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

2   Short title: Effect of temperature on Cobia digestion

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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 34℃ 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 34℃.

Pepsin activity showed a daily rhythm at 30 ℃ with maximum in the middle of the light period, while at 34℃ 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 ℃ (6.1 and 7.3 U mg^{-1} BW at 30 and 34 ℃ 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 ℃ 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 ℃. In addition, the transit was notably faster at 34 ℃ 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 ℃, 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 ℃ grew faster and showed ~~better~~ a more favorable feed conversion ratio than those at elevated temperature (34 ℃). The present results indicate that at 34 ℃, a subtle increase of proteolytic activity cannot compensate for the faster gut transit rate. Therefore, 30 ℃ is more appropriate temperature for the early on-growing

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

57

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

59

60 **Introduction**

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

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

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

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

88 information.

89 Other species-specific digestive characteristics may also strongly affect the digestion process. That is

90 the case of the gut luminal pH that conditions the activation of proenzymes in the gut, which may vary

91 among fish, particularly within the stomach (Bucking and Wood, 2009; Papastamatiou and Lowe, 2005;

92 Papastamatiou et al., 2007; Secor and Carey, 2016; Yúfera et al., 2004, 2007, 2012; Solovyev et al.,

93 2018). Optimum temperature for growth does not necessarily coincide with maximum feed intake,

94 highest digestion efficiency or optimal feeds utilization. In fact, from the point of view of aquaculture,

95 the aim is to optimize all these factors to obtain the better juvenile quality and weight gain at a

96 reasonable cost and with the lowest environmental impact.

97 Cobia (*Rachycentron canadum*), is a fast-growing species inhabiting tropical and subtropical waters

98 with a broad geographical distribution over several continents. The species may reach up to 60 Kg and

99 has a high-quality white flesh, being considered an excellent marine fish for aquaculture. It is being

100 produced mainly in Asian-Pacific coast and in a lesser extent in the Gulf of Mexico with a world

101 production above 40.000 tons during the last years (FAO, 2018; Tveteras, 2016). In Vietnam, it is one

102 of the main marine fish in large scale commercial aquaculture (Nhu et al., 2011). In South Vietnam and

103 other Southeast Asian regions water temperature in ponds and tanks ranges between 27 and 30 °C,

104 but may reach up to 36 °C during the daytime in the warmer season. For this reason, it is necessary to

105 understand the responses to increased temperatures, particularly in this region in the scenario of

106 global warming (IPCC 2015). Effect of rearing temperature on growth in cobia juveniles has been

107 examined by Sun and Chen (2009, 2014). In these studies, cobia juveniles were reared in the rage 20

108 to 35 °C and highest growth rates were observed in the range 27-31 °C. An unsolved question is how

109 these temperature changes affect the digestion process.

110 Therefore, in this study we have examined the effects of temperature on the digestive function from

111 a global perspective in order to advance in the physiological basis and mechanisms behind digestive

112 efficiency and the corresponding effects on growth in cobia juveniles. Specifically, the aim of this study

113 was to elucidate whether a temperature increase from 30 to 34 °C affects gastrointestinal pH,

114 enzymatic proteolytic digestive activity and feed transit over the whole day period, in early juveniles

115 of this fast growing fish.

116

117 **Material and Methods**

118 *Fish rearing and sampling*

119   This study was part of a larger experiment examining growth performance in juveniles fed three

120   different diets (Nguyen et al., 2019). We used the tanks from control treatment for the present study.

121   Cobia juveniles were obtained from a local hatchery in Nha Trang, Vietnam, and acclimatized to final

122   experimental temperatures in two indoor fiberglass 5000-L tanks at Nha Trang University facilities

123   during one week. During the period of acclimatization, water temperature in one tank increased at a

124   rate of 1℃ per day up to 34℃, while temperature in the other tank was kept constant at 30°.

125   Acclimatized juveniles with 3.7 ± 0.4 g wet body weight, were randomly distributed to 6 experimental

126   200-L tanks (60 fish tank^{-1}) and reared under a light/dark cycle at two temperatures (30 and 34 ℃,

127   three tanks for each temperature) in recirculation systems. Water salinity was 29.0 ± 3.1 g L^{-1}, pH 7.8–

128   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

129   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

130   common procedure in the local hatcheries (Nguyen, 2013; 2014). ~~with an~~ The experimental diet was

131   produced at SPAROS Lda (Olhão, Portugal) containing 47% protein and 10% lipid (Table 1). ~~The~~

132   ~~experimental diet and was formulated based on previous results in cobia (Nguyen et al., 2014)~~. The

133   fish were fed to nearly satiety (until most of the fish losing their appetite) by hand. Eventual uneaten

134   feed and removed fish were recorded for the calculation of daily feed intake and feed conversion ratio,

135   parameters considered in the companion study (Nguyen et al., 2019). After 2 weeks under these

136   conditions, 3 fish per tank (wet body weight: ca. 6-8 g range) were sampled every 4 hours during 24

137   hours, for gut pH, digestive enzyme activity and feed transit determinations. Dissected gut were freeze-

138   dried and sent to Spain for the analyses of enzyme activities at the University of Almeria and the gut

139   transit at the ICMAN-CSIC. All experimental procedures complied with the Guidelines of the European

140   Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed

141   and approved by the Spanish National Research Council (CSIC) bioethical committee.

142   *Measurements of gut pH*

143   The gastrointestinal pH was measured in nine individuals at each sampling point and for each

144   temperature condition immediately after sampling using a pH microelectrode (Thermo Orion, Thermo

145   Fisher Scientific Inc) following the procedure described in Yúfera et al. (2012). In short, the fish were

146   dissected to make the digestive track accessible. Next, the tip of the microelectrode (diameter 1.7 mm)

147   was inserted in small slits made in the stomach, anterior intestine, medium intestine and posterior

148   intestine (Fig. 1).

149   *Digestive enzyme activity analyses*

150   The complete digestive tract of three individuals of each sampling point and temperature were

151   dissected, immediately frozen at -80 ℃ and later freeze-dried. Enzyme extracts were prepared for

152 enzyme activity measurement from these samples. Stomach and intestine samples were dissected and

153 homogenized separately. Samples were manually homogenized in 3 mL distilled water and centrifuged

154 for ten minutes at 4 °C at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from

155 the stomach samples were measured for pepsin activity, and the supernatants from the intestine

156 samples were analyzed for trypsin activity.

157 Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1

158 mL of 0.5% acid-denatured bovine hemoglobin diluted in 0.2 M HCl-Glycine buffer. Assays were carried

159 out at the specific gastric pH determined in each sampling point. In this way we are determining

160 actually activated pepsin instead of pepsinogen (Yúfera et al., 2012). After incubation at 25°C for 30

161 minutes, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 °C

162 for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes. The absorbance of the resulting

163 supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the

164 reaction mixture just after the TCA. Trypsin activity was determined using BAPNA (N-benzoyl-DL-

165 arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate at 25°C. 0.5 mM BAPNA was dissolved in

166 1mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50mM, pH 8.5, containing

167 20 mM $CaCl_2$. Reactions were started in 96-well microplates by the addition of 15 µL of the enzyme

168 extract to 200 µL of the respective substrate and liberation of p-nitroanilline was kinetically followed

169 at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA). The activities were

170 provided as units per weight unit of fish to prevent the variability due to gut content.

171 *Gut content and feed transit time measurements*

172 The feed content in the stomach and the intestine was estimated from their respective weight

173 determined in the gut samples used for enzyme and transit determinations. Average of empty guts

174 were subtracted from these values. The values were normalized as dry weight of feed content per g of

175 wet body weight (BW) to account for size differences among individuals.

