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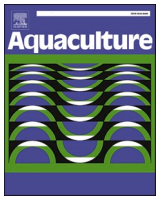

# Morphophysiological responses of *Octopus tehuelchus* juveniles during the transition period between endogenous and exogenous feeding

Ramiro Braga [a,b], Silvina Van der Molen [a], Yamila E. Rodriguez [c], Analía V. Fernández-Giménez [c], Nicolás Battini [d], Carlos Rosas [e], Nicolás Ortiz [a,*]

[a] *Laboratorio de Cefalópodos, Instituto de Biología de Organismos Marinos, IBIOMAR- CONICET, Boulevard Brown 2915, 9120 Puerto Madryn, Chubut, Argentina*
[b] *Universidad Nacional de la Patagonia San Juan Bosco, Ciudad Universitaria, 25 de Mayo km 4-, 9000 Comodoro Rivadavia, Chubut, Argentina*
[c] *Instituto de Investigaciones Marinas y Costeras, FCEyN, UNMdP-CONICET, CC1260, 7600 Mar del Plata, Buenos Aires, Argentina*
[d] *Grupo de Ecología en Ambientes Costeros, Instituto de Biología de Organismos Marinos, IBIOMAR-CONICET, Boulevard Brown 2915, 9120 Puerto Madryn, Chubut, Argentina*
[e] *Unidad Multidisciplinaria de Docencia e Investigación, Facultad de Ciencias, Universidad Nacional Autónoma de México, Puerto de abrigo s/n, Sisal, 97350 Hunucmá, Yucatán, Mexico*

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ABSTRACT

In cephalopod hatchlings there is a transitional period considered critical, in which the digestive system undergoes morphophysiological changes associated with the passage from the consumption of vitelline reserves to an independent feeding. During this period, the characterization of the digestive dynamics and growth is key information to define their nutritional requirements under culture. *Octopus tehuelchus*, which has become increasingly interesting for cultivation, is a species endemic to South America with holobenthic development and large juveniles. In this work, the enzymatic and cytological changes of the digestive gland (DG) along with the variation in body weight and size of *O. tehuelchus* hatchlings were evaluated when reared at 16 °C under two feeding treatments: fed with the isopod *Exosphaeroma* sp. and starved. In fed juveniles, the predatory activity began a few hours after hatching; and up to 6 days post hatching (DPH) there was neither somatic growth nor cellular differentiation of the DG, but there was an activation of acid enzymes. From 6 to 10 DPH, there was an increase in the size and weight of juveniles, and the maturation of the DG began with the appearance of heterolysosomes and heterophagosomes. Furthermore, lipases activity reached its maximum level and there was a considerable increase in both alkaline phosphatases and peptidases. From 15 to 25 DPH, the DG became fully mature and the lipase activity reached minimum levels, while the alkaline peptidases continued increasing. At the end of the experiment, at 25 DPH, fed juveniles doubled their weight. In the starved juveniles, neither somatic growth nor cytological differentiations of the DG were registered. Besides, in this treatment, acid phosphatases and lipase activities (i.e. enzymes related to yolk consumption) remained at basal levels or decreased over time, even with yolk platelets still present, suggesting that the consumption of yolk reserves would be delayed, regulating the survival time. In fed juveniles, the ingestion of isopods seems to act as a modulating factor of the digestive activity, triggering an increment in the activity of lipases, acid phosphatases, as well as alkaline peptidases which are related to the digestion of exogenous food. It is concluded that morphological and physiological changes during the digestive maturation are related to the ingestion of *Exosphaeroma* sp. In this sense, this isopod species would be a suitable food which promotes the rapid growth of *O. tehuelchus* during their early post-hatching stage under culture.

## 1. Introduction

In cephalopods, the post hatching period is critical because it is when the transition between consumption of the internal yolk reserves and independent feeding occurs. In this period, the hatchlings undergo physiological and morphological changes in both external and internal organs, which give them the ability to feed on exogenous resources (Boletzky, 1989; Martínez et al., 2011; Moguel et al., 2010; Vecchione,

* Corresponding author.
*E-mail address:* nicortiz@cenpat-conicet.gob.ar (N. Ortiz).

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1987). During this transition, understanding the processes involved in the absorption and assimilation of nutrients under different feeding conditions could provide useful information for the development of suitable protocols for cephalopod hatchlings' nutrition, a key bottleneck for their mass production (Gallardo et al., 2020; Iglesias et al., 2007; Sykes et al., 2017; Uriarte et al., 2018; Vidal et al., 2014).

The digestive processes in cephalopods consist of two major events: extracellular alkaline digestion and intracellular acid digestion. The extracellular digestion is generated by the mechanical action of the beak and the radula together with the enzymatic secretion (mainly chymotrypsin) of the salivary glands on the prey, forming the chyme. Then, this chyme is ingested through the mouth, passing to the anterior stomach (crop) and almost simultaneously to the posterior stomach, the caecum, and to the digestive gland (DG), where the intracellular digestion takes place (Boucher-Rodoni et al., 1987; Gallardo et al., 2017; Ibarra-García et al., 2018a). The lipids, proteins and polysacharides by intracellular digestion are hydrolyzed and transformed into acyl-glycerides, amino acids and carbohydrates, respectively (Boucaud-Camou et al., 1976; Boucher-Rodoni et al., 1987; Budelmann et al., 1997; Gallardo et al., 2017), which are then transported through the hemolymph for their use as an energy source for growth and tissue restoration, while the undigested material is eliminated (Boucher-Rodoni et al., 1987; Gallardo et al., 2017; Ibarra-García et al., 2018a). During the first days after hatching, the DG undergoes a series of important structural and functional changes. First, it acts as a vitelline reserve, and later as the main organ for intracellular digestion of ingested food through the action of digestive cells and the secretion of enzymes (Boucaud-Camou and Boucher-Rodoni, 1983; Ibarra-García et al., 2018a; Martínez et al., 2011; Moguel et al., 2010; Perrin et al., 2004; Pollero and Iribarne, 1988).

Digestive physiology is considered a crucial aspect in octopus aquaculture (Gallardo et al., 2017; Ibarra-García et al., 2018a). In these animals, whose natural diet is based mainly on crustaceans, fish and mollusks, the main metabolic substrate are proteins (Alejo-Plata et al., 2009; Estefanell et al., 2013; Gallardo et al., 2017; Krstulovíc and Vrgoc, 2009). The activity of peptidases is determinant in the effectiveness of the digestive processes (Boucaud-Camou and Boucher-Rodoni, 1983; Ibarra-García et al., 2018a; Morote et al., 2005). Alkaline peptidases (trypsin and chymotrypsin) contribute to the extracellular digestion, while acid peptidases (intracellular cathepsins) contribute to the intracellular digestion (Hamdan et al., 2014; Ibarra-García et al., 2018a; Martínez et al., 2011; Safi et al., 2018). Likewise, immediately after hatching there is a high activity of lipases and acid phosphatases that are associated with the degradation of yolk reserves (Ibarra-García et al., 2018b; Lacoue-Labarthe et al., 2010; Moguel et al., 2010; Pasteels, 1973; Perrin et al., 2004; Vidal et al., 2005), which are mainly composed of proteins, carbohydrates and lipids (Craig, 2001; Matozzo et al., 2015). This physiological response gives the juveniles the possibility of surviving during the first days, when food may be limited (Braga et al., 2021; Moguel et al., 2010; Segawa and Hanlon, 1988).

In cephalopods, unlike other animals such as fish, both intracellular and extracellular digestion occur simultaneously during all life stages (Boucaud-Camou and Roper, 1995; Lazo et al., 2007; O'Dor and Weber, 1986). The combination of both digestive processes results in a digestive system that is especially efficient in the assimilation of nutrients, and causes the rapid growth of individuals (Boucaud-Camou and Roper, 1995; Safi et al., 2018; Swift et al., 2005). However, studies carried out on squids such as *Loligo opalescens* (Forsythe and Van Heukelem, 1987; Vidal et al., 2002) and octopus as *O. maya* (Moguel et al., 2010) showed that immediately after hatching there was a time space where weight and size did not vary, followed by exponential growth. During this period, known as “no net growth”, the weight lost during the absorption of yolk reserves is recovered only through exogenous feeding, equaling the initial weight of the hatchlings (Parra et al., 2000; Vidal et al., 2002). This is a period where high mortality can be observed due to starvation's effects, eventually provoked when hatchlings do not have enough food to restore energy that allows initiating the growth, indicating the inability of hatchlings to resist starvation (Moguel et al., 2010; Uriarte et al., 2011; Vidal et al., 2002).

*Octopus tehuelchus* is a species endemic to South America that is distributed in the Atlantic Ocean (16°–45° S) from the intertidal area down to 100 m deep (Ré, 1998; Narvarte et al., 2006; Storero et al., 2010). In northern Argentinean Patagonia, this octopus species is an important artisanal fishing resource (Braga et al., 2021; Narvarte et al., 2006; Storero et al., 2010). Its holobenthic development and large juveniles, added to the biological and fishery knowledge of this species, facilitate the experimentation and the maintenance and growth of specimens in captivity, increasing their potential as a species for cultivation (Braga et al., 2021).

In the natural environment, the isopod *Exosphaeroma* sp. constitutes an important fraction of the juveniles' diet (Ré and Gómez-Simes, 1992). In previous experiments, carried out under controlled aquarium conditions, it was observed that juveniles of *O. tehuelchus* began hunting *Artemia* sp. the third day after hatching (Ré, 1989). Recently, it was recorded that in starved juveniles, the thermal regime modulates the use of vitelline reserves, the survival times, and the size of diverse body structures (Braga et al., 2021). However, what type and amount of food is required and how food impacts the digestive dynamics and growth during the first days of the life cycle is still unknown. In this context, knowing the morphophysiology dynamics of the DG under different feeding conditions is key to define the nutritional requirements and food characteristics that guarantee the viability of the *O. tehuelchus* hatchlings (Arvy, 1960; Gallardo et al., 2017; Mangold and Young, 1998; Martínez et al., 2011; Rosas et al., 2008). To characterize the *O. tehuelchus* digestive dynamics and growth during the early post-hatching period, we evaluated the following features in juveniles fed with live prey (fed) or not fed at all (starved): Enzymatic activity of lipases, acid and alkaline phosphatases, and aspartic, cysteine and alkaline peptidases; the ontogenetic changes of the DG, and changes in wet weight and dorsal mantle length.

## 2. Materials and methods

### 2.1. Collection and artificial incubation of eggs

*O. tehuelchus* spawning females along with egg masses were collected during the spawning period between April and August of 2018, in the Nuevo Gulf (42° 42′S 65° 36′ W), northern Patagonia, Argentina. Specimens were collected using traps and were transported to the Experimental Aquarium at the CCT CONICET-CENPAT (Centro Científico Tecnológico, Consejo Nacional de Investigaciones Científicas y Técnicas - Centro Nacional Patagónico) in 60-l containers with aerated seawater. A total of nine complete egg clutches were separated from the females after acclimatization and were artificially incubated in independent five-liter glass aquariums at 16 ± 1 °C, following the protocols for the incubation of eggs (Braga et al., 2021; Ortiz and Ré, 2011).

### 2.2. Rearing and maintenance of juveniles

Immediately after hatch, each hatchling was placed in an individual three-liter aquarium with two shelters (shells) at 16 °C in aerated, filtered (10, 5, and 1 μm), and UV-sterilized seawater. Seawater in the aquariums was maintained with dissolved oxygen >5 mg/l, pH of 7.5–8.4, total ammonia concentration of <1 mg/l, and salinity of 33–35 ppt. The photoperiod was 12 light: 12 dark. To maintain the seawater quality stable every 2 days 3/4 of the water volume was replaced.

### 2.3. Experimental design

To test the effects of feeding and age on the morphometric parameters, the cytological structure and the DG enzymatic dynamics of the juveniles, a factorial design was applied. Juveniles were randomly

assigned to two feeding treatments: starved and fed ad libitum once a day with isopods (*Exosphaeroma* sp.) obtained from the intertidal rocky areas of the Nuevo Gulf. The food consisted of live isopods of both sexes from 5 to 15 mm in length and from 0.003 to 0.333 g of weight. Six sampling points were selected for the starved juveniles (2, 4, 6, 8, 10, and 15 days post hatching, DPH) and 8 for fed juveniles (2, 4, 6, 8, 10, 15, 20, and 25 DPH). The starved treatment lacked samples 20 and 25 DPH because in previous experiences at 16 °C the hatchlings' survival time without feeding was, on average, 17 DPH (Braga et al., 2021). Additionally, measurements at 0 DPH were performed to provide an initial value for every variable. In total, 366 individually reared juveniles were used either for histological or enzymatic procedures, among them a random sample was previously used to measure the morphometric parameters. Before sampling, all the octopuses from the fed treatment were deprived of food for 12 h. (Moguel et al., 2010). In the morning, each juvenile was anesthetized with seawater at 2 °C and 1% alcohol (Butler-Struben et al., 2018; Fiorito et al., 2015) in order to perform all analyses considering animal welfare (Moltschaniwskyj et al., 2007).

### 2.4. Morphometric measurements

The wet weight (WW) in grams and dorsal mantle length (ML) in mm were measured in 150 juveniles: 10 individuals from 0 DPH plus 10 individuals from each feeding treatment in each sampling time. Juveniles were photographed under a stereomicroscope and ML was measured using the ImageJ software (Schneider et al., 2012). Each juvenile was considered a replicate.

### 2.5. Histological procedures

Cytological changes in the DG for each feeding treatment were evaluated on 3 individuals from each DPH. Animals were anesthetized and dissected to separate the DG from the rest of the body structures. Each DG was fixed in Bouin's solution for 24 h (Ávila-Poveda and Baqueiro-Cárdenas, 2009; Martínez et al., 2011) and preserved in 70% ethanol with 0.1% glycerin (Ávila-Poveda et al., 2009; Sweeney and Roper, 1983). Transversal cuts of DG were made with a manual rotary microtome (Leica RM 2225) following Ávila-Poveda et al. (2009). The 5 μm serial sections were stained with hematoxylin and eosin, and Masson's trichromic. Images for the identification of cellular structures were obtained with a Leica DFC 450 camera mounted on a Leica DM 2500 microscope. Cell structures from different DPH were identified following the classification and terminology proposed by Martínez et al. (2011).

### 2.6. Enzyme activity

To determine the specific enzymatic activity of digestive enzymes, a total of 42 (0 DPH), 153 (starved treatment), and 126 (fed treatment) DGs were used. For each enzyme, between 3 and 4 replicates per DPH per feeding treatment were analyzed. To achieve an adequate volume, each replica consisted of a pool of 3 DGs. All measurements were made in triplicate. After dissection, the DGs were immediately stored at −80 °C until analysis. Samples were freeze-dried and then transported to the Laboratory of "Fisiología de Organismos Acuáticos y Biotecnología Aplicada" (IIMyC, Mar del Plata, Argentina). Dried organs were resuspended in distilled water at 4 °C overnight. Then, the homogenates were prepared with distilled water 1:3 (*w/v*) and centrifuged at 10,000 ×*g* at 4 °C for 30 min. The soluble protein concentration of the supernatants was analyzed following the Bradford (1976) procedure.

The determination of lipase activity was made following the modified method of Nolasco-Soria et al. (2018). In a 96-well microplate, 10 μl of sodium taurocholate (100 mM), 50 μl of Tris-HCl buffer (200 mM) pH 8, and 10 μl of substrate β-naphthyl caprylate (20 mM) in dimethylsulfoxide (DMSO) were added to 10 μl of protein extract. After incubating for 9 min at 25 °C, 10 μl of fast blue (20 mM) in DMSO was added. Finally, 110 μl TCA-SDS (2% w/v TCA and 12.5% w/v SDS) was incorporated and the mix was stirred at 1500 rpm for 1 min. The absorbance of each sample was read at 540 nm. For the activity of acid and alkaline phosphatases, the methods of Moyano et al. (1996) and Principato et al. (1982) were followed. Ten microliters of 2% *p*-nitrophenyl-phosphate was added as a substrate in 1 M Tris HCl pH 3 (acid phosphatases) or 1 M Tris HCl pH 10 (alkaline phosphatases) buffer to 10 μl of homogenate poured on flat bottom plates. After incubating for 30 min at 25 °C, 100 μl of 1 M NaOH were added. The absorbance was measured at 405 nm.

The activity of acidic (pH 4) peptidases, possibly aspartic peptidases, was analyzed following Bonete et al. (1984). For this, 250 μl of 0.4 M acetate buffer (pH 4) and 250 μl of hemoglobin (0.5% w/v), as substrate, were added to 5 μl of homogenate. The reaction was incubated at 37 °C for 60 min and then 250 μl of TCA (20% w/v) was added. The sample cooled down for 10 min, and next centrifuged at 10,000 ×*g* for 5 min. The absorbance was measured at 280 nm. The activity of mild acidic (pH 6) peptidase, possibly cysteine peptidase, was measured following the modified protocol reported by Cárdenas-López and Haard (2005), where 5 μl of homogenate and 250 μl of 100 mM sodium phosphate buffer (pH 6) containing 1 mM of ethylenediamine tetra-acetic acid (EDTA) were added to the reaction tube. Then, 250 μl of azocasein substrate (0.5% w/v) was added and next incubated at 37 °C for 60 min. Afterward, 250 μl of TCA (20% w/v) was added, the reaction was cooled for 10 min, and then centrifuged at 10,000 ×*g* for 5 min. The absorbance was measured at 366 nm (González-Zamorano et al., 2013). The alkaline peptidases activity was determined following the method described by Sarath et al. (1989) modified by Nolasco-Soria (2021). To 5 μl of homogenate, 250 μl of 0.05 M Tris-HCl/10 mM $CaCl_2$ (pH 8.1) buffer and 250 μl of azocasein (0.5% w/v) as substrate were added. After incubation at 37 °C for 60 min, 250 μl TCA (20% w/v) was added to stop the reaction. The reaction was cooled for 10 min and then tubes were centrifuged at 10,000 ×*g* for 5 min. The absorbance was measured at 366 nm. For all peptidases, the temperature used to measure enzyme activity was standardized based on Safi et al. (2018) protocols.

