# FEEDING ENTRAINMENT OF LOCOMOTOR ACTIVITY RHYTHMS, DIGESTIVE ENZYMES AND NEUROENDOCRINE FACTORS IN GOLDFISH

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**ABSTRACT**

L.M. VERA, N. DE PEDRO, E. GÓMEZ-MILÁN, M.J. DELGADO, M.J. SÁNCHEZ-MUROS, J.A. MADRID, F.J. SÁNCHEZ-VÁZQUEZ. Feeding entrainment of locomotor activity, digestive enzymes and neuroendocrine factors in goldfish. PHYSIOL BEHAV 90 (2-3) 518-524, 2007. The existence of food anticipatory activity (FAA) in animals subjected to daily feeding schedules seems to be mediated by a feeding-entrainable oscillator (FEO). Such an FEO may help in anticipating meal time and so optimizing food acquisition and nutrient utilization. In this study we investigated the existence of FAA and whether digestive enzymes, plasma cortisol, hypothalamic NPY and gastrointestinal tract (GIT) and plasma melatonin were entrained by periodic feeding in goldfish. We observed that periodically fed goldfish showed FAA in locomotor activity as well as in amylase and NPY. Alkaline protease and GIT melatonin were higher after feeding, whereas plasma cortisol levels were reduced. Plasma melatonin remained unmodified before and after meal time. These results suggested that scheduled feeding entrained both behavioral and certain physiological patterns in goldfish, FAA being of adaptive value to anticipate a meal and prepare the digestive physiology of fish.

**Keywords:** goldfish, FEO, locomotor activity, amylase, protease, NPY, cortisol, melatonin.

## 1. INTRODUCTION

Most animals show food-anticipatory activity (FAA) when they are subjected to daily schedules of restricted food availability. This phenomenon is usually characterized by an increase in locomotor activity and core body temperature several hours before feeding [1]. When the mechanisms explaining FAA in mammals were reviewed, the conclusions were that external cues are not necessary for FAA, and that activity is triggered each day when energy depletion reaches some threshold prior to feeding time. It was also seen that a self-sustaining oscillator entrained by food can persist in the absence of a feeding cycle [2].

The light/dark (LD) cycle has been considered the most powerful environmental factor synchronizing biological rhythms. This entrainment is mediated by a light-entrainable oscillator (LEO). However, the existence of FAA in several species is thought to be mediated by an internal timing system or biological clock [3], in other words a feeding-entrainable oscillator (FEO). Some studies carried out in goldfish have demonstrated that scheduled feeding acts as a zeitgeber in entraining locomotor activity rhythms in this species. Indeed, several properties, such the anticipation to feeding time and the appearance of free-running rhythms following the end of periodic feeding, suggest the participation of a self-sustained FEO [4,5].

As regards the biological significance of feeding entrainment (FAA), as biological rhythms in general, provides animals with the ability to anticipate a recurrent event (i.e. feeding). Animals optimize digestive and metabolic processes which allow them to concentrate the intake of food and water in a short time period to reduce the risk of predation in the wild [6]. Physiologically, if the organism is able to anticipate an approaching meal, food acquisition and nutrient utilisation may be improved [7]. Several studies have been carried out into this physiological adaptation. For example,

some enzymatic activities have been observed to increase in the intestinal tract of the mouse prior to the expected intake of the daily food. These anticipatory responses are probably under endogenous control since they persist in fasted animals and they free-run when the animal is kept under constant conditions [8]. However, this issue has been scarcely explored in fish species.

Neuropeptide Y is distributed through the central nervous system in fish, and several studies have shown its important role in feeding regulation [9]. In goldfish, for example, it acts as an orexigenic neuropeptide [10, 11], and increases in central NPY as a response to 3-days food deprivation have been reported in this species, while the effect was reversed by refeeding (1-3 h) [12]. These reports suggest that NPY in goldfish may act as a signal of feeding anticipation, although the possible role of feeding as entrainment of this neuropeptide remains to be studied.

Cortisol is a key hormone in the stress responses, and shows regulatory actions on feeding and growth in fish [13]. Daily rhythms in plasma cortisol levels exist in goldfish [14], although the relative roles of different factors, such as light/dark cycle, locomotor activity and feeding time as possible synchronizers are unknown.

Melatonin has been widely reported as a hormone involved in regulation of circadian and seasonal rhythms in animals [15]. The pineal organ was originally described as the main source of melatonin, although several extrapineal sources have been identified to date: the retina, Harderian gland and the gastrointestinal tract (GIT) [e.g. 16,17]. However, the anticipatory production of GIT melatonin prior to meal has not been investigated.

In the present study, we further investigated FAA, evaluating whether digestive enzymes (amylase and alkaline protease), plasma cortisol, hypothalamic NPY and GIT and circulating melatonin were affected by scheduled feeding in goldfish.

