Feeding regimes affected the circadian rhythms of pancreatic digestive enzymes and somatic growth in flathead grey mullet (*Mugil cephalus*) fry

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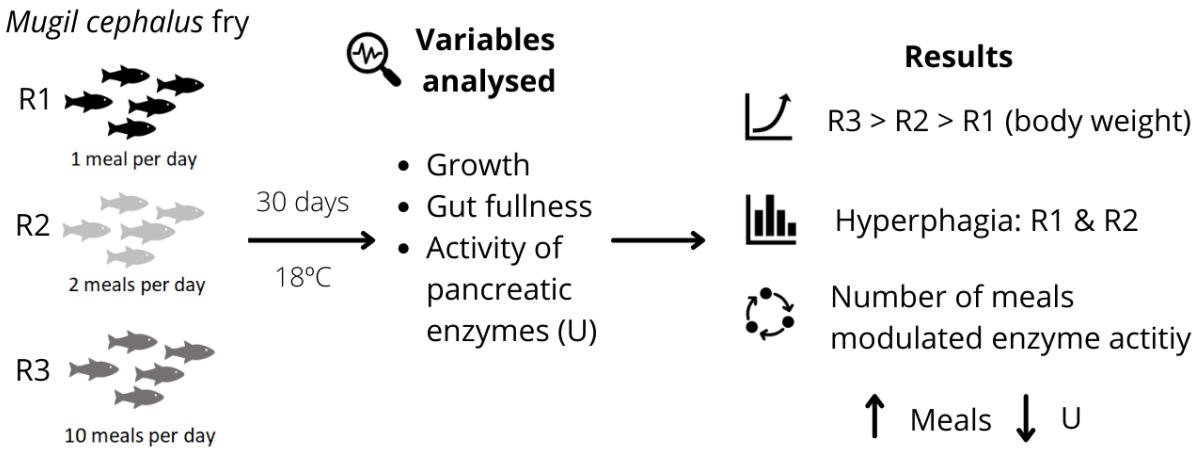
Highlights

- Feeding regimens directly affected growth performance in grey mullet fry.
- The number of daily meals modulated feeding behaviour
- The number of daily meals affected digestive enzyme activity levels.
- Feeding grey mullet fry just one or two meals per day promoted hyperphagia
- Feeding grey mullet fry continuously during daylight hours enhances feed digestion and somatic growth.

Graphical Abstract



Abstract

The effect of different feeding regimes on the circadian rhythms of pancreatic digestive enzyme activities was evaluated in *Mugil cephalus* fry weighting 0.34 ± 0.01 g. Feeding regimes (feeding ration = 3% stocked biomass) differed on the number of meals offered per day: one, two and ten meals per day (R1, R2 and R3, respectively). The number of meals per day affected somatic growth; in particular, fry from the R3 group (0.80 ± 0.01 g) grew better than their congeners from the R2 (0.70 ± 0.01 g) and R1 (0.63 ± 0.01 g) groups ($P < 0.05$). Feeding behaviour was modulated by the feeding regime, being the maximal gut fullness values found just after meal distribution in R1 and R2 groups, whereas this trend was not observed when feed was offered continuously during light hours (R3). Fry from the R1 and R2 groups showed hyperphagia as they tended to store in their gut as much as possible feed particles to be later digested due to the limited daily meals. This strategy negatively affected feed digestion due to inappropriate enzyme to substrate ratio, changes in digestive enzyme activities and chyme transit times, which ultimately impaired growth performance. Enzyme activities were modulated by the number of meals, the more frequent the meals offered, the lower enzyme activities, supporting the hypothesis that digestive function is adapted to obtain a maximum benefit of the ingested nutrients. Present results showed that feeding grey mullet fry continuously during day light hours optimized feed digestion and promoted fry growth.

Keywords: circadian rhythm; pancreatic digestive enzymes; feeding regime; grey mullet.

1. Introduction

Growth performance is a key performance indicator of economic importance in animal production that is influenced by many different factors (i.e., genotype, production technology, husbandry and feeding conditions, health management, etc.). Among them, feeding protocol is among of the most important ones and its standardization and application should be based on the deep knowledge of different aspects of feeding behaviour, general physiology and ecology of farmed animals. According to many studies, one of the ways to improve a feeding protocol is to take into account as many as possible factors that could significantly influence animal nutrition. One of such factors is the rhythmicity of different physiological processes, including digestion (Delahunty et al., 1978; Boujard et al., 1996; Kohbara et al., 2003; Navarro et al., 2009; Flood et al., 2011; Gilannejad et al., 2021a, b). For many farmed fish species, it has been shown that feeding frequency and timing must be incorporated in feeding protocol in order to improve feed efficiency and maximize growth performance. It is generally advised that the best results in terms of feed efficiency and growth performance indicators may be obtained by the application of a feeding regime that most closely reflects the natural feeding rhythms of the species at the stage of development considered (Lanteri et al., 2016). In this sense, there are many studies that have revealed the rhythmicity patterns (*e.g.* circadian, seasonal, annual, etc.) associated with feeding behaviour and digestive physiological parameters (i.e. secretion of hydrochloric acid, bile, pancreatic digestive enzymes, etc.) in both marine and freshwater fish species (Delahunty et al., 1978; Aranda et al., 1999; Nordgarden et al., 2003; Kousoulaki et al., 2015; Navarro-Guillén et al., 2018). The rhythmicity of the above-mentioned patterns under natural conditions is dependent on a broad range of different biotic and abiotic factors that may be listed as water temperature (Fraser et al., 1993), the light/dark ratio (Cuenca, de la Higuera, 1993; Aranda et al., 1999), light intensity (Fraser, Metcalfe, 1997) and spectrum (Choi et al., 2014), feeding regime (Yúfera et al., 2014), concentration of dissolved gases in water, food density and availability (Modica et al., 2014), competition with other fish species and among different individuals of the same species, among others. Under aquaculture conditions, the number of these factors tends to be lower because many of the above-mentioned factors could be easily controlled and/or their range of variability substantially reduced. Thus, the influence of feeding regimes on different physiological parameters has received a lot of attention in farmed fish. Using an inappropriate feeding schedule may lead to miss the period of active feeding as fish show different levels of appetite along the day (Ma et al., 2006; Wang et al., 2008; Cowan et al., 2017). Feeding strategies based on circadian rhythms may dramatically reduce feed wastage; thus, directly impacting on feed conversion ratios as well as reducing the impact on the environment (Hossain et al., 2001; Amirkolaie, 2011). Hence, the optimization of daily feeding schedule

according to physiological fish demands and developmental stages may be a good strategy for optimizing somatic growth performance and reducing waste (non-ingested feed) in farmed fish.

The digestive process is tightly linked to feeding regime in fish. Thus, Yúfera et al. (2014) demonstrated that when fish was feeding with only one daily meal, the postprandial digestive process was fitted within a 24-hour period, but when several meals were offered, a more complex pattern resulting from overlapping of the different digestive processes (i.e., activity of enzymes, pH level in stomach, level of gut fullness) was found. In this context, the development of optimal feeding protocols designed for promoting a more efficient food digestion requires a deeper knowledge on how the feeding time and frequency may influence different digestive factors (*e.g.*, feed transit, gastric and intestinal pH, molecular expression of enzyme precursors and pepsin activity, etc) and to what extent the circadian cycle affects and modulates the whole digestive function. Thus, the synchronization between food distribution and the feeding demand of fish in tanks may significantly improve farming procedures in fish. Unfortunately, for many fish species the daily feeding rhythms have not been properly investigated and consequently, feeding practices are based on feed distribution in one or several times per day regardless of the real fish circadian rhythms. Regarding their feeding behaviour, most teleost fish are either diurnal or nocturnal feeders (Reebs, 2002) and, similarly to other vertebrates, the photocycle is a signal of primary importance in the synchronization of their daily feeding rhythms (Pando et al., 2001). While day and night cycle defines the predatory activity and food intake in juvenile and adult fish, the postprandial processing of the ingested food may last many hours independently of illumination conditions, which is also of special relevance when evaluating the digestive capacities of the organism (López-Olmeda and Sánchez-Vázquez, 2010).

