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# 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 enables animals to anticipate feeding times and so maximizes 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 or the dark (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 ± 0.2% of their total daily activity during daytime). In addition, the locomotor activity rhythm of PF fish showed a statistically significant daily rhythm ($p<0.05$) for a period of 24 h, whereas RF fish did not display a statistically significant rhythm. Blood glucose levels were higher in RF fish during the 8 h 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 h 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.



## 1. 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 (López-Olmeda and Sánchez-Vázquez, 2010). 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 behavioural 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).

Daily and seasonal variations in feeding behaviour have been reported in self-fed seabream (Paspatis et al., 2000; Velázquez et al., 2004). Daily rhythms of locomotor activity, as well as hormones (cortisol and melatonin) have been reported in seabream (López-Olmeda et al., 2009b; Sánchez et al., 2009) but, to date, little is known on digestive rhythms in this species. 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 seabream (*Sparus aurata*) takes place first in the stomach through the action of pepsin in an acidic environment. Progressive acidification in the lumen of the stomach has been reported to occur from late larvae to juveniles in several teleosts such as barramundi (*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 et al., 2004) and red porgy (*Pagrus pagrus*) (Darias et al., 2005), although no such decreasing pattern with age has always been observed (Yúfera and Darias, 2007). Two different digestion strategies have been described in vertebrates, including fish.

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Some groups or species maintain a permanent low pH 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 (Papastamatiou 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-Jiménez et al., 2009). The activity of the main digestive enzymes such as proteases and amylase may be one of the most important parameters that determine 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). 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., 2009b). Glucose daily rhythms in this fish species have been previously described (Pavlidis et al., 1997) but, to date, the effect of a single meal provided either periodic or randomly remains unknown.

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

## 2. Materials and methods

### 2.1. Animals and housing

Seabream (n = 72) of 83 ± 4.80 g initial mean body weight 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.

### 2.2. 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% (wet weight) 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 40% protein, 15% fat, 20% carbohydrate, 2% vitamins and minerals, 4.6% sodium alginate as binder and 15.5% cellulose as filler. Casein and gelatine (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 h as PF fish.

Seabream were maintained under these experimental conditions for two weeks and, after this period, samples began to be collected. Sampling was performed every 4 h during a 24 hour cycle (6 sampling points), with the first sampling point being 1 h after food delivery for each experimental group. As two aquaria were used per group, sampling was performed alternately after 8 h. 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 concentration of 50 μl/l. Blood was collected by caudal puncture with heparinised sterile syringes. Blood samples were collected in less than 5 min to avoid the increase in glucose levels originated by manipulation (Rotllant 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 (λ>600 nm).

### 2.3. Data analyses

Blood glucose concentration was measured immediately after its extraction by means of a glucometer (Glucocard G-meter, Menarini, Italy), which used the method of the glucose oxidase, as reported by López-Olmeda et al. (2009b). 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 μm) was inserted in a small slit made in the stomach. Tissue samples of stomach and midgut for the enzymatic analyses were collected, removing food under digestion when it was present. Tissues were homogenized by means of a potter with distilled water (250 mg tissue/ml) at 4 °C. The homogenates were centrifuged twice at 12,000 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



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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. This location of the photocell was selected according to previous research which showed that FAA and food synchronisation are best observed near the water surface (Sánchez-Vázquez et al., 1997). 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, mean waveforms and periodograms with chronobiology software *El Temps* (version 1,228; Prof. Díez-Noguera, University of Barcelona). The periodogram analysis relies on the chi-square distribution to distinguish stochastic oscillations from true rhythms, providing $Q_P$ value for a period (P) that has a probability distribution of chi-square with P-1 degrees of freedom (Refinetti, 2004). $Q_P$ indicates the percentage of variance of the rhythm explained by the period. The level of significance was set at $p<0.05$. The periodogram indicates the percentage of variance of the rhythm explained by each analyzed period within a range of 20 to 28 h. The highest percentage is associated with the real value of the period (tau). 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 ($\tau$) 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 glucose, gastric pH and enzymatic activity of both treatments, 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 glucose, gastric pH and enzymatic activity were compared between feeding groups (random *vs.* periodic) by means of a t-test. Values are reported as the mean + S.E.M., and were obtained from direct measurements of individual fish ($n=6$).