176 For feed transit assessment, the day of the sampling, the feed labeled with containing of Yttrium oxide

177 (200 mg Kg^{-1}) was provided for the first meal (8:00 h) while the standard feed without the marker was

178 provided in the second meal (16:00 h). Yttrium content within the gut was analyzed at the ICMAN

179 (Spain) by inductivity-coupled plasma mass spectroscopy (Thermo Scientific iCAP Q ICP-MS) separately

180 in the stomach and the intestine of three individuals collected at each sampling point and temperature.

181 Two technical subsamples were performed for each analysis. Yttrium content, normalized as mg g^{-1} of

182 fish BW was plotted as a function of time.

183 The residence time of ingested feed within the gut were estimated as the period of time from when

184 half of the stomach or intestine were filled with the marked feed to when half of the corresponding

185   section was emptied. Previously, in order to calculate the total amount of feed accessing the stomach

186   in the first 4-h period, the total amount of yttrium in the stomach and intestine were considered,

187   assuming that most of offered feed was ingested during the first hours of feeding. The yttrium content

188   in each sampling point was converted to percentage of maximum measured in each section and

189   temperature. The partial transit rates for each inter-sampling period were calculated as the difference

190   of the relative fullness percentage between two consecutive time-points (considering the absolute

191   values). Results have been presented as percentage of the maximum measured capacity entering or

192   leaving each compartment for each 4h-period.

193   *Statistical analyses*

194   Two-way analysis of variance (ANOVA) was used to compare differences between the postprandial

195   time and temperature for each section of the digestive tract. A post-hoc Tukey honest significant

196   difference (HSD) test was used when ANOVA results revealed significant differences ($P < 0.05$). The

197   homogeneity of variances was previously tested using Levene's test, and all parameters expressed as

198   percentages were subjected to arcsin square root transformation. Data are presented as the mean of

199   nine or three replicates ± sem. All statistical tests were performed in IBM SPSS Statistics 18 software

200   (IBM Corp., USA).

201

202   **Results**

203   The pH within the stomach was permanently acidic with mean values ranging from 2.76 to 4.74 (Fig.

204   2) although a significant increase ($P < 0.05$) was observed after each meal. The two way-ANOVA suggest

205   that the gastric pH values change during the daily cycle but not in relation to temperature ($P > 0.05$). On

206   the other hand, the intestinal pH ranged from 6.05 to 7.69 (Fig. 3). An increase was observed after the

207   first meal and this slight alkaline condition was maintained for several hours before declining to neutral

208   or slightly acidic values at the end of the day ($P < 0.05$). Furthermore, the maximum measured pH values

209   were progressively higher when moving from proximal to distal part of the intestine ($P < 0.05$). A slightly

210   higher acidity was observed in the anterior and medium sections of the intestine at 34°C ($P < 0.05$).

211   Pepsin activity showed a daily rhythm at 30 °C with a maximum in the middle of the light period and a

212   minimum at the beginning of the dark period, while at 34°C some hourly changes with a decrease after

213   each meal were observed, but without so clear daily trend during the 24-h period were observed. (Fig.

214   4). The trypsin activity exhibited a clear daily rhythm at both temperatures with a patent increase after

215   morning feeding to reach a maximum several hours later (Fig. 4). Overall the activity of pepsin during

216   the daily cycle was slightly higher at 34 °C (average of the seven sampling points: 6.1 and 7.3 U·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·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

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250　(8h:18min vs 5h:54min, respectively). On the other hand, the residence time of this first meal at 30 ℃

251　was longer in the stomach, while at 34 ℃ it was longer in the intestine.

252

253　**Discussion**

254　The present study was performed in parallel with other study analyzing growth and feed conversion

255　ratio differences in the same batch of juveniles fed different diet formulations at these two

256　temperatures for six weeks (Nguyen et al. 2017, 2019). In that report, we found that cobia reared at

257　30°C grew faster and showed bettera more favorable feed conversion ratio (FCR) than those at

258　elevated temperature (34°C), being in both cases fed the same daily ration. We used the control tanks

259　for the present study on digestion. The sampling was intentionally performed two weeks after the start

260　of the experiment to determine the digestion status in the middle of the growth experiment, when

261　both feed intake and growth were assessed to be high. Cobia is a voracious carnivorous fish with a

262　large stomach and a short intestine (Fig. 1). This species feeds on small fish, crustaceans and squids

263　(Franks et al., 1996). These feeding habits and gut anatomy have consequences in the mode of

264　digestion as we observed in the different parameters examined. It seems evident that the stomach is

265　very important for digestion and maybe more so than the intestine in this species, based on their

266　respective volumes, luminal ionic values, proteolytic activities and transit rates results. Most research

267　on postprandial response in fish has been done considering only one morning meal in order to examine

268　the results without interferences of subsequent meals. In our study we considered two meals

269　according to the customary use in hatcheries for this species in this region (Nguyen, 2013). With this

270　feeding protocol we found more realistic overview of the digestive function but with additional

271　complications to interpret results because fish may change the feeding behavior and daily digestive

272　patterns when have the possibility to choose among different daily meals (Montoya et al., 2010; Yúfera

273　et al., 2014).

274　In relation to luminal pH of the digestive tract, two gastric acidification strategies have been reported

275　for vertebrates. One is to maintain a permanent acidic environment in the stomach with independence

276　of the presence or absence of ingested feed, as observed for instance in mammals and birds; the other

277　is to maintain a neutral pH in the lumen of the stomach between meals and with a decline only after

278　the ingestion of feed (Papastamatiou and Lowe, 2005; Secor and Carey, 2016). Most teleostean fish

279　analyzed up to date exhibited this second strategy (Hlophe et al., 2014; Nikolopoulou et al., 2011;

280　Yúfera et al., 2004, 2012, Solovyev et al., 2016). However, our study reveals that cobia juveniles

281　maintain a permanent gastric acidification. This is an interesting finding because such a strategy has

282　been previously described only in rainbow trout *Oncorhynchus mykiss* (Bucking and Wood, 2009) and

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

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

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

397   of the same weight range, although it was higher at 35 ℃. 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

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541

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

18

570   4h-period. ~~SEM was omited for clarity~~. Different letters denote significant differences at the different

571   sampling times for each temperature. Asterisks denote significant differences between temperatures.

572

573   Fig. 8. Residence time ~~of the digesta~~ of the first meal in the stomach and intestine of *R. canadum*

574   juveniles at the two experimental temperatures. Results are presented as percentage of the maximum

575   feed content (mean and SEM) at each sampling time. Arrows represent the period of time from the

576   50% of the maximum acceded to each gut compartment to the 50% is evacuated from the same

577   compartment. Values in the insets indicate the residence time according to this criterion.

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

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

2   Short title: Effect of temperature on Cobia digestion

3

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21

22

23   **Abstract**

24   The present study is focused to elucidate the main characteristics of the digestive function of this

25   carnivorous fast-growing fish living at high temperatures. With this aim, we have examined the effects

26   of an increased temperature from 30 to 34°C on the daily pattern of gastrointestinal pH, enzymatic

27   proteolytic digestive activity and the feed transit time in early juveniles of cobia (*Rachycentron*

28   *canadum*), a species living in tropical and subtropical waters with an increasing aquaculture

29   production. Fish were fed two meals a day. Gastric luminal pH was permanently acidic (mean pH

30   values: 2.76 - 4.74) while the intestinal pH increased from neutral/slightly acidic to slightly alkaline

31   when the digesta was present, with an increasing alkalinity from proximal to distal intestine (mean pH

32   values: 6.05 to 7.69). The temperature did not affect the gastric pH but a slightly higher acidity was

33   induced in the intestine at 34°C.

