**Feeding time synchronises daily rhythms of behaviour and digestive physiology in
Gilthead seabream (*Sparus aurata*)**

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ABSTRACT

Feeding cycles entrain biological rhythms, which enabling animals to anticipate feeding times and so maximize food utilization. In this article the effect of mealtime on locomotor activity, blood glucose, gastric pH and digestive enzymes was studied in two groups of seabream (*Sparus aurata*): one group received a single daily meal at random times either during the light and the dark period (random feeding, RF), whereas the other group received the meal during the light period every day at the same time (periodic feeding, PF). PF fish showed strong synchronisation of locomotor activity to the light phase ($97.9 \pm 0.2\%$ of their total daily activity during daytime). In addition, the locomotor activity rhythm of PRF fish showed a statistically significant daily rhythm ($p < 0.05$) for a period of 24 h, whereas the locomotor activity of RF fish was not statistically significant for any period. Blood glucose levels were higher in RF fish during the 8 hours following feeding. Gastric pH showed a postprandial decrease in both groups, but RF fish showed a lower daily average value (4.31 ± 0.21 compared with 5.52 ± 0.20). Amylase and alkaline protease activity increased some hours before mealtime in PF fish, whereas amylase activity increased 1 hour after feeding and alkaline protease showed no statistically significant differences in RF fish. Acid protease activity showed no statistically significant differences in any group. Taken together, these results demonstrate that altering the feeding time affects the physiology and behaviour of seabream, which have the capacity to prepare themselves for a forthcoming meal.

INTRODUCTION

The light-dark and feeding cycles are the most important factors that entrain biological rhythms in animals. In wild conditions, food is not continuously available, but is restricted in both place and time. When meals are delivered at the same time every day, an increase in the locomotor activity may be observed several hours before the mealtime. This phenomenon is known as food anticipatory activity (FAA) and persists even with the lack of food (Mistlberger, 1994). FAA not only involves behaviour but also other physiological variables which allow the animals to optimise their digestive and metabolic processes (Davidson and Stephan, 1999; Stephan, 2002). If the organism is able to anticipate an approaching meal, food acquisition and nutrient utilisation will be improved. Indeed, several fish species maintained under a periodic feeding regime have shown synchronization of their behavioral and physiological rhythms to mealtimes (López-Olmeda and Sánchez-Vázquez, 2010). For instance, goldfish (*Carassius auratus*) showed their anticipation to feeding time by increasing their locomotor activity, amylase activity and secretion of neuropeptide Y a few hours before mealtime (Vera et al., 2007).

Under farming conditions, food availability is often restricted to a single meal a day and the efficient use of nutrients has economic as well as environmental implications (food waste). This situation is easily reproducible in the laboratory by establishing a feeding cycle. As in other carnivorous teleosts and vertebrates, the proteolysis of ingested food in gilthead seabream (*Sparus aurata*) takes place first in the stomach through the action of pepsin in an acid environment. Progressive acidification in the lumen of the stomach has been reported to occur from late larvae to juveniles in several teleosts such as sea bass (*Lates calcarifer*) (Walford and Lam, 1993), Japanese flounder

(*Paralichthys olivaceus*) (Rønnestad et al., 2000), turbot (*Scophthalmus maximus*) (Hoehne-Reitan et al., 2001), gilthead seabream (Yúfera and Darías, 2004) and red porgy (*Pagrus pagrus*) (Darias et al., 2005), although no such decreasing pattern with age has always been observed (Yúfera et al., 2007). Two different digestion strategies have been described in vertebrates, including fish. Some groups or species exhibit a permanent acid luminal environment in the stomach in both fasted and fed animals, while others tend to recover a neutral pH after the digestion and between meals (Papastamiou and Lowe, 2004). A decline in gastric pH from nearly-neutral values after food ingestion has been described in a few species of Cottids (Western, 1971), Sparids (Deguara et al., 2003; Yúfera et al., 2004) and Salmonids (Sugiura et al., 2006; Bucking and Wood, 2009). In mammals, circadian variations of digestive variables, including gastric pH, have been widely reported (Zabielski, 2004), but in teleost fish, neither the daily rhythms of gastric pH nor the effect of feeding time on gastric pH variations have been described to date.