## 3. Results

### 3.1. Locomotor activity rhythms

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

### 3.2. Blood glucose daily rhythms

No statistical differences could be observed in the blood glucose daily rhythm of seabream subjected to periodic feeding (ANOVA, $p>0.05$) (Fig. 2). RF fish, in contrast, showed an increase in blood glucose 4 h after feeding, which was maintained 8 h after the mealtime. Glucose levels in this group returned to basal values 12 h after feeding (Fig. 2). A blood glucose daily rhythm was observed in the RF group (COSINOR, $p<0.05$), with the acrophase located 7 h after feeding (Table 1). In addition, the daily average blood glucose concentration of the RF group was higher than in the PF group ($4.36 \pm 0.33$ and $3.24 \pm 0.23$ mmol/l for RF and PF fish, respectively) (t-test, $p<0.05$).

### 3.3. Digestive physiology

#### 3.3.1. Gastric pH

Gastric pH of fish subjected to periodic feeding showed a decrease 4 h after feeding time and in the middle of the dark cycle, to $4.51 \pm 0.62$ and $3.87 \pm 0.30$, respectively. The pH values ranged from 6 to 7 the rest of the day (ANOVA, $p<0.05$) (Fig. 3). Fish fed randomly showed a decrease in their gastric pH 4 h after feeding, as also observed in the PF group, with the pH reaching values of $3.50 \pm 0.29$. The low pH levels were maintained longer in the RF group, until the end of the dark cycle. RF fish showed a 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 ($5.52 \pm 0.20$ and $4.31 \pm 0.21$ for PF and RF fish, respectively) (t-test, $p<0.05$).

#### 3.3.2. Amylase activity

Fish subjected to periodic feeding anticipated the mealtime in the form of amylase secretion, with the highest amylase activity being observed 4 h before feeding ($186.16 \pm 37.99$ U/mg protein) (Fig. 4). In this group, a decrease in amylase was observed after feeding until the middle of the dark phase (ANOVA, $p<0.05$) (Fig. 4). A 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 h after mealtime ($96.74 \pm 16.71$ U/mg protein) (ANOVA, $p<0.05$). RF fish showed an arrhythmic pattern in amylase production, with lower amplitude than PF fish (Table 1) (COSINOR, $p>0.05$). In addition, PF fish showed higher daily amylase activity levels than RF fish (t-test, $p<0.05$) ($116.06 \pm 9.79$ and $74.07 \pm 8.45$ U/mg protein) for PF and RF groups, respectively).

#### 3.3.3. Alkaline protease activity

The PF group showed higher alkaline protease activity during the light phase, with the highest levels being found 1 h after mealtime ($1.36 \pm 0.50$ U/mg protein) (ANOVA, $p<0.05$) (Fig. 5). In addition, anticipation to mealtime in alkaline protease secretion was observed in this group, with the levels of this enzyme increasing 4 h before feeding ($1.24 \pm 0.59$ U/mg protein). A daily rhythm in alkaline protease was found in this group (COSINOR, $p<0.05$), with the acrophase located 2 h after light onset. In contrast, in the random feeding group, no statistically significant differences 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, $p<0.05$) ($0.82 \pm 0.17$ and $0.34 \pm 0.05$ U/mg protein for PF and RF fish, respectively).

#### 3.3.4. Acid protease activity

No statistically significant differences were found neither in daily acid protease levels (ANOVA, $p>0.05$) nor in the daily average acid protease activity between the two experimental groups (t-test, $p>0.05$). In addition, no daily rhythms in the acid protease activity were observed (COSINOR, $p>0.05$). The daily average acid protease activity was $8.88 \pm 1.14$ and $9.56 \pm 0.92$ (U/mg protein) for PF and RF fish, respectively.



