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CIRCADIAN CYCLE OF DIGESTIVE ENZYME PRODUCTION AT FASTING AND FEEDING CONDITIONS IN NILE TILAPIA, *OREOCHROMIS NILOTICUS* (ACTINOPTERYGII: PERCIFORMES: CICHLIDAE)

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Background. Fish have photoperiod-dependent rhythms (circadian cycle of about 24 h), including the enzymatic secretion cycles, and this information has not been available for Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758). The knowledge on the digestive enzymes in Nile tilapia may have practical implications in fish nutrition. The aim of the presently reported study was to determine the circadian cycle of digestive enzyme production of Nile tilapia, in fasting and feeding regimes, in hope to receive information that might improve feeding schedules of this fish in aquaculture.

Materials and methods. In juvenile Nile tilapia the circadian cycle of concentrations of total soluble protein, protease, pepsin-like, trypsin, chymotrypsin, amylase, and lipase were determined. The baseline (fasting) and feeding conditions (ad libitum) were sampled and monitoring every hour for 24 h.

Results. The basal peak of enzyme activity in the intestine occurred at 18:44 h for amylase, at 19:57 h for proteases, and 20:29 for trypsin. The minimal activity for most enzymes, appeared between 4:51 h (amylase) and 10:13 h (lipases). In the feeding treatment (ad libitum), stomach activity (pepsin-like) had maximal activity at 20:06 h and minimal activity 05:46 h. Intestinal amylase activity covered an extended period of low enzymatic activity beginning at the 05:46 h and ending at 12:59 h. The peak of digestive enzyme activity occurred within 18:44–20:29 h. In general, secretion of digestive enzymes was positively stimulated by food, for all enzymes assayed.

Conclusion. Nile tilapia has a higher digestive enzyme activity at night than during the day. Knowledge of the circadian cycle of digestive enzymes, and modifications initiated by food, is useful to establish feeding times. If feeding schedules are adjusted to coincide with maximum natural peaks, feed efficiency will increase, which will be reflected in weight gain of the fish and provide more profitable yields for aquaculture.

Keywords: enzyme activity, physiology, feeding schedule

INTRODUCTION

The Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), is a freshwater, omnivorous cichlid fish native to Africa, but currently available worldwide (Eknath and Hulata 2009). It represents the second largest group of farmed fish, which production quadrupled within the past decade. Its rapid success as a useful food species has been related to a number of factors, such as: tolerance to overcrowding, rapid growth, high commercial value, stable market prices, relatively easy spawning in captivity throughout the year, high resistance to diseases, suitability for polyculture, and inexpensive diets of processed plant sources (El-Sayed 1999, Shiau and Hsu 2002, Anonymous 2011).

To continue its rapid expansion, Nile tilapia farming must incorporate more sustainable practices, without compromising product quality and optimizing nutrient sources. The largest cost item of farming operations is the cost of fish feed. It is necessary to consider nutritional needs and factors that influence these requirements and implementation of the most appropriate feeding strategies. Digestive enzymes are among the most important factors influencing food consumption (Jun-Sheng et al. 2006). Several enzymes are involved in digestion in tilapia, such

as pepsin-like, amylase, trypsin, alkaline phosphatase, and esterase (Nagase 1964, Cockson and Bourne 1972, Moriarty 1973, Klaren et al. 1993, Li and Fan 1997, Tengjaroenkul et al. 2000). Circadian cycles are controlled by the timing of the light and dark phases; the pineal organ acts as a photo transducer, responsible for the regulation of the circadian clock of fish, conveying photoperiod information to the brain via neural pathways and release of indoleamines, primarily melatonin (Ekstrzm and Meissl 1997). Melatonin cycles provide the animal with information about the time of day and also the time of year, synthesizing and releasing melatonin into the bloodstream during the dark phase (Reiter 1993). Although melatonin is synthesized in several tissues (Cassone 1990), its rhythmic synthesis is primarily localized in the pineal organ and the retina (Falcón et al. 1992, Cassone 1998). The photoperiod is the most important factor that entrains animal rhythms, including enzymatic secretion cycles, tied to the daily melatonin rhythms (Bromage et al. 2001). Fish exhibit photoperiod-dependent circadian cycles of several processes, including locomotion (zebrafish; Zhdanova et al. 2001), development (zebrafish; Danilova et al. 2004), growth (rainbow trout; Falcón et al. 2003), reproduction (salmon; Amano et al. 2000, 2004; Senegal sole; Vera et al. 2007), control and regulation of reproductive hormones and melatonin (European seabass; Bayarri et al. 2004), and digestive enzyme activity (Yúfera et al. 2012). Also, they have oscillators and circadian photo transduction drag mechanisms, suggesting that circadian pacemaker functions can be spread throughout the animal (zebrafish; Cahill 2002).

Understanding the circadian cycle of digestive enzymes can be used to optimize feeding schedules of Nile tilapia, *Oreochromis niloticus*, under culture conditions.

MATERIALS AND METHODS

Fish rearing and sampling. Specimens of the Nile tilapia, *Oreochromis niloticus*, (70.9 ± 10.3 g) from the same progeny were cultivated at CIIDIR-IPN Mexico (25°32′41″N, 108°28′38″W). Growing conditions were 14 h light : 10 h dark, 27 ± 1°C, and >4 mg · L^{-1} dissolved oxygen, 250 fish per tank (5000 L), and provided a diet containing 30% crude protein fed ad libitum (Nutripec, Nutrimentos Purina, Guadalajara, Mexico).

Two experiments were performed to determine the circadian cycle of digestive enzyme activity:
- Basal circadian cycle established under fasting conditions; and
- Circadian cycle established with ad libitum feeding conditions.

Tilapias were kept 24 h fasting before being sampled. Random samples of 3 tilapias were collected every hour for 24 h (*n* = 144 fish; 72 per experiment) (Nolasco-Soria and Vega-Villasante 1998, Casillas-Hernández et al. 2006). The fish were sacrificed by immersion in cold water and then ice. Wet weight (±0.0001 g) of the fish was recorded (EX324, Ohaus, Parsippany, NJ), stomach and intestine were removed, weighted, and individually stored at –20°C for air transport to facilities at CIBNOR for analysis. All

the work involving the use of the fish during his study was approved by the Institutional Animal Care and Use Committee, CIIDIR-IPN Sinaloa.

Preparation of enzyme extracts. The wet weight (±0.1 mg) of the stomach and intestines of each specimen was recorded (APX-200, formerly Denver Instrument, Sartorius, Göttingen, Germany). All samples were kept on ice during this procedure to reduce enzyme denaturation or damage. Stomachs and intestines were separated and then separately pooled with distilled water (tissue/distilled water, w/v 1 : 3) before being homogenized (Potter PRO 250, PRO Scientific, Oxford, CT). The mixtures were double centrifuged at 15 294 × *g* at 4°C for 10 min (5810-R, Eppendorf, Hamburg, Germany). The supernatants were stored at –20°C, for future analysis.

Enzyme activity analyses. The concentration of soluble protein in stomach and intestine extracts was determined (Bradford 1976), using bovine serum albumin (#A4503, Sigma-Aldrich) as a standard. Activity of acid proteases (pepsin-like) in the stomach extract was measured (Anson 1938) with slight modifications. In brief, the reaction mixture consisted of 1 mL of haemoglobin (#H2625, Sigma-Aldrich) (1% in glycine-HCl, 100 mmol · L^{-1} buffer, pH 2) and 20 µL of stomach multienzyme extract from tilapia, which was incubated at 25°C for 30 min. The reaction was stopped with 500 µL 20% trichloroacetic acid (TCA) and was left to stand at 4°C for 15 min. The samples were centrifuged at 15 300 × *g*, under the same standing conditions. The supernatants were collected and diluted with distilled water (1 : 10) to take a reading of absorbance at 280 nm in a spectrophotometer (Jenway, Model 6405) using quartz cell (1 cm light path). One unit of activity was defined as the amount of enzyme needed to catalyse the liberation of an equivalent of 1 µmol tyrosine per minute.

Activity of the alkaline proteases in the intestine multi-enzyme extract was measured (García-Carreño 1992), using 500 µL of azocasein (#A2765, Sigma-Aldrich) (1% w/v, in Tris-HCl 100 mmol · L^{-1} buffer, pH 9), as substrate, 500 µL Tris-HCl (100 mmol + 10 mmol CaCl$_2$, pH 9.0) and 40 µL of intestine multi-enzyme extract from tilapia. The mixture was incubated at 25°C for 20 min. The reaction was stopped with 500 µL of 20% TCA. The samples were centrifuged and absorbance was read at 440 nm. One unit of alkaline protease activity was defined as the amount of enzyme required to increase 0.01 units Abs · min^{-1}.

