Full-length paper - CBP MS27590 Part A R.1

# Rhythmicity and plasticity of digestive physiology in a euryhaline teleost fish, permit (*Trachinotus falcatus*)

Carlo C. Lazado[a,b*], Per Bovbjerg Pedersen[a], Quang Huy Nguyen[c], Ivar Lund[a]

[a] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, DK-9850 Hirtshals, Denmark

[b] Nofima, The Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway

[c] Research Institute for Aquaculture No.1 (RIA1), Ministry of Agriculture and Rural Development, Dinh Bang – Tu Son – Bac Ninh – Vietnam

*Corresponding author:
C.C. Lazado
Tel: + 47 64970114
E-mail: carlolazado@yahoo.com | carlo.lazado@nofima.no

**Abstract**

Digestive physiology is considered to be under circadian control, but there is little evidence in teleost fish. The present study explored the rhythmicity and plasticity to feeding schedules of enzymatic digestion in a candidate aquaculture fish, the permit (*Trachinotus falcatus*). The first experiment identified the rhythms of digestive factors throughout the light-dark (LD) cycle. Gastric luminal pH and pepsin activity showed significant daily variation albeit not rhythmic. These dynamic changes were likewise observed in several digestive enzymes, in which the activities of intestinal protease, chymotrypsin and lipase exhibited significant daily rhythms. In the second experiment, the existence of feed anticipatory activity in the digestive factors was investigated by subjecting the fish to either periodic or random feeding. Anticipatory gastric acidification prior to feeding was identified in periodically fed fish. However, pepsin activity did not exhibit such anticipation but a substantial postprandial increase was observed. Intestinal protease, leucine aminopeptidase and lipase anticipated periodic mealtime with elevated enzymatic activities. Plasma melatonin and cortisol demonstrated robust daily rhythms but feeding time manipulations revealed no significant impact. Plasma ghrelin level remained constant during the LD cycle and appeared to be unaffected by differing feeding regimes as well. Taken together, the digestive factors of permit were highly dynamic during the LD cycle. Periodic feeding entrained digestive physiology and mediated anticipatory gastric acidification and intestinal enzymatic activities. This knowledge will be essential in developing feeding protocols and husbandry-related welfare strategies that will further advance this candidate finfish as an aquaculture species.

**Keywords:** aquaculture, circadian rhythm, digestive enzyme, fish, food anticipatory activity

## 1. Introduction

Biological rhythms enable almost all life forms to adapt to dynamic and periodic changes in the environment. This evolutionarily conserved mechanism regulates the rhythms of physiology and behavior, providing the organism a significant adaptive advantage by scheduling fundamental biological processes to occur at optimal times of the daily or annual cycle (Vaze and Sharma, 2013; Yerushalmi and Green, 2009). Two of the most important environmental cues that entrain biological rhythms in animals include the light–dark (LD) and feeding cycles (López-Olmeda et al., 2009; Montoya et al., 2010a). The entrainment is mediated either by a light-entrainable oscillator (LEO) or by feeding-entrainable oscillator (FEO). In fish, feeding behavior is primarily driven by FEO which appears to be coupled to the LEO (Sánchez-Vázquez and Madrid, 2001).

The rhythmicity of digestive physiology has been widely described in mammalian models. The secretions and activities of key enzymes in the digestion process have been documented to display daily rhythms (Asher and Sassone-Corsi, 2015; Bron and Furness, 2009; Glasbrenner et al., 1992; Keller and Layer, 2002; Maouyo et al., 1995). In fish, the rhythmic functions of digestive factors are barely explored. Knowledge on feeding rhythms is mostly based on behavioral observations and less attention has been directed to the underlying enzymatic mechanisms in the gastrointestinal (GI) tract. In Nile tilapia (*Oreochromis niloticus*), the activity of alkaline protease in the midgut showed daily rhythm with the acrophase at the beginning of the dark phase, but such a dynamic activity was not observed in acid protease and amylase (Guerra-Santos et al., 2017). Amylase displayed daily rhythm in European seabass (*Dicentrarchus labrax*) with dual (diurnal/nocturnal) feeding behavior (del Pozo et al., 2012). On the other hand, acid protease but not amylase activity in the gut of European eel (*Anguilla anguilla*) displayed significant daily oscillation (López-Olmeda et al., 2012a). Rhythmic digestive function likely participates in the homeostasis and adaptability of the gut during a daily cycle, ensuring that the breakdown, adsorption and eventual utilization of dietary components are optimized (Guerra-Santos et al., 2017; López-Olmeda et al., 2012b; Montoya et al., 2010b; Vera et al., 2007).

Most animals, including fish, have the ability to anticipate mealtime, particularly in instances where food availability is under restricted schedule. One of the defining features of food anticipatory activity (FAA) is an increase in locomotor activity hours prior to feed delivery (Sánchez-Vázquez and Madrid, 2001). Feed anticipation is biologically significant, especially in the wild, as it allows the animal to optimize the digestive and metabolic processes thereby concentrating feed intake in a short period to reduce the risk of predation. Most strikingly, physiological anticipation to scheduled feeding facilitates improved food acquisition and nutrient utilization as biochemical activation prepares the host for the forthcoming meal. The dynamics of GI anticipation is scarcely available in fish, though a few studies have indicated that digestive enzymatic process exhibits a high degree of plasticity towards feeding at a scheduled regime (Montoya et al., 2010b; Vera et al., 2007).

In this study, we investigated the daily rhythms and feeding plasticity of digestive physiology (*i.e.,* gastric acidification and intestinal enzymes) in a euryhaline teleost fish, the permit (*Trachinotus falcatus*). Since hormonal regulation is likely involved in the responses to these exogenous manipulations, the levels of key circadian-related hormones were also quantified. The model fish species is being developed as an aquaculture commodity in Asia, particularly in Vietnam. Knowledge on the circadian physiology of this fish species is important in developing husbandry protocols to optimize feed utilization and promote good animal welfare.

## 2. Materials and methods

### *2.1. Ethics statement*

All fish handling procedures in the study were in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

### *2.2. Experimental fish and rearing conditions*

Hatchery-produced permit fish (*Trachinotus falcatus*) juveniles were provided by the Aquaculture Research Sub-Institute in North Central (ARSINC), Research Institute for Aquaculture No. 1, Cua Lo, Nghe An, Viet Nam and shipped by air cargo. They were quarantined for 2 weeks following their arrival at the DTU Aqua facility in Hirtshals, Denmark. Thereafter, the fish were transferred to fiberglass holding tanks in a flow-through system. During an ongrowing period, the husbandry conditions were as follows: water temperature 27-28°C, dissolved oxygen levels above 80 % saturation; average salinity 33 g $L^{-1}$; pH 7.3 – 7.4, constant illumination (average water surface light intensity of 150 lux) and one daily ration of a high-protein commercial diet (EFICO Sigma 870, BIOMAR, Denmark).

### *2.3. Experiment 1: Rhythmicity of digestive physiology*

Apparently healthy fish with an average weight of 130±15 g (mean±SD) were selected and stocked to each of ten 189-L, cylindrical-conical, thermoplastic tanks in a recirculation system at a density of 8 fish per tank. To ensure minimal disturbance and potential stress during sample collection, two tanks were exclusively dedicated to a single sampling point. It was previously observed that this fish displayed burst swimming activity (Lund et al., unpublished), thus, black plastic was used to cover the tank to minimize tank wall collisions. Water temperature was controlled at 28$^{o}$C and dissolved oxygen levels were above 80% saturation. Seawater (average salinity: 33 ppt) flow rate in each tank was 40 L $h^{-1}$. White LED light with a maximum water surface intensity of 350 lux was provided in each tank and the photoperiod was set at 12L:12D with lights on at 07:00 AM (*Zeitgeber* Time, ZT, 0). A high protein diet (EFICO Sigma 870) was delivered once daily (09:00 AM, ZT2) at a ration corresponding to 2% (w/w)

of initial total body weight by an automated rotary feeder. Fish were subjected to these conditions for 15 days. No mortality was recorded over the 2-week period.

### *2.4. Experiment 2: Feeding plasticity of digestive physiology*

Fish with an initial average weight of 155±10 g (mean±SD) were divided into two groups: one group was fed in a periodic scheme while the second group was subjected to random feeding. Each treatment group included 6 tanks, each of which was stocked with 8 fish. Rotary feeders equipped with a timer were employed to deliver a commercial diet (EFICO Sigma 870) with a single daily ration of 2% (w/w) body weight, adjusted according to expected biomass in that particular period. The periodically fed group received the diet at 09:00 AM (ZT2) every day. Dietary provision in the other group was delivered in 3-4 portions at random times of the day. Both groups were subjected to a 24L:0D (LL) photoperiod cycle for 6 weeks. Tank specifications and water quality parameters were similar to those described in Section 2.3. To avoid the effect of different feeding times on the day of sampling, both groups received the diet at 09:00 AM.

