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Evaluation of physiological stress and nutritional deficiency related to cannibalism in early paralarvae of Patagonian red octopus *Enteroctopus megalocyathus*

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ABSTRACT

Patagonian red octopus *Enteroctopus megalocyathus* is one of the two octopuses' species of great commercial importance in Chile. At the present time, the life cycle of this species in captivity has been closed at experimental level; however, high mortalities at the early paralarvae stage are a limiting factor for attaining sustainable production. Cannibalism has been identified as one of the main mortality causes at the early paralarvae rearing. Stress has been proposed as an important cause of cannibalism in cephalopods. Likewise, nutritional deficiency has been suggested as a potential cause to onset cannibalism. However, neither of both factors has been directly assessed in cephalopod paralarvae. In the present study, we aimed to elucidate if physiological stress due to a food shortage and/or to a high rearing density of paralarvae trigger cannibalism in *E. megalocyathus*. An experiment was carried out with newly hatched paralarvae, under conditions that trigger cannibalism with densities of 10 and 15 paralarvae L^{-1} combined with two measures of prey availability: 0 and 2 prey paralarva^{-1} day^{-1}. Paralarvae alone in the aquarium fed ad libitum was used as a control to avoid cannibalism. Nutritional status was assessed as the total protein and energy content per paralarvae; and the 70 kD heat shock protein (*HSP70*) transcript level was used as a bioindicator of physiological stress, documented as a suitable stress biomarker for cephalopods. No differences were observed between the treatments and control in the absolute protein levels and energy content. There was a strong induction in *HSP70* transcriptional level when high density and prey availability where combined, proving that there are only a few paralarvae rearing combinations that cause critical stress and trigger *HSP70* transcription. In experimental conditions, nutritional status indicators showed that early paralarvae, with similar reserves of protein and energy, exhibited cannibalism. Results suggest that mortality as well as cannibalism is associated with competition for prey and with nutritional and energy imbalance due to food shortage that determines the expression of the related stress gene.

1. Introduction

The cephalopod rearing has not attained commercial production because several constraints, and the reliable production of viable numbers of juveniles is one of them (Vidal et al., 2014). In Chile, there are two species of octopuses with commercial importance, which are cought through artisanal fishing. One of them is the Patagonian red octopus *Enteroctopus megalocyathus*, and recently its life cycle under captivity has been completed studied at experimental level (Uriarte et al., 2017). However, the high mortality that occurs in the paralarvae phase, reaching 70–80% in the first 30 days (Farías et al., 2016), makes difficult to obtain the necessary juveniles for scaling its production in captivity (Uriarte and Farías, 2014). Among the causes of this high mortality, cannibalism accounts for up to 60% during the first 10–15 days post-hatching (Uriarte et al., 2013).

Cannibalism is likely to be an important mechanism of density-dependent regulation in aquatic animals, including cephalopods. Among cephalopods, