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Daily rhythms of intestinal cholecystokinin and pancreatic proteases activity in Senegalese sole juveniles with diurnal and nocturnal feeding

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ABSTRACT

The influence of diurnal and nocturnal feeding on daily rhythms of gut levels of cholecystokinin (CCK) and the activity of two key pancreatic proteases, trypsin and chymotrypsin, were examined in juveniles of Senegalese sole (Solea senegalensis), a species with nocturnal habits. Four feeding protocols were performed: P1) One morning meal; P2) Six meals during the light period; P3) Six meals during the dark period; and P4) 12 meals during 24 h. Daily activity patterns of both proteases were remarkably similar and showed a high correlation in all the experimental protocols. In P1, daily patterns of CCK and digestive enzymes showed a single maximum. In P2, CCK levels exhibited two peaks. Digestive enzymes activities showed slightly delayed peaks compared to CCK, although their daily fluctuations were not significant. In P3, intestinal CCK concentration exhibited two peaks at the end of light and dark periods, but only the second one was significant. The first maximum level of chymotrypsin activity occurred 4 h after the first CCK peak, while the second one coincided with the second CCK peak. Fluctuations of trypsin activity were not significant. In P4, CCK concentration showed three small peaks. Digestive enzymes daily fluctuations were not significant, although they showed an inverted trend with respect to CCK. The daily pattern of the gut CCK content in our study is in agreement with the anorexigenic function of this hormone. Our results support the existence of a negative feedback regulatory loop between CCK and pancreatic proteolytic enzymes in Senegalese sole juveniles.

1. Introduction

Cholecystokinin (CCK) is a peptide hormone that plays an important role in regulating the intestinal phase of digestion in teleosts. CCK produced by enteroendocrine cells (EECs) in the

gastrointestinal tract (GIT) is involved in regulation of bile release, secretion of pancreatic enzymes, gastric emptying and gut motility (Einarsson et al., 1997; Murashita et al., 2007; Tillner et al., 2013a; Rønnestad et al., 2017). Free amino acids, short peptides, and most likely also fatty acids in the chyme entering the anterior intestine act as triggering factors for releasing CCK, which stimulates discharge of pancreatic digestive enzymes and bile into the intestinal lumen (Liddle, 2006; Koven et al., 2002; Kofuji et al., 2007; Rønnestad et al., 2017). In addition, CCK serves as an anorexic factor that inhibits appetite in mammals and fish. Studies in mammals have shown that the released CCK stimulate vagal afferent fibers that communicate with appetite controlling centers in the hypothalamus (Rehfeld, 2017). In fish, the same anorectic effect of CCK has been demonstrated in several species (i.e. G´elineau and Boujard, 2001; Volkoff et al., 2003; Rubio et al., 2008; Kang et al., 2010; Yuan et al., 2014; Volkoff, 2016; White et al., 2016).

The main aim of digestion is to degrade ingested feed into absorbable units. Trypsin and chymotrypsin are the main pancreatic proteases in fish (Dabrowski, 1983; Ueberscha¨r, 1995). The activity of these intes- tinal endopeptidases changes during the feeding and fasting cycles, and is correlated with the amount of digesta in the intestine (Gilannejad et al., 2021). Aside from the influence of abiotic factors, their activity levels have also been related to the levels and quality of dietary protein (Kuz'mina et al., 2011; Srichanun et al., 2014) and presence of anti-nutritional factors and protease inhibitors in the diet (Alarco´n et al., 1999; Chong et al., 2002; Santigosa et al., 2008). These two proteases seem to have a complementary role in the intestinal digestion process. Chymotrypsin is activated by trypsin (Zhou et al., 2011) and the relative proportion of both proteases varies under different feeding scenarios. In this sense, an increased trypsin/chymotrypsin ratio has been related to increased digestibility and growth (Sunde et al., 2001; Cara et al., 2007). Nevertheless, relatively few studies, (Atlantic salmon (Salmo salar), yellowtail (Seriola quinqueradiata), goldfish (Carassius auratus), gilthead seabream (Sparus aurata), cobia (Rachycentron canadum), have exam- ined variations in the daily activity of digestive enzymes (Einarsson et al., 1996; Murashita et al., 2007; Vera et al., 2007; Montoya et al., 2010; Yúfera et al., 2014, 2019; Gilannejad et al., 2021).

Within-one-day fluctuations of CCK and trypsin activity have been addressed in some fish larvae, Atlantic herring (Clupea harengus), Atlantic cod (Gadus morhua), sea bass (Dicentrarchus labrax) (Rojas- García et al., 2011; Tillner et al., 2013b, 2014), demonstrating a regu- latory loop between them in the intestine. However, periprandial in- teractions of CCK with pancreatic enzymes in the juvenile stage and under different feeding schedules have scarcely been studied (Murashita et al., 2006, 2008).

Senegalese sole (Solea senegalensis) is a flatfish species inhabiting the temperate coasts of the Northeast Atlantic Ocean and West Mediterra- nean Sea. This species has a great interest for marine aquaculture in- dustry in South-western Europe (Morais et al., 2014). Juveniles and adults of this species have benthic habits (Martínez et al., 1999; Ribeiro et al., 1999; Fern´andez-Díaz et al., 2001; Conceiça˜o et al., 2007; Padro´s et al., 2011) and preferably nocturnal feeding (Bayarri et al., 2004; Can˜avate et al., 2006; Navarro et al., 2009). It is an omnivorous species with an atypical small stomach with limited acidification capacity and without pyloric caeca (Yúfera and Darias, 2007), and the main part of digestion occurs in its long intestine.