All enzyme activities were calculated in the same way to unify units and thus expressed as U mg-1 protein, where one unit of enzyme activity was defined as the change of absorbance per min.

### 2.7. Statistical analyses

The morphometric variables (WW and ML) and the specific enzymatic activity were compared among feeding treatments for each age (DPH) using linear models, with a statistical significance of $p < 0.05$. Given that most variables did not show a linear relationship with age, age was considered categorical. Data of 0 DPH was not used in the analyses, and neither were the data from fed juveniles of 20 and 25 DPH, as there was not a starved treatment for those DPH. Whenever heteroscedasticity was detected, a power variance structure was applied to the model. When the interaction between feeding treatments and DPH was significant, the response variables from both feeding treatments were compared for each DPH using similar linear models. In cases where significant differences were detected only for DPH, multiple comparisons were performed using Tukey tests. All the analyses were performed in R version 4.0.3. (R Core Team, 2020) using package 'nlme' (Pinheiro et al., 2016). The results obtained are reported as mean values and standard deviations.

## 3. Results

### 3.1. Morphometric changes and behavior in juveniles of O. tehuelchus

For both morphometric variables, the significant differences were explained by the interaction between age (DPH) and feeding treatment (Table 1). The ML and WW at hatching were 6.68 ± 0.74 mm and 0.16 ± 0.04 g, respectively. Regardless of the feeding treatment, both

**Table 1**
Results of the linear models. Effects of feeding treatment, age (DPH), and their interaction with morphometric parameters of *Octopus tehuelchus* juveniles.

| Source of variation | DF | WW | | ML | |
|---|---|---|---|---|---|
| | | F value | *p*-Value | F value | p-Value |
| Feeding Treatment | 1 | 27.4915 | <0.0001 | 16.280 | 0.0001 |
| Age (DPH) | 5 | 5.7587 | <0.0001 | 1.9428 | 0.0931 |
| Age (DPH) × Feeding Treatment | 5 | 6.3272 | <0.0001 | 3.918 | 0.0027 |

morphometric variables behaved similarly over time (Fig. 1). For the starved treatment, no substantial changes were observed throughout the experience. Instead, in the fed treatment, both variables progressively increased over the days, registering their maximum peaks at 20 DPH for ML (7.35 ± 1.16) and at 25 DPH for WW (0.34 ± 0.08 g), doubling the WW from hatching. For both morphometric variables, significant differences between feeding treatments were registered at the same time of development, being higher in juveniles fed with isopods (Fig. 1). For WW, significant differences were observed at 6 DPH ($\chi 2 = 9.6486$, $p = 0.0018$), 8 DPH ($\chi 2 = 4.3067$, $p = 0.0379$), 10 DPH ($F = 37.137$, $p < 0.0001$), and 15 DPH ($F = 14.824$, $p = 0.0011$) (Fig. 1a). Differences in ML were found at 6 DPH ($F = 8.046$, $p = 0.0109$), 10 DPH ($F = 10.537$, $p = 0.0044$), and 15 DPH ($F = 14.378$, $p = 0.0013$) (Fig. 1b). For both treatments, juveniles exhibited a benthic behavior as soon as they hatched, placing themselves under the shelters or exploring the bottom of the aquarium. In the fed treatment, juveniles reacted to visual stimuli such as the erratic movement and fast swimming of *Exosphaeroma* sp. on the bottom; they began to feed a few hours after hatching (i.e. between 0 and 1 DPH).

### 3.2. Cytological changes in the digestive gland

In DG histological sections of the starved juveniles no cellular differentiation was seen, unlike what was observed in the hatchlings fed with isopods (Fig. 2). In newborn juveniles (0 DPH), the DG was completely covered with yolk platelets (Fig. 2a). For both feeding treatments, up to 6 DPH, the poorly differentiated tubules of the DG contained large yolk platelets covering the entire surface, though in some tubules from fed juveniles it was observed a small central lumen (Fig. 2b, c). In starvation, after 6 DPH, the central lumen of the tubules expanded and the size and amount of the yolk platelets decreased until they were almost entirely consumed between 10 and 15 DPH (Fig. 2d, f). On the contrary, in the fed treatment from 6 to 10 DPH, the cellular differentiation began and the heterolysosomes appeared. Besides, the central lumen expanded considerably while the yolk platelets decreased in amount and size. Likewise, heterophagosomes appeared and the lateral membrane in the tubules became visible (Fig. 2e). At 15 DPH, the amount of yolk in fed juveniles was completely reduced, and the secretory lysosomes and residual bodies began to appear (Fig. 2g). Finally, at 25 DPH, the microvilli of the digestive cells of the tubules became visible (Fig. 2h). At the end of comparison (15 DPH), it was observed that the DG from the starved treatment had more yolk and larger yolk platelets compared to the fed treatment (Fig. 2f, g).

### 3.3. Enzymatic activity in the digestive gland

The different enzymes showed varied responses to age (DPH) and feeding treatment. The specific activity of lipases was significantly affected only by age. Differences in acid and alkaline phosphatases and in cysteine and alkaline peptidases were explained by the interaction between both factors. The acid peptidase (defined in this study as aspartic peptidase) activity was not significantly affected by any of the factors nor by their interaction (Table 2).

In the starved treatment, lipases registered a maximum peak of specific activity at 4 DPH, 6 days earlier than for the fed treatment. After those peaks, in both feeding treatments, the lipase activity decreased until the end of the assay (Fig. 3a). The Tukey test showed that 4 and 10 DPH were significantly different from 15 DPH. In the starved treatment, the activity of both phosphatases remained low and with little variation during the 15 days of the experiment (Fig. 3b, c). On the contrary, for the fed treatment, the phosphatases activity increased over time, reaching the maximum activity at 15 DPH for acid phosphatases, and between 10 and 20 DPH for alkaline phosphatases (Fig. 3b, c). When comparing between feeding treatments, a significantly higher activity was observed in fed juveniles in the following DPH: 4 ($\chi^2 = 24.514$, $p < 0.0001$), 6 ($\chi^2 = 35.392$, $p < 0.0001$), 10 ($\chi^2 = 52.6445$, $p < 0.0001$) and 15 ($F = 1676$ and $p < 0.0001$) for acid phosphatases, and 4 ($F = 7.324$, $p = 0.0068$), 8 ($\chi^2 = 11.375$, $p = 0.0007$), 10 ($\chi^2 = 18.609$, $p < 0.0001$) and 15 ($\chi^2 = 17.596$, $p < 0.0001$) for alkaline phosphatases (Fig. 3b).

For the aspartic peptidases, no significant differences were detected between feeding treatments (Fig. 3d). Regarding mild acid peptidase (defined in this study as cysteine peptidase), unlike the rest of the enzymes, the specific activity in the fed treatment remained low and with slight variations, in contrast to the starved treatment. There were

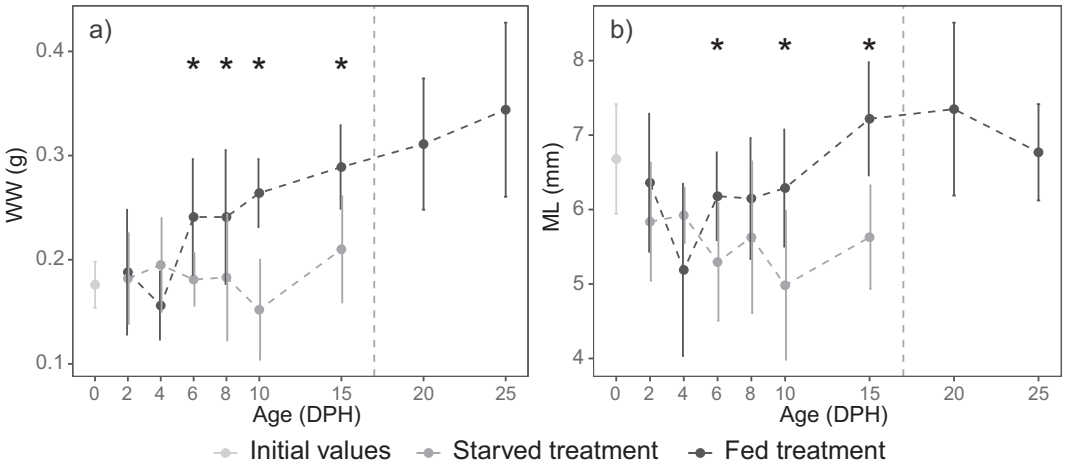


**Fig. 1.** Relationship between the juvenile morphometric parameters and age (DPH) of *Octopus tehuelchus* as a function of the starved (dark gray points) and fed (black points) treatments. The initial values are shown as light gray points. a) Wet weight (WW), b) dorsal mantle length (ML). Vertical bars indicate the standard deviation. Asterisks mark significant differences between feeding treatments for each DPH. The vertical dotted line indicates the average survival time in starvation (days) of juveniles (Braga et al., 2021).

