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Advances in the *Octopus maya* digestive physiology
Avances en la fisiología digestiva de *Octopus maya*

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ABSTRACT

Red octopus (*Octopus maya*) is an important fishery resource in Mexico, due to its high commercial value and domestic and foreign demand. An alternative to fishing is the mariculture. Cephalopods digestion is a two-part process: extracellular and intracellular. This paper describes the progress made in *Octopus maya* digestive physiology study, particularly, in the digestion temporality, absorption and assimilation of crab in an attempt to assess the nutrients dynamic and usage along the digestive tract and, how these are transported, stored and used as an energy source for growth.

RESUMEN

El pulpo rojo (*Octopus maya*) es un importante recurso pesquero en México, debido a su alto valor comercial y demanda interna y externa. Una alternativa a la pesca es la maricultura. La digestión de los cefalópodos es un proceso de dos partes: extracelular e intracelular. El presente trabajo describe los progresos realizados en el estudio de la fisiología digestiva de *Octopus maya*, en particular la temporalidad de la digestión, la absorción y asimilación de carne de cangrejo, en un intento por evaluar la dinámica de nutrientes y el paso a lo largo del tracto digestivo y, cómo éstos son transportados, almacenados y utilizados como fuente de energía para el crecimiento.

INTRODUCTION

Red octopus (*Octopus maya*) is an important fishery resource in Mexico. Therefore, it confronts a great catch strain and in recent years has showed an inconsistent production. An alternative to fishing is the mariculture. This species has great advantages for cultivation. However, reliance on natural diets and the lack of a specific and economical feed have slowed the production development of this and other cephalopods at commercial scale round the world. There is a consensus that several crustaceans are the most common diet for octopus species, in both juveniles (Aguado-Giménez & García-García, 2002; Boucaud-Camou & Boucher-Rodoni, 1983; Pérez, López, Águila & González, 2006; Prato, Portacci & Biandolino, 2010; Rosas *et al.*, 2013; Zuñiga, Olivares & Ossandón, 1995) and paralarvae (Roura, González, Redd & Guerra, 2012; Uriarte *et al.*, 2010). Cerezo-Valverde *et al.* (2013) studied 33 marine species and their nutrition, and concluded that arginine and leucine were the limiting amino acids in most of the samples analysed, suggesting that the inclusion of those amino acids in diets comprised of crustacean meals could improve fish or animal meal in diets for *Octopus maya*. It is interesting to note that arginine (Arg), along with other amino acids (aa), is one of the most abundant in the free amino acid pool in the crab *Callinectes sapidus*; these are widely distributed in the muscle and haemolymph because of their role as osmotic effectors in this and other euryhaline crustacean species (Lynch & Webb, 1973). As Arg and other amino acids

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play an important role in octopus nutrition, its ingestion may act to accelerate the uptake of other nutrients in the octopus digestive gland, as has been described in terrestrial animals (Herzberg & Lerner, 1973).

Cephalopods digestion is a two-part process: extracellular and intracellular. Extracellular takes place along the digestive tract, which begins in the mouth, follows the anterior stomach (crop), the posterior stomach, cecum and ending with the digestive gland (DG) where the chyme is absorbed. Intracellular develops inside the DG where nutrients hydrolyzed to make them bioavailable. Therefore, this organ plays an important role in this process (Boucher-Rodoni, 1976; Boucher-Rodoni, Boucaud-Camou & Mangold, 1987; Budelmann, Schipp & Boletzky, 1997). To date, studies on *Octopus maya* digestive physiology were not been carried out in depth. Develop a diet is a fundamental requirement to understand cephalopods digestive physiology, especially octopuses. This will allow designing experimental diets according to the species in order to achieve a highly digestible diet and suitable for cultivation that promotes growth. Using *Octopus maya* as a model, the obtained results allow general schedules of digestive physiology. These could become a proposal to apply for other tropical species, being an important contribution towards the development and management of a balanced feed.

Digestive Gland

Digestive Gland (DG) has a fundamental role in the cephalopods digestive process, provides digestive enzymes and stores nutrients (Bustamante, 1998). Especially, those that are used as a metabolic energy source (Hatfield, Rodhouse & Barber, 1992). Glucose is the main cells energy source and it is stored as glycogen (Murray *et al.*, 2013) in the digestive gland. *O. maya* DG is represented by a single cell type, organized as acini: the digestive cell. The maturation process of this organ is characterized by the gradual disappearance of yolk platelets in contrast to the gradual appearance of heterophagosomes, heterolysosomes and residual bodies within the cells; at the same time, complete and mature digestive cells develop gradually evolving the same columnar structure and function of a acinar-tubule organ (Martínez *et al.*, 2011a). Similar to what was observed in *S. officinalis*, the consumed food affects the digestive cells kinetics that forms the *O. maya* DG, which are affected by the presence of food and change during digestion (Boucaud-Camou, 1968; Boucaud-Camou, 1973; Perrin, 2004; Perrin, Le Bihan & Koueta, 2004).