In a previous study with Senegalese sole during the first month after hatching, we found that both pelagic larvae and benthic post-larvae after eye migration displayed the above-mentioned regulatory loop (Navarro-Guill´en et al., 2017). We also found increased CCK levels when the gut was emptied. At that early age, the nocturnal feeding habits have not completely been acquired (Navarro-Guill´en et al., 2015) and the fish were maintained with a permanent availability of live prey during the selected feeding period in each of the tested protocols.

The aim of the present work was to evaluate the influence of diurnal and nocturnal feeding on daily rhythm of gut levels of CCK and the activity of two key pancreatic proteases in Senegalese sole juveniles in order to advance in the understanding of the regulation of digestion in a species with a long intestine when full nocturnal feeding habits have been attained.

2. Materials and methods

2.1. Experimental design

Senegalese sole juveniles were obtained from a local hatchery and maintained at the ICMAN experimental facilities (REGA ES110280000311). Fish were randomly distributed into four groups, with two replicates for each (N 84 in each group, with an initial body mass of 51.61 15.73 g, mean and SD). Fish were maintained in quadrangular flat-bottom 250-L tanks with flow-through water system, at 19.5 1.0 ◦C, and a natural photoperiod (11L/13D). Using automatic feeders, the four groups were fed the same experimental diet and daily ration, close to the satiation level (3% of fresh body mass), but different daily feeding protocols (P). P1) One meal at 8:30 h (local time); P2) Six meals during the light period, at 08:30, 10:00, 12:00, 14:00, 16:00, and 18:00 h; P3) Six meals during the dark period, at 20:00, 22:00, 24:00, 02:00, 04:00 and 06:00 h; and P4) 12 meals during 24 h, at times mentioned for P2 and P3. Within each feeding protocol, daily ration was equally distributed among feeding times. The feeding experimental period lasted for three weeks, after which sampling was performed. The experimental design with feeding and sampling times for the different protocols are illustrated in Fig. 1. Composition of the experimental diet (SPAROS Lda., Olha˜o, Portugal) is shown in Table 1.

2.2. Sampling procedure

All the experimental and sampling procedures were carried out in compliance with the European Union Council Guidelines (2010/63/EU) and the Spanish legislation for the use of laboratory animals, with approval of Bioethics Committee of the Spanish National Research Council for project EFISHDIGEST (AGL2014-52888-R).

For each group, fish were sampled every 4 h, at 09:00, 13:00, 17:00, 21:00, 01:00, 05:00, and 08:30 h. The first sampling (at 09:00 h) was performed after the first daily meal, while the last sampling (at 08:30 h) was done before supplying the first daily meal (Fig. 1). Ten individuals were sampled per feeding protocol and sampling time, four for CCK analyses (two per tank) and six (three per tank) for enzymes activity analyses. The fish were anaesthetized (250 ppm) and then killed (600 ppm) with a 2-phenoxyethanol

overdose. The whole GIT was carefully dissected, immediately transferred to liquid nitrogen, and then stored at -80 ◦C until being processed.

2.3. CCK measurements

In order to determine the CCK distribution along the digestive tract, one GIT sample (individual belonged to Protocol 1 and sampled at 13:00 h) was split into six segments; stomach was considered as 'segment 1' and intestine was divided into five parts of equal length, 'segments 2 to 6' (Fig. 2). Although we might have lost the inter-individual variation, according to our experience, a single sample could provide a strong indication of the spatial GIT CCK distribution profile. In the rest of GIT samples, only the proximal intestine corresponding to 'segments 2 and 3', were used for CCK measurements, as they showed the highest levels of this hormone in the aforementioned analysis (Figs. 2, 3).
In order to prepare the tissue homogenates for CCK measurements, the selected intestinal segment of each individual was cut open and with the help of forceps was vigorously agitated in ice-cold 1 Phosphate Buffered Saline solution (PBS; Sigma-Aldrich, P5493), to wash away any remaining food or digesta. Epithelial tissue (mainly the mucosa layer) was scraped off the intestinal wall (muscle layers) and transferred into pre-weighted 5 mL tube containing 1/10 volume of 1 PBS. Samples were homogenized with a motorized pestle and then centrifuged at 4 ◦C, 1500 g for 15 min. Supernatants were aliquoted and stored at 20 ◦C until further processing.
CCK concentration was quantified by using a sandwich Fish CCK-8 Enzyme-Linked Immunosorbent Assays (ELISA) Kit (Ref. No. MBS069488; MyBioSource, USA), according to the manufacturer's in-structions. Samples were assayed in duplicate, and their concentrations extrapolated from a standard curve (15.6–500 pg.mL-1) and expressed as 'pg per g of fish fresh body mass (pg.g-1 BM)', considering both the total amount of tissue used for CCK determination and the fresh body mass (including the gut content) of the corresponding individual.