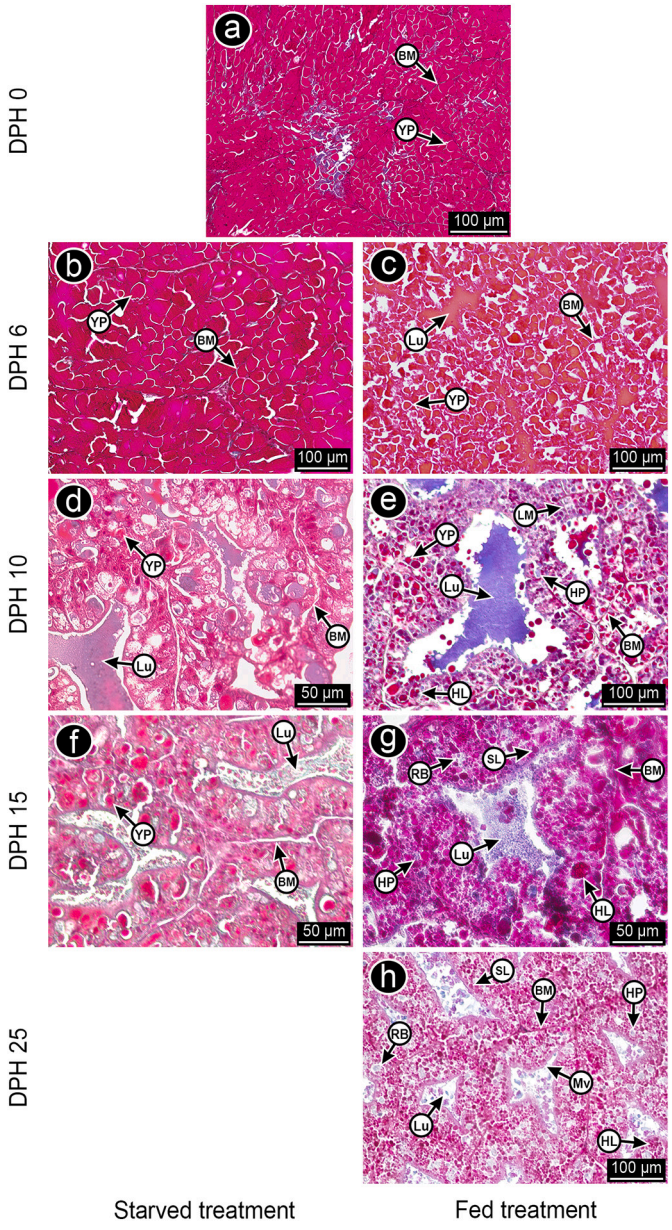


**Fig. 2.** Cytological changes of the digestive gland in juveniles of *Octopus tehuelchus* at different days post hatching (DPH), at birth (A), in starved (B, D and F) and fed (C, E, G and H) treatment. BM: basement membrane, HL: heterolysosomes, HP: heterophagosomes, LM: lateral membrane, Lu: lumen, Mv: microvilli, RB: residual bodies, SL: secretory lysosomes YP: yolk platelets. The sections were stained with Hematoxylin and Eosin (A, B, C, and D) and with Masson's trichromic (E, F, G, and H).

significant differences between feeding treatments at 8 ($\chi^2 = 5.338$, $p = 0.0209$) and 15 DPH ($\chi^2 = 4.159$, $p = 0.0414$), being higher in the starved specimens (Fig. 3e). For the alkaline peptidases, the starved treatment showed uniform activity throughout the days. In the fed treatment, the activity increased to its maximum levels at 20 DPH (Fig. 3f). A higher and significant activity was registered at 2 DPH ($\chi^2 = 5.847$, $p = 0.0156$) in starved juveniles and at 10 DPH ($\chi^2 = 8.980$, $p = 0.0027$) in fed juveniles (Fig. 3f).

## 4. Discussion

During the early post-hatching period, many species of cephalopods go through critical phases until the hatchlings reach the full juvenile stage. A high lipid metabolism (Boucher-Rodoni et al., 1987; Segawa and Hanlon, 1988; Vidal et al., 2002), a no net growth period (Vidal et al., 2002), an immature DG without cell differentiation (Safi et al., 2018; Vecchione and Hand, 1989), and absent or erratic prey consumption (Boletzky, 1975; Moguel et al., 2010; Rosas et al., 2007, 2008; Wells, 1958) are some of the characteristics present during this period. Moreover, in a holobenthic species such as *O. maya*, a nectobenthic behavior was observed during the first weeks after hatching (Moguel et al., 2010). During the first 6 DPH, *O. tehuelchus* shows certain characteristics typical of an early post-hatching phase: a no net growth period, an immature DG denoted by the presence of yolk platelets only, and certain attributes typical of a full juvenile stage such as a totally benthic behavior and the regular consumption of live prey from the first day of life. The no net growth period in *O. tehuelchus* lasts as long as in *S. officinalis* and is shorter than in *O. maya*, *L. opalescence*, *L. vulgaris reynaudii* and *Robsonella fontaniana* (Espinoza et al., 2017; Moguel et al., 2010; Safi et al., 2018; Vidal et al., 2002, 2005). In *O. tehuelchus*, the duration of this period could be related to the type of food offered. In this sense, certain characteristics such as food palatability and the visual and chemical stimuli generated by prey could lead to an increase in the predatory behavior of hatchlings (Langridge, 2009; Moguel et al., 2010; Querol et al., 2015; Uriarte et al., 2018). These food characteristics along with the nutritional composition (Caamal-Monsreal et al., 2015; Iglesias et al., 2000) would promote the activation of digestive enzymes and the subsequent growth of hatchlings (Moguel et al., 2010; Safi et al., 2018). Regarding the digestive activity, only an increase in lipase and acid phosphatase activity is evidenced in our study; these enzymes are responsible for the degradation of yolk reserves (Ibarra-García et al., 2018b; Lacoue-Labarthe et al., 2010; Moguel et al., 2010; Pasteels, 1973; Perrin et al., 2004; Vidal et al., 2005). Lipases reach their maximum activity in starved juveniles but remain at basal levels in fed juveniles, while the acid phosphatase activity only increased in fed juveniles. This pattern indicates that enzymatic dynamics at this time in juveniles would be related to yolk consumption, although conditioned by food ingestion. However, during this period, this enzymatic activity is not apparently reflected in the cytological differentiation of the DG nor in the juveniles' somatic growth.

Cephalopod hatchlings must be supplied with food that allows them to develop normally before they exhaust their last vitelline reserves (Vidal et al., 2002). At this stage the morphology of the DG and the digestive enzymatic activity change, allowing the use of nutrients from endogenous and exogenous food (Gallardo et al., 2017; Moguel et al., 2010; Morote et al., 2005; Rosas et al., 2008; Solorzano et al., 2009). In fed *O. tehuelchus,* between 6 and 10 DPH, the maximum peak in lipases activity and the presence of heterolysosomes and heterophagosomes, which are involved in the digestion of nutrients in the DG and their mobilization through the hemolymph (Gallardo et al., 2017; Linares et al., 2015; Martínez et al., 2011), begin to be observed. Furthermore, the activity of alkaline peptidases and phosphatases increases. These enzymes are linked to the extracellular digestion of exogenous resources (Boucaud-Camou and Roper, 1995; Hamdan et al., 2014; Ibarra-García et al., 2018a; Martínez et al., 2011; Perrin et al., 2004), and also to the absorption and active transport of nutrients (Boucaud-Camou and Roper, 1995). These findings show that food ingestion increases the consumption of yolk reserves and triggers the morphophysiological maturation of the DG, resulting in juveniles suitable for the transformation of the endogenous and exogenous resources into biomass. Between 15 and 25 DPH, the presence of secretory lysosomes, residual bodies, microvilli of digestive cells, and the depletion of yolk platelets associated with the lowest lipase activity and maximum alkaline enzymes activities indicate that the DG of *O. tehuelchus* has reached its full maturity. A similar maturity pattern was also observed in *O. maya* (López-Ripoll, 2010; Martínez et al., 2011; Rosas et al., 2007). In consequence, at around 20 DPH *O. tehuelchus* will depend exclusively on exogenous food to meet its energy demands.

Previous studies have shown that cephalopods hatchlings can

**Table 2**
Results of the linear models showing the main effects of feeding treatment and age (DPH) and their interaction on the enzymatic activity of each enzyme in the digestive gland of juvenile *Octopus tehuelchus*.

| Source of variation | DF | Lipases | | Acid phosphatases | | Alkaline phosphatases | |
|---|---|---|---|---|---|---|---|
| | | $\chi 2$ value | *p*-Value | $\chi 2$ value | *p*-Value | $\chi 2$ value | *p*-Value |
| Feeding Treatment | 1 | 0.565 | 0.4522 | 140.32 | <0.0001 | 2.392 | 0.1219 |
| Age (DPH) | 5 | 17.832 | 0.0032 | 1310.95 | <0.0001 | 5.864 | 0.2096 |
| Age (DPH) × Feeding Treatment | 5 | 10.200 | 0.0698 | 298.02 | <0.0001 | 19.690 | 0.0006 |
| | | Aspartic proteases | | Cystein proteases | | Alkaline proteases | |
| Feeding Treatment | 1 | 0.1086 | 0.7440 | 7.436 | 0.0064 | 0.071 | 0.7900 |
| Age (DPH) | 5 | 2.125 | 0.0887 | 12.128 | 0.0331 | 16.884 | 0.0047 |
| Age (DPH) × Feeding Treatment | 5 | 1.034 | 0.4154 | 13.471 | 0.0193 | 13.577 | 0.0185 |