34   Pepsin activity showed a daily rhythm at 30 °C with maximum in the middle of the light period, while

35   at 34°C some hourly changes coinciding with feed adding without a clear daily trend during the 24-h

36   period were observed. The trypsin activity exhibited a daily rhythm at both temperatures with an

37   increase after morning feeding to reach a maximum several hours later. Average pepsin activity during

38   the daily cycle was slightly higher at 34 °C (6.1 and 7.3 U mg^{-1} BW at 30 and 34 °C respectively), but

39   values were significantly different only at 8 and 24 h after the morning meal. Similarly, the trypsin

40   activity was significantly affected by the temperature only at 8 and 16 h after the morning meal, but

41   daily activity averages were similar (1.20 and 1.29 U g^{-1} BW at 30 and 34 °C respectively).

42   The partial transit rates of the first meal in the stomach for each period inter-samplings were higher

43   during the first 4-h period and decreased progressively along the rest of the 24-h cycle at both

44   temperatures, but no significant differences were detected at 30 °C. In addition, the transit was notably

45   faster at 34 °C particularly during the first 8 h after feeding, with rates between 100 and 65% of total

46   volume displaced (intake or released) during each 4-h period. In the intestine the transit rate was

47   relatively constant and similar at both temperatures during 12 h after feeding. Then the rates remained

48   very low during the following 12 h.

49   Residence time of the first meal was longer at 30 than at 34 °C, particularly in the stomach (12h:02min

50   vs 4h:54min respectively). In the intestine the difference was not so large (8h:18min vs 6h:24min

51   respectively). In a parallel study with under same conditions, cobia reared at 30 °C grew faster and

52   showed a more favorable feed conversion ratio than those at elevated temperature (34 °C). The

53   present results indicate that at 34 °C, a subtle increase of proteolytic activity cannot compensate for

54   the faster gut transit rate. Therefore, 30 °C is more appropriate temperature for the early on-growing

55   of cobia because at higher temperatures the digestion efficiency decrease being one of the causes for

56   a lower growth.

57

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

59

60   **Introduction**

61   Water temperature is a key factor affecting metabolic rates in fish and therefore has an evident impact

62   on feed intake, nutrient utilization and growth (Brett, 1979; Buentello et al., 2000). To cope with the

63   wide range of temperatures in the oceans depending on the geographic location and environmental

64   cycles, the various fish species have adapted their feeding behavior and physiology to the temperature

65   conditions of their particular habitat (Brett, 1979; Somero, 2004, 2010). Many studies have examined

66   different perspectives of physiological responses to changes in temperature.

67   Particularly relevant is the way the ingested nutrients are digested before their incorporation into

68   growing tissues. In spite of a large research effort, the effect of temperature on fish digestion is far

69   from being well understood. The digestive function includes different processes from feed capture to

70   assimilation of nutrients that may be affected in different manners by temperature changes. Generally,

71   the feed intake increases with increased temperatures up to levels close to the upper tolerance limits

72   (Fernández-Montero et al., 2018; Pérez-Casanova et al., 2009). Digestive enzyme activity has been

73   traditionally assessed in two ways. On one side, *in vitro* experiments for the enzyme characterization

74   performed with enzyme extracts show that activity increases with increasing temperature usually up

75   to values exceeding those representative of their natural habitats, and also beyond lethal levels

76   (Alarcón et al., 1998; Fernández et al., 2001; Gelman et al., 2008; Tanji et al., 1988). On the other hand,

77   information about digestive enzyme activities analyzed in live fish at different temperatures is also

78   available (Bowyer et al., 2014; Hani et al., 2018; Mazumder et al., 2018; Miegel et al., 2010; Sharma et

79   al., 2017). However, these studies are based on only one sampling point during the postprandial

80   response and, also report contradictory responses among the different studied species.

81   Gut evacuation rate also increases at increasing temperatures up to a certain limit, leading to lower

82   residence time in the digestive tract (De et al., 2016; Fernández-Montero et al., 2018; Handeland et

83   al., 2008; Temming and Herrmann, 2001). However, the estimation of evacuation rate has usually been

84   performed under unrealistic feeding conditions in which the fish has been refed until satiation after a

85   starvation period. Digestion efficiency will depend on the relation between enzymatic activity and gut

86   transit time that are not short punctual facts but long dynamic cyclic processes usually occurring along

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

88   information.

89   Other species-specific digestive characteristics may also strongly affect the digestion process. That is

90   the case of the gut luminal pH that conditions the activation of proenzymes in the gut, which may vary

91   among fish, particularly within the stomach (Bucking and Wood, 2009; Papastamatiou and Lowe, 2005;

92   Papastamatiou et al., 2007; Secor and Carey, 2016; Yúfera et al., 2004, 2007, 2012; Solovyev et al.,

93   2018). Optimum temperature for growth does not necessarily coincide with maximum feed intake,

94   highest digestion efficiency or optimal feeds utilization. In fact, from the point of view of aquaculture,

95   the aim is to optimize all these factors to obtain the better juvenile quality and weight gain at a

96   reasonable cost and with the lowest environmental impact.

97   Cobia (*Rachycentron canadum*), is a fast-growing species inhabiting tropical and subtropical waters

98   with a broad geographical distribution over several continents. The species may reach up to 60 Kg and

99   has a high-quality white flesh, being considered an excellent marine fish for aquaculture. It is being

100   produced mainly in Asian-Pacific coast and in a lesser extent in the Gulf of Mexico with a world

101   production above 40.000 tons during the last years (FAO, 2018; Tveteras, 2016). In Vietnam, it is one

102   of the main marine fish in large scale commercial aquaculture (Nhu et al., 2011). In South Vietnam and

103   other Southeast Asian regions water temperature in ponds and tanks ranges between 27 and 30 °C,

104   but may reach up to 36 °C during the daytime in the warmer season. For this reason, it is necessary to

105   understand the responses to increased temperatures, particularly in this region in the scenario of

106   global warming (IPCC 2015). Effect of rearing temperature on growth in cobia juveniles has been

107   examined by Sun and Chen (2009, 2014). In these studies, cobia juveniles were reared in the rage 20

108   to 35 °C and highest growth rates were observed in the range 27-31 °C. An unsolved question is how

109   these temperature changes affect the digestion process.

110   Therefore, in this study we have examined the effects of temperature on the digestive function from

111   a global perspective in order to advance in the physiological basis and mechanisms behind digestive

112   efficiency and the corresponding effects on growth in cobia juveniles. Specifically, the aim of this study

113   was to elucidate whether a temperature increase from 30 to 34 °C affects gastrointestinal pH,

114   enzymatic proteolytic digestive activity and feed transit over the whole day period, in early juveniles

115   of this fast growing fish.

116

117   **Material and Methods**

118   *Fish rearing and sampling*

119   This study was part of a larger experiment examining growth performance in juveniles fed three

120   different diets (Nguyen et al., 2019). We used the tanks from control treatment for the present study.

121   Cobia juveniles were obtained from a local hatchery in Nha Trang, Vietnam, and acclimatized to final

122   experimental temperatures in two indoor fiberglass 5000-L tanks at Nha Trang University facilities

123   during one week. During the period of acclimatization, water temperature in one tank increased at a

124   rate of 1°C per day up to 34°C, while temperature in the other tank was kept constant at 30°.