2.4. Enzyme activity analyses

In order to prepare the enzyme extracts, anterior intestine of each individual, corresponding to 'segments 2, 3, and 4' (Fig. 2), was sepa- rately homogenized in distilled water (1:3 w/v), and centrifuged at 4 ◦C, 19,000 g for 10 min. Supernatants were aliquoted and stored at 20 ◦C until being processed.
Trypsin and chymotrypsin activities were measured according to Erlanger et al. (1961) and (1966), using 0.5 mM Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) and 0.5 mM N-Glutaryl-L- phenylalanine p-nitroanilide (GAPNA) as substrates, respectively. The substrate stocks of trypsin and chymotrypsin were brought into the

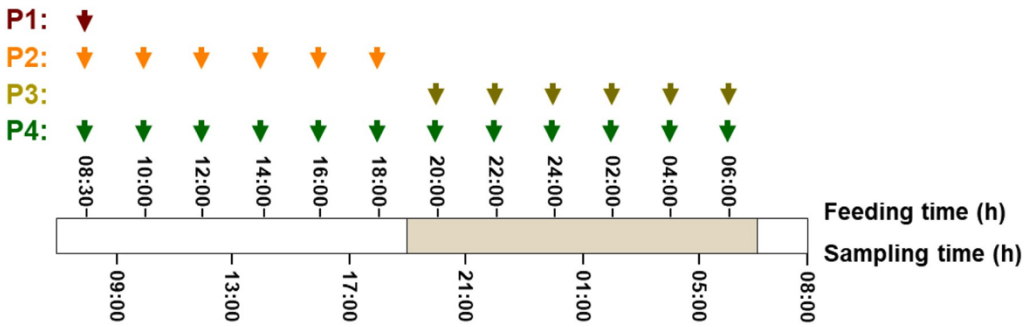

Fig. 1. Detailed schema for feeding and sampling schedule in S. senegalensis with different daily experimental feeding protocols. Red, orange, yellow, and green arrows indicate the feed supply in protocols 1, 2, 3, and 4, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

working concentration by a 1/10th dilution using 50 mM Tris-HCl and 20 mM CaCl2 buffer (pH 8.5), respectively. For each enzyme, changes in absorbance at 405 were measured, for 10 to 15 µL of the enzyme extract and 200 µL of the corresponding substrate per microplate well, over 10 min. One unit of activity was defined as 1 µmol p-nitroaniline released per minute using coefficients of molar extinction of 8270 M-1 cm-1 at 405 nm. For each enzyme, samples were assayed in duplicate and ac- tivities were expressed as 'units per g of fish fresh body mass (U.g-1 BM)', considering both the total amount of tissue used for enzyme activity determination and the fresh body mass (including the gut content) of each individual.

2.5. Statistics

Statistical analyses were performed using GraphPad Prism v. 6.01. Data were analysed for possible outliers using the Grubbs' test (ESD method). To estimate the feeding protocol and sampling time as inde- pendent factors, values obtained for CCK concentrations and enzymatic activities were subjected to two-way ANOVA, followed by a post-hoc Tukey test. One-way ANOVA followed by a post-hoc Tukey test were used to assess the differences in final body mass among groups, as well as differences among sampling times for the studied parameters within each feeding protocol. All data were previously checked for normality of distribution and homogeneity of variance. Data were considered statis- tically significant when $p < 0.05$ and were visualized as mean stan- dard error of the mean (SEM), using SigmaPlot v.13. The correlation between CCK concentration with trypsin and chymotrypsin activities was analysed by a Pearson 2-tailed significance correlation, in each feeding protocol ($p < 0.05$) (Supplementary Table 1).

Existence of daily rhythms was tested by cosinor analysis, using the chronobiology software EL TEMPS® v.292 (from Prof. A. Díez Noguera, University of Barcelona, Spain). Following this method, it was evaluated whether fluctuations of a data set, CCK concentration or enzyme activ- ity, over time were fitted into a cosine curve, applying the following equation. Y Mesor Amplitude cos [2π (t Acrophase)/Period], where, Mesor (MES) is the mean of the rhythm, Amplitude (AMP) is the maximum variation, t is the phase of the rhythm, Acrophase (ACR) is the time of the peak value, and Period is the duration of one cycle (24 h). In this analysis,

sampling points were considered as Zeitgeber time (ZT). The ZT 0 was defined as the time of the light on (08:00 h local time) and light was switched off at ZT 11 (19:00 h local time). Rhythms were assumed significant when AMP differed from zero ($p < 0.05$).

3. Results

After the experimental period, body mass (66.6 2.8, 75.8 3.4, 81.6 3.4, and 78.2 2.8 g from P1 to P4, respectively) was signifi- cantly lower in fish from P1 compared to P3 and P4.

No significant differences were detected among the other feeding protocols ($p < 0.05$). Daily patterns of CCK concentration and digestive enzymes activity in response to different feeding protocols are shown in Fig. 4 and Sup- plementary Table 2. Daily activity patterns of these two digestive en- zymes were remarkably similar and showed a high correlation in all the experimental protocols ($p < 0.0001$). Such correlation was not detected between CCK with trypsin or chymotrypsin activities (Supplementary Table 1).
In P1, daily patterns of CCK and digestive enzymes showed a single peak (Fig. 4). Besides, these patterns were similar, although a slight advancement of CCK with respect to the activity of digestive enzymes was detected. In other words, while maximum and minimum CCK concentration levels were respectively found at 08:00 h (before the first daily meal) and 17:00 h, highest and lowest enzymatic activities were respectively obtained at 09:00 h (after the first daily meal) and at 21:00 h (Fig. 4). According to the cosinor analysis, CCK, trypsin, and chymo- trypsin presented significant circadian rhythms, with ACR at 06:27 h (ZT 22:27), 07:54 h (ZT 23:54), and 09:07 h (ZT 01:07), respectively (Supplementary Table 3).