### *2.5. Sampling strategies*

For experiment 1, fish were fasted for one day and sampling was carried out at a 6-h (ZT 0, 6, 12, 18 and 24) intervals over a period of 24 h. Samples taken at ZT0 were collected immediately after the light reached its maximal intensity (350 lux), while those at ZT24 were collected just before the transition to the light phase. ZT12 samples were collected during the transition period. Six fish, 3 from each representative tank, were taken at every sampling point and euthanized with an overdose of ethylene glycol-monophenyl ether (Merck, Darmstadt, Germany). Sampling during the dark phase was conducted in a room with illumination not exceeding 3 lux and exposure of anesthetized fish to this lighting condition was no longer than 5 min. For experiment 2, sampling took place, 8 h (− 8 h) and 2 h (− 2 h) before food delivery, and at 4 h (+ 4 h) post-feeding. Six fish were collected, 3 from each of the two tanks exclusively dedicated to a particular sampling point.

Blood was withdrawn from the caudal vein using a 2 ml heparinized syringe fitted with a 21-G needle. The tubes with blood samples were centrifuged for 5 min at 3,000 rpm and plasma was carefully pipetted out, aliquoted and stored at -80°C until analysis. Thereafter, the intestinal tract was dissected out. The collected tissue was placed on an aluminum foil and immediately snap-frozen in liquid nitrogen. Samples were stored at −80 °C until tissue extraction. Luminal gastric pH was measured according to the previously published protocol (Yúfera et al., 2012). Thereafter, the stomach was removed, placed in liquid nitrogen and stored at −80°C until analysis. Skeletal muscle from the dorsal region was dissected and washed with cold 5% ethanol before placing in liquid nitrogen and eventually stored at −80°C.

### *2.6. Preparation of intestinal and gastric tissue extracts*

Intestinal and gastric homogenates were prepared following a previously published protocol for fish (Lazado et al., 2012), with minor modifications. All steps involved in the preparation of tissue extracts were performed at 4°C. Cold, sterile 1 × phosphate buffered saline (PBS, pH 7.4) was used as the homogenization diluent. Tissues were mixed (ratio 1:3 for intestine and 1:2 for stomach) with PBS and homogenized in an Ultra-Turrax® tissue grinder (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 5 min. The tissue homogenate was centrifuged for 30 min at 10,000 rpm. Thereafter, the resulting supernatant was filtered (Millex-GV unit 0.22 µm pore size, Millipore), aliquoted and stored at −80°C until analysis. Soluble protein content in the tissue extracts was determined using bovine serum albumin as a standard (Thermoscientific, Illinois, USA).

### *2.7. Digestive enzyme assays*

Activities of digestive enzymes in the tissue extracts were determined following standard spectrophotometric-based enzyme assay protocols. Protease activity was quantified using casein as substrate (Walter, 1984). One unit of protease activity was defined as the amount of enzyme able to hydrolyze casein to produce color equivalent to 1.0 µmole of tyrosine per minute. L-Leucinamide hydrolysis was

used to quantify leucine aminopeptidase activity (Mitz and Schlueter, 1958). One unit of leucine aminopeptidase activity was defined as the amount of enzyme able to hydrolyze 1.0 µmole of L-leucine *p*-nitroanilide to L-leucine and *p*-nitroaniline per minute. Cellulase activity was determined by its effect on microcrystalline cellulose with respect to glucose formation (Worthington, 1988). One unit of cellulase activity was defined as the release of 0.01 mg glucose per hour from micro-crystalline cellulose. Chymotrypsin was analyzed using N-Benzoyl-L-tyrosine ethyl ester (BTEE) as substrate (Wirnt and Bergmeyer, 1974). One unit chymotrypsin activity was defined as the amount of enzyme able to hydrolyze 1.0 µmole of BTEE per minute. Lipase activity was determined using a commercial kit (Sigma) based on a coupled enzyme reaction. One unit of lipase activity was defined as the amount of enzyme able to generate 1.0 µmole of glycerol from triglycerides per minute.

### *2.8. Gastric pepsin activity*

Pepsin activity was based on the stop-point assay of hemoglobin degradation developed by Anson (Anson, 1938). One unit of pepsin activity was defined as 1 µg of tyrosine released per minute. Assays were performed at standard pH 2 and at the actual luminal gastric pH that was experimentally determined at each sampling point (Section 2.5).

### *2.9. Quantification of plasma hormones*

Plasma hormones were quantified by commercially available EIA/ELISA kits: melatonin (IBL, Hamburg, Germany), cortisol (Neogen, Kentucky, USA) and ghrelin (BertinPharma, Montigny-le-Bretonneux, France).

### *2.10. Proximate analyses*

Dry matter, crude protein, crude lipid and ash of the skeletal white muscle were determined following the procedures of the Association of Official Analytical Chemists (AOAC, 2005). Dry matter was determined after oven drying for 24 h at 105 °C (Memmert UN110). Ash contents were calculated from the weight loss after

incineration of the samples for 6 h at 550 °C in a muffle furnace (Hareaus Instruments K1252). Crude protein levels were determined from the Kjeldahl method (Foss Kjeltec 2200) and crude lipid by the method of Bligh and Dyer (1959).

### *2.11. Statistical analyses*

Significant differences in the daily activity of the studied parameters were analyzed with the SigmaStat statistical package (Systat Software, London, UK). A one-way ANOVA was performed on data sets that passed the tests of normality and equal variance, and Tukey's multiple comparison test followed to delineate differences between time points. For data sets that did not follow a Gaussian distribution or did not meet the equal variance requirements, Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparison test were used instead. The level of significance was set at $P < 0.05$.

COSINOR was employed to determine the parameters defining the rhythmicity and the significance of daily oscillation. Analysis was performed by fitting a periodic sinusoidal function to the activity values of a studied factor across the five ZTs, using the formula: $f(t) = M + A\cos(t/pi/12 - \phi)$, where $f(t)$ is the level of the parameter at given time, mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, $t$ is time in hours and $\varphi$ is the acrophase. For a studied parameter to be characterized as exhibiting a significant daily rhythmicity, it had to pass the level of significance set for both ANOVA ($P < 0.05$) and COSINOR ($p < 0.05$) (Lazado et al., 2015).

## 3. Results

### *3.1. Daily dynamics in gastric luminal pH and pepsin activity*

The pH of the stomach lumen (**Fig. 1A**) showed significant variations during the LD cycle, though these changes were revealed to be not rhythmic by COSINOR. Gastric pH was relatively acidic from mid-light to mid-dark phase. The lowest gastric pH of around 3.94±0.45 (mean±SE) was registered at ZT12.

When analyzed at standard assay pH of 2, pepsin activity did not exhibit significant changes during the LD cycle (**Fig. 1B**, **Table 1**). However, significant differences were identified when the actual gastric pH was used during the assay. Pepsin activity significantly increased by no less than 77% from ZT0 to ZT6. Thereafter, constantly elevated level of pepsin activity until ZT18 was observed. Lower pepsin activity was observed at the beginning of the light phase and at the end of the dark phase.

### *3.2. Daily rhythms of intestinal enzyme activities*

Intestinal protease, leucine aminopeptidase, chymotrypsin and lipase activities displayed significant variations during the LD cycle (**Fig. 2A-F**). COSINOR analysis further revealed that the activities of protease, chymotrypsin and lipase exhibited significant daily rhythms (**Table 1**). Leucine aminopeptidase activity was not rhythmic despite showing a significant temporal variation with ANOVA. Intestinal amylase and cellulase activities did not display significant temporal differences in both statistical algorithms.

The acrophases of intestinal enzymes differed remarkably during the LD cycle (**Table 1**). Protease (ZT 20.2) and amylase (ZT 21.1) displayed peak of activity during the late hours of the dark phase whereas acrophases of leucine aminopeptidase (ZT 17.1) and lipase (ZT 15.2) were found to be in the early hours of the same phase. The peak of activity of cellulase (ZT 3.24) and chymotrypsin (ZT 5.42) was identified in the early hours of the light phase.