The alkaline digestion stage in fish is carried out in the intestine by means of hydrolytic enzymes (lipase, carbohydrase and alkaline protease) synthesized in the pancreas and secreted into the lumen. The activity of digestive enzymes in fish has been extensively studied in relation with the influence of diet composition, food quantity and the feeding habits of the species on its digestive enzyme system (Reimer, 1982; Hidalgo et al., 1999; Zambonino-Infante and Cahu, 2007; Pérez-Jimenez et al., 2009). The activity of the main digestive enzymes such as proteases and amylase may be one of the most important parameters that determines the effectiveness of a given diet, optimising growth and food utilization (Lemieux et al., 1999; Debnath et al., 2007; Mohanta et al., 2008). On the other hand, very few studies have focused on the effect of mealtime on the daily profile of digestive enzymes (Vera et al., 2007). In fish, anticipation of

amylase activity, but not proteases, to feeding time has previously been described in goldfish (*Carassius auratus*) fed periodically, though the daily rhythms of these enzymes were not described (Vera et al., 2007).

Carbohydrates are the cheapest source of energy for terrestrial animals, although the use of dietary carbohydrates by fish appears to be related to their digestive and metabolic systems, since herbivorous and omnivorous fish utilize higher levels of carbohydrates than carnivorous fish, such as Salmonids (Wilson, 1994). Gilthead seabream is one of the most important Mediterranean cultured species and has been described as a carnivorous fish (Gamito et al., 2003). A recent study reported higher blood glucose levels in seabream fed randomly compared with fish fed periodically (Sánchez et al., 2009). When seabream were allowed to self-feed either during the dark or the light phase, however, no effect of feeding time on glucose levels was reported (López-Olmeda et al., 2009a). Glucose daily rhythms in this fish species have been previously described (Pavlidis et al., 1997) but, to date, the effect of random feeding remains unknown.

Thus, the aim of this study was to investigate the effect of meal timing (periodic vs. random) on gilthead seabream behaviour (daily rhythms of locomotor activity) and daily rhythms of food utilization indicators such as blood glucose, gastric pH and the activity of the digestive enzymes, amylase, alkaline protease and acid protease.

MATERIALS AND METHODS

Animals and housing

Gilthead seabream (n=72) of 83 ± 4.80 g initial mean bodyweight were obtained from a local farm (Culmarex S.A., Aguilas, Murcia) and were reared at the facilities of the University of Murcia located at the Naval Base of Algameca (E.N.A., Cartagena,

Spain). Fish were kept in 500-l tanks supplied with aeration and filtered seawater from an open system. The photoperiod was set at 12:12 h light:dark (LD) with lights on at 8:00 h and water temperature at 18° C.

Experimental design

Fish were reared and manipulated following the Spanish legislation on Animal Welfare and Laboratory Practices. The experimental protocol was approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

Fish were divided into 4 tanks (18 fish per tank) and two experimental groups (2 tanks per group) were designed with different feeding schedules: fish were fed once a day at 14:00 h (PF group) (in the middle of the light phase) or once a day at a random time (RF group). Fish were fed 1% of the biomass once a day with an experimental diet that was formulated according to the macronutrient requirements of this species (Couto et al., 2008) and contained 46.5 % protein, 20 % fat and 33.5 % carbohydrate. Casein and gelatin (6:1) were used as protein sources, dextrin as carbohydrate and a mixture of fish oil and soybean oil (3:1) as fat. In addition, the diet was supplemented with vitamins and minerals and had sodium alginate as binder and cellulose as filler. Each tank was equipped with an automatic feeder (EHEIM, model 3581, Germany). Random feeding times were programmed weekly using a timer (Data Micro, Orbis, Spain), which set feeding interval between 12 and 36 h, so on average RF-fish received the same amount of food per 24 hours as PF-fish.

Gilthead seabream were maintained under these experimental conditions for two weeks and, after this period, samples began to be collected. Sampling was performed every 4 hours during a 24 hour cycle (6 sampling points), with the first sampling point

being 1 hour after food delivery for each experimental group. In order to avoid the effect of different feeding times, animals of the RF group were fed at 14:00 h on the sampling day. Fish were anesthetized with eugenol (clove oil essence, Guinama, Valencia, Spain) dissolved in water at a dose of 50 μ l/l. Blood was collected by caudal puncture with heparinized sterile syringes. Blood samples were collected in less than 5 min to avoid the increase in glucose levels originated by manipulation (Rotland and Tort, 1997). Blood was centrifuged at 3000 rpm for 15 min at 4°C and, after centrifugation, plasma was separated and frozen at -80°C until analysis. After blood collection, fish were sacrificed by decapitation, gastric pH was measured and samples from stomach and intestine for enzymatic analyses were collected and stored at -80°C. Sampling during the dark phase was performed under a dim red light ($\lambda > 600\text{nm}$).

