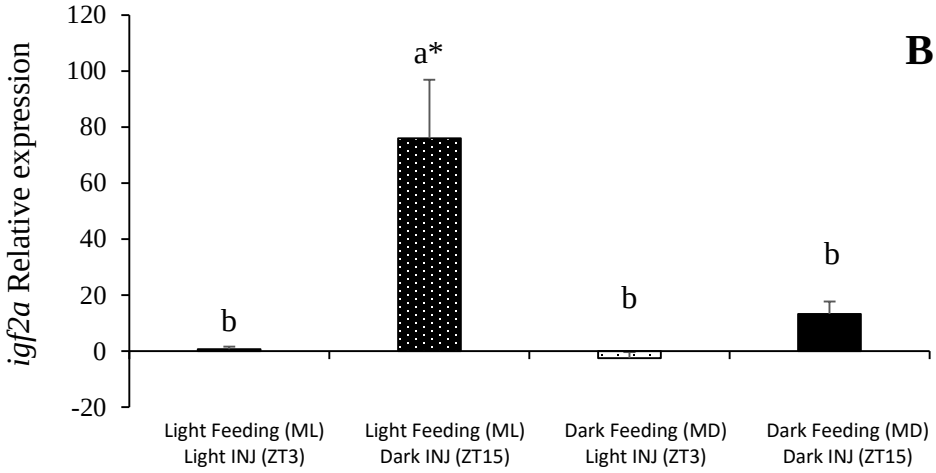
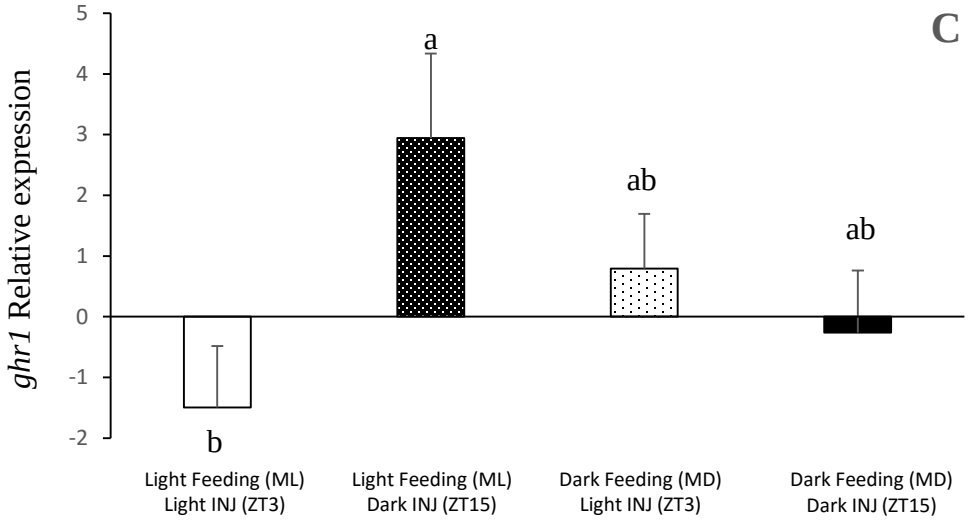

A
gh Relative expression
1.5
1
0.5
0
-0.5
-1
-1.5
-2
a
ab
ab
b
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)
B
pacap1b Relative expression
3
2
1
0
-1
-2
-3
-4
a
ab
b
ab
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)

Fig.4


A
igf1 Relative expressoin
7
6
5
4
3
2
1
0
-1
-2
a*
b
b
b
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)


B
igf2a Relative expression
120
100
80
60
40
20
0
-20
a*
b
b
b
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)


C
ghr1 Relative expression
5
4
3
2
1
0
-1
-2
a
ab
ab
b
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)