In P2, CCK daily fluctuation was characterized by two peaks, the first and smaller peak at 17:00 h and the second and sharper peak at 05:00 h (second part of the dark period) (Fig. 4). The lowest CCK levels were observed at 09:00 h (after the first daily meal) and at the beginning of the dark period (21:00 h). Trypsin and chymotrypsin showed a slightly delayed activity patterns compared to CCK, although their daily fluc- tuations were not significant (Fig. 4). Cosinor analysis did not show significant circadian rhythm in any of these parameters in this feeding protocol (Supplementary Table 3).
In P3, CCK concentration in the intestinal tissue increased at the end of the dark period (05:00 h) and reached the maximum values at 08:00 h, although a trend of high values was also found at the end of the light period (17:00 h), as well. The lowest levels of the hormone were detected from 09:00 to 13:00 h (Fig. 4). Of the two digestive enzymes, only chymotrypsin activity showed significant changes during the daily cycle, with their first maximum level at 021:00 h (4 h after the first CCK peak). However, the second peak (08:00 h) coincided with the second CCK peak (Fig. 4). According to the cosinor analysis, neither CCK nor digestive enzymes exhibited significant circadian rhythm in this feeding protocol (Supplementary Table 3).
In P4, CCK concentration also showed daily fluctuations, being characterized by three small peaks, at 13:00, 01:00 and 08:00 h (Fig. 4). Digestive enzymes daily fluctuations were not significant, although an inverted trend with respect to CCK, with higher activity levels at 09:00, 21:00 and 05:00 h, was detected (Fig. 4). Cosinor analysis did not show significant circadian rhythm in any of these parameters in this feeding protocol (Supplementary Table 3).

Daily average values for each one of the studied parameters are summarized in Table 2. According to the two-way ANOVA, both CCK

Table 1
Formulation and proximate composition of the experimental diet.

Ingredients (%)	
Fishmeal LT70[1]	47.0
Fish protein concentrate[2]	5.0
Squid meal[3]	5.0
Porcine gelatin[4]	2.0
Soy protein concentrate[5]	7.0
Pea protein concentrate[6]	5.0
Wheat gluten[7]	5.0
Wheat meal[8]	7.7
Fish oil[9]	5.7
Vitamin and mineral premix[10]	1.0
Lutavit E50[11]	0.05
Soy lecithin[12]	2.0
Binder (guar gum)[13]	0.2
Binder (zeolite)[14]	0.5
Antioxidant[15]	0.2
Sodium propionate[16]	0.1
Monocalcium phosphate[17]	3.0
Encapsulated taurine[18]	0.5
Glycerol[19]	3.0
Composition	
Crude protein (% DM)	57.3
Crude fat (% DM)	12.5
Fibre (% DM)	0.5
Ash (% DM)	8.1
Gross Energy ($MJ \cdot kg^{-1}$ DM)	19.3
Total phosphorus (% DM)	1.7

[1] NORVIK 70: 70.6% crude protein (CP), 5.8% crude fat (CF), Soprop^eche, France.
[2] CPSP90: 84% CP, 9.6% CF, SOPROPE^CHE, France.
[3] 82% CP, 7.1% CF, SOPROPE^CHE, France.
[4] 89% CP, Weishardt, Switzerland.
[5] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[6] Lysamine: 86% CP, 1% CF, ROQUETTE Fr`eres, France.
[7] VITAL: 83.7% CP, 1.6% CF, ROQUETTE Fr`eres, France.
[8] 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal.
[9] SAVINOR UTS, Portugal.
[10] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.
[11] ROVIMIX E50, DSM Nutritional Products, Switzerland.
[12] Lecico P700IPM, LECICO GmbH, Germany.
[13] Seah International, France.
[14] Kieselguhr, LIGRANA GmbH, Germany.
[15] Paramega PX, Kemin Europe NV, Belgium.
[16] Disproquímica, Portugal.
[17] 22% P, 18% Ca, Fosfitalia, Italy.
[18] ORFFA, The Netherlands.
[19] Belgosuc, Belgium.

and trypsin activities were significantly influenced by the feeding pro- tocol ($p < 0.05$), while sampling time only affected the CCK concen- tration. The effect of interaction of these two factors was significant on the activity of both digestive enzymes. According to this analysis,

P1 had the highest CCK concentration, while it showed the lowest trypsin ac- tivity. The lowest CCK concentration and the highest trypsin activity were respectively found in P4 and P3 (Table 2).

4. Discussion

This study aimed to describe to what extent different feeding pro- tocols influence the daily pattern of gut CCK content and its interaction with the most important pancreatic proteolytic enzymes in a juvenile fish. In the present study, average wet body mass of the juvenile fish ranged from 66.6 to 78.2 g. Although the aim of the current work was not to conduct a growth trial and the experimental duration was not long enough to draw firm conclusions, the group with a single diurnal feeding (P1) showed lower weight gain than the other protocols as expected. Nevertheless, these differences were not important enough to cause bias in our findings by the size factor.