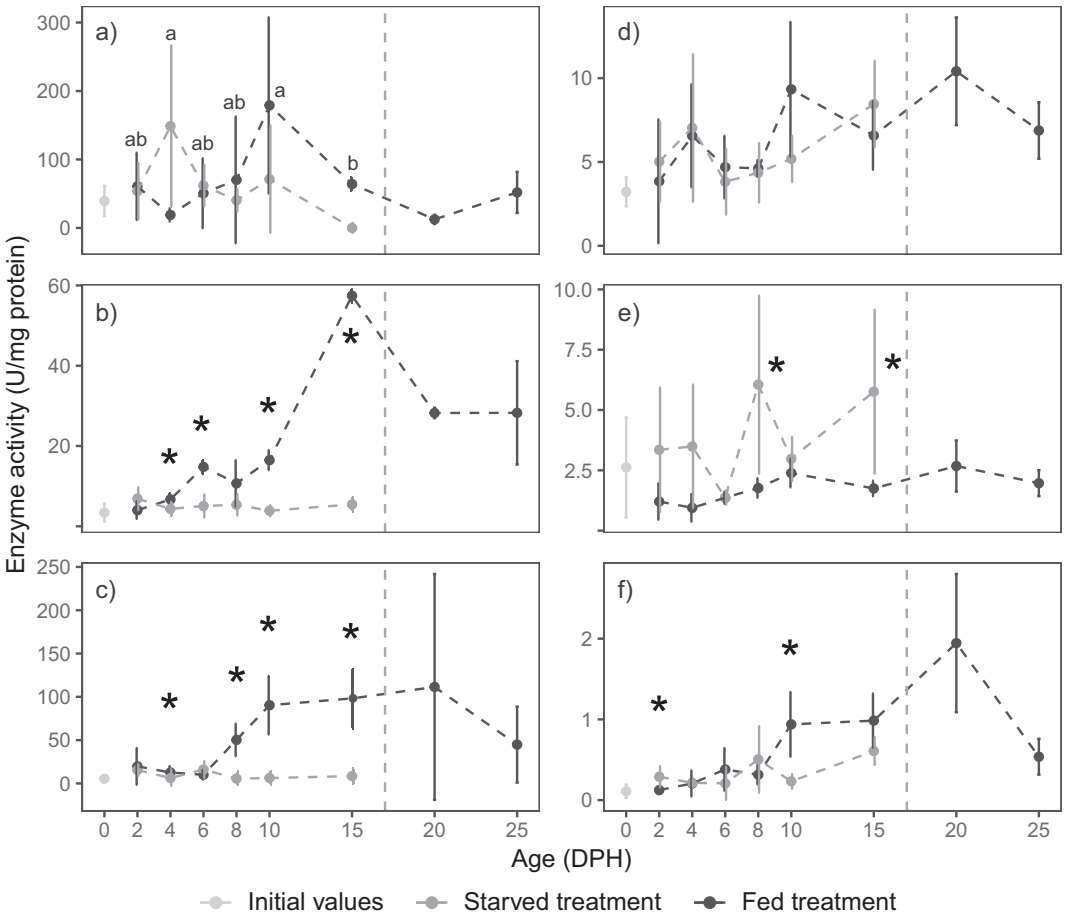


**Fig. 3.** Relationship between the juveniles DG enzymatic activity and age (DPH) of *Octopus tehuelchus* as a function of the starved (dark gray points) and fed (black points) treatments. The initial values are shown as light gray points. a) Lipases, b) acid phosphatases, c) alkaline phosphatases, d) aspartic peptidases, e) cysteine peptidases, f) alkaline peptidases. The vertical bars indicate the standard deviation. The asterisks mark significant differences between feeding treatments for each DPH. The vertical dotted line indicates the average survival time in starvation (days) of the juveniles (Braga et al., 2021). Similar letters indicate no significant differences between DPH of the same feeding treatment.

regulate the use of their yolk reserves based on their feeding conditions (Boletzky, 2003; Vidal et al., 2002). Particularly, under starvation conditions, hatchlings undergo morphophysiological changes and decrease their metabolic activity to survive using their reserves until food is available (Espinoza et al., 2017; Gebauer et al., 2010; Rosas et al., 2011). Considering that *O. tehuelchus* can survive starvation up to 59 days at 16 °C (Braga et al., 2021) and that in starved juveniles of 15 DPH lipase activity declines to minimal levels while yolk platelets are still present, it is suggested that the consumption of yolk reserves would be delayed mainly by the reduction of the lipase activity, which would help extend the survival time. Even more, cero changes on WW and ML recorded in *O. tehuelchus* during the 15 days of starvation indicates that the consumption of yolk reserves was destined to the maintenance of body structures and not for body growth, as was observed in other cephalopods species (Espinoza et al., 2017; Rosas et al., 2011; Vidal et al., 2006).

Another interesting enzymatic response is the high and erratic activity of cysteine peptidases in starved juveniles compared to the activity in the fed ones. This enzyme has been little studied. In starved juveniles of *O. tehuelchus,* the high activity of cysteine peptidases seems to be related to the intracellular acid digestion, as it occurs in *Dosidicus gigas* squid (Cárdenas-López and Haard, 2005, 2009), and to the digestion of the proteins present in the yolk reserves, as in *Xenopus laevis* frog

(Yoshizaki and Yonezawa, 1998). Moreover, in squid *Euprymna scolopes*, this enzyme has been related to the degradation of tissues associated with infections (Peyer et al., 2018). Thus, considering the evidence in other taxa, it is suggested that the increase in cysteine peptidases after the depletion of yolk reserves in *O. tehuelchus* juveniles could be pathological, as a response to a condition such as an undernutrition.

Several studies carried out in cephalopod hatchlings have classified the relationship between digestive processes and the age after hatch into several steps considering multiple factors; for example, the determination of stages according to the cytological ontogeny of the DG (López-Ripoll, 2010; Martínez et al., 2011), or the delimitation of events based on the type of digestion and enzymatic dynamics (Boucher-Rodoni et al., 1987; Gallardo et al., 2017). Based on the aforementioned findings, three developmental phases were recognized in *O. tehuelchus* when reared at 16 °C. Phase I, from birth to 6 DPH, is characterized by no net-growth, an immature DG and a prevalence of intracellular acid digestion, mainly related to the degradation of vitelline reserves. Phase II, between 6 and 10 DPH, when hatchling growth and the beginning of the DG's maturation are evident. This phase is considered a transition period between an endogenous and an exogenous feeding (Martínez et al., 2011; Moguel et al., 2010) linked to the enzymatic adjustments from an acid intracellular digestion to an alkaline extracellular digestion. Finally, phase III, between 15 and 25 DHP, is determined by the continuous growth of juveniles, full maturation of the DG, and the predominance of extracellular alkaline digestion. Starved juveniles did not overcome phase I. These developmental phases provide an integrating framework to understand the maturation of the digestive processes, and to perform comparative studies on the digestive ontogeny and juvenile performance.

Assessing the activity of digestive enzymes is a widely used procedure in studies of the maturation and digestive capacity of the digestive tract in species of aquaculture importance (Caruso et al., 2009; Gallardo et al., 2017; Nimrat et al., 2013). However, enzymatic assays are not standardized and the methodology chosen may affect the quantification of enzymatic activity and bias absolute values (Bisswanger, 2014; Nolasco-Soria, 2021). For example, when the azocasein substrate is hydrolyzed, it releases soluble compounds with the associated azo pigment but also non-chromatophoric pigments. This does not allow us to know how many peptide bonds were hydrolyzed, so the enzyme activity would be underestimated (Nolasco-Soria, 2021). Besides, in the present study, the peptidase activity assays were performed at a temperature (37 °C) higher than the range of temperature to which the species is adapted (8–24 °C) (Ciotti et al., 1995; Rivas and Beier, 1990; Williams et al., 2018). This would increase the rate of enzyme reaction, giving an overestimation of real enzymatic activity values (Bisswanger, 2014; Nolasco-Soria, 2021). Notwithstanding the above, same as in other studies on cephalopods (Gallardo et al., 2020; Morote et al., 2005; Perrin et al., 2004; Semmens, 2002; Solorzano et al., 2009), in the present work the enzyme essays were performed under the same experimental conditions for both treatments; thus, enabling a reliable comparative evaluation of the digestive dynamics between treatments.

As expected, the morphological and physiological changes associated to the DG maturation of *O. tehuelchus* are related to age, as in *O. maya* (López-Ripoll, 2010; Martínez et al., 2011); but also to the ingestion of appropriate food. In this sense, this is the first work that feeds isopods to cephalopods hatchlings and our feeding trials showed that *Exosphaeroma* sp. is a suitable live prey under culture conditions that supports a fast juvenile growth. With this live food, juveniles of *O. tehuelchus* can feed endogenously and exogenously simultaneously and successfully combine acid intracellular digestion and alkaline extracellular digestion, doubling their weight 25 days after hatching. At this age, the apparent decrease in DML could be related to an allometric growth or, as in other cephalopod species, to the high individual variability in relation to growth (Alford and Jackson, 1993; Domain et al., 2000; Ho et al., 2004; Semmens et al., 2004). Our findings are the starting point for identifying optimal dietary formulations (natural or artificial) and studying feeding efficiency, which will be necessary to develop hatchery technologies for *O. tehuelchus*.