## 2. MATERIALS AND METHODS

### 2.1. Animals and housing

For this study one hundred twenty-six goldfish of 18.0 ± 0.6 g bw were used. The animals were held in 60L glass aquaria. Water was aerated and recirculated by a biological filter (EHEIM). Illumination was provided by a fluorescent tube placed over each aquarium (GRO-LUX, 40 W, Germany). Throughout the experiment goldfish were maintained under constant light conditions (LL) to avoid the entraining effect of light / dark cycle and possible interferences with feeding entrainment.

### 2.2. Experimental design

Fish were divided into three groups. Animals that belonged to group I (n=54) were fed every day at the same time for three weeks, goldfish from group II (n=53) were fed at random, while goldfish from group III (n=19) were fed at 18:00 h for three weeks before being submitted to fasting for two days. Goldfish from each experimental group were distributed into 5 aquaria (4-5 individuals per aquarium).

Half of fish from groups I and II were used to study enzyme anticipation to food and the other half for NPY, cortisol and GIT melatonin. Goldfish from group III were used to test the effect of fasting on digestive enzymes.

To deliver food, an automatic timer feeder (EHEIM, model 3581, Germany) was placed in each aquarium. Commercial food for goldfish was used (TetraAniMin® Goldfish Energy, Germany), the ration provided being 1.5% of body weight. For groups I and III, the feeder was programmed to deliver food every day at the same time (18:00). For group II, the time of feeding was chosen randomly by a computer, and a digital timer connected to the feeder. The feeding interval was set between twelve and thirty-

six hours, so on average they received the same amount of food per 24 hours as group I and III. To avoid the effect of different feeding times, when animals from groups I and II were sampled on day 21, food was delivered at the same time (18:00) to all fish.

Locomotor activity from all the groups was registered. To this end, an infrared photocell (OMROM E3S-AD62, Japan) was placed in each aquarium facing the automatic feeder to record food anticipatory activity. All photocells were connected to a computer, in which data where stored for further analysis.

Goldfish from groups I and II were sampled after three weeks under experimental conditions. Sampling took place, eight hours (-8h) and two hours (-2h) before food delivery, and at four hours (+4h) postfeeding. At each sampling time animals were obtained from different aquaria to avoid stressing fish.

On day 24 the animals from group III, which had been fasting for two days, were sampled at the same times.

### 2.3. Digestive enzymes, Neuropeptide Y, cortisol, GIT and plasma melatonin analysis

For digestive enzyme and GIT melatonin analysis, the digestive tract from each fish was removed, introduced into an Eppendorf tube, frozen on solid $CO_2$ and kept at –80 ºC until analysis. For this, the digestive tubes were first homogenized with distilled water (250 mg/ml) at 4ºC and extracts were used to measure amylase and alkaline protease activities. For melatonin, Tris-HCl buffer (500 mg/ml) was used [18]. The concentration of soluble protein in samples was determined by the Bradford method using bovine serum albumin as a standard [19].

Amylase activity was determined according to the Somogy-Nelson method using soluble starch 2% as substrate [20]. One unit of activity was defined as the amount of enzyme able to produce 1 mg of maltose per minute and mg of protein.

Alkaline protease activity was measured using the casein method [21, 22], using casein 1% as substrate. One unit of enzyme activity was defined as 1 mg of tyrosine released per minute and mg of protein.

The hipothalami were dissected, immediately frozen in 250 µl of 2 M acetic acid + aprotinin 200 pg/ml, and stored at –80 ºC until analysis. The hypothalamic NPY content was determined using a commercial radioimmunoassay (RIA) kit (Peninsula Laboratories, Inc.), previously validated for goldfish hypothalamic samples [23].

Fish were anaesthetized in water containing ice and weighed, and then blood was taken from the caudal vessel using 1 ml heparinized syringes (TERUMO, Leuven, Belgium). Blood was centrifuged and plasma samples were frozen on solid $CO_2$ and finally stored at – 80 ºC until analysis. Plasma cortisol levels were determined using a commercial RIA kit (DRG Diagnostics, Germany), previously validated in this species [24].

To measure GIT melatonin, digestive homogenates were extracted using C18 extraction cartridges (IBL, Germany) and finally reconstituted with PBS (0.14 M NaCl, 2.70 mM KCl, 6.40 mM $Na_2HPO_4.12H_2O$, 0.88 mM $KH_2PO_4$, pH=7.2) . After this, GIT and plasma melatonin was determined by a commercial RIA kit (IBL, Hamburg, Germany) [25].

**2.4. Data analysis**

Locomotor activity records were stored in a computer and analysed using chronobiology software (EL TEMPS® Prof. Antoni Díez Noguera, University of

Barcelona). Excel and SPSS were used for data analysis. The statistical differences between mean enzyme activities, hypothalamic NPY, plasma cortisol and GIT melatonin were analysed by analysis of variance (ANOVA) followed by Duncan test, with $P < 0.05$ taken as the statistically significant threshold.