In *Octopus maya*, digestive cells work asynchronous responding to the food presence in the digestive tract. During this process, the digestive cell varies according to the role played depending on the time in the postprandial period, either as an enzymes synthesizing cell or receiving the produced chyme for absorption and assimilation (Martínez *et al.*, 2011a). Similar to other cephalopods species, *Octopus maya* DG performs intracellular digestion and release enzymes to carry out extracellular digestion (Boucaud-Camou & Boucher-Rodoni, 1983; Martínez *et al.*, 2011a; Martínez *et al.*, 2011b). Consumed food affects the digestive cells kinetics that forms the *Octopus maya* DG. Which are, at the same time, affected by the food presence and change during the digestive process. Digestive cells work asynchronous responding to the food presence in the digestive tract. During this process, the digestive cell varies according to the role played depending on the time in the postprandial period, either as an enzymes synthesizing cell or receiving the produced chyme for absorption and assimilation. Many studies have discussed the utility of the digestive gland of *Octopus maya* and other cephalopods as a tool for monitoring the nutritional condition of animals fed different types of food (Águila y Elvira, 2007; Cerezo-Valverde *et al.*, 2013; Estefanell *et al.*, 2013). To date, we found that the type of diet strongly influenced biochemical characteristics in the DG of *Octopus maya*. Águila y Elvira (2007), fed octopuses with a diet made out of fishmeal and fish hydrolysed protein, and found that the energy content and total lipid values were lower than in crabmeat fed animals. In that study, they also found that there was an inverse relationship between soluble protein and DG glycogen in animals fed crab, suggesting that DG glycogen is the result of a gluconeogenic pathway. As consequence, crab fed octopuses observed higher levels of hemolymph glucose in comparison with octopuses fed elaborate diets. Those results and other experiments performed using *Octopus maya* (Baesa-Rojano *et al.*, 2012; Martínez *et al.*, 2011b; Rosas *et al.*, 2011) confirmed that fed crab animals used ingested protein as a source of energy via a gluconeogenic pathway, facilitating the accumulation of glycogen and its posterior release as glucose. DG protein and glycogen of *O. maya* followed an inverse relationship along the digestive process, confirming the role of protein as a source of energy during that process. These results suggest that glycogen accumulation must occur between meals, once the DG´s glycogen level has returned to normal and nutrients are newly available for the next meal.

Digestive Enzymes

Morishita (1972) observed first the role of acidic proteases during digestion in octopus, when reported acidic enzymes in the crop, stomach and DG of *O. vulgaris*. In *O. maya*, the characterization of DG proteases shows that these enzymes are essentially acidic (Martínez *et al.*, 2011b), similar to those reported in squid and cuttlefish (Cárdenas-López & Haard, 2005; 2009; Perrin *et al.*, 2004). Gastric juice (GJ) enzymes are exopeptidases from the cathepsin family which requires an acidic environment to develop their maximum activity, thus, lyophilization allows the protein structure used to be bioavailable (native state) so these enzymes can break the amino acids bonds (Martínez *et al.*, 2014), opposite if the raw materials were dried by heat. The significance of the partial enzymatic characterization by means of pH is that extracellular digestive enzymes are the first to be in contact with food. The pH at which a diet is prepared affects the digestive environment; therefore, it affects directly the digestive enzymes activity. The maximum activity of the *Octopus maya* DG and GJ enzymes occur at different pH, with optimum values of 3 to 4 and 6, respectively, suggesting that the intracellular and extracellular enzymes are different and *Octopus maya* DG proteases show maximum activity at acidic pH, indicating the presence of lysosomal enzymes associated with chyme intracellular digestion. 72% of the observed inhibition when using an aspartic proteases inhibitor suggested cathepsin D presence in the DG, while in the crop 18% of the observed inhibition in crop's GJ suggests that these enzymes are released with the cell portions in the apocrine secretion process that happens in the DG and reaches the crop (Martínez *et al.*, 2011b).