Trypsin activity was determined using specific substrate (#B4875, Sigma-Aldrich) (Erlanger et al. 1961), with slight modification. In brief, the reaction mixture consisted of 10 µL of intestine multi-enzyme extract from tilapia + 160 Tris-HCl (60 mmol, pH 8) + 10 µL calcium chloride (192 mmol Tris-HCl, 60 mmol, pH 8), starting the reaction with 10 µL BAPNA. The control was inactivated crude extract (water bath at 95°C for 5 min). Absorbance was measured at 405 nm every 15 s for 30 min. One unit of trypsin activity was defined as the amount of enzyme required to release 1 µmol 4-nitroaniline · min^{-1}.

Chymotrypsin activity was determined (Del Mar et al. 1979), with slight modifications, as described for

trypsin, but using SAPNA (#S7388, Sigma-Aldrich) as the substrate in DMSO and 60 mmol Tris-HCl, pH 8 and $CaCl_2$, pH 8. For controls, the crude extract was inactivated (water bath at 95°C for 5 min). One unit of chymotrypsin activity was defined as the enzyme required to release 1 μmol of 4-nitroanilide · min^{-1}.

Lipase activity was determined using β-naphthyl caprylate as substrate (Versaw et al. 1989). The reaction mixture consisted of 100 μL sodium taurocholate (100 mmol), 1900 μL Tris HCl (200 mmol, pH 8), 10 μL enzyme extract and 10 μL β-naphthyl caprylate (#N8875, Sigma-Aldrich) (100 mmol in DMSO), as substrate. The mixture was incubated at 25°C for 9.5 min. Then 10 μL Fast Blue BB (100 mmol in DMSO) was added and the mixture was incubated at the same temperature for 0.5 min. The reaction was stopped by adding 200 μL 0.72 N TCA, then 2.70 mL reagent ethyl acetate-ethanol (1 : 1, v : v) was added, and the tubes were stirred in vortex. Absorbance was read at 540 nm. For the controls, the crude extract was applied after stopping the reaction with the TCA reagent. One unit of lipase activity was defined as the enzyme required to release 1 μmol of β-naphtol · min^{-1}.

Amylase activity was determined using soluble starch (#S9765, Sigma-Aldrich) as substrate (Vega-Villasante et al. 1993). The reaction mixture contained 490 μL Tris HCl (50 mmol at pH 8), 10 μL enzyme extract, and 500 μL of the starch solution (1% Tris HCl, 100 mmol at pH 8), as substrate. The mixture was incubated at 25°C for 10 min, then 200 μL $NaCO_3$ (2 N) and 1500 μL dinitrosalicylic acid reagent (DNS) was added to stop the reaction. Colour development took place in water bath at 90°C for 15 min. Colour solution was diluted to 10 mL final volume. Absorbance at 540 nm was read. For the controls, the crude extract was applied after adding DNS reagent. One unit of amylase activity was defined as the enzyme required to release 1 μmol glucose · min^{-1}.

The activity of proteases, lipases, and amylases are expressed as the number of units of enzyme per fish (total units; TU). All determinations were performed in quadruplicate. With these data for 24 h, regression analysis yielded a circadian cycle to represent the behaviour of each enzyme, to determine a straight-line behaviour and polynomial of second and third degree. Polynomial regression analysis generalizes analysis of covariance in nonlinear situations. The main advantage of this approach is to compare hourly enzyme activities (Draper and Smith 1981, Freud and Little 1981, Snedecor and Cochran 1981).

For these polynomials (linear equations, $P < 0.05$), patterns were calculated. To represent each enzyme, enzyme activity was averaged every two hours on the same graphics that show the polynomial lines.

Statistical analysis. Normality and variance homogeneity of data were analysed with the Lilliefor and Bartlett tests (Sokal and Rohlf 1995). Regression analysis was used to determine significance of the relation between TU and time. Linear regression and exponential and quadratic equations were used to represent the behaviour of each enzyme during the circadian cycle. When equation coefficients were

significant and residual variance of regression decreased, they were compared with simple linear regression. One-way ANOVA was used to test mean TU every two hours throughout the sampling, followed by post hoc Tukey's test was used to separate significantly different mean values and to compare treatments (starvation versus ad libitum feeding). Significance was set at $P < 0.05$. All analyses were performed with Statistica 7.0.