## 3. RESULTS

### 3.1. Food anticipatory activity

Goldfish maintained under LL conditions and fed once a day at the same time (group I) displayed most locomotor activity during the hours just before and after meal time. Three hours before food was provided, animals started increasing their activity (FAA), reaching a peak just before feeding. After feeding, activity dropped dramatically but around thirty minutes later activity was resumed and then decreased gradually, reaching a minimum around five hours after the meal, a level which was maintained until the increase before the next feeding event (Fig. 1A, 1B). FAA was calculated as the interval in which the activity of fish increased 50% over the baseline without subsequent inflections until the feeding time.

Fish that were submitted to a random feeding schedule (Group II) displayed a continuous pattern of locomotor activity during the day, with no activity peaks at any particular time (Fig. 1C, 1D). The total activity displayed by goldfish in this group was slightly higher than that observed in group I (2430 vs 2016 counts/day) although no statistically significant differences were found (t-Student, $P>0.05$).

### 3.2. Digestive enzymes

Amylase activity in goldfish from group I showed statistically significant differences between the different sampling times: eight hours before feeding amylase

activity dropped to the lowest levels (23.4 ± 4.2 U/mg prot), while two hours prior to feeding amylase activity increased up to 56.8 ± 17.6 U/mg prot, and remained high for four hours after feeding (54.9 ± 4.92 U/mg prot). When amylase activity was measured at the same time in animals fed following a random schedule (group II), no statistically significant differences were found among sampling times nor between this treatment and groups I and III, and values for enzymatic activity was around 39 U/mg prot in all cases. In the case of the group III, in which fish were fasted for two day, eight hours before the meal had been supplied (before the fasting phase) amylase activity was low, 14.0 ± 2.9 U / mg prot, while two hours before meal time amylase activity increased up to 28.1 ± 6.2 U / mg prot, reaching 42.2 ± 9.3 U / mg prot four hours after the meal was due, although food was not provided. However, differences within the three measurement times of amylase activity did not reach statistical significance (ANOVA, $P>0.05$) as previously seen in group I ((Fig. 2A).

Alkaline protease activity in goldfish from group I did not show statistically significant differences between the different sampling points. Two hours prior to food being provided, alkaline protease activity reached the lowest levels, 12.0 ± 1.2 U / mg prot. However, by four hours after feeding this activity had increased again to 22.4 ± 2.6 U / mg prot. As regards groups II and III, a similar trend was observed (lowest levels at -2h), and statistically significant differences were found between the different times at which alkaline protease activity was measured within treatment II and between +4h from this group and +4h values in group I. Values ranged from 17 to 37 U / mg prot in the first case and from 17to 27 U / mg prot in the second (ANOVA, $P<0.05$) (Fig 2B).

### 3.3. Neuropeptide Y (NPY)

Hypothalamic NPY in goldfish from group I showed statistically significant differences between the different sampling times: the highest concentration was measured two hours prior to feeding (6.2 ± 0.3 ng/hyp), while eight hours before and four hours after food was provided, NPY values were around 4.5 ng/hyp. As regards group II, no statistically significant differences between the different sampling times were observed, with NPY concentrations being around 5.0 ng/hyp in all cases, however -2h NPY levels from this group statistically differed from levels observed at the same time in group I (ANOVA, $P < 0.01$) (Fig. 3).

### 3.4. Cortisol

Plasma cortisol in goldfish under scheduled feeding did not show statistically significant differences between the different sampling times, however cortisol levels were around 160.0 ng/ml eight and two hours before meal time, although this dropped to 85.7 ± 19.9 ng/ml four hours after feeding. In fish fed at random time during the experiment, plasma cortisol ranged from 214.0 to 256.9 ng/ml eight and two hours prior to meal, whereas by four hours after feeding it had decreased significantly to 93.2 ± 19.8 ng/ml. Indeed, plasma cortisol levels at -8h statistically differed between both groups (ANOVA, $P<0.01$) (Fig. 4).

Note that the average plasma cortisol concentration from group II (190.3 ± 22.7 ng/ml) was substantially higher (43%) than that observed in group I (133.7 ± 14.8 ng/ml) (t-Student, $P<0.05$).

### 3.5. GIT and plasma melatonin

GIT levels of melatonin in goldfish fed every day at the same time (group I) showed statistically significant differences between sampling times: eight and two hours

pre-feeding GIT melatonin ranged from 10 to 30 pg/g of digestive tissue. However, four hours after fish were fed GIT melatonin had raised to 785.7 ± 163.8 pg/g. In group II, a similar pattern of GIT melatonin variation was found, with low levels before meal (10-37 pg/g) and high titers after feeding (985.1 ± 42.1 pg/g). Statistically significant differences were found between +4h GIT melatonin values from groups I and II and the rest of sampling points (ANOVA, $P<0.01$ ) (Fig.5A).

Plasma melatonin concentrations were similar at all sampling times in both groups I and II, with values ranging between 130 and 158 pg/ml, indicating that the feeding regime did not affect plasma melatonin. (Fig 5B).

