



Gut transit of daily consecutive meals in greater amberjack juveniles reared at different temperatures

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ABSTRACT

Feed passage time throughout the digestive tract of three daily consecutive meals containing three different inert markers respectively and the gut luminal ionic conditions generated during a daily cycle have been examined in juveniles of greater amberjack (*Seriola dumerili*) growing at 18, 22 and 26 °C of water temperature. As expected, transit time tended to decrease with increasing temperature in the tested range. However, each meal progressed in a different manner affecting the ionic daily pattern within the tract lumen and the residence time within the different gut segments, two parameters of primary importance for the digestion efficiency. At 22 and 26 °C the gastric acidification effort was focused on the first meal whilst at 18 °C it was on the second and third meal. Intestinal alkalisation was maintained during more hours at 22 °C than at 18 °C and 26 °C coinciding with the moments of highest chyme content in the intestine. At 26 °C, the three meals transited fast enough to progress clearly as separate batches. On the contrary, at 18 and 22 °C the consecutive meals became mixed in the stomach and intestine. Transit time was progressively shorter from the first to third meals at 18 and 22 °C, while at 26 °C it was shorter for the second meal and similar for the first and third meals when 95% total gut evacuation was considered. Total transit time ranged from 12 to 24 h approximately and the posterior intestine was completely emptied before the first morning meal in all cases. Interestingly, the residence time of ingested food in the middle intestine was longer at 22 °C than at the other two temperatures.

1. Introduction

The efficiency with which the on-growing fish feeds are digested is a key factor determining the feed conversion ratio and, therefore, the profitability and environmental cost of the production process (Dias et al., 2010; Piedecausa et al., 2010; Wu et al., 2019). Feed quality, namely physical-chemical structure and biochemical composition, as well as luminal environmental conditions within the digestive tract, mainly pH and temperature, are well-known factors influencing the rates at which the nutrients are hydrolysed and absorbed (Fountoulaki et al., 2005; Adamidou et al., 2009; Gilannejad et al., 2018; Mazumder et al., 2018; Volkoff and Rønnestad, 2020; Murashita et al., 2021). Other decisive factor determining the digestion efficiency is the way in which the ingested feed transits throughout the digestive tract, and more specifically, the residence time of the digesta within the different gut segments under the action of the different digestive enzymes

(Gilannejad et al., 2019). Furthermore, luminal pH may vary in relation to temperature and transit rate (Solovyev et al., 2018; Yúfera et al., 2019) affecting consequently both digestion (Yúfera et al., 2012) and peptides absorption (Con et al., 2017).

Gastrointestinal transit time of the ingested food may vary in relation to feed quality and feeding frequency (Bromley, 1987; Jobling, 1987; Riche et al., 2004; Aas et al., 2017; Ballester-Moltó et al., 2017; Moutinho et al., 2017; Bonvini et al., 2018), but probably water temperature is one of the factors inducing more drastic changes in the feed transit and gut evacuation (Jobling, 1980; Temming and Herrmann, 2001; Handeland et al., 2008; Miegel et al., 2010; De et al., 2016; Das et al., 2018; Gonçalves de Sandre et al., 2016; Fernández-Montero et al., 2018; Nakagawa, 2018; Mazumder et al., 2020; Kounna et al., 2021). Different methodologies have been utilised to measure the gastrointestinal transit in fish. In general, it has been mostly estimated from the evacuation of a single meal after a previous starvation period. In many cases, only

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gastric evacuation was measured (Hop and Tonn, 1998; Temming and Herrmann, 2001; Das et al., 2018; Yokoyama et al., 2020), although residence time in the intestine was also provided in some studies (Adamidou et al., 2009; Dias et al., 2010; Nikolopoulou et al., 2011; Aas et al., 2017; Bonvini et al., 2018).

Nevertheless, it is necessary to consider that farmed fishes are usually fed several times per day. The estimation of gut transit time under such routine feeding schedule during on-growing, able to better reproduce the actual conditions for digestion, is a more laborious task scarcely addressed (Storebakken et al., 1999; Sveier et al., 1999; Riche et al., 2004; Gonçalves de Sandre et al., 2016; Gilannejad et al., 2021). But, in addition, in a scenario of multiple meals per day, it is also necessary to elucidate whether or not successive meals are processed in similar way or, if by contrast, their transit and digestion differ from each other. This is an important aspect that has not been examined before.

Greater amberjack (*Seriola dumerili*) is a marine fish species with circumglobal distribution inhabiting from temperate to tropical areas preferring temperatures from 19 to 28 °C approximately (Nakada, 2000; Fishbase, 2021; Aquamaps, 2021). This is a species of great interest for aquaculture industry worldwide (Sicuro and Luzzana, 2016) due to its fast growth and excellent flesh quality, currently being farmed in the Mediterranean and Eastern Asian countries. This species exhibits a voracious appetite and needs to be feed several times a day during the juvenile stage to maintain high growth rates and to prevent excessive aggressiveness. Feeding studies in greater amberjack have mainly assessed the effects of diet quality on growth performance (Jover et al., 1999; Takakuwa et al., 2006; Papadakis et al., 2008; Tomas-Vidal et al., 2008; Uyan et al., 2009; Monge-Ortiz et al., 2018a, 2018b; Bordignon et al., 2020; Yokoyama et al., 2020). However, the digestive processing of ingested feed has been scarcely studied, mainly focusing on digestive enzymes at early stages (Navarro-Guillén et al., 2019; Pérez et al., 2020; Gamberoni et al., 2021), and on the digestibility assessment of experimental on-growing diets (Tomás-Vidal et al., 2019; Yokoyama et al., 2020). On the other hand, gut transit of a single meal has been studied by Fernández-Montero et al. (2018).

A first step for understanding in what way successive meals are being processed within the digestive tract in on-growing juveniles is to determine their respective transit times. We hypothesise firstly, that the passage time of the digesta throughout the digestive tract varies among the different meals supplied during the day affecting therefore the efficiency with which each meal is digested. Secondly, that water temperature may change not only the transit time of digesta but also the gut transit pattern of the successive meals. Therefore, in the present study, we have examined separately the progress throughout the digestive tract of three different daily meals containing three different inert markers in greater amberjack juveniles growing at three different water temperatures. The final aim was to elucidate what are the luminal conditions and circumstances that would determine the efficiency at which successive meals are digested in the gastrointestinal tract. This knowledge could help to optimize the on-growing feeding practices in fish farming.

