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FEATURE ARTICLE

Timing of digestion, absorption and assimilation in octopus species from tropical (*Octopus maya*) and subtropical-temperate (*O. mimus*) ecosystems

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ABSTRACT: Culture of octopuses is still in an experimental stage due to a lack of knowledge about their digestive physiology. This study aimed to determine the temporality of nutrient digestion, absorption and assimilation in 2 species of octopus: the tropical *Octopus maya* (habitat temperature 22 to 26°C) and the subtropical-temperate *O. mimus* (15 to 21°C). In both species, the chyme volume displayed 2 peaks over time along the digestive tract: one at the beginning of digestion and a second at the end of the digestion process. These phases were observed in gastric juice (GJ) enzymes, in the flow of nutrients in the digestive gland (DG), in the appearance of metabolites in the hemolymph, and in muscle protein and glycogen levels. The soluble nutrient flow throughout the digestive tract into the DG was similar in both species. However, intracellular digestion was different in *O. mimus* compared to *O. maya*; lipids were mobilized faster than proteins in *O. mimus*, whereas proteins were mobilized faster than lipids in *O. maya*. This suggests that at low temperatures, the mobilization of lipids could be a biological priority due to their role in membrane protection. In addition, *O. mimus* displayed more digestive enzyme activity in the GJ and DG compared with *O. maya*, suggesting that adaptive differences may be related to environmental temperatures.

KEY WORDS: *Octopus maya* · *Octopus mimus* · Digestive physiology · Digestion timing · Enzyme activity · Gastric juice · Free amino acid pool · Hemolymph

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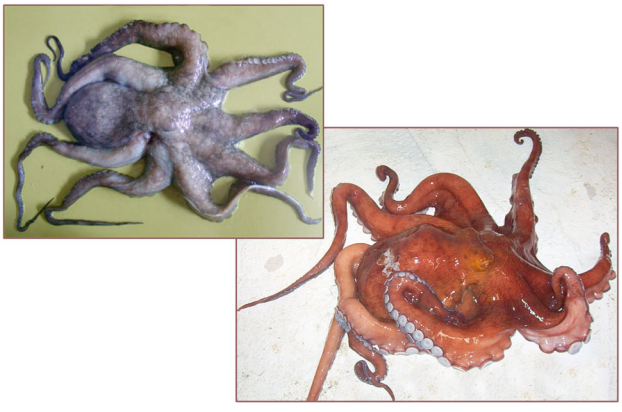

Top left: *Octopus mimus* from the Antofagasta coastal zone, Chile (subtropical environment); bottom right: *O. maya* from the continental shelf of the Yucatán Peninsula (tropical).

Photos: A. Olivares & C. Rosas

INTRODUCTION

Proteins are the main metabolic substrate for cephalopods. They are characterized by a low oxygen:nitrogen ratio and are derived from a natural diet based mainly on crustaceans, mollusks and fish (Segawa & Hanlon 1988, Olivares et al. 1996, Carrasco & Guisado 2010, Rosas et al. 2011). Roura et al. (2012) demonstrated the importance of crustaceans in the diet of octopods, reporting 19 species of crus-

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taceans in the diet of wild *Octopus vulgaris* paralarvae. *O. maya* and *O. mimus* are not exceptions; these carnivorous species also use protein as their main energy source (Van Heukelem 1977, Rosas et al. 2007a,b). *O. maya* and *O. mimus* adapt well to captivity, eat freeze-dried diets, and can reach high market value (Zuñiga et al. 1995, Salas et al. 2006), thus making both species good candidates for marine aquaculture (Iglesias et al. 2014). However, one of the main bottlenecks in octopus aquaculture is the lack of elaborated diets that cover their nutritional requirements (Aguado Giménez & García García 2002, Prato et al. 2010, Villanueva et al. 2014). Recently, a semi-humid paste made with squid and crab meat was created as a successful diet for *O. maya*, and is the only diet that has been tested in octopus juveniles (Martínez et al. 2014). In most cases, octopus culture is at an experimental phase given the general lack of knowledge about their digestive physiology and the way proteins are digested. Protein digestion is a key aspect of digestibility in both invertebrates and vertebrates, and is an important determinant of the suitability of diets.

Digestion in cephalopods, and particularly in octopods, begins in the buccal mass (Boucaud-Camou & Boucher-Rodoni 1983). As the prey is broken down mechanically, the posterior salivary glands secrete alkaline enzymes and the food then passes through the esophagus to the anterior stomach (crop) (Nixon 1979, 1980, Boucaud-Camou & Boucher-Rodoni 1983). The food is further broken down in the crop, where it combines with acidic enzymes secreted by the digestive gland (DG) to form the chyme, initiating extracellular digestion (Morishita 1972, Bocaud-Camou & Boucher-Rodoni 1983). The chyme passes to the stomach (St), and from there to the caecum (Ce) where in some cephalopods (e.g. *Sepia officinalis* and *O. vulgaris*) a small quantity of chyme is absorbed (Boucaud-Camou & Boucher-Rodoni 1983). The chyme then moves directly from the Ce towards the DG, where intracellular digestion is carried out (Boucaud-Camou & Boucher-Rodoni 1983). The DG is the largest gland in the cephalopod body and performs many functions, such as synthesis and secretion of digestive enzymes, re-absorption and metabolism of nutrients, and synthesis and storage of glycogen (Boucaud-Camou et al. 1976, Rosa et al. 2005b, Martínez et al. 2011a,b).

Digestive enzymes in *O. vulgaris* are released by apocrine secretion into the lumen of the DG tubules where they are sent as zymogens to the digestive tract (Budelmann et al. 1997, Semmens 2002, Martínez et al. 2011a). Most of these enzymes are acidic, and are present in secretory lysosomes in octopods (e.g. *O. vulgaris* and *O. maya*), and in the loliginid squid *Sepioteuthis lessoniana* (Morishita 1972, Semmens 2002, Martínez et al. 2011b). Depending on the species, the number of secretory lysosomes increases several times throughout the feeding process, suggesting that extracellular digestive enzymes are released in pulses. Therefore, the release and activity of digestive enzymes may occur in response to the phase of the digestive cycle. In *O. maya*, the diameter and density of secretory lysosomes in the DG change according to the postprandial period, increasing significantly between 2 and 4 h after feeding (Martínez et al. 2011b). In *O. vulgaris*, the presence of food in the crop activates the DG and the secretion of enzymes in the posterior salivary glands (Best & Wells 1984).

In studies examining the chemical composition of 33 marine species, Cerezo Valverde et al. (2012, 2013) concluded that arginine (Arg) and leucine (Leu) were the limiting amino acids (AA) in most of the analyzed samples of *O. vulgaris* diet. Thus, the authors concluded that the inclusion of those AA in crustacean-based diets could benefit the culture of *O. vulgaris*. A better understanding of AA kinetics during the digestive process may also help to elucidate how the type of diet modulates the use of proteins and octopus growth.

Perez-Losada et al. (2002) suggested that *O. mimus* and *O. maya* belong to a different confamilial genus than *O. vulgaris* due to allopatric speciation associated with the geomorphologic lifting of Central America. Apparently, the formation of the Panama Isthmus during the late Miocene and early Pliocene interrupted the genetic flow between Pacific and Atlantic octopus populations, separating populations and allowing speciation to occur (Porta 2003). Indeed, *O. mimus* is distributed from northern Peru (Tumbes) to central Chile (Bahía de San Vicente) where temperatures are lower (15 to 21°C; Uriarte et al. 2012) than the temperatures within the geographic range of *O. maya* (22 to 26°C; Noyola et al. 2013). Even though both species could have the same evolutionary origin and a similar mechanism of digesting food, the way in which environmental conditions modulate the use of the ingested nutrients may differ. Hence, the nutritional requirements for each species may be related to the specific environmental conditions in their respective habitats.

