Effect of increased rearing temperature on digestive function in cobia early juvenile

Short title: Effect of temperature on Cobia digestion

M. Yúfera[a], M.V. Nguyen[b], C. Navarro-Guillén[a1], F.J. Moyano[c], A-E.O. Jordal[d], M. Espe[e], L.E.C. Conceição[f], S. Engrola[g], M.H. Le[b], and I. Rønnestad[d]

[a]Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC), Campus Universitario Rio San Pedro s/n, 11519 Puerto Real, Spain
[b]Institute of Aquaculture, Nha Trang University, 02 Nguyen Dinh Chieu st, Nha Trang, Vietnam
[c]Department of Biology and Geology, University of Almería, 04120 Almería, Spain
[d]Department of Biological Sciences, University of Bergen, NO-5020, Bergen, Norway
[e]Institute of Marine Research, Bergen, Norway
[f]Sparos Lda, Olhão, Portugal
[g]Centre of Marine Sciences of Algarve (CCMAR), University of Algarve, Campus de Gambelas, University of Algarve, 8005-139 Faro, Portugal

Corresponding author: manuel.yufera@icman.csic.es

[1] Present address: Centro de Ciências do Mar do Algarve (CCMAR), Universidade do Algarve, Faro, Portugal.

Abstract

The present study is focused to elucidate the main characteristics of the digestive function of this carnivorous fast-growing fish living at high temperatures. With this aim, we have examined the effects of an increased temperature from 30 to 34ºC on the daily pattern of gastrointestinal pH, enzymatic proteolytic digestive activity and the feed transit time in early juveniles of cobia (*Rachycentron canadum*), a ~~fast-growing carnivorous fish~~ species living in tropical and subtropical waters with an increasing aquaculture production. Fish were fed two meals a day. Gastric luminal pH was permanently acidic (mean pH values: 2.76 - 4.74) while the intestinal pH increased from neutral/slightly acidic to slightly alkaline when the digesta was present, with an increasing alkalinity from proximal to distal intestine (mean pH values: 6.05 to 7.69). The temperature did not affect the gastric pH but a slightly higher acidity was induced in the intestine at 34ºC.

Pepsin activity showed a daily rhythm at 30 ºC with maximum in the middle of the light period, while at 34ºC some hourly changes coinciding with feed adding without a clear daily trend during the 24-h period were observed. The trypsin activity exhibited a daily rhythm at both temperatures with an increase after morning feeding to reach a maximum several hours later. Average pepsin activity during the daily cycle was slightly higher at 34 ºC (6.1 and 7.3 U mg^{-1} BW at 30 and 34 ºC respectively), but values were significantly different only at 8 and 24 h after the morning meal. Similarly, the trypsin activity was significantly affected by the temperature only at 8 and 16 h after the morning meal, but daily activity averages were similar (1.20 and 1.29 U g^{-1} BW at 30 and 34 ºC respectively).

The partial transit rates of the first meal in the stomach for each period inter-samplings were higher during the first 4-h period and decreased progressively along the rest of the 24-h cycle at both temperatures, but no significant differences were detected at 30 ºC. In addition, the transit was notably faster at 34 ºC particularly during the first 8 h after feeding, with rates between 100 and 65% of total volume displaced (intake or released) during each 4-h period. In the intestine the transit rate was relatively constant and similar at both temperatures during 12 h after feeding. Then the rates remained very low during the following 12 h.

Residence time of the first meal was longer at 30 than at 34 ºC, particularly in the stomach (12h:02min vs 4h:54min respectively). In the intestine the difference was not so large (8h:18min vs 6h:24min respectively). In a parallel study with under same conditions, cobia reared at 30 ºC grew faster and showed ~~better~~ a more favorable feed conversion ratio than those at elevated temperature (34 ºC). The present results indicate that at 34 ºC, a subtle increase of proteolytic activity cannot compensate for the faster gut transit rate. Therefore, 30 ºC is more appropriate temperature for the early on-growing

of cobia because at higher temperatures the digestion efficiency decrease being one of the causes for a lower growth.

Key words: Temperature, GIT luminal pH, Digestive enzyme, Gut transit time, *Rachycentron canadum*

Introduction

Water temperature is a key factor affecting metabolic rates in fish and therefore has an evident impact on feed intake, nutrient utilization and growth (Brett, 1979; Buentello et al., 2000). To cope with the wide range of temperatures in the oceans depending on the geographic location and environmental cycles, the various fish species have adapted their feeding behavior and physiology to the temperature conditions of their particular habitat (Brett, 1979; Somero, 2004, 2010). Many studies have examined different perspectives of physiological responses to changes in temperature.

Particularly relevant is the way the ingested nutrients are digested before their incorporation into growing tissues. In spite of a large research effort, the effect of temperature on fish digestion is far from being well understood. The digestive function includes different processes from feed capture to assimilation of nutrients that may be affected in different manners by temperature changes. Generally, the feed intake increases with increased temperatures up to levels close to the upper tolerance limits (Fernández-Montero et al., 2018; Pérez-Casanova et al., 2009). Digestive enzyme activity has been traditionally assessed in two ways. On one side, *in vitro* experiments for the enzyme characterization performed with enzyme extracts show that activity increases with increasing temperature usually up to values exceeding those representative of their natural habitats, and also beyond lethal levels (Alarcón et al., 1998; Fernández et al., 2001; Gelman et al., 2008; Tanji et al., 1988). On the other hand, information about digestive enzyme activities analyzed in live fish at different temperatures is also available (Bowyer et al., 2014; Hani et al., 2018; Mazumder et al., 2018; Miegel et al., 2010; Sharma et al., 2017). However, these studies are based on only one sampling point during the postprandial response and, also report contradictory responses among the different studied species.

Gut evacuation rate also increases at increasing temperatures up to a certain limit, leading to lower residence time in the digestive tract (De et al., 2016; Fernández-Montero et al., 2018; Handeland et al., 2008; Temming and Herrmann, 2001). However, the estimation of evacuation rate has usually been performed under unrealistic feeding conditions in which the fish has been refed until satiation after a starvation period. Digestion efficiency will depend on the relation between enzymatic activity and gut transit time that are not short punctual facts but long dynamic cyclic processes usually occurring along

a whole day. Consequently, only experiments performed in routine feeding may provide realistic information.

Other species-specific digestive characteristics may also strongly affect the digestion process. That is the case of the gut luminal pH that conditions the activation of proenzymes in the gut, which may vary among fish, particularly within the stomach (Bucking and Wood, 2009; Papastamatiou and Lowe, 2005; Papastamatiou et al., 2007; Secor and Carey, 2016; Yúfera et al., 2004, 2007, 2012; Solovyev et al., 2018). Optimum temperature for growth does not necessarily coincide with maximum feed intake, highest digestion efficiency or optimal feeds utilization. In fact, from the point of view of aquaculture, the aim is to optimize all these factors to obtain the better juvenile quality and weight gain at a reasonable cost and with the lowest environmental impact.

Cobia (*Rachycentron canadum*), is a fast-growing species inhabiting tropical and subtropical waters with a broad geographical distribution over several continents. The species may reach up to 60 Kg and has a high-quality white flesh, being considered an excellent marine fish for aquaculture. It is being produced mainly in Asian-Pacific coast and in a lesser extent in the Gulf of Mexico with a world production above 40.000 tons during the last years (FAO, 2018; Tveteras, 2016). In Vietnam, it is one of the main marine fish in large scale commercial aquaculture (Nhu et al., 2011). In South Vietnam and other Southeast Asian regions water temperature in ponds and tanks ranges between 27 and 30 ºC, but may reach up to 36 ºC during the daytime in the warmer season. For this reason, it is necessary to understand the responses to increased temperatures, particularly in this region in the scenario of global warming (IPCC 2015). Effect of rearing temperature on growth in cobia juveniles has been examined by Sun and Chen (2009, 2014). In these studies, cobia juveniles were reared in the rage 20 to 35 ºC and highest growth rates were observed in the range 27-31 ºC. An unsolved question is how these temperature changes affect the digestion process.

Therefore, in this study we have examined the effects of temperature on the digestive function from a global perspective in order to advance in the physiological basis and mechanisms behind digestive efficiency and the corresponding effects on growth in cobia juveniles. Specifically, the aim of this study was to elucidate whether a temperature increase from 30 to 34 ºC affects gastrointestinal pH, enzymatic proteolytic digestive activity and feed transit over the whole day period, in early juveniles of this fast growing fish.

Material and Methods

Fish rearing and sampling

This study was part of a larger experiment examining growth performance in juveniles fed three different diets (Nguyen et al., 2019). We used the tanks from control treatment for the present study.

Cobia juveniles were obtained from a local hatchery in Nha Trang, Vietnam, and acclimatized to final experimental temperatures in two indoor fiberglass 5000-L tanks at Nha Trang University facilities during one week. During the period of acclimatization, water temperature in one tank increased at a rate of 1°C per day up to 34°C, while temperature in the other tank was kept constant at 30°. Acclimatized juveniles with 3.7 ± 0.4 g wet body weight, were randomly distributed to 6 experimental 200-L tanks (60 fish $tank^{-1}$) and reared under a light/dark cycle at two temperatures (30 and 34 °C, three tanks for each temperature) in recirculation systems. Water salinity was 29.0 ± 3.1 g L^{-1}, pH 7.8–8.3, oxygen level 4.6 ± 0.5 mg L^{-1} and NH_3<0.03 mg L^{-1}. Fish were maintained with a 12:00 h illumination period from 6:00 to 18:00 h and fed twice a day (8:00 and 16:00 h local time) according to the most common procedure in the local hatcheries (Nguyen, 2013; 2014). ~~with an~~ The experimental diet was produced at SPAROS Lda (Olhão, Portugal) containing 47% protein and 10% lipid (Table 1)~~. The experimental diet and was formulated based on previous results in cobia (Nguyen et al., 2014)~~. The fish were fed to nearly satiety (until most of the fish losing their appetite) by hand. Eventual uneaten feed and removed fish were recorded for the calculation of daily feed intake and feed conversion ratio, parameters considered in the companion study (Nguyen et al., 2019). After 2 weeks under these conditions, 3 fish per tank (wet body weight: ca. 6-8 g range) were sampled every 4 hours during 24 hours, for gut pH, digestive enzyme activity and feed transit determinations. Dissected gut were freeze-dried and sent to Spain for the analyses of enzyme activities at the University of Almeria and the gut transit at the ICMAN-CSIC. All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved by the Spanish National Research Council (CSIC) bioethical committee.

Measurements of gut pH

The gastrointestinal pH was measured in nine individuals at each sampling point and for each temperature condition immediately after sampling using a pH microelectrode (Thermo Orion, Thermo Fisher Scientific Inc) following the procedure described in Yúfera et al. (2012). In short, the fish were dissected to make the digestive track accessible. Next, the tip of the microelectrode (diameter 1.7 mm) was inserted in small slits made in the stomach, anterior intestine, medium intestine and posterior intestine (Fig. 1).

Digestive enzyme activity analyses

The complete digestive tract of three individuals of each sampling point and temperature were dissected, immediately frozen at -80 °C and later freeze-dried. Enzyme extracts were prepared for

enzyme activity measurement from these samples. Stomach and intestine samples were dissected and homogenized separately. Samples were manually homogenized in 3 mL distilled water and centrifuged for ten minutes at 4 °C at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from the stomach samples were measured for pepsin activity, and the supernatants from the intestine samples were analyzed for trypsin activity.

Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1 mL of 0.5% acid-denatured bovine hemoglobin diluted in 0.2 M HCl-Glycine buffer. Assays were carried out at the specific gastric pH determined in each sampling point. In this way we are determining actually activated pepsin instead of pepsinogen (Yúfera et al., 2012). After incubation at 25°C for 30 minutes, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 °C for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes. The absorbance of the resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after the TCA. Trypsin activity was determined using BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate at 25°C. 0.5 mM BAPNA was dissolved in 1mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50mM, pH 8.5, containing 20 mM $CaCl_2$. Reactions were started in 96-well microplates by the addition of 15 µL of the enzyme extract to 200 µL of the respective substrate and liberation of p-nitroanilline was kinetically followed at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA). The activities were provided as units per weight unit of fish to prevent the variability due to gut content.

Gut content and feed transit time measurements

The feed content in the stomach and the intestine was estimated from their respective weight determined in the gut samples used for enzyme and transit determinations. Average of empty guts were subtracted from these values. The values were normalized as dry weight of feed content per g of wet body weight (BW) to account for size differences among individuals.

For feed transit assessment, the day of the sampling, the feed labeled with containing of Yttrium oxide (200 mg Kg^{-1}) was provided for the first meal (8:00 h) while the standard feed without the marker was provided in the second meal (16:00 h). Yttrium content within the gut was analyzed at the ICMAN (Spain) by inductivity-coupled plasma mass spectroscopy (Thermo Scientific iCAP Q ICP-MS) separately in the stomach and the intestine of three individuals collected at each sampling point and temperature. Two technical subsamples were performed for each analysis. Yttrium content, normalized as mg g^{-1} of fish BW was plotted as a function of time.

The residence time of ingested feed within the gut were estimated as the period of time from when half of the stomach or intestine were filled with the marked feed to when half of the corresponding

section was emptied. Previously, in order to calculate the total amount of feed accessing the stomach in the first 4-h period, the total amount of yttrium in the stomach and intestine were considered, assuming that most of offered feed was ingested during the first hours of feeding. The yttrium content in each sampling point was converted to percentage of maximum measured in each section and temperature. The partial transit rates for each inter-sampling period were calculated as the difference of the relative fullness percentage between two consecutive time-points (considering the absolute values). Results have been presented as percentage of the maximum measured capacity entering or leaving each compartment for each 4h-period.