2. Materials and methods

2.1. Fish rearing and experimental design

Fish were provided by Futuna Blue España SL and were maintained at the ICMAN experimental facilities (REGA ES110280000311). Juvenile fish ($n = 90$, 34.32 ± 15.54 g of body weight) were randomly distributed into three RAS units, each one with three 900-L tanks. Each RAS unit was maintained at one of the three experimental temperatures (18, 22 and 26 °C). This is the prevalent range of temperatures in the Mediterranean and North Atlantic areas in which this species is farmed. Fish were acclimatised from 22 °C to final experimental temperatures along one week. In all tanks, the water salinity was $34 \text{ g}\cdot\text{L}^{-1}$, and the pH ranged between 7.8 and 8.0, oxygen saturation was maintained above 87% and $\text{NH}_3 < 0.03 \text{ mg}\cdot\text{L}^{-1}$. The photoperiod was adjusted to 12 h light

and 12 h dark (illumination period from 08:00 to 20:00 h). Fish were fed by hand until apparent satiation three times a day during the light period (at 8:00, 12:00 and 16:00 h), usually it took <5 min. Three experimental diets with the same formulation but including three different inert markers respectively, yttrium oxide (Y_2O_3), lanthanum oxide (La_2O_3) and ytterbium oxide (Yb_2O_3), were utilised (Table 1). Diet marked with yttrium was offered at 8:00, the diet with lanthanum at 12:00 h and the diet with ytterbium at 16:00 h. The experimental diets (extruded pellets with diameter 2 mm) were manufactured by SPAROS Lda (Olhão, Portugal) and were designed to be similar to the common commercial feeds for this species (Table 1).

2.2. Sampling and analytical procedure

Fish were maintained under the experimental conditions for three more weeks after acclimatization. At the end of the experiment, fish were sampled at 8:05, 10:05, 12:05, 16:05, 20:05, 00:05, 04:05 and 07:55 (three individuals per temperature at each sampling time). All samples coinciding with meal times were taken after the feed supply, except the sample taken at 7:55 that was performed just before the first morning meal. To prevent excessive stress, fish were randomly collected along 6 days at the different scheduled hours. Fish were anaesthetized (250 ppm), and then euthanized (600 ppm) by 2-phenoxyethanol overdose. After determination of body mass, the fish were immediately frozen for posterior analysis. 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 and Spanish National Veterinary Authority (REF: 02/07/2019/107).

2.3. Digestive tract pH measurements

Luminal pH within the gastrointestinal tract was measured in freshly thawed fish immediately after dissection in different sections of the gastrointestinal tract using a pH microelectrode with a tip diameter of 1.7 mm (Thermo Orion, Thermo Fisher Scientific Inc), following the procedure described in Yúfera et al. (2012, 2019). Briefly, the tip of the microelectrode was inserted in small slits made in the stomach, anterior intestine, middle intestine, and posterior intestine (Fig. 1). For the pH measurements, the room temperature was adjusted at the same experimental water temperatures ($\approx 18, 22$ and 26 °C). The microelectrode was calibrated before each fish measurement, using standard buffer

Table 1
Formulation and proximate composition of experimental diets (g per 100 g).

Ingredients	1st meal	2nd meal	3rd meal
Fishmeal LT70	40.0	40.0	40.0
Fish protein concentrate	3.0	3.0	3.0
Squid meal	5.0	5.0	5.0
Krill meal	3.0	3.0	3.0
Soy protein concentrate	6.0	6.0	6.0
Pea protein concentrate	7.5	7.5	7.5
Wheat gluten	9.0	9.0	9.0
Wheat meal	13.8	13.8	13.8
Vitamin and mineral premix	1.0	1.0	1.0
Antioxidant	0.2	0.2	0.2
Fish oil coating	11.5	11.5	11.5
Yttrium oxide	0.02		
Lanthanum oxide		0.02	
Ytterbium oxide			0.02
Crude protein, % feed	55.1	55.1	55.1
Crude fat, % feed	18.1	18.1	18.1
Crude fat (no oils)	6.7	6.7	6.7
Fiber, % feed	0.9	0.9	0.9
Starch, % feed	9.4	9.4	9.4
Ash, % feed	9.3	9.3	9.3
Gross Energy, MJ/kg feed	21.7	21.7	21.7

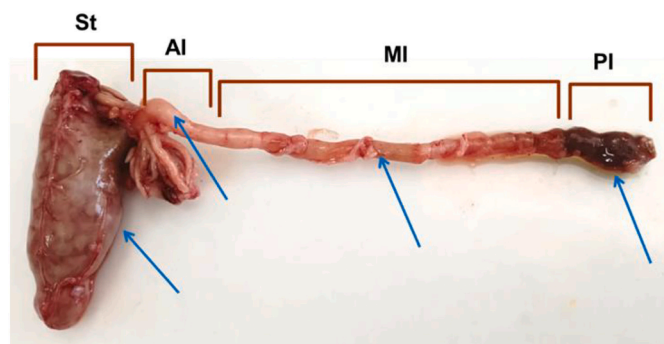


Fig. 1. Digestive tract of *Seriola dumerili* showing the different segment considered in this study. Arrows indicate the place the pH microelectrode were inserted. St: stomach; AI: anterior intestine; MI: middle intestine; PI: posterior intestine.

solutions at pH 4 and 7.

2.4. Feed transit throughout the digestive tract

To determine the filling and evacuation pattern of the digestive tract with the successive meals, sampled juveniles were thawed and the different tract segments (stomach, anterior intestine, middle intestine and posterior intestine) were dissected according to Fig. 1. Each segment (mucosa + gut content) was processed separately. Three individuals per treatment (temperature) at each sampling time were processed. The content of yttrium, lanthanum and ytterbium in each digestive tract section, as well as in the feeds, was measured using inductivity-coupled plasma mass spectroscopy (Thermo Scientific iCAP Q ICP-MS). For this purpose, approximately 100 mg of freeze-dried sample were digested in nitric acid at 95 °C during one hour. Two analytical replicates of the homogenized tissue were performed for each sample. Additionally, the relative daily ingestion for each meal (ng marker per mg gut) was estimated from the amount of the corresponding marker within the whole digestive tract (tissue + gut content) determined in the samples taken just after each meal. The actual marker concentration (g per 100 g of feed) analysed in the feeds ($Y = 0.018$, $La = 0.017$, $Yb = 0.018$) were utilised for all calculations.