### *3.3. Plasticity of digestive physiology in the stomach and intestine*

Prior to feed delivery, gastric pH in periodically fed fish demonstrated a decreasing trend (**Fig. 3A**). From pH 5.6 at 8 h before mealtime, pH significantly dropped to 4.6 at 2 h before mealtime in this group. Such an anticipatory gastric acidification was not observed in the randomly fed group. Post-prandial acidification was observed in both groups with gastric luminal pH of around 4, hours after feeding.

No significant changes were observed in the gastric pepsin activity when assayed at pH 2 before and after feed delivery in both groups (**Fig. 3B**). When analysis was performed at the actual luminal pH, no significant pre-prandial changes were observed, though an increasing pattern was quite apparent in both groups. Pepsin activity increased substantially 4 h post feeding and was significantly different compared with the values measured at 8 h but not at 2 h prior to feed delivery in both groups.

Anticipatory secretions of several enzymes were demonstrated in fish subjected to periodic feeding scheme (**Fig. 4**). There was a significant increase in the activity of protease, leucine aminopeptidase and lipase 2 hours prior to feed delivery in fish subjected to periodic feeding (**Fig. 4A,B,F**). From 8 h to 2 h before mealtime, the activity increased by 12.5% in protease, 41.6% in leucine aminopeptidase and a remarkable 136% in lipase. Such anticipatory increments were not observed in fish subjected to random feeding. A significant post-prandial (+4 h after mealtime) increase was observed in leucine aminopeptidase (**Fig. 4B**) and chymotrypsin (**Fig. 4E**) in fish under periodic feeding. Similar post-prandial increase was identified in amylase (**Fig. 4C**) and lipase (**Fig. 4F**) activities of randomly fed fish. Only protease in periodically fed group displayed a significant post-prandial decrease with approximately 6.3% activity reduction relative to the activity at 2 h before feeding.

Further, the impact of feeding schemes on the level of intestinal enzyme activity at a specific time point was explored. At 2 hours before feed delivery, the activities of protease, leucine aminopeptidase and chymotrypsin were significantly lower in randomly fed group than the group under periodic feeding. For instance, chymotrypsin activity in randomly fed group was around 60% lower than in periodically fed fish at 2 h before mealtime. Two apparent patterns were observed in post-prandial responses. Activities of amylase and lipase were significantly higher by 72% and 131%, respectively, in randomly fed fish than in periodically fed group at 4 h after feeding. On the other hand, leucine aminopeptidase and chymotrypsin activities were significantly lower in randomly fed than in periodically fed group at 4 h after feeding.

### *3.4. Daily rhythms of plasma melatonin and cortisol*

The plasma levels of melatonin and cortisol showed significant temporal variations and these changes likewise displayed significant daily rhythms (**Fig. 5**, **Table 2**). Plasma melatonin level decreased progressively from the start of the light phase until the mid-light phase (**Fig. 5A**). Thereafter, the level began to increase gradually until the mid-dark phase. The acrophase was registered at ZT 19.4. Plasma cortisol was highest during the mid-light phase (**Fig. 5B**) where the acrophase was identified at ZT 5.5. From the light-dark transition to the end of the dark phase, the level of plasma cortisol remained constant.

### *3.5. Responses of plasma melatonin and cortisol to different feeding regimes*

Plasma melatonin level before and after feeding did not show significant changes regardless of the feeding schedules (**Fig. 6A**). A similar observation was identified in plasma cortisol (**Fig. 6B**). Nonetheless, the level of plasma cortisol in randomly fed fish was almost two-fold higher compared with the fish under periodic feeding regardless of the sampling point.

### *3.6. Unaffected plasma ghrelin levels*

Plasma ghrelin showed no significant variations during the LD cycle both in ANOVA and in COSINOR (**Table 2**), though a decreasing tendency was observed as light-dark transition approached (**Fig. 7A**). There were no significant pre-prandial changes in plasma ghrelin regardless of the feeding schedules. Both groups displayed a seeming decrease in the level of plasma ghrelin after feeding, however, the changes were not considered to be statistically significant. At 2 h before feed delivery, plasma ghrelin in periodically fed group was around 60% higher than for the fish under random feeding.

### *3.7. Growth performance and biochemical composition of fish subjected to periodic and random feeding*

There was no mortality during the 6-week feeding trial. In addition, there were no significant differences in the % weight gain (periodic: 72.6%, random: 60.4%) and specific growth rate (SGR; periodic: 1.35±0.10, random: 1.17±0.09) between periodically and randomly fed fish. The biochemical composition of the white muscle was similar in both groups (**Supplementary Table 1**).

## 4. Discussion

Light-dark and feeding cycles have pervasive influence in the physiology of fish, including the fundamental functions of digestion. In the present study, digestive physiology characterized by gastric and intestinal enzyme dynamics has been demonstrated to exhibit robust rhythm during the LD cycle. In addition, its plasticity has been highlighted by the capability of periodic feeding to entrain the digestive factors, allowing them to prepare the gastrointestinal tract for the forthcoming meal by performing anticipatory secretions. It was likewise shown that circadian-related hormones, including melatonin and cortisol demonstrated daily rhythms. The trends are in agreement with the known functions of these hormones in the mediation of physiological rhythms in fish.

One model of gastric acidification in teleost suggests that there is a continuous acidic secretion and low pH is maintained during feeding and fasting. Another model indicates that neutral pH is maintained during fasting and hydrochloric acid is only released following ingestion of the meal (Yúfera et al., 2012). Our results revealed that permit followed the gastric acidification process described by the latter model during the LD cycle (**Fig. 1**). This postprandial pattern of abrupt decrease in gastric pH has been earlier described in other teleost fish (Nikolopoulou et al., 2011; Yúfera et al., 2004). In this study, it was observed that the gastric pH dropped to around 4 and maintained this level for at least 10 hours post feeding. Although the observed changes were not rhythmic, the temporal dynamics suggest that gastric acidification was probably influenced by the LD cycle as well. Pepsin is the predominant gastric enzymes in teleost fish. It is synthesized and secreted in the gastric membrane in an inactive state called pepsinogen, but exposure to the

hydrochloric acid auto-catalytically activates it to pepsin (Raufman, 2004). Yúfera and colleagues (Yúfera et al., 2012) earlier raised the concerns about that performing the pepsin assay on fish samples using the standard pH 2 and not the actual luminal pH. The present study corroborated this concern as there were discrepancies in the results of pepsin activity at standard assay and at actual pH values. Gastric pepsin activity assayed at pH 2 appeared to be stable during the LD cycle but revealed a strong dynamic daily pattern when the actual luminal pH was employed. When the luminal pH was relatively neutral, the values obtained at standard pH 2 was at least two-fold higher than the value obtained at actual luminal pH. This indicates that assay pH had a profound catalytic impact in the gastric pepsin and should be considered in future studies involving the model fish.

The rhythmicity in protease, chymotrypsin and lipase activities clearly shows that intestinal enzymatic physiology was strongly influenced by the LD cycle. Proteolysis in the intestine of the model fish seems to be the most influenced enzymatic process by the LD cycle as two proteolytic enzymes (*i.e.,* protease and chymotrypsin) demonstrated robust daily rhythms. Our preliminary results indicate that permit juveniles have a high protein requirement, 49.1 % crude protein or 39.3 % digestible protein in diet (Nguyen et al., In Press) hence diet containing 54% crude protein (EFICO Sigma 870) was provided in the experiments. The high protein requirement necessitates a proteolytic system that is efficient in breakdown of large proteins into biologically active peptides. The observed rhythmic proteolytic activities implies an adaptive response by allowing the system to have a period when the activity is low, and a period when it is high and optimal ensuring a more effective proteolytic action. This feature is likely more beneficial compared to maintaining constant elevated levels which may pose higher metabolic cost. It is also interesting to note that the two rhythmic proteolytic enzymes displayed opposite acrophases: protease activity increased during the dark phase, chymotrypsin during the light phase. This poses the possibility that by having two proteolytic enzymes at their most active state at different times of the day, proteolysis would be maximized. There may be other proteolytic enzymes that may be involved in this process, but the distinctive daily

rhythms of these two factors offer an intriguing hypothesis that proteolysis in the intestine of the model fish may be under circadian clock control. The presence of clock components in fish gut lends support to this hypothesis (Lazado et al., 2014; Peyric et al., 2013; Velarde et al., 2009). Lipid metabolism is known to be under clock control and many associated metabolic factors exhibit circadian rhythm (Betancor et al., 2014; Gnocchi et al., 2015). While we were unable to affirmatively establish lipid metabolic rhythm-circadian clock relationship, the rhythmic activity of lipase observed in the present study implies a probable temporal control of lipid metabolism in the model fish. Some prospects to be explored in the future include the changes in lipid digestibility in relation to feeding time and/or the impact of the time spent in the digestive tract in the observed temporal variability. To date, there are only a few intestinal enzymes that have been identified with rhythmic activity in fish (del Pozo et al., 2012; Guerra-Santos et al., 2017; López-Olmeda et al., 2012a). The observations in the present study offer valuable insights into the rhythm of intestinal enzymes and how they play a part in the digestive metabolic process during the LD cycle in fish.