## 4. DISCUSSION

This study showed that when goldfish were submitted to scheduled feeding both behavioural patterns and physiological processes were entrained to feeding. Goldfish that were fed every day at the same time showed FAA, whereas fish fed randomly did not. As regards digestive and neuroendocrine physiology, the anticipatory secretion of amylase and NPY was observed two hours before feeding, whereas alkaline protease and GIT melatonin showed a post-prandial high. Plasma melatonin levels remained constant at all the sampling times.

In goldfish it was described that gut distension may influence feeding entrainment, meaning that an almost empty stomach is probably a stronger synchronizing signal for the FEO than a full one [26]. In our experiments we further investigated FAA in goldfish and observed that not only behavioural patterns were entrained by food but also physiological parameters were affected by the feeding schedule. In mammals, digestive enzymes increase their activity before the expected intake of daily food. This anticipation would indicate the existence of endogenous

control since they persist in fasted mice and free run under constant conditions [27]. In the rat, similar results have been found, suggesting that rhythms in digestive enzymes are not a direct and passive response to food intake, but are controlled by the anticipatory mechanism existing when animals are expecting to be fed [28]. In our study we observed a significant increase in amylase activity two hours prior to food intake in goldfish fed periodically, an increase that was also observed in fasted animals. This might indicate, on the one hand, the existence of anticipating mechanisms to scheduled feeding and, on the other hand, that this anticipation is endogenously controlled, since it persists after two days of fasting. The fact that amylase activity in goldfish fed randomly remained equal at all sampling times indicates the importance of periodic feeding as a zeitgeber entraining the secretion of this enzyme. Some studies carried out in fish species lend support to these findings: when amylase activity was measured in wild jaraqui from the Amazon (*Semaprochilodus taeniurus*), a clear daily rhythm was found, with the highest values obtained when the fish had emptied their stomachs, reflecting a possible preparation phase for feeding activity [29]. In contrast, we found that alkaline protease activity in goldfish from group I showed the lowest levels two hours prior to feeding, whereas a post-prandial increase was observed. In African catfish (*Clarias gariepinus*) larvae fed only one meal a day, the protease activity increased after feeding, reaching a maximum level 12 h after food was supplied; no significant change in enzymatic activity was found for starved catfish larvae [30]. In our study, as in catfish, no significant differences were observed between sampling times in fasted goldfish.

NPY increased 2 h before meal in feeding-scheduled, but not in randomly fed fish, which strongly suggests that scheduled feeding time can act as entrainment of NPY production in goldfish. Our results support previous data which showed that goldfish

following a feeding schedule have significant increases in NPY mRNA levels in different brain regions after 72 h of food deprivation [12]. Overall, all these data agree with the role of this peptide as a mediator of feeding stimulation by fasting in goldfish [11]. The existence of a possible functional relationship between FAA and NPY synthesis before feeding behaviour remains to be investigated.

The similarity in circulating cortisol profile found in both groups, scheduled and randomly fed, indicates a lack of entrainment of this hormone to feeding time in this species. This assertion agrees with a recent report in channel catfish [31], but differs from results in other fish [32, 33]. Other factors, apart from feeding, must be considered in an analysis of the entrainment of circulating cortisol, e.g. light-dark cycle, rest-activity, which would explain such different results.

A complex relationship exists between feeding pattern and circulating cortisol, depending on species, nutritional status, and many other variables. In our study, the cortisol reduction observed four hours after feeding, regardless of feeding regime, may be a direct consequence of the physiological postprandial status of fish (on the sampling day food was provided at the same time to both groups I and II). In fact, a sharp fall in plasma cortisol levels also occurs after feeding in other teleosts [34, 31].

The fact that the average values of plasma cortisol were significantly higher in goldfish from group II than in group I might indicate higher stress in goldfish fed randomly. Moreover, goldfish from this group showed a higher total daily activity, which supports a stress induced by the lack of feeding time as entrainer. It is to be noted that those fish kept in the absence of external time cues showed arrhythmic (continuous) locomotor activity patterns, which may be stressful.

GIT melatonin production in mammals and fish is influenced by different feeding conditions [35, 36], regardless of light. The relationship between food intake

and melatonin production in the GIT tract has been demonstrated in several studies [37, 38]. Although variations of night time plasma melatonin seem to be controlled by photoperiodic changes, basal daytime melatonin levels appear to be controlled by nutritional factors [39]. In our study we found that both groups I and II showed a sharp increase of GIT melatonin 4 hours after food was supplied. This result suggests the existence of direct relationship between GIT melatonin production and food intake in goldfish, and reinforces the role of melatonin as an anorectic signal in the feeding regulation in this species [40]. Several studies indicate that daytime plasma melatonin could have a gastrointestinal origin. Some authors found melatonin in the plasma of goldfish after pinealectomy combined with a bilateral removal of eyes, concluding that the residual concentrations of plasma melatonin had a gastrointestinal origin [41]. However, in our experiments plasma melatonin remained equal (around 150 pg/ml) at all sampling times and in all experimental groups, suggesting that GIT melatonin is not secreted into blood. Previous studies described how plasma melatonin in goldfish under LL remained at low levels (75-225 pg/ml) and failed to exhibit daily rhythms [41], which is in accordance with our findings. Further studies will be needed to elucidate the source of plasma melatonin under LL.