125   Acclimatized juveniles with 3.7 ± 0.4 g wet body weight, were randomly distributed to 6 experimental

126   200-L tanks (60 fish tank^{-1}) and reared under a light/dark cycle at two temperatures (30 and 34 °C,

127   three tanks for each temperature) in recirculation systems. Water salinity was 29.0 ± 3.1 g L^{-1}, pH 7.8–

128   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

129   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

130   common procedure in the local hatcheries (Nguyen, 2013; 2014). The experimental diet was produced

131   at SPAROS Lda (Olhão, Portugal) containing 47% protein and 10% lipid (Table 1). The fish were fed to

132   nearly satiety (until most of the fish losing their appetite) by hand. Eventual uneaten feed and removed

133   fish were recorded for the calculation of daily feed intake and feed conversion ratio, parameters

134   considered in the companion study (Nguyen et al., 2019). After 2 weeks under these conditions, 3 fish

135   per tank (wet body weight: ca. 6-8 g range) were sampled every 4 hours during 24 hours, for gut pH,

136   digestive enzyme activity and feed transit determinations. Dissected gut were freeze-dried and sent to

137   Spain for the analyses of enzyme activities at the University of Almeria and the gut transit at the

138   ICMAN-CSIC. All experimental procedures complied with the Guidelines of the European Union Council

139   (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved

140   by the Spanish National Research Council (CSIC) bioethical committee.

141   *Measurements of gut pH*

142   The gastrointestinal pH was measured in nine individuals at each sampling point and for each

143   temperature condition immediately after sampling using a pH microelectrode (Thermo Orion, Thermo

144   Fisher Scientific Inc) following the procedure described in Yúfera et al. (2012). In short, the fish were

145   dissected to make the digestive track accessible. Next, the tip of the microelectrode (diameter 1.7 mm)

146   was inserted in small slits made in the stomach, anterior intestine, medium intestine and posterior

147   intestine (Fig. 1).

148   *Digestive enzyme activity analyses*

149   The complete digestive tract of three individuals of each sampling point and temperature were

150   dissected, immediately frozen at -80 °C and later freeze-dried. Enzyme extracts were prepared for

151   enzyme activity measurement from these samples. Stomach and intestine samples were dissected and

152   homogenized separately. Samples were manually homogenized in 3 mL distilled water and centrifuged

153   for ten minutes at 4 °C at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from

154   the stomach samples were measured for pepsin activity, and the supernatants from the intestine

155   samples were analyzed for trypsin activity.

156   Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1

157   mL of 0.5% acid-denatured bovine hemoglobin diluted in 0.2 M HCl-Glycine buffer. Assays were carried

158   out at the specific gastric pH determined in each sampling point. In this way we are determining

159   actually activated pepsin instead of pepsinogen (Yúfera et al., 2012). After incubation at 25°C for 30

160   minutes, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 °C

161   for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes. The absorbance of the resulting

162   supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the

163   reaction mixture just after the TCA. Trypsin activity was determined using BAPNA (N-benzoyl-DL-

164   arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate at 25°C. 0.5 mM BAPNA was dissolved in

165   1mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50mM, pH 8.5, containing

166   20 mM $CaCl_2$. Reactions were started in 96-well microplates by the addition of 15 µL of the enzyme

167   extract to 200 µL of the respective substrate and liberation of p-nitroanilline was kinetically followed

168   at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA). The activities were

169   provided as units per weight unit of fish to prevent the variability due to gut content.

170   *Gut content and feed transit time measurements*

171   The feed content in the stomach and the intestine was estimated from their respective weight

172   determined in the gut samples used for enzyme and transit determinations. Average of empty guts

173   were subtracted from these values. The values were normalized as dry weight of feed content per g of

174   wet body weight (BW) to account for size differences among individuals.

175   For feed transit assessment, the day of the sampling, the feed labeled with containing of Yttrium oxide

176   (200 mg Kg^{-1}) was provided for the first meal (8:00 h) while the standard feed without the marker was

177   provided in the second meal (16:00 h). Yttrium content within the gut was analyzed at the ICMAN

178   (Spain) by inductivity-coupled plasma mass spectroscopy (Thermo Scientific iCAP Q ICP-MS) separately

179   in the stomach and the intestine of three individuals collected at each sampling point and temperature.

180   Two technical subsamples were performed for each analysis. Yttrium content, normalized as mg g^{-1} of

181   fish BW was plotted as a function of time.

182   The residence time of ingested feed within the gut were estimated as the period of time from when

183   half of the stomach or intestine were filled with the marked feed to when half of the corresponding

184   section was emptied. Previously, in order to calculate the total amount of feed accessing the stomach

185   in the first 4-h period, the total amount of yttrium in the stomach and intestine were considered,

186   assuming that most of offered feed was ingested during the first hours of feeding. The yttrium content

187   in each sampling point was converted to percentage of maximum measured in each section and

188   temperature. The partial transit rates for each inter-sampling period were calculated as the difference

189   of the relative fullness percentage between two consecutive time-points (considering the absolute

190   values). Results have been presented as percentage of the maximum measured capacity entering or

191   leaving each compartment for each 4h-period.

192   *Statistical analyses*

193   Two-way analysis of variance (ANOVA) was used to compare differences between the postprandial

194   time and temperature for each section of the digestive tract. A post-hoc Tukey honest significant

195   difference (HSD) test was used when ANOVA results revealed significant differences ($P < 0.05$). The

196   homogeneity of variances was previously tested using Levene's test, and all parameters expressed as

197   percentages were subjected to arcsin square root transformation. Data are presented as the mean of

198   nine or three replicates ± sem. All statistical tests were performed in IBM SPSS Statistics 18 software

199   (IBM Corp., USA).

200

201   **Results**

202   The pH within the stomach was permanently acidic with mean values ranging from 2.76 to 4.74 (Fig.

203   2) although a significant increase ($P < 0.05$) was observed after each meal. The two way-ANOVA suggest

204   that the gastric pH values change during the daily cycle but not in relation to temperature ($P > 0.05$). On

205   the other hand, the intestinal pH ranged from 6.05 to 7.69 (Fig. 3). An increase was observed after the

206   first meal and this slight alkaline condition was maintained for several hours before declining to neutral

207   or slightly acidic values at the end of the day ($P < 0.05$). Furthermore, the maximum measured pH values

208   were progressively higher when moving from proximal to distal part of the intestine ($P < 0.05$). A slightly

209   higher acidity was observed in the anterior and medium sections of the intestine at 34°C ($P < 0.05$).

210   Pepsin activity showed a daily rhythm at 30 °C with a maximum in the middle of the light period and a

211   minimum at the beginning of the dark period, while at 34°C some hourly changes with a decrease after

212   each meal were observed, but without so clear daily trend during the 24-h period. (Fig. 4). The trypsin

213   activity exhibited a clear daily rhythm at both temperatures with a patent increase after morning

214   feeding to reach a maximum several hours later (Fig. 4). Overall the activity of pepsin during the daily

215   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

216   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 °C was longer in the stomach, while at 34 °C it was longer in the intestine.