Many organisms including fish exhibit feed anticipatory activity before mealtime (Davidson et al., 2003), however, knowledge on how the digestive tract anticipates the forthcoming meal is poorly understood. There is some evidence when fish are fed at a single specific time of the day, GI enzymes increase their activity prior to feeding thereby increasing feed digestion and feed efficiency (Guerra-Santos et al., 2017; Montoya et al., 2010a; Vera et al., 2007). In the present study, this anticipatory mechanism characterized by a significant increase in enzymatic activities 2 h before delivery of scheduled meals was demonstrated in protease, leucine aminopeptidase, and lipase in fish provided with single periodic ration. These results indicate that intestinal enzymatic mechanism exhibit plasticity and can be entrained by recurring cycle of feed delivery. The anticipatory secretion suggests that periodic feeding acts as a potent *zeitgeber* in entraining the digestive physiological processes and correspondingly provides an indication that a food-entrainable oscillator may be present in the GI intestinal tract of the model fish. Besides the anticipatory activity in periodically fed fish, it was also observed that this fish group exhibited a significantly

higher enzymatic activity (*i.e.*, protease, leucine aminopeptidase and chymotrypsin) compared with their counterparts in randomly fed group, especially the pre-prandial levels. Feeding entrainment is not only important in allowing the system to prepare but at the same time may be beneficial in improving the enzymatic capacity for digestion, thus can be explored in strategies aiming at modulating the metabolic functions. Postprandial effects of periodic and random feeding were not very marked and the pattern of changes were too stochastic to draw a clear single deduction. This implies that the plasticity to scheduled feeding may have a stronger influence in the pre-prandial than in the post-prandial activities.

Melatonin is a major output of the vertebrates' circadian clocks and has a role in conveying the rhythmic information. The pineal organ produces melatonin at night, hence, the levels are high at night and low during the day (Falcón et al., 2010). Plasma melatonin of the model fish displayed this pattern, where acrophase was identified at ZT 19.4. Cortisol is closely related to the activity phase of the animal: peaking during early morning in diurnal animals, while the peak is during early evening in nocturnal animals (Dickmeis, 2009). *Trachinotus* spp. are diurnal fish species (Bellinger and Avault, 1971; Lazado et al., 2015), so the acrophase of cortisol at ZT 5.5 corresponds to the model organism's activity phase. Previous studies have shown that the levels of melatonin and cortisol are impacted by feed and feeding time (Kulczykowska and Sánchez Vázquez, 2010; López-Olmeda et al., 2009; Montoya et al., 2010a), and this relationship has been implicated in the feed entrainment mechanism. In the present study, temporal profiles in melatonin and cortisol levels appeared to be unaffected by periodic and random feeding. It is possible that these hormones may not be directly involved in the physiological responses to scheduled feeding in the model fish, which contradicts earlier observations in other fish species (Falcón et al., 2010; Montoya et al., 2010a; Vera et al., 2007). Nonetheless, the observed significantly elevated cortisol levels in the randomly fed group compared to the periodically fed group in all sampling points is salient and has been observed in previous studies (López-Olmeda et al., 2012b; Sánchez et al., 2009; Vera et al., 2007). One probable cause of elevated cortisol level is that when fish are fed randomly, their preying

behavior, an energy-demanding process, is always active. During this period, cortisol plays a role in the mobilization of energy reserves (*e.g.*, glucose) to cope up with the increased metabolic rate (Mommsen et al., 1999). At present, it could not be ascertained the extent of the impact of elevated cortisol level in the model fish, though, we could conjecture that it was not that pronounced as growth performance indicators remained the same in both groups.

Ghrelin, a hormone controlling food intake and metabolism (Jönsson, 2013), has been suggested to be an input to food entrainable oscillators (Nisembaum et al., 2014). Hence, we speculated that it may be involved as well in the FAA observed in the digestive physiology in the model fish to periodic feeding. Interestingly, plasma ghrelin did not show anticipatory activity and remained constant during the LD cycle. This observation indicates that neither LD cycle nor feeding time has a regulatory impact in the circulating ghrelin levels in the model fish, at least in the duration of the present study. Anticipatory activity of appetite regulation may be modulated by other hormones such as neuropeptide Y and orexin, which have not been explored in the present study.

We hypothesized that since periodically fed fish exhibited anticipatory secretion and elevated levels of intestinal enzymes, these adaptive features may have an impact in the growth performance and the biochemical composition of the skeletal muscle. No significant differences were observed between the two groups in weight gain, SGR and muscle biochemical composition. This suggests that though periodic feeding remarkably affected the enzymatic physiology in the GI tract, it may have less influence in the downstream consequences of metabolic process such as growth and tissue composition. Nonetheless, we could not eliminate the possibility that the feeding duration was not long enough to identify significant phenotypic changes. Other parameters (*e.g.* digestibility, ammonia excretion profiles) that may shed insights into the metabolic consequences of modulated intestinal enzymatic functions should be explored in future studies.

In conclusion, the present study describes for the first time the gastric and intestinal digestive physiology of permit, a candidate euryhaline finfish for

aquaculture. The digestive physiology of permit is greatly impacted by the LD cycle and feeding schedule, supporting the importance of these environmental cues in the physiological processes in fish. We have provided evidence that several intestinal enzymes exhibit robust rhythmicity and dynamic plasticity, which likely participate in providing the temporal homeostasis and adaptive nature of the digestive process. The enzymatic activity profile would aid in developing optimized diets that consider the metabolic capacities of the GI tract in this fish species. Successful fish domestication requires a better understanding of the underlying physiological mechanisms of fish to develop efficient husbandry protocols. Hence, the information presented here will lay both fundamental and practical knowledge that potentiate the prospect of permit as an aquaculture species of biological and economic significance.

**Acknowledgments**

This work has been supported by DTU Aqua, Section for Aquaculture project (Døgnrytmefysiologi, Grant number 39269) and partly by Danida Fellowship Centre (Grant number 11-PO2-VIE). The technical assistance of Ulla Sproegel, Brian Møller, Remko Oosterveld, Rasmus Frydenlund Jensen and Ole Madvig Larsen at DTU Aqua is also acknowledged. The help of Attila Hadnagy during sampling is likewise appreciated.

**References**

Anson, M., 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. Journal of General Physiology 22, 79.

AOAC, 2005. (Association of Official Analytical Chemists) Official Methods of Analysis of the Official Association of Analytical Chemists (18th ed.). Association of the Official Analytical Chemists, Arlington, VA.

Asher, G., Sassone-Corsi, P., 2015. Time for food: The intimate interplay between nutrition, metabolism, and the circadian clock. Cell 161, 84-92.

Bellinger, J.W., Avault, J.W., 1971. Food habits of juvenile pompano, *Trachinotus carolinus*, in Louisiana. Transactions of the American Fisheries Society 100, 486-494.

Betancor, M.B., McStay, E., Minghetti, M., Migaud, H., Tocher, D.R., Davie, A., 2014. Daily rhythms in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). PLOS ONE 9, e106739.

Bron, R., Furness, J.B., 2009. Rhythm of digestion: keeping time in the gastrointestinal tract. Clinical and Experimental Pharmacology and Physiology 36, 1041-1048.

Davidson, A.J., Poole, A.S., Yamazaki, S., Menaker, M., 2003. Is the food-entrainable circadian oscillator in the digestive system? Genes, Brain and Behavior 2, 32-39.

del Pozo, A., Montoya, A., Vera, L.M., Sánchez-Vázquez, F.J., 2012. Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass. Physiology & Behavior 106, 446-450.

Dickmeis, T., 2009. Glucocorticoids and the circadian clock. Journal of Endocrinology 200, 3-22.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. General and Comparative Endocrinology 165, 469-482.