Other enzymes working in acid environments are also part of the GJ, suggesting that perhaps other cathepsins are present in the DG apocrine secretions conducting extracellular digestion. Recent studies have shown that cathepsin L is present in the squid's DG (Cárdenas-López & Haard, 2009) suggesting that this and other cathepsins may also be present in the *O. maya* GJ. *O. maya* observed a digestive acidic environment in both in the GJ and the DG (Martínez *et al.*, 2011a). Identified aspartic acid proteinases (cathepsin D) were in both sites as well (Martínez *et al.*, 2011b). Although cathepsins have been identified as digestive intracellular enzymes in invertebrates (Mukhin, Smirnova & Novikov, 2007), that acidic proteases were activated in the GJ during the extracellular digestion of the meal in both species. During the process, the pH of *O. maya* fluctuated between 5 and 6, favouring the activity of aspartate proteases (like cathepsin D), which along with a weakly alkaline activity, completed the digestion of complex ingested nutrients (i.e. myofibrillar protein) (Martínez *et al.*, 2011a; 2011b). The GJ enzyme activity of *O. maya* had several peaks throughout the digestive process, indicating that the release of digestive enzymes from the DG occurred in two pulses (20 min - 60 min and 100 min - 180 min) (Martínez *et al.*, 2011b; Semmens, 2002).

Digestive Process:
Morphometric and Biochemistry

The digestive process in *O. maya* involves two large steps: a first characterised by soluble nutrients passing rapidly along the digestive tract, which were absorbed and used for muscle protein synthesis; and a second slower step where complex nutrients were digested and transformed into useful molecules that were transported to the muscle or stored temporarily in the DG. At the date, there is a successful diet for *Octopus maya* growth (Martínez *et al.*, 2014). This diet was designed using morpho-physiological data derived of the studies showed in the actual paper. When a new species must be cultured, this type of studies are highly recommended before test any diet, because with this type of information the probability of design a diet according with physiological functioning of animals will be higher. The digestion process starts when the digestive tract is ready to receive a meal. From the results obtained by Martínez *et al.* (2011a; 2011b; 2012) a conceptual model in which the most important aspects of the *Octopus maya* digestive physiology has been developed (figure 1). A summary of the digestive process occurring in each section of the digestive tract of *O. maya* shown in table 1. In this summary, the type of response observed (namely response) and its function as a part of the digestion and assimilation processes (namely function) is included in an attempt to explain the sequence of events that occur at the same time along the digestive tract during digestion.

Octopus maya prepare its digestive tract for digestion. Before ingestion, digestive enzymes and zymogens in GJ were located in the crop, stomach, caecum and digestive gland. At the same time, protein, fatty acids and cholesterol in the haemolymph were considered a baseline and interpreted as nutrients required to maintain the fasting octopus

period (Rosas *et al.*, 2011) (table 1). In this sense, García-Garrido, Hachero-Cruzado, Domingues, López & Rosas (2011) showed that a fasting time of 3 days is enough in *O. vulgaris* to provoke mobilisation of lipids, Chol, AG, total protein and amino acids of octopus mantle to be use as a source of energy. In *O. maya*, mobilisation of nutrients occurred in animals undergoing 24 h fasting, suggesting that octopus species are well adapted to tolerate short fasting periods. In fact, George-Zamora, Viana, Rodríguez, Espinoza & Rosas (2011) showed that a period of 10 days of fasting is totally reversible in *Octopus maya*; in that study was observed that amino acids were used to sustain the energy metabolism of animals. Inside *Octopus maya* digestive cell under fasting conditions, none or few heterolysosomes, secretory lysosomes, residual bodies and heterophagosomes were observed. Showing that cells are passive, as there is no food in the gastrointestinal tract that stimulates synthesis and release of enzymes (secretory lysosomes). Verified by observing the acid and alkaline proteases activity in the GJ from the fasting DG, since these enzymes showed a lower activity compared to that observed at 60 and 120 min after feeding.

Once the crab was offer to the octopus, they immediately reacted to it. According to previous studies we considered that *O. maya* with a wet weight of around 900 g need 20 min to ingest a crab of around 100 g in wet weight (Martínez *et al.*, 2011b). As was observed by Martínez *et al.* (2012), when crab caught, the octopus injects saliva and waits for the enzymes to open the crab. After, chyme containing partially digested crab flesh was ingested, the digestion process started. High concentrations of soluble protein recorded in chyme, suggesting that nutrients rapidly transported from the digestive tract to the DG, where digestion began. Chyme formed with the consumed and macerated food together with the salivary glands secretion (figure 1a). During the first and second hours after alimentary bolus is transport to the crop (anterior stomach) where the digestive environment under fasting conditions is acidic and the extracellular digestion takes place. The role of the salivary glands in the external digestion of cephalopods is recognise as a source of proteases and venoms (Boucaud-Camou & Boucher-Rodoni, 1983; Undehim *et al.*, 2010). Actual results indicate that, the first chyme pulses formed in the crab and in the digestive tract during the first minutes after the meal (mixing salivary enzymes and soluble protein from the crab) play a key role in activating the digestion of *Octopus maya*.