Gastric evacuation rate of each meal was calculated from the decreasing curves of the marker content in the stomach, starting from the highest measured value in each case up to the value in the last sample of the daily cycle. We assumed that the decreasing trend follows an exponential decay pattern:

$$M_t = M_0 e^{-rt}$$

where, M_t is the gut marker content (yttrium, lanthanum or ytterbium; ng marker per mg gut) at time t after each meal, r is the gastric evacuation rate, and M_0 the initial marker content after each meal.

Average transit time of each meal was calculated as the time between the maximum content of marker in the stomach (corresponding with the meal time) and the maximum in the following gut segments. Total transit time was considered as the time between the maximum content of marker in the stomach and when only 5% of the maximum recorded in the posterior intestine remained to be evacuated.

Residence time of digesta in each gut segment was calculated as the time during which the marker content was equal or higher than 50% of the maximum amount recorded in the same segment (Gilannejad et al., 2019; Yúfera et al., 2019). Residence time was calculated over the daily pattern elaborated with the mean values at each sampling point.

2.5. Statistical analysis

Significant differences among final body mass and among total daily

relative ingestion at each water temperature were determined using One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test in case of significance ($p \leq 0.05$). Differences of relative ingestion among meals and water temperatures were determined using Two-way ANOVA, considering meal time and temperature as independent factors. Differences in luminal pH values among postprandial times and water temperatures were determined using Two-way ANOVA. Gastric evacuation rates were compared by F-test. All the data were tested for normality of distribution and homogeneity of variance before the analyses. Differences were considered statistically significant when $p \leq 0.05$. Data were visualized as mean \pm standard deviation (SD) or standard error of the mean (SEM).

3. Results

The body mass of individuals sampled at the end of the experiment was similar at the three temperatures (ANOVA, $p = 0.724$). The final body mass was 79.00 ± 33.70 , 82.29 ± 35.06 and 87.59 ± 39.21 g (mean \pm SD), implying a weight gain of 44.69, 47.97 and 53.27 g during the whole experimental period (including the acclimation period) for 18, 22 and 26 °C, respectively.

The relative total daily ingestion including the three meals, estimated from sum of marker content at each meal time, was progressively higher with the increase of temperature from 18 to 26 °C (One-way ANOVA, $p \leq 0.05$), although the values were not significantly different between 18 and 22 °C (Table 2). Regarding the marker amounts ingested in each meal, highest mean values were recorded in the third meal at 18 and 22 °C, while at 26 °C the ingestion mean values were similar in the first and third meals. The lower mean values were always observed in the second meal (Table 2). However, the two-way ANOVA revealed that neither the meal order nor temperature significantly affected the relative ingestion of each meal ($p > 0.05$).

Daily patterns of luminal pH in the stomach are shown in Fig. 2. The gastric pH declined after the first morning meal in all cases ($p \leq 0.05$), but while at 22 and 26 °C the minimum was detected 2 h after the first meal, at 18 °C the lowest value was recorded 8 h after the first meal. Then, the gastric pH recovered their initial resting values, close to 6, between 2 and 6 h later.

In the middle and posterior intestine (Fig. 3) the luminal pH was affected by both water temperature and postprandial time (Two-way ANOVA, $p \leq 0.05$). Although with a similar daily pattern, no effect of these two parameters were detected in the pH of the anterior intestine ($p > 0.05$). Lowest values (pH 6.5 to 6.9 approximately) were measured at the time of first meal (8:00 h local time; 0 h post-feeding), increasing afterwards although with different patterns at each temperature. In the middle intestine, the luminal alkalisation after the first morning meal occurred faster at 22 °C and slower at 18 °C. *maximum* mean values (above pH 7.0) were recorded between 12 and 16 h after first morning

Table 2

Total daily relative ingestion (ng marker \cdot mg gut⁻¹) and relative ingestion of each meal at the three tested temperatures (mean \pm SD).

Temperature	1st meal	2nd meal	3rd meal	Whole day $p = 0.026$
18 °C	91.60 \pm 21.52	52.02 \pm 89.91	126.34 \pm 110.64	269.97 \pm 4.94 ^b
22 °C	94.03 \pm 72.89	75.66 \pm 65.49	161.93 \pm 43.44	331.62 \pm 46.71 ^b
26 °C	202.36 \pm 7.60	73.14 \pm 105.12	212.44 \pm 121.08	487.94 \pm 48.32 ^a
Two-way ANOVA		Temperature $p = 0.557$ Meal number $p = 0.087$ Interaction $p = 0.793$		

Different superscript letters denote significant statistical differences of total daily ingestion among temperatures (One-way ANOVA, $p \leq 0.05$). No significant differences among meals at each temperature were detected (Two-way ANOVA $p > 0.05$).

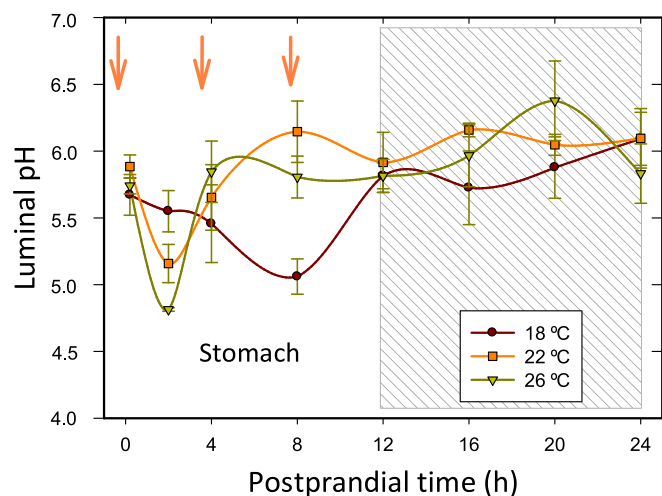


Fig. 2. Daily patterns of luminal pH in the stomach of juveniles maintained at the three tested temperatures. Means \pm SEM. Postprandial time indicates the hours from the first morning meal supplied at 8:00 h of local time. Arrows indicate the three feeding times. Dashed area indicates the dark period.