RESULTS

Nile tilapia, *Oreochromis niloticus*, under starvation conditions increased pepsin-like activity with the time of day (Fig. 1). Alkaline proteases, trypsin, and amylase activity decline in the early hours of the morning and maximum activity at night (Figs. 2, 3). Chymotrypsin activity was not affected by phases of light and darkness. Lipase activity declined during midday (Fig. 4). Tilapia under ad libitum feeding conditions increased pepsin-like activities, had a secondary peak in the morning and a major peak at night (Fig. 1). Alkaline proteases, trypsin, chymotrypsin, and amylase maintained high activity at most hours, but lower activity at midday (Figs. 2, 3).

Enzyme activity is summarized in Table 1. Peak of enzyme activity in the intestine, appeared at 18.43:48 h for amylases, 19:57:36 h for proteases, and 20:29:24 h for trypsin; minimum activity 4:51:00 h for amylase, but 10:13:12 h for lipase.

Under standard feeding conditions, show pepsin-like activity in the stomach reached maximum activity at 20:06:00 h and minimum activity at 5:46:12 h. In the intestine, digestive enzyme activity had an extended period of low activity beginning at 5:46:12 h and ending at 12:58:48 h for amylase.

DISCUSSION

Basal digestibility (fasting conditions). The photoperiod is considered the most important factor entraining animal rhythms. In particular, melatonin rhythms provide animals
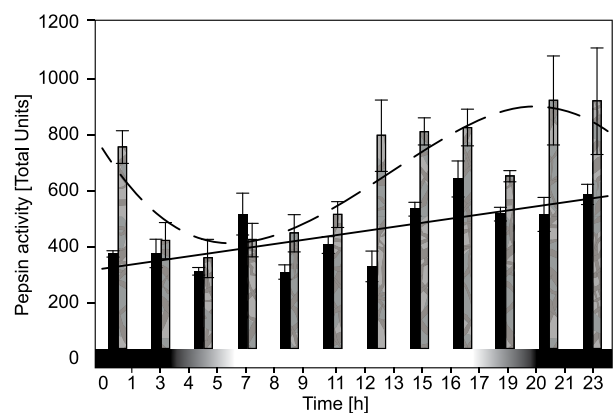

Fig. 1. Pepsin-like activity in Nile tilapia, *Oreochromis niloticus*; Black bars represent basal conditions (fasting fish) with solid line ($P < 0.05$); Marbled bars with dashed lines represent ad libitum feeding conditions ($P < 0.05$); Black section in the bar at the bottom of the figure represent night time; Values are mean ± SE
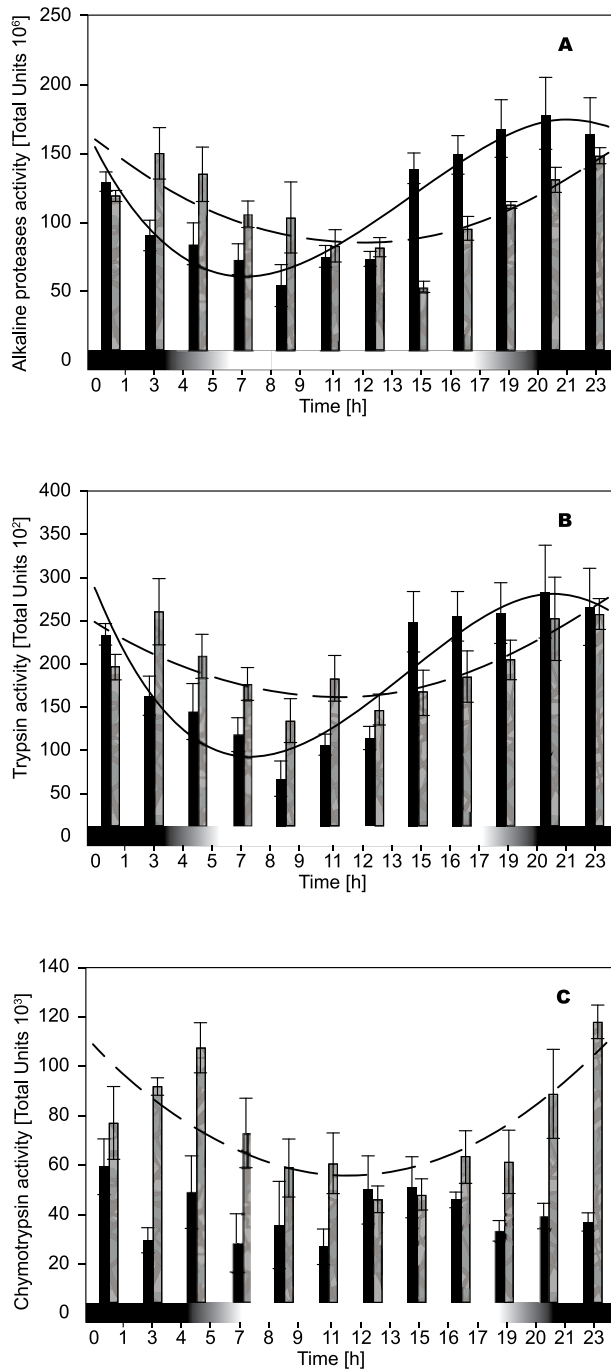
Fig. 2. Activity of alkaline proteases in Nile tilapia, *Oreochromis niloticus*, under fasting and ad libitum conditions (**A**); Trypsin-like activity (**B**), with same pattern as proteases Chymotrypsin-like activity (**C**) in tilapia fed ad libitum; Black bars represent basal digestive enzyme activity (fasting fish) and marbled bars represent activity of enzymes of tilapia fed ad libitum; Solid line represents the pattern of secretion under basal conditions and dashed line represents the enzyme pattern under ad libitum conditions ($P < 0.05$); Black section in the bar at the bottom of the figure represent night time; Values are mean ± SE
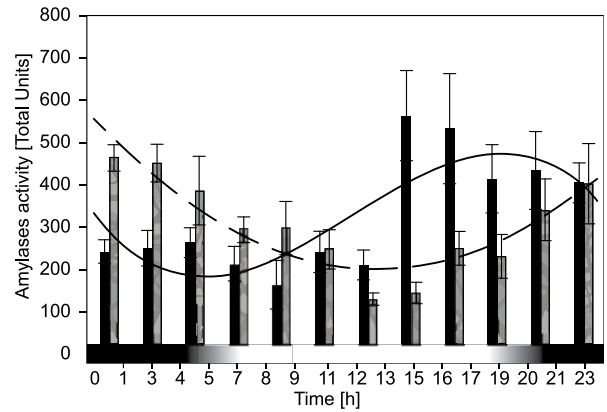