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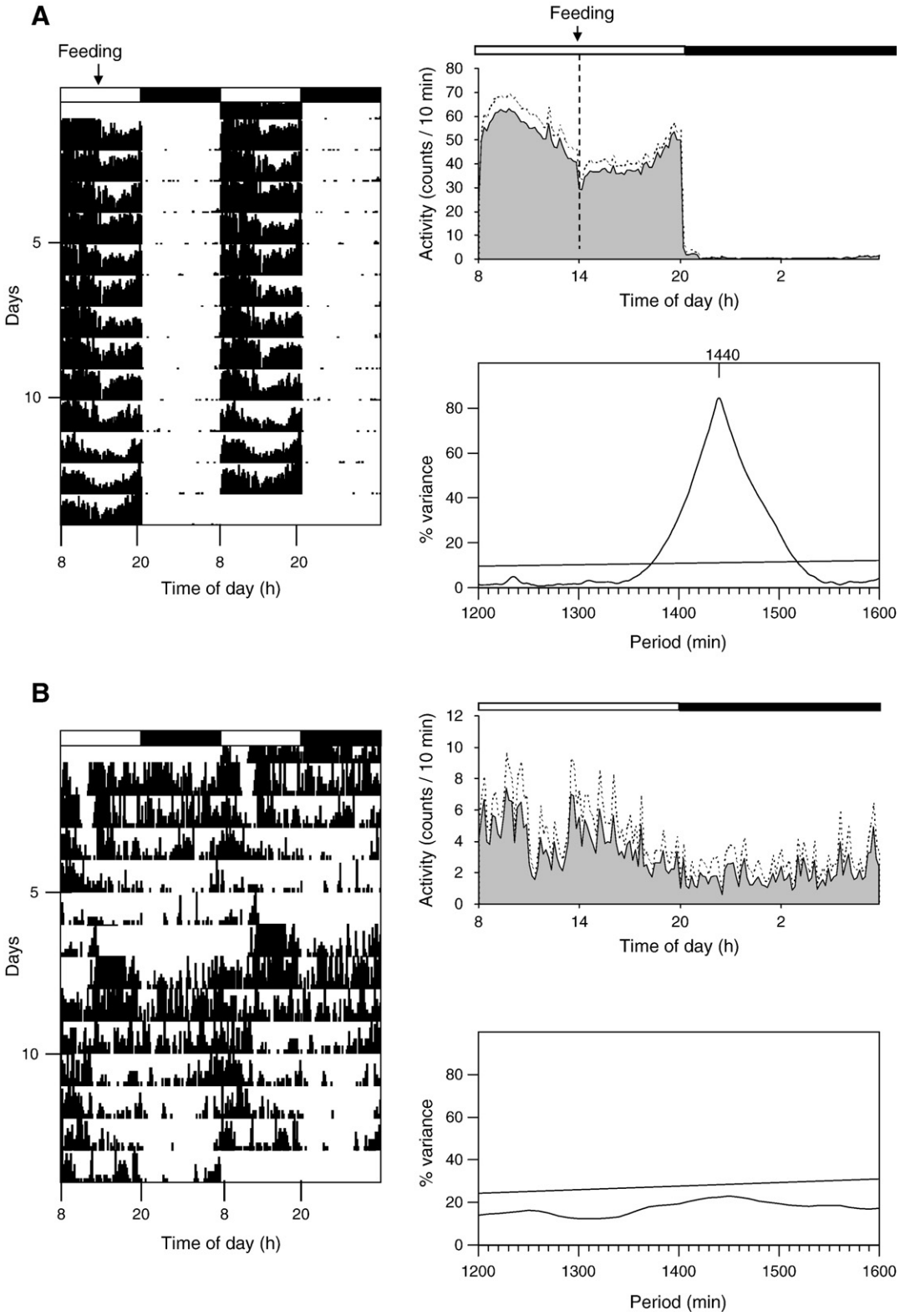


**Fig. 1.** Representative actograms, mean waveforms and chi-square periodograms of locomotor activity of seabream subjected to periodic feeding (A) and random feeding (B). For convenient visualization, data in the actograms have been double-plotted (48 h). The y-axis progresses in single days with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). 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 + S.D. Bottom right: the chi-square 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 periodogram indicates the percentage of variance of the rhythm explained by each analyzed period within a range of 20 to 28 h. The horizontal line represents the threshold of significance, set at $p = 0.05$. 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.