Grey mullets (family *Mugilidae*) are a group of omnivorous and detritivorous species that are candidates for aquaculture diversification in the Mediterranean region, as well as in other regions of the world (Republic of Korea, Taiwan Province of China, South Africa). Regardless of the interest on this group of species, little is known about their feeding and nutritional requirements for their proper cultivation (Whitfield et al., 2012). Under aquaculture conditions, these species demonstrate good adaptation to captivity, rapid growth, omnivorous feeding habits and high market price of its salt-cured and dried eggs named "bottarga" (Whitfield et al., 2012). A previous field study in conducted in several grey mullet species, *Liza ramada*, *L. saliens*, *Chelon labrorus* showed that they had a diurnal feeding behaviour, although *C. labrosus* showed a peak in feeding activity at dusk (Gisbert et al., 1997). This information may be of value for improving current feeding protocols for another cultured grey mullet species, the flathead grey mullet (*Mugil cephalus*). Thus, the main aim of the study was to estimate the activities of main pancreatic

enzymes in the intestine of grey mullet fry reared under three different feeding regimes that differed on the number of meals per day.

2. Materials and Methods

2.1 Animals and rearing conditions

Wild grey mullet fry (24.2 ± 0.8 mm in standard length, SL; 0.202 ± 0.05 g in wet body weight, BW; N = 3,500) were caught as described in Gisbert and López (2008) and obtained from Pescados y Mariscos Roset S.L. (Deltebre, Spain). Fry were transported to IRTA facilities at St. Carles de la Rapita (IRTA-SCR, Spain) where they were acclimated to 17 °C for 21 days in brackish water (14‰). Once acclimated, fry were distributed among 9 cylindroconical 100-L tanks (*n* = 350 per tank) connected to a recirculation system IRTAmar® in order to maintain water quality through UV, biological, and mechanical filtration. Water quality conditions during the experimental period were as follows: temperature, 18.1 ± 0.3 °C (mean ± standard deviation, SD); salinity, 1.2 ± 0.2‰; dissolved oxygen, 6.5 ± 0.4 mg L^{-1} (~90% saturation); NH_4^+, 0.20–0.29 mg l^{-1}; NO_2^-, 0.001 mg l^{-1}, and the photoperiod was 10 h light:14 h darkness (light hours: 08:00 to 18:00 h). Water temperature and salinity were chosen considering the physicochemical characteristics of estuarine waters where wild fry were captured for experimental purposes. The initial BW and SL of fry used in this study was 0.34 ± 0.01 g and 24.0 ± 0.02 mm, respectively. During this period, fish were fed at 3% of the biomass per day (apparent satiation) with a compound diet (36% crude protein, 15.9% crude fat, 1,771 J kg^{-1}; see Gisbert et al. (2016) for diet formulation and ingredient list).

2.2 Experimental design and sampling

Experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. In order to evaluate the impact of feeding regimes on the circadian rhythm of enzyme production and secretion, a 30-days trial was conducted in which fry were offered three different feeding regimes that differed on the number of meals offered per day, but at a fixed feeding ratio of 3% of the stocked biomass. The first treatment consisted on offering feed in just one single meal per day, between 9 and 10 h (R1). Another experimental treatment consisted on offering feed in two meals per day between 9 and 10:00 h and between 14:00 and 15:00 h (R2), whereas the third group of fish was fed every hour during light hours (08:00 to 18:00 h; ten meals per day) (R3). All treatments were tested in triplicate. Regardless of the dietary treatment, feed was distributed every 10 minutes. Fish were kept under these feeding regimes for 30 days. At the end of this period, fry (n = 100 per tank) were captured with a scoop-net, gently anesthetized (50 mg L^{-1} tricaine methane sulfonate. MS-222,

Sigma-Aldrich, Madrid, Spain) and individually measured in body weight (BW) and standard length (SL) to the nearest 0.1 g and 1 mm, respectively. These values were also used for calculating individual Fulton's condition (K) values using the following formula: $K = 100 \times (BW/SL^3)$. Fry specific growth rates (SGR) were calculated as follows: SGR (% BW day^{-1}) = [(ln BWf- ln BWi) / t] x 100, were BWf and BWi are the final and initial BW values, respectively, and *t* the number of days that the trial lasted. In addition, forty fry were sampled every three hours (04:00, 07:00, 10:00, 13:00, 15:00, 18:00, 21:00, and 24:00 h) from each tank and feeding condition to assess the level of fullness of their gut (n = 10), and activity of pancreatic digestive enzymes (n = 30). For such purposes, fish were sacrificed with an overdose of anaesthetic (300 mg MS-222 L^{-1}). Fry used for evaluating the level of gut fullness were fixed in ethanol (70%) and stored at 4 ºC until further examination, while the specimens sampled for assessing the activity of pancreatic digestive enzymes were frozen at -80 ºC.

2.3 Evaluation of gut fullness

Gut fullness was determined in the stomach and intestine every three hours (04:00, 07:00, 10:00, 13:00, 15:00, 18:00, 21:00, and 24:00 h). Firstly, the whole stomach was detached from the alimentary system by removing the posterior part of the oesophagus and intestine and its weight (accuracy: 0.1 mg), including its content, measured using an electronic microbalance MX5 (Mettler Toledo, USA). Then, the stomach was dissected and the weight of the stomach content was also measured. In addition, the level of stomach fullness was expressed as a ratio between the stomach content weight and fry BW, expressed as a percentage. In addition to the stomach, the level of fullness of the intestine was also assessed. The degree of fullness of the intestine was assessed qualitatively using arbitrary units (AU) ranging from 0 (empty intestine) to 5 (full intestine), as it was not possible to accurately separate the intestinal mucosa from the intestinal content. Total consumed food was calculated as a sum of average value of consumed food in fish stomach for every sampling point adjusted to 1,000 mg of fish body weight.

2.4 Determination of pancreatic digestive enzyme activities

Fish were dissected (head and caudal part of body discarded) on a glass plate at 0–4 °C and the digestive region, including digestive tract and hepatopancreas, and dorsal musculature were removed and frozen at -80 ºC until their analysis. Samples were homogenized in 5 volumes v/w of distillated water (1–2 min at 0–4 °C) and sonicated; then, samples were centrifuged (3,300 x *g*, 3 min at 4 °C) and the supernatant was collected, aliquoted and frozen at −80 °C for the determination of pancreatic enzymes (Gisbert et al., 2009). Samples were processed and handled

following the indications of Solovyev and Gisbert (2016) in order to prevent their degradation during their storage and handling.

Total alkaline proteases were assayed after 15 min of incubation at 25 °C (endpoint measurement) using 0.5 % (w/v) azo-casein as substrate in 50mM Tris-HCl buffer (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 μmol azo-casein hydrolyzed min^{-1} mL^{-1} of extract at λ = 366 nm (García-Careño and Haard, 1984). Trypsin (E.C. 3.4.21.4) was assayed at 25 °C (kinetic measurement) using BAPNA as a substrate in 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer (pH 8.2). One unit of trypsin per ml (U) was defined as 1 μmol BAPNA hydrolyzed min^{-1} mL^{-1} of extract at λ = 407 nm (Holm et al., 1988). Chymotrypsin (EC. 3.4.21.1) activity was quantified at 25 °C (kinetic measurement) using BTEE as substrate in 80 mM Tris-HCl, 100 mM $CaCl_2$ buffer (pH 7.8). Chymotrypsin activity (U) corresponded to the μmol BTEE hydrolyzed min^{-1} mL^{-1} of extract at λ = 256 nm (Worthington, 1991). Alpha-amylase (E.C. 3.2.1.1) was measured using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate (Métais and Bieth, 1968) and its activity (U) was defined as the mg of starch hydrolyzed during 3 min mL^{-1} of extract at 25 °C at λ = 580 nm (endpoint measurement). Bile salt-activated lipase (E.C. 3.1.1) activity was assayed for 20 min at 25 °C (endpoint measurement) using p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl (pH 7.9), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone: *n*-heptane (5:2), the extract centrifuged (6,080g, 2 min at 4 °C) and the absorbance of the supernatant read at λ = 405 nm. The activity of bile salt-activated lipase (U) was defined as the μmol of substrate hydrolyzed min^{-1} mL^{-1} of extract (Iijima et al., 1998). The determination of pancreatic enzyme activities was performed along the experimental period (4, 7, 10, 13, 15, 18, 21, and 24 h) and expressed as total activity (U per one gram of tissue). In order to compare activity of different enzymes among different feeding groups at day and night light hours, the average activity for all day (all measurements) and during light hours (sampling points: 10, 13, 15, and 18 h) and night hours (sampling points: 4, 7, 21, and 24 h) was calculated for each studied enzyme.