Statistical analyses

Two-way analysis of variance (ANOVA) was used to compare differences between the postprandial time and temperature for each section of the digestive tract. A post-hoc Tukey honest significant difference (HSD) test was used when ANOVA results revealed significant differences ($P < 0.05$). The homogeneity of variances was previously tested using Levene's test, and all parameters expressed as percentages were subjected to arcsin square root transformation. Data are presented as the mean of nine or three replicates ± sem. All statistical tests were performed in IBM SPSS Statistics 18 software (IBM Corp., USA).

Results

The pH within the stomach was permanently acidic with mean values ranging from 2.76 to 4.74 (Fig. 2) although a significant increase ($P<0.05$) was observed after each meal. The two way-ANOVA suggest that the gastric pH values change during the daily cycle but not in relation to temperature ($P>0.05$). On the other hand, the intestinal pH ranged from 6.05 to 7.69 (Fig. 3). An increase was observed after the first meal and this slight alkaline condition was maintained for several hours before declining to neutral or slightly acidic values at the end of the day ($P<0.05$). Furthermore, the maximum measured pH values were progressively higher when moving from proximal to distal part of the intestine ($P<0.05$). A slightly higher acidity was observed in the anterior and medium sections of the intestine at 34°C ($P<0.05$).

Pepsin activity showed a daily rhythm at 30 °C with a maximum in the middle of the light period and a minimum at the beginning of the dark period, while at 34°C some hourly changes with a decrease after each meal were observed, but without so clear daily trend during the 24-h period ~~were observed~~. (Fig. 4). The trypsin activity exhibited a clear daily rhythm at both temperatures with a patent increase after morning feeding to reach a maximum several hours later (Fig. 4). Overall the activity of pepsin during the daily cycle was slightly higher at 34 °C (average of the seven sampling points: 6.1 and 7.3 $U \cdot mg^{-1}$

BW at 30 and 34 °C respectively), but values were significantly different only at 8 and 24 hours after the morning meal. Similarly, the trypsin activity was significantly affected by the temperature only at 8 and 16 hours after the morning meal, but considering globally all daily samples the activity was quite similar (averages 1.20 and 1.29 $U \cdot g^{-1}$ BW at 30 and 34 °C respectively). Considering all hourly data together, the two-way ANOVA indicates that the temperature is not affecting the trypsin and pepsin activities.

The daily pattern of the estimated feed content within the stomach and the intestine is shown in Figure 5. The patterns were clearly different at each temperature. At 30 °C the amount of digesta within the stomach increased continuously from the morning feeding up to 8 hours after the second feeding. Contrarily, at 34 °C the pattern showed two peaks, the first one 4 hours after the first meal and the second one 8 hours after the second meal. Feed content of the intestine at both temperatures was dramatically lower compared to the stomach and also showed two maxima at the same times observed in the stomach at 34 °C.

Postprandial pattern of yttrium content within the gut is shown in Figure 6 (only the first meal contained yttrium oxide). At 30 °C the yttrium content in the stomach reached the maximum value at 8 h after the first meal, while in the intestine the maximum was observed only 4 h after the first meal ($P<0.05$) maintaining similar high content at 8 h post-feeding. At 34 °C the maximum yttrium content was observed 4 h after the first meal in the stomach and at 8 h post-feeding in the intestine ($P<0.05$), although an important amount of yttrium was already observed in the intestine at 4 h.

The partial transit rates of the first meal in the stomach for each period inter-samplings were higher during the first 4-h period and decreased progressively along the rest of the 24-h cycle, although no significant differences were detected at 30 °C (Fig. 7). In addition, the transit was notably faster at 34 °C particularly during the first 8 h after feeding, with rates between 100 and 65% of total volume displaced (intake or released) during each 4-h period. In the intestine the transit rate was relatively constant and similar at both temperatures during 12 h after feeding (Fig. 7). Then the rates dropped and remained very low during the following 12 h.

The residence time within the gut of the first meal was calculated from Figure 8. In this figure, the total amount of labelled feed accessing the stomach during the first 4-h period was considered, including the amount already transferred to and analyzed in the intestine. This criterion considers the time period from 50% of maximum measured label (yttrium) accessed the stomach or intestine up to the time when 50 % of this material has disappeared from the same compartment. Thus, the time the first meal spent in the stomach and the intestine was longer at 30 than at 34 °C, particularly in the stomach (12h:02min vs 4h:54min, respectively) (Fig. 8). In the intestine, the difference was not so large

(8h:18min vs 5h:54min, respectively). On the other hand, the residence time of this first meal at 30 ºC was longer in the stomach, while at 34 ºC it was longer in the intestine.

Discussion

The present study was performed in parallel with other study analyzing growth and feed conversion ratio differences in the same batch of juveniles fed different diet formulations at these two temperatures for six weeks (Nguyen et al. ~~2017,~~ 2019). In that report, we found that cobia reared at 30ºC grew faster and showed ~~better~~a more favorable feed conversion ratio ~~(FCR)~~ than those at elevated temperature (34ºC), being in both cases fed the same daily ration. We used the control tanks for the present study on digestion. The sampling was intentionally performed two weeks after the start of the experiment to determine the digestion status in the middle of the growth experiment, when both feed intake and growth were assessed to be high. Cobia is a voracious carnivorous fish with a large stomach and a short intestine (Fig. 1). This species feeds on small fish, crustaceans and squids (Franks et al., 1996). These feeding habits and gut anatomy have consequences in the mode of digestion as we observed in the different parameters examined. It seems evident that the stomach is very important for digestion and maybe more so than the intestine in this species, based on their respective volumes, luminal ionic values, proteolytic activities and transit rates results. Most research on postprandial response in fish has been done considering only one morning meal in order to examine the results without interferences of subsequent meals. In our study we considered two meals according to the customary use in hatcheries for this species in this region (Nguyen, 2013). With this feeding protocol we found more realistic overview of the digestive function but with additional complications to interpret results because fish may change the feeding behavior and daily digestive patterns when have the possibility to choose among different daily meals (Montoya et al., 2010; Yúfera et al., 2014).

In relation to luminal pH of the digestive tract, two gastric acidification strategies have been reported for vertebrates. One is to maintain a permanent acidic environment in the stomach with independence of the presence or absence of ingested feed, as observed for instance in mammals and birds; the other is to maintain a neutral pH in the lumen of the stomach between meals and with a decline only after the ingestion of feed (Papastamatiou and Lowe, 2005; Secor and Carey, 2016). Most teleostean fish analyzed up to date exhibited this second strategy (Hlophe et al., 2014; Nikolopoulou et al., 2011; Yúfera et al., 2004, 2012, Solovyev et al., 2016). However, our study reveals that cobia juveniles maintain a permanent gastric acidification. This is an interesting finding because such a strategy has been previously described only in rainbow trout *Oncorhynchus mykiss* (Bucking and Wood, 2009) and

some elasmobranchian species (Papastamatiou and Lowe 2005; Papastamatiou et al., 2007). Some clues about the same strategy have also been reported for southern bluefin tuna *Thunnus maccoyii* examining fed and starved fish although a postprandial response was not scrutinized (Leef et al., 2012). Unfortunately, the list of teleostean species examined in detail is too short to know if this strategy is less common or we need still to explore more species, particularly those with strict carnivorous feeding habits, to get a more complete figure of the acidification strategy in teleosteans. To maintain a neutral gastric environment during fasting has been associated to infrequent feeding in snakes and sharks (Papastamatiou and Lowe, 2005; Secor et al., 2012), but in teleostean with daily feeding habits this rule remains uncertain. In fact, an erratic daily feeding by changing randomly the moment of feed delivery every day may also alter the daily pattern from neutral/acid alternation to permanent acidification in gilthead seabream *Sparus aurata* (Montoya et al., 2010). A constant low gastric pH enables this voracious species to be always ready to activate pepsinogen to start the hydrolysis of the ingested prey. The small increase of gastric pH after meals has been attributed to the dilution effect of the ingesting feed, possibly in parallel with some water; drinking water for osmoregulatory purposes, as well as to the buffering capacity of feeds (Márquez et al., 2012) and also the buffer capacity of the slightly alkaline seawater itself.

We found that increased temperature did not affect to gastric pH but it led to decreased luminal pH in the anterior intestine and to a lesser extent the mid intestine. This effect is probably related to higher transit rates observed at 34 ºC in which the acidic chyme pass quickly to the short intestine. A similar effect on the intestinal pH was described at increased temperature in channel catfish *Ictalurus punctatus* (Page et al., 1976). The same effect in the anterior intestine was also detected in other species when feed only one meal (Bucking and Hood, 2009; Rosero, 2013; Yúfera et al., 2014). A decrease of the intestinal pH has been reported for different freshwater species associated to seasonal increased temperatures (Solovyev et al., 2018). The authors explained this decrease as an adaptation to enable fish to regulate and optimize the activity of their digestive pancreatic enzymes. Our results would indicate, that in addition, the changes in the water temperature alter the feeding behavior and feed processing along the day. ~~To our knowledge this is the first time that the luminal pH has been examined at different water temperatures~~.

Pepsin activity showed hourly variations along the 24-h period ~~cycle~~ although a clear daily rhythm was observed only at 30 ºC. Considering that the minor variations of gastric pH are practically no affecting to pepsinogen activation as reported in other species (Yúfera et al., 2012), these changes should be interpreted in relation to the amount of substrate. The lower activity practically coincides with the higher amount of digesta in the stomach (Fig. 4) that is consuming the active enzyme. Contrary to this, the pancreatic trypsin activity exhibited a daily cycle with the expected increase associated with the

317 intestinal alkalization when the chyme is released from the stomach. Such daily pattern has been
318 already reported in other fish species (Rosero, 2013; Yúfera et al., 2014). It is also interesting to note
319 that the proteolytic activity in the stomach was much higher than in the intestine. We found that the
320 proteolytic activity of pepsin and trypsin was hardly affected by the 4 °C increase of temperature, at
321 least when the standard incubation temperature were used for the analytical protocols. It could be
322 interesting to explore analytical methodologies adapted to different temperatures. Miegel et al. (2010)
323 did not find differences of intestinal proteases activity in fed individuals of yellowtail kingfish *Seriola*
324 *lalandi* maintained at 12.6 and 20.8 °C. However, Bowyer et al. (2014) found higher tryptic activity at
325 intermediate temperatures in the range 21 to 27 °C in starved individuals of the same species. Similar
326 results were observed by Hani et al. (2018) in starved threespine stickleback *Gasterosteus aculeatus* in
327 the range 16 to 21 °C, by Sharma et al. (2017) in Indian major carp *Catla catla* in the range 10 to 35 °C,
328 as well as by Zhao et al. (2009) in the range 20 to 32 °C in Chinese longsnout catfish *Leiocassis*
329 *longirostris*. On the other hand, Mazamder et al. (2018) reported higher pepsin activity at 30 °C in the
330 range 22 to 34 °C in fasted Malabar blood snapper *Lutjanus malabaricus*. Comparison of these results
331 is difficult due to differences in fish size, experimental protocols and analytical methods, and in
332 addition, the fish for those analyses were collected at only one time and any postprandial patterns was
333 not explored. In our study and with independence of the similarity of the global daily averages of the
334 lytic activity, the postprandial patterns showed maximum and minimum values that are not coincident
335 at both temperatures. These results indicate that a single daily sample is not enough to characterize
336 the enzymatic activity under different temperature conditions. Such data must be interpreted in
337 relation to gut content as mentioned above but also in relation to transit results.

338 Transit rate assessment is a challenging task when more than one meal is offered. The postprandial
339 responses overlap and the patterns are harder to interpret. A key factor in our study is to recognize
340 that some ingested feed may pass to the intestine before the second sampling (Figs. 4 and 5) and
341 therefore the estimation of the ingestion during the first period should include both sections. The gut
342 content on weight basis gives only indicative information because it is representing the balance
343 between digesta input and output. To obtain a more complete information it is necessary to estimate
344 the temporal rates for the gut filling and evacuation under this feeding protocol in each gut
345 compartment. An interesting result has been to verify whether the transit velocity of digesta
346 throughout the digestive tract is changing along the daily cycle (Fig. 6), something perhaps obvious but
347 never examined in fish. Thus, the transit rates were maxima during 8 h after feeding at 34 °C. In this
348 period the whole stomach volume was filled during the first 4-h period and emptied in a great part
349 during the following 4 h before the next meal. The rest of the day the transit of remaining chyme was
350 notably slower. At 30 °C the transit rates during the first hours were significantly lower than at 34 °C
351 and the posterior decrease was smoother and not statistically significant. In the intestine no effect of

temperature was observed and the transit was relatively fast during the first 12 h during which most part of the first meal is evacuated, the remaining digesta moved at notably slower rate. In our study, the second meal was not labeled and therefore these transit rates are only referring to the first meal when a second meal is pushing 8 h later.

Evacuation rates have been determined in many species usually based on fish with the stomach already full and without further feeding, such an approach gives an incomplete understanding of transit time in the stomach but in many cases the pass of the digesta through the intestine was properly assessed (Adamidou et al., 2009; Bonvini et al., 2018). While the evacuation of the stomach may last less than one day, the evacuation of the intestine may last 36 to 48 h. These values are only indicative for median sized farmed fish with daily feeding. Different factors such as feeding frequency, ration size, feed quality, body size and water temperature have been described to affect transit time in fish (Miegel et al., 2010), particularly the last one (De et al., 2016; Fernández-Montero et al., 2018; Handeland et al., 2008; Temming and Herrmann, 2001). According to these studies, transit time increases with the temperature except at extremely high values. Our results however showed an increase at very high temperatures that is probably close to tolerance limit.