Glasbrenner, B., Dürrschnabel, L., Büchler, M., Malfertheiner, P., 1992. Nonparallel patterns of circadian pancreatic and biliary secretions in fasting rats. International Journal of Pancreatology 11, 169-177.

Gnocchi, D., Pedrelli, M., Hurt-Camejo, E., Parini, P., 2015. Lipids around the clock: Focus on circadian rhythms and lipid metabolism. Biology 4, 104-132.

Guerra-Santos, B., López-Olmeda, J.F., de Mattos, B.O., Baião, A.B., Pereira, D.S.P., Sánchez-Vázquez, F.J., Cerqueira, R.B., Albinati, R.C.B., Fortes-Silva, R., 2017. Synchronization to light and mealtime of daily rhythms of locomotor activity, plasma glucose and digestive enzymes in the Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 204, 40-47.

Jönsson, E., 2013. The role of ghrelin in energy balance regulation in fish. General and Comparative Endocrinology 187, 79-85.

Keller, J., Layer, P., 2002. Circadian pancreatic enzyme pattern and relationship between secretory and motor activity in fasting humans. Journal of Applied Physiology 93, 592-600.

Kulczykowska, E., Sánchez Vázquez, F.J., 2010. Neurohormonal regulation of feed intake and response to nutrients in fish: aspects of feeding rhythm and stress. Aquaculture Research 41, 654-667.

Lazado, C.C., Caipang, C.M.A., Kiron, V., 2012. Enzymes from the gut bacteria of Atlantic cod, *Gadus morhua* and their influence on intestinal enzyme activity. Aquaculture Nutrition 18, 423-431.

554 Lazado, C.C., Kumaratunga, H.P.S., Nagasawa, K., Babiak, I., Giannetto, A., Fernandes,
555 J.M.O., 2014. Daily rhythmicity of clock gene transcripts in Atlantic cod fast skeletal
556 muscle. PLOS ONE 9, e99172.

557 Lazado, C.C., Lund, I., Pedersen, P.B., Nguyen, H.Q., 2015. Humoral and mucosal
558 defense molecules rhythmically oscillate during a light–dark cycle in permit,
559 *Trachinotus falcatus*. Fish & Shellfish Immunology 47, 902-912.

560 López-Olmeda, J.F., Egea-Álvarez, M., Sánchez-Vázquez, F.J., 2009. Glucose tolerance in
561 fish: Is the daily feeding time important? Physiology and Behavior 96, 631-636.

562 López-Olmeda, J.F., López-García, I., Sánchez-Muros, M.J., Blanco-Vives, B., Aparicio,
563 R., Sánchez-Vázquez, F.J., 2012a. Daily rhythms of digestive physiology, metabolism
564 and behaviour in the European eel (*Anguilla anguilla*). Aquaculture International 20,
565 1085-1096.

566 López-Olmeda, J.F., Noble, C., Sánchez-Vázquez, F.J., 2012b. Does feeding time affect
567 fish welfare? Fish Physiology and Biochemistry 38, 143-152.

568 Maouyo, D., Guan, D., Rivard, N., Adelson, J.W., Morisset, J., 1995. Stability of circadian
569 and minor cycles of exocrine pancreatic secretion in atropine- and MK-329-infused
570 rats. American Journal of Physiology - Gastrointestinal and Liver Physiology 268, G251-
571 G259.

572 Mitz, M., Schlueter, R., 1958. Direct spectrophotometric measurement of the peptide
573 bond: application to the determination of Acylase 1. Biochim Biophys Acta 27, 168.

574 Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics,
575 mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries
576 9, 211-268.

577 Montoya, A., López-Olmeda, J.F., Garayzar, A.B.S., Sánchez-Vázquez, F.J., 2010a.
578 Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and
579 thyroid hormones to feeding in gilthead seabream *(Sparus aurata*) under a light-dark
580 cycle. Physiology and Behavior 101, 101-107.

581 Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez,
582 F.J., 2010b. Feeding time synchronises daily rhythms of behaviour and digestive
583 physiology in gilthead seabream (*Sparus aurata*). Aquaculture 306, 315-321.

584 Nguyen, H.Q., Chu, T.C., Nguyen, T.T.L., Lund, I., In Press. Effects of dietary digestible
585 protein and energy levels on growth performance, feed utilization, and body
586 composition of juvenile permit, *Trachinotus falcatus* (Linnaeus, 1758). Journal of the
587 World Aquaculture Society, doi: 10.1111/jwas.12433.

588 Nikolopoulou, D., Moutou, K.A., Fountoulaki, E., Venou, B., Adamidou, S., Alexis, M.N.,
589 2011. Patterns of gastric evacuation, digesta characteristics and pH changes along the

gastrointestinal tract of gilthead sea bream (*Sparus aurata* L.) and European sea bass *(Dicentrarchus labrax* L.). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 158, 406-414.

Nisembaum, L.G., de Pedro, N., Delgado, M.J., Isorna, E., 2014. Crosstalking between the "gut-brain" hormone ghrelin and the circadian system in the goldfish. Effects on clock gene expression and food anticipatory activity. General and Comparative Endocrinology 205, 287-295.

Peyric, E., Moore, H.A., Whitmore, D., 2013. Circadian clock regulation of the cell cycle in the zebrafish intestine. PLOS ONE 8, e73209.

Raufman, J., 2004. Pepsin. Academic Press, Amsterdam

Sánchez-Vázquez, F.J., Madrid, J.A., 2001. Feeding anticipatory activity. Food intake in fish, 216-232.

Sánchez, J.A., López-Olmeda, J.F., Blanco-Vives, B., Sánchez-Vázquez, F.J., 2009. Effects of feeding schedule on locomotor activity rhythms and stress response in sea bream. Physiology & Behavior 98, 125-129.

Vaze, K.M., Sharma, V.K., 2013. On the adaptive significance of circadian clocks for their owners. Chronobiology International 30, 413-433.

Velarde, E., Haque, R., Iuvone, P.M., Azpeleta, C., Alonso-Gómez, A.L., Delgado, M.J., 2009. Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of Period and Cryptochrome transcripts in retina, liver, and gut. Journal of Biological Rhythms 24, 104-113.

Vera, L.M., De Pedro, N., Gómez-Milán, E., Delgado, M.J., Sánchez-Muros, M.J., Madrid, J.A., Sánchez-Vázquez, F.J., 2007. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiology & Behavior 90, 518-524.

Walter, H.E., 1984. Proteinases: methods with hemoglobin, casein, and azocoll as substrates, in: H.U. Bergmeyer (Ed.), Methods in Enzymatic Analysis. Verlag Chemie, Weinheim, 270–277.

Wirnt, R., Bergmeyer, H.U., 1974. Chymotrypsin, Methods of Enzymatic Analysis. Academic Press, New York, 1009-1012.

Worthington, C.E., 1988. Worthington Enzyme Manual. Worthington Biochemical Corporation, Freehold, NJ.

Yerushalmi, S., Green, R.M., 2009. Evidence for the adaptive significance of circadian rhythms. Ecology Letters 12, 970-981.

624 Yúfera, M., Fernández-Díaz, C., Vidaurreta, A., Cara, J.B., Moyano, F.J., 2004.
625 Gastrointestinal pH and development of the acid digestion in larvae and early juveniles
626 of *Sparus aurata* (Pisces: Teleostei). Marine Biology 144, 863-869.

627 Yúfera, M., Moyano, F.J., Astola, A., Pousão-Ferreira, P., Martínez-Rodríguez, G., 2012.
628 Acidic digestion in a teleost: postprandial and circadian pattern of gastric pH, pepsin
629 activity, and pepsinogen and proton pump mRNAs expression. PLoS ONE 7, e33687.

630

631

## Figure captions

**Figure 1. Gastric acidic digestion in permit**. **A**: Variations in luminal pH of the stomach throughout the LD cycle. The red broken line is the periodic sinusoidal function of the pH level constructed from COSINOR rhythmicity parameters. **B**: Changes in pepsin activity during the LD cycle. The broken lines are the periodic sinusoidal function from COSINOR analysis. Blue solid and broken lines represent the pepsin activity determined at standard pH 2. On the other hand, the red solid and broken lines show the pepsin activity measured at the actual luminal pH of that particular ZT. Significant differences are indicated by different letters. Asterisk (*) in Fig. 1B shows that the pepsin activities, analyzed in two different assay conditions, were significantly different at that particular ZT. The values presented are mean±SEM of 6 individual fish. The bar at the top of each graph indicates the LD photoperiod employed: white bar = light phase, black bar = dark phase.