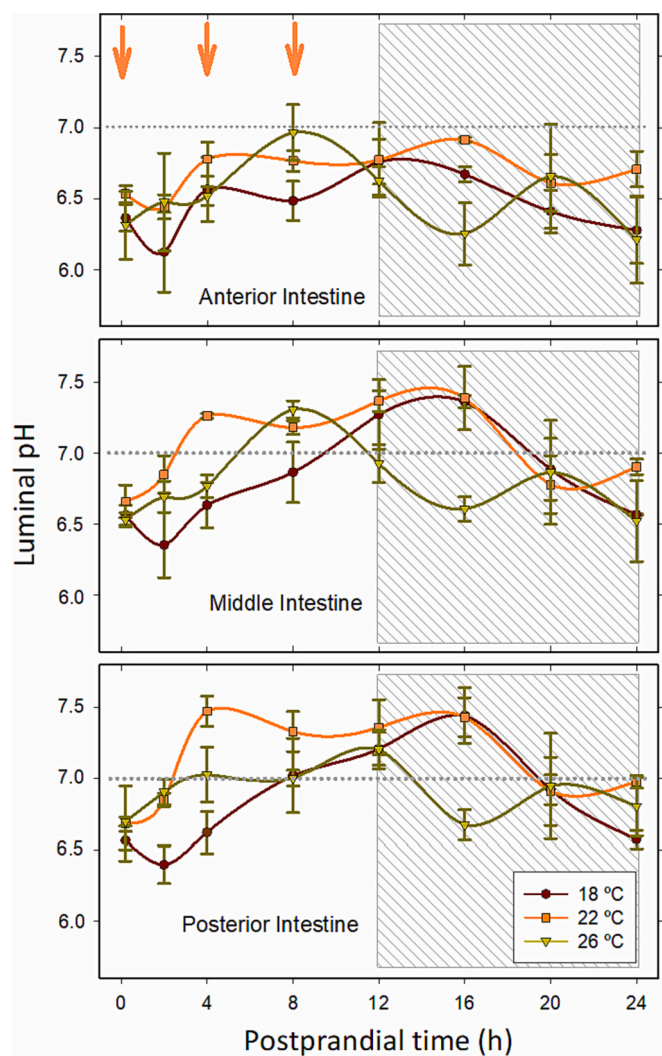


Fig. 3. Daily patterns of luminal pH in the intestine of juveniles maintained at the three tested temperatures. Means \pm SEM. Arrows indicate the three feeding times. Dashed area indicates the dark period.

meal in fish maintained at 18 °C, between 4 and 16 h post-feeding at 22 °C, and at 8 h post-feeding at 26 °C. Very similar patterns were recorded in the posterior intestine, except that the highest value at 26 °C occurred at 12 h post-feeding.

Overall, the values at in the anterior intestine were lower than in the other intestine segments, with averages below pH 7 at all sampling times, while in the middle intestine and the posterior intestine the maximum average values ranged between 7.2 and 7.4 approximately.

Transit pattern of the digesta through the different segments of the digestive tract is shown in Fig. 4. Fish immediately filled the stomach with each meal in all cases and then started the progressive gastric evacuation following somehow a decreasing exponential (Table 3). However, in some cases, the ingestion of the second and third meals altered this trend resulting in lower correlation coefficients and non-significant slopes (r) for the second meal. Global F-test comparison of gastric evacuation rate among all treatments evidenced a significant effect of both, meal number and temperature ($p \leq 0.05$). Specific comparisons for each meal and each temperature revealed significant differences for the first meal in relation to temperature and for the meal order at 26 °C.

The transit from one given gut segment to the next one is difficult to assess due to mixing of the different meals. Comparing the times at which the maximum marker content was reached it is possible to make some estimations. It can be observed that the transit from stomach to middle intestine occurred at progressively shorter time with the temperature increase from 18 to 26 °C. However, there is no clear trend when comparing the different daily meals. Thus, it was the third meal that reached faster the middle intestine at 18 °C (12 h, 12 h and 8 h for 1st, 2nd and 3rd meal, respectively) while at 26 °C it was the first meal (2 h, 4 h and 4 h, respectively).

Total transit time of each meal has been estimated considering the time elapsed between the moment of ingestion and the evacuation of the 95% of the maximum amount of corresponding marker recorded in the posterior intestine (Table 4). Total transit time ranged from a minimum of 08 h 26 min (2nd meal at 26 °C) and a maximum of 20 h 23 min (1st meal at 18 °C). Overall, total transit time was progressively shorter with the increase of temperature, but differences were only significant for the second meal ($p < 0.05$). No statistical differences were found among the successive meals for each temperature, although a decreasing trend from first to third meal was observed at 18 and 22 °C.

Residence time in each segment (Fig. 5) was estimated from the daily pattern of the means markers content at each sampling time in Fig. 4. Different trends were observed for each temperature and digestive tract section, being longer for the second meal at 18 °C and the first meal at 26 °C in the stomach, and for the second meal at 18 °C and the third meal at 26 °C in the intestine. In relation to temperature, residence time in the intestine was shorter with the temperature increase for the first and second meals but no clear changes were observed in the third meal.

The sum of the three markers of each sampling time revealed the temporal pattern of gut content along the daily cycle (Fig. 6). In the stomach, highest content at 18 °C was recorder at the time of the third meal; at 22 °C during the second and third meals; and at 26 °C at the time of the first and third meals. In the middle intestine, the highest content at 18 °C was recorder from 12 to 16 h postprandial; at 22 °C from 8 to 16 h postprandial; and at 26 °C from 8 to 12 h postprandial. At the three temperatures, the stomach and the intestine were emptied at the end of the daily cycle.