2.5 Statistical analysis

All data are presented as means ± standard error of the mean (SEM). As fry BW and SL were normally distributed (Shapiro-Wilk test, $P < 0.001$), differences in growth among experimental groups were analysed using an one-way ANOVA with Tukey's post hoc test. As the activity of digestive enzymes was not normal (Shapiro-Wilk test, $P > 0.05$), data were compared by means of a non-parametric test. In particular, the level of activity of digestive enzymes between different sampling points (hours) for each feeding regime and between different regimes (R1, R2, R3) for every sampling point were compared by the non-parametric Dunn's *post hoc* test. To assess the

statistical significance of the effects of the feeding regimes and the night/light hours (time of sampling) as factors, the two (and one)-way PERMANOVAs were applied (Hammer et al., 2001). The coefficient of determination for every factor was estimated according to following formula: $R^2 = (1–SS_{res}/SS_{tot}) \times 100$, where SS_{res} – residual sum of squares, SS_{tot} – total sum of squares and the data for calculation were obtained from two-way PERMANOVA. The correlation analysis between activities of different enzymes was conducted by means of the R Spearman rank test. All calculations were performed using the STATISTICA software package, version 8 (StatSoft Inc., Tulsa, OK; www.statsoft.com) and PAST 4.03 software. In all tests, the significance level was set at $P < 0.05$.

3. Results

3.1 Effect of feeding regimes on somatic growth

Differences in BW and SL of grey mullet fry subjected to different feeding regimes are shown in Table 1. The effect of feeding regime on fry's BW, SL, K and SGR values was significant (one-way ANOVA, $P < 0.05$). In particular, values of BW, SL, K, and SGR fry from the R3 group were higher than in the R1 and R2 groups (Tukey *post hoc* test, $P < 0.05$).

Table 1. Body size in terms of standard length (SL), wet body weight (BW), specific growth rate (SGR, % BW day^{-1}) and Fulton's condition factor (K) of grey mullet (*M. cephalus*) fry fed a fixed feed ration distributed in different meals per day (R1: 1 meal; R2: 2 meals and R3: every hour during day light hours, ten meals). Different lowercase letters designate the significant differences among feeding regimes (Tukey HSD test, at $P \leq 0.05$).

Feeding regime	SL (mm)	BW (g)	K	SGR (% BW day^{-1})
R1	31.9 ± 0.19^a	0.63 ± 0.01^a	0.19 ± 0.002^a	1.93 ± 0.06^a
R2	32.9 ± 0.23^b	0.70 ± 0.01^b	0.19 ± 0.002^a	2.24 ± 0.07^b
R3	34.0 ± 0.22^c	0.80 ± 0.01^c	0.20 ± 0.003^b	2.74 ± 0.06^c

3.2 Filling and evacuation gut patterns

Gut fullness levels of grey mullet fry offered different feeding regimes are presented in Figure 2. In the R1 and R3 groups, general feed ingestion patterns were similar and showed a single daily peak of fullness for both stomach and intestine (Fig. 2a, c), whereas two peaks of gut fullness were registered in the R2 group (Fig. 2b). The maximum level of stomach fullness coincided with

feeding time for the R1 (10:00 h) and R2 groups (10:00 and 15:00 h), whereas for the R3 group (the feed ration was distributed in ten meals along day light hours) the maximum was noted at 13:00 h. The maximum levels of fullness in the intestine were registered at 5 h after the maximum level of stomach fullness in the R1 group, whereas this occurred between 3 and 4 h post maximum level of stomach fullness in the R2 and R3 groups, respectively. The differences of fullness in the stomach among different feeding regimes were statistically significant at 13:00 h (R1 *vs*. R2, R2 *vs.* R3) and at 18:00 h (one-way PERMANOVA, $P < 0.05$). There was a delay between stomach and intestine filling in the R1 and R2 groups, whereas in the peaks of stomach and intestine fullness coincided in fry from the R3 group. The fullness of stomach significantly decreased from the R1 group (3.5 AU) to the R2 (2.5-3.0 AU) and R3 (2.5 AU) groups. Regarding the intestine, this pattern was absent and fullness levels were stable (around 4.0 AU) independently to the feeding regime. Total consumed feed (mg of feed in stomach per 1000 mg of fry weight) was similar (one-way PERMANOVA, $P > 0.05$) among different feeding groups. The level of stomach fullness decreased steadily over time, followed by an increase in the content of intestinal digesta. In particular, most part of the stomach content was evacuated during the light-hours period in the R1 group, whereas the evacuation time for the R2 and R3 groups was longer (3 and 6 hours after maximum stomach fullness values, respectively). The evacuation time for intestinal digesta was similar among groups (one-way PERMANOVA, $P > 0.05$). No food was found in stomach as well as in intestine in all three groups after 24:00 h.

3.3 Circadian activity of pancreatic digestive enzymes in R1, R2, and R3 feeding regimes

The total activities of studied pancreatic enzymes in grey mullet fry fed according to different feeding regimes are shown in Figure 3. All studied enzymes had similar total activity profiles for the R1 and R2 groups. In particular, there was a peak of activity before first feeding and day light hours (07:00 h) for all alkaline proteases and α-amylase, whereas for bile salt-activated lipase this peak was recorded later at 10:00 h. Then, the total activity dropped between 10:00 and 13:00 h, and remained stable until 18:00 h, whereas total activity increased again at 21:00 and 24:00 h. The complete list of significant differences according to Dunn's test ($P < 0.05$) is presented in Supplementary file 1.

Regarding total activity values of pancreatic digestive enzymes in grey mullet fry from the R3 group, the activity of all enzymes was maximal at 24:00 h. In the case of α-amylase, its total activity progressively decreased from 07:00 h to its minimum value at 18:00 h and, then, it progressively increased until 24:00 h. The total activity of bile salt-activated lipase progressively dropped from 07:00 h to its minimum value at 15:00 h, whereas total activity values were recovered by 18:00 h. The activity of chymotrypsin progressively increased from 07:00 to 24:00

h. In contrast, trypsin (Dunn's test, $P = 0.95$) and total alkaline proteases (Dunn's test, $P = 0.83$) activities were constant along all sampling times ($P > 0.05$).

When considering the average all-day-activity values for all the assayed enzymes, they were significantly lower for the R3 group in comparison to the R1 and R2 groups (Table 1). During the night hours, the average activity values of all studied enzymes was also significantly higher in the R1 group than in R3 group (one-way PERMANOVA, $P < 0.05$), whereas during light hours these significant differences were absent with the exception of chymotrypsin (one-way PERMANOVA, $P < 0.05$) (Table 1). The complete list of significant differences is presented in Supplementary file 2.

The average activity of α-amylase, trypsin and total proteases was significantly higher during night hours than during the day light hours in the R1 group, whereas for the R2 group only significant differences were found for α-amylase and total alkaline proteases (one-way PERMANOVA, $P < 0.05$). There were no significant differences between night and light hours for the studied enzymes in the R3 group, although α-amylase was the exception (one-way PERMANOVA, $P < 0.05$) (Table 1). The complete list of significant differences is presented in Supplementary file 3).

Table 1. Total activity values of pancreatic digestive enzymes (mean ± SEM) in grey mullet (*M. cephalus*) fry during day light and night conditions fed a fixed feed ration distributed in different meals per day (1 meal: R1; 2 meals: R2 and every hour during day light hours, ten meals: R3).