## CRediT authorship contribution statement

**Ramiro Braga:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Silvina Van der Molen:** Conceptualization, Investigation, Resources, Writing – review & editing, Project administration, Funding acquisition. **Yamila E. Rodriguez:** Investigation, Writing – review & editing, Formal analysis. **Analía V. Fernández-Giménez:** Investigation, Writing – review & editing, Formal analysis. **Nicolás Battini:** Investigation, Writing – review & editing, Formal analysis. **Carlos Rosas:** Investigation, Writing – review & editing. **Nicolás Ortiz:** Conceptualization, Methodology, Validation, Investigation, Resources, Supervision, Formal analysis, Writing – review & editing, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## References

Alejo-Plata, M.C., Gómez-Marquez, J.L., Ramos-Carrillo, S., Herrera- Galindo, J.E., 2009. Reproducción, dieta y pesquería del pulpo *Octopus* (Octopus) *hubbsorum* (Mollusca: Cephalopoda) en la costa de Oaxaca, México. Int. J. Trop. Biol. 57, 63–78. https://doi.org/10.15517/rbt.v57i1-2.11291.

Alford, R.A., Jackson, G.D., 1993. Do cephalopods and larvae of other taxa grow asymptotically? Am. Nat. 141 (5), 717–728.

Arvy, L., 1960. Histoenzymological data on the digestive tract of *Octopus vulgaris* Lamarck (Cephalopoda). Ann. N. Y. Acad. Sci. 90, 929–949.

Ávila-Poveda, O.H., Baqueiro-Cárdenas, E.R., 2009. Reproductive cycle of *Strombus gigas* Linnaeus 1758 (Caenogastropoda: Strombidae) from Archipelago of San Andrés, Providencia and Santa Catalina, Colombia. Invertebr. Reprod. Dev. 53, 1–12. https://doi.org/10.1080/07924259.2009.9652284.

Ávila-Poveda, O.H., Colin-Flores, R.F., Rosas, C., 2009. Gonad development during the early life of *Octopus maya* (Mollusca: Cephalopoda). Biol. Bull. 216, 94–102. https://doi.org/10.1086/BBLv216n1p94.

Bisswanger, H., 2014. Enzyme assays. Perspect. Sci. 1 (1–6), 41–55. https://doi.org/10.1016/j.pisc.2014.02.005.

Boletzky, S.V., 1975. A contribution to the study of yolk absorption in the Cephalopoda. Z. Morphol. Tiere. 80, 229–246.

Boletzky, S.V., 1989. Recent studies on spawning, embryonic development, and hatching in the Cephalopoda. Adv. Mar. Biol. 25, 85–115. https://doi.org/10.1016/S0065-2881(08)60188-1.

Boletzky, S.V., 2003. Biology of early life in cephalopod molluscs. Adv. Mar. Biol. 44, 144–203.

Bonete, M.J., Manjon, A., LIorca, F., Iborra, J.L., 1984. Acid proteinase activity in Fish – I. Comparative study of extraction of cathepsins B and D from *Mujil auratus*. Comp. Biochem. Physiol. B 78 (1), 203–206. https://doi.org/10.1016/0305-0491(84)90169-X.

Boucaud-Camou, E., Boucher-Rodoni, R., 1983. Feeding and digestion in cephalopods. In: Saleuddin, A.S.M., Wilbur, K.M. (Eds.), The Mollusca. Physiology, Vol. 5. Academic Press, New York, pp. 149–187.

Boucaud-Camou, E., Roper, C.F., 1995. Digestive enzymes in paralarval cephalopods. Bull. Mar. Sci. 57, 313–327.

Boucaud-Camou, E., Boucher-Rodoni, R., Mangold, K., 1976. Digestive absorption in *Octopus vulgaris* (Cephalopoda: Octopoda). J. Zool. 179, 261–271. https://doi.org/10.1111/j.1469-7998.1976.tb02295.x.

Boucher-Rodoni, R., Boucaud-Camou, E., Mangold, K., 1987. Feeding and digestion. In: Boyle, P.R. (Ed.), Cephalopod Life Cycles. Academic Press London, London, pp. 85–108.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Braga, R., Van der Molen, S., Pontones, J., Ortiz, N., 2021. Embryonic development, hatching time and newborn juveniles of *Octopus tehuelchus* under two culture