4.1. GIT CCK distribution

The GIT is the largest endocrine organ in vertebrates (Holst et al., 1996), and produces a range of peptide hormones that act in complex signalling pathways, that also interact with the enteric, peripheral, and central nervous systems, to regulate the food intake and digestion pro- cesses (reviewed by Volkoff, 2016). CCK polypeptides of various lengths are derived from proCCK in endocrine cells and neurons (Chandra and Liddle, 2018; Le et al., 2019). In the intestine, CCK is produced and released from EECs in the brush border of mucosa (Rønnestad et al., 2007; Pereira et al., 2017, 2019). The C-terminus octapeptide of CCK (CCK-8) is the smallest and one of the most potent forms of CCK with complete biological activity, which is often used in assessing CCK digestive functions in mammals (Liddle et al., 1986; Chandra and Liddle, 2018).
In our study, CCK-8 was detected in all GIT segments, including the stomach and hindgut. Although some cross-reaction between CCK and gastrin may occur when using commercial ELISA kits, we still believe that the signal we obtained strongly reflects the CCK response. Several GIT distribution patterns of this peptide have been reported in fish species, being attributed to the differences in the anatomy of their digestive system or feeding habits, i.e. carnivore, omnivore or herbivore (Pan et al., 2000; Pereira et al., 2015; Lin et al., 2017). Similarly, analysis of CCK protein or transcripts, measured by radioimmunoassay, in situ hybridization, or qPCR, has shown important species-specific differences in appearance of CCK-producing cells along the larval GIT (Rojas-García et al., 2011). According to these studies, in species with larval stages with a coiled gut, e.g. Atlantic halibut (Hippoglossus hippoglossus) and bluefin tuna (Thunnus thynnus), CCK-producing cells were mainly concentrated in the anterior intestine (close to the stomach). Whereas in larvae with a straight gut, e.g. Atlantic herring and ayu (Plecoglossus altivelis), these cells were more uniformly distributed along the whole length of GIT (Rojas-García et al., 2011; Rønnestad et al., 2013).

The GIT of S. senegalensis is characterized by a small stomach, a long S-shaped intestine, and the lack of pyloric caeca (Yúfera and Darias, 2007). The only visually detectable morphological feature, besides the pyloric sphincter, is the ileocaecal valve that divides the anterior and the posterior intestinal regions (Valente et al., 2019), which in spite

of their visual anatomical differences show similar histological characteristics (Arellano et al., 2002). Therefore, the ubiquitous GIT detection of CCK-8 in our study is in accordance with previous findings in agastric species or with a simple tubular GIT (Le et al., 2019). Furthermore, due to a re- sidual gastric acidification and pepsin activity, the stomach plays a negligible role in protein hydrolysis of Senegalese sole and the intestinal proteolysis phase is of primordial importance in the digestion process (Yúfera and Darias, 2007; Gilannejad et al., 2017). Therefore, the CCK-8 distribution pattern in our study is closely related to those generally found in fish with omnivorous feeding habits (Barrios et al., 2020).
This characteristic distribution of CCK-producing cells has been associated with CCK specific roles in regulating the gut motility in different GIT segments and local signalling functions, and may underlie the relatively fast transit of undigested food, due to the lack of the

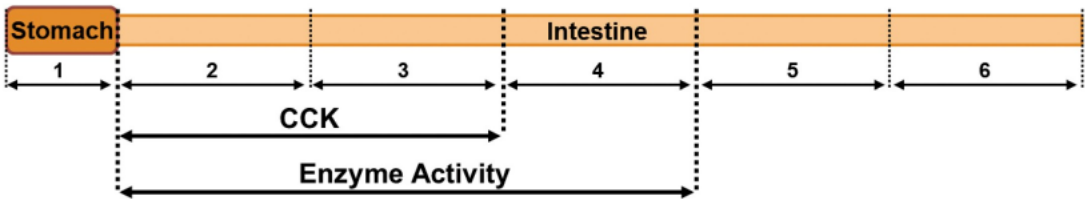

Fig. 2. Schema of S. senegalensis digestive tract divided into different segments, where the segments used for measurement of CCK concentration and digestive enzyme activities are identified.

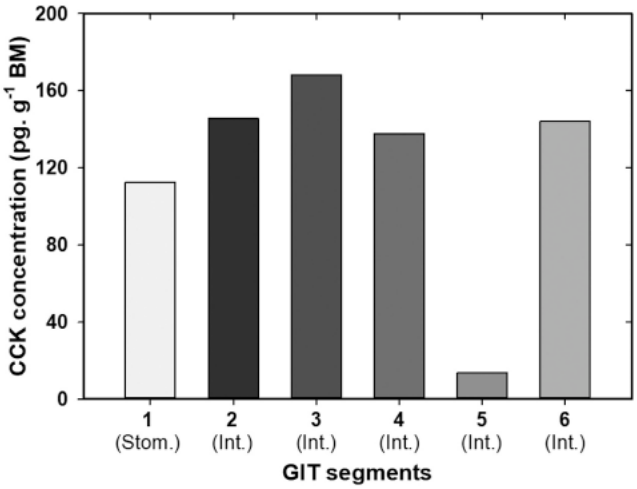

Fig. 3. Concentration of CCK in different segments of S. senegalensis digestive tract. GIT, gastrointestinal tract; Stom., stomach; Int., intestine.

controlling role of a functional stomach acting as a short term reservoir (Hartviksen et al., 2009; Gra¨ns and Olsson, 2011; Lie et al., 2018; Le et al., 2019). Therefore, besides the previously mentioned reasons, the extremely low consistency of the feces in this species, could justify the presence of CCK in all GIT segment. In fish with a functional stomach CCK inhibits the gastric motility and passage rate to adjust the gut transit rate, while a recent study in the agastric fish ballan wrasse (Labrus bergylta) has shown that CCK plays different

roles. In the foregut section, CCK acts as a “physiological sphincter” that, besides inhibiting the motility, stimulates the retrograde ripples to permit more time for digesting the ingested food. However, in the hindgut, it induces non- propagating and propagating contractions, regulating water absorp- tion and defecation of waste products (Le et al., 2019).