Enzymes	**Feeding regimes**								
	R1			**R2**			**R3**		
	N	**L**	**All day**	**N**	**L**	**All day**	**N**	**L**	**All day**
α-amylase	189.9±11.6*^{A}	98.1±7.7	144.0±11.7^{b}	146.1±12.5*^{B}	84.0±3.4	115.1±9.1^{b}	105.6±6.2*^{C}	82.0±5.0	93.8±4.6^{a}
Bile salt-activated lipase	0.30±0.01^{A}	0.25±0.02	0.28±0.01^{b}	0.28±0.01^{A}	0.23±0.03	0.26±0.02^{b}	0.23±0.01^{B}	0.20±0.01	0.22±0.01^{a}
Chymotrypsin	4.6±0.26^{A}	4.0±0.21^{A}	4.3±0.18^{b}	4.1±0.35^{AB}	3.7±0.12^{A}	3.9±0.19^{b}	3.2±0.28^{B}	3.2±0.16^{B}	3.2±0.16^{a}
Trypsin	1.1±0.09^{A}	0.8±0.09	0.98±0.07^{b}	1.0±0.06*^{A}	0.7±0.03	0.87±0.04^{b}	0.7±0.04^{B}	0.7±0.04	0.7±0.03^{a}
Total proteases	3.0±0.21*^{A}	2.2±0.19	2.59±0.16^{bA}	2.7±0.14*^{A}	2.0± 0.06	2.37±0.11^{b}	2.0±0.07^{B}	1.9±0.11	1.96±0.06^{a}

Lowercase letters denote the differences among different feeding regimes for all day; uppercase letters denote the differences among different feeding regimes for night "N" and light "L" hours, whereas the asterisk (*) denotes statistically significant differences between night and light hours for each feeding regime (one-way PERMANOVA). Day light period: 08:00 to 18.00 h. *Abbreviations*: D, night hours; L, day light hours.

3.4 Relationships between feeding regime, night/light hours, time of sampling and total activity of digestive enzymes

The feeding regime, night/light hours, and time of sampling were factors that significantly affected the total activity of all studied pancreatic enzymes (two-way PERMANOVA, $P < 0.05$), although bile salt-activated lipase was an exception (night/light hours as a factor was not significant, two-way PERMANOVA, $P = 0.18$). The percent of explained dispersion was highest for the factor "sampling time" and minimum for the factor "night/light hours" (Table 2). The interaction between studied factors was significant only for activity of α-amylase (two-way PERMANOVA, $P = 0.001$) (Table 2).

Table 2. Coefficient of determination values (R^2) for all studied pancreatic enzymes for feeding regime and night/light hours as one pair of factors and feeding regime and sampling time as another ones (two-way PERMANOVA).

Factors	**α-amylase**	**Bile salt-activated lipase**	**Trypsin**	**Chymotrypsin**	**Total alkaline proteases**
Feeding regime	18.6	12.3	17.8	21.6	17.4
Night/Light hours	25.5	2.2	11.0	4.9	19.4
Interaction	1.3	2.2	0.2	-9.6	1.5
Total	**45.4**	**16.7**	**29.1**	**26.5**	**38.2**
Feeding regime	18.6	12.3	17.8	21.6	17.4
Sampling time	48.1	28.1	25.1	26.1	35.4
Interaction	16.7	18.8	15.1	12.4	16.2
Total	**83.4**	**59.1**	**58.1**	**60.1**	**68.9**

According to the two-way PERMANOVA results ($P < 0.05$), during the night hours the feeding regime was a significant factor for explaining changes in the activity patterns observed for all studied enzymes. Based on one-way PERMANOVA, the different for each enzyme were as follow: bile-salt activated lipase (R1, R2 *vs*. R3, at $P = 0.0004$ and 0.002, respectively), total alkaline proteases (R1, R2 *vs*. R3, at $P = 0.0002$ and 0.0003, respectively), α-amylase (R1 *vs*. R2 *vs*. R3, at $P = 0.0001$ and 0.0002, respectively), chymotrypsin (R1 *vs*. R3, at $P = 0.003$), and trypsin (R1, R2 *vs.* R3, at $P = 0.0008$ and 0.0004, respectively). The complete list of significant differences according to one-way PERMANOVA ($P < 0.05$) is presented in Supplementary file 2. In contrast, the sampling time was only significant as a factor (two-way PERMANOVA) for explaining the

activity of total alkaline proteases ($P < 0.008$), α-amylase ($P < 0.004$) and chymotrypsin ($P < 0.003$).

Considering the day light hours, the feeding regime based on two-way PERMANOVA was a significant factor for explaining changes in the activity patterns observed for chymotrypsin ($P = 0.01$) and α-amylase ($P = 0.03$). Based on one-way PERMANOVA, the different for each enzyme were as follow: α-amylase (R1 *vs*. R3, at $P = 0.08$) and chymotrypsin (R1, R2 *vs*. R3, at $P = 0.009$ and 0.03, respectively). The complete list of significant differences according to one-way PERMANOVA ($P < 0.05$) is presented in Supplementary file 2. In the same time, the sampling time was only significant as a factor (two-way PERMANOVA) for explaining the activity of bile salt-activated lipase ($P = 0.03$) and total alkaline proteases ($P = 0.03$) (Table 3).

Table 3. Coefficient of determination values (R^2) for studied pancreatic enzymes for feeding regime and sampling time as pair of factors for night/light hours (two-way PERMANOVA). The asterisk (*) indicate that the considered factor significantly influences the variable measured (two-way PERMANOVA, $P < 0.05$).

Factors	**AMY**		**BAL**		**TRYP**		**CHYM**		**TAP**	
	N	L	N	L	N	L	N	L	N	L
Feeding regime	49.5*	12.8	34.4*	5.3	34.8	9.6	24.3*	23.9*	41.3*	6.5
Sampling time	17.7*	2.9	16.4	20.9*	13.4	15.1	28.8*	6.5	19.8*	22.2*
Interaction	8.0	44.1	4.2	27.6	8.1	20.1	8.8	18.2	7.0	19.9
Total	**75.2**	**59.7**	**55.0**	**53.8**	**56.3**	**44.8**	**61.8**	**48.6**	**68.1**	**48.7**

Abbreviations: D, night hours; L, day light hours; AMY, α-amylase; TRYP, trypsin; BAL, bile salt-activated lipase; CHYM, chymotrypsin; TAP, total alkaline proteases.

As indicated in Figure 1, all studied pancreatic enzymes had similar profiles within each feeding regime. In general terms, the correlation coefficients (R) for all studied pancreatic enzymes were higher within the R1 group (1 meal per day) when compared to R values of fry from the R3 group (feed was available during all day light hours, ten meals per day) (Table 4). Particularly, a high and significant positive correlation (R values up to 0.93; Spearman rank test, $P < 0.05$) was found between the activity of α-amylase and alkaline proteases like trypsin, chymotrypsin and total proteases in grey mullet fry from the R1 group (Table 4). Considering the R2 group, a significant positive correlation was also found among total activity values of α-amylase, trypsin,

and total proteases, and between trypsin and chymotrypsin, even though R values were not as higher than in the R1 group (Spearman rank test, $P < 0.05$). No significant correlations were found between the total activities of the bile salt-activated lipase and all other enzymes in the R1 and R2 groups. For the R3 group, no significant correlations were found among the total activities of all studied pancreatic enzymes (Spearman rank test, $P > 0.05$).

Table 4. Spearman rank correlation coefficients (R) among all studied pancreatic enzymes in grey mullet (*M. cephalus*) fry fed under different feeding regimes (R1/R2/R3). R1 corresponds to the group just fed just one meal per day; R2 to fry fed two meals per day; and R3 to fed every hour during day light hours (10 meals per day). The asterisk (*) denotes a significant correlation ($P < 0.05$) between enzyme total activity values (Spearman rank test).

Enzymes	AMY	BAL	TRYP	CHYM	TAP
AMY	1	0.61/0.45/0.45	0.74*/0.76*/0.40	0.81*/0.43/-0.14	0.86*/0.74*/-0.33
BAL	0.61/0.45/0.45	1	0.51/0.45/0.57	0.68/0.31/0.26	0.51/0.10/0.36
TRYP	0.74*/0.76*/0.40	0.51/0.45/0.57	1	0.81*/0.71*/0.38	0.93*/0.83*/0.50
CHYM	0.81*/0.43/-0.14	0.68/0.31/0.26	0.81*/0.71*/0.38	1	0.86*/0.57/0.60
TAP	0.86*/0.74*/-0.33	0.51/0.10/0.36	0.93*/0.83*/0.50	0.86*/0.57/0.60	1

Abbreviations: AMY, α-amylase; TRYP, trypsin; BAL, bile salt-activated lipase; CHYM, chymotrypsin; TAP, total alkaline proteases.