250

Discussion

252 The present study was performed in parallel with other study analyzing growth and feed conversion
253 ratio differences in the same batch of juveniles fed different diet formulations at these two
254 temperatures for six weeks (Nguyen et al. 2019). In that report, we found that cobia reared at 30°C
255 grew faster and showed a more favorable feed conversion ratio than those at elevated temperature
256 (34°C), being in both cases fed the same daily ration. We used the control tanks for the present study
257 on digestion. The sampling was intentionally performed two weeks after the start of the experiment
258 to determine the digestion status in the middle of the growth experiment, when both feed intake and
259 growth were assessed to be high. Cobia is a voracious carnivorous fish with a large stomach and a short
260 intestine (Fig. 1). This species feeds on small fish, crustaceans and squids (Franks et al., 1996). These
261 feeding habits and gut anatomy have consequences in the mode of digestion as we observed in the
262 different parameters examined. It seems evident that the stomach is very important for digestion and
263 maybe more so than the intestine in this species, based on their respective volumes, luminal ionic
264 values, proteolytic activities and transit rates results. Most research on postprandial response in fish
265 has been done considering only one morning meal in order to examine the results without
266 interferences of subsequent meals. In our study we considered two meals according to the customary
267 use in hatcheries for this species in this region (Nguyen, 2013). With this feeding protocol we found
268 more realistic overview of the digestive function but with additional complications to interpret results
269 because fish may change the feeding behavior and daily digestive patterns when have the possibility
270 to choose among different daily meals (Montoya et al., 2010; Yúfera et al., 2014).

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

9

284   less common or we need still to explore more species, particularly those with strict carnivorous feeding

285   habits, to get a more complete figure of the acidification strategy in teleosteans. To maintain a neutral

286   gastric environment during fasting has been associated to infrequent feeding in snakes and sharks

287   (Papastamatiou and Lowe, 2005; Secor et al., 2012), but in teleostean with daily feeding habits this

288   rule remains uncertain. In fact, an erratic daily feeding by changing randomly the moment of feed

289   delivery every day may also alter the daily pattern from neutral/acid alternation to permanent

290   acidification in gilthead seabream *Sparus aurata* (Montoya et al., 2010). A constant low gastric pH

291   enables this voracious species to be always ready to activate pepsinogen to start the hydrolysis of the

292   ingested prey. The small increase of gastric pH after meals has been attributed to the dilution effect of

293   the ingesting feed, possibly in parallel with some water; drinking water for osmoregulatory purposes,

294   as well as to the buffering capacity of feeds (Márquez et al., 2012) and also the buffer capacity of the

295   slightly alkaline seawater itself.

296   We found that increased temperature did not affect to gastric pH but it led to decreased luminal pH in

297   the anterior intestine and to a lesser extent the mid intestine. This effect is probably related to higher

298   transit rates observed at 34 °C in which the acidic chyme pass quickly to the short intestine. A similar

299   effect on the intestinal pH was described at increased temperature in channel catfish *Ictalurus*

300   *punctatus* (Page et al., 1976). The same effect in the anterior intestine was also detected in other

301   species when feed only one meal (Bucking and Hood, 2009; Rosero, 2013; Yúfera et al., 2014). A

302   decrease of the intestinal pH has been reported for different freshwater species associated to seasonal

303   increased temperatures (Solovyev et al., 2018). The authors explained this decrease as an adaptation

304   to enable fish to regulate and optimize the activity of their digestive pancreatic enzymes. Our results

305   would indicate, that in addition, the changes in the water temperature alter the feeding behavior and

306   feed processing along the day.

307   Pepsin activity showed hourly variations along the 24-h period ~~cycle~~ although a clear daily rhythm was

308   observed only at 30 °C. Considering that the minor variations of gastric pH are practically no affecting

309   to pepsinogen activation as reported in other species (Yúfera et al., 2012), these changes should be

310   interpreted in relation to the amount of substrate. The lower activity practically coincides with the

311   higher amount of digesta in the stomach (Fig. 4) that is consuming the active enzyme. Contrary to this,

312   the pancreatic trypsin activity exhibited a daily cycle with the expected increase associated with the

313   intestinal alkalization when the chyme is released from the stomach. Such daily pattern has been

314   already reported in other fish species (Rosero, 2013; Yúfera et al., 2014). It is also interesting to note

315   that the proteolytic activity in the stomach was much higher than in the intestine. We found that the

316   proteolytic activity of pepsin and trypsin was hardly affected by the 4 °C increase of temperature, at

317   least when the standard incubation temperature were used for the analytical protocols. It could be

318   interesting to explore analytical methodologies adapted to different temperatures. Miegel et al. (2010)

319   did not find differences of intestinal proteases activity in fed individuals of yellowtail kingfish *Seriola*

320   *lalandi* maintained at 12.6 and 20.8 °C. However, Bowyer et al. (2014) found higher tryptic activity at

321   intermediate temperatures in the range 21 to 27 °C in starved individuals of the same species. Similar

322   results were observed by Hani et al. (2018) in starved threespine stickleback *Gasterosteus aculeatus* in

323   the range 16 to 21 °C, by Sharma et al. (2017) in Indian major carp *Catla catla* in the range 10 to 35 °C,

324   as well as by Zhao et al. (2009) in the range 20 to 32 °C in Chinese longsnout catfish *Leiocassis*

325   *longirostris*. On the other hand, Mazamder et al. (2018) reported higher pepsin activity at 30 °C in the

326   range 22 to 34 °C in fasted Malabar blood snapper *Lutjanus malabaricus*. Comparison of these results

327   is difficult due to differences in fish size, experimental protocols and analytical methods, and in

328   addition, the fish for those analyses were collected at only one time and any postprandial patterns was

329   not explored. In our study and with independence of the similarity of the global daily averages of the

330   lytic activity, the postprandial patterns showed maximum and minimum values that are not coincident

331   at both temperatures. These results indicate that a single daily sample is not enough to characterize

332   the enzymatic activity under different temperature conditions. Such data must be interpreted in

333   relation to gut content as mentioned above but also in relation to transit results.

334   Transit rate assessment is a challenging task when more than one meal is offered. The postprandial

335   responses overlap and the patterns are harder to interpret. A key factor in our study is to recognize

336   that some ingested feed may pass to the intestine before the second sampling (Figs. 4 and 5) and

337   therefore the estimation of the ingestion during the first period should include both sections. The gut

338   content on weight basis gives only indicative information because it is representing the balance

339   between digesta input and output. To obtain a more complete information it is necessary to estimate

340   the temporal rates for the gut filling and evacuation under this feeding protocol in each gut

341   compartment. An interesting result has been to verify whether the transit velocity of digesta

342   throughout the digestive tract is changing along the daily cycle (Fig. 6), something perhaps obvious but

343   never examined in fish. Thus, the transit rates were maxima during 8 h after feeding at 34 °C. In this

344   period the whole stomach volume was filled during the first 4-h period and emptied in a great part

345   during the following 4 h before the next meal. The rest of the day the transit of remaining chyme was

346   notably slower. At 30 °C the transit rates during the first hours were significantly lower than at 34 °C

347   and the posterior decrease was smoother and not statistically significant. In the intestine no effect of

348   temperature was observed and the transit was relatively fast during the first 12 h during which most

349   part of the first meal is evacuated, the remaining digesta moved at notably slower rate. In our study,

350   the second meal was not labeled and therefore these transit rates are only referring to the first meal

351   when a second meal is pushing 8 h later.