This study aimed to describe the temporality of nutrient digestion, absorption and assimilation in *O. maya* and *O. mimus* fed with crab. This approach allowed us to evaluate the dynamics of nutrient use along the digestive tract, including how the nutrients

are transported and stored for use as a source of energy and growth. Using *O. maya* and *O. mimus* as model species, this study proposes temporal patterns of digestive physiology that may extend to other tropical and subtropical-temperate octopods.

MATERIALS AND METHODS

Octopus collection

Wild *Octopus maya* adults (n = 66; 810 ± 116 g wet weight [ww]) were caught in Sisal harbor (Yucatán, México) using artisan lines without hooks and with fresh crabs as bait. Captured octopuses were transported to the laboratory, located 300 m inland, in a 400 l circular tank filled with sea water. In the laboratory, animals were placed individually into 80 l green tanks containing sea water. All individuals were acclimated for 12 d at 28 ± 1°C, 34 PSU salinity, dissolved oxygen levels >5.5 mg l^{-1} and pH 8.2 ± 0.1; a 10 cm diameter PVC tube was offered as shelter. During the acclimation period, octopus were fed frozen crabs *Callinectes sapidus* at a ratio of 4% d^{-1} of the octopus ww. A 12 h light:12 h dark photoperiod was maintained during the experiments.

Wild *O. mimus* (n = 27; 1048 ± 180 g ww) were caught by SCUBA divers in the Coloso zone, Antofagasta, Chile (23° 45′ 29″ S, 70° 27′ 41″ W). Animals were transported individually in sea water and placed in 3.8 m^3 tanks at a density of 3 ind. m^{-3}. Tanks were covered with a black mesh to attenuate light by 50%; an open-flow sea water system maintained the temperature at 14 ± 2°C, oxygen levels >6 mg l^{-1} and pH >8. Animals were fed frozen crabs *Cancer setosus* ad libitum for 3 d before proceeding with the sampling.

Experimental design

A total of 61 *O. maya* individuals were sampled at 9 different time points: 0 (fasting), 20, 40, 80, 120, 160, 240, 360 and 480 min after feeding. *O. mimus* specimens (n = 28) were sampled at 8 time points: 0, 30, 90, 150, 210, 270, 330 and 390 min after feeding (see Table 1 for details of the number sampled at each time). The times after feeding at which the animals were sampled were determined from previous experiments with both species, and by taking into account that the digestive process may be faster at the beginning in the tropical *O. maya* than in the temperate *O. mimus* (Carrasco & Guisado 2010, Martínez et al. 2012). Crabs were deposited in the tanks and octopuses were left with the prey for 20 to 30 min, which was sufficient time for them to ingest the crabs (Martínez et al. 2012). Crab carapaces were then retrieved and octopuses were held in their tanks until the sampling time.

As an ethical consideration (Mather & Anderson 2007) and to ensure the welfare of octopuses during manipulation (Moltschaniwskyj et al. 2007), each animal was immersed in seawater at 15°C (for *O. maya*) and at 4°C (for *O. mimus*) for 3 min to reduce their metabolism (anesthesia) before dissection. Octopuses were covered with a clean piece of cloth, the arms of the animal were positioned at rest and the mantle exposed for surgery. The hemolymph was sampled and anesthetized animals were immediately euthanized by cutting the brain behind the eyes to minimize suffering.

Hemolymph samples, metabolites and amino acids

Approximately 2 to 3 ml of hemolymph was obtained from each animal using a pre-chilled catheter (24G × $^3/_4$ inch and 22G × 1 inch Punzocat) inserted into the dorsal artery after the octopus was dried with a paper towel. The individual weight of each animal (±0.05 g) was recorded. The hemolymph was centrifuged (8000 × *g*, 4°C) for 5 min to obtain plasma, which was placed immediately in liquid nitrogen and stored at −40°C until analysis. Commercial kits were used for the determination of acylglycerols (AG) (Ellitech TGML5415), cholesterol (Chol) (Ellitech CHSL5505) and glucose (Ellitech GPSL0507). Plasma was further diluted 1:300 for soluble protein determination using a commercial kit (Bio-Rad; Cat. 500-0006) based on the Bradford (1976) method. Determinations were adapted to a microplate using 20 µl of plasma and 200 µl of enzyme chromogen reagent. Absorbance was recorded using a microplate reader (Benchmark Plus; Bio-Rad) and concentrations were calculated from a standard substrate solution.

The free AA content (g AA per 100 g protein) of hemolymph was determined by ethanol extraction of lyophilized hemolymph (80% v/v; Dooley et al. 2002). Solids were diluted with 20 mM HCl to obtain a concentration of 1 mg ml^{-1}. AA profiles were determined following the Waters AccQ-Tag™ procedure as follows: samples were (1) filtered (0.45 µm) and maintained at −20°C until used, (2) derivatized using the Waters system AccQ-Tag™ and (3) chromatographed through a reverse phase (3.9 × 150 mm) 4 µm Nova Pak™ C-18 column, using a water-

acetonitrile gradient recommended by the Waters AccQ-Tag™ methodology (Milford), in a Waters™ HPLC system with a 2475 fluorescence detector (excitation and emission wavelengths 250 and 395 nm, respectively). Analyses were conducted at a constant temperature of 39°C. HPLC signal calibration and standard curves were obtained by using an AA standard solution at 3 different concentrations containing 18.75 to 150 pmol of each AA. These results were taken with caution given that methionine is partially destroyed by acid hydrolysis.

Chyme digestive tract sampling and enzymatic activity

The digestive tracts of both species were completely extracted and separated into crop, St, Ce and DG using surgical clamps to avoid mixing the digestive contents of each section. DG sections were dissected, frozen in liquid nitrogen and stored at −40°C until analysis. At the same time, the volume of the chyme contained in each section was measured (±1 ml), immediately frozen in liquid nitrogen and stored at −40°C until analysis. Samples of *O. mimus* were freeze dried and stored at −20°C until analysis.

Gastric juice (GJ) enzymatic activity was quantified in the chyme from each section of the digestive tract obtained throughout the digestion process. Chyme samples were thawed at 24°C (laboratory temperature) and centrifuged (8000 × *g*; 4°C) for 5 min; the supernatant was used to determine digestive enzyme activity and soluble protein. The frozen DG sections were homogenized at 4°C in 500 µl ice-cold, pyrogen-free water. Homogenates were immediately used for enzymatic analysis. Total soluble protein was evaluated with the Coomassie blue dye method (Bradford 1976) using serum bovine albumin as the standard.

Activity of total acidic proteinases was evaluated using Stauffer's universal buffer (Stauffer 1989) prepared at pH 6 for GJ samples and pH 3 for DG samples (Martínez et al. 2011a). Acidic proteinase and alkaline proteinase activities were assayed using hemoglobin (1%) or casein (1%) as the substrate (Anson 1938). Briefly, 20 µl of the enzyme extract (dilution 1:10) was mixed with 0.5 ml of buffer, and 0.5 ml of freshly prepared substrate in buffer at the corresponding pH (6 or 3 for acidic proteinases; 8 for alkaline proteinases), and incubated for 10 min at 40°C for GJ samples and 45°C for DG samples. The reaction was stopped by adding 0.5 ml of 20% (w/v) trichloroacetic acid (TCA) and cooling for 15 min at 4°C. The precipitated undigested substrate was centrifuged for 15 min at 13 370 × *g*. The absorbance of the supernatants was measured spectrophotometrically (Genesys 10UV; Thermo Scientific) at 280 nm against the substrate without the enzyme extract (blank). All determinations were done in triplicate and included blanks, which consisted of buffer, substrate, and TCA without enzyme extracts. Blanks were incubated as previously described.