4. Discussion

The flathead grey mullet is an economically important fish species along its distribution area (Whitfield et al., 2012; Koven et al., 2020). Studying the different aspects of feeding behaviour and digestive physiology of this species may be important for the aquaculture industry as well as for natural ecosystem management. In aquaculture, this information helps optimizing feeding protocols (Yúfera et al., 2014; Gilannejad et al., 2021a,b), whereas in natural environments, it clarifies the role of this species in food webs, providing insight into trophic relationships among species (Solovyev et al., 2014). The circadian rhythmicity of feeding behaviour has been registered for many different fish species (Ceinos et al., 2019; Kulczykowska and Sánchez-Vázquez, 2010; Reis et al., 2019). In many cases, fish do not feed constantly and show a specific rhythm that can synchronize to other rhythmic cues interacting with food utilization, which may also be modified by food availability (Velázquez et al., 2006; Modica et al., 2014).

In the present study, we have used a constant photoperiod (10 h light:14 h darkness) for rearing grey mullet fry for 30 days that allowed us to control this factor and just focus on the effects

of feeding frequency on several digestive physiological parameters. The feeding activity of grey mullet fry measured by the level of stomach fullness was strongly correlated to the daily feeding patterns (one or two meals per day), being the maximal gut fullness values found just after the meal distribution. In particular, fry from the R1 group showed maximal stomach fullness values at 10:00 h and at 10:00 and 15:00 h when the feeding schedule was based on two meals (R2). However, when the feed was available during all day light hours, a single peak of stomach fullness was registered at 13:00 h. Wild grey mullet fry are considered as zooplanktivorous, foraging on a wide range of food items, including copepods, cladocerans, bivalves, algae, rotifers, adult chironomids, nematodes, polychaetes, detritus, silt and sand grains (Zismann et al., 1975; Tosi and Torricelli, 1988; Eggold and Motta, 1992; Gisbert et al., 1995; Salvarina et al., 2016). Although this species at fry stage is considered as a visual feeder, different studies have reported different daily feeding activity patterns. For instance, De Silva and Wijeyaratne (1977) reported two peaks of feeding activity, a first one at around midday and the other at the sunset. However, Torricelli et al. (1981–1982) found an increase in feeding activity coinciding to dusk and during the first night hours, whereas in a third study, feeding activity was predominantly registered during the morning and at the sunset (Tosi and Torricelli, 1988). For fry of other members of the family Mugilidae (*L. saliens*, *L. ramada*, *L. aurata*, and *C. labrosus*), the daily feeding activity values were found to maximal during the day and/or at the sunset (De Silva and Wijeyaratne, 1977; Torricelli et al., 1981; Gisbert et al., 1997). Differences in feeding activity under natural conditions of Mugilidae fry may be explained by their fish size associated to dietary shifts on prey selectivity (Albertini-Berhaut, 1979; Torricelli et al., 1981; Tosi and Torricelli, 1988; Eggold and Motta, 1992), results that have also been reported in other fish species like the loach (*Misgurnus anguillicaudatus*) (Wang et al., 2008) and tongue sole (*Cynoglossus semilaevis*) (Ma et al., 2006).

Feeding frequency affects the time and pattern of filling, retention, and evacuation of feed particles in the gut (Gilannejad et al., 2019; Reis et al., 2019). In the present study, the stomach filling time was faster and its peak's shape was sharper in groups with one (R1) and two (R2) meals per day when compared to their congeners that were fed regularly (10 meals per day) during day light hours (R3). These results indicated that sporadic feeding or a limited number of meals per day stimulates fish to ingest as much as possible feed particles, whereas when feed is constantly available, feed is ingested depending on the appetite level of fish (Jobling et al., 2012). Similar to our results, Gilannejad et al. (2019) also found that the juveniles of gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) showed the fastest gut filling rates when they were fed one meal per day when compared to other feeding strategies. Under current experimental conditions, the filling time of the intestine until reaching its maximum replenishment values in grey mullet fry ranged from 3 to 5 h, depending on the feeding protocol (3 h in fry from R1 and

R2 groups, and 5 h in fry from the R3 group). In particular, we observed a sort of hyperphagia in grey mullet fry from the R1 and R2 groups as they tended to store as much feed particles as possible in their gut to be later digested due to the limited daily meals. Although there exist data on this variable from other species (Ortiz-Monís et al., 2018; Gilannejad et al., 2019, 2021a,b), results are not directly comparable due to species-specific differences between studies in terms of developmental stages (larvae *vs.* fry), gut size and morphology, feeding protocols (live prey *vs.* compound feeds) and rearing conditions among others.

Differences in gut filling times of fish may be affected by neuroendocrine regulators that modulate satiation (Jobling et al., 2012) as well as intrinsic biochemical parameters linked to the digestion process such as enzyme:substrate ration and pH values (Moyano et al., 2015; Gilannejad et al., 2021a). Indeed, the acid stage of digestion in the stomach is very important for gastric fish species since hydrochloric acid and pepsin start to denature and digest dietary proteins. As proteins are molecules with positive and negative charges, they may regulate the pH level in fish gut. In particular, the amount of protein in the chyme may partially neutralize the secretion of hydrochloric acid in the stomach and, consequently, modulate pepsin activation (Solovyev et al., 2018; Nolasco et al., 2020). Another factor that affects the digestive efficiency is the enzyme:substrate ratio (Moyano et al., 2015). Thus, an excess of food quantity in the gut due to hyperphagia may lead to an inappropriate enzyme to substrate ratio in chyme and, consequently, a decrease in the digestive efficiency. These facts may explain the differences in somatic growth observed in grey mullet fry from the R1 group in comparison to their congeners from the R2 and R3 groups. In this sense, although fish from different groups were offered the same feed ration per day, grey mullet fry from the R1 and R2 groups showed a worst growth performance when compared to fry from the R3 group. These results may be attributed to an inappropriate enzyme to substrate ratio in the R1 and R2 groups, since no remarkable differences in digestive enzyme activities were found among groups after meals. This would lead into a relatively high content of undigested nutrients in faeces and, consequently, less nutrients and energy for promoting somatic growth.

In addition, we have found that the feeding frequency modulated the profile and activity levels of key pancreatic digestive enzymes in grey mullet fry under controlled night/light and water temperature conditions. The effect of feeding regime on the activity of different digestive enzymes has been evaluated in different species such as malabar grouper (*Epinephelus malabaricus*) (Fujii et al., 2007), *S. aurata* (Montoya et al., 2010; Zeytin et al., 2016; Busti et al., 2020; Gilannejad et al., 2021a) and *S. senegalensis* (Gilannejad et al., 2021b), whereas some authors have found that the activity of some enzyme was independent from feeding regime in tambaqui (*Colossoma macropomum*) (Reis et al., 2019). Under current experimental conditions, feeding regimes had a

significant modulatory effect on the level of activity of all studied pancreatic enzymes such as α-amylase, bile salt-activated lipase, trypsin, chymotrypsin, and total alkaline proteases. Thus, the activity of all studied enzymes decreased when the feeding frequency increased from a single (R1 group) to several meals per day (R3 group) in grey mullet fry. Similarly, the feeding frequency in *S. aurata* significantly affected circadian trypsin activity. Moreover, there was a tendency showed that the enzymatic activity was decreased when the feeding frequency increased from one to five times a day (Gilannejad et al., 2021a). These results supported our findings for the R3 group. In contrast, in another study in *S. aurata*, the effect of feeding frequency on the activity of pancreatic enzymes reported inconclusive results regarding the effect of feeding patterns on digestive enzyme activities (Busti et al., 2020). Such different responses between studies might be also related to different diets, since different dietary composition may impact on the regulation of enzyme secretion and activity (i.e., vegetal protein sources may inhibit the activity of alkaline proteases like trypsin) (Busti et al., 2020). In the present study, the peak of activity of pancreatic proteases (trypsin, chymotrypsin, and total alkaline proteases) and bile salt-activated lipase coincided with maximum levels of intestinal fullness (15.00 h) in fry fed a single meal per day (R1 group). As it was previously indicated by Yúfera et al. (2014), our results confirmed that the feeding protocol has a marked effect in the digestive strategy of grey mullet fry, supporting the hypothesis that the digestive function is adapted in order to obtain a maximum benefit of the ingested nutrients at a minimum digestive cost. Furthermore, the similar results related to a peak in trypsin activity coinciding with maximum level of intestinal fullness were found in *S. aurata* (Gilannejad et al., 2021a). Furthermore, we found a peak in enzyme activity at night in fry from R1 and R2 groups. In contrast, a peak in α-amylase activity for *C. macropomum* was found at night hours regardless the feeding time (day- or night-time feeding) (Reis et al., 2019). It is well known that the circadian rhythms are species specific and depend on feeding activity (diurnal/nocturnal/crepuscular). In the present study, we used the fixed light/night ratio and, consequently, we are not able to estimate this factor on the activity of studied enzymes.