Fig. 3. Amylase activity in Nile tilapia, *Oreochromis niloticus*; Black bars represent basal (fasting) conditions with solid line ($P < 0.05$); marbled bars represent enzyme activity under ad libitum feeding conditions with dashed line ($P < 0.05$); Black section in the bar at the bottom of the figure represent night time; Values are mean ± SE
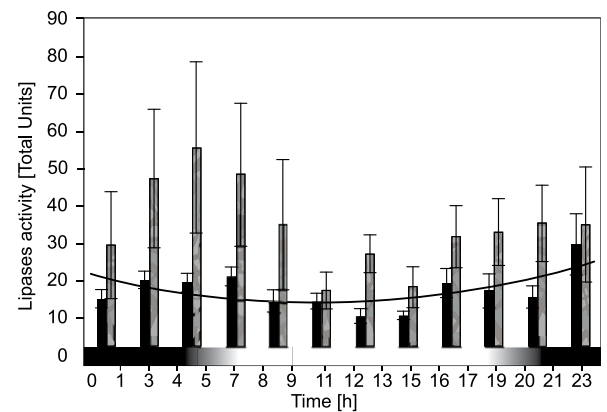

Fig. 4. Lipases activity in Nile tilapia, *Oreochromis niloticus*; Black bars represent basal (fasting) conditions with solid line ($P < 0.05$); marbled bars represent enzyme activity under ad libitum feeding conditions ($P < 0.05$); Black section in the bar at the bottom of the figure represent night time; Values are mean ± SE

information about the time of day, synthesizing and releasing high levels of melatonin into the bloodstream during the night (Reiter 1993). The influence and control of these regulators on basal digestive enzyme secretion in fish has not yet been clarified. The majority of studies have related secretion of digestive enzymes to food stimuli in species-specific experiments. However, basal responses of fish should be determined, as well as responses to frequencies and feeding schedules. Boujard (1995) demonstrated that fish could be trained to feeding schedules; however, voluntary intake is reduced. As we expected, the circadian cycle of the digestive enzyme secretions in the tilapia under basal conditions was consistent with López-Olmeda et al. (2006). In general, low digestive enzyme secretions occur in the early morning, immediately before

Table 1

Pattern of digestive enzymes during the circadian cycle of Nile tilapia, *Oreochromis niloticus*