Hence, the highest CCK-8 levels in the proximal intestine in our study could be explained by the importance of this region in the digestion process since this is the location of openings of the biliary and pancreatic ducts into the intestinal lumen (Sarasquete et al., 2019). On the other hand, the lowest CCK levels in the stomach and segment 5 could be due to the presence of the pyloric sphincter and ileocecal valve, which re- duces the need of this hormone in regulating the GIT transit in these regions.

4.2. Daily patterns of gut CCK content and digestive enzymes

To the best of our knowledge, the presence or absence of CCK circadian rhythms in fish species have not previously been assessed by proper statistical methods, e.g. cosinor analysis, and have been rather made based on the visual interpretation of daily fluctuations graphs. In our study, the group with a single daily meal (P1) displayed a significant circadian pattern, but more remarkably, the rest of the feeding protocols with several daily meals, presented apparent ultradian patterns of approximately 12 h. This shows that several feeding events could result in complex overlapped responses that prevent the detection of a clear circadian rhythm. Similarly, a recent study in human has shown that three daily meals could produce plasma CCK rhythms that could be fitted into circadian or ultradian patterns (Period 8, 12, o 24 h) (Rehfeld et al., 2020). Interestingly, in our study, a maximum protease activity just after first daily meal, as could be expected after a period of resting, was not observed in diurnal feeding (P2). In addition, the feedback response between CCK and tryptic activity was more evident when nocturnal feeding was available (P3 and P4). Such results would indicate that the digestive proteolytic ability better adapted to the nocturnal feeding preference of this species.

Most of the studies addressing the CCK in vertebrates, including human and rat, use blood samples, i.e. secreted CCK into circulation (Rojas-García and Rønnestad, 2002). However, this method has some limitations. Firstly, the stress induced by consecutive daily samplings may adversely affect the physiological responses. Secondly, the extremely short half-life of this hormone in the plasma, around 1.3 0.1 min in dogs as an example (Hoffmann et al., 1993). Therefore, in this study, we have assessed the levels of this hormone in tissue of the proximate intestine. The cellular content or storage of CCK is the net balance between the secretion and re-synthesis rates (Rojas-García and Rønnestad, 2002). RIA analysis of CCK hormonal levels on different body compartments in the Atlantic herring larvae showed that a drop in gut CCK levels 1 h after feeding was concurrent with an increase of this hormone in the body (head excluded). After 2 h, CCK returned to pre- feeding levels, suggesting that the released CCK had been re- synthesized in the gut EECs (Rojas-García et al., 2011). The gradual release of CCK from EECs and its clearance from plasma, will tend to maintain target levels of circulating CCK to regulate sufficient pancre- atic secretion of trypsin for several hours to ensure proper digestion (Rojas-García and Rønnestad, 2002).

In line with this, the large increase in gut CCK content during the dark hours in P1 indicates re-synthetizing the reserves of CCK in EECs during the non-feeding period, while the gradual decrease after feeding would be explained by the secretion of this hormone into the blood

stream, in accordance with its involvement in controlling digestion and satiety. In the other feeding protocols, although such clear patterns could not be observed, a tendency to increase gut CCK levels at the end of the unfed period, that is very short in P4, is evident. Such response could be a part of the physiological modifications related to the Food Anticipatory Activity (FAA), stocking the EECs with sufficient synthe- tized CCK to be released by the time food enters the GIT. FAA in response to periodic and predictable events, e.g. light-dark and feeding-fasting cycles, adjusts the behavioural, physiological, and metabolic processes to optimize nutrient utilization (Stephan, 2002; Strubbe and Van Dijk, 2002).
In addition, maximum CCK levels at the end of the dark and/or the beginning of the light period, regardless of the feeding protocol or gut transit pattern (Gilannejad et al., 2019), could highlight the nocturnal feeding preference of this species at this life stage, potentially driving higher CCK synthesis rate during the late dark period. Similarly, in a study conducted in rats, significant circadian plasma CCK patterns with acrophase during the dark period were obtained even in unfed animals.