For both acidic and alkaline proteinases, one unit of enzymatic activity was defined as the change in absorbance min^{-1} mg^{-1} protein of the enzyme used in this assay (UI mg^{-1} protein). All enzymatic evaluations were also expressed as a percentage of the maximum activity (100%) registered in a particular sampling time along the digestive process.

Digestive gland and muscle metabolites

DG samples and 2 arms of each sampled animal were frozen in liquid nitrogen and then stored at −80°C until analysis. From DG samples, soluble protein, AG, Chol, glucose and glycogen were measured. Muscle soluble protein and glycogen were evaluated from the octopus arms. Total soluble protein was evaluated with the Coomassie blue dye method (Bradford 1976) adapted to a microplate method using a commercial chromogen reagent (Bio-Rad; Cat. 500-0006) and bovine serum albumin as the standard. The same commercial kits described above were used for glucose, AG and Chol measurements. Determinations were adapted to a microplate using 10 ml DG extract and GJ (dilution 1:10) and 200 µl of the chromogen reagent. Absorbance was recorded on a microplate reader and the concentrations were calculated from a standard substrate solution.

Glycogen was determined using the method described by Carroll et al. (1956). Glycogen in the DG and arms was extracted with 5% TCA and determined through the reaction with sulfuric acid and phenol. Sections of the DG and arms were weighed (20 to 30 mg) and homogenized in TCA for 2 min at 4550 × *g*, then 100 µl of supernatant was pipetted into a tube and mixed with 500 µl of 95% ethanol. Tubes were placed in an oven at 37°C for 3 h. After precipitation, the tubes were centrifuged at 4550 × *g* for 15 min. The supernatant was discarded, leaving the glycogen as a pellet. Glycogen was dissolved by adding 1 ml concentrated sulfuric acid and 200 µl of 5% phenol; 200 µl was then transferred from the solution to a microplate and read at 490 nm on an ELISA plate reader (Bio-Rad 550). The total weight of the DG was also recorded.

Statistical analyses

Assumptions of normality and homogeneity of variances were verified using the Kolmogorov-Smirnov and Levene tests, respectively. Data in percentages were transformed using the arcsin transformation (Zar 1999). A 1-way analysis of variance (ANOVA) was used to test if there were statistically significant differences ($p < 0.05$) between sampling times for each biochemical parameter measured. *A posteriori* comparison was performed using the Student-Newman-Keuls (SNK) test. All statistical analyses were performed using Statistica v.8.0 (StatSoft).

RESULTS

Wet weight and hepatosomatic index

The ww of *Octopus maya* was not significantly different among sampling times (Table 1; $p > 0.05$). The hepatosomatic index (HSI) was higher in animals sampled 360 and 480 min after feeding compared with the other sampling times (Table 1, Fig. 1; $p < 0.001$). The ww of *O. mimus* specimens showed no significant differences among animals sampled throughout the experiment (Table 1; $p > 0.05$). The HSI was higher at 270 and 330 min after feeding (Table 1, Fig. 1; $p < 0.001$).

Table 1. Wet weight of animals used to evaluate the digestive physiology of *Octopus mimus* and *O. maya*. n = number of animals sampled. Different letters indicate significant differences at the $p < 0.05$ level

Time (min)	n	Wet weight (g) Mean	SD	HSI Mean	SD	Sig.
Octopus maya						
0	6	893.5	481.5	2.60	0.20	a
20	7	685.1	188.1	3.08	0.10	b
40	7	769.3	209.3	3.46	0.12	c
80	7	954.3	517.6	2.54	0.15	a
120	7	769.7	337.4	3.04	0.09	b
180	8	923.9	470.8	3.54	0.39	c
240	10	797.4	414.7	3.30	0.22	c
360	6	896.6	476.6	4.46	0.37	d
480	3	608.7	211.3	4.07	0.06	d
Octopus mimus						
0	5	987.20	275.78	2.98	0.30	a
30	3	1056.50	163.53	3.00	0.41	a
90	5	1171.20	121.64	2.98	0.29	a
150	3	1159.20	137.71	3.01	0.28	a
210	3	1002.23	81.57	2.58	0.14	a
270	3	1094.67	272.58	3.84	0.48	b
330	3	992.53	179.23	4.11	0.29	b
390	3	948.87	158.70	3.55	0.42	ab

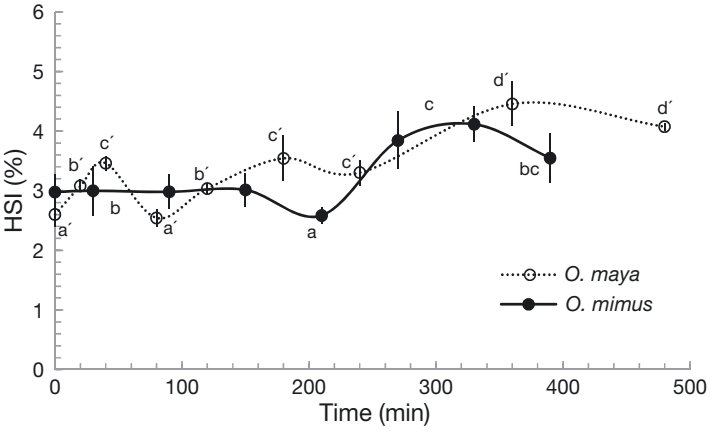

Fig. 1. Hepatosomatic index (HSI, %) of *Octopus maya* and *O. mimus* during the digestion process. Values are means ± SD. In Figs. 1–5 & 8, different letters indicate significant differences ($p < 0.05$) between sampling times. Letters for *O. maya* are distinguished by an apostrophe

Extracellular digestion: chyme volume

Chyme volumes varied along the digestive tract and throughout the digestion process in both species. In *O. maya* these changes were most evident in crop sections where 2 peaks were detected: one 20 min after feeding and another 120 min after feeding (Fig. 2; $p < 0.001$). In *O. mimus*, 50% of the GJ was recorded in the crop, 40% in the St and 10% in the Ce at the beginning of the experiment (Fig. 2).

Soluble protein in chyme

After feeding, the soluble protein concentration in the crop of *O. maya* was unchanged, increasing significantly until 80 min after feeding (Fig. 3; $p > 0.05$). *O. mimus* had more soluble protein in the GJ in the crop before feeding compared with the other sampling times (Fig. 3; $p < 0.001$). In addition, there were 2 peaks of soluble proteins in the St at 40 and 240 min in *O. maya* and at 30 and 150 min in *O. mimus* (Fig. 3; $p < 0.001$).