4. Discussion

A basic premise for the digestive function is that the duration and physical-chemical conditions of the enzymatic reaction affect the efficiency at which the feed macronutrients are hydrolysed (Moyano and Savoie, 2001; de la Parra et al., 2007; Hollebeek et al., 2013; Gilan-nejad et al., 2017, 2018; Navarro-Guillén et al., 2022). In this study, we have examined the way in which greater amberjack juvenile is

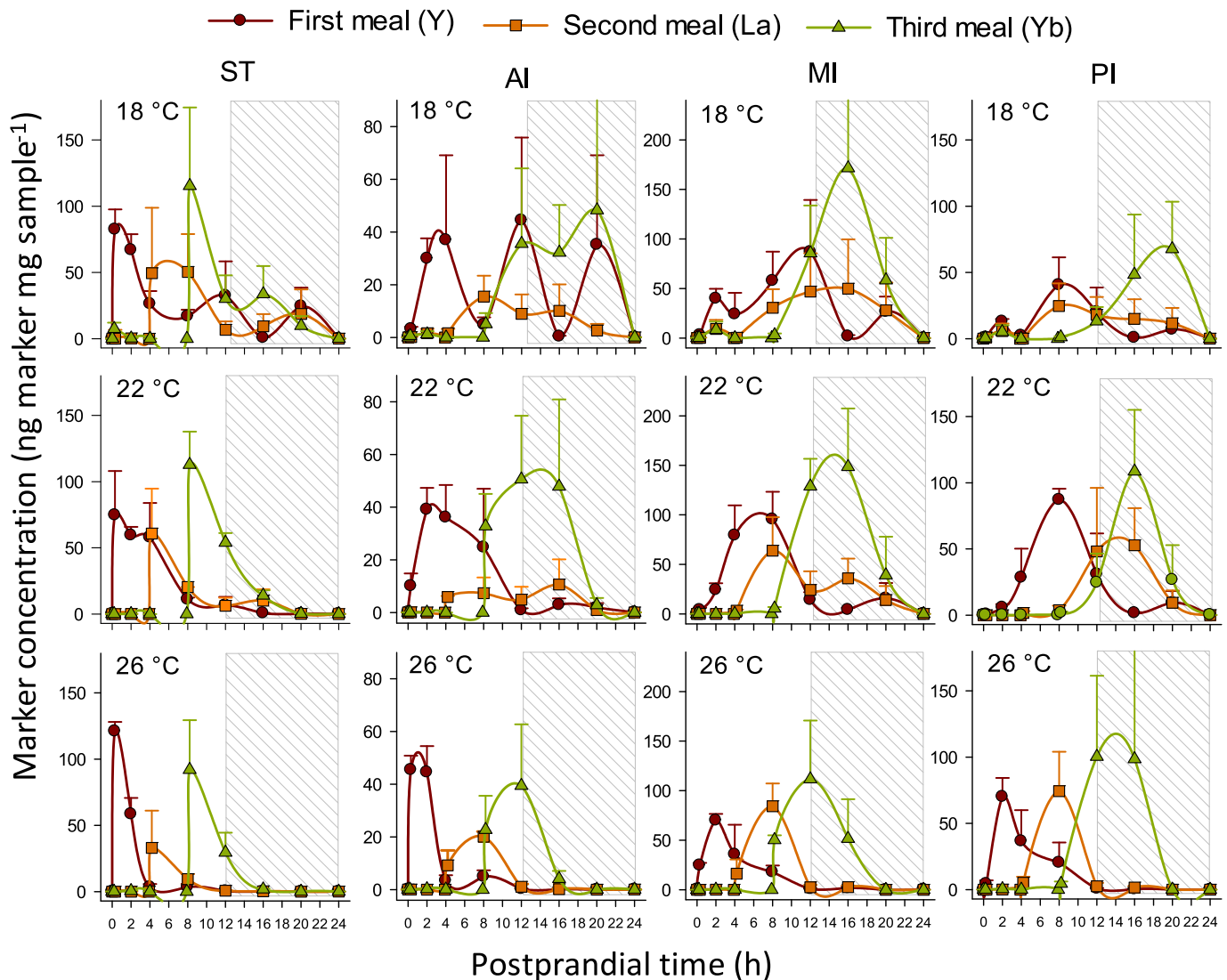


Fig. 4. Daily pattern of markers of the three daily meals in the gut of greater amberjack juveniles at the three tested temperatures ($\text{ng marker} \cdot \text{mg sample}^{-1}$). Means \pm SEM. Y = yttrium oxide; L = lanthanum oxide; Yb = ytterbium oxide. ST = stomach, AI = anterior intestine, MI = middle intestine, PI = posterior intestine. Postprandial time indicates the hours from the first morning meal supplied at 8:00 h local time. Dashed area indicates the dark period.

processing the feed ingested in three consecutive daily meals, and whether different environmental temperatures may affect their respective gut transits and the ionic conditions for nutrient hydrolysis. In the trial, fish were maintained under the different experimental temperatures for three weeks in order to allow the adaptation of the digestive machinery to the new conditions. Although the average body weight of juveniles at the end of the experiment was progressively higher with increasing water temperature, the differences were not statistically significant. Therefore, we are comparing fish of similar body mass and no biases in the results are expected in relation to fish body size.

We found that each meal progresses in a different manner, affecting to some degree two factors of importance for the digestion, the daily pattern of environmental pH within the gut and the transit of digesta throughout the digestive tract. Nevertheless, the feeding behaviour of greater amberjack juveniles is not homogeneous among individuals due to hierarchy established in the rearing tanks; larger and more aggressive individuals ate first and smaller individuals use to eat later than the others. This fact may increase the variance of the measurements and limits the statistical significance of the observed trends.