Under present experimental condition, feeding frequency significantly affected somatic growth in grey mullet fry. Our findings are in agreement with results obtained for different fish species such as hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (Liu, Liao, 1999), African catfish (*Clarias gariepinus*) (Okomoda et al., 2019), sutchi catfish (*Pangasius hypophthalmus*), silver carp (*Hypophthalmichthys molitrix*) (Ali et al., 2005), Atlantic halibut (*Hippoglossus hippoglossus*) (Schnaittacher et al., 2005) and *S. senegalensis* (Gilannejad et al., 2019). It is known that the distribution of the same amount of daily food, but with different frequency may influence on feed conversion ratio (Kousoulaki et al., 2015). In this sense, we assume that the combination of different factors, such as optimal production levels of digestive enzymes and effective feed

(substrate)/enzyme ratios, which would be necessary for maintaining the required levels of enzymatic activity without their overproduction when food is constantly available in optimal amount, it would save energy that may be diverted for promoting somatic growth. These results are of relevance and may applied for improving actual of feeding practices, since feeding regimes that exceed the digestive capacity of fish may not be economically viable (Ueberschär et al., 2018).

5. Conclusions

Feeding regimes have a direct effect on grey mullet fry growth performance. In particular, fry fed continuously during light hours grew better than their congeners fed the same feed ration but only one or two times per day. Feeding activity of fry measured by the level of stomach fullness was correlated to the daily feeding patterns (one or two meals per day), being the maximal gut fullness values found just after meal distribution, whereas this trend was not observed when feed was offered continuously during light hours. These results indicated that feeding behaviour was modulated by the feeding protocol and in particular, by feeding frequency. Fry fed just one and two meals per day showed hyperphagia as they tended to store as much feed particles as possible in their gut to be later digested due to the limited daily meals. However, this strategy negatively affected feed digestion (i.e., inappropriate enzyme to substrate ratio in chyme, changes in digestive enzyme activities and chyme transit times), impairing somatic growth performance. The levels of enzyme activity were modulated by the number of meals, the more frequent the meals offered, the lower the activity of pancreatic enzymes, supporting the hypothesis that digestive function is adapted to obtain a maximum benefit of the ingested nutrients at a minimum digestive cost. Considering our results, we recommend feeding grey mullet fry continuously during day light hours in order to optimize feed digestion and promote fry condition and growth.

Author contributions

Conceptualization, E.G.; methodology, E.G., M.S.; validation, E.G., M.S. formal analysis, M.S.; visualization, E.G., M.S.; supervision, writing and original draft preparation, M.S.; writing, review and editing, all authors; E.G.; project administration, E.G.; funding acquisition, E.G. Both authors have read and agreed to the published version of the manuscript.

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Figure captions

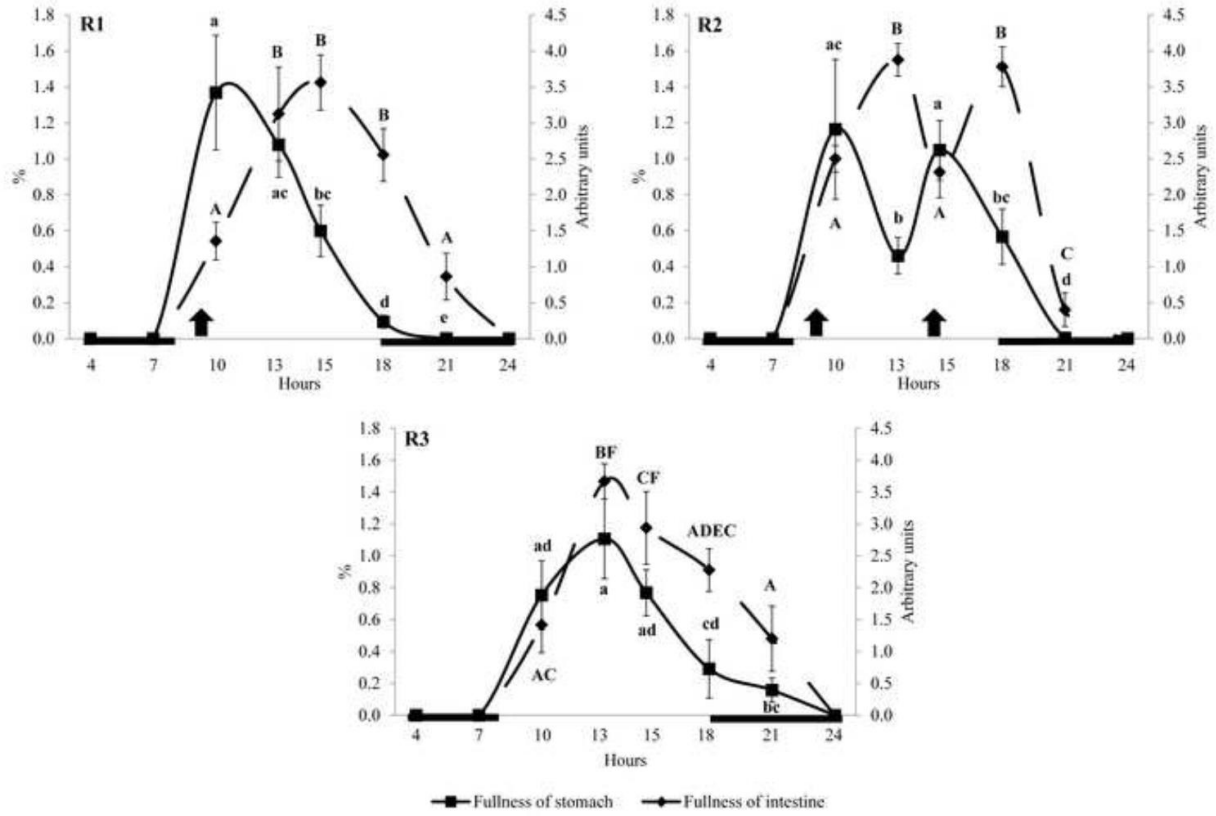

Fig. 1. Fullness level of the stomach and intestine in grey mullet (*M. cephalus*) fry during day light and night conditions fed a fixed feed ration distributed in different meals per day (1 meal: R1; 2 meals: R2 and every hour during day light hours, 10 meals per day: R3). Upper case letters denote the significant differences among different sampling points for the intestine (one-way PERMANOVA, $P < 0.05$). Lower case letter denote the significant differences among different sampling points for the stomach (one way PERMANOVA, $P < 0.05$). The black bands indicate darkness hours.