temperatures. Aquaculture 530, 735778. https://doi.org/10.1016/j.aquaculture.2020.735778.
Budelmann, B.U., Schipp, R., Boletzky, S.V., 1997. Cephalopoda. In: Harrison, F.W., Kohn, A. (Eds.), Microscopic Anatomy of Invertebrates. Willey-Liss, New York, NY, pp. 119–414.
Butler-Struben, H.M., Brophy, S.M., Johnson, N.A., Crook, R.J., 2018. In vivo recording of neural and behavioral correlates of anesthesia induction, reversal, and euthanasia in cephalopod molluscs. Front. Physiol. 9, 109. https://doi.org/10.3389/fphys.2018.00109.
Caamal-Monsreal, C., Mascaró, M., Gallardo, P., Gallardo, P., Rodriguez, S., Noreña-Barroso, E., Rosas, C., 2015. Effects of maternal diet on reproductive performance of *O. maya* and its consequences on biochemical characteristics of the yolk, morphology of embryos and hatchlings quality. Aquaculture 441, 84–94. https://doi.org/10.1016/j.aquaculture.2015.01.020.
Cárdenas-López, J.L., Haard, N.F., 2005. Cysteine proteinase activity in jumbo squid (*Dosidicus gigas*) hepatopancreas extracts. J. Food Biochem. 29, 171–186. https://doi.org/10.1111/j.1745-4514.2005.00009.x.
Cárdenas-López, J.L., Haard, N.F., 2009. Identification of a cysteine proteinase from jumbo squid (*Dosidicus gigas*) hepatopancreas as cathepsin L. Food Chem. 112, 442–447. https://doi.org/10.1016/j.foodchem.2008.05.100.
Caruso, G., Denaro, M., Genovese, L., 2009. Digestive enzymes in some teleost species of interest for Mediterranean aquaculture. Open Fish Sci. J. 2 (1) https://doi.org/10.2174/1874401X00902010074.
Ciotti, Á.M., Odebrecht, C., Fillmann, G., Moller, O.O., 1995. Freshwater outflow and subtropical convergence influence on phytoplankton biomass on the southern Brazilian continental shelf. Cont. Shelf Res. 15, 1737–1756. https://doi.org/10.1016/0278-4343(94)00091-Z.
Craig, S., 2001. Environmental Conditions and Yolk Biochemistry: Factors Influencing Embryonic Development in the Squid *Loligo forbesi* (Cephalopoda: Loliginidae) Steenstrup 1856. University of Aberdeen (United Kingdom).
Domain, F., Jouffre, D., Caveriviere, A., 2000. Growth of *Octopus vulgaris* from tagging in Senegalese waters. J. Mar. Biol. Assoc. UK 80, 699–705. https://doi.org/10.1017/S0025315400002526.
Espinoza, V., Viana, M.T., Rosas, C., Uriarte, I., Farías, A., 2017. Effect of starvation on the performance of baby octopus (*Robsonella fontaniana*) paralarvae. Aquac. Res. 48, 5650–5658. https://doi.org/10.1111/are.13387.
Estefanell, J., Roo, J., Guirao, R., Afonso, J.M., Fernández-Palacios, H., Izquierdo, M., 2013. Efficient utilization of dietary lipids in *Octopus vulgaris* (Cuvier 1797) fed fresh and agglutinated moist diets based on aquaculture by-products and low price trash species. Aquac. Res. 44, 93–105. https://doi.org/10.1111/j.1365-2109.2011.03014.x.
Fiorito, G., Affuso, A., Basil, J., Cole, A., de Girolamo, P., D'angelo, L., Andrews, P.L., 2015. Guidelines for the care and welfare of cephalopods in research–a consensus based on an initiative by CephRes, FELASA and the Boyd Group. Lab. Anim. 49, 1–90. https://doi.org/10.1177/0023677215580006.
Forsythe, J.W., Van Heukelem, W.F., 1987. Growth. In: Boyle, P.R. (Ed.), Cephalopod Life Cycles. Academic Press, London, pp. 135–155.
Gallardo, P., Olivares, A., Martínez-Yáñez, R., Caamal-Monsreal, C., Domingues, P.M., Mascaró, M., Rosas, C., 2017. Digestive physiology of *Octopus maya* and *O. mimus*: temporality of digestion and assimilation processes. Front. Physiol. 8, 355. https://doi.org/10.3389/fphys.2017.00355.
Gallardo, P., Villegas, G., Rosas, C., Domingues, P., Pascual, C., Mascaró, M., Rodríguez, S., 2020. Effect of different proportions of crab and squid in semi-moist diets for *Octopus maya* juveniles. Aquaculture 524, 735233. https://doi.org/10.1016/j.aquaculture.2020.735233.
Gebauer, P., Paschke, K., Anger, K., 2010. Seasonal variation in the nutritional vulnerability of first-stage larval porcelain crab, *Petrolisthes laevigatus* (Anomura: Porcellanidae) in southern Chile. J. Exp. Mar. Biol. Ecol. 386, 103–112. https://doi.org/10.1016/j.jembe.2010.02.016.
González-Zamorano, M., Navarrete del Toro, M.A., García-Carreño, F.L., 2013. Exogenous proteinases as feed supplement for shrimp: in vitro evaluation. Aquac. Nutr. 19 (5), 731–740. https://doi.org/10.1111/anu.12020.
Hamdan, M., Tomás-Vidal, A., Martínez, S., Cerezo-Valverde, J., Moyano, F.J., 2014. Development of an in vitro model to assess protein bioavailability in diets for common octopus (*Octopus vulgaris*). Aquac. Res. 45, 2048–2056. https://doi.org/10.1111/are.12155.
Ho, J.D., Moltschaniwskyj, N.A., Carter, C.G., 2004. The effect of variability in growth on somatic condition and reproductive status in the southern calamary *Sepioteuthis australis*. Mar. Freshw. Res. 55, 423–428. https://doi.org/10.1071/MF03149.
Ibarra-García, L.E., Mazón-Suástegui, J.M., Rosas, C., Tovar-Ramírez, D., Bárcenas-Pazos, G., Civera-Cerecedo, R., Campa-Córdova, A.I., 2018a. Morphological and physiological changes of *Octopus bimaculoides*: from embryo to juvenile. Aquaculture 497, 364–372. https://doi.org/10.1016/j.aquaculture.2018.07.069.
Ibarra-García, L.E., Tovar-Ramírez, D., Rosas, C., Campa-Córdova, Á.I., Mazón-Suástegui, J.M., 2018b. Digestive enzymes of the Californian two-spot octopus, *Octopus bimaculoides* (Pickford and McConnaughey, 1949). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 215, 10–18. https://doi.org/10.1016/j.cbpb.2017.10.001.
Iglesias, J., Sánchez, F.J., Otero, J.J., Moxica, C., 2000. Culture of octopus (*Octopus vulgaris*, Cuvier). Present knowledge, problems and perspectives. Cah. Options Méditerr. 47, 313–321. http://om.ciheam.org/article.php?IDPDF=600632.
Iglesias, J., Sánchez, F.J., Bersano, J.G.F., Carrasco, J.F., Dhont, J., Fuentes, L., Villanueva, R., 2007. Rearing of *Octopus vulgaris* paralarvae: present status, bottlenecks and trends. Aquaculture 266, 1–15. https://doi.org/10.1016/j.aquaculture.2007.02.019.
Krstulovíc, S., Vrgoc, N., 2009. Diet and feeding of the musky octopus, *Eledone moschata*, in the northern Adriatic Sea. J. Mar. Biol. Assoc. UK 89, 413–419. https://doi.org/10.1017/S0025315408002488.
Lacoue-Labarthe, T., Le Bihan, E., Borg, D., Koueta, N., Bustamante, P., 2010. Acid phosphatase and cathepsin activity in cuttlefish (*Sepia officinalis*) eggs: the effects of Ag, Cd, and Cu exposure. ICES J. Mar. Sci. 67, 1517–1523. https://doi.org/10.1093/icesjms/fsq044.
Langridge, K.V., 2009. Cuttlefish use startle displays, but not against large predators. Anim. Behav. 77, 847–856. https://doi.org/10.1016/j.anbehav.2008.11.023.
Lazo, J.P., Mendoza, R., Holt, G.J., Aguilera, C., Arnold, C.R., 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). Aquaculture 265, 194–205. https://doi.org/10.1016/j.aquaculture.2007.01.043.
Linares, M., Caamal-Monsreal, C., Olivares, A., Sánchez, A., Rodríguez, S., Zúñiga, O., Rosas, C., 2015. Timing of digestion, absorption and assimilation in octopus species from tropical (*Octopus maya*) and subtropical-temperate (*O. mimus*) ecosystems. Aquat. Biol. 24, 127–140. https://doi.org/10.3354/ab00642.
López-Ripoll, E.R., 2010. Descripción del Desarrollo y Efecto de la Alimentación en la Estructura de la Glándula Digestiva de los Juveniles Tempranos de *Octopus maya* (Mollusca: Cephalopoda) Voss y Solís-Ramirez (1966). B.Sc. Thesis. Universidad de Bogotá Jorge Tadeo Lozano, Santa Marta, Colombia, 119 pp.
Mangold, K.M., Young, R.E., 1998. The systematic value of the digestive organs. Sm. C. Zool. 586, 21–30.
Martínez, R., López-Ripoll, E., Ávila-Poveda, O.H., Santos-Ricalde, R., Mascaró, M., Rosas, C., 2011. Cytological ontogeny of the digestive gland in post-hatching *Octopus maya*, and cytological background of digestion in juveniles. Aquat. Biol. 11, 249–261. https://doi.org/10.3354/ab00305.
Matozzo, V., Conenna, I., Riedl, V.M., Marin, M.G., Marčeta, T., Mazzoldi, C., 2015. A first survey on the biochemical composition of egg yolk and lysozyme-like activity of egg envelopment in the cuttlefish *Sepia officinalis* from the Northern Adriatic Sea (Italy). Fish Shellfish Immunol. 45 (2), 528–533. https://doi.org/10.1016/j.fsi.2015.04.026.
Moguel, C., Mascaró, M., Ávila-Poveda, O.H., Caamal-Monsreal, C., Sanchez, A., Pascual, C., Rosas, C., 2010. Morphological, physiological and behavioral changes during post-hatching development of *Octopus maya* (Mollusca: Cephalopoda) with special focus on the digestive system. Aquat. Biol. 9, 35–48. https://doi.org/10.3354/ab00234.
Moltschaniwskyj, N.A., Hall, K., Lipinski, M.R., Marian, J.E., Nishiguchi, M., Sakai, M., Warnke, K., 2007. Ethical and welfare considerations when using cephalopods as experimental animals. Rev. Fish Biol. Fish. 17, 455–476. https://doi.org/10.1007/s11160-007-9056-8.
Morote, E., Rodríguez, M., Muñoz, J.L., Mancera, J.M., Moyano, F.J., 2005. Digestive Enzymes Indicating Nutritional Condition in Octopus Paralarvae *Octopus vulgaris* Cuvier, 1797. Boletín - Instituto Español de Oceanografía, pp. 177–186.
Moyano, F.J., Díaz, M., Alarcón, F.J., Sarasquete, M.C., 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). Fish Physiol. Biochem. 15, 121–130. https://doi.org/10.1007/BF01875591.
Narvarte, M., González, R., Fernández, M., 2006. Comparison of Tehuelche octopus (*Octopus tehuelchus*) abundance between an open-access fishing ground and a marine protected area: evidence from a direct development species. Fish. Res. 79, 112–119. https://doi.org/10.1016/j.fishres.2006.02.013.
Nimrat, S., Tanutpongpalin, P., Sritunyalucksana, K., Boonthai, T., Vuthiphandchai, V., 2013. Enhancement of growth performance, digestive enzyme activities and disease resistance in black tiger shrimp (*Penaeus monodon*) postlarvae by potential probiotics. Aquac. Int. 21 (3), 655–666. https://doi.org/10.1007/s10499-012-9600-y.
Nolasco-Soria, H., 2021. Improving and standardizing protocols for alkaline protease quantification in fish. Rev. Aquac. 13 (1), 43–65. https://doi.org/10.1111/raq.12463.
Nolasco-Soria, H., Moyano-López, F., Vega-Villasante, F., del Monte-Martínez, A., Espinosa-Chaurand, D., Gisbert, E., Nolasco-Alzaga, H.R., 2018. Lipase and phospholipase activity methods for marine organisms. In: Sandoval, G. (Ed.), Lipases and Phospholipases. Methods in Molecular Biology, Vol. 1835. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-8672-9_7.
O'Dor, R.K., Weber, D.M., 1986. Constraints on cephalopods: why squid aren't fish. Can. J. Zool. 64, 1591–1605. https://doi.org/10.1139/z86-241.
Ortiz, N., Ré, M.E., 2011. The eggs and hatchlings of the octopus *Robsonella fontaniana* (Cephalopoda: Octopodidae). J. Mar. Biol. Assoc. UK 91, 705–713. https://doi.org/10.1017/S0025315410001232.
Parra, G., Villanueva, R., Yúfera, M., 2000. Respiration rates in late eggs and early hatchlings of the common octopus, *Octopus vulgaris*. J. Mar. Biol. Assoc. UK 80, 557–558. https://doi.org/10.1017/S0025315400002319.
Pasteels, J.J., 1973. Acid phosphatase and thiolacetic esterase activities studied with the electron microscope in the gill epithelium and eggs of lamellibranchial molluscs. Bull. Assoc. Anat. 57, 603–606.
Perrin, A., Le Bihan, E., Koueta, N., 2004. Experimental study of enriched frozen diet on digestive enzymes and growth of juvenile cuttlefish *Sepia officinalis* L. (Mollusca Cephalopoda). J. Exp. Mar. Biol. Ecol. 311, 267–285. https://doi.org/10.1016/j.jembe.2004.05.012.
Peyer, S.M., Kremer, N., McFall-Ngai, M.J., 2018. Involvement of a host Cathepsin L in symbiont-induced cell death. Microbiol. Open 7, e00632. https://doi.org/10.1002/mbo3.632.
Pinheiro, J.C., Bates, D.J., DebRoy, S., Sakar, D., 2016. R Core Team, 2016. Linear and Nonlinear Mixed Effects Models. R package 3.1–128. http://CRAN.R-project.org/package=nlme.