Data analyses

Blood glucose concentration was measured immediately after its extraction by means of a glucometer (Glucocard G-meter, Menarini, Italy) as reported by López-Olmeda et al. (2009a). Gastric pH measurements were taken immediately after fish slaughter by means of a pH microelectrode (WPI, Minicombo, pH 660) (Yúfera et al., 2004). The tip of the microelectrode (diameter 660 μ m) was inserted in a small slit made in the stomach. Samples of stomach and intestine for the enzymatic analyses were homogenized by means of a potter with distilled water (250 mg tissue/ml) at 4°C. The homogenates were centrifuged twice at 12000 rpm for 15 min at 4°C and the supernatants were collected for use in the assays to measure enzymatic activities. Samples from stomach were used to measure acid protease activity, and samples from intestine were used to measure amylase and alkaline protease. The concentration of soluble protein in samples was determined by the Bradford method, using bovine serum

albumin as standard (Bradford, 1976). Amylase activity was determined according to the Somogy-Nelson method using soluble starch (2%) as substrate (Robyt and Whelan, 1968). Alkaline protease activity was measured by the casein method, using 1% casein as substrate (Kunitz, 1947; Walter, 1984). Acid protease activity was determined with a similar method to that used for alkaline protease, using 0.5% haemoglobin as substrate. The extracts were incubated at pH 2 (Anson, 1938). One unit of amylase activity was defined as the amount of enzyme able to produce 1 mg of maltose per minute and mg of protein. One unit of protease activity was defined as 1 mg of tyrosine released per minute and mg of protein.

Locomotor activity was measured by means of an infrared photocell (Omron, mod E3S-AD62, Kyoto Japan) immersed in the tank under the feeder and 3 cm from the water surface. A computer connected to the photocells counted and stored the number of lightbeam interruptions in 10 min intervals. Locomotor activity records were analysed and are represented as actograms and mean waveforms with chronobiology software *El Temps* (Version 1,228; Prof. Díez-Noguera, University of Barcelona). Data of glucose, gastric pH, amylase and proteases from each group were subjected to Cosinor analysis to test for the existence of statistically significant daily rhythms in each parameter. Cosinor analysis is based on least squares approximation of time series data with a cosine function of known period of the type $Y = \text{Mesor} + \text{Amplitude} * \cos((2\pi(t - \text{Acrophase})/\text{Period}))$, where Mesor is the time series mean; amplitude is a measure of the amount of temporal variability explained by the rhythm; period (τ) is the cycle length of the rhythm, i.e., 24 h for circadian rhythms; and acrophase is the time of the peak value relative to the designated time scale. Cosinor analysis also provided a statistical value for a null hypothesis of zero amplitude. Therefore, if for a statistical significance of $p < 0.05$, this null hypothesis was rejected, the amplitude could be considered as differing

from 0, thereby constituting evidence for the existence of a statistically significant rhythm of the given period under consideration.

Statistical analyses were performed using SPSS[®] software. Data from the daily rhythms of cortisol, glucose and thyroid hormones were subjected to a Levene's test to check for homogeneity of variances, and then, were subjected to one way ANOVA followed by Duncan's *post hoc* test. In addition, daily average values for each hormone and enzyme were compared between feeding groups (random vs. Scheduled) by means of a t-test. Values are reported as the mean $\pm$ S.D.

RESULTS

Locomotor activity rhythms

PF fish showed a strong synchronization to the light phase of the LD cycle, displaying 97.9 ± 0.2 % of the total daily activity during daytime with a periodicity of 24 h (Figure 1A). In contrast, RF-fish did not show a clear daily activity pattern (Figure 1B), displaying 72.6 ± 4.6 % of their total daily activity during the light phase and an arrhythmic pattern (Figure 1B). Fish fed periodically displayed more activity during the lightphase than those fed randomly (t-test, $p < 0.05$).