**Figure 2. Daily rhythms of intestinal enzymatic activities. A**: protease; **B**: leucine aminopeptidase; **C**: amylase; **D**: cellulase; **E**: chymotrypsin; **F**: lipase. The values presented are mean±SEM of 6 individual fish. Significant temporal differences are indicated by different letter notations. The red broken line is the periodic sinusoidal function of the enzyme activity in the LD cycle constructed from the rhythmicity parameters revealed by COSINOR. The bar above the graphs show the photoperiod regime: white block represents the light phase while the black counterpart is the dark phase. Asterisk (*) in the graph indicates that the enzymatic activity exhibits significant daily rhythm.

**Figure 3. Gastric changes in response to periodic and random feeding. A:** Gastric luminal pH and **B:** Pepsin activity. Determinations were performed both pre-prandial (-8 and -2 h before mealtime) and post-prandial (+4 h after feeding). The values presented are mean±SEM of 6 individual fish. Pepsin activity was determined at standard assay pH 2 and at actual luminal pH (as in Fig. 1). In Fig. 3A, different letters indicate significant differences between time points within a feeding group. In Fig. 3B,

different numbers indicate significant differences between time points within a feeding group when assayed at standard pH 2, whereas different letters refer to significant differences between time points within a feeding group when assayed at actual luminal pH (bars with red margin). The red broken line with a triangular head indicates the feeding time. The white bar at the top of each graph specifies the photoperiod (LL).

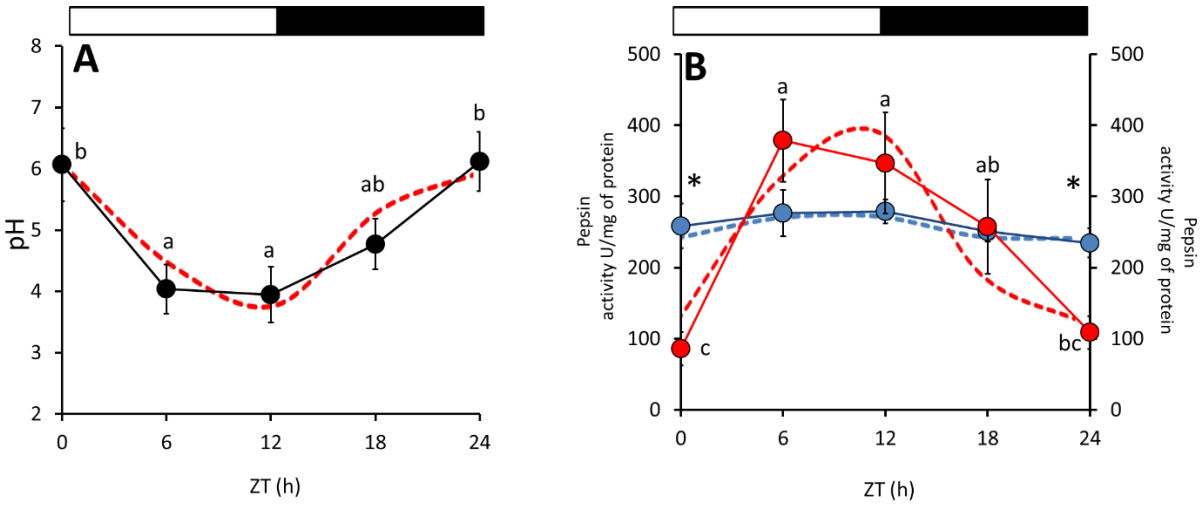
**Figure 4. Altered intestinal enzymatic activities following periodic and random feeding for 6 weeks. A**: protease; **B**: leucine aminopeptidase; **C**: amylase; **D**: cellulase; **E**: chymotrypsin; **F**: lipase. Intestinal samples were taken -8 and -2 h before and +4 h after feeding. The values presented are mean+SEM of 6 individual fish. Significant differences are indicated by different letter notations. Asterisk (*) indicates significant difference in the enzyme activity between periodically and randomly fed fish at a particular sampling point. For uniformity purposes, notation of statistical significance is designated only on the column bar of randomly fed fish. The white block above the graphs shows the LL photoperiod employed in the experiment. The red broken line with a triangular head indicates the feeding time.

**Figure 5. Daily rhythms of plasma A: melatonin and B: cortisol.** The values presented are mean+SEM of 6 individual fish. Other details of the graphs are provided in Fig. 1.

**Figure 6. Plasma (A) melatonin and (B) cortisol levels in fish subjected to periodic and random feeding**. The values presented are mean+SEM of 4 individual fish. Other graph details are provided in Figure 2. In Fig. 6B, asterisk (*) indicates that the level at that particular time point is significantly different from the value at the same time point in the other feeding group.

**Figure 7. Plasma ghrelin levels in the model fish. A**: Level of ghrelin in the plasma throughout the LD cycle. Additional details about the graph are given in Fig. 1. **B**: Changes in plasma ghrelin levels in fish subjected to periodic and random feeding

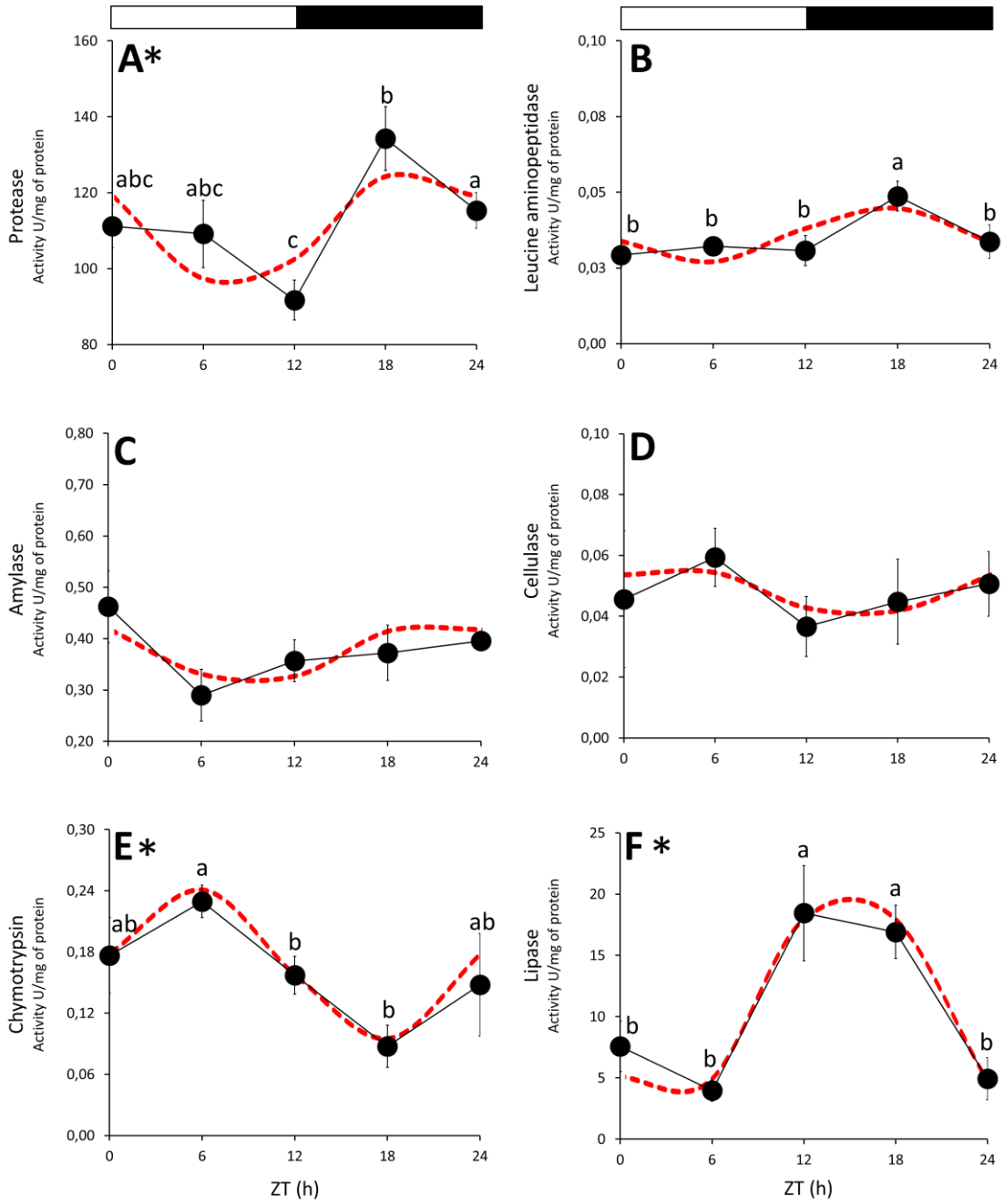
692 schemes. Refer to Fig. 3 for additional information about Fig. 7B. The values presented
693 are mean±SEM of 4 individual fish.
694