The lumen of the stomach showed the post-feeding acidification usually found in many fish species (Nikolopoulou et al., 2011; Yúfera

et al., 2012, 2014; Hlophe et al., 2014; Solovyev et al., 2016), though the gastric pH declined only moderately reaching minimum levels near to 5. The minimum pH value was reached quickly at 22 and 26 °C. In addition, the duration of this relative acidification was very short at these two temperatures. By contrast, at 18 °C, minimum pH was observed much later and the period of relative acidification is longer than in the other two temperatures. However, pH values turned back to pre-feeding values in few hours in all cases. The fast recovery of neutral values could be due to the difficulty for maintaining the chloride acid production in relation to the daily amount of ingested food and to the buffering capacity of the feeds. A similar effect was seen in Japanese yellowtail, *Seriola quinqueradiata*, with gastric pH values above 6.5 (Nakada, 2002). Thus, at 22 and 26 °C the acidification effort seems to be concentrated in the first meal, while at 18 °C it is more focused towards the second and third meals. Likewise, the alkalisation in the intestine (above pH 7) was detected at different moments for each temperature. At 22 °C, the alkaline values were observed earlier and maintained more hours than at 18 and 26 °C. There is not a clear reason for this response but it is probably related to the food transit at each temperature and the moment at which the chyme reaches the intestinal segment. Interestingly, these highest intestinal pH values coincide with the period of maximum

Table 3

Parameters of exponential regressions fitted to the gastric evacuation of the three successive meals at the three tested temperatures.

Meal number	Mo	r	R ²	p	F-test
18 °C – 1st	86.85 ± 13.34	0.2284 ± 0.1111	0.5495	0.005	A
	53.71 ± 17.61	0.1017 ± 0.1251			
18 °C – 2nd	113.3 ± 19.42	0.2558 ± 0.1221	0.5401	0.009	
	79.40 ± 11.43	0.1318 ± 0.0744			
22 °C – 1st	60.59 ± 10.85	0.2353 ± 0.1634	0.6664	0.004	A
	114.40 ± 5.87	0.2139 ± 0.0289			
22 °C – 2nd	121.10 ± 4.50	0.8226 ± 0.1176	0.9657	<0.0001	B a
	33.17 ± 8.526	0.3284 ± 0.2495			
26 °C – 1st	92.41 ± 12.19	0.3017 ± 0.1177	0.6944	0.0051	b

Mean ± SEM. r indicates the evacuation rate. p = significance of the slope (r). Different letters in F-test indicates significant differences among temperatures (capital letters) or among meals (small letters).

Table 4

Total transit time from ingestion up to 95% digesta evacuation (mean ± SD) of the three successive meals at the three tested temperatures.

	1st meal	2nd meal	3rd meal
18 °C	20 h 23 min ± 04 h 04 min	16 h 41 min ± 04 h 04 min ^a	14 h 28 min ± 02 h 19 min
	18 h 55 min ± 07 h 19 min	17 h 07 min ± 02 h 20 min ^a	13 h 08 min ± 02 h 19 min
22 °C	10 h 50 min ± 04 h 30 min	08 h 26 min ± 01 h 05 min ^b	11 h 48 min ± 02 h 35 min

Different superscript letters indicate significant statistical differences for a meal among temperatures ($p \leq 0.05$).

content of digesta within the intestine at each temperature (Fig. 6). The relative low acidification and alkalisation capacity observed in the present study could also be due to the voracity of this species and the consequent quick transfer of chyme to the intestine. Results indicate that the stomach content was quickly evacuated to the intestine dragging the gastric chloride acid and masking the intestinal alkalisation. In fact, the anterior intestine clearly showed this effect with values below pH 7 during the whole daily cycle.

Different postprandial pattern for gut acidification and alkalisation have been described in relation to feed quality (Murashita et al., 2021) and feeding frequency (Yúfera et al., 2014; Gilannejad et al., 2021). Here we show that water temperature may also affect the daily luminal pH pattern in agreement with results reported in perch *Perca fluviatilis* (Solovyev et al., 2016). This modulation of luminal pH is probably the physiological response to changes in feed intake and residence time within the gut in order to optimize the activation of digestive enzymes. Navarro-Guillén et al. (2022) found that the optimal pH for pepsin activity of this species is 2.5, and no response was observed at the physiological pH found in the present study (> pH 4.7). As observed in other species like Atlantic salmon, *Salmo salar* (Krogdahl et al., 2015), and gilthead seabream, *Sparus aurata* (Gilannejad et al., 2021), only a residual activity of gastric proteases would be displayed under some feeding conditions questioning the role of the stomach in relation to acidic digestion of proteins (Márquez et al., 2012). Unlike in the stomach, Navarro-Guillén et al. (2022) observed a good digestive activity for pancreatic and intestinal proteases (trypsin, chymotrypsin and leucine amino peptidase) at the current alkaline physiological values. It is worth noting that the optimum pH described by these authors for leucine

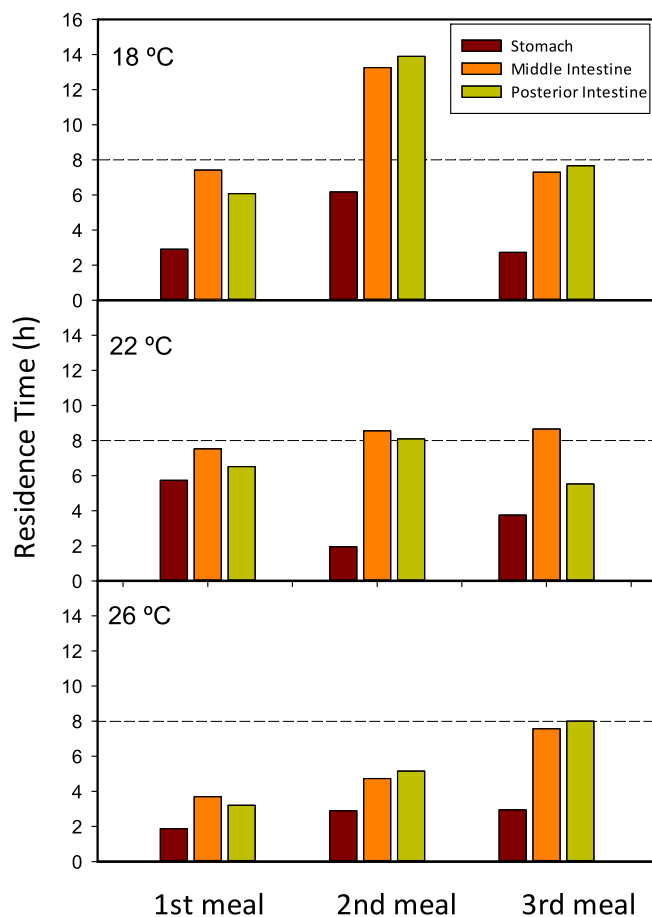


Fig. 5. Residence time (hours) of the three daily meals in the stomach, middle intestine and posterior intestine of greater amberjack at the different tested temperatures. Residence time has been estimated from average markers content presented in Fig. 4, considering the values above the 50% of the maximum.

amino peptidase is 7.5, very close to the maximum alkalisation level maintained during 12 h at 22 °C in the present study.