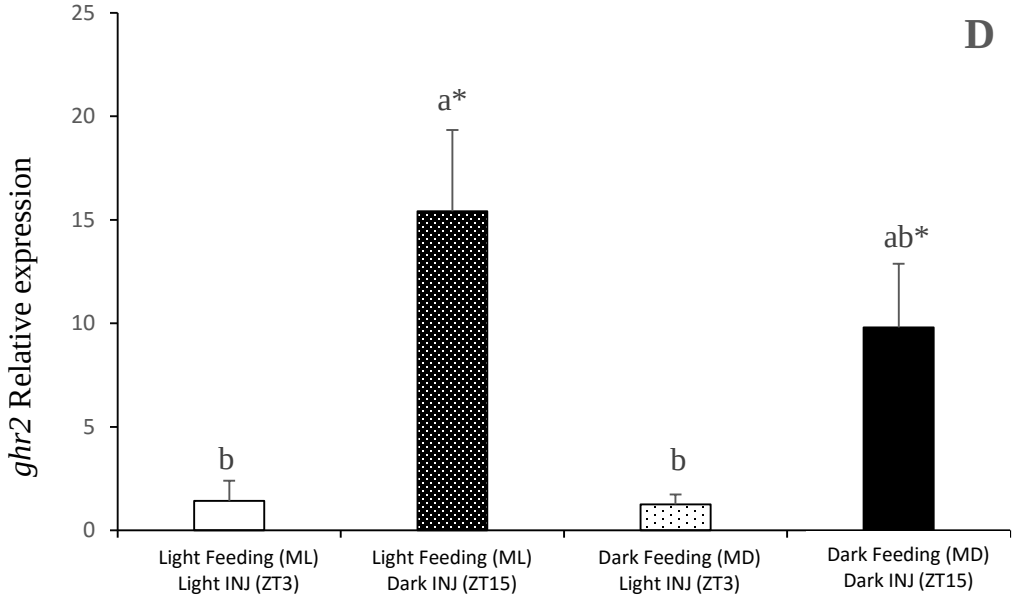
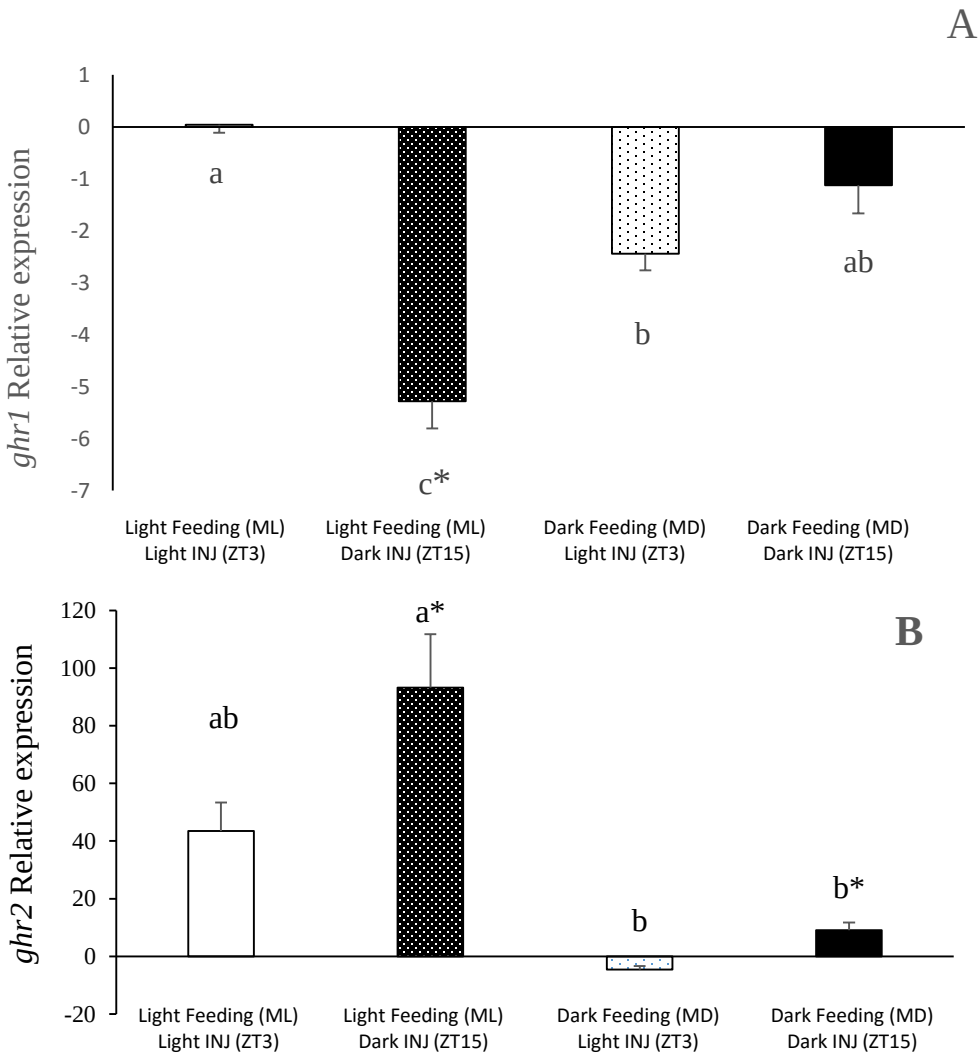

D
ghr2 Relative expression
25
20
15
10
5
0
a*
ab*
b
b
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)

Fig. 5


A
ghr1 Relative expression
1
0
-1
-2
-3
-4
-5
-6
-7
a
c*
b
ab
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)
B
ghr2 Relative expression
120
100
80
60
40
20
0
-20
ab
a*
b
b*
Light Feeding (ML) Light INJ (ZT3)
Light Feeding (ML) Dark INJ (ZT15)
Dark Feeding (MD) Light INJ (ZT3)
Dark Feeding (MD) Dark INJ (ZT15)