352 Evacuation rates have been determined in many species usually based on fish with the stomach already

353 full and without further feeding, such an approach gives an incomplete understanding of transit time

354 in the stomach but in many cases the pass of the digesta through the intestine was properly assessed

355 (Adamidou et al., 2009; Bonvini et al., 2018). While the evacuation of the stomach may last less than

356 one day, the evacuation of the intestine may last 36 to 48 h. These values are only indicative for median

357 sized farmed fish with daily feeding. Different factors such as feeding frequency, ration size, feed

358 quality, body size and water temperature have been described to affect transit time in fish (Miegel et

359 al., 2010), particularly the last one (De et al., 2016; Fernández-Montero et al., 2018; Handeland et al.,

360 2008; Temming and Herrmann, 2001). According to these studies, transit time increases with the

361 temperature except at extremely high values. Our results however showed an increase at very high

362 temperatures that is probably close to tolerance limit.

363 Probably the most useful information is the time the digesta spent within the different sections and

364 being hydrolyzed by the corresponding digestive enzymes. In routine feeding, the ingested feed is

365 mixed up with the feed of the previous and the next meal(s) and its complete evacuation from the gut

366 may last longer than expected due to the residual amount that can be detected for many hours, even

367 days, later than most part of the digesta was evacuated. The criterion explained above allows an

368 estimation of the residence time that can be compared between compartments and temperatures

369 (Fig. 7). The most evident result is that the residence time was shorter at 34 °C. The increase of 4 °C

370 induced a faster filling and evacuation in the stomach as commented above but also a lower residence

371 time, that was less than half of the period at 30 °C. In the intestine the effect was not as dramatic but

372 the reduction of digesta residence time was still important. The lower period of time for the proteolytic

373 work of the digestive proteases brings on lower dietary protein utilization and is one of the reasons for

374 the lower weight gain and higher feed conversion ratio observed at 34 °C (Nguyen et al., 2019).

375 Furthermore, a relevant aspect is that the first meal transited almost simultaneously ~~than~~ in the

376 stomach and intestine, when certain temporal displacement would be expected as determined in

377 other species (Bonvini et al., 2018). The ingested pellets of the morning meal in our experiment passed

378 directly into the intestine, and this segment was filled almost at same time as the stomach, working

379 more like an extension of the stomach than like a different digestive tract compartment.

380 Unfortunately, our experimental protocol does not allow to evaluate the transit time of the second

381 meal that not necessarily may follow the same pattern but that we can assume it is similar to that of

382 the first meal. It is likely that the feeding protocols for the voracious and carnivorous cobia in

383 aquaculture where pelleted feed particles are offered in large amounts results in a digestive process

384 that is progressing differently from nature where cobia ingest larger and intact prey. However, given

385 the artificial feeding conditions in aquaculture, it is important to understand how the digestive system

386 that is evolutionary adapted to natural conditions perform under different feeding regimes.

387   In summary, the present results indicate that at 34 ℃, a subtle increase of proteolytic activity cannot

388   compensate for the faster gut transit rate. The reduced time the dietary proteins are available for

389   hydrolysis when compared with fish maintained at 30 ℃ can explain the lower growth observed at this

390   temperature (Nguyen et al. 2019). Another reason for the lower growth could be an unfavorable

391   energetic balance at the higher temperature but the studies by Sun and Chen (2009, 2014) showed no

392   evident variations of the feed energy allocated to metabolism in the range 27-33℃ in cobia juveniles

393   of the same weight range, although it was higher at 35 ℃. Furthermore, this study shows a general

394   appraisal of digestion in the 24-h temporal horizon as correspond to a daily feeding protocol,

395   demonstrating the importance of observing inter-hourly changes in the different digestion parameters

396   to characterize the digestive potential under given temperature conditions.