Digestive process involves two main steps: the first is characterised by soluble nutrients passing rapidly along the digestive tract, where they are absorbed and used for muscle protein synthesis, while the second is slower, and involves complex nutrients being digest and transform into useful molecules that are transport to the muscle or store temporarily in the DG. Due to the first digestion acidic environment, cathepsin D may be present. The GJ's pH observed in the cecum and GJ secreted by DG indicates that these organs are ready to receive the produced chyme. An acidic digestive environment is conducive to proteins hydrolysis, because the proteases type (lysosomal) cathepsins are release and are most active in low pH.

The GJ's pH observed during the chyme formation through the digestive tract is between 5 and 6. According to Boucaud-Camou & Boucher-Rodoni (1983), octopus protein digestion starts when the posterior salivary glands excrete enzymes that initiate the external digestion of the prey. In *O. maya*, chymotrypsin activity in the posterior salivary glands is involved at the beginning of the external digestion, facilitating the digestion of soluble protein in the crab before its ingestion. Between 60 min - 120 min of the postprandial period, the consumed food reaches the crop (figure 1a) presenting a pH increase up to 5.9. This pH change suggests that the crop digestion begins with the release of acidic extracellular enzymes (figure 1b). The high activity of GJ's proteases from the DG observed after feeding indicates that digestive enzymes are secrete into the DG lumen probably to be used during the chyme formation in the crop. To this point, it appears that some of the DG acini shown chyme in the lumen and *O. maya* digestive cell has few heterolysosomes inside, an increase occur in the secretory lysosomes production and the consequent release of them and their content (enzymes) to acinar lumen by apocrine secretion. The high density of secretory lysosomes in *Octopus maya* DG indicates that extracellular digestive enzymes were release after the first hour of consuming the food. Apparently, relationship between food and the secretory lysosomes production is a species-specific process and a sharp increase in the density of this type of lysosomes was observed 60 min post-feeding and remains constant through the digestive cycle.

Once the external digestion process initiated, soluble proteins forming the chyme pass from the mouth to the DG in a short time, peaking in the crop, stomach and caecum 40 min - 80 min after the meal. Although we do not know the exact mechanism of action of the soluble protein into the chyme, we can hypothesise that it has several key roles. One of them could be to activate zymogens of acidic and alkaline enzymes located in the crop and other sections of the digestive tract where zymogens were stored in preparation for the next meal. Besides polypeptides, it is highly probable that there are other enzymes in the chyme, both from the crab and from

the salivary glands injected during ingestion of food (Boucaud-Camou & Boucher-Rodoni, 1983). In fact, it is possible to hypothesise that there are enzymes in the chyme that can activate zymogens of enzymes located along the digestive tract, functioning as a signal to start the digestion of more complex nutrients, as has been described in general as the mechanism of activation of zymogens of this type of enzyme (Hedstrom, 2002). Zymogens in the digestive tract of cephalopods have been previously reported. Studying the cytological structure of the digestive gland during feeding of *Sepioteuthis lessoniana*, Semmens (2002) observed that digestive enzymes were released one hour after feeding, concluding that those enzymes were stored in the stomach as zymogens to increase digestive efficiency. Results obtained in the present study suggest that the role of chyme as an activator of zymogens and other digestive process can be extend at the absorption sites, for activation enzymes and in the mobilisation of reserves in the DG (table 1). With the first chyme pulse, soluble protein and amino acids can stimulate the brush wall of the octopus digestive gland cells, absorbing nutrients and activating endocytosis. Although we do not know how those mechanisms operate in the octopus, in terrestrial animals, it has been observed that some free amino acids can facilitate the absorption of other amino acids, depending on the metabolic relationship between them or whether some specific amino acids are pre-loaded in the intestine during *in vitro* assays (Herzberg & Lerner, 1973). In *O. maya*, the first pulse of chyme could be the pre-loaded amino acids and other nutrients to facilitate, as in terrestrial animals, the absorption of the bulk of nutrients coming from the digestion of complex nutrients to be absorbed.

Octopus maya in fasting or postprandial period, the diameter of secretory lysosomes is constant, suggesting that not only the number but also the size of the latter are maintained through digestion. This implies that *Octopus maya* could be ready for continuous enzymes production in preparation for feeding. Once the chyme has been absorbed and entered into the DG cells, soluble protein can also stimulate zymogens of acidic intracellular enzymes located in the heterophagosomes of the DG (Martínez *et al.*, 2011b), enabling the intracellular digestion of more complex nutrients. At the same time and with all of the system activated, heterophagosomes initiate the mobilisation of energetic molecules that permit the transportation of molecules to the haemolymph and finally to muscle tissue (table 1). Results obtained in *Octopus maya* provided evidence that this activating mechanism included the rapid mobilisation of nutrients, presumably directed at fuel for intracellular digestion. Reductions in DG glycogen along with an increase of DG soluble glucose was observed

in *Octopus maya*, showing that once the digestion had been initiated, energetic reserves were used. Between 60 min and 120 min postprandial, glycogen concentration in *Octopus maya* DG decreases about 50%, showing that energy reserves are mobilize for food capture and consumption.