Probably the most useful information is the time the digesta spent within the different sections and being hydrolyzed by the corresponding digestive enzymes. In routine feeding, the ingested feed is mixed up with the feed of the previous and the next meal(s) and its complete evacuation from the gut may last longer than expected due to the residual amount that can be detected for many hours, even days, later than most part of the digesta was evacuated. The criterion explained above allows an estimation of the residence time that can be compared between compartments and temperatures (Fig. 7). The most evident result is that the residence time was shorter at 34 ºC. The increase of 4 ºC induced a faster filling and evacuation in the stomach as commented above but also a lower residence time, that was ~~even~~ less than half of the period at 30 ºC. In the intestine the effect was not as dramatic but the reduction of digesta residence time was still important. The lower period of time for the proteolytic work of the digestive proteases brings on lower dietary protein utilization and is one of the reasons for the lower weight gain and higher feed conversion ratio observed at 34 ºC (Nguyen et al., 2019). ~~Nevertheless~~Furthermore, a relevant aspect is that the ~~transit of the~~ first meal transited almost simultaneously ~~than~~ in the stomach and intestine, when certain temporal displacement would be expected as determined in other species (Bonvini et al., 2018). The ingested pellets of the morning meal in our experiment passed directly into the intestine, and this segment was filled almost at same time as the stomach, working more like an extension of the stomach than like a different digestive tract compartment. Unfortunately, our experimental protocol does not allow to evaluate the transit time of the second meal that not necessarily may follow the same pattern but that we can assume it

386 is similar to that of the first meal. It is likely that the feeding protocols for the voracious and carnivorous
387 cobia in aquaculture where pelleted feed particles are offered in large amounts results in a digestive
388 process that is progressing differently from nature where cobia ingest larger and intact prey. However,
389 given the artificial feeding conditions in aquaculture, it is important to understand how the digestive
390 system that is evolutionary adapted to natural conditions perform under different feeding regimes.

391 In summary, the present results indicate that at 34 °C, a subtle increase of proteolytic activity cannot
392 compensate for the faster gut transit rate. The reduced time the dietary proteins are available for
393 hydrolysis when compared with fish maintained at 30 °C can explain the lower growth observed at this
394 temperature (Nguyen et al. 2019). Another reason for the lower growth could be an unfavorable
395 energetic balance at the higher temperature but the studies by Sun and Chen (2009, 2014) showed no
396 evident variations of the feed energy allocated to metabolism in the range 27-33°C in cobia juveniles
397 of the same weight range, although it was higher at 35 °C. Furthermore, this study shows a general
398 appraisal of digestion in the 24-h temporal horizon as correspond to a daily feeding protocol,
399 demonstrating the importance of observing inter-hourly changes in the different digestion parameters
400 to characterize the digestive potential under given temperature conditions.

401

402 **Acknowledgements:** The project WISEFEED received funding by the European Union's H2020
403 programme (Marie Skłodowska-Curie grant No 691150). Additional funding from project EFISHDIGEST
404 AGL2014-52888 (MINECO, Spain + FEDER/ERDF contribution). S.E. acknowledges a Foundation for
405 Science and Technology of Portugal (FCT) investigator grant IF/00482/2014/CP1217/CT0005 funded
406 by the European Social Fund, the Operational Programme Human Potential and FCT. This work also
407 received national funds through FCT through project UDI/Multi/04326/2013 and Norwegian Agency
408 for Development Cooperation NORHED, No. QZA-0485 SRV-13/0010.

409 **References**

410 Adamidou, S., Nengas, I., Alexis, M., Foundoulaki, E., Nikolopoulou, D., Campbell, P., Karacostas, C.,
411 Rigos, G., Bell, G.J., Jauncey, K., 2009. Apparent nutrient digestibility and gastrointestinal evacuation
412 time in European seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes.
413 Aquaculture 289, 106-112.

414 Alarcón, F.J., Díaz, M., Moyano F.J., Abellan, E., 1998. Characterization of functional properties in two
415 sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol.
416 Biochem. 19, 257–267.

417 Brett, JR., 1979. Environment factors and growth. In W.S. Hoar, et al. (Eds.), Fish Physiology, vol. 8,
418 Academic Press, New York (1979), pp. 599-675.

419 Bonvini, E., Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Grandi, M., Fontanillas, R., Viroli, C., Gatta,
420 P.P., 2018. Feeding European sea bass with increasing dietary fibre levels: Impact on growth, blood
421 biochemistry, gut histology, gut evacuation. Aquaculture 494, 1-9.

Bowyer, J.N., Booth, M.A, Qin, J. G., D'Antignana, T., Thomson, M. J. S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquacult. Res. 45, 2010–2020.

Bucking C, Wood CM., 2009. The effect of postprandial changes in pH along the gastrointestinal tract on the distribution of ions between the solid and fluid phases of chime in rainbow trout. Aquacult. Nutr. 15, 282–296.

Buentello, J. A., Gatlin, D. M., Neill, W. H., 2000. Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilization and growth of channel catfish (*Ictalurus punctatus*). Aquaculture 182, 339-352.

De, M., Ghaffar, M.A, Bakar, Y., Das, S.K., 2016. Effect of temperature and diet on growth and gastric emptying time of the hybrid, *Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂. Aquacult. Rep. 4, 118–124.

FAO 2018. Cultured Aquatic Species Information Programme – *Rachycentron canadum*. Available at www.fao.org/fishery/culturedspecies/Rachycentroncanadum/en. Accessed on 15 September 2018.

Fernández-Montero, A., Caballero, M.J., Torrecillas, S., Tuset, V.M., Lombarte, A., Ruiz Ginés, R., Izquierdo, M., Robaina, L., Montero, D., 2018. Effect of temperature on growth performance of greater amberjack (*Seriola dumerili* 1810) Juveniles. Aquacult. Res. (in press).

Fernández, I., Moyano, F.J., Dıaz, M., Martınez, T., 2001. Characterization of a-amylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei). J. Exp. Mar. Biol. Ecol. 262, 1–12.

Franks, J.S., Garber, N.M., Warren, J.R., 1996. Stomach contents of juvenile cobia, *Rachycentron canadum*, from the Northern Gulf of Mexico. Fish. Bull. 94, 374–380.

Gelman A, Kuz'mina V, Drabkin V, Glatman L., 2008. Temperature adaptation pf digestive enzymes in fish. In: JEP Cyrino, DP Bureau, BG Kapoor eds., Feeding and digestive functions in fished. Science Publishers, Enfield NH, pp. 155-225.

Handeland, S.O., Imsland, A.K., Stefansson, S.O., 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon postsmolts. Aquaculture, 283, 36–42.

Hani, Y.M.I., Marchand, A., Turies, C., Kerambrun, E., Palluel, O., Bado-Nilles, A., Beaudouin, R., Porcher, J.M., Geffard, A., Dedourge-Geffard, O., 2018. Digestive enzymes and gut morphometric parameters of threespine stickleback (*Gasterosteus aculeatus*): Influence of body size and temperature. PLoS ONE 13(4): e0194932.

Hlophe, S.N., Moyo, N.A.G., Ncube,I., 2014. Postprandial changes in pH and enzyme activity from the stomach and intestines of *Tilapia rendalli* (Boulenger, 1897), *Oreochromis mossambicus* (Peters, 1852) and *Clarias gariepinus* (Burchell, 1822). J. Appl. Ichthyol. 30, 35–41.

IPCC 2015. Climate change 2014: Synthesis report. The intergovernmental panel on climate change Geneva, Switzerland, 151.

458 Márquez, L., Robles, R., Morales, G.A., Moyano, F.J., 2012. Gut pH as a limiting factor for digestive
459 proteolysis in cultured juveniles of the gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 38,
460 859-69.

461 Miegel, R.P., Pain, S.J., van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water
462 temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail
463 kingfish (*Seriola lalandi*). Aquaculture 308, 145–151.

464 Mazumder, S.K., Dasa, S.K., Rahim, S.M., Ghaffar, M.A., 2018. Temperature and diet effect on the
465 pepsin enzyme activities, digestive somatic index and relative gut length of Malabar blood snapper
466 (*Lutjanus malabaricus* Bloch & Schneider, 1801). Aquacult. Rep. 9, 1–9.

467 Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez, F.J., 2010.
468 Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream
469 (*Sparus aurata*). Aquaculture. 306, 315–21.

470 Nguyen, M.V., 2013. The impact of lysine to arginine ratios in plant-based protein diets on appetite,
471 growth performance and gene expression of brain neuropetide Y (NPY) and cholecystokinin (CCK) in
472 juvenile cobia (*Rachycentron canadum*). PhD thesis, University of Bergen, 74 pp.

473 Nguyen, M.V., Rønnestad, I., Buttle, L., LAI, H.V., Espe, M., 2014. Imbalanced lysine to arginine ratios
474 reduced performance in juvenile cobia (*Rachycentron canadum*) fed high plant protein diets. Aquacult.
475 Nutr. 20, 25-35.

476 ~~Nguyen, M.V., Espe, M., Conceição, L., Le, M.H., Yúfera, M., Engrola, S., Jordal, A-E.O., Pham, Q.H.,~~
477 ~~Rønnestad, I., 2017. Growth, metabolism and N-retention in cobia at elevated water temperatures –~~
478 ~~The role of dietary methionine levels. Aquaculture Europe 2017, 17-20 October, Dubrovnik, Croatia.~~

479 Nguyen, M.V., Espe, M., Conceição, L., Le, M.H., Yúfera, M., Engrola, S., Jordal, A-E.O., Pham, Q.H.,
480 Rønnestad, I., 2019. The role of dietary methionine concentrations on growth, metabolism and N-
481 retention in cobia (*Rachycentron canadum*) at elevated water temperatures. Aquacult. Nutr. (in press).

482 Nhu, V.C., Nguyen, Q.H., Le, T.L., Tran, M.T., Sorgeloos, P., Dierckens, K., Reinertsen, H., Kjørsvik, E.,
483 Svennevig, N., 2011. Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and
484 prospects. Aquaculture 315, 20-25.

485 Nikolopoulou, D., Moutou, K.A., Fountoulaki, E., Venou, B., Adamidou, S., Alexis, N.M., 2011. Patterns
486 of gastric evacuation, digesta characteristics and pH changes along the gastrointestinal tract of gilthead
487 sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Comp. Biochem. Physiol.
488 A 158, 406-414.

489 Page, J.W., Andrews, J.W., Murai, T., Murray, M.W., 1976. Hydrogen ion concentration in the
490 gastrointestinal tract of channel catfish. J. Fish Biol. 8, 225–228.

491 Papastamatiou, Y.P., Lowe, C.G., 2005. Variations in gastric acid secretion during periods of fasting
492 between two species of shark. Comp. Biochem. Physiol. A 141, 210-214.

493 Papastamatiou, Y.P., Purkis, S.J., Holland, K.N., 2007. The response of gastric pH and motility to fasting
494 and feeding in free swimming blacktip reef sharks, *Carcharhinus melanopterus*. J. Exp. Mar. Biol. Ecol.
495 345, 129-140.

Pérez-Casanova, J.C., Lall, S.P., Gamperl, A.K., 2009. Effect of feed composition and temperature on food consumption, growth and gastric evacuation of juvenile Atlantic cod (*Gadus morhua* L.) and haddock (*Melanogrammus aeglefinus* L.). Aquaculture 294, 228-235.

Rosero, A., 2013. Comparación de la fisiología digestiva entre un pez carnívoro (Corvina, *Argyrosomus regius*) y un pez omnívoro (Liseta, *Chelon labrosus*), e influencia de la inclusión de un acidificante en el pienso sobre el proceso digestivo. Master Thesis, Universidad de Cádiz.

Secor, S.M., Carey, H.V., 2016. Integrative Physiology of fasting. Compr. Physiol. 6, 773-825.

Secor, S.M., Taylor, J.R., Grosell, M., 2012. Selected regulation of gastrointestinal acid–base secretion and tissue metabolism for the diamondback water snake and Burmese python. J. Exp. Biol. 215, 185-196.

Sharma, J.G., Singhb, S.P., Chakrabarti, P., 2017. Effect of temperature on digestive physiology, immune-modulatory parameters, and expression level of Hsp and LDH genes in *Catla catla* (Hamilton, 1822). Aquaculture 479, 134-141.

Solovyev, M.M., Kashinskaya, E.N., Rusinek, O.T., Izvekova, G.I., 2016. Physiological pH values in the digestive tract of perch *Perca fluviatilis* from different habitats. J. Ichthyol. 56 (2), 312-318.

Solovyev, M.M., Izvekova, G., Kashinskaya, E., Gisbert, E., 2018. Dependence of pH values in the digestive tract of freshwater fishes on some abiotic and biotic factors. Hydrobiologia 807, 67–85.

Somero, G.N., 2004. Adaptation of enzymes to temperature: Searching for basic “strategies.” Comp. Biochem. Physiol. B 139, 321-333.

Somero GN., 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers”, J. Exp. Biol. 213, 912-20.

Sun, L., Chen, H., 2009. Effects of ration and temperature on growth and energy budget of juvenile cobia (*Rachycentron canadum*). Aquaculture 292, 197-206.

Sun, L., Chen, H., 2014. Effects of water temperature and fish size on growth and bioenergetics of cobia (*Rachycentron canadum*). Aquaculture 426-427, 172-180.

Tanji, M., Kageyama, T., Takahashi, K., 1988. Tuna pepsinogens and pepsins. Eur. J. Biochem. 177, 251-255.

Temming, A., Herrmann, J.-P., 2001. Gastric evacuation in horse mackerel. I. The effects of meal size, temperature and predator weight. J. Fish Biol. 58, 1230-1245.

Tveteras, R., 2016. Global fish production data & analysis. GOAL 2016, September 19-22, Guangzhou, China.

Yúfera, M., Fernández-Díaz, C., Vidaurreta, A., Cara, J.B., Moyano, F.J., 2004. Gastrointestinal pH and development of the acid digestion in larvae and early juveniles of *Sparus aurata* L. (Pisces: teleostei). Mar. Biol. 144, 863-869.

Yúfera, M., Darias, M.J., 2007. Changes in the gastrointestinal pH from larvae to adult in Senegal sole (*Solea senegalensis*). Aquaculture 267, 94-99.

532 Yúfera, M., Moyano, F.J., Astola, A., Pousão-Ferreira, P., Martínez-Rodríguez, G., 2012. Acidic digestion
533 in a teleost: Postprandial and circadian pattern of gastric pH, pepsin activity, and pepsinogen and
534 proton pump mRNAs expression. PLoS ONE, 7(3) e33687.