695

696 **Figure 1.**

697




698

699

700 **Figure 2.**

701

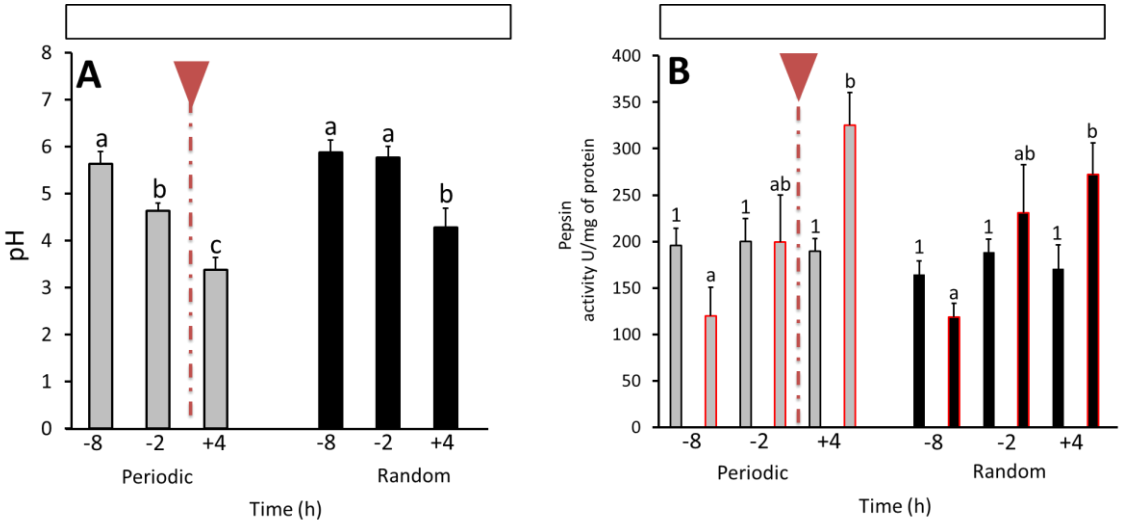
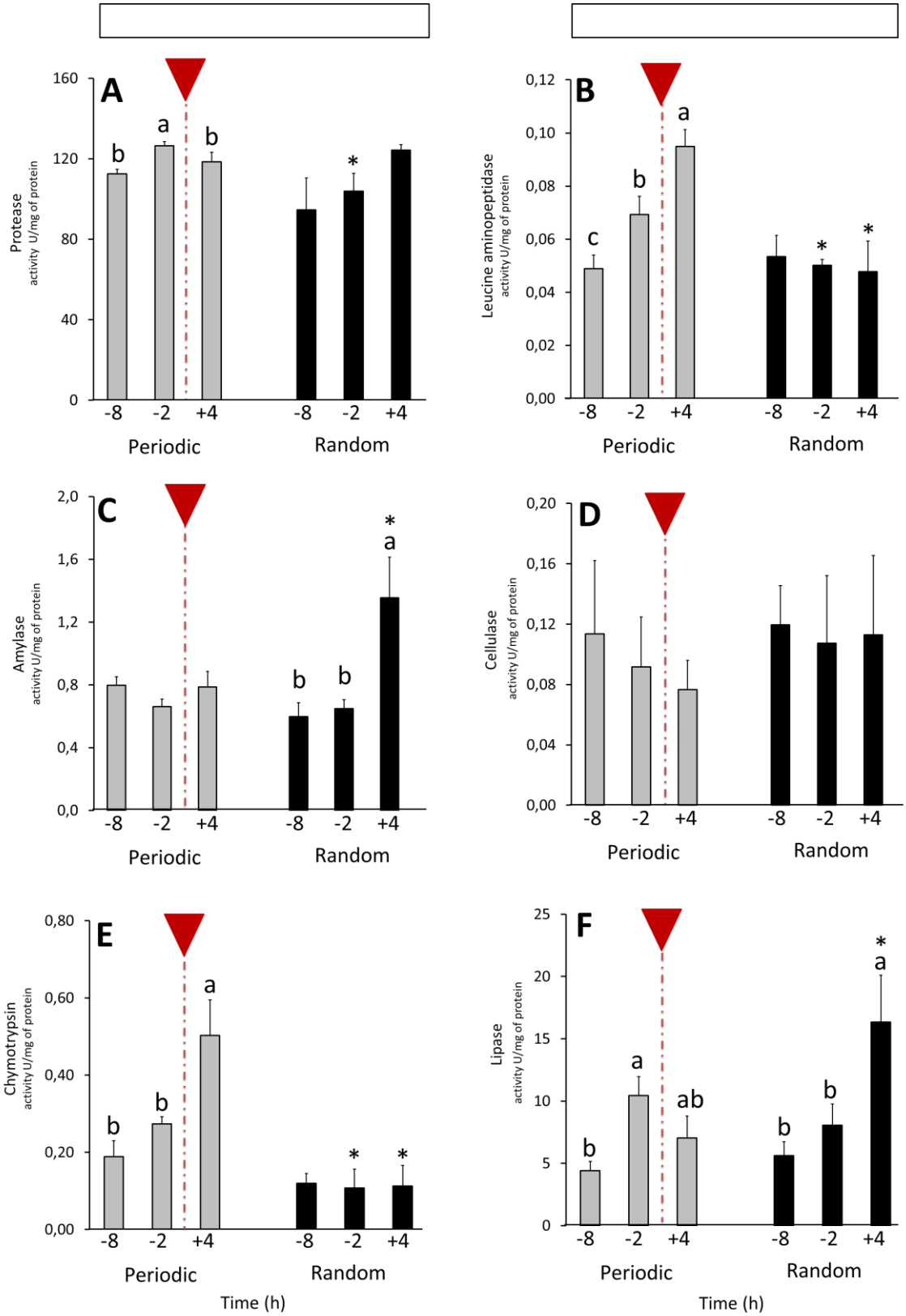

A
pH
a
b
c
a
a
b
-8
-2
+4
-8
-2
+4
Periodic
Random
Time (h)
B
Pepsin activity U/mg of protein
1
a
1
ab
1
b
1
a
1
ab
1
b
-8
-2
+4
-8
-2
+4
Periodic
Random
Time (h)