Enzyme activity

Extracellular digestion. Acidic and alkaline proteases showed 2 peaks at 40 and 180 min in *O. maya* (Fig. 4; $p < 0.001$). In contrast, *O. mimus* showed one well-defined peak of acidic and one of alkaline protease activity along the digestive tract at 120 and 240 min after feeding, respectively (Fig. 4; $p < 0.001$). Activity of alkaline proteases in the stomach of *O.*

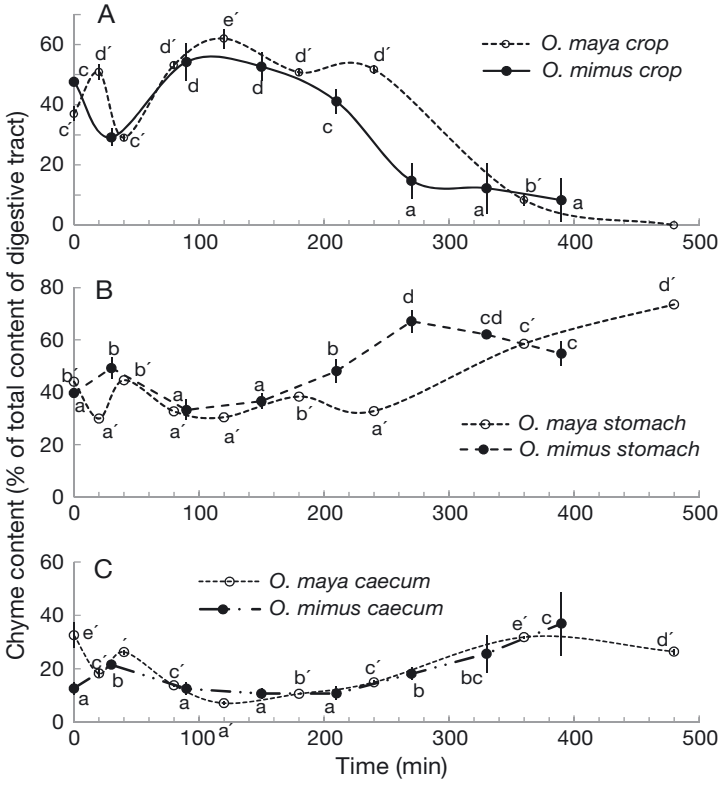

Fig. 2. Mean (±SD) variation in chyme volume in the (A) crop, (B) stomach, and (C) caecum of adult *Octopus maya* and *O. mimus* during the digestion process

mimus was marginal, with a small increase at the end of the digestive process (Fig. 4; $p < 0.01$). In general, enzyme activity in the GJ measured along the digestive tract and during the digestive process was greater in *O. mimus* than in *O. maya* (Table 2).

Intracellular digestion in the digestive gland. Two peaks of acidic proteases were detected in the DG of *O. maya* at 0 and 360 min after feeding, whereas multiple peaks of alkaline proteases were also detected after feeding (Fig. 4; $p < 0.01$). *O. mimus* had 2 peaks of acidic and alkaline proteases in the DG at 30 and 210 min after feeding (Fig. 4; $p < 0.001$). Alkaline and acidic protease activity in DG measured during the digestive process was also greater in *O. mimus* than in *O. maya* (Table 3).

Soluble metabolites in the DG and hemolymph. Peaks of soluble metabolites in the DG of *O. maya* were followed by a decrease, or by stable levels of soluble metabolites in the hemolymph. Only glucose levels in the hemolymph registered similar peaks to those found in DG glycogen (Fig. 5; $p < 0.001$). In contrast, Soluble metabolites in *O. mimus* showed peaks in the DG that were followed by peaks in the hemolymph (Fig. 5; $p < 0.001$).

Plasma AA in *O. maya*. Essential (EAA) and non-essential (NEAA) amino acids showed 2 peaks. The first was observed 20 to 40 min after feeding and the second was detected 120 to 180 min after feeding (Fig. 6; $p < 0.001$). The Factor 1 axis of the principal component analysis conducted using all AA data explained 63.8% of the total variance, correlating negatively ($p < 0.01$) to isoleucine (Ileu), serine (Ser) and proline (Pro), and separating sampling times of 40, 120 and 180 min after feeding. The Factor 2 axis explained only 17.7% of the variance, with lysine (Lys) separating the sampling times of 240 and 360 min after feeding (Fig. 7).

Muscle soluble protein and glycogen. Two peaks of soluble protein were detected in muscle samples from *O. maya* during the digestion process: one peak 40 min after feeding (22.1 mg g^{-1}) and another peak 180 min after feeding (20.6 mg g^{-1}) (Fig. 8; $p < 0.005$). In contrast, only 1 peak was recorded for muscle glycogen. *O. mimus* muscle glycogen had 2 peaks: one peak 270 min after feeding (4.15 mg g^{-1}) and a second peak 390 min after feeding (6.1 mg g^{-1}) (Fig. 8; $p < 0.001$).

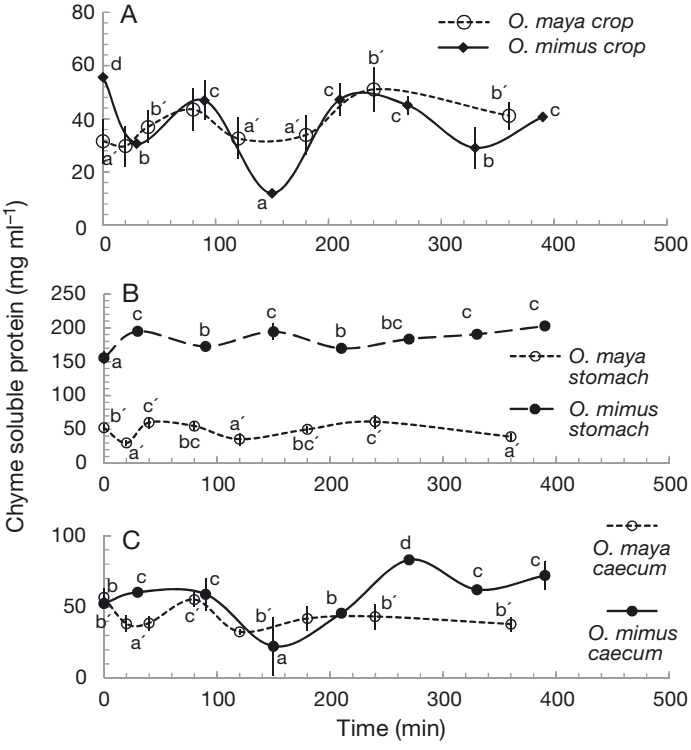

Fig. 3. Mean (±SD) variation in soluble protein (mg ml^{-1}) in the (A) crop, (B) stomach, and (C) caecum of adult *Octopus maya* and *O. mimus* during the digestion process