535 Yúfera, M., Romero, M.J., Pujante, I.M. Astola, A., Mancera, J.M., Sánchez-Vázquez, F.J., Moyano, F.J.,
536 Martínez-Rodríguez, G., 2014. Effect of feeding frequency on the daily rhythms of acidic digestion in a
537 teleost fish (gilthead seabream). Chronobiol. Int. 31,1024–1033.

538 Zhao, H., Han, D., Xie, S., Zhu, X., Yang, Y., 2009. Effect of water temperature on the growth
539 performance and digestive enzyme activities of Chinese longsnout catfish (*Leiocassis longirostris*
540 Günther). Aquacult. Res. 40, 1864-1872.

541

542 **Figure captions**

543 Fig. 1. Digestive tract of an early *R. canadum* juvenile indicating the places for the gut pH
544 determinations. ST: stomach; AI: anterior intestine; MI: medium intestine; PI: posterior intestine.

545

546 Fig. 2. Postprandial changes in gastric pH (mean and SEM) of *R. canadum* juveniles at the two
547 experimental temperatures. Different letters denote statistical difference at the different sampling
548 times. Arrows indicate the time for the two feed supplies. Shaded area indicates the dark period.

549

550 Fig. 3. Postprandial changes in the luminal pH (mean and SEM) of the different section of the intestine
551 of *R. canadum* juveniles at the two experimental temperatures. Arrows indicate the time for the two
552 feed supplies. Dashed line at pH 7 was included for a better comparison between both temperatures.
553 Shaded area indicates the dark period.

554

555 Fig. 4. Postprandial changes of pepsin and trypsin activities (mean and SEM) in the stomach and
556 intestine of *R. canadum* juveniles at the two experimental temperatures. Arrows indicate the time for
557 the two feed supplies. Shaded area indicates the dark period. Different letters denote significant
558 differences at the different sampling times for each temperature. Asterisks denote significant
559 differences between temperatures.

560

561 Fig. 5. Postprandial changes of gut content within the stomach (grey) and intestine (black) of *R.*
562 *canadum* juveniles at the two experimental temperatures. Shaded area indicates the dark period.

563

564 Fig. 6. Postprandial changes of yttrium content (mean and SEM) within the stomach and intestine of
565 *R. canadum* juveniles at the two experimental temperatures. Shaded area indicates the dark period.

566

567 Fig. 7. Partial transit rates of digesta for each inter-sampling period (4 h) in the stomach and intestine
568 of *R. canadum* juveniles at the two experimental temperatures. Results are presented as percentage
569 of the maximum (mean and SEM) measured capacity entering or leaving each compartment for each

4h-period. ~~SEM was omited for clarity~~. Different letters denote significant differences at the different sampling times for each temperature. Asterisks denote significant differences between temperatures.

Fig. 8. Residence time ~~of the digesta~~ of the first meal in the stomach and intestine of *R. canadum* juveniles at the two experimental temperatures. Results are presented as percentage of the maximum feed content (mean and SEM) at each sampling time. Arrows represent the period of time from the 50% of the maximum acceded to each gut compartment to the 50% is evacuated from the same compartment. Values in the insets indicate the residence time according to this criterion.

578 **Tables**

579 Table 1. Formulation (g kg^{-1} dry matter basis) and proximate analysis of the diet.

Ingredients	**g kg^{-1}**
Krill meal	50.0
Wheat meal	175.3
Fish meal	250.0
Soy protein concentrate	100.0
Pea protein concentrate	134.0
CPSP 90	50.0
DL methionine	5.5
Betaine HCl	5.0
Encapsuled taurine	5.0
Encapsuled tryptophane	5.0
Fish oil	28.0
Krill oil	30.0
Pea starch	100.0
Vitamin & mineral mix	20.0
Lutavit E50	0.2
Calcium carbonate	10.0
Mono ammonium phosphate	30.0
Antioxidant (Paramega)	2.0
Proximate composition	
Dry matter	958.0
Energy (MJ kg^{-1})	20.1
Crude protein	465.0
Crude fat	103.0

580

Highlights

1- Cobia exhibits a permanent gastric acidification

2- Water temperature (30 and 34 ºC) does not substantially affect the digestive proteolytic activities

3- Both stomach and intestine are filled almost simultaneously

4- Transit time was much faster and the residence time lower at 34ºC than at 30 ºC

Effect of increased rearing temperature on digestive function in cobia early juvenile

Short title: Effect of temperature on Cobia digestion

M. Yúfera[a], M.V. Nguyen[b], C. Navarro-Guillén[a1], F.J. Moyano[c], A-E.O. Jordal[d], M. Espe[e], L.E.C. Conceição[f], S. Engrola[g], M.H. Le[b], and I. Rønnestad[d]

[a]Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC), Campus Universitario Rio San Pedro s/n, 11519 Puerto Real, Spain
[b]Institute of Aquaculture, Nha Trang University, 02 Nguyen Dinh Chieu st, Nha Trang, Vietnam
[c]Department of Biology and Geology, University of Almería, 04120 Almería, Spain
[d]Department of Biological Sciences, University of Bergen, NO-5020, Bergen, Norway
[e]Institute of Marine Research, Bergen, Norway
[f]Sparos Lda, Olhão, Portugal
[g]Centre of Marine Sciences of Algarve (CCMAR), University of Algarve, Campus de Gambelas, University of Algarve, 8005-139 Faro, Portugal

Corresponding author: manuel.yufera@icman.csic.es

[1] Present address: Centro de Ciências do Mar do Algarve (CCMAR), Universidade do Algarve, Faro, Portugal.

Abstract

The present study is focused to elucidate the main characteristics of the digestive function of this carnivorous fast-growing fish living at high temperatures. With this aim, we have examined the effects of an increased temperature from 30 to 34ºC on the daily pattern of gastrointestinal pH, enzymatic proteolytic digestive activity and the feed transit time in early juveniles of cobia (*Rachycentron canadum*), a species living in tropical and subtropical waters with an increasing aquaculture production. Fish were fed two meals a day. Gastric luminal pH was permanently acidic (mean pH values: 2.76 - 4.74) while the intestinal pH increased from neutral/slightly acidic to slightly alkaline when the digesta was present, with an increasing alkalinity from proximal to distal intestine (mean pH values: 6.05 to 7.69). The temperature did not affect the gastric pH but a slightly higher acidity was induced in the intestine at 34ºC.

Pepsin activity showed a daily rhythm at 30 ºC with maximum in the middle of the light period, while at 34ºC some hourly changes coinciding with feed adding without a clear daily trend during the 24-h period were observed. The trypsin activity exhibited a daily rhythm at both temperatures with an increase after morning feeding to reach a maximum several hours later. Average pepsin activity during the daily cycle was slightly higher at 34 ºC (6.1 and 7.3 U mg^{-1} BW at 30 and 34 ºC respectively), but values were significantly different only at 8 and 24 h after the morning meal. Similarly, the trypsin activity was significantly affected by the temperature only at 8 and 16 h after the morning meal, but daily activity averages were similar (1.20 and 1.29 U g^{-1} BW at 30 and 34 ºC respectively).

The partial transit rates of the first meal in the stomach for each period inter-samplings were higher during the first 4-h period and decreased progressively along the rest of the 24-h cycle at both temperatures, but no significant differences were detected at 30 ºC. In addition, the transit was notably faster at 34 ºC particularly during the first 8 h after feeding, with rates between 100 and 65% of total volume displaced (intake or released) during each 4-h period. In the intestine the transit rate was relatively constant and similar at both temperatures during 12 h after feeding. Then the rates remained very low during the following 12 h.

Residence time of the first meal was longer at 30 than at 34 ºC, particularly in the stomach (12h:02min vs 4h:54min respectively). In the intestine the difference was not so large (8h:18min vs 6h:24min respectively). In a parallel study with under same conditions, cobia reared at 30 ºC grew faster and showed a more favorable feed conversion ratio than those at elevated temperature (34 ºC). The present results indicate that at 34 ºC, a subtle increase of proteolytic activity cannot compensate for the faster gut transit rate. Therefore, 30 ºC is more appropriate temperature for the early on-growing

of cobia because at higher temperatures the digestion efficiency decrease being one of the causes for a lower growth.

Key words: Temperature, GIT luminal pH, Digestive enzyme, Gut transit time, *Rachycentron canadum*

Introduction

Water temperature is a key factor affecting metabolic rates in fish and therefore has an evident impact on feed intake, nutrient utilization and growth (Brett, 1979; Buentello et al., 2000). To cope with the wide range of temperatures in the oceans depending on the geographic location and environmental cycles, the various fish species have adapted their feeding behavior and physiology to the temperature conditions of their particular habitat (Brett, 1979; Somero, 2004, 2010). Many studies have examined different perspectives of physiological responses to changes in temperature.

Particularly relevant is the way the ingested nutrients are digested before their incorporation into growing tissues. In spite of a large research effort, the effect of temperature on fish digestion is far from being well understood. The digestive function includes different processes from feed capture to assimilation of nutrients that may be affected in different manners by temperature changes. Generally, the feed intake increases with increased temperatures up to levels close to the upper tolerance limits (Fernández-Montero et al., 2018; Pérez-Casanova et al., 2009). Digestive enzyme activity has been traditionally assessed in two ways. On one side, *in vitro* experiments for the enzyme characterization performed with enzyme extracts show that activity increases with increasing temperature usually up to values exceeding those representative of their natural habitats, and also beyond lethal levels (Alarcón et al., 1998; Fernández et al., 2001; Gelman et al., 2008; Tanji et al., 1988). On the other hand, information about digestive enzyme activities analyzed in live fish at different temperatures is also available (Bowyer et al., 2014; Hani et al., 2018; Mazumder et al., 2018; Miegel et al., 2010; Sharma et al., 2017). However, these studies are based on only one sampling point during the postprandial response and, also report contradictory responses among the different studied species.

Gut evacuation rate also increases at increasing temperatures up to a certain limit, leading to lower residence time in the digestive tract (De et al., 2016; Fernández-Montero et al., 2018; Handeland et al., 2008; Temming and Herrmann, 2001). However, the estimation of evacuation rate has usually been performed under unrealistic feeding conditions in which the fish has been refed until satiation after a starvation period. Digestion efficiency will depend on the relation between enzymatic activity and gut transit time that are not short punctual facts but long dynamic cyclic processes usually occurring along

a whole day. Consequently, only experiments performed in routine feeding may provide realistic information.

Other species-specific digestive characteristics may also strongly affect the digestion process. That is the case of the gut luminal pH that conditions the activation of proenzymes in the gut, which may vary among fish, particularly within the stomach (Bucking and Wood, 2009; Papastamatiou and Lowe, 2005; Papastamatiou et al., 2007; Secor and Carey, 2016; Yúfera et al., 2004, 2007, 2012; Solovyev et al., 2018). Optimum temperature for growth does not necessarily coincide with maximum feed intake, highest digestion efficiency or optimal feeds utilization. In fact, from the point of view of aquaculture, the aim is to optimize all these factors to obtain the better juvenile quality and weight gain at a reasonable cost and with the lowest environmental impact.

Cobia (*Rachycentron canadum*), is a fast-growing species inhabiting tropical and subtropical waters with a broad geographical distribution over several continents. The species may reach up to 60 Kg and has a high-quality white flesh, being considered an excellent marine fish for aquaculture. It is being produced mainly in Asian-Pacific coast and in a lesser extent in the Gulf of Mexico with a world production above 40.000 tons during the last years (FAO, 2018; Tveteras, 2016). In Vietnam, it is one of the main marine fish in large scale commercial aquaculture (Nhu et al., 2011). In South Vietnam and other Southeast Asian regions water temperature in ponds and tanks ranges between 27 and 30 ºC, but may reach up to 36 ºC during the daytime in the warmer season. For this reason, it is necessary to understand the responses to increased temperatures, particularly in this region in the scenario of global warming (IPCC 2015). Effect of rearing temperature on growth in cobia juveniles has been examined by Sun and Chen (2009, 2014). In these studies, cobia juveniles were reared in the rage 20 to 35 ºC and highest growth rates were observed in the range 27-31 ºC. An unsolved question is how these temperature changes affect the digestion process.

Therefore, in this study we have examined the effects of temperature on the digestive function from a global perspective in order to advance in the physiological basis and mechanisms behind digestive efficiency and the corresponding effects on growth in cobia juveniles. Specifically, the aim of this study was to elucidate whether a temperature increase from 30 to 34 ºC affects gastrointestinal pH, enzymatic proteolytic digestive activity and feed transit over the whole day period, in early juveniles of this fast growing fish.

Material and Methods

Fish rearing and sampling

This study was part of a larger experiment examining growth performance in juveniles fed three different diets (Nguyen et al., 2019). We used the tanks from control treatment for the present study. Cobia juveniles were obtained from a local hatchery in Nha Trang, Vietnam, and acclimatized to final experimental temperatures in two indoor fiberglass 5000-L tanks at Nha Trang University facilities during one week. During the period of acclimatization, water temperature in one tank increased at a rate of 1°C per day up to 34°C, while temperature in the other tank was kept constant at 30°. Acclimatized juveniles with 3.7 ± 0.4 g wet body weight, were randomly distributed to 6 experimental 200-L tanks (60 fish $tank^{-1}$) and reared under a light/dark cycle at two temperatures (30 and 34 °C, three tanks for each temperature) in recirculation systems. Water salinity was 29.0 ± 3.1 g L^{-1}, pH 7.8–8.3, oxygen level 4.6 ± 0.5 mg L^{-1} and NH_3<0.03 mg L^{-1}. Fish were maintained with a 12:00 h illumination period from 6:00 to 18:00 h and fed twice a day (8:00 and 16:00 h local time) according to the most common procedure in the local hatcheries (Nguyen, 2013; 2014). The experimental diet was produced at SPAROS Lda (Olhão, Portugal) containing 47% protein and 10% lipid (Table 1). The fish were fed to nearly satiety (until most of the fish losing their appetite) by hand. Eventual uneaten feed and removed fish were recorded for the calculation of daily feed intake and feed conversion ratio, parameters considered in the companion study (Nguyen et al., 2019). After 2 weeks under these conditions, 3 fish per tank (wet body weight: ca. 6-8 g range) were sampled every 4 hours during 24 hours, for gut pH, digestive enzyme activity and feed transit determinations. Dissected gut were freeze-dried and sent to Spain for the analyses of enzyme activities at the University of Almeria and the gut transit at the ICMAN-CSIC. All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved by the Spanish National Research Council (CSIC) bioethical committee.