Pollero, R.J., Iribarne, O.O., 1988. Biochemical changes during the reproductive cycle of the small Patagonian octopus, *Octopus tehuelchus,* D'Orb. Comp. Biochem. Physiol. B 90, 317–320. https://doi.org/10.1016/0305-0491(88)90080-6.

Principato, G.B., Aisa, M.C., Biagioni, M., Giovannini, E., 1982. Partial purification and characterization of an alkaline phosphatase in *Helix nemoralis* and in *Octopus vulgaris*. Comp. Biochem. Physiol. B 72 (2), 325–328. https://doi.org/10.1016/0305-0491(82)90055-4.

Querol, P., Gairín, J.I., Guerao, G., Monge, R., Jover, M., Tomás, A., 2015. Effect of two extruded diets with different fish and squid meal ratio on growth, digestibility and body composition of *Octopus vulgaris* (Cuvier, 1797). Aquac. Res. 46, 2481–2489. https://doi.org/10.1111/are.12407.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.

Ré, M.E., 1989. Estudios ecológicos sobre el crecimiento y la alimentación de *Octopus tehuelchus* (d'Orbigny) en Puerto Lobos, Golfo San Matías. Tesis Doctoral. Universidad Nacional de La Plata, Argentina, 368 pp. http://sedici.unlp.edu.ar/handle/10915/4699.

Ré, M.E., 1998. Pulpos octopódidos (Cephalopoda, Octopodidae). In: Boschi, E.E. (Ed.), El Mar Argentino y sus Recursos Pesqueros, Tomo 2. Instituto Nacional de Investigación y Desarrollo Pesquero, Mar del Plata, pp. 69–98.

Ré, M.E., Gómez-Simes, E., 1992. Hábitos alimentarios del pulpo (*Octopus tehuelchus*). I. Análisis cuali-cuantitativos de la dieta en el intermareal de Puerto Lobos, Golfo San Matías (Argentina). Frente Marítimo 11, 119–128.

Rivas, A.L., Beier, E.J., 1990. Temperature and salinity fields in the north patagonian gulfs. Oceanol. Acta 13 (1), 15–20.

Rosas, C., Cuzon, G., Pascual, C., Gaxiola, G., Chay, D., López, N., Domingues, P.M., 2007. Energy balance of *Octopus maya* fed crab or an artificial diet. Mar. Biol. 152, 371–381. https://doi.org/10.1007/s00227-007-0692-2.

Rosas, C., Tut, J., Baeza, J., Sánchez, A., Sosa, V., Pascual, C., Cuzon, G., 2008. Effect of type of binder on growth, digestibility, and energetic balance of *Octopus maya*. Aquaculture 275, 291–297. https://doi.org/10.1016/j.aquaculture.2008.01.015.

Rosas, C., Sánchez, A., Pascual, C., Aguila, J., Maldonado, T., Domingues, P., 2011. Effects of two dietary protein levels on energy balance and digestive capacity of *Octopus maya*. Aquac. Int. 19, 165–180. https://doi.org/10.1007/s10499-010-9350-7.

Safi, G., Martínez, A.S., Le Pabic, C., Le Bihan, E., Robin, J.P., Koueta, N., 2018. Digestive enzyme ratios are good indicators of hatchling yolk reserve and digestive gland maturation in early life stages of cuttlefish *Sepia officinalis* L.: application of these new tools in ecology and aquaculture. J. Comp. Physiol. B. 188, 57–76. https://doi.org/10.1007/s00360-017-1115-4.

Sarath, G., De La Motte, R.S., Wagner, F.W., 1989. Protease assay methods. In: Beynon, R., Bond, J. (Eds.), Proteolytic Enzymes: A Practical Approach. IRL Press, Oxford, pp. 25–56.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to ImageJ: 25 yearsof image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Segawa, S., Hanlon, R.T., 1988. Oxygen consumption and ammonia excretion rates in *Octopus maya*, *Loligo forbesi* and *Lolliguncula brevis* (Mollusca: Cephalopoda). Mar. Freshw. Behav. Physiol. 13, 389–400. https://doi.org/10.1080/10236248809378687.

Semmens, J.M., 2002. Changes in the digestive gland of the loliginid squid *Sepioteuthis lessoniana* (Lesson 1830) associated with feeding. J. Exp. Mar. Biol. Ecol. 274 (1), 19–39. https://doi.org/10.1016/S0022-0981(02)00165-X.

Semmens, J.M., Pecl, G.T., Villanueva, R., Joufre, D., Sobrino, I., Wood, J.B., Rigby, B.R., 2004. Understanding octopus growth: patterns, variability and physiology. Mar. Freshw. Res. 55, 367–377. https://doi.org/10.1071/MF03155.

Solorzano, Y., Viana, M.T., López, L.M., Correa, J.G., True, C.C., Rosas, C., 2009. Response of newly hatched *Octopus bimaculoides* fed enriched *Artemia salina*: growth performance, ontogeny of the digestive enzyme and tissue amino acid content. Aquaculture 289, 84–90. https://doi.org/10.1016/j.aquaculture.2008.12.036.

Storero, L.P., Ocampo-Reinaldo, M., González, R.A., Narvarte, M.A., 2010. Growth and life span of the small octopus *Octopus tehuelchus* in San Matías Gulf (Patagonia): three decades of study. Mar. Biol. 157, 555–564. https://doi.org/10.1007/s00227-009-1341-8.

Sweeney, M.J., Roper, C.F.E., 1983. Techniques for fixation, preservation, and curation of cephalopods. Mem. Nat. Mus. Victoria 44, 29–47.

Swift, K., Johnston, D., Moltschaniwskyj, N., 2005. The digestive gland of the southern dumpling squid (*Euprymna tasmanica*): structure and function. J. Exp. Mar. Biol. Ecol. 315, 177–186. https://doi.org/10.1016/j.jembe.2004.09.017.

Sykes, A.V., Almansa, E., Cooke, G.M., Ponte, G., Andrews, P.L., 2017. The digestive tract of cephalopods: a neglected topic of relevance to animal welfare in the laboratory and aquaculture. Front. Physiol. 8, 492. https://doi.org/10.3389/fphys.2017.00492.

Uriarte, I., Iglesias, J., Domingues, P., Rosas, C., Viana, M.T., Navarro, J.C., Zuñiga, O., 2011. Current status and bottleneck of octopod aquaculture: the case of American species. J. World Aquacult. Soc. 42, 735–752. https://doi.org/10.1111/j.1749-7345.2011.00524.x.

Uriarte, I., Astorga, M., Navarro, J.C., Viana, M.T., Rosas, C., Molinet, C., Farías, A., 2018. Early life stage bottlenecks of carnivorous molluscs under captivity: a challenge for their farming and contribution to seafood production. Rev. Aquac. 11, 431–457. https://doi.org/10.1111/raq.12240.

Vecchione, M., 1987. Juvenile ecology. In: Boyle, P.R. (Ed.), Cephalopod Life Cycle: Comparative Reviews. Academic Press, London, pp. 61–84.

Vecchione, M., Hand, V.A., 1989. Digestive-gland histology in paralarval squids (Cephalopoda; Loliginidae). Fish. Bull. 87, 995–1000.

Vidal, E.A., DiMarco, F.P., Wormuth, J.H., Lee, P.G., 2002. Influence of temperature and food availability on survival, growth and yolk utilization in hatchling squid. Bull. Mar. Sci. 71, 915–931.

Vidal, E.A., Roberts, M.J., Martins, R.S., 2005. Yolk utilization, metabolism and growth in reared *Loligo vulgaris reynaudii* paralarvae. Aquat. Living Resour. 18, 385–393. https://doi.org/10.1051/alr:2005040.

Vidal, E.A., DiMarco, P., Lee, P., 2006. Effects of starvation and recovery on the survival, growth and RNA/DNA ratio in loliginid squid paralarvae. Aquaculture 260, 94–105. https://doi.org/10.1016/j.aquaculture.2006.05.056.

Vidal, E.A., Villanueva, R., Andrade, J.P., Gleadall, I.G., Iglesias, J., Koueta, N., Wood, J., 2014. Cephalopod culture: status of main biological models and research priorities. Adv. Mar. Biol. 67, 1–98. https://doi.org/10.1016/B978-0-12-800287-2.00001-9.

Wells, M.J., 1958. Factors affecting reactions to Mysis by newly hatched Sepia. Behaviour 13, 96–111.

Williams, G.N., Solís, M.E., Esteves, J.L., 2018. Satellite-measured phytoplankton and environmental factors in north Patagonian Gulfs. In: Plankt. Ecol. Southwest. Atl. From Subtrop. Subantarctic Realm, pp. 307–325. https://doi.org/10.1007/978-3-319-77, 869- 3_15.

Yoshizaki, N., Yonezawa, S., 1998. Cysteine proteinase plays a key role for the initiation of yolk digestion during development of *Xenopus laevis*. Develop. Growth Differ. 40, 659–667.