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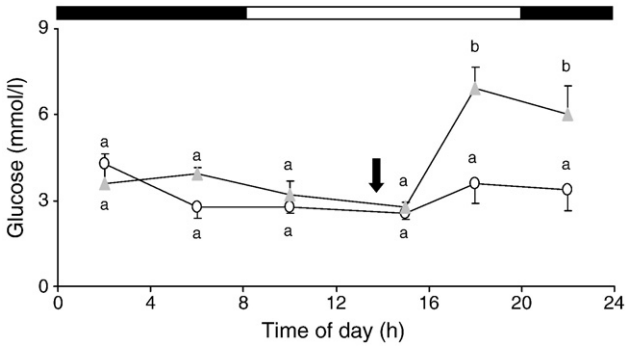


**Fig. 2.** Daily profiles of blood glucose in seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean + S.E.M. (RF) or the mean-S.E.M. (PF) (n = 6/time point). White and black bars above the graph represent light and darkness, respectively. Black arrow indicates time of food delivery. Different letters indicate statistically significant differences between times of day and treatments (one-way ANOVA, $p<0.05$).

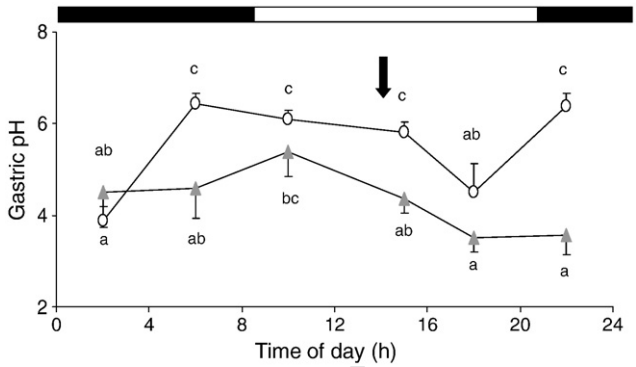


**Fig. 3.** Daily profiles of gastric pH in seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean + S.E.M. (PF) or the mean-S.E.M. (RF) (n = 6/time point). White and black bars above the graph represent light and darkness, respectively. Black arrow indicates time of food delivery. Different letters indicate statistically significant differences between times of day and treatments (one-way ANOVA, $p<0.05$).

## 4. Discussion

Our findings point to a strong effect of the feeding schedule on the daily behavioural and digestive physiology of seabream. Fish subjected to PF-feeding synchronized their locomotor activity to the light phase with a periodicity of 24 h, whereas RF fish did not show such a clear diurnal behaviour and displayed sustained activity along the 24 h. Furthermore, blood glucose levels were higher during the 8 h 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 close to the water surface, which synchronized to the feeding phase. In contrast, the randomly fed group showed an arrhythmic pattern of locomotor activity at this place of the tank as well as a sustained activity along the 24 h of the day, which could indicate that fish were continuously expecting or searching for food. 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. In addition, the photocell in the study by Sánchez et al. (2009) was located at the bottom of the tank, while in our study the photocell was located at the water surface, where FAA could be observed more clearly (Sánchez-Vázquez et al., 1997). 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 behavioural 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 to diet composition, meal frequency and feeding habits of the fish species (Moon, 2001). Daily variations of blood glucose have previously been described in seabream fed periodically 3 times a day at a daily rate of 2–3% of body weight,

**Table 1**
Cosinor values for blood glucose, gastric pH and digestive enzyme activity in the two experimental groups: RF, randomly fed; and PF, 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.

| | | PF-feeding | RF-feeding |
|---|---|---|---|
| Glucose | Acrophase (h:m) | 23:47 ± 5:25 | 20:51 ± 3:33 |
| | Amplitude (mmol/l) | 0.66 ± 0.66 | 1.57 ± 0.80 |
| | Mesor (mmol/l) | 3.19 ± 0.45 | 4.32 ± 0.57 |
| | p | N.S. | * |
| Gastric pH | Acrophase (h:m) | 10:16 ± 5:53 | 8:41 ± 3:31 |
| | Amplitude | 0.57 ± 0.58 | 0.85 ± 0.55 |
| | Mesor | 5.53 ± 0.4 | 4.35 ± 0.39 |
| | p | N.S. | * |
| Amylase | Acrophase (h:m) | 8:16 ± 2:20 | 18:24 ± 5:36 |
| | Amplitude (U/mg prot) | 47.80 ± 23.01 | 20.93 ± 22.86 |
| | Mesor (U/mg prot) | 125.58 ± 16.61 | 72.59 ± 16.99 |
| | p | ** | N.S. |
| Alcaline protease | Acrophase (h:m) | 9:58 ± 3:52 | 17:47 ± 3:19 |
| | Amplitude (U/mg prot) | 0.63 ± 0.43 | 0.06 ± 0.16 |
| | Mesor (U/mg prot) | 0.95 ± 0.34 | 0.34 ± 0.12 |
| | p | * | N.S. |
| Acid protease | Acrophase (h:m) | 1:26 ± 4:54 | 17:06 ± 3:34 |
| | Amplitude (U/mg prot) | 0.07 ± 0.06 | 0.019 ± 0.05 |
| | Mesor (U/mg prot) | 0.15 ± 0.4 | 0.17 ± 0.04 |
| | p | N.S. | N.S. |

N.S. non significant.
*$p<0.05$.
**$p<0.01$.