Measurements of gut pH

The gastrointestinal pH was measured in nine individuals at each sampling point and for each temperature condition immediately after sampling using a pH microelectrode (Thermo Orion, Thermo Fisher Scientific Inc) following the procedure described in Yúfera et al. (2012). In short, the fish were dissected to make the digestive track accessible. Next, the tip of the microelectrode (diameter 1.7 mm) was inserted in small slits made in the stomach, anterior intestine, medium intestine and posterior intestine (Fig. 1).

Digestive enzyme activity analyses

The complete digestive tract of three individuals of each sampling point and temperature were dissected, immediately frozen at -80 °C and later freeze-dried. Enzyme extracts were prepared for enzyme activity measurement from these samples. Stomach and intestine samples were dissected and

homogenized separately. Samples were manually homogenized in 3 mL distilled water and centrifuged for ten minutes at 4 ºC at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from the stomach samples were measured for pepsin activity, and the supernatants from the intestine samples were analyzed for trypsin activity.

Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1 mL of 0.5% acid-denatured bovine hemoglobin diluted in 0.2 M HCl-Glycine buffer. Assays were carried out at the specific gastric pH determined in each sampling point. In this way we are determining actually activated pepsin instead of pepsinogen (Yúfera et al., 2012). After incubation at 25ºC for 30 minutes, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 ºC for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes. The absorbance of the resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after the TCA. Trypsin activity was determined using BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate at 25ºC. 0.5 mM BAPNA was dissolved in 1mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50mM, pH 8.5, containing 20 mM $CaCl_2$. Reactions were started in 96-well microplates by the addition of 15 µL of the enzyme extract to 200 µL of the respective substrate and liberation of p-nitroanilline was kinetically followed at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA). The activities were provided as units per weight unit of fish to prevent the variability due to gut content.

Gut content and feed transit time measurements

The feed content in the stomach and the intestine was estimated from their respective weight determined in the gut samples used for enzyme and transit determinations. Average of empty guts were subtracted from these values. The values were normalized as dry weight of feed content per g of wet body weight (BW) to account for size differences among individuals.

For feed transit assessment, the day of the sampling, the feed labeled with containing of Yttrium oxide (200 mg Kg^{-1}) was provided for the first meal (8:00 h) while the standard feed without the marker was provided in the second meal (16:00 h). Yttrium content within the gut was analyzed at the ICMAN (Spain) by inductivity-coupled plasma mass spectroscopy (Thermo Scientific iCAP Q ICP-MS) separately in the stomach and the intestine of three individuals collected at each sampling point and temperature. Two technical subsamples were performed for each analysis. Yttrium content, normalized as mg g^{-1} of fish BW was plotted as a function of time.

The residence time of ingested feed within the gut were estimated as the period of time from when half of the stomach or intestine were filled with the marked feed to when half of the corresponding section was emptied. Previously, in order to calculate the total amount of feed accessing the stomach

in the first 4-h period, the total amount of yttrium in the stomach and intestine were considered, assuming that most of offered feed was ingested during the first hours of feeding. The yttrium content in each sampling point was converted to percentage of maximum measured in each section and temperature. The partial transit rates for each inter-sampling period were calculated as the difference of the relative fullness percentage between two consecutive time-points (considering the absolute values). Results have been presented as percentage of the maximum measured capacity entering or leaving each compartment for each 4h-period.

Statistical analyses

Two-way analysis of variance (ANOVA) was used to compare differences between the postprandial time and temperature for each section of the digestive tract. A post-hoc Tukey honest significant difference (HSD) test was used when ANOVA results revealed significant differences ($P < 0.05$). The homogeneity of variances was previously tested using Levene's test, and all parameters expressed as percentages were subjected to arcsin square root transformation. Data are presented as the mean of nine or three replicates ± sem. All statistical tests were performed in IBM SPSS Statistics 18 software (IBM Corp., USA).

Results

The pH within the stomach was permanently acidic with mean values ranging from 2.76 to 4.74 (Fig. 2) although a significant increase ($P<0.05$) was observed after each meal. The two way-ANOVA suggest that the gastric pH values change during the daily cycle but not in relation to temperature ($P>0.05$). On the other hand, the intestinal pH ranged from 6.05 to 7.69 (Fig. 3). An increase was observed after the first meal and this slight alkaline condition was maintained for several hours before declining to neutral or slightly acidic values at the end of the day ($P<0.05$). Furthermore, the maximum measured pH values were progressively higher when moving from proximal to distal part of the intestine ($P<0.05$). A slightly higher acidity was observed in the anterior and medium sections of the intestine at 34ºC ($P<0.05$).

Pepsin activity showed a daily rhythm at 30 ºC with a maximum in the middle of the light period and a minimum at the beginning of the dark period, while at 34ºC some hourly changes with a decrease after each meal were observed, but without so clear daily trend during the 24-h period. (Fig. 4). The trypsin activity exhibited a clear daily rhythm at both temperatures with a patent increase after morning feeding to reach a maximum several hours later (Fig. 4). Overall the activity of pepsin during the daily cycle was slightly higher at 34 ºC (average of the seven sampling points: 6.1 and 7.3 $U \cdot mg^{-1}$ BW at 30 and 34 ºC respectively), but values were significantly different only at 8 and 24 hours after the morning

meal. Similarly, the trypsin activity was significantly affected by the temperature only at 8 and 16 hours after the morning meal, but considering globally all daily samples the activity was quite similar (averages 1.20 and 1.29 $U \cdot g^{-1}$ BW at 30 and 34 ºC respectively). Considering all hourly data together, the two-way ANOVA indicates that the temperature is not affecting the trypsin and pepsin activities.

The daily pattern of the estimated feed content within the stomach and the intestine is shown in Figure 5. The patterns were clearly different at each temperature. At 30 ºC the amount of digesta within the stomach increased continuously from the morning feeding up to 8 hours after the second feeding. Contrarily, at 34 ºC the pattern showed two peaks, the first one 4 hours after the first meal and the second one 8 hours after the second meal. Feed content of the intestine at both temperatures was dramatically lower compared to the stomach and also showed two maxima at the same times observed in the stomach at 34 ºC.

Postprandial pattern of yttrium content within the gut is shown in Figure 6 (only the first meal contained yttrium oxide). At 30 ºC the yttrium content in the stomach reached the maximum value at 8 h after the first meal, while in the intestine the maximum was observed only 4 h after the first meal ($P<0.05$) maintaining similar high content at 8 h post-feeding. At 34 ºC the maximum yttrium content was observed 4 h after the first meal in the stomach and at 8 h post-feeding in the intestine ($P<0.05$), although an important amount of yttrium was already observed in the intestine at 4 h.

The partial transit rates of the first meal in the stomach for each period inter-samplings were higher during the first 4-h period and decreased progressively along the rest of the 24-h cycle, although no significant differences were detected at 30 ºC (Fig. 7). In addition, the transit was notably faster at 34 ºC particularly during the first 8 h after feeding, with rates between 100 and 65% of total volume displaced (intake or released) during each 4-h period. In the intestine the transit rate was relatively constant and similar at both temperatures during 12 h after feeding (Fig. 7). Then the rates dropped and remained very low during the following 12 h.

The residence time within the gut of the first meal was calculated from Figure 8. In this figure, the total amount of labelled feed accessing the stomach during the first 4-h period was considered, including the amount already transferred to and analyzed in the intestine. This criterion considers the time period from 50% of maximum measured label (yttrium) accessed the stomach or intestine up to the time when 50 % of this material has disappeared from the same compartment. Thus, the time the first meal spent in the stomach and the intestine was longer at 30 than at 34 ºC, particularly in the stomach (12h:02min vs 4h:54min, respectively) (Fig. 8). In the intestine, the difference was not so large (8h:18min vs 5h:54min, respectively). On the other hand, the residence time of this first meal at 30 ºC was longer in the stomach, while at 34 ºC it was longer in the intestine.

Discussion

The present study was performed in parallel with other study analyzing growth and feed conversion ratio differences in the same batch of juveniles fed different diet formulations at these two temperatures for six weeks (Nguyen et al. 2019). In that report, we found that cobia reared at 30ºC grew faster and showed a more favorable feed conversion ratio than those at elevated temperature (34ºC), being in both cases fed the same daily ration. We used the control tanks for the present study on digestion. The sampling was intentionally performed two weeks after the start of the experiment to determine the digestion status in the middle of the growth experiment, when both feed intake and growth were assessed to be high. Cobia is a voracious carnivorous fish with a large stomach and a short intestine (Fig. 1). This species feeds on small fish, crustaceans and squids (Franks et al., 1996). These feeding habits and gut anatomy have consequences in the mode of digestion as we observed in the different parameters examined. It seems evident that the stomach is very important for digestion and maybe more so than the intestine in this species, based on their respective volumes, luminal ionic values, proteolytic activities and transit rates results. Most research on postprandial response in fish has been done considering only one morning meal in order to examine the results without interferences of subsequent meals. In our study we considered two meals according to the customary use in hatcheries for this species in this region (Nguyen, 2013). With this feeding protocol we found more realistic overview of the digestive function but with additional complications to interpret results because fish may change the feeding behavior and daily digestive patterns when have the possibility to choose among different daily meals (Montoya et al., 2010; Yúfera et al., 2014).

In relation to luminal pH of the digestive tract, two gastric acidification strategies have been reported for vertebrates. One is to maintain a permanent acidic environment in the stomach with independence of the presence or absence of ingested feed, as observed for instance in mammals and birds; the other is to maintain a neutral pH in the lumen of the stomach between meals and with a decline only after the ingestion of feed (Papastamatiou and Lowe, 2005; Secor and Carey, 2016). Most teleostean fish analyzed up to date exhibited this second strategy (Hlophe et al., 2014; Nikolopoulou et al., 2011; Yúfera et al., 2004, 2012, Solovyev et al., 2016). However, our study reveals that cobia juveniles maintain a permanent gastric acidification. This is an interesting finding because such a strategy has been previously described only in rainbow trout *Oncorhynchus mykiss* (Bucking and Wood, 2009) and some elasmobranchian species (Papastamatiou and Lowe 2005; Papastamatiou et al., 2007). Some clues about the same strategy have also been reported for southern bluefin tuna *Thunnus maccoyii* examining fed and starved fish although a postprandial response was not scrutinized (Leef et al., 2012). Unfortunately, the list of teleostean species examined in detail is too short to know if this strategy is

less common or we need still to explore more species, particularly those with strict carnivorous feeding habits, to get a more complete figure of the acidification strategy in teleosteans. To maintain a neutral gastric environment during fasting has been associated to infrequent feeding in snakes and sharks (Papastamatiou and Lowe, 2005; Secor et al., 2012), but in teleostean with daily feeding habits this rule remains uncertain. In fact, an erratic daily feeding by changing randomly the moment of feed delivery every day may also alter the daily pattern from neutral/acid alternation to permanent acidification in gilthead seabream *Sparus aurata* (Montoya et al., 2010). A constant low gastric pH enables this voracious species to be always ready to activate pepsinogen to start the hydrolysis of the ingested prey. The small increase of gastric pH after meals has been attributed to the dilution effect of the ingesting feed, possibly in parallel with some water; drinking water for osmoregulatory purposes, as well as to the buffering capacity of feeds (Márquez et al., 2012) and also the buffer capacity of the slightly alkaline seawater itself.

We found that increased temperature did not affect to gastric pH but it led to decreased luminal pH in the anterior intestine and to a lesser extent the mid intestine. This effect is probably related to higher transit rates observed at 34 ºC in which the acidic chyme pass quickly to the short intestine. A similar effect on the intestinal pH was described at increased temperature in channel catfish *Ictalurus punctatus* (Page et al., 1976). The same effect in the anterior intestine was also detected in other species when feed only one meal (Bucking and Hood, 2009; Rosero, 2013; Yúfera et al., 2014). A decrease of the intestinal pH has been reported for different freshwater species associated to seasonal increased temperatures (Solovyev et al., 2018). The authors explained this decrease as an adaptation to enable fish to regulate and optimize the activity of their digestive pancreatic enzymes. Our results would indicate, that in addition, the changes in the water temperature alter the feeding behavior and feed processing along the day.