Blood glucose daily rhythms

Gilthead seabream subjected to periodic feeding displayed subtle variations in glucose values, with no statistically significant differences being observed (ANOVA, $p > 0.05$) (Figure 2). RF fish, in contrast, showed an increase in blood glucose 4 hours after feeding, which was maintained 8 hours after the mealtime. Glucose levels in this group returned to basal values 12 hours after feeding (Figure 2). A statistically significant blood glucose daily rhythm was observed in the RF group (COSINOR, $p < 0.05$), with the acrophase located 7 hours after feeding (Table 1). In addition, the

daily average blood glucose concentration of the RF group was higher than in the PF group (4.36 ± 0.33 and 3.24 ± 0.23 mmol/l for RF and PF fish, respectively) (t-test, $p < 0.05$).

Digestive physiology

Gastric pH

Gastric pH of fish subjected to periodic feeding showed a decrease 4 hours after feeding time and in the middle of the dark cycle, to 4.51 ± 0.62 and 3.87 ± 0.30 , respectively. The pH values ranged from 6 to 7 the rest of the day (ANOVA, $p < 0.05$) (Figure 3). Fish fed randomly showed a decrease in their gastric pH 4 hours after feeding, as also observed in the PF group, with the pH reaching values of 3.50 ± 0.29 . The low pH levels were maintained longer in the RF group, until the end of the dark cycle. RF-fish showed a statistically significant gastric pH daily rhythm (COSINOR, $p < 0.05$), with the acrophase fixed at the beginning of the light cycle (Table 1). Daily gastric pH values for both experimental groups differed statistically (5.52 ± 0.20 and 4.31 ± 0.21 for PF and RF fish, respectively) (t-test, $p < 0.05$).

Amylase activity

Fish subjected to periodic feeding anticipated the mealtime in the form of amylase secretion, with the highest amylase activity being observed 4 hours before feeding (186.16 ± 37.99 U/mg protein) (Figure 4). In this group, a decrease in amylase was observed after feeding until the middle of the dark phase (ANOVA, $p < 0.05$) (Figure 4). A statistically significant daily rhythm in amylase activity was observed in this group (COSINOR, $p < 0.05$), with the acrophase located at light onset (Table 1). In contrast, fish subjected to random feeding showed highest amylase activity 1 hour after mealtime (96.74 ± 16.71 U/mg protein) (ANOVA, $p < 0.05$) and a daily profile with a

lower amplitude and non-significant daily variations (Table 1) (COSINOR, $p>0.05$). In addition, PF fish showed higher daily amylase activity levels than RF fish (t-test, $p<0.05$) (116.06 ± 9.79 and 74.07 ± 8.45 U/mg protein) for PF and RF groups, respectively).

Alkaline Protease Activity

The PF group showed higher alkaline protease activity during the light phase, with the highest levels being found 1 hour after mealtime (1.36 ± 0.50 U/mg protein) (ANOVA, $p<0.05$) (Figure 5). In addition, an anticipation to mealtime in alkaline protease secretion was observed in this group, with the levels of this enzyme increasing 4 hours before feeding (1.24 ± 0.59 U/mg protein). A statistically significant daily rhythm was found in this experimental group (COSINOR, $p<0.05$), with the acrophase located 2 hours after light onset. In contrast, in the random feeding group, no statistically significant differences (ANOVA, $p>0.05$) could be reported in the alkaline protease activity levels (ANOVA, $p>0.05$). The daily average of alkaline protease levels in the RF fish were lower than the levels reported for PF fish (t-test, $p<0.05$) (0.82 ± 0.17 and 0.34 ± 0.05 U/mg protein for PF and RF fish, respectively).

Acid Protease Activity

No statistically significant differences were found in daily acid protease levels (ANOVA, $p>0.05$) or in the daily average acid protease activity between the two experimental groups (t-test, $p>0.05$). In addition, no statistically significant daily rhythm in the acid protease activity was observed (COSINOR, $p>0.05$). The daily average acid protease activity was 8.88 ± 1.14 and 9.56 ± 0.92 (U/mg protein) for PF and RF fish, respectively.