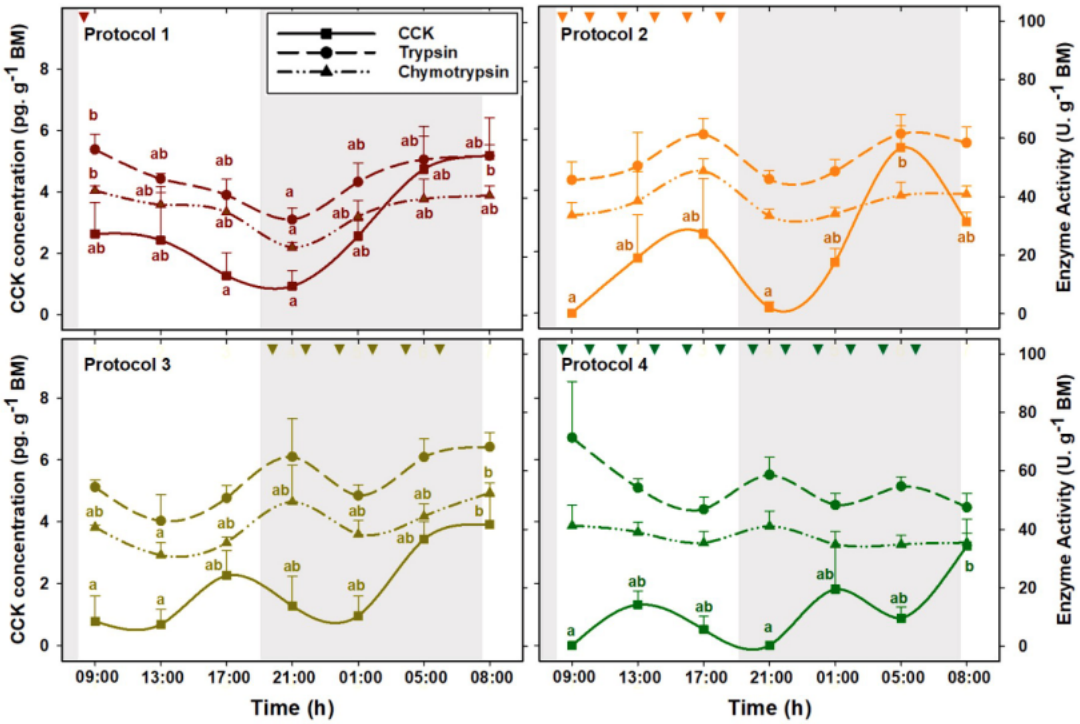

Fig. 4. Daily patterns of CCK concentration, and activity of trypsin and chymotrypsin in S. senegalensis with different feeding protocols. For each parameter, different letters denote significant differences between sampling times (for each feeding protocol and sampling time N = 4 and 6 individuals respectively for CCK and digestive enzymes, one-way ANOVA, $p < 0.05$). Grey areas indicate the dark period and coloured triangles on the top margin of each plots represent the feed supply in the corresponding feeding protocol.

Table 2
Daily average values for CCK concentration and trypsin and chymotrypsin activities in Senegalese sole juveniles with different daily feeding protocols.

	CCK (pg. g $^{-1}$ BM)	Trypsin (U. g $^{-1}$ BM)	Chymotrypsin (U. g $^{-1}$ BM)
Two-way ANOVA[1]			
Effect of protocol	0.001	0.029	0.087
Effect of time	< 0.0001	0.036	0.238
Interaction	0.071	0.025	0.005
Post-hoc Tukey test[2]			
Protocol 1	2.83 ± 0.41 ^{b}	47.06 ± 2.23 ^{a}	36.17 ± 1.79
Protocol 2	2.16 ± 0.44 ^{ab}	53.11 ± 2.23 ^{ab}	38.52 ± 1.70
Protocol 3	1.90 ± 0.33 ^{ab}	56.69 ± 2.59 ^{b}	41.60 ± 1.99
Protocol 4	1.15 ± 0.29 ^{a}	54.53 ± 2.93 ^{ab}	37.41 ± 1.47

[1] Effect of the two main factors (protocol and time) and their interactions are expressed as decimal p-values, with significant effects marked in bold ($p < 0.05$).

[2] Values are as mean ± SEM (for each feeding protocol $N = 28$ and 42 individuals respectively for CCK and digestive enzymes) and different superscripts indicate significant differences ($p < 0.05$) among feeding protocols.

The authors attributed this pattern to the nocturnal feeding habit in this species and the anticipatory release of this peptide at regular meal times, and suggested the existence of endogenous control mechanisms that are not completely dependent of the presence of food in the GIT (Pasley and Rayford, 1990). The presence of natural endogenous CCK and/or trypsin rhythms, independent of food intake has been also suggested in larval stage of some fish species (Tillner et al., 2013b, 2014).

Although the gut content was not measured in the present study, according to our recent experiment on GIT transit in smaller Senegalese sole juveniles (approx. 9 g) under the same feeding protocols (Gilan- nejad et al., 2019), periods of high GIT content are roughly followed by decreasing CCK levels. This suggest that the arrival of chyme/digesta could not be directly compared with their measurements, as they have been made in the body content (head excluded), and therefore, puta- tively correspond to both gut and circulating CCK levels.

As mentioned previously, the alkaline protease trypsin and chymo- trypsin are the most relevant proteolytic enzymes in Senegalese sole (Santigosa et al., 2008; Gilannejad et al., 2017). In our study, trypsin and chymotrypsin displayed daily activity fluctuations that, although not significant in all the cases, were remarkably similar and showed extremely high correlation in all feeding protocols. Similar to our re- sults, a recent study in yellowtail showed highly comparable post- prandial trypsin and chymotrypsin activity, regardless of the dietary composition and/or GIT segment in which they were measured (Mura- shita et al., 2019).

Similar to CCK, the storage level of pancreatic digestive proenzymes (trypsinogen and chymotrypsinogen) pools depends on the rate of the de novo synthesis and release into the anterior intestine (Einarsson et al., 1997; Uebersch¨ar et al., 2018; Murashita et al., 2007, 2019). Therefore, the pulse-like pattern of these enzymes in the present study could indicate the time needed for their biosynthesis and secretion. Accord- ingly, the incapacity of maintaining high trypsin and chymotrypsin ac- tivities for long time after the single daily meal in P1 can be explained by the temporary exhaustion of pancreatic zymogens, together with the gradual reduction of the food substrate in the gut. The protease activity