The FAA observed in goldfish fed once a day agrees with previous results obtained in this species, which indicated that scheduled feeding acts as a potent zeitgeber in entraining locomotor activity. Some authors have suggested the existence of separate but tightly coupled LEO and FEO, or a single oscillator entrainable by both light and food, in which feeding entrainment could overcome light entrainment in some cases [4]. Concerning this last theory, a multi-oscillatory system entrainable by lights and influenced by food that drives the development of FAA has been proposed [5]. In our study, animals were kept under LL to avoid the effect of light entrainment, so that

locomotor activity rhythms would be controlled exclusively by an FEO in fish fed periodically. In contrast, goldfish under LL and fed randomly showed an arrhythmic pattern of locomotor activity, which reflected the lack of external time cues entraining endogenous oscillators (i.e. LEO and FEO).

In mammals such an LEO is located in the suprachiasmatic nuclei (SCN) of the hypothalamus [42]. In fish, as well in mammals, the location of the FEO and the physiological mechanisms involved in the transduction of feeding entrainment remain unknown.

To conclude, our results showed that scheduled feeding entrains behavioural and physiological patterns in goldfish, which may optimize nutritional functions. Animals fed at the same time each day showed FAA: amylase secretion was synchronized by periodical food delivery and hypothalamic NPY production anticipated the feeding time. Plasma cortisol and GIT melatonin were modified by food intake, with a postprandial reduction in cortisol and a postprandial increase in GIT melatonin.

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## FIGURE LEGENDS

**Figure 1.** Actograms (left) and average diel profile (right) of locomotor activity from representative goldfish showing FAA when submitted to scheduled feeding (upper panels), and a continuous pattern of locomotion when submitted to random feeding (lower panels). Actograms are double-plotted for better visualization. The white bars at the top of each graph indicate the photoperiod (LL).

**Figure 2.** Amylase (A) and protease (B) activity from goldfish submitted to scheduled feeding (dark grey bars), random feeding (light grey bars) and two days fasting (white bars). Samples were taken eight hours before (-8h), two hours before (-2h) and four hours after (+4h) meal time of group I. Values represent the mean ± SEM. Numbers in

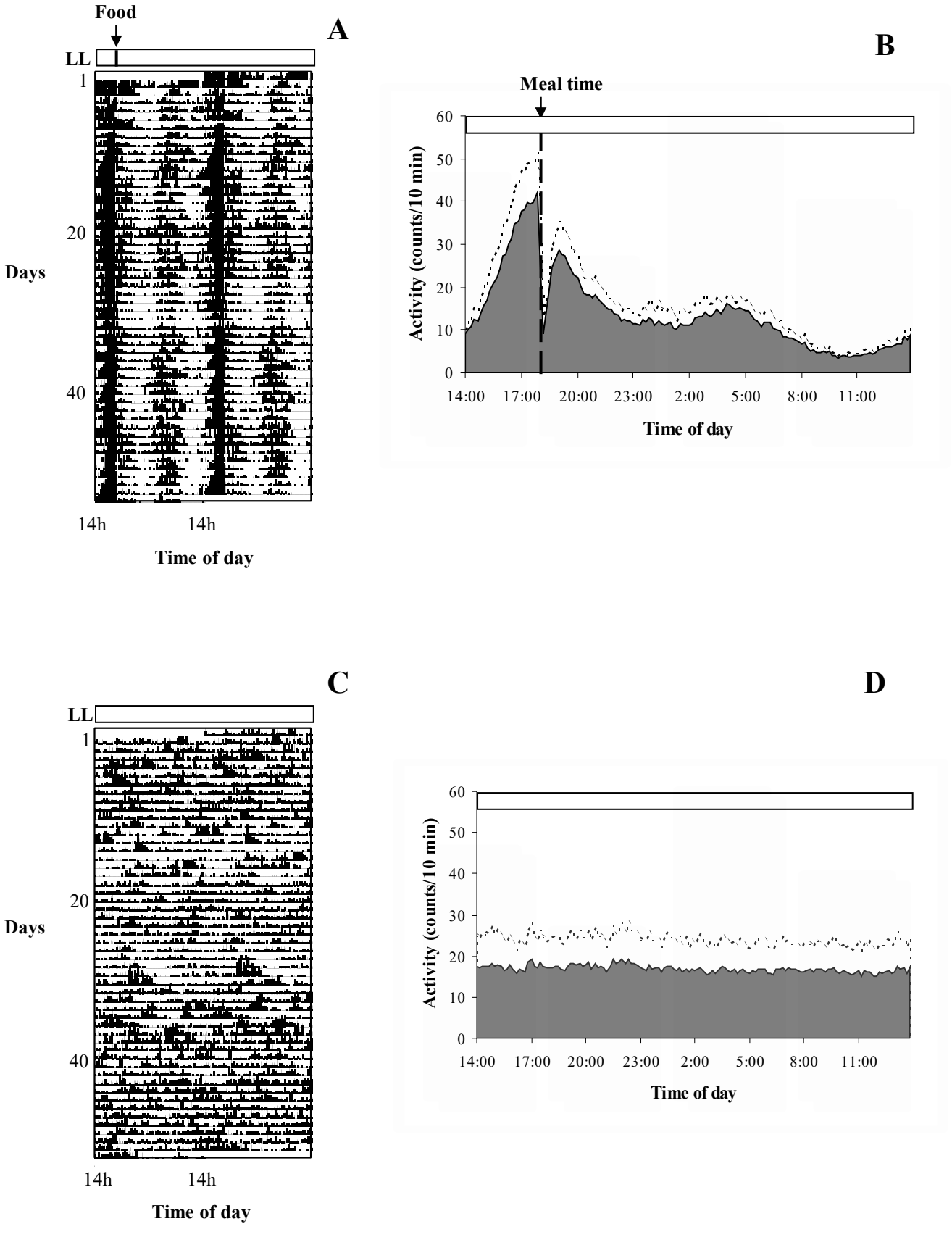
brackets indicate the number of replicates per group. Different letters indicate statistically significant differences between sampling points (ANOVA, Duncan's test, $P<0.05$).