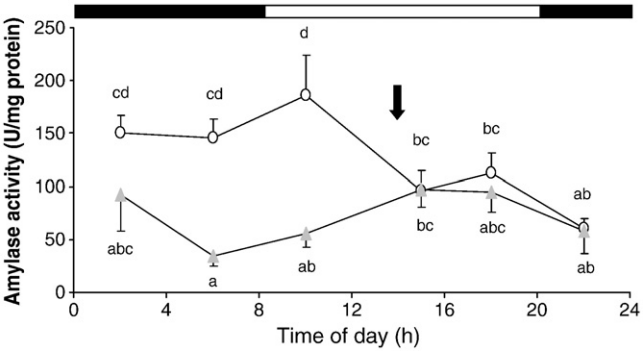


**Fig. 4.** Daily profiles of amylase in seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean + S.E.M. (PF) or the mean-S.E.M. (RF) (n = 6/time point). White and black bars above the graph represent light and darkness, respectively. Black arrow indicates time of food delivery. Different letters indicate statistically significant differences between times of day and treatments (one-way ANOVA, $p<0.05$).

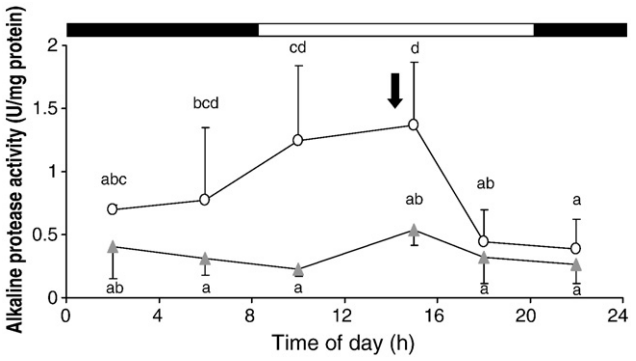


**Fig. 5.** Daily profiles of alkaline protease in seabream subjected to periodic feeding (open circles) and random feeding (grey triangles). Values represent the mean + S.E.M. (PF) or the mean-S.E.M. (RF) (n = 6/time point). White and black bars above the graph represent light and darkness, respectively. Black arrow indicates time of food delivery. Different letters indicate statistically significant differences between times of day and treatments (one-way ANOVA, $p<0.05$).

with values ranging from 4 and 6 mmol/l and a peak between 5 and 9 h 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 $2.5 \pm 0.2$ and $4.6 \pm 0.8$ mmol/l, which are close to values reported previously. 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., 2009a). Although the daily rhythm in glucose tolerance in seabream 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 decreases pH, inducing the conversion of pepsinogen to pepsin. A postprandial decrease in gastric pH has been described in adults and early juvenile of seabream (Deguara et al., 2003; Yúfera et al., 2004). In the present research, the daily gastric pH profile of both groups decreased significantly 4 h after mealtime coinciding with the observations made in a previous study carried out in seabream (Deguara et al., 2003). However, 8 h after mealtime gastric pH increased in the PF group, but not in the RF group, and it decreased again 12 h after the meal. Such 12 h 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 elasmobranches revealed that leopard sharks (*Triakis semifasciata*) (Papastamatiou and Lowe, 2004) and blacktip reef sharks (*Carcharhinus melanopterus*) (Papastamatiou et al., 2007) fed continuously in the wild and display continuous gastric acid secretion, which enables them to be in a state of continuous physiological readiness.

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 synthesised 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 seabream 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 24 h cycle (García-Ortega et al., 2000). These larvae were fed along the LD cycle (every 4 h), which could explain the lack of feeding synchronization observed in that study.

Taken together, these results emphasize 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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