Enzyme source	Enzyme	Conditions	Pattern equation	P values	Turning points Max	Min
Stomach	Pepsin-like	Starvation	$y = 10.527x + 326.53$	0.000003		
		Ad libitum	$y = -0.3532x^3 + 13.706x^2 - 122.9x + 720.98$	0.002000	20:06:00	5:46:12
Intestine	Proteases	Starvation	$y = -75105x^3 + 3 \times 10^{\wedge}6x^2 - 3 \times 10^{\wedge}7x + 1 \times 10^{\wedge}8$	0.000060	19:57:36	6:40:12
		Ad libitum	$y = 502560x^2 - 1 \times 10^{\wedge}7x + 2 \times 10^{\wedge}8$	0.000006		9:57:00
	Trypsin-like	Starvation	$y = -14.331x^3 + 585.88x^2 - 5958.9x + 27192$	0.000100	20:29:24	6:45:36
		Ad libitum	$y = 70.118x^2 - 1495x + 24224$	0.000400		10:39:36
	Chymotrypsin-like	Starvation				
		Ad libitum	$y = 377.3x^2 - 8673.9x + 105840$	0.000020		11:29:24
	Amylases	Starvation	$y = -0.214x^3 + 7.5695x^2 - 58.317x + 308.64$	0.009000	18:43:48	4:51:00
		Ad libitum	$y = 2.0381x^2 - 52.917x + 540.82$	0.000000		12:58:48
	Lipases	Starvation	$y = 0.0748x^2 - 1.5284x + 21.353$	0.006000		10:13:12
		Ad libitum				

sunrise, remaining at this level during most of the day until late afternoon. Digestive enzyme activity starts at sunset. Since the majority of basal enzymatic secretions occur at night, feeding strategies can be modified to take advantage of high-level enzyme secretion. Or changing the photoperiods will change melatonin level (Randall et al. 1995), which will synchronize feeding times with cyclic secretion of digestive enzymes to provide better and faster hydrolysis and increased feed efficiency. López-Vásquez et al. (2000) suggest that when amylase activity is highest, about two hours before food intake, then it is practical to start the feeding period at the peak of maximum of trypsin secretion. In larvae of the north African catfish, *Clarias gariepinus* (Burchell, 1822), that were fed one meal a day, protease activity increased after feeding and reached a peak 12 h after food was supplied, while no significant change in enzymatic activity occurred in starved catfish larvae (García-Ortega et al. 2000).

Circadian enzyme activity with feeding stimulation. In vertebrates, after food intake, several neural and hormonal mechanisms are activated that mediate postprandial secretion, to stomach distension, and nutrients in the small intestine. Both systems contribute precise adjustments of the secretory response to increase efficient food digestion (Kelley 1992). Montoya et al. (2010) demonstrate that altering feeding time affects the physiology and behaviour of gilthead seabream, *Sparus aurata* Linnaeus, 1758, where they modify their secretory capacity to prepare themselves for a forthcoming meal. For example, amylase and alkaline protease activity increased some hours before mealtime in periodic feeding, whereas in random feeding, amylase activity increased 1 h after feeding and alkaline protease showed no statistically significant differences. Acid protease activity was not significantly different in any group. Navarro-Guillén et al. (2015), studying food intake and digestive enzyme production of post-metamorphic larvae of Senegalese sole, *Solea senegalensis* Kaup, 1858, demonstrated that larvae has the higher food intake during the night, but continued to feed during the day, they also found that lipase activity increased before feeding, however trypsin activity was constant during the day.

In our study, tilapia digestive activity in the feeding treatment was higher than activity found under fasting basal conditions (difference was statistically significant). Under basal conditions the enzymatic activity showed minimum (at noon) and maximum (at night) peaks. Under ad libitum conditions, enzyme activities were maintained at high level, with a slight decrease at noon, but both experiments showed a markedly high nocturnal enzymatic activity. Sousa et al. (2012), however, compared night-, daytime-, and mixed feeding schedules, getting lower feed efficiency at night, because of that they proposed to optimize feeding offering feed before the sunset, to close-synchronize the moment of the highest digestive enzymes activity with the filling of gastrointestinal tract of fish.

CONCLUSIONS

Nile tilapias, *Oreochromis niloticus*, have circadian natural rhythms for digestive enzyme activity, regulated by endogenous systems, and which can be measured under fasting conditions. The natural rhythms may be stimulated by food, increasing digestive enzyme activity. If feeding schedules are adjusted to coincide with maximum natural peaks, feed efficiency will increase, which will be reflected

in weight gain of the fish and provide more profitable yields for aquaculture. Nile tilapia has a higher digestive enzyme activity at night than during the day.

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