As expected, water temperature modulated the food transit by decreasing the passing time throughout the digestive tract with temperature increase from 18 to 26 °C. In our study, all the tested temperatures are within the thermal tolerance range described for this species (Jover et al., 1999; Nakada, 2000; Takakuwa et al., 2006; Fernández-Montero et al., 2018; Jerez et al., 1918; Yokoyama et al., 2020) and therefore a decay of the ingestion or an inconsistent food transit by excessive temperature were not detected. In addition, the tested range of temperatures fall within the optimal values for the activation of the principal digestive proteases in this species (Navarro-Guillén et al., 2022). Many studies with teleosts showed a similar decreasing trend of transit time with the temperature increase within the tolerance range (see for instance: Handeland et al., 2008; Bernreuther et al., 2009; Miegel et al., 2010; Das et al., 2018; Gonçalves de Sandre et al., 2016; Fernández-Montero et al., 2018; Nakagawa, 2018; Mazumder et al., 2020; Kounna et al., 2021; Liu et al., 2022; Pham et al., 2022), although most of gut transit studies merely refer to gastric evacuation and were performed with only one daily meal.

Nevertheless, gastric evacuation provides only partial information about the digesta transit throughout the digestive tract. For a more complete understanding of this process, it is necessary to assess also the patterns of gut filling, total transit time and residence time of a given meal within the gut segments (Yúfera et al., 2019; Gilannejad et al., 2019). In the present study, the stomach was immediately filled in all cases and no differences among treatments could be detected. On the

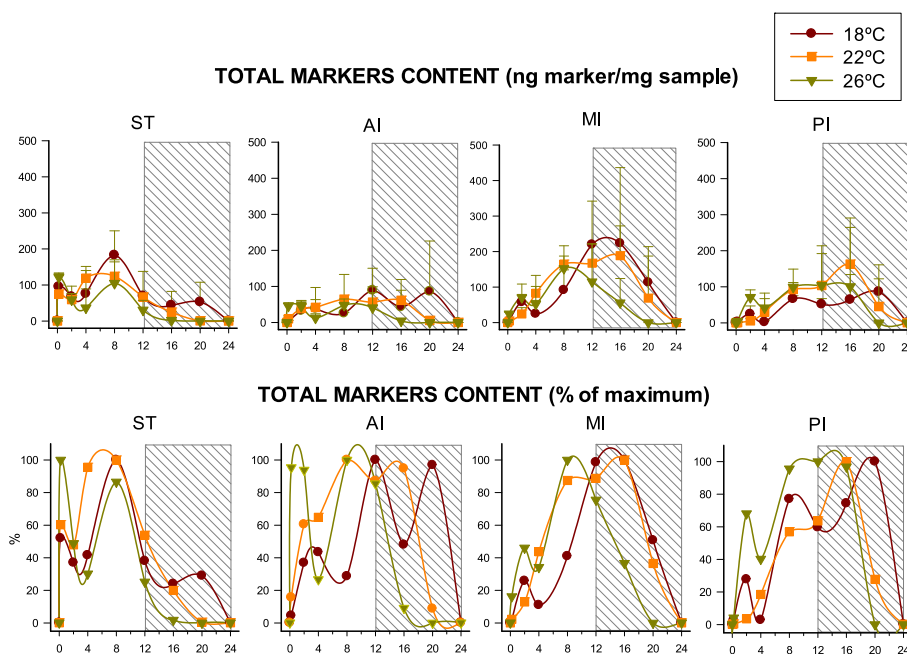


Fig. 6. Total content of markers (sum of the three markers) in each digestive tract segment during the daily cycle at the three tested temperatures. A) ng markers \cdot mg sample⁻¹; means \pm SD. B) Percentage of the maximum amount recorded. ST = stomach, AI = anterior intestine, MI = middle intestine, PI = posterior intestine.

contrary, gastric evacuation, total transit time and residence time were affected in different manner by temperature. Here we show for the first time that not all meals ingested during the same day have the same transit rate and that they are affected in a different way by the temperature increase. At 26 °C the meals transited fast enough to progress clearly as separate batches, one behind the other, through the tract. That is, the stomach had been practically emptied by the time of the following meal. In addition, at 26 °C the feed was ingested so fast that reached directly the middle intestine at the meal time, particularly in the third meal of the day. On the contrary, at 18 and 22 °C, the consecutive meals became mixed in the stomach and in the intestine. The first consequence of this mixing is that gastric evacuation and intestinal transit exhibited irregular postprandial patterns with some waves associated to the entry of new digesta in the stomach and intestine. These waves or peaks of digesta along the daily cycle can be more clearly observed at 18 °C in all segments, but particularly in the anterior intestine. The reason of this pattern could be that a great part of the second and the third meals were transferred to the intestine in detriment of the first meal during the following hours of being ingested. Then, when the different meals became well mixed in the stomach, the first meal continued the transit to the intestine forming another peak.

Time for total gastric evacuation (usually $\geq 95\%$ stomach content) in juvenile fish may range from several days or weeks (Pääkkönen and Marjomäki, 1997; Hop and Tonn, 1998) to only few hours (Das et al., 2018; Gao et al., 2022). Aside from the species-specific characteristics, transit results are greatly influenced by the experimental conditions and measurement methodology. Therefore, comparisons among species and studies should be taken with caution. In the case of greater amberjack, Fernández-Montero et al. (2018) with one-single-meal experiment after a starvation period found that gastric evacuation time varied between 16 and 30 h approximately with decreasing temperature from 26 to 17 °C (body weight: 100 to 400 g). In the present study, gastric evacuation required between 4 and almost 24 h (body weight: 60 to 100 g) being affected by both, temperature and the mixing of digesta from successive meals. In any case, the response pattern against the water temperature increase was similar in both studies having a comparable range of temperatures.