At the beginning of the digestion process (20 min - 80 min after feeding), soluble AG and Chol were also used as a source of energy in *Octopus maya*. This is the first time that those soluble nutrients have been identified to participate in digestion in *O. maya*. It is interesting to note that AG had one peak 180 min after the meal in *Octopus maya*, while Chol showed two peaks (one 80 min and the other 180 min after feeding). Those differences are in accordance with the form in which different nutrients were stored in prey. Acylglycerols in crustaceans are mainly stored in the DG and muscle of the crab (D'Abramo, 1997), from where octopus enzymes should extract it. Results obtained in the present study suggest that AG appears in the DG when more complex nutrients are digested, 180 min after the meal, because AG was extracted from crab tissues only until that time. In crustaceans, Chol is an essential nutrient that is stored in the DG, muscle and haemolymph, from where it is mobilised to be use as a precursor of moulting hormones or growth (Pascual, Gaxiola & Rosas, 2003; Rosa, Costa & Nunes, 2004; Rosas *et al.*, 2007; Teshima, 1997). Those characteristics of Chol clearly explain why this molecule showed two peaks in *Octopus maya* DG, where it is evident that Chol was obtained from the meal on two occasions: from soluble nutrients in the first chyme pulse (probably soluble cholesterol found in haemolymph and other tissues) and after, when the more complex nutrients were digested. It is interesting to note that both nutrients were maintain in the DG, where they were presumably used as a source of energy, because neither of the nutrients was significantly released into the blood during the digestion process.

At this stage, there is a lack of information concerning phospholipids and its role in for example the repartition of circulating cholesterol in tissues (hemolymph). In fact, only haemolymph glucose levels changed significantly during the digestive process of *O. maya*, suggesting that those molecules were release to support the energy source in the muscle and other organs during that time. As in other invertebrates, glycogen in cephalopods is a precursor of metabolic intermediates in the production of energy and non-essential amino acids, as it is part of the muscle energy pathways (Rosa, Pereira & Nunes, 2005b), and a source of energy for gonadal development (Rosa *et al.*, 2004; Rosa, Costa, Bandarra & Nunes, 2005a).

Table 1.
Evolution of the digestive process, absorption and assimilation in wild pre-adults of *O. maya*.