is re-established during the nocturnal period waiting for the next meal. This situation, imposed by the low feeding frequency, could lead to a poor fish performance, as demonstrated by the minimum growth ob- tained in this group compared to the other feeding protocols in the present study.
Moreover, one (P1) and several (P2, P3, and P4) daily meals respectively produced one and several daily CCK peaks that were fol- lowed by subsequent protease activity maximum levels. In other words, a general temporal displacement was obtained between CCK and digestive enzymes in each one of the feeding protocols, with CCK peaks preceding the maximum enzymatic activity levels. The antagonistic behaviour of CCK and tryptic activity in fish larvae has been associated with a regulating feedback mechanism (Cahu et al., 2004; Drossou, 2006). Therefore, the general daily pattern of the studied parameters in our study, could confirm the existence of a negative feedback regulatory loop between CCK-8 and pancreatic proteolytic enzymes in Senegalese sole juveniles, as it was previously documented in the larval stage of this species (Navarro-Guill´en et al., 2017).
Furthermore, we found that the functionality of this loop with tem- poral displacement between maximum levels depended on the feeding protocol, i.e. increasing the feeding frequency seemed to prolong the delay between peaks in CCK secretion and proteases activity. Accord- ingly, while CCK and digestive enzymes daily patterns in P1 were apparently similar, cosinor analysis revealed a general phase shift, with acrophase of CCK, trypsin, and chymotrypsin occurring at 06:27, 07:54, and 09:07 h, respectively. Besides, an approximate phase shift of 4 and 8 h between CCK and the pancreatic proteases were respectively observed in the groups with 6 (P2 and P3) and 12 (P4) daily meals. In agreement with this, it has been suggested that several factors contribute to set-up of this feedback mechanism in fish larvae, including feeding frequency, species-specific feeding habits and GIT morphology, feed content and physical-chemical characteristics (Uebersch¨ar et al., 2018).

4.3. Daily average values of gut CCK content and digestive enzymes

The fish with single diurnal (P1) and continuous daily (P4) meals exhibited respectively the highest and lowest daily average CCK values. Previously, we also demonstrated that the fish fed these two feeding protocols had the fastest and slowest gut filling rates, respectively (Gilannejad et al., 2019). This could point to the potential role of CCK in modulating gut motility and transit in this species, similar to what has been suggested for Ballan wrasse (Le et al., 2019). Moreover, a general and inverse trend was observed in the absolute daily average values for gut CCK and trypsin activity levels, e.g. the group with a single daily meal (P1) with highest CCK levels showed the minimum trypsin activity. Although this trend was not observed in all feeding regimes, i.e. the group with nocturnal feeding (P3) showed the

maximum daily average of trypsin activity but not the lowest average of CCK concentration. Probably, additional endocrine or neuroendocrine regulatory pathways are involved in this process (Konturek et al., 2003; Raybould, 2007).
The fact that increasing food availability (number of meals) along the whole daily cycle (P4) did not lead to highest digestive enzymes activity and growth performance, might imply that fish digestive capacity has limits, whose borders (maximal rates) could not be pushed beyond (Tillner et al., 2014; Zeytin et al., 2016; Ueberscha¨r et al., 2018). Among these limitations could be the time needed to restore the hormonal, metabolic, and enzymatic resources (Yúfera et al., 2018) or the existence of species-specific exogenous rhythms that drive the daily pattern of many digestive physiological aspects (Tillner et al., 2014; Yúfera et al., 2017; Gilannejad et al., 2021). In fact, despite the constant food avail- ability in P4, smaller Senegalese sole juveniles showed a gut transit pattern similar to P2 (Gilannejad et al., 2019).
Considering the anorexic function of CCK, the highest gut CCK levels in P1, which is probably parallel to low circulating levels in body fluids, could indicate that the daily rate of food intake is not adequate in this feeding protocol, something otherwise expected. In fact, we found the lowest food digestibility capacity and the poorest growth performance with this protocol, in this study as well as in smaller juveniles (Gilan- nejad et al., 2019). On the other hand, high activity levels of proteolytic enzymes have been attributed to a better digestive performance, nutri- tional status, and growth efficiency in fish (Debnath et al., 2007; Mohanta et al., 2008). Therefore, high daily average value together with a better synchrony of the daily pattern of pancreatic digestive enzymes with the feeding time in P3 (higher levels during the dark period), which is in line with the nocturnal feeding preference of this species at this life stage, could explain the better growth performance in this feeding protocol.

5. Conclusions

CCK-8 is present in all GIT segments in Senegalese sole, but with higher levels in the anterior intestine. This is in accordance with the anatomy of the digestive system and omnivorous feeding habit of the species. The spatial distribution of CCK along the GIT support the key role of this hormone in regulating gut motility to increase the transit time and digestive efficiency. The daily pattern of the gut CCK content in our study is in agreement with the anorexigenic function of this hor- mone. Besides, our results suggest that although the feeding protocol plays an important role in the daily fluctuations of gut CCK levels pu- tatively through modifications in the feed transit and gut content, the nocturnal feeding habit of this species is also decisive in defining these tendencies. Furthermore, the general asynchrony between CCK and digestive enzymes peaks suggests the existence of a negative feedback regulatory loop, whose functionality depend on the feeding protocol, i.e. increasing the feeding frequency prolonged the delay between CCK and proteases activity peaks. The general inverse trend in the daily average values for gut CCK and trypsin activity, underlines the existence of the aforementioned negative regulatory feedback loop in Senegalese sole juveniles, although the presence of more complex endocrine or neuronal regulatory pathways are also plausible. Overall, according to the daily average of digestive enzymes and their circadian pattern, we could suggest that exclusively nocturnal (P3) and a single diurnal (P1) feeding could lead to the best and worst digestive and growth performance, respectively.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2020.110868.

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