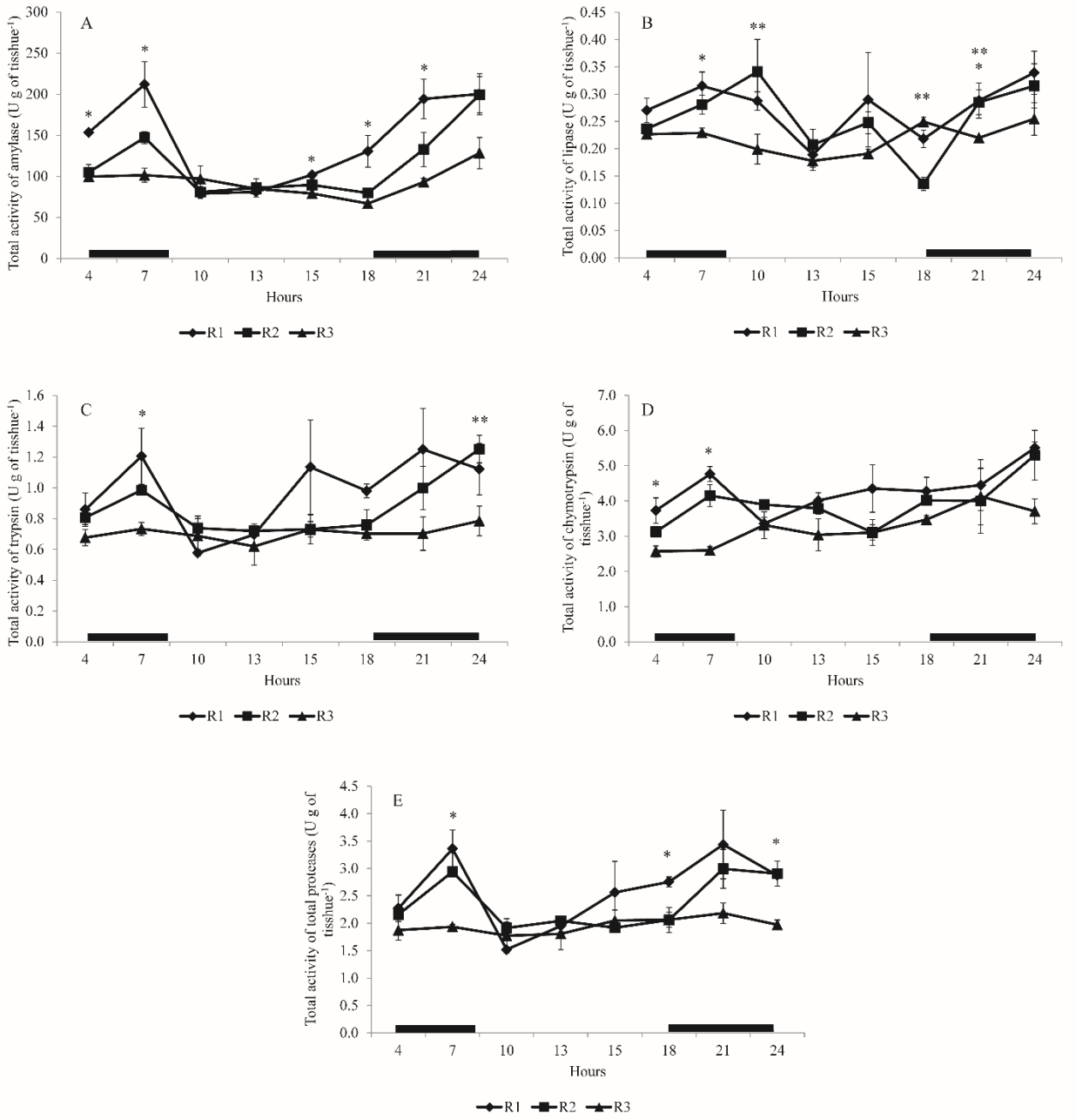

Fig. 2. Total activity (U/ g tissue) of several pancreatic digestive enzymes in grey mullet (*M. cephalus*) fry during day light and night conditions fed a fixed feed ration distributed in different meals per day (1 meal: R1; 2 meals: R2 and every hour during day light hours, 10 meals per day: R3). A) α– amylase, B) bile salt-activated lipase, C) trypsin, D) chymotrypsin and E) total alkaline proteases. The asterisk (*) and double asterisks (**) denote the significant differences for R1 vs R3 and R2 *vs.* R3 respectively. The black bands indicate the darkness hours.

Results ***3.3 Circadian activity of pancreatic digestive enzymes in R1, R2, and R3 feeding regimes***

"p" values among different time points (hours) for each feeding regime (Figure 3)

Dunn's post hoc test

Amylase

Feeding regimes R1

Hours	4	7	10	13	15	18	21
4							
7	0,25						
10	0,07	**0,003**					
13	0,08	**0,004**	0,95				
15	0,39	**0,04**	0,36	0,39			
18	0,73	0,13	0,15	0,17	0,60		
21	0,39	0,77	**0,01**	**0,01**	0,08	0,23	
24	0,27	0,95	**0,004**	**0,005**	**0,05**	0,15	0,82

Feeding regimes R2

Hours	4	7	10	13	15	18	21
4							
7	0,27						
10	0,25	**0,02**					
13	0,36	**0,04**	0,82				
15	0,45	0,06	0,69	0,86			
18	0,17	**0,01**	0,82	0,64	0,53		
21	0,42	0,77	**0,05**	0,08	0,12	**0,03**	
24	0,11	0,60	**0,01**	**0,01**	**0,02**	**0,003**	0,42

Feeding regimes R3

Hours	4	7	10	13	15	18	21
4							
7	0,98						
10	0,80	0,82					
13	0,27	0,29	0,40				
15	0,17	0,17	0,26	0,77			
18	**0,02**	**0,02**	**0,04**	0,23	0,36		
21	0,56	0,58	0,75	0,60	0,42	0,08	
24	0,42	0,40	0,29	0,06	**0,03**	**0,002**	0,17

BSA Lipase

Feeding regimes R1

Hours	4	7	10	13	15	18	21
4							
7	0,39						
10	0,88	0,47					
13	0,13	**0,02**	0,10				
15	0,77	0,56	0,88	0,07			
18	0,25	**0,04**	0,19	0,73	0,15		
21	0,73	0,60	0,84	0,06	0,95	0,13	
24	0,31	0,88	0,39	**0,01**	0,47	**0,03**	0,51

Feeding regimes R2

Hours	4	7	10	13	15	18	21
4							
7	0,20						
10	0,10	0,71					
13	0,79	0,12	0,06				
15	0,79	0,31	0,16	0,60			
18	0,21	**0,01**	**0,004**	0,32	0,13		
21	0,17	0,93	0,77	0,10	0,27	**0,01**	
24	0,11	0,75	0,95	0,06	0,18	**0,005**	0,82

Feeding regimes R3

Hours	4	7	10	13	15	18	21
4							
7	1,00						
10	0,38	0,38					
13	0,07	0,07	0,35				
15	0,12	0,12	0,48	0,82			
18	0,86	0,86	0,29	**0,048**	0,08		
21	0,82	0,82	0,52	0,12	0,18	0,68	
24	0,73	0,73	0,22	**0,03**	0,05	0,86	0,56

Trypsin

Feeding regimes R1

Hours	4	7	10	13	15	18	21
4							
7	0,30						
10	0,12	**0,01**					
13	0,33	**0,04**	0,56				
15	0,49	0,73	**0,02**	0,09			
18	0,75	0,47	0,06	0,19	0,71		
21	0,36	0,91	0,01	0,06	0,82	0,54	
24	0,47	0,75	**0,02**	0,09	0,98	0,69	0,84

Feeding regimes R2

Hours	4	7	10	13	15	18	21
4							
7	0,24						
10	0,45	0,05					
13	0,47	0,06	0,98				
15	0,49	0,06	0,95	0,98			
18	0,49	0,06	0,95	0,98	1,00		
21	0,36	0,79	0,09	0,10	0,11	0,11	
24	0,06	0,47	**0,01**	**0,01**	**0,01**	**0,01**	0,33

Feeding regimes R3

Hours	4	7	10	13	15	18	21
4							
7	0,58						
10	0,79	0,77					
13	0,62	0,30	0,45				
15	0,56	0,98	0,75	0,28			
18	0,79	0,77	1,00	0,45	0,75		
21	0,79	0,77	1,00	0,45	0,75	1,00	
24	0,37	0,73	0,52	0,17	0,75	0,52	0,52

Chymotrypsin

Feeding regimes R1

Hours	4	7	10	13	15	18	21
4							
7	0,13						
10	0,69	0,05					
13	0,60	0,31	0,36				
15	0,42	0,47	0,23	0,77			
18	0,51	0,39	0,29	0,89	0,89		
21	0,30	0,62	0,15	0,60	0,82	0,71	
24	**0,01**	0,31	**0,003**	**0,04**	0,08	0,06	0,13

Feeding regimes R2

Hours	4	7	10	13	15	18	21
4							
7	**0,04**						
10	0,11	0,69					
13	0,20	0,45	0,73				
15	1,00	**0,04**	0,11	0,20			
18	**0,04**	0,95	0,64	0,42	**0,04**		
21	0,27	0,36	0,60	0,86	0,27	0,33	
24	**0,01**	0,45	0,25	0,13	**0,01**	0,49	0,09