Pepsin activity showed hourly variations along the 24-h period ~~cycle~~ although a clear daily rhythm was observed only at 30 ºC. Considering that the minor variations of gastric pH are practically no affecting to pepsinogen activation as reported in other species (Yúfera et al., 2012), these changes should be interpreted in relation to the amount of substrate. The lower activity practically coincides with the higher amount of digesta in the stomach (Fig. 4) that is consuming the active enzyme. Contrary to this, the pancreatic trypsin activity exhibited a daily cycle with the expected increase associated with the intestinal alkalization when the chyme is released from the stomach. Such daily pattern has been already reported in other fish species (Rosero, 2013; Yúfera et al., 2014). It is also interesting to note that the proteolytic activity in the stomach was much higher than in the intestine. We found that the proteolytic activity of pepsin and trypsin was hardly affected by the 4 ºC increase of temperature, at least when the standard incubation temperature were used for the analytical protocols. It could be

interesting to explore analytical methodologies adapted to different temperatures. Miegel et al. (2010) did not find differences of intestinal proteases activity in fed individuals of yellowtail kingfish *Seriola lalandi* maintained at 12.6 and 20.8 ºC. However, Bowyer et al. (2014) found higher tryptic activity at intermediate temperatures in the range 21 to 27 ºC in starved individuals of the same species. Similar results were observed by Hani et al. (2018) in starved threespine stickleback *Gasterosteus aculeatus* in the range 16 to 21 ºC, by Sharma et al. (2017) in Indian major carp *Catla catla* in the range 10 to 35 ºC, as well as by Zhao et al. (2009) in the range 20 to 32 ºC in Chinese longsnout catfish *Leiocassis longirostris*. On the other hand, Mazamder et al. (2018) reported higher pepsin activity at 30 ºC in the range 22 to 34 ºC in fasted Malabar blood snapper *Lutjanus malabaricus*. Comparison of these results is difficult due to differences in fish size, experimental protocols and analytical methods, and in addition, the fish for those analyses were collected at only one time and any postprandial patterns was not explored. In our study and with independence of the similarity of the global daily averages of the lytic activity, the postprandial patterns showed maximum and minimum values that are not coincident at both temperatures. These results indicate that a single daily sample is not enough to characterize the enzymatic activity under different temperature conditions. Such data must be interpreted in relation to gut content as mentioned above but also in relation to transit results.

Transit rate assessment is a challenging task when more than one meal is offered. The postprandial responses overlap and the patterns are harder to interpret. A key factor in our study is to recognize that some ingested feed may pass to the intestine before the second sampling (Figs. 4 and 5) and therefore the estimation of the ingestion during the first period should include both sections. The gut content on weight basis gives only indicative information because it is representing the balance between digesta input and output. To obtain a more complete information it is necessary to estimate the temporal rates for the gut filling and evacuation under this feeding protocol in each gut compartment. An interesting result has been to verify whether the transit velocity of digesta throughout the digestive tract is changing along the daily cycle (Fig. 6), something perhaps obvious but never examined in fish. Thus, the transit rates were maxima during 8 h after feeding at 34 ºC. In this period the whole stomach volume was filled during the first 4-h period and emptied in a great part during the following 4 h before the next meal. The rest of the day the transit of remaining chyme was notably slower. At 30 ºC the transit rates during the first hours were significantly lower than at 34 ºC and the posterior decrease was smoother and not statistically significant. In the intestine no effect of temperature was observed and the transit was relatively fast during the first 12 h during which most part of the first meal is evacuated, the remaining digesta moved at notably slower rate. In our study, the second meal was not labeled and therefore these transit rates are only referring to the first meal when a second meal is pushing 8 h later.

Evacuation rates have been determined in many species usually based on fish with the stomach already full and without further feeding, such an approach gives an incomplete understanding of transit time in the stomach but in many cases the pass of the digesta through the intestine was properly assessed (Adamidou et al., 2009; Bonvini et al., 2018). While the evacuation of the stomach may last less than one day, the evacuation of the intestine may last 36 to 48 h. These values are only indicative for median sized farmed fish with daily feeding. Different factors such as feeding frequency, ration size, feed quality, body size and water temperature have been described to affect transit time in fish (Miegel et al., 2010), particularly the last one (De et al., 2016; Fernández-Montero et al., 2018; Handeland et al., 2008; Temming and Herrmann, 2001). According to these studies, transit time increases with the temperature except at extremely high values. Our results however showed an increase at very high temperatures that is probably close to tolerance limit.

Probably the most useful information is the time the digesta spent within the different sections and being hydrolyzed by the corresponding digestive enzymes. In routine feeding, the ingested feed is mixed up with the feed of the previous and the next meal(s) and its complete evacuation from the gut may last longer than expected due to the residual amount that can be detected for many hours, even days, later than most part of the digesta was evacuated. The criterion explained above allows an estimation of the residence time that can be compared between compartments and temperatures (Fig. 7). The most evident result is that the residence time was shorter at 34 ℃. The increase of 4 ℃ induced a faster filling and evacuation in the stomach as commented above but also a lower residence time, that was less than half of the period at 30 ℃. In the intestine the effect was not as dramatic but the reduction of digesta residence time was still important. The lower period of time for the proteolytic work of the digestive proteases brings on lower dietary protein utilization and is one of the reasons for the lower weight gain and higher feed conversion ratio observed at 34 ℃ (Nguyen et al., 2019). Furthermore, a relevant aspect is that the first meal transited almost simultaneously ~~than~~ in the stomach and intestine, when certain temporal displacement would be expected as determined in other species (Bonvini et al., 2018). The ingested pellets of the morning meal in our experiment passed directly into the intestine, and this segment was filled almost at same time as the stomach, working more like an extension of the stomach than like a different digestive tract compartment. Unfortunately, our experimental protocol does not allow to evaluate the transit time of the second meal that not necessarily may follow the same pattern but that we can assume it is similar to that of the first meal. It is likely that the feeding protocols for the voracious and carnivorous cobia in aquaculture where pelleted feed particles are offered in large amounts results in a digestive process that is progressing differently from nature where cobia ingest larger and intact prey. However, given the artificial feeding conditions in aquaculture, it is important to understand how the digestive system that is evolutionary adapted to natural conditions perform under different feeding regimes.

In summary, the present results indicate that at 34 ℃, a subtle increase of proteolytic activity cannot compensate for the faster gut transit rate. The reduced time the dietary proteins are available for hydrolysis when compared with fish maintained at 30 ℃ can explain the lower growth observed at this temperature (Nguyen et al. 2019). Another reason for the lower growth could be an unfavorable energetic balance at the higher temperature but the studies by Sun and Chen (2009, 2014) showed no evident variations of the feed energy allocated to metabolism in the range 27-33℃ in cobia juveniles of the same weight range, although it was higher at 35 ℃. Furthermore, this study shows a general appraisal of digestion in the 24-h temporal horizon as correspond to a daily feeding protocol, demonstrating the importance of observing inter-hourly changes in the different digestion parameters to characterize the digestive potential under given temperature conditions.

Acknowledgements: The project WISEFEED received funding by the European Union's H2020 programme (Marie Skłodowska-Curie grant No 691150). Additional funding from project EFISHDIGEST AGL2014-52888 (MINECO, Spain + FEDER/ERDF contribution). S.E. acknowledges a Foundation for Science and Technology of Portugal (FCT) investigator grant IF/00482/2014/CP1217/CT0005 funded by the European Social Fund, the Operational Programme Human Potential and FCT. This work also received national funds through FCT through project UDI/Multi/04326/2013 and Norwegian Agency for Development Cooperation NORHED, No. QZA-0485 SRV-13/0010.

References

Adamidou, S., Nengas, I., Alexis, M., Foundoulaki, E., Nikolopoulou, D., Campbell, P., Karacostas, C., Rigos, G., Bell, G.J., Jauncey, K., 2009. Apparent nutrient digestibility and gastrointestinal evacuation time in European seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes. Aquaculture 289, 106-112.

Alarcón, F.J., Díaz, M., Moyano F.J., Abellan, E., 1998. Characterization of functional properties in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol. Biochem. 19, 257–267.

Brett, JR., 1979. Environment factors and growth. In W.S. Hoar, et al. (Eds.), Fish Physiology, vol. 8, Academic Press, New York (1979), pp. 599-675.

Bonvini, E., Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Grandi, M., Fontanillas, R., Viroli, C., Gatta, P.P., 2018. Feeding European sea bass with increasing dietary fibre levels: Impact on growth, blood biochemistry, gut histology, gut evacuation. Aquaculture 494, 1-9.

Bowyer, J.N., Booth, M.A, Qin, J. G., D'Antignana, T., Thomson, M. J. S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquacult. Res. 45, 2010–2020.

Bucking C, Wood CM., 2009. The effect of postprandial changes in pH along the gastrointestinal tract on the distribution of ions between the solid and fluid phases of chime in rainbow trout. Aquacult. Nutr. 15, 282–296.

Buentello, J. A., Gatlin, D. M., Neill, W. H., 2000. Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilization and growth of channel catfish (*Ictalurus punctatus*). Aquaculture 182, 339-352.

De, M., Ghaffar, M.A, Bakar, Y., Das, S.K., 2016. Effect of temperature and diet on growth and gastric emptying time of the hybrid, *Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂. Aquacult. Rep. 4, 118–124.

FAO 2018. Cultured Aquatic Species Information Programme – *Rachycentron canadum*. Available at www.fao.org/fishery/culturedspecies/Rachycentroncanadum/en. Accessed on 15 September 2018.

Fernández-Montero, A., Caballero, M.J., Torrecillas, S., Tuset, V.M., Lombarte, A., Ruiz Ginés, R., Izquierdo, M., Robaina, L., Montero, D., 2018. Effect of temperature on growth performance of greater amberjack (*Seriola dumerili* 1810) Juveniles. Aquacult. Res. (in press).

Fernández, I., Moyano, F.J., Dıaz, M., Martınez, T., 2001. Characterization of a-amylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei). J. Exp. Mar. Biol. Ecol. 262, 1–12.

Franks, J.S., Garber, N.M., Warren, J.R., 1996. Stomach contents of juvenile cobia, *Rachycentron canadum*, from the Northern Gulf of Mexico. Fish. Bull. 94, 374–380.

Gelman A, Kuz'mina V, Drabkin V, Glatman L., 2008. Temperature adaptation pf digestive enzymes in fish. In: JEP Cyrino, DP Bureau, BG Kapoor eds., Feeding and digestive functions in fished. Science Publishers, Enfield NH, pp. 155-225.

Handeland, S.O., Imsland, A.K., Stefansson, S.O., 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon postsmolts. Aquaculture, 283, 36–42.

Hani, Y.M.I., Marchand, A., Turies, C., Kerambrun, E., Palluel, O., Bado-Nilles, A., Beaudouin, R., Porcher, J.M., Geffard, A., Dedourge-Geffard, O., 2018. Digestive enzymes and gut morphometric parameters of threespine stickleback (*Gasterosteus aculeatus*): Influence of body size and temperature. PLoS ONE 13(4): e0194932.

Hlophe, S.N., Moyo, N.A.G., Ncube,I., 2014. Postprandial changes in pH and enzyme activity from the stomach and intestines of *Tilapia rendalli* (Boulenger, 1897), *Oreochromis mossambicus* (Peters, 1852) and *Clarias gariepinus* (Burchell, 1822). J. Appl. Ichthyol. 30, 35–41.

IPCC 2015. Climate change 2014: Synthesis report. The intergovernmental panel on climate change Geneva, Switzerland, 151.

Márquez, L., Robles, R., Morales, G.A., Moyano, F.J., 2012. Gut pH as a limiting factor for digestive proteolysis in cultured juveniles of the gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 38, 859-69.

Miegel, R.P., Pain, S.J., van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). Aquaculture 308, 145–151.

460 Mazumder, S.K., Dasa, S.K., Rahim, S.M., Ghaffar, M.A., 2018. Temperature and diet effect on the
461 pepsin enzyme activities, digestive somatic index and relative gut length of Malabar blood snapper
462 (*Lutjanus malabaricus* Bloch & Schneider, 1801). Aquacult. Rep. 9, 1–9.

463 Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vázquez, F.J., 2010.
464 Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream
465 (*Sparus aurata*). Aquaculture. 306, 315–21.

466 Nguyen, M.V., 2013. The impact of lysine to arginine ratios in plant-based protein diets on appetite,
467 growth performance and gene expression of brain neuropetide Y (NPY) and cholecystokinin (CCK) in
468 juvenile cobia (*Rachycentron canadum*). PhD thesis, University of Bergen, 74 pp.

469 Nguyen, M.V., Rønnestad, I., Buttle, L., LAI, H.V., Espe, M., 2014. Imbalanced lysine to arginine ratios
470 reduced performance in juvenile cobia (*Rachycentron canadum*) fed high plant protein diets. Aquacult.
471 Nutr. 20, 25-35.

472 Nguyen, M.V., Espe, M., Conceição, L., Le, M.H., Yúfera, M., Engrola, S., Jordal, A-E.O., Pham, Q.H.,
473 Rønnestad, I., 2019. The role of dietary methionine concentrations on growth, metabolism and N-
474 retention in cobia (*Rachycentron canadum*) at elevated water temperatures. Aquacult. Nutr. (in press).

475 Nhu, V.C., Nguyen, Q.H., Le, T.L., Tran, M.T., Sorgeloos, P., Dierckens, K., Reinertsen, H., Kjørsvik, E.,
476 Svennevig, N., 2011. Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and
477 prospects. Aquaculture 315, 20-25.

478 Nikolopoulou, D., Moutou, K.A., Fountoulaki, E., Venou, B., Adamidou, S., Alexis, N.M., 2011. Patterns
479 of gastric evacuation, digesta characteristics and pH changes along the gastrointestinal tract of gilthead
480 sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Comp. Biochem. Physiol.
481 A 158, 406-414.

482 Page, J.W., Andrews, J.W., Murai, T., Murray, M.W., 1976. Hydrogen ion concentration in the
483 gastrointestinal tract of channel catfish. J. Fish Biol. 8, 225–228.

484 Papastamatiou, Y.P., Lowe, C.G., 2005. Variations in gastric acid secretion during periods of fasting
485 between two species of shark. Comp. Biochem. Physiol. A 141, 210-214.

486 Papastamatiou, Y.P., Purkis, S.J., Holland, K.N., 2007. The response of gastric pH and motility to fasting
487 and feeding in free swimming blacktip reef sharks, *Carcharhinus melanopterus*. J. Exp. Mar. Biol. Ecol.
488 345, 129-140.

489 Pérez-Casanova, J.C., Lall, S.P., Gamperl, A.K., 2009. Effect of feed composition and temperature on
490 food consumption, growth and gastric evacuation of juvenile Atlantic cod (*Gadus morhua* L.) and
491 haddock (*Melanogrammus aeglefinus* L.). Aquaculture 294, 228-235.