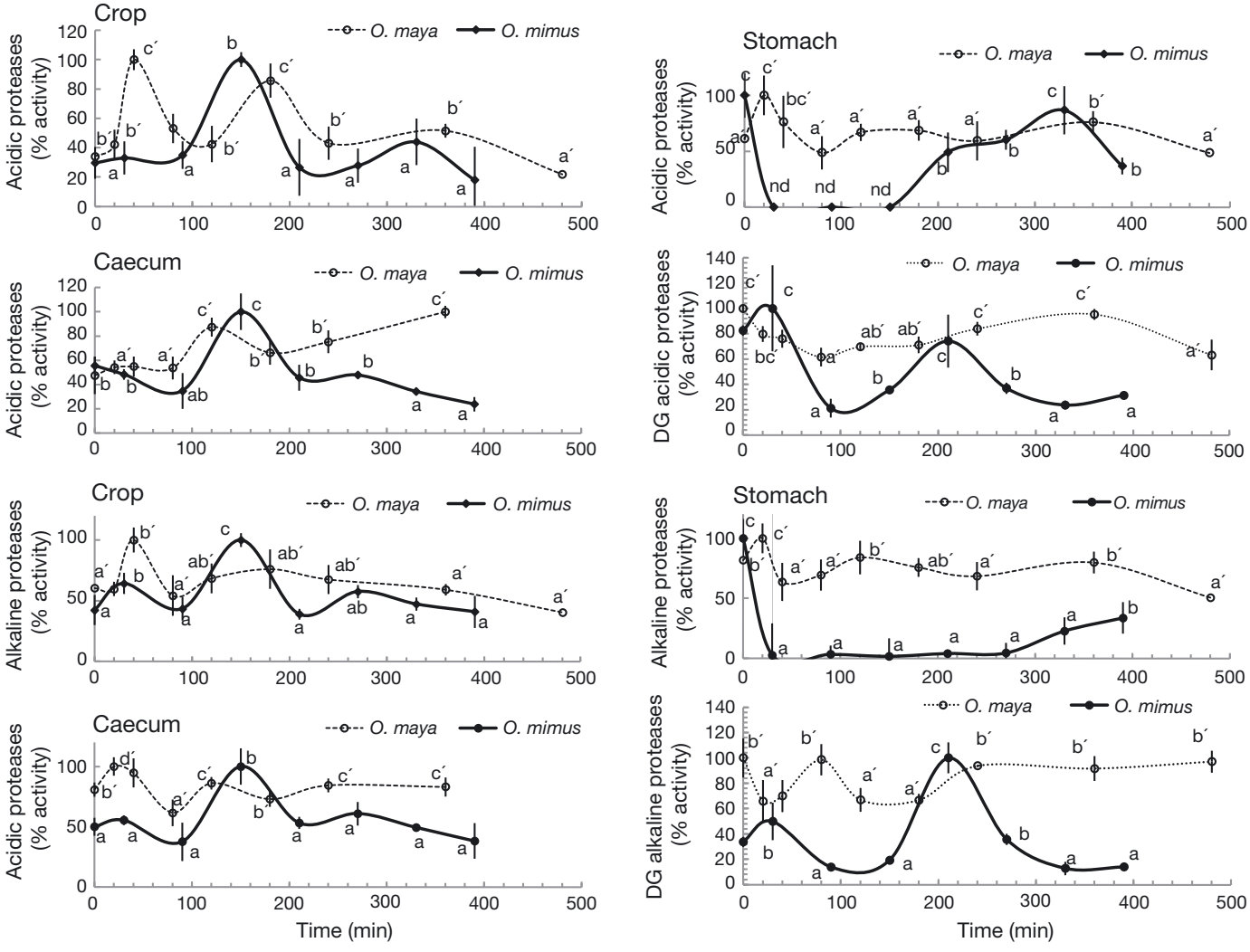

Fig. 4. Mean (±SD) variations in gastric juice acidic and alkaline proteases (% activity) along the digestive tract during the digestion process of adult *Octopus maay* and *O. mimus*. DG: digestive gland

DISCUSSION

We provide, for the first time, a detailed description of the digestion, absorption and assimilation of soluble and complex nutrients by tropical (i.e. *Octopus maya*) and subtropical-temperate (*O. mimus*) octopuses. We also provide valuable insights on the effect of environmental conditions on the digestion and final use of nutrients by these 2 species (Rosas et al. 2013), and presumably other species of octopods as well (Farías et al. 2011, Estefanell et al. 2012, Cerezo Valverde et al. 2013). The process of digestion begins when the digestive tract is ready to receive a meal. In this study, octopuses were fasted for 36 h, which is sufficient time to guarantee that all experimental animals would ingest the meal when it was offered (George-Zamora et al. 2011).

Extracellular digestion

Our results indicated that *O. maya* and *O. mimus* prepared their digestive tracts for digestion. Digestive enzymes (zymogens) in the GJ were detected in the crop, St, Ce and DG before ingestion. At the same time, protein, AG and Chol levels in the hemolymph appeared to be at baseline levels, with nutrients maintaining the octopus during the fasting period. George-Zamora et al. (2011) showed that a fasting period of 10 d is totally reversible in *O. maya* as individuals use energetic AA to sustain their metabolism.

Octopuses immediately reacted to the offered food (crabs). We estimated that an individual *O. maya* of ~900 g ww would need ca. 20 min to ingest a ~100 g ww crab (Martínez et al. 2012), whereas a period of

Table 2. Variations in gastric juice enzyme activity (general proteases, GP) along the digestive tract during the digestion process of adult *Octopus maya* and *O. mimus*. ND = not detected. Different letters indicate significant differences ($p < 0.05$) between sampling times

	Total acidic GP (UI mg^{-1} protein) Mean ± SD × 10^5	Sig.	Total alkaline GP (UI mg^{-1} protein) Mean ± SD × 10^5	Sig.
Octopus maya				
Crop				
0	0.03 ± 0.000	cd	0.08 ± 0.00	c
20	0.05 ± 0.025	b	0.12 ± 0.00	d
40	0.08 ± 0.006	a	0.16 ± 0.00	e
80	0.04 ± 0.009	bc	0.07 ± 0.00	b
120	0.03 ± 0.012	cd	0.09 ± 0.00	c
180	0.06 ± 0.017	b	0.11 ± 0.04	d
240	0.03 ± 0.007	c	0.21 ± 0.00	f
360	0.04 ± 0.002	bc	0.08 ± 0.00	b
480	0.02 ± 0.000	d	0.05 ± 0.00	a
Stomach				
0	0.04 ± 0.001	ab	0.17 ± 0.00	e
20	0.06 ± 0.014	c	0.23 ± 0.00	f
40	0.05 ± 0.015	ab	0.08 ± 0.00	b
80	0.04 ± 0.012	ab	0.09 ± 0.00	b
120	0.05 ± 0.015	ab	0.11 ± 0.00	c
180	0.04 ± 0.013	ab	0.13 ± 0.00	d
240	0.04 ± 0.010	ab	0.09 ± 0.00	b
360	0.05 ± 0.005	b	0.10 ± 0.02	c
480	0.03 ± 0.000	a	0.06 ± 0.00	a
Caecum				
0	0.03 ± 0.005	a	0.08 ± 0.01	a
20	0.04 ± 0.007	a	0.10 ± 0.02	b
40	0.04 ± 0.014	b	0.10 ± 0.01	ab
80	0.03 ± 0.007	a	0.09 ± 0.02	ab
120	0.07 ± 0.053	d	0.09 ± 0.02	a
180	0.07 ± 0.050	d	0.11 ± 0.01	b
240	0.06 ± 0.027	c	0.09 ± 0.01	a
360	0.07 ± 0.019	c	0.08 ± 0.01	a
480	ND			ND
Octopus mimus				
Crop				
0	2.6 ± 0.3	a	3.5 ± 0.4	a
30	2.9 ± 0.3	a	5.3 ± 0.5	b
90	3.0 ± 0.3	a	3.6 ± 0.3	a
150	8.7 ± 0.4	b	8.2 ± 0.5	c
210	2.3 ± 0.4	a	3.2 ± 0.1	a
270	2.4 ± 0.3	a	4.7 ± 0.3	ab
330	3.8 ± 0.6	a	3.9 ± 0.2	a
390	1.6 ± 0.4	a	3.4 ± 0.4	a
Stomach				
30	643 ± 97	c	33.5 ± 5.4	d
60	ND			a
120	ND			a
180	ND			a
240	317 ± 56	b	1.3 ± 0.01	a
300	388 ± 34	b	1.5 ± 0.1	a
360	559 ± 56	c	7.6 ± 1.8	b
420	238 ± 103	b	11.3 ± 1.5	bc
Caecum				
30	2.1 ± 0.2	b	2.9 ± 0.2	b
60	1.9 ± 0.1	b	3.2 ± 0.1	b
120	1.3 ± 0.2	a	2.2 ± 0.6	a
180	3.9 ± 0.6	c	5.9 ± 0.9	c
240	1.8 ± 0.2	b	3.1 ± 0.2	b
300	1.8 ± 0.0	b	3.6 ± 0.0	b
360	1.3 ± 0.0	b	2.9 ± 0.0	b
420	0.9 ± 0.4	a	2.2 ± 1.1	a

Table 3. Enzymatic activity (general proteases, GP) in the digestive gland evaluated during the digestive process in *Octopus maya* and *O. mimus*. Different letters indicate significant differences ($p < 0.05$) between treatments