Time, min.	Location	Response	Function
0	Digestive tract	Gastric juice (GJ) present in the crop, stomach and caecum with low enzymatic activity. Also, low enzymatic activity in the DG. High levels of soluble protein, Acyl glycerides (AG) and cholesterol; low levels of glucose in the DG.	Zymogens in the GJ and DG in preparation for digestion. High levels of metabolites in the plasma suggesting that energetically useful molecules were mobilized. In muscle, low levels of metabolites indicate muscles in fasting conditions.
	Plasma	High levels of soluble protein. Relatively low levels of AG, glucose, cholesterol and AA.	
	Muscle	Low levels of soluble protein and glycogen.	
External digestion 0 – 20		Chymotrypsin and other alkaline enzymes from posterior salivary glands acting on the flesh of crabs (Morishita 1972; Águila y Elvira, 2007).	Starts chime formation into the crab. External digestion promoting formation of soluble nutrients (Boucaud-Camou and Boucher-Rodoni 1983).
20 – 80	Digestive tract	Peaks of chime volume along digestive tract along with peaks of soluble protein, enzyme activity in the GJ and DG along with protein, glucose and cholesterol. Reduction in AG and Cholesterol.	Once the chime arrives in the digestive tract, soluble nutrients are digested and absorbed into the DG. Presumably glycogen and AG of the DG are used as a fuel for this first digestion. Glucose, coming from glycogen degradation is presumably sent more quickly to the haemolymph to be used as a fuel for protein synthesis in muscle.
	Plasma	Plasma protein reduction. Peak of glucose. Marginal increments of AG and cholesterol. Peak of AAs.	
	Muscle	Peak of soluble protein. Glycogen levels remain low and almost constant during this period.	
80 – 180	Digestive tract	Second peaks of chime in the crop and stomach. Strong reduction of chime volume in the caecum. Second peaks of soluble protein in chime and enzyme activity in the GJ and DG. Peak of glycogen and progressive increment of soluble protein and glucose. Peaks of AG and cholesterol.	Chime is progressively digested in the crop and stomach. A second pulse of enzymes from the DG is sustaining external digestion of more complex ingested nutrients. Gluconeogenesis is activated and lipids accumulating in the DG are sent to the plasma where they are maintained constant. AAs from digestion are sent to the haemolymph to be distributed and used presumably for protein muscle synthesis.
	Plasma	Protein, cholesterol and AG are maintained constant during this period. A peak of glucose was registered. A second peak of AAs was registered.	
	Muscle	Peak of soluble proteins was registered. Glycogen remained almost constant.	
180 – 360	Digestive tract	Reduction of chime volume in the crop while an increment of volume is registered in the stomach and caecum. Reduction of soluble protein along the digestive tract. Reduction of acid and alkaline enzyme activity in the crop and stomach was observed. Peaks of acid and alkaline enzyme activity in the caecum and DG were observed. Progressive increment of soluble protein while glucose, AG, cholesterol and glycogen remained constant.	External digestion is located mainly in the caecum where a new pulse of enzymes coming from the DG sustains that activity. Soluble proteins accumulate in the DG presumably to be used as new digestive enzymes and for glycogen synthesis. The DG passes nutrients to the haemolymph to be transported to tissues. Lipids are apparently more slowly digested than proteins. Nutrients are stored in muscle; proteins are used for glycogen synthesis.
	Plasma	Reduction in protein, AAs and glucose levels. AG and cholesterol remain constant.	
	Muscle	Reduction in soluble protein and peak of glycogen concentration.	
480	Digestive tract	Stomach reaches its maximum chime volume while a reduction in chime volume of the caecum is observed. Soluble protein along the digestive tract is reduced along with enzyme activity in the GJ and DG. Proteins reach their maximum level in the DG while glucose, AG, cholesterol and glycogen remain constant.	At the end of digestion, a pulse of GJ is launched presumably to be sent to the crop for the next meal. That pulse helps to end the external digestion in the caecum. Synthesis of new enzymes starts in the DG where soluble proteins are stored. Internal digestion of the last nutrients occurs in the DG where lipids appear as the most difficult nutrients to digest. End of the digestion, absorption and assimilation processes.
	Plasma	Increment of proteins reaching values similar to those observed before feeding. AG and cholesterol remained constant, while low values of AAs and glucose were registered.	
	Muscle	Low values of soluble protein and glycogen were registered, similar to those observed before feeding.	

Source: Linares *et al.* (2015).

Octopus maya Digestive Physiology Conceptual Model

MOUTH
- Food consumption
- Digestive enzymes arrival via anterior and posterior salivary glands
- Food maceration
- Alimentary bolus formation

a — Esophagus transports bolus to

CROP (Anterior stomach) FASTING
- Digestive environment pH 5.2 - 5.3
60 - 120 Min AF
- Consumed food arrival increases pH to 5.9
- Extracellular digestion takes place
240 Min AF
- Emptying and pH descend

b — Enzyme release by apocrine secretion

DIGESTIVE GLAND (DG) FASTING
- DG gastric juice (GJ) pH 5.3 - 5.5
- None or few heterolysosomes, secretory lysosomes, residual bodies and heterophagosomes (passive cells)
60 – 120 min AF
- Glycogen concentration decreases 50%
- Secretory lysosomes Increase and release via
- apocrine secretion
240 Min AF
- DG's GJ Acidic and alkaline proteases activity increases
- Heterophagosomes and heterolysosomes formation
- Intracellular digestion begins (lysosomes)
360 Min AF
- pH increases due to nutrients arrival
- DG's GJ Acidic and alkaline proteases activity is kept
- Acinar lumen empty, Digestive cell with high number of heterolysosomes
480 Min AF
- Organ's pH decreases due to nutrients passage to blood
- DG's GJ Acidic and alkaline proteases activity decreases
- Glycogen tissue concentration restored
- Residual bodies in high numbers
- Digestive cells ready to start a new digestive cycle

c — Produced chyme is transported

POSTERIOR STOMACH
240 Min AF
- Chyme mixture

d — Chyme transport

CECUM FASTING
- Digestive environment pH 5.4 - 5.5
240 Min AF
-Chyme arrival increases pH
360 Min AF
- Some chyme still present in organ
- pH is kept
480 Min AF
- Emptying and pH descend