397

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534

Figure captions

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

538

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

542

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

547

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

553

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

556

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

559

560 Fig. 7. Partial transit rates of digesta for each inter-sampling period (4 h) in the stomach and intestine
561 of *R. canadum* juveniles at the two experimental temperatures. Results are presented as percentage
562 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
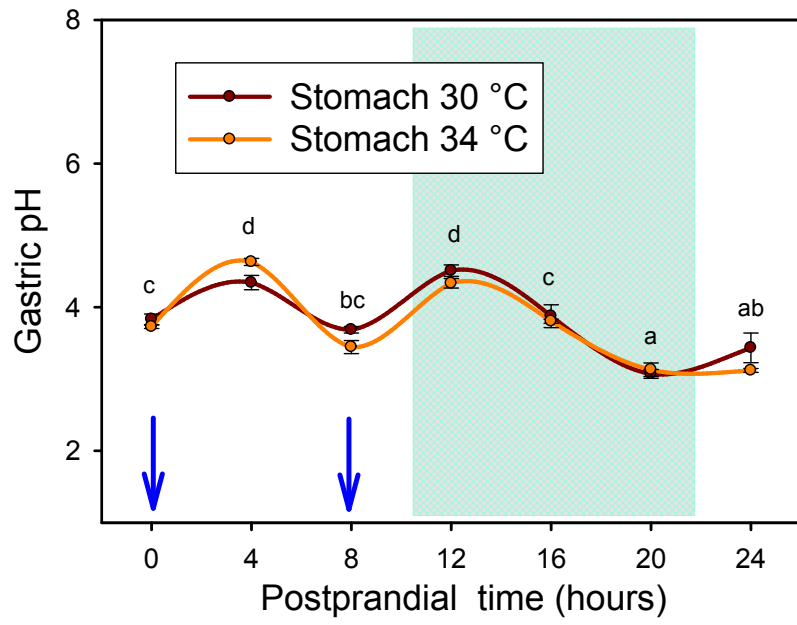
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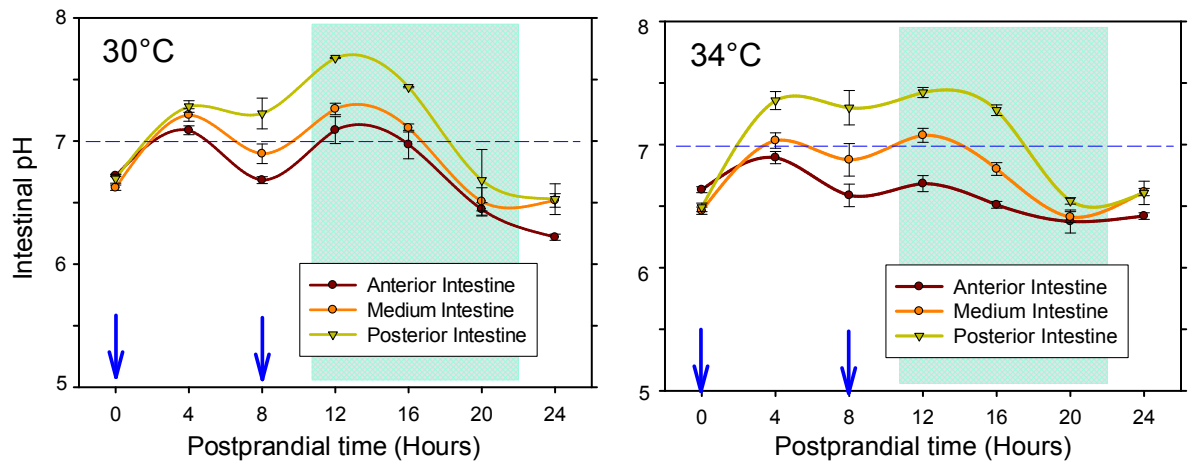
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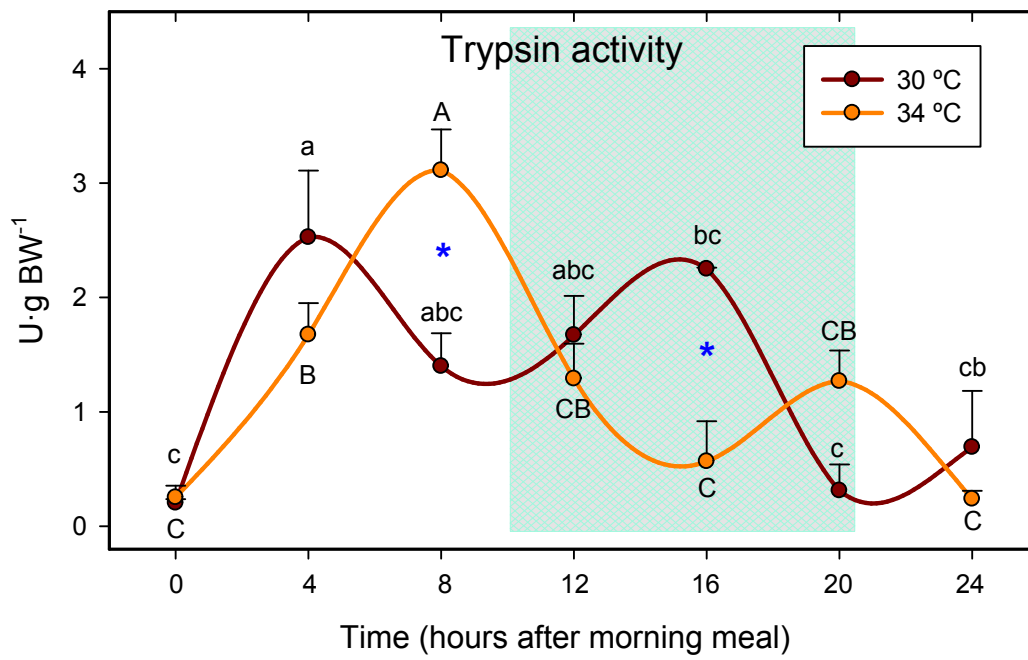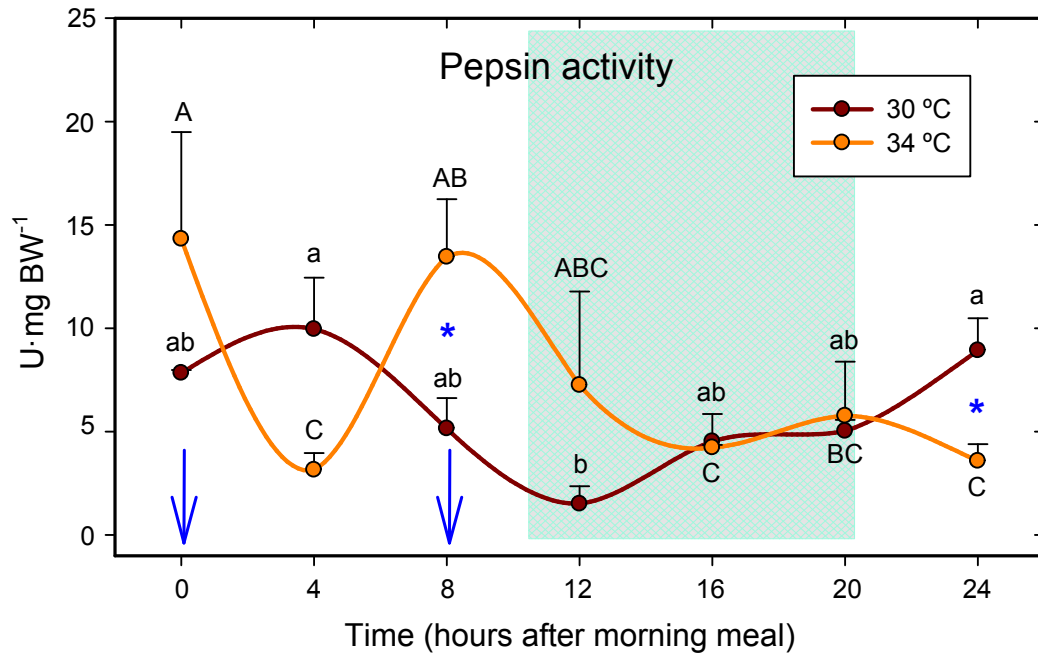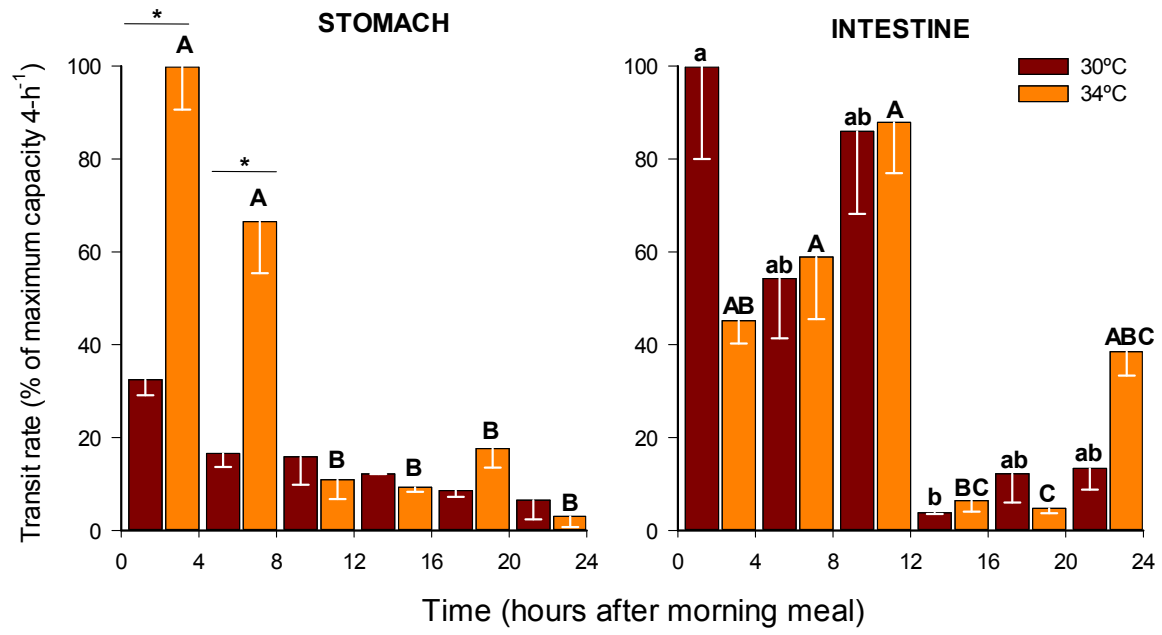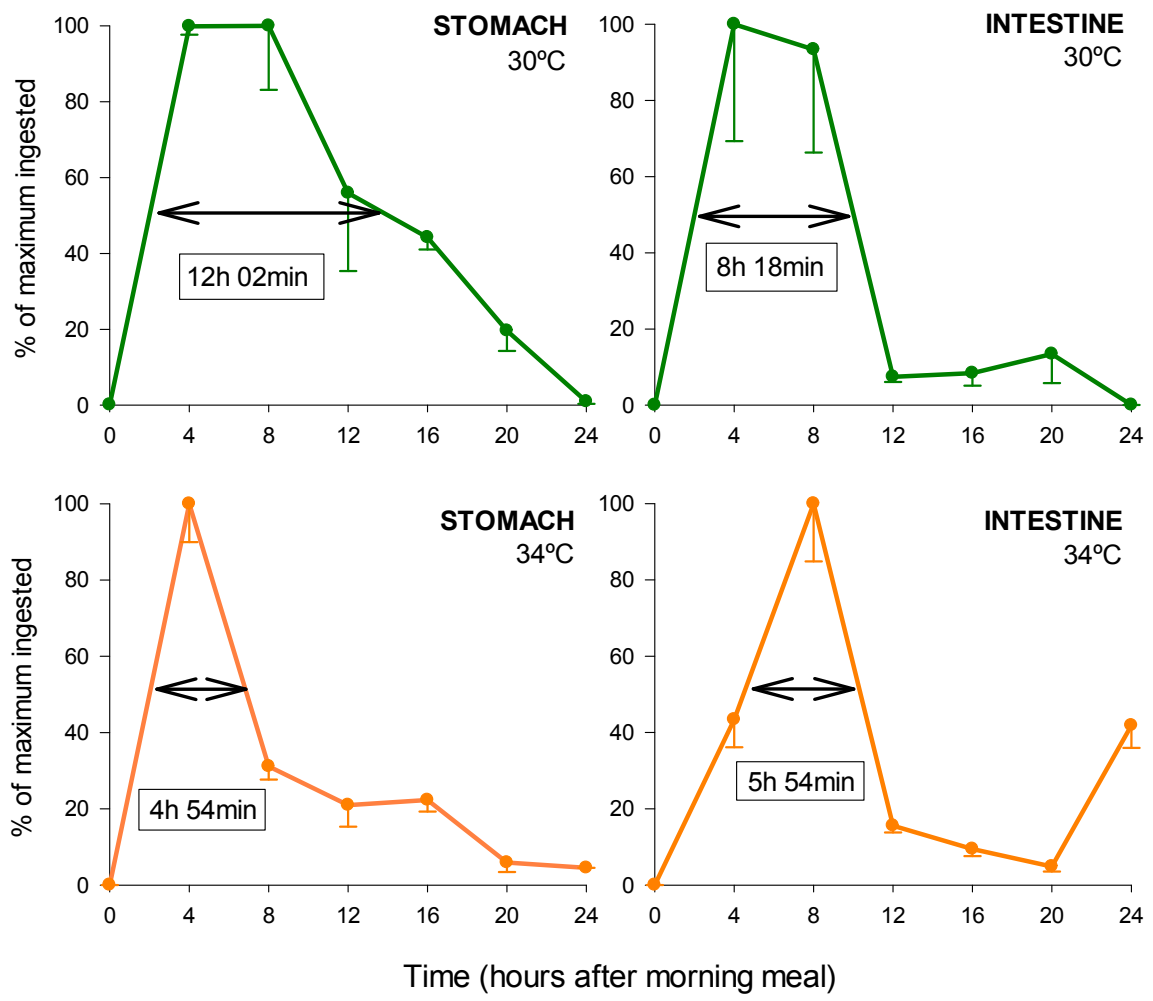
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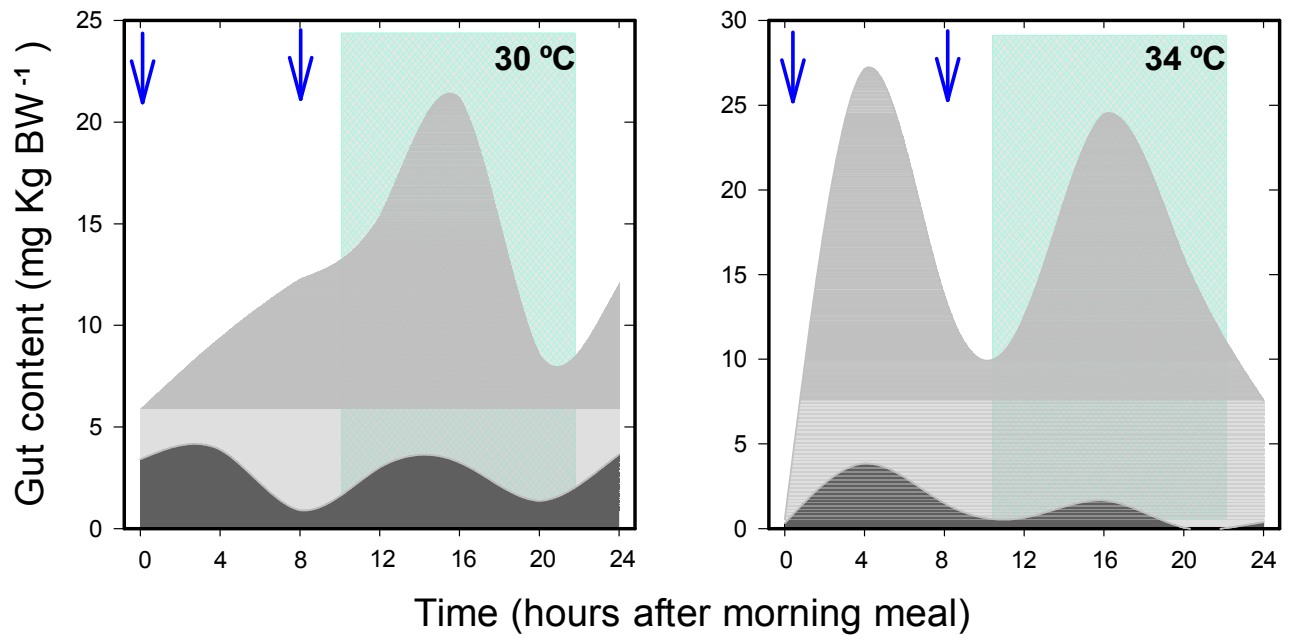
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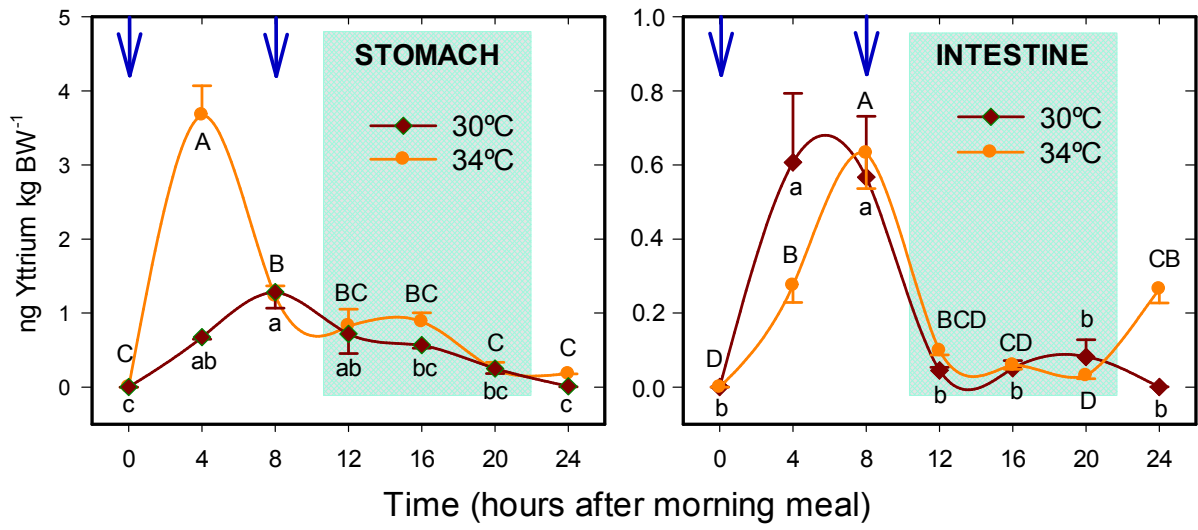
Fig. 6

Fig. 7

Fig. 8.

Conflicts of interest

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