	Total acidic GP (UI mg^{-1} protein) Mean ± SD × 10^5	Sig.	Total alkaline GP (UI mg^{-1} protein) Mean ± SD × 10^5	Sig.
Octopus maya				
0	0.049 ± 0.00	c	2.1 ± 0.5	b
20	0.036 ± 0.01	bc	1.4 ± 0.4	a
40	0.042 ± 0.01	bc	1.5 ± 0.5	a
80	0.028 ± 0.01	a	2.1 ± 0.9	b
120	0.035 ± 0.01	ab	1.4 ± 0.3	a
180	0.031 ± 0.01	ab	1.4 ± 0.2	a
240	0.042 ± 0.01	c	2.0 ± 0.2	b
360	0.051 ± 0.01	c	1.9 ± 0.2	b
Octopus mimus				
0	13.9 ± 0.7	c	6.5 ± 0.8	b
30	16.8 ± 5.7	c	9.6 ± 2.8	b
90	3.6 ± 1.3	a	2.7 ± 0.2	a
150	6.0 ± 0.2	b	3.7 ± 0.3	a
210	12.5 ± 3.5	c	19.2 ± 8.1	c
270	6.2 ± 0.8	b	6.9 ± 0.9	b
330	4.0 ± 0.6	a	2.5 ± 1.0	a
390	5.3 ± 0.4	a	2.7 ± 0.1	a

30 min might be required for *O. mimus* to ingest the crab (Carrasco & Guisado 2010). Martínez et al. (2012) observed that octopods inject saliva into their prey and wait for the enzymes contained in the saliva to open the prey (i.e. the crab). The chyme and partially digested flesh of the crab are then ingested, beginning the digestion process. Although this process is similar in both *O. maya* and *O. mimus*, our results indicate that external digestion is a more important process in *O. mimus*. A high concentration of soluble protein was recorded in the chyme of *O. mimus*, indicating that nutrients were quickly transported from the digestive tract to the DG where digestion started. It is interesting to note that lipid levels peaked in the DG of *O. mimus* before *O. maya*, suggesting that *O. mimus* may favor the digestion of lipids over proteins at the beginning of the digestion process. Differences in the digestion of lipids between the species could be related to the relationship between lipids and temperature. The geographic range of *O. mimus* includes temperatures between 15 and 21°C, whereas *O. maya* lives in tropical zones where temperatures oscillate between 21 and 30°C. The essentiality of *n*-3 highly unsaturated fatty acids (HUFA) in the diet of fish is directly related to the role of HUFA, as components of phospholipid biomembranes, in response to changes in environmental temperature (Tocher 2003). Such adaptation could occur in octopuses as well, allowing temperate spe-

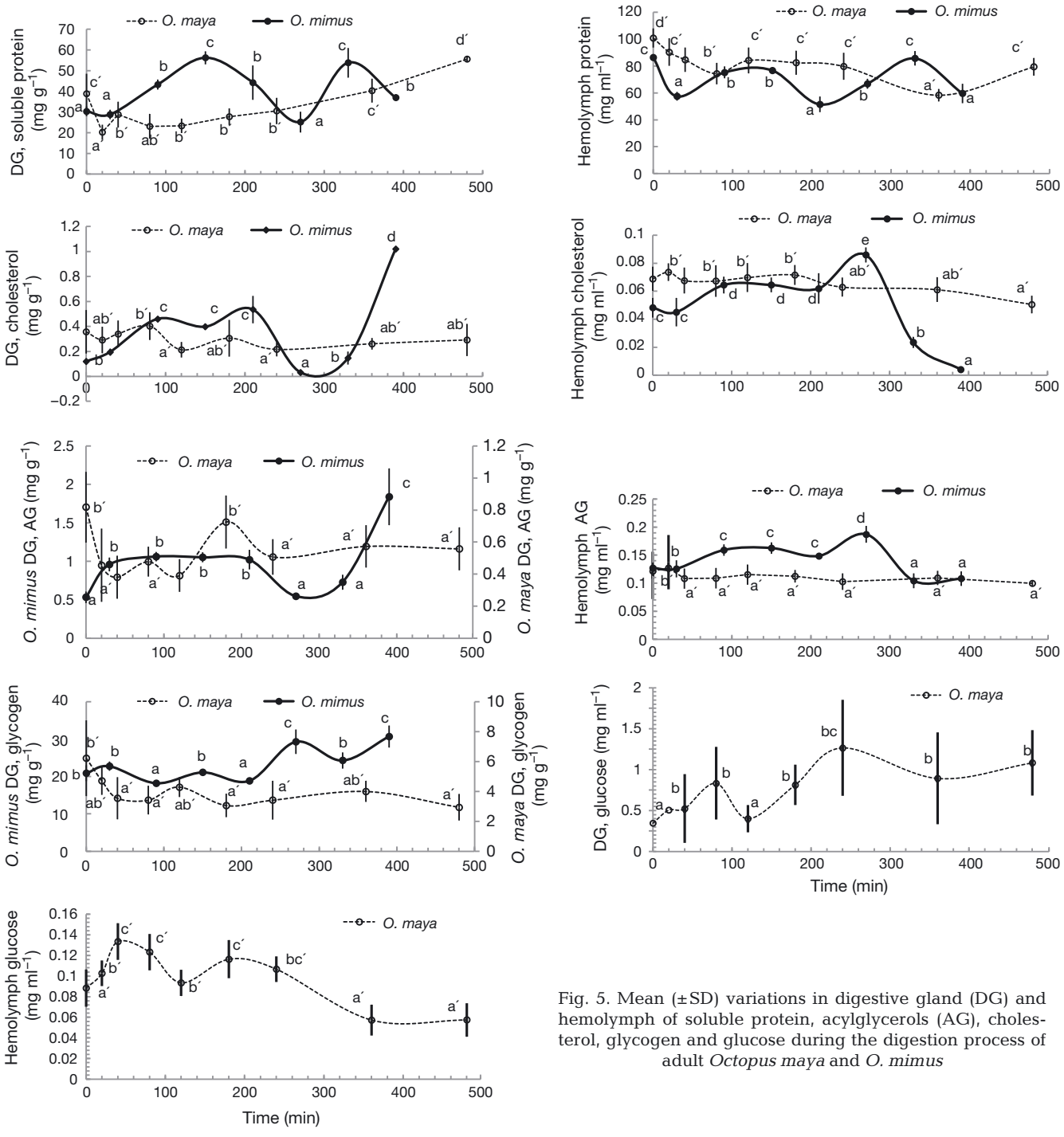

Fig. 5. Mean (±SD) variations in digestive gland (DG) and hemolymph of soluble protein, acylglycerols (AG), cholesterol, glycogen and glucose during the digestion process of adult *Octopus maya* and *O. mimus*

cies to use lipids in a different way than tropical octopuses. An increase in the demand for eicosapentanoic acid (EPA) and docosohexanoic acid (DHA) occurs in *O. vulgaris* maintained at low temperature (15°C), indicating that these fatty acids (FA) are used for biomembrane synthesis during growth (Miliou et al. 2006). In contrast, the use of FA by *O. vulgaris* maintained at higher temperatures (i.e. 20 to 25°C) changes with temperature in an inverse relationship (Miliou et al. 2005).