**Figure 3.** Hypothalamic NPY content in goldfish submitted to scheduled (dark grey bars) and random (light grey bars) feeding. Graph definitions as given in Fig. 2.

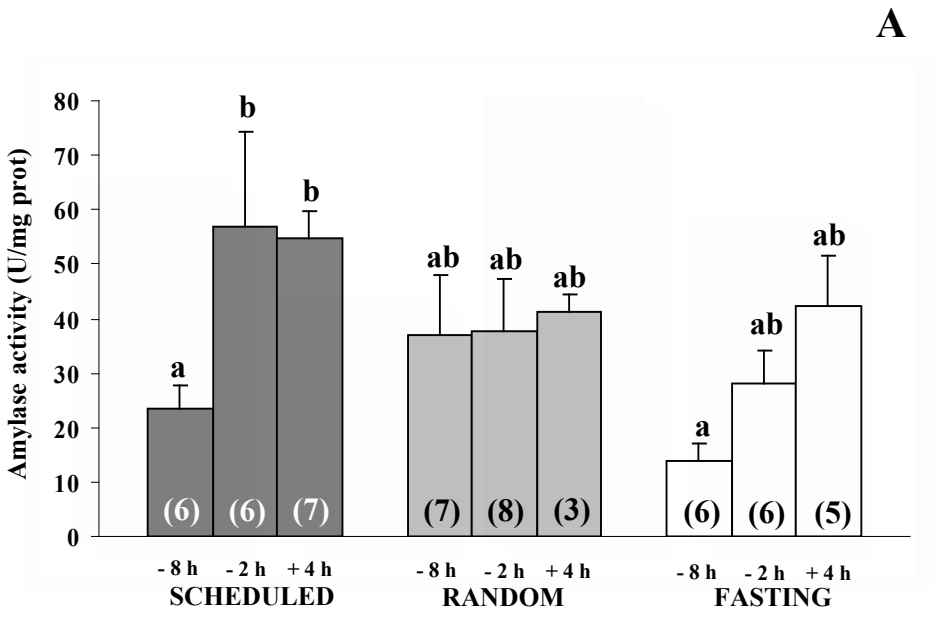
**Figure 4.** Plasma cortisol levels in goldfish submitted to scheduled (black bars) and random (light grey bars) feeding. Graph definitions as given in Fig. 2.

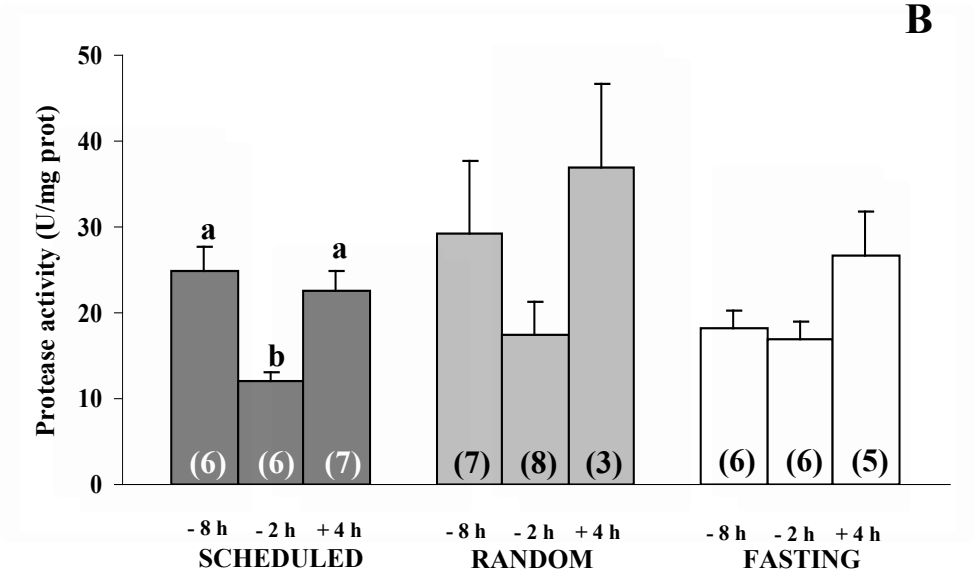
**Figure 5.** GIT (A) and plasma (B) melatonin concentrations in goldfish submitted to scheduled (dark grey bars) and random (light grey bars) feeding. Graph definitions as given in Fig. 2.