492 Rosero, A., 2013. Comparación de la fisiología digestiva entre un pez carnívoro (Corvina, *Argyrosomus*
493 *regius*) y un pez omnívoro (Liseta, *Chelon labrosus*), e influencia de la inclusión de un acidificante en el
494 pienso sobre el proceso digestivo. Master Thesis, Universidad de Cádiz.

495 Secor, S.M., Carey, H.V., 2016. Integrative Physiology of fasting. Compr. Physiol. 6, 773-825.

Secor, S.M., Taylor, J.R., Grosell, M., 2012. Selected regulation of gastrointestinal acid–base secretion and tissue metabolism for the diamondback water snake and Burmese python. J. Exp. Biol. 215, 185-196.

Sharma, J.G., Singhb, S.P., Chakrabarti, P., 2017. Effect of temperature on digestive physiology, immune-modulatory parameters, and expression level of Hsp and LDH genes in *Catla catla* (Hamilton, 1822). Aquaculture 479, 134-141.

Solovyev, M.M., Kashinskaya, E.N., Rusinek, O.T., Izvekova, G.I., 2016. Physiological pH values in the digestive tract of perch *Perca fluviatilis* from different habitats. J. Ichthyol. 56 (2), 312-318.

Solovyev, M.M., Izvekova, G., Kashinskaya, E., Gisbert, E., 2018. Dependence of pH values in the digestive tract of freshwater fishes on some abiotic and biotic factors. Hydrobiologia 807, 67–85.

Somero, G.N., 2004. Adaptation of enzymes to temperature: Searching for basic “strategies.” Comp. Biochem. Physiol. B 139, 321-333.

Somero GN., 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers”, J. Exp. Biol. 213, 912-20.

Sun, L., Chen, H., 2009. Effects of ration and temperature on growth and energy budget of juvenile cobia (*Rachycentron canadum*). Aquaculture 292, 197-206.

Sun, L., Chen, H., 2014. Effects of water temperature and fish size on growth and bioenergetics of cobia (*Rachycentron canadum*). Aquaculture 426-427, 172-180.

Tanji, M., Kageyama, T., Takahashi, K., 1988. Tuna pepsinogens and pepsins. Eur. J. Biochem. 177, 251-255.

Temming, A., Herrmann, J.-P., 2001. Gastric evacuation in horse mackerel. I. The effects of meal size, temperature and predator weight. J. Fish Biol. 58, 1230-1245.

Tveteras, R., 2016. Global fish production data & analysis. GOAL 2016, September 19-22, Guangzhou, China.

Yúfera, M., Fernández-Díaz, C., Vidaurreta, A., Cara, J.B., Moyano, F.J., 2004. Gastrointestinal pH and development of the acid digestion in larvae and early juveniles of *Sparus aurata* L. (Pisces: teleostei). Mar. Biol. 144, 863-869.

Yúfera, M., Darias, M.J., 2007. Changes in the gastrointestinal pH from larvae to adult in Senegal sole (*Solea senegalensis*). Aquaculture 267, 94-99.

Yúfera, M., Moyano, F.J., Astola, A., Pousão-Ferreira, P., Martínez-Rodríguez, G., 2012. Acidic digestion in a teleost: Postprandial and circadian pattern of gastric pH, pepsin activity, and pepsinogen and proton pump mRNAs expression. PLoS ONE, 7(3) e33687.

Yúfera, M., Romero, M.J., Pujante, I.M. Astola, A., Mancera, J.M., Sánchez-Vázquez, F.J., Moyano, F.J., Martínez-Rodríguez, G., 2014. Effect of feeding frequency on the daily rhythms of acidic digestion in a teleost fish (gilthead seabream). Chronobiol. Int. 31,1024–1033.

531 Zhao, H., Han, D., Xie, S., Zhu, X., Yang, Y., 2009. Effect of water temperature on the growth
532 performance and digestive enzyme activities of Chinese longsnout catfish (*Leiocassis longirostris*
533 Günther). Aquacult. Res. 40, 1864-1872.

534

Figure captions

Fig. 1. Digestive tract of an early *R. canadum* juvenile indicating the places for the gut pH determinations. ST: stomach; AI: anterior intestine; MI: medium intestine; PI: posterior intestine.

Fig. 2. Postprandial changes in gastric pH (mean and SEM) of *R. canadum* juveniles at the two experimental temperatures. Different letters denote statistical difference at the different sampling times. Arrows indicate the time for the two feed supplies. Shaded area indicates the dark period.

Fig. 3. Postprandial changes in the luminal pH (mean and SEM) of the different section of the intestine of *R. canadum* juveniles at the two experimental temperatures. Arrows indicate the time for the two feed supplies. Dashed line at pH 7 was included for a better comparison between both temperatures. Shaded area indicates the dark period.

Fig. 4. Postprandial changes of pepsin and trypsin activities (mean and SEM) in the stomach and intestine of *R. canadum* juveniles at the two experimental temperatures. Arrows indicate the time for the two feed supplies. Shaded area indicates the dark period. Different letters denote significant differences at the different sampling times for each temperature. Asterisks denote significant differences between temperatures.

Fig. 5. Postprandial changes of gut content within the stomach (grey) and intestine (black) of *R. canadum* juveniles at the two experimental temperatures. Shaded area indicates the dark period.

Fig. 6. Postprandial changes of yttrium content (mean and SEM) within the stomach and intestine of *R. canadum* juveniles at the two experimental temperatures. Shaded area indicates the dark period.

Fig. 7. Partial transit rates of digesta for each inter-sampling period (4 h) in the stomach and intestine of *R. canadum* juveniles at the two experimental temperatures. Results are presented as percentage of the maximum (mean and SEM) measured capacity entering or leaving each compartment for each

563 4h-period. Different letters denote significant differences at the different sampling times for each
564 temperature. Asterisks denote significant differences between temperatures.

565

566 Fig. 8. Residence time of the first meal in the stomach and intestine of *R. canadum* juveniles at the two
567 experimental temperatures. Results are presented as percentage of the maximum feed content (mean
568 and SEM) at each sampling time. Arrows represent the period of time from the 50% of the maximum
569 acceded to each gut compartment to the 50% is evacuated from the same compartment. Values in
570 the insets indicate the residence time according to this criterion.

571 **Tables**

572 Table 1. Formulation (g kg^{-1} dry matter basis) and proximate analysis of the diet.

Ingredients	**g kg^{-1}**
Krill meal	50.0
Wheat meal	175.3
Fish meal	250.0
Soy protein concentrate	100.0
Pea protein concentrate	134.0
CPSP 90	50.0
DL methionine	5.5
Betaine HCl	5.0
Encapsuled taurine	5.0
Encapsuled tryptophane	5.0
Fish oil	28.0
Krill oil	30.0
Pea starch	100.0
Vitamin & mineral mix	20.0
Lutavit E50	0.2
Calcium carbonate	10.0
Mono ammonium phosphate	30.0
Antioxidant (Paramega)	2.0
Proximate composition	
Dry matter	958.0
Energy (MJ kg^{-1})	20.1
Crude protein	465.0
Crude fat	103.0

573

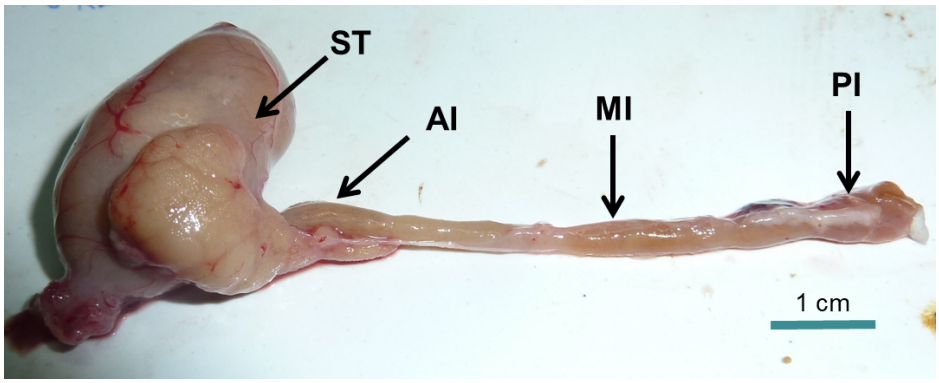

Fig. 1

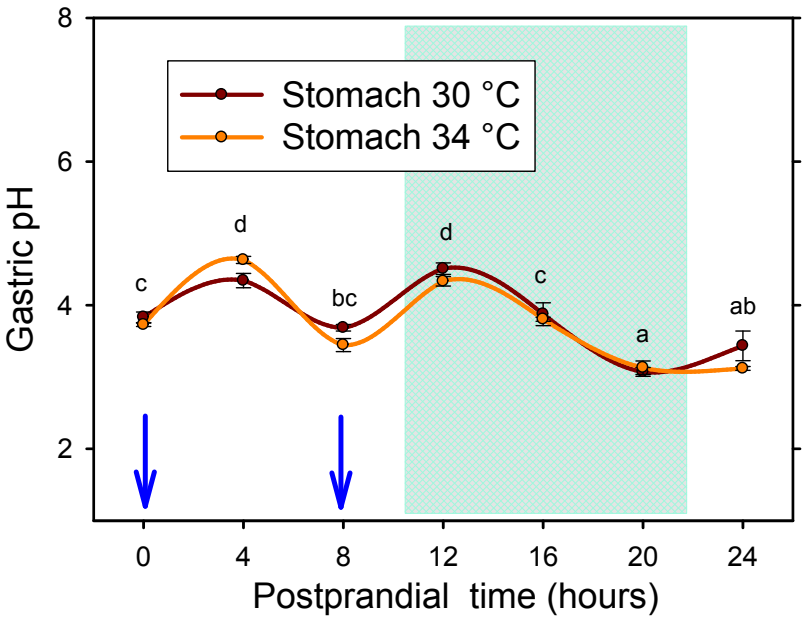
Stomach 30 °C
Stomach 34 °C
Gastric pH
Postprandial time (hours)
8
6
4
2
0
4
8
12
16
20
24
c
d
bc
d
c
a
ab

Fig. 2

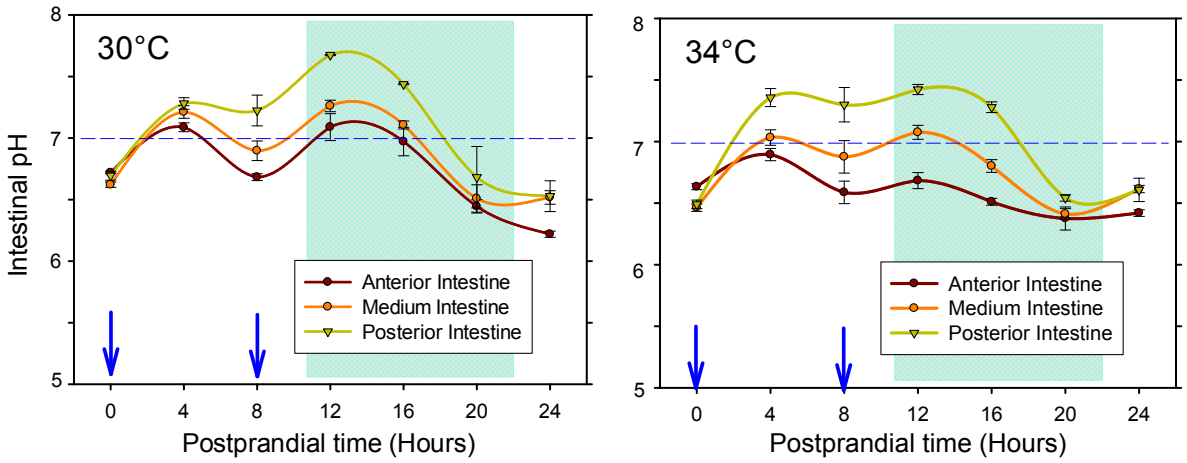
30°C
34°C
Intestinal pH
Postprandial time (Hours)
Anterior Intestine
Medium Intestine
Posterior Intestine
8
7
6
5
0
4
8
12
16
20
24