We observed that the digestive processes occurred in 2 major steps: the first was characterized by soluble nutrients passing quickly along the digestive tract, where nutrients were absorbed and used for muscle protein synthesis. The second, slower step involved the digestion and transforma-

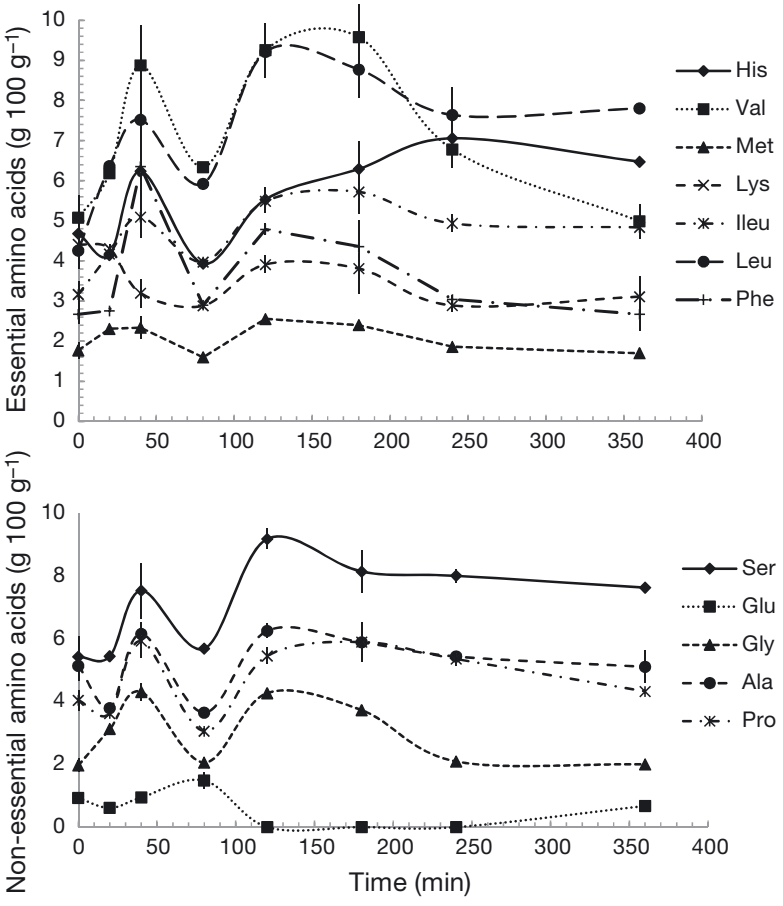

Fig. 6. Mean (±SD) variations in hemolymph free amino acids during the digestion process of adult *Octopus maya*. His: histidine; Val: valine; Met: methionine; Lys: lysine; Ileu: Isoleucine; Leu: leucine; Phe: phenylalanine; Ser: serine; Gln: glutamine; Gly: glycine; Ala: alanine; Pro: proline

tion of complex nutrients into useful molecules that were subsequently transported to muscles or temporarily stored in the DG. Despite the flow of soluble nutrients through the digestive tract into the DG, intracellular digestion in the DG of *O. mimus* was slower than in *O. maya*. Differences in the habitat temperatures of each species could affect the activity of digestive enzymes, slowing the digestive process in temperate species. It is interesting to note that the digestive enzyme activity in the GJ and DG of *O. mimus* was higher than that observed in *O. maya*. The slower digestion in temperate species may therefore be compensated for by high enzymatic activity, as has been observed in several fishes (Kuz'mina et al. 2015). In accordance with this finding, Pörtner (2006) suggested that temperate species tend to have more enzymes in order to counterbalance the high enzymatic activation energy required in temperate environments.

Intracellular digestion

Boucaud-Camou & Boucher-Rodoni (1983) found that protein digestion in octopuses starts when the posterior salivary glands excrete enzymes that initiate the process of external digestion. We detected chymotrypsin activity in the posterior salivary glands of *O. maya* at the beginning of external digestion, which facilitates the digestion of soluble proteins before the ingestion of crabs (Aguila 2007). In both octopuses examined, the soluble proteins that form the chyme rapidly passed from the mouth to the DG after the external digestion process was initiated; soluble proteins then peaked in the crop, St and Ce 40 to 80 min after the octopuses were fed. The mechanism of action of the soluble protein in the chyme is unknown, but we hypothesize that it has several key roles, one of which could be to activate zymogens (acidic and alkaline enzymes) located in the crop and other sections of the digestive tract where zymogens are stored. Besides polypeptides, it is likely that enzymes injected during the ingestion of food are present in the chyme (Boucaud-Camou & Boucher-Rodoni 1983, Hedstrom 2002). Indeed, zymogens have also been found in the digestive tract of other cephalopods. When studying the cytological structure of the digestive gland during the feeding of *Sepioteuthis lessoniana,* Semmens (2002) observed that digestive enzymes were released after the first hours following feeding and concluded that these enzymes were stored in the stomach as zymogens to increase digestive efficiency. Our results suggest that the role of chyme as an activator of zymogens and other digestive processes can be extended at absorption sites, and can activate enzymes and reserve mobilization in the DG. With the first pulse of chyme, soluble proteins and amino acids could stimulate the brush wall of the cells in the octopus DG to absorb nutrients and activate endocytosis (Martínez et al. 2011a). It is not known how these mechanisms operate in octopuses; however, in terrestrial animals some free AA can facilitate the absorption of other AA, depending on the metabolic relationship between them or if specific AA are pre-loaded in the intestine (Herzberg & Lerner 1973).The first pulse of chyme in *O. maya* and *O. mimus* may pre-load AA

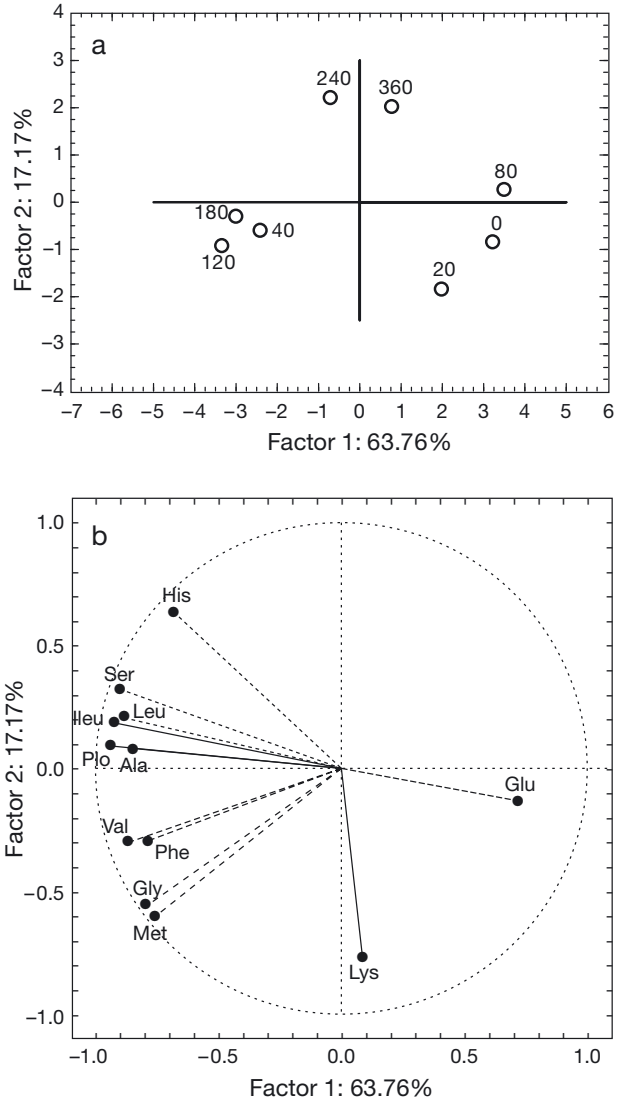

Fig. 7. Principal components analysis by cases (sampling time) and variables (amino acids) of hemolymph free amino acids obtained (n = 3) during the digestion process in *Octopus maya*. Numbers indicate sampling time. Correlation analysis indicates that along the axis of Factor 1, Ileu (−0.93), Ser (−0.90) and Pro (−0.94) had a greater influence in the distribution of animals sampled at different times, placing animals sampled at 40, 120, 180 min after feeding at the left of the Cartesian plane. The same analysis indicated that along the axis for Factor 2, Lys (−0.77) and His (0.64) were the amino acids responsible for the separation of animals sampled 20 min after feeding (down) and 240 and 360 min in after feeding (up) on the Cartesian plane. See Fig. 6 legend for amino acid abbreviations