Feeding regimes R3

Hours	4	7	10	13	15	18	21
4							
7	0,91						
10	0,17	0,20					
13	0,29	0,34	0,75				
15	0,27	0,33	0,77	0,98			
18	0,06	0,07	0,60	0,40	0,42		
21	**0,03**	**0,04**	0,42	0,26	0,27	0,77	
24	**0,03**	**0,04**	0,44	0,27	0,29	0,80	0,98

Total proteases

Feeding regimes R1

Hours	4	7	10	13	15	18	21
4							
7	0,07						
10	0,27	**0,004**					
13	0,69	**0,03**	0,49				
15	0,51	0,25	0,08	0,29			
18	0,36	0,37	**0,04**	0,18	0,80		
21	0,11	0,84	**0,01**	**0,04**	0,34	0,49	
24	0,18	0,62	**0,02**	0,08	0,51	0,69	0,77

Feeding regimes R2

Hours	4	7	10	13	15	18	21
4							
7	0,18						
10	0,19	**0,01**					
13	0,45	**0,04**	0,58				
15	0,16	**0,01**	0,91	0,51			
18	0,42	**0,03**	0,62	0,95	0,54		
21	0,25	0,86	**0,01**	0,06	**0,01**	**0,0496**	
24	0,27	0,82	**0,02**	0,06	**0,01**	0,06	0,95

Feeding regimes R3

Hours	4	7	10	13	15	18	21
4							
7	0,71						
10	0,75	0,95					
13	0,91	0,79	0,84				
15	0,36	0,58	0,54	0,42			
18	0,39	0,62	0,58	0,45	0,95		
21	0,20	0,37	0,34	0,25	0,73	0,69	
24	0,60	0,89	0,84	0,69	0,69	0,73	0,45

Results ***3.3 Circadian activity of pancreatic digestive enzymes in R1, R2, and R3 feeding regimes***

"p" values among different feeding regimes for each night and light hours (Figure 3)

Dunn's post hoc

Amylase

Feeding regimes		R1	R2
Hours (4)	R1		
	R2	0,07	
	R3	**0,03**	0,65
Hours (7)	R1		
	R2	0,18	
	R3	**0,007**	0,18
Hours (10)	R1		
	R2	0,88	
	R3	0,46	0,55
Hours (13)	R1		
	R2	0,55	
	R3	0,46	0,88
Hours (15)	R1		
	R2	0,18	
	R3	**0,03**	0,37
Hours (18)	R1		
	R2	0,18	
	R3	**0,007**	0,18
Hours (21)	R1		
	R2	0,30	
	R3	**0,01**	0,14
Hours (24)	R1		
	R2	0,77	
	R3	0,053	0,10

BSA Lipase

Feeding regimes		R1	R2
Hours (4)	R1		
	R2	0,31	
	R3	0,12	0,58
Hours (7)	R1		
	R2	0,46	
	R3	**0,02**	0,10
Hours (10)	R1		
	R2	0,71	
	R3	0,08	**0,04**
Hours (13)	R1		
	R2	0,59	
	R3	0,76	0,39
Hours (15)	R1		
	R2	1,00	
	R3	0,18	0,18
Hours (18)	R1		
	R2	0,07	
	R3	0,65	**0,02**
Hours (21)	R1		
	R2	0,88	
	R3	**0,03**	**0,049**
Hours (24)	R1		
	R2	0,50	
	R3	0,12	0,37

Trypsin

Feeding regimes		R1	R2
Hours (4)	R1		
	R2	0,65	
	R3	0,07	0,18
Hours (7)	R1		
	R2	0,65	
	R3	**0,03**	0,07
Hours (10)	R1		
	R2	0,10	
	R3	0,29	0,55
Hours (13)	R1		
	R2	0,88	
	R3	0,46	0,55
Hours (15)	R1		
	R2	0,37	
	R3	0,37	1,00
Hours (18)	R1		
	R2	0,07	
	R3	0,07	1,00
Hours (21)	R1		
	R2	0,55	
	R3	0,06	0,20
Hours (24)	R1		
	R2	0,65	
	R3	0,12	**0,04**

Chymotrypsin

Feeding regimes		R1	R2
Hours (4)	R1		
	R2	0,46	
	R3	**0,02**	0,10
Hours (7)	R1		
	R2	0,30	
	R3	**0,01**	0,14
Hours (10)	R1		
	R2	0,10	
	R3	0,88	0,14
Hours (13)	R1		
	R2	0,65	
	R3	0,07	0,18
Hours (15)	R1		
	R2	0,23	
	R3	0,30	0,88
Hours (18)	R1		
	R2	0,77	
	R3	0,053	0,10
Hours (21)	R1		
	R2	0,46	
	R3	0,55	0,88
Hours (24)	R1		
	R2	0,77	
	R3	0,10	0,053

Total proteases

Feeding regimes		R1	R2
Hours (4)	R1		
	R2	0,46	
	R3	0,14	0,46
Hours (7)	R1		
	R2	0,55	
	R3	**0,02**	0,09
Hours (10)	R1		
	R2	0,14	
	R3	0,23	0,77
Hours (13)	R1		
	R2	0,60	
	R3	0,76	0,41
Hours (15)	R1		
	R2	0,37	
	R3	0,65	0,65
Hours (18)	R1		
	R2	0,053	
	R3	**0,04**	0,88
Hours (21)	R1		
	R2	0,65	
	R3	0,07	0,18
Hours (24)	R1		
	R2	0,88	
	R3	**0,04**	0,053

Amylase

Feeding regime	Night hours R1	vs	Light hours R1
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	7,61E+04	
	Within-group sum of squares:	2,56E+04	
	F:	43,5	
	p (same):	**0,0001**	

Feeding regime	Night hours R2	vs	Light hours R2
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	4,53E+04	
	Within-group sum of squares:	2,22E+04	
	F:	23,0	
	p (same):	**0,0002**	

Feeding regime	Night hours R3	vs	Light hours R3
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	1,18E+04	
	Within-group sum of squares:	8454,0	
	F:	8,7	
	p (same):	**0,005**	

BSA Lipase

Feeding regime	Night hours R1	vs	Light hours R1
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	0,12	
	Within-group sum of squares:	0,10	
	F:	4,4	
	p (same):	0,052	

Feeding regime	Night hours R2	vs	Light hours R2
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	0,13	
	Within-group sum of squares:	0,12	
	F:	2,6	
	p (same):	0,13	

Feeding regime	Night hours R3	vs	Light hours R3
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	0,03	
	Within-group sum of squares:	0,03	
	F:	3,2	
	p (same):	0,08	

Trypsin

Feeding regime	Night hours R1	vs	Light hours R1
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	2,8	
	Within-group sum of squares:	2,3	
	F:	3,9	
	p (same):	0,06	

Feeding regime	Night hours R2	vs	Light hours R2
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	1,0	
	Within-group sum of squares:	0,6	
	F:	16,6	
	p (same):	**0,0004**	

Feeding regime	Night hours R3	vs	Light hours R3
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	0,42	
	Within-group sum of squares:	0,41	
	F:	0,47	
	p (same):	0,49	

Chymotrypsin

Feeding r	Night hours R1	vs	Light hours R1
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	17,5	
	Within-group sum of squares:	15,3	
	F:	3,2	
	p (same):	0,09	

Feeding r	Night hours R2	vs	Light hours R2
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	19,1	
	Within-group sum of squares:	17,9	
	F:	1,4	
	p (same):	0,24	

Feeding r	Night hours R3	vs	Light hours R3
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	13,9	
	Within-group sum of squares:	13,9	
	F:	0,003	
	p (same):	0,96	

Total proteases

Feeding r	Night hours R1	vs	Light hours R1
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	14,7	
	Within-group sum of squares:	11,0	
	F:	7,5	
	p (same):	**0,01**	

Feeding r	Night hours R2	vs	Light hours R2
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	6,5	
	Within-group sum of squares:	3,0	
	F:	26,1	
	p (same):	**0,0001**	

Feeding r	Night hours R3	vs	Light hours R3
	one-way PERMANOVA		
	Permutation N:	9999	
	Total sum of squares:	2,29	
	Within-group sum of squares:	2,26	
	F:	0,29	
	p (same):	0,61	