DISCUSSION

Our findings point to a strong effect of the feeding schedule on the daily behavioral and digestive physiology of sea bream. Fish subjected to PF feeding synchronized their locomotor activity to the light phase with a periodicity of 24h, whereas RF fish did not show such a clear diurnal behavior and displayed an arrhythmic pattern. Furthermore, blood glucose levels were higher during the 8 hours following mealtime in the RF fish. In addition, a postprandial decrease in the gastric pH could be observed in both groups, although fish fed randomly showed a lower daily average level. Moreover, an increase in amylase and in alkaline protease could be detected some hours before mealtime in PF fish, but not in RF fish.

Fish subjected to periodic feeding showed a strong light synchronization and a rhythmic pattern of locomotor activity, which synchronized to the feeding phase. In contrast, the randomly fed group showed an arrhythmic pattern of locomotor activity. This result contrasts with those of a previous study in which seabream subjected to random feeding displayed strict diurnal activity (Sánchez et al., 2009). However, it should be noted that in that study seabream were fed randomly only during the day, while in our trial fish were fed randomly either during the day or at night. The stronger influence of feeding than of LD cycles was previously reported in sea bass (*Dicentrarchus labrax*), when both synchronizers were provided with different periods (conflicting *zeitgebers*) (Sánchez-Vázquez et al., 1995). In that report feeding restricted to the light or dark phase shifted the daily pattern of behaviour to diurnal or nocturnal, respectively. These results highlight the strong influence of feeding time on fish behavioral patterns, which may cause arrhythmicity when food is randomly distributed during the day or night.

Daily variations in blood glucose in fish strongly depend on feeding, since they disappear in fasted fish (Polakof et al., 2007). Traditionally, the magnitude and duration

of the glucose peak after feeding has been related with diet composition, meal frequency and feeding habits of the fish species (Moon, 2001). Daily variations of blood glucose have previously been described in gilthead seabream fed periodically 3 times a day at a daily rate of 2-3% of body weight, with values ranging from 4 and 6 mmol/l and a peak between 5 and 9 hours after the first meal (Pavlidis et al., 1997). In our study, fish were fed a single daily meal of 1% of body weight, and showed blood glucose levels ranging from 2.5 ± 0.2 and 4.6 ± 0.8 mmol/l, which are close to previously reported values. Nevertheless, RF fish showed a two fold increase in blood glucose after feeding, which did not occur in PF fish. This result is in agreement with the study carried out by Sánchez et al., 2009, where higher glucose levels were reported in seabream fed randomly, suggesting poor regulation of blood glucose. Fish have been described as glucose intolerant, since hyperglycaemia persists several hours after feeding and in many cases reduces growth (Moon, 2001). Daily rhythms in glucose may be related with daily rhythms in glucose tolerance. In fact, a recent study carried out in goldfish reported differences in glucose tolerance with regard to mealtime (López-Olmeda et al., 2009b). Although the daily rhythm in glucose tolerance in sea bream remains unexplored, the hyperglycaemia observed in RF fish could be the result of a loss of synchronization of the glucose tolerance in these randomly fed fish. Further research is required to test this hypothesis.

In fish with a stomach, the enzymatic digestion starts in this section of the digestive tract, where the gastric glands secrete the pepsinogen and hydrochloric acid which decrease pH, inducing the conversion of pepsinogen to pepsin. A postprandial decrease in gastric pH has been described in adults and early juvenile of gilthead seabream (Deguara et al., 2003; Yúfera et al., 2004). In the present research, the daily gastric pH profile of both groups decreased significantly 4 hours after mealtime

coinciding with the observations made in a previous study carried out in seabream (Deguara et al., 2003). However, 8h after mealtime gastric pH increased in the PF group, but not in the RF group, and it decreased again 12h after the meal. Such 12h fluctuation in gastric pH has been reported in rainbow trout (Sugiura et al., 2006), although the driving mechanisms remain unclear. In our trials, seabream subjected to random feeding showed a significant daily rhythm of acid secretion with the acrophase around lights on. This group showed lower pH levels than the PF fish, which indicated increased acid secretion in order to be ready to digest the unpredictable forthcoming meal. Studies carried out on gastric pH variations in free swimming elasmobranchs revealed that leopard sharks (*Triakis semifasciata*) (Papastamatiou and Lowe, 2004) and blacktip reef sharks (*Carcharhinus melanopterus*) (Papastamatiou et al., 2007) feed continuously in the wild and display continuous gastric acid secretion, which enables them to be in a state of continuous physiological readiness.