Fig. 3

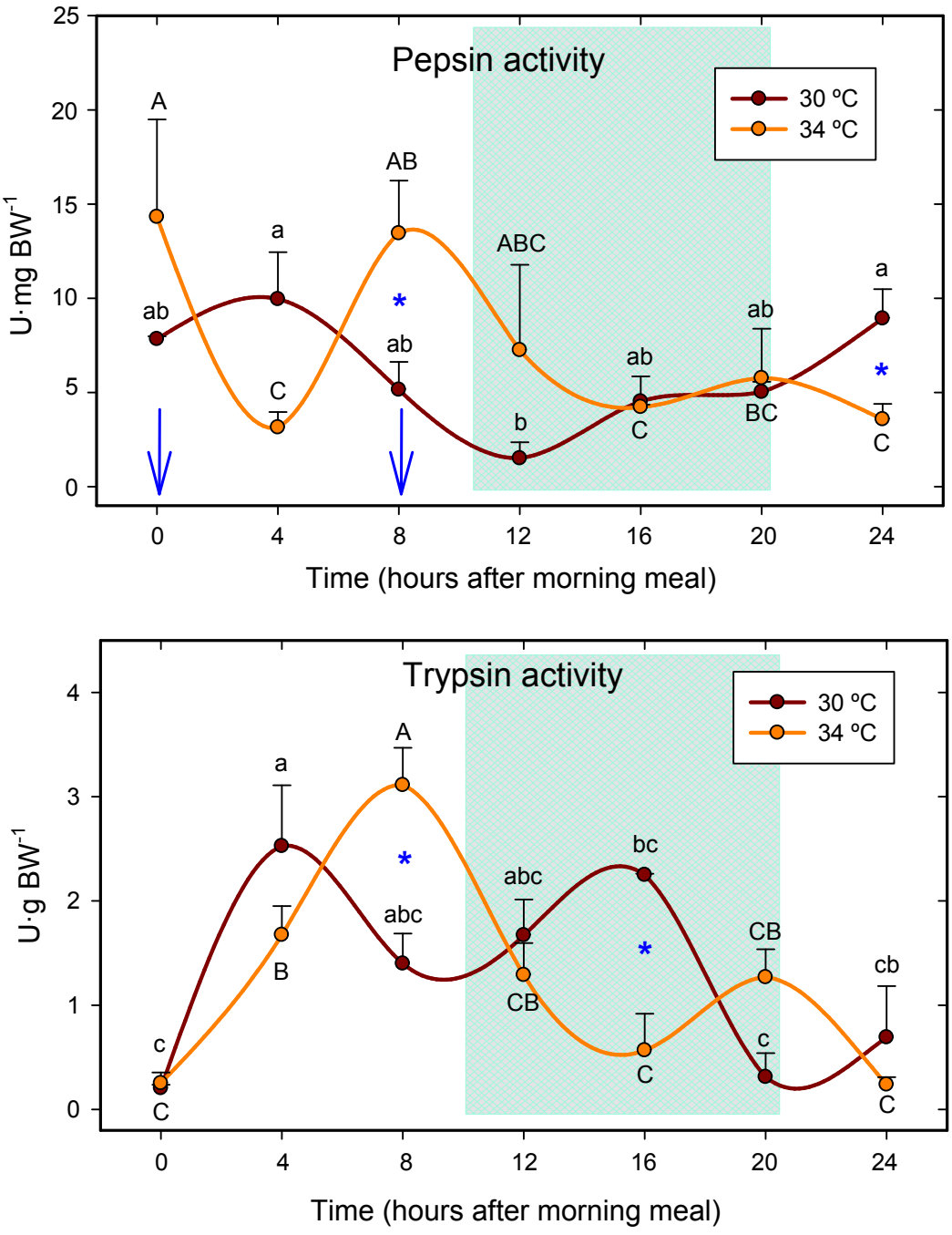
Pepsin activity
30 °C
34 °C
U·mg BW-1
Time (hours after morning meal)
Trypsin activity
30 °C
34 °C
U·g BW-1
Time (hours after morning meal)

Fig. 4

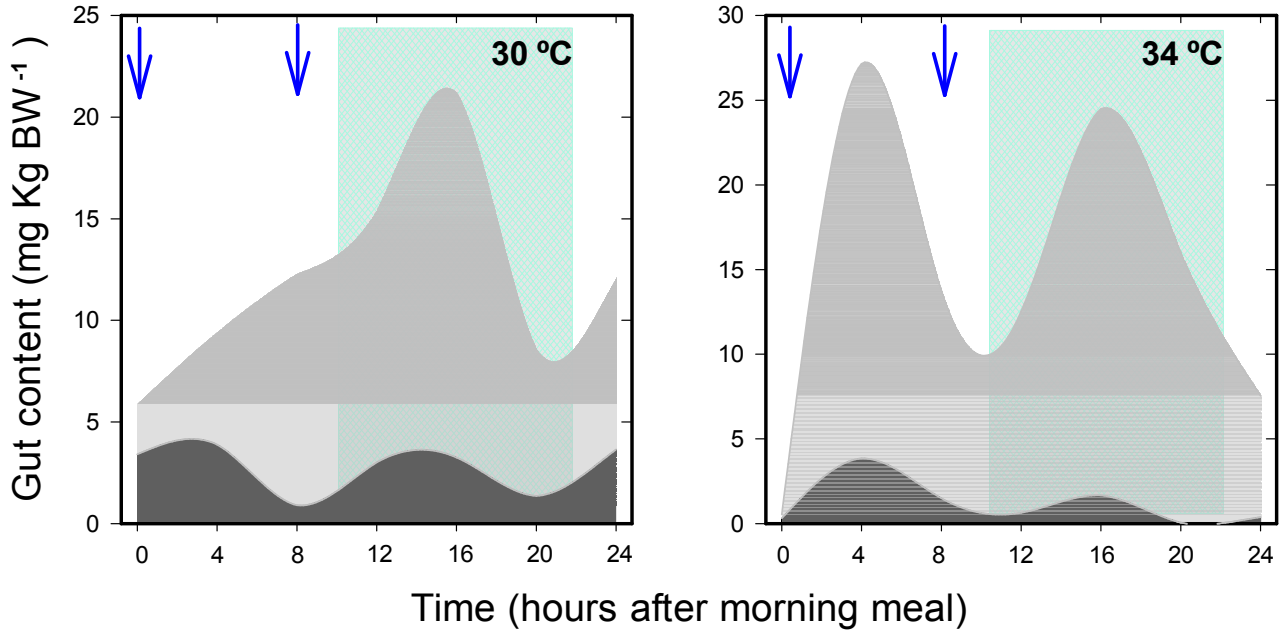
30 ºC
34 ºC
Gut content (mg Kg BW-1)
Time (hours after morning meal)
0
4
8
12
16
20
24
0
5
10
15
20
25
30

Fig. 5.

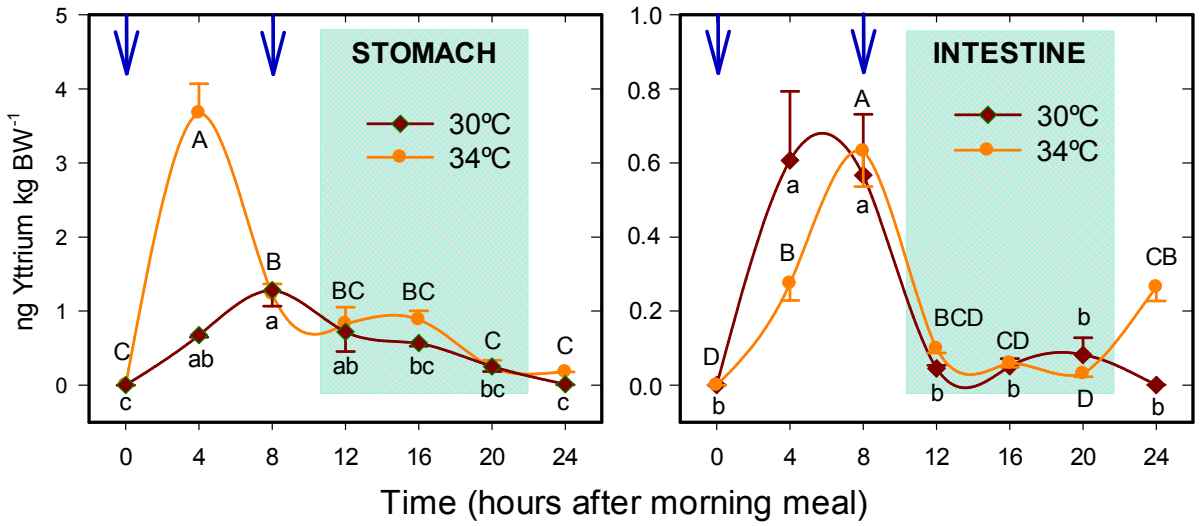
STOMACH
INTESTINE
30ºC
34ºC
ng Yttrium kg BW-1
Time (hours after morning meal)
0
4
8
12
16
20
24
C
ab
A
B
a
BC
ab
BC
bc
C
bc
C
c
D
b
a
A
a
B
BCD
b
CD
b
b
D
CB
b

Fig. 6

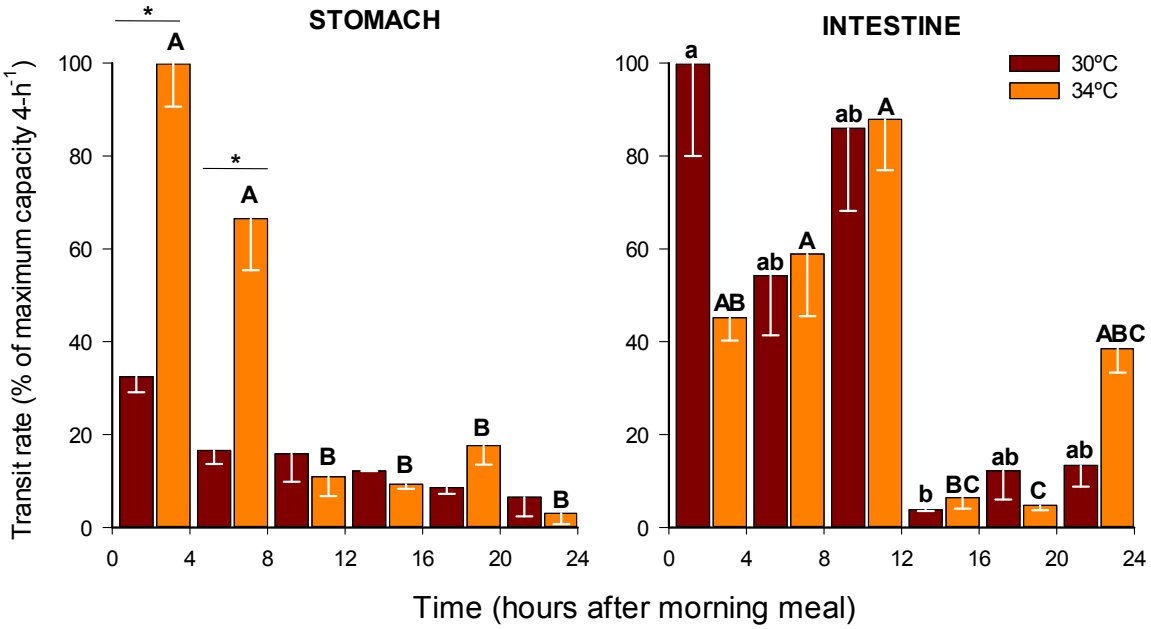
STOMACH
INTESTINE
30ºC
34ºC
Transit rate (% of maximum capacity 4-h-1)
Time (hours after morning meal)
*
*
A
A
B
B
B
B
a
AB
ab
A
ab
A
b
BC
ab
C
ab
ABC
0
20
40
60
80
100
0
4
8
12
16
20
24

Fig. 7

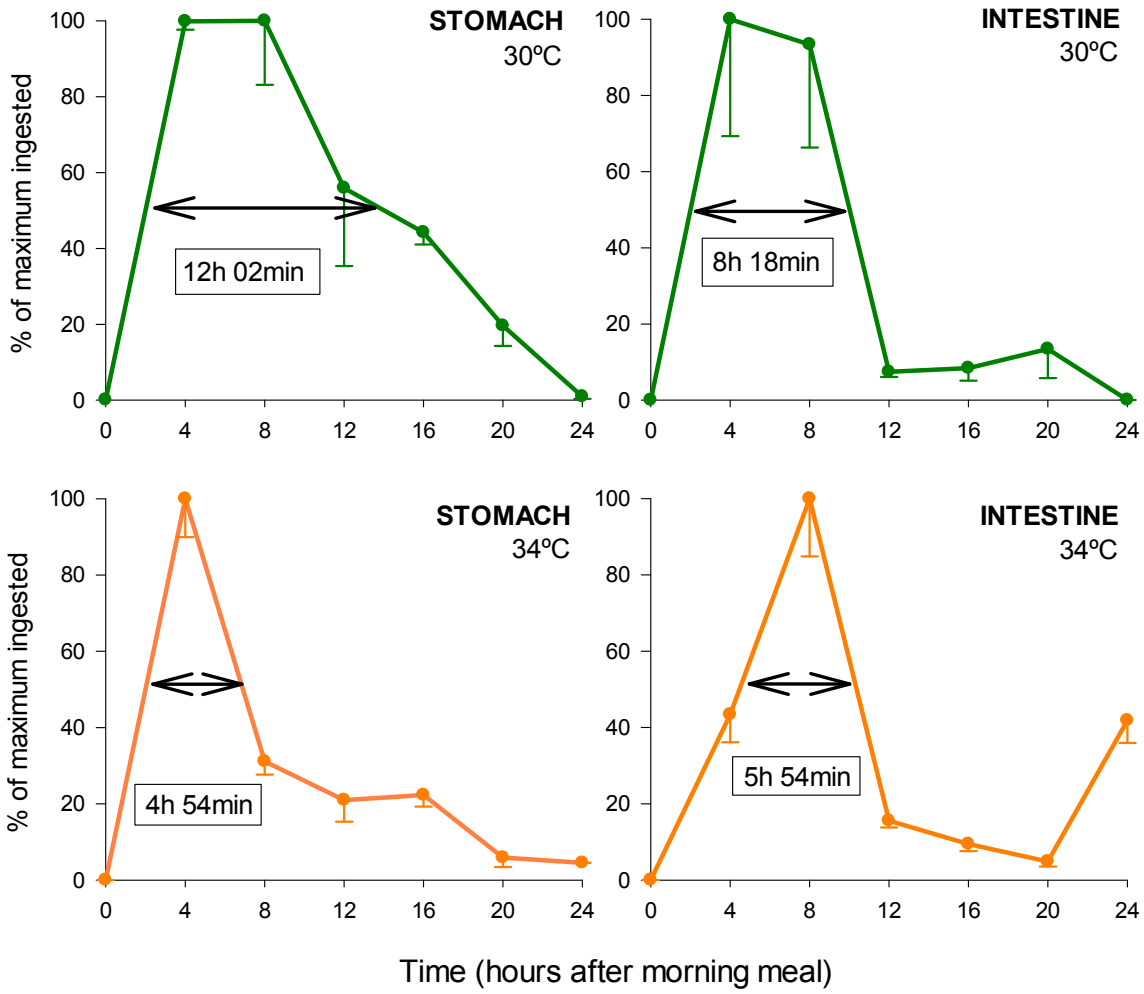
STOMACH
30ºC
INTESTINE
30ºC
STOMACH
34ºC
INTESTINE
34ºC
% of maximum ingested
% of maximum ingested
12h 02min
8h 18min
4h 54min
5h 54min
0
20
40
60
80
100
0
4
8
12
16
20
24
Time (hours after morning meal)

Fig. 8.

Conflicts of interest

The authors declare no conflicts of interest in relation to the present investigation.