# FIGURE 1

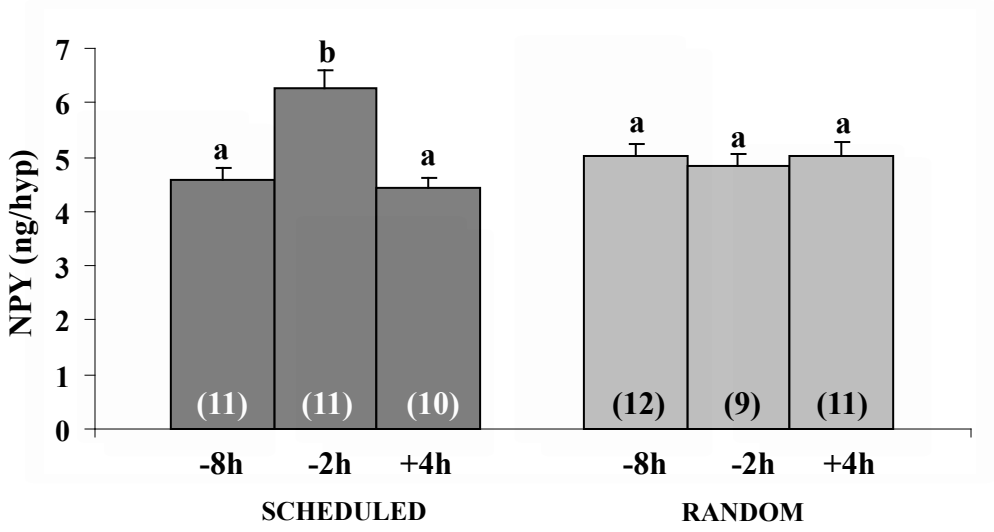

A
Food
LL
1
20
40
Days
14h
14h
Time of day
B
Meal time
Activity (counts/10 min)
60
50
40
30
20
10
0
14:00
17:00
20:00
23:00
2:00
5:00
8:00
11:00
Time of day
C
LL
1
20
40
Days
14h
14h
Time of day
D
Activity (counts/10 min)
60
50
40
30
20
10
0
14:00
17:00
20:00
23:00
2:00
5:00
8:00
11:00
Time of day

# FIGURE 2

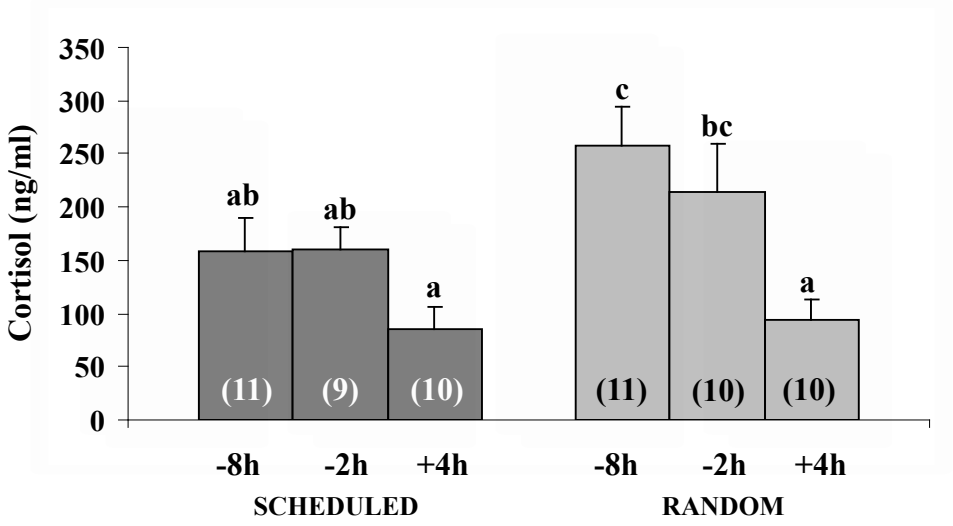

A
Amylase activity (U/mg prot)
80
70
60
50
40
30
20
10
0
a
b
b
ab
ab
ab
a
ab
ab
(6)
(6)
(7)
(7)
(8)
(3)
(6)
(6)
(5)
- 8 h
- 2 h
+ 4 h
SCHEDULED
RANDOM
FASTING


B
Protease activity (U/mg prot)
50
40
30
20
10
0
a
b
a
(6)
(6)
(7)
(7)
(8)
(3)
(6)
(6)
(5)
- 8 h
- 2 h
+ 4 h
SCHEDULED
RANDOM
FASTING