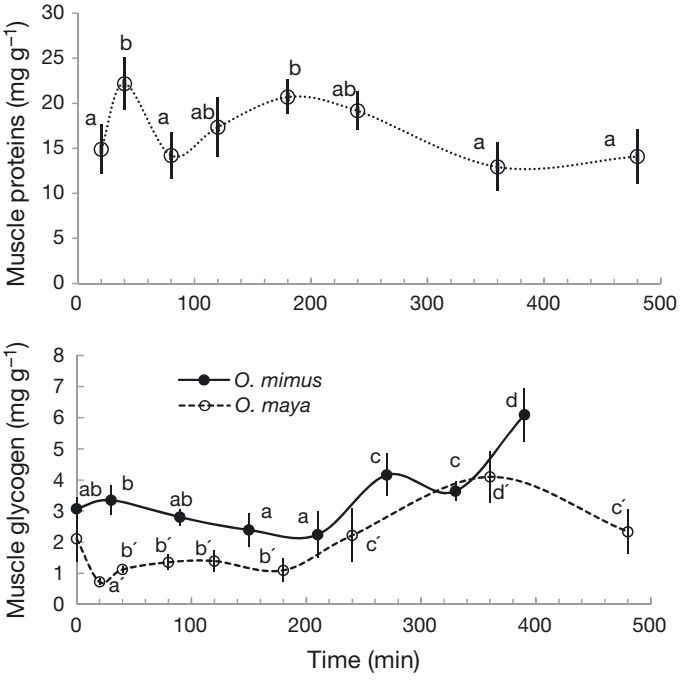

Fig. 8. Mean (±SD) variations in muscle protein for *Octopus maya* and glycogen for *O. mimus* and *O. maya* during the digestion process

and other nutrients to facilitate the absorption of the bulk of nutrients coming from the digestion of complex nutrients. To our knowledge, this is the first time that AA variations have been described during the digestion process in cephalopods.

Morishita (1972) reported acidic enzymes in the crop, St and DG of *O. vulgaris*, describing for the first time the role of acidic proteases during digestion in an octopod. Acidic proteases were several times more active than alkaline proteases in *O. maya* and *O. mimus*. This finding supports previous studies suggesting that the main role of acidic protease (presumably cathepsines) in octopus digestion is as extracellular digestive protease (Martínez et al. 2011a, 2012). We observed that GJ enzyme activity in *O. maya* exhibited several peaks throughout the digestive process, indicating that the release of digestive enzymes from the DG occurred in 2 pulses (Semmens 2002, Martínez et al. 2012). Aside from the initial enzymes stored in the crop before digestion, there was only one peak of enzyme activity in the GJ of *O. mimus*, suggesting that there was only one more pulse of enzymes from the DG during the digestion process. These differences could be due to the environmental temperature at which each species was studied. Enzymes released to GJ appear to be more frequent in tropical species (e.g. *O. maya*) than in subtropical or temperate species (*O. mimus*). Therefore, temperature is likely to govern not only digestive activity through intracellular digestion in the DG, but also through enzyme production.

Nutrient metabolism

Previous experiments performed with *O. maya* confirmed that animals fed with crab used ingested protein as a source of energy via the gluconeogenic pathway, which facilitated the accumulation of glycogen and its posterior release as glucose (Rosas et al. 2011, Martínez et al. 2012, Baeza-Rojano et al. 2013). In the present study, DG protein and glycogen levels in *O. maya* and *O. mimus* followed an inverse relationship throughout the digestive process, confirming the role of protein as a source of energy. These results suggest that glycogen accumulation must occur between meals, once the DG has recovered from the digestive process and nutrients are newly available for the next meal.

Soluble AG and Chol were also used as a source of energy for *O. maya*, whereas glycogen was the first source of energy for *O. mimus*. This is the first record detailing the role of soluble nutrients in the digestion process for both species examined. It is interesting that in *O. maya*, AG levels peaked once (180 min after feeding), whereas Chol peaked twice (80 and 180 min after feeding). These differences may be due to the form in which diverse nutrients were stored in the prey. AG are mainly stored in the DG and muscle of crustaceans (e.g *Callinectes* spp. or *Cancer* spp.; D'Abramo 1997), where octopus enzymes should be able to extract them. Our results suggest that AG appears in the DG when more complex nutrients are digested 180 min after feeding; for instance when AG was extracted from crab tissues. In crustaceans, Chol is an essential nutrient stored in the DG, muscle and hemolymph where it is mobilized and used as a precursor for molting and growth hormones (Teshima 1997, Pascual et al. 2003, Rosas et al. 2004, 2007a). The characteristics of Chol explain why this molecule showed 2 peaks in the *O. maya* DG, as it was obtained from the food on 2 occasions: from soluble nutrients in the first chyme pulse (probably soluble cholesterol found in the hemolymph and other tissues) and later, when more complex nutrients were digested. It is interesting to note that both nutrients were maintained in the DG, where they were presumably used as a source of energy, because neither of these nutrients was released into the blood during the digestion process. In fact, only hemolymph glucose levels changed significantly throughout the digestive process in *O. maya* and *O. mimus*. These molecules may therefore be mobilized as an energy source in muscle and other organs during the digestive process (Rosa et al. 2004, 2005a).

Amino acid metabolism

Following the chyme pulses, EAA and NEAA showed 2 peaks in *O. maya* hemolymph, indicating that there was AA flow during the first chyme pulse (40 min after feeding) and during the peak digestion of complex protein (140 to 180 min after feeding). Based on our results, we hypothesize that AA peaks were, at least in part, mobilized to be used for growth and glycogen synthesis in the muscle. Peaks of soluble protein observed at the same time as the AA peaks support this finding. Rosa et al. (2005a) demonstrated that AA, and in particular Pro and Arg, are the basis of energetic metabolism of pelagic, nektobenthic, benthic and benthopelagic cephalopods. Cephalopod muscle biochemistry includes complex mechanisms to use protein as a source of energy and for the accumulation of biomass at the same time (Boucaud-Camou & Boucher-Rodoni1983). In cephalopods, as in other invertebrates, glycogen is mainly derived from dietary proteins, via amino acid metabolism (Rosas et al. 2002, Miliou et al. 2005). In the present study, we observed that glycogen peaks followed the peak of soluble protein, suggesting that these proteins were first synthesized from AA coming from the hemolymph to be used later as a source of energy via the gluconeogenesis pathway.

CONCLUSIONS

Although the flow of soluble nutrients through the digestive tract was similar in *Octopus mimus* and *O. maya,* intracellular digestion in the DG differed between these species. Whilst lipids were mobilized faster than proteins in *O. mimus,* proteins were mobilized faster than lipids in *O. maya*. This finding suggests that the mobilization of lipids in temperate octopuses is different from the metabolic process that occurs in tropical octopuses (Mukhin et al. 2007). The digestive enzyme activity in the GJ (mainly acidic proteases) and DG was greater in *O. mimus* than in *O. maya.* Adaptive differences may therefore be associated with the environmental temperatures in the regions where those species occur, which should be taken into account by the nutritionists who design dry foods for octopuses.

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