e — Particles separation

f — Nutrients

g — Waste products

h

SIPHON
360 – 480 min AF
- Stools formation

BLOOD STREAM
240 – 360 min AF
- Nutrients arrival from the intracellular digestion for distribution within the organism
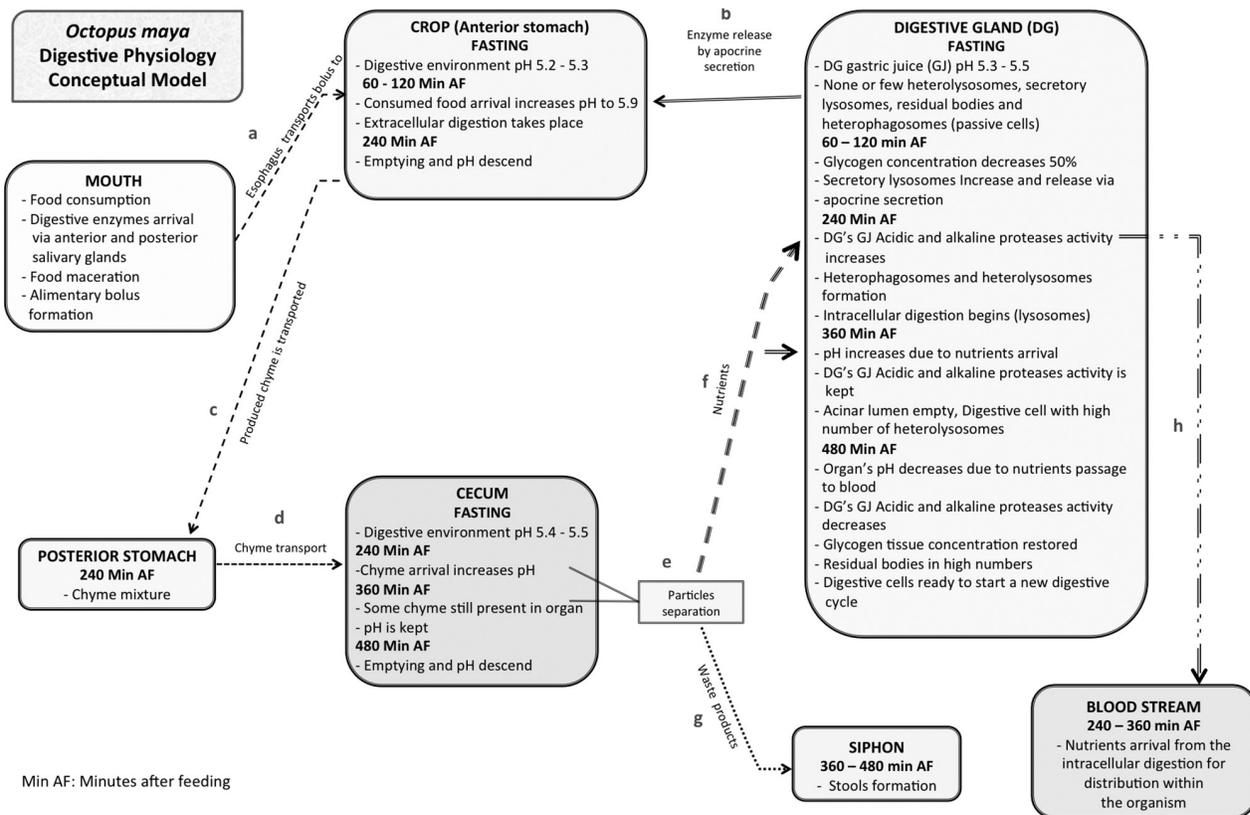
Min AF: Minutes after feeding

Figure 1. *Octopus maya* digestive physiology. Conceptual model.
Source: Author's own elaboration.

Following the chyme pulses, essential amino acids and non-essential amino acids showed two peaks in *Octopus maya* haemolymph, indicating that there was amino acids flow during the first chyme pulse (40 min after meal) and another during the peak digestion of complex proteins (140 min – 180 min after meal). According to our records, this is the first time that such AA variations during the digestion process have been shown in cephalopods. The results suggest that, at least using crab as a diet, all of the amino acids passed to haemolymph to be mobilised in other tissues. Previous studies performed in *Octopus maya* demonstrated that Phe, Ile, Ala, Glu, and Ser are use as metabolic fuel, while His, Arg and Lys are accumulate during starvation (George-Zamora *et al.*, 2011). In this sense, and according to the present results, we can hypothesise that AA peaks were, at least in part, mobilise to be use for growth and for glycogen synthesis in the muscle. Peaks of soluble protein observed at the same time as AA peaks support that finding. When studying 14 cephalopod species it was demonstrated that

AA, and in particular Pro and Arg, are the basis of the energy metabolism of pelagic, nektobenthonic, benthic and benthopelagic cephalopods (Rosa *et al.*, 2005b).