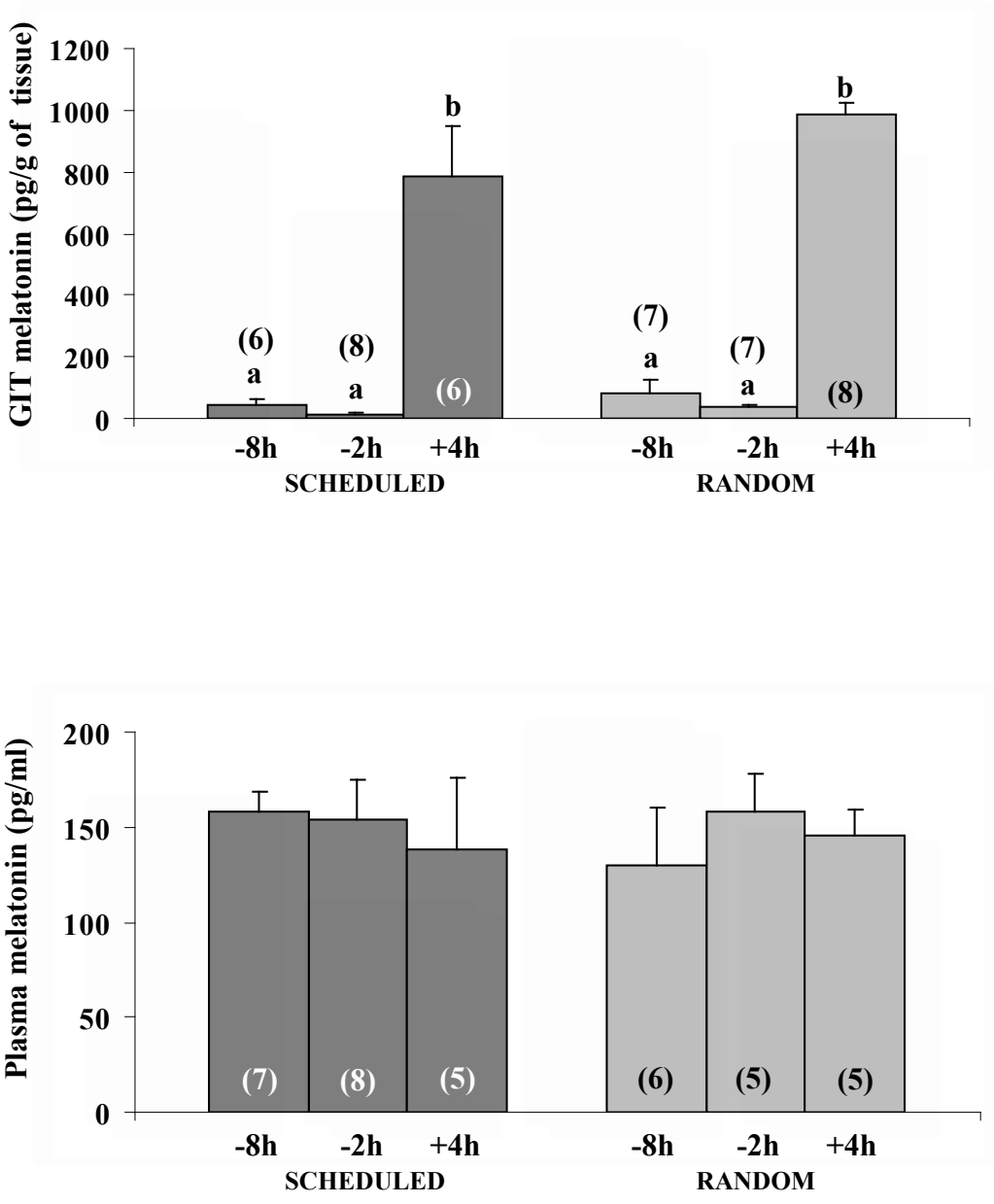
# FIGURE 3


NPY (ng/hyp)
7
6
5
4
3
2
1
0
a
b
a
a
a
a
(11)
(11)
(10)
(12)
(9)
(11)
-8h
-2h
+4h
-8h
-2h
+4h
SCHEDULED
RANDOM

# FIGURE 4


Cortisol (ng/ml)
350
300
250
200
150
100
50
0
ab
ab
a
c
bc
a
(11)
(9)
(10)
(11)
(10)
(10)
-8h
-2h
+4h
-8h
-2h
+4h
SCHEDULED
RANDOM

# FIGURE 5


GIT melatonin (pg/g of tissue)
1200
1000
800
600
400
200
0
(6)
a
(8)
a
b
(6)
(7)
a
(7)
a
b
(8)
-8h
-2h
+4h
-8h
-2h
+4h
SCHEDULED
RANDOM
Plasma melatonin (pg/ml)
200
150
100
50
0
(7)
(8)
(5)
(6)
(5)
(5)
-8h
-2h
+4h
-8h
-2h
+4h
SCHEDULED
RANDOM