The alkaline digestion stage in fish is carried out in the intestine by means of hydrolytic enzymes (lipase, carbohydrase and alkaline protease) synthesized in the pancreas and secreted into the lumen. In this study, an increase in both amylase and alkaline protease activity was observed before mealtime in fish fed periodically, whereas fish subjected to random feeding did not show such anticipation. As both enzymes are synthesized in the pancreas, the similar alkaline protease and amylase activity profiles obtained suggest synchronization of pancreatic secretion to mealtime. Our results concerning amylase activity agree with a study carried out in goldfish, in which amylase activity increases in anticipation of meal time in fish fed periodically, but not in fish fed randomly (Vera et al., 2007). In that report, however, alkaline protease did not show anticipation to meal time, whereas a postprandial increase was observed in both groups. It should be noted that differences between reports in alkaline

protease activity may be related to the fact that goldfish is omnivorous, while sea bream is a carnivorous species.

In the present research, a significant daily rhythm both in amylase and in alkaline protease activity was detected in PF fish, but not in RF fish. These results suggest that scheduled feeding provides temporal integration and entrains both amylase and alkaline protease activity. Reports on daily rhythms in amylase or protease activity are scarce. A study carried out in larvae of African catfish (*Clarias gariepinus*), which had been feeding exogenously for 7 days, failed to detect the rhythmic production of proteolytic activity during a 24h cycle (Garcia-Ortega et al., 2000). The feeding methods (5 times a day every 4 hours) as well as the poorly developed digestive system of the fish were the probable cause of this.

Taken together, these results emphasise the importance of mealtime in fish behaviour, metabolism and digestion: locomotor activity, blood glucose, gastric pH, amylase and protease digestive enzymes were all affected by the feeding regime (periodic vs random). These findings should be given proper consideration when designing meal timetables for seabream in aquaculture.

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FIGURE LEGENDS

Table 1. Cosinor values for blood glucose, gastric pH and digestive enzyme activity in the two experimental groups: RD, randomly fed; and PR, periodically fed fish. Mesor and amplitude are expressed in mmol/l for glucose and U/mg protein for digestive enzymes. The reference phase for the acrophase is referred to the time of day and is expressed in hours.

Figure 1. Representative actograms, mean waveforms and chi-periodograms of locomotor activity of sea bream subjected to periodic feeding (1A) and random feeding (1B). For convenient visualization, data in the actograms have been double-plotted on a time scale of 48 h. In the actograms, each horizontal line corresponds to one experimental day, represented along the ordinate axis. Top right: the waveform, where the height of each point represents the number of infrared lightbeam interruptions for each period of 10 min during the 24 h cycle, is represented as the mean $\pm$ S.D. Bottom right: the chi-periodogram (confidence-level 95%), where the periodicity of the locomotor daily rhythm is represented as the significant peak at the top of the periodogram (min). The bars above each actogram and waveform represent the light regime: white and black bars represent the light and dark phase of the cycle (12:12 LD), respectively.

Figure 2. Daily profiles of blood glucose in gilthead seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean $\pm$ S.E.M. (n=6)/time point. White and black bars above the graph represent light and darkness, respectively. Different letters indicate statistically significant differences (one-way ANOVA, $p < 0.05$).

Figure 3. Daily profiles of gastric pH in gilthead seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean $\pm$ S.E.M. (n=6)/time point. White and black bars above the graph represent light and darkness, respectively. Different letters indicate statistically significant differences (one-way ANOVA, $p<0.05$).

Figure 4. Daily profiles of amylase in gilthead seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean $\pm$ S.E.M. (n=6)/time point. White and black bars above the graph represent light and darkness, respectively. Different letters indicate statistically significant differences (one-way ANOVA, $p<0.05$).

Figure 5. Daily profiles of alkaline protease in gilthead seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean $\pm$ S.E.M. (n=6)/time point. White and black bars above the graph represent light and darkness, respectively. Different letters indicate statistically significant differences (one-way ANOVA, $p<0.05$).

Figure 1A

B

Figure 1B

Figure 2

Figure 3

Figure 4

Figure 5