The second consequence of mixing the successive digesta is a prolongation of residence time of some meals, mainly the second meal, in

both the stomach and the intestine. We found that the residence time in the middle intestine varied from almost 4 h up to 14 h. Nevertheless, it is difficult to discern which has been the relative contribution of the digesta mixing and of the temperature in the observed differences among the meals. This range of values is in the line of other studies using the same methodology. In cobia juveniles, the intestinal residence time decreased from 8 h 18 min to 5 h 54 min with increasing the temperature from 30 to 34 °C (Yúfera et al., 2019). In gilthead seabream juveniles reared at 20 °C, intestinal residence time varied between 10 and 20 h depending on the feeding frequency (Gilannejad et al., 2019). However, residence times longer than 24 h were found in one-single-meal experiments with Atlantic salmon at 13.5 °C (Aas et al., 2017) and European sea bass, *Dicentrarchus labrax*, at 22 °C (Bonvini et al., 2018). Though a longer residence time may benefit the nutrient hydrolysis, a lower enzyme/substrate ratio by the combination of two or three digesta batches may decrease the digestion efficiency (Gilannejad et al., 2017, 2018). On the other hand, a lower residence time at increased temperatures probably is compensated by the higher digestive activity found at 26 °C (Navarro-Guillén et al., 2022). To elucidate to what extent these and other factors are affecting the digestion efficiency it is necessary to examine in detail the daily pattern of enzymatic activities under the current feeding protocol.

Interestingly, the transit of the last meal was less affected by temperature. This fact could be related to a longer remaining time until the next morning meal, when no other meal is pushing the digestive content. We found that in all cases, the posterior intestine was completely emptied every day before the first morning meal, which means that the feed processing is organised on the basis of 24 h cycles when fish are being fed under a routine daily protocol, at least for this body size. Similar daily transit schedule has been described in other fish species such as cobia (Yúfera et al., 2019), gilthead seabream (Gilannejad et al., 2019), pacu, *Piaractus mesopotamicus* (Gonçalves de Sandre et al., 2016) or hybrid grouper *Epinephelus* spp. (De et al., 2016). In large yellowtail kingfish, *Seriola lalandi*, (body weight > 2Kg) a similar 24 h schedule for food processing was observed at summer temperatures (21 °C) but at winter temperatures (13 °C) the gut transit time was longer than 24 h (Miegel et al., 2010). Transit times longer than one day have also been described in other species like European seabass (Adamidou et al., 2009; Bonvini et al., 2018), Atlantic salmon (Aas et al., 2021) or meagre,

Argyrosomus regius (Kounna et al., 2021). Probably, factors such as body size, relative intestine length, previous experimental feeding conditions, feed quality, and some others are also behind these differences (Bromley, 1994).

Feed intake and gut transit are regulated by a complex system of gastrointestinal hormones and neuropeptides that determine the hunger and satiation feeling (Volkoff, 2016; Soengas et al., 2018). Grove et al. (1978) pointed out that in rainbow trout, *Oncorhynchus mykiss*, return of the appetite appears when most of the stomach content was evacuated. On the other hand, Riche et al. (2004) found that the gastric evacuation in tilapia juveniles, *Oreochromis niloticus* was incomplete at the time of the next meal and that the amount of ingesta was related to the percentage of remaining gastric content. Nevertheless, in our study these premises were not always accomplished. Thus, the level of ingestion in the second meal was similar at the three temperatures, but at 18 and 22 °C the stomach content was only partially evacuated at the meal time. Similarly, no clear correlation between previous evacuation level and ingestion level was observed in the third meal at the different temperatures. These discrepancies are partially due to inter-species differences in the appetite/satiation signalling system and subsequent feeding response (Rønnestad et al., 2017; Soengas et al., 2018) but also to the different daily feeding schedule. The short-term signalling system is working within the framework of the established circadian feeding rhythms. Light/dark cycle and feeding time works as entrainment cues for the circadian clocks (Delgado et al., 2017) that on turn are driving the digestive function. Return of the appetite depends not only on gastric evacuation but also from metabolic signals derived from digestion and assimilation occurring in the intestine. The alteration of daily transit pattern of the successive meals due to changes in the temperature may therefore have a direct effect on feed intake along the daytime (Volkoff and Rønnestad, 2020). In the present study, no statistical differences in the feed intake among meals were found, although a trend to prioritise feeding in different moments of the day at each temperature can be observed. Finally, these trends resulted in significant differences in the whole day ingestion. Similar alteration of the daily feeding strategy related to temperature increase was also found in cobia (Yúfera et al., 2019). In addition to the enhancement of the digestive enzymes activity with the temperature increase (Navarro-Guillén et al., 2022), these changes in ingestion and gut transit of successive meals may have important repercussion in the whole day nutrient utilisation and, in the long-term, in growth performance. The weight gain differences during the short experimental period of the present study were not significant, but reflect somehow this effect.

5. Conclusions

Greater amberjack juveniles fed three times a day showed an increasing trend of the daily ingestion with the increase of temperature from 18 to 26 °C, though the increase was not statistically significant between 18 and 22 °C. Overall, gastric evacuation, total transit time throughout the gastrointestinal tract and residence time of digesta within the intestine tended to decrease with the increase of water temperature. Nevertheless, the transit of digesta differed among daily meals and temperatures. At 26 °C, the different daily meals transited through the tract fast enough to progress clearly as separate batches. On the contrary, at 18 and 22 °C the consecutive meals became mixed in the stomach and the intestine.

The observed changes in the transit rate also affected the luminal pattern of acidification and alkalisation. At 22 and 26 °C the gastric acidification effort was focused on the first meal whilst at 18 °C it was on the second and third meals. Intestinal alkalisation was maintained during more hours at 22 °C than at 18 °C and 26 °C coinciding with the moments of highest chyme content in the intestine.

These findings contribute to explain the effect of water temperature on gut transit of successive meals in routinely fed greater amberjack juveniles. This is a necessary step for advancing in the understanding of

the interrelations among feed intake, digesta transit, metabolic signals and return of the appetite in this voracious species, and ultimately to design more efficient feeding protocols.

Declaration of Competing Interest

None.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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