Cephalopod muscle biochemistry includes complex mechanisms for the use of protein as a source of energy and, at the same time, an accumulation of biomass. Muscle growth in cephalopods involves two processes: *i)* hyperplasia, the generation of new muscle fibres; and *ii)* hypertrophy, the increase in size of those fibres that are already in existence (Semmens, Doubleday, Hoyle & Pecl, 2011). In their study, Semmens *et al.* (2011), observed the hyperplasia process in *O. pallidus* through the histological analysis of mantle muscle fibres. Poor and rich mitochondrial muscle fibres were observe even in octopus adults, demonstrating a mechanism by which octopus species maintain non-asymptotic growth during their life cycle. Although hyperplasia has not yet been demonstrated in *Octopus maya*, we can assume that a similar growth process occurs in those species and that those processes have a high demand for nutrients and energy for protein

synthesis. In cephalopods, as in other invertebrates, glycogen is mainly derived from dietary proteins, via amino acid metabolism (Miliou, Fintikaki, Kountouris & Verriopoulos, 2005; Rosas *et al.*, 2002). In the present study, we observed that glycogen peaks follow a peak of soluble protein, suggesting that those proteins were first synthesised from AA coming from haemolymph to be use later as a source of energy via the gluconeogenesis pathway.

At 240 min of the postprandial period, chyme slowly reaches the cecum from the crop and subsequent stomach (figure 1c and 1d), crop's pH returns to its initial acidic environment, possibly preparing for the next digestion or an adjustment is made releasing new enzymes to maintain the acidic environment during transit of chyme through the digestive tract. Observing pH changes during the digestion process, it is evident that when the chyme reaches the cecum a pH change in the environment occurs from 5.4 to 5.9, suggesting again the effects of digestion in the environment of the gastrointestinal tract. Considering pH data, a regulatory mechanism could be operating to keep the digestive environment around 6, the right level where GJ's enzymes have their maximum activity observed. To maintain the acidic environment and at the same time the activity of the acidic enzymes *Octopus maya* may release more GJ during the digestion process to keep the maximum activity of these enzymes, which are more active than alkaline digestive enzymes.

Between 240 min and 360 min postprandial period chyme from the cecum reaches the DG (figure 1c) and a separation of particles is performed (figure 1e), nutrients reach the DG (figure 1f) and now heterophagosomes are formed apically and heterolysosomes in the middle part of the digestive cell, decreasing the secretory lysosomes density. This indicates that the extracellular digestion phase is completed and intracellular digestion and chyme absorption from the cecum carried out. Stools formation starts from 360 min postprandial (figure 1g). DG Intracellular digestion is carry out by lysosomes, which essentially contain acidic enzymes in its interior. Interestingly, the maximum alkaline digestive enzymes activity at 240 min postprandial were only observed in cultured octopuses, suggesting that these youth may show a faster metabolism than wild pre adults, suggesting that the size (wet weight) and age could affect the food transit in the cephalopods digestive tract.

The DG's GJ maximum activity observed 360 min after feeding indicating the arrival of chyme to DG from the cecum (figures 1f). The digestive cell is re-plete with heterolysosomes, reducing the heterophagosomes and begins forming residual bodies, while continuing the nutrients passage within the blood for distribution (figure 1h). This step took at least 120 min, during which the cecum still maintaining a relatively high pH. Interestingly, the maximum protease activity observed 6 h after feeding indicating that enzymes present in the chyme are still active when they reach the DG lumen. In *Octopus maya*, 480 min post-feeding a pH reduction suggests that the DG activity gradually reduced along with the enzymatic activity reduction in the DG lumen. At this time, a new release of digestive enzymes could be responsible for the low pH observed in the crop and DG in preparation for a new digestive cycle, suggesting that a reserve of active extracellular enzymes and an appropriate digestive environment pH being kept. At 360 min, postprandial DG acinar lumen observed empty. The absorption and nutrients passage from the digestive tract into the blood for distribution within the rest of the body indicated that the intracellular digestion is completed (figure 1h).

As expected, the residual bodies are absent in the digestive cell during fasting and growing as digestion takes place. A peak in the residual bodies density was observed in animals 360 min after feeding, indicating that the rests removal process has reached its maximum level. At 480 min postprandial period, a decrease in the DG acid and alkaline proteases activity were observed along with a very low residual bodies density stating that the digestive cycle has ended. Once intracellular digestion completed at 480 min postprandial, digestive cells are ready to start a new digestive cycle. Is observed a heterolysosomes reduction inside the digestive cell, thus, these stocks can be used for the glucose production. Considering that the cephalopods metabolism is essentially protein based, it is logical that the glycogen stored in the DG is synthesize via gluconeogenesis taking amino acids as substrate. The glycogen decreasing in the DG at 120 min after feeding points out that used as an energy source for the digestive process. The glycogen concentration restoration in the DG 480 min post-feeding shows that this nutrient was again stored in preparation for the next digestive cycle.

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