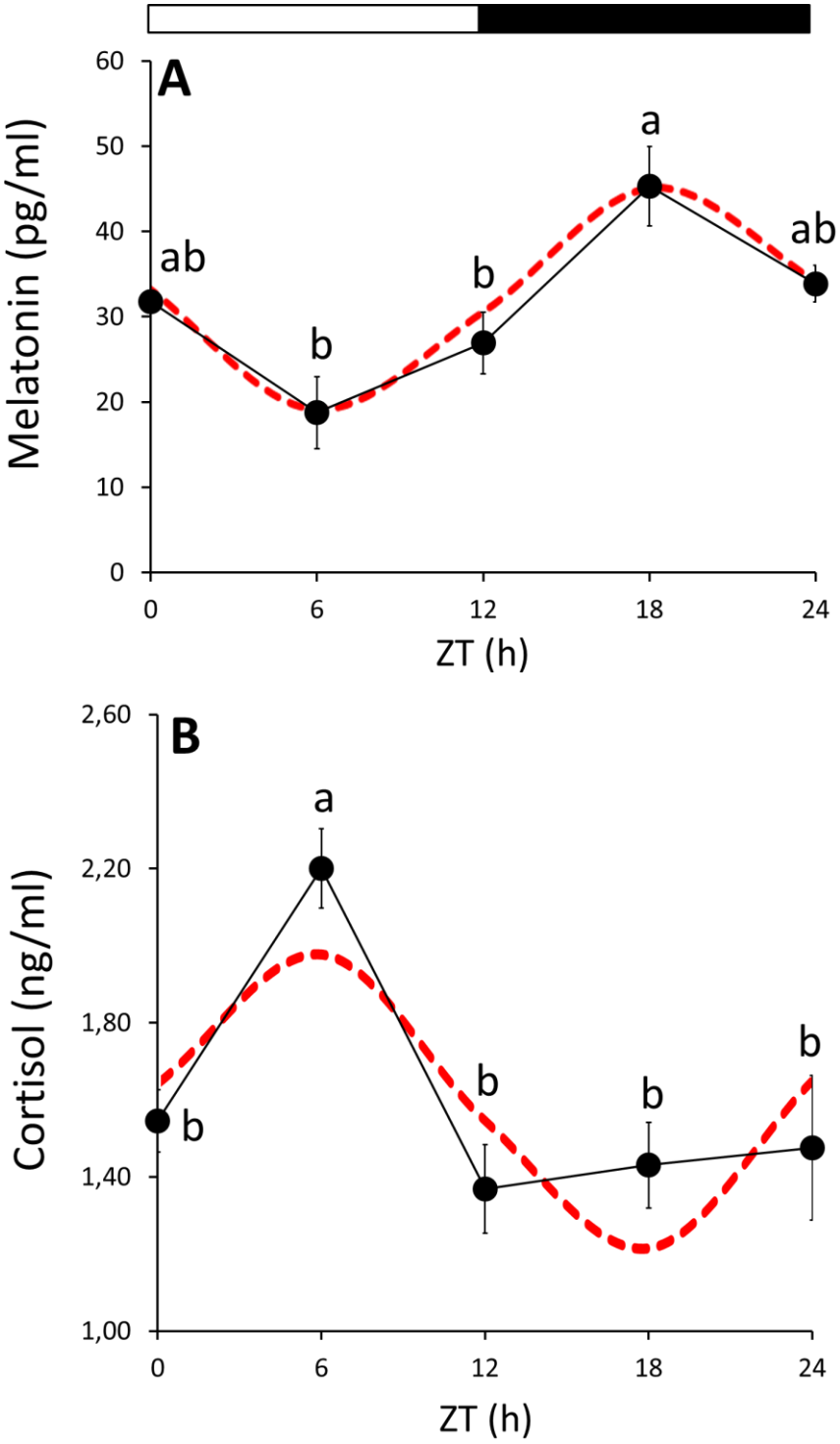
**Figure 3.**




705

706 **Figure 4.**

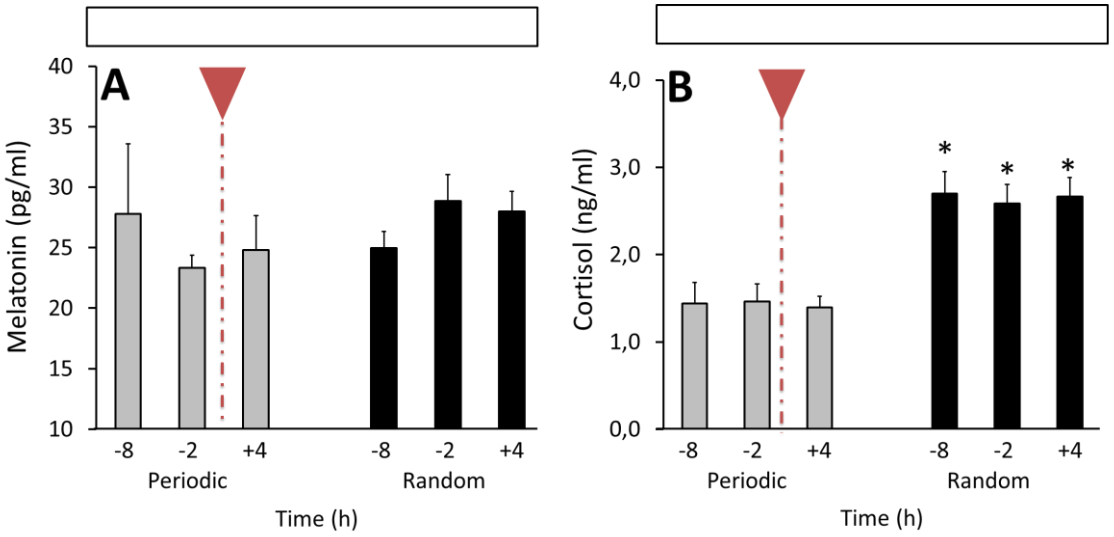
707




708

709 **Figure 5.**

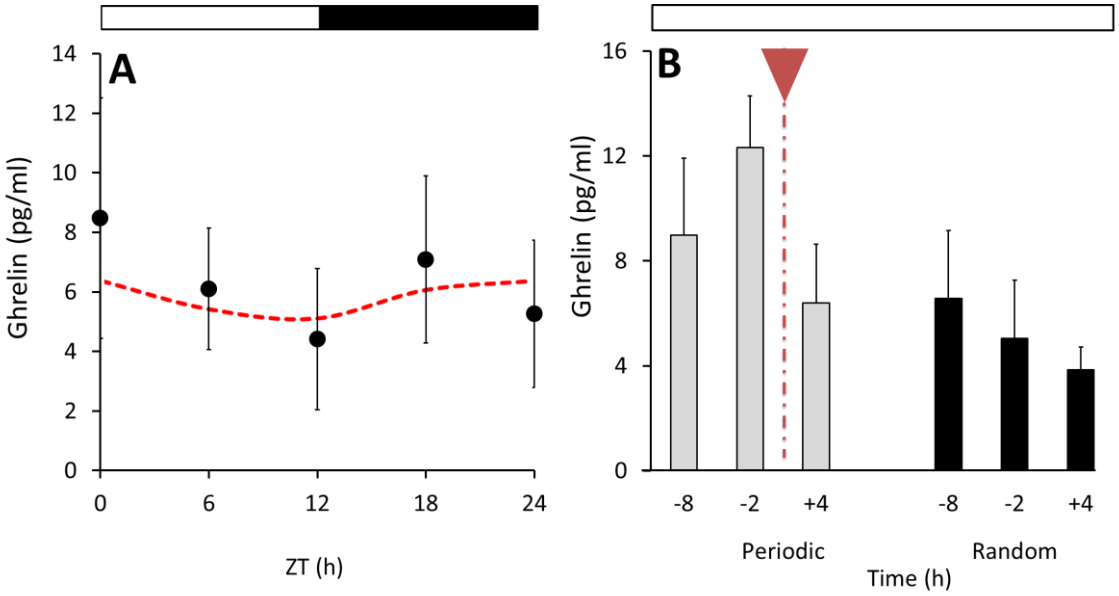
710




711

712 **Figure 6.**

713




714

715 **Figure 7.**

716

**Table 1.** Indicators of rhythmicity[1] of the gastric and intestinal enzymes during the LD cycle.

| Enzyme | COSINOR | | ANOVA |
|---|---|---|---|
| | Acrophase (h) | p value | P value |
| Pepsin | 9.3[2];10.3[3] | 0.22[2];0.15[3] | 0.71[2];0.38[3] |
| Protease | 20.2 | 0.02 | 0.003 |
| Leucine aminopeptidase | 17.1 | 0. 43 | 0.03 |
| Amylase | 21.1 | 0.18 | 0.16 |
| Cellulase | 3.24 | 0.68 | 0.38 |
| Chymotrypsin | 5.42 | 0.05 | 0.05 |
| Lipase | 15.2 | 0.04 | <0.001 |

[1]Enzyme activity is considered daily rhythmic under LD when p value in COSINOR is <0.05 and P value in ANOVA is <0.05.
[2]Values generated when assay was performed at standard assay pH 2.
[3]Values generated when assay was performed at the actual luminal pH.

**Table 2.** Indicators of rhythmicity [1] of neuroendocrine and appetite-related hormones in the plasma during the LD cycle.

| Hormone | COSINOR | | ANOVA |
|---|---|---|---|
| | Peak of activity/ Acrophase (h) | p value | P value |
| Melatonin | 19.4 | 0.006 | 0.009 |
| Cortisol | 5.5 | 0.02 | 0.005 |
| Ghrelin | 22.2 | 0.63 | 0.28 |

[1]Hormonal activity is considered daily rhythmic under LD regime when p value in COSINOR is <0.05 and P value in ANOVA is <0.05.

746 **Supplementary Table 1.** Biochemical composition (%) of white muscle from fish
747 subjected to either periodic or random feeding.

| Composition | Periodic | Random |
|---|---|---|
| Dry matter | 28.5 ± 0.94 | 27.8 ± 0.68 |
| Ash | 1.34 ± 0.07 | 1.37 ± 0.10 |
| Crude protein | 20.8 ± 0.36 | 21.1 ± 0.21 |
| Crude lipid | 7.86 ± 1.23 | 6.77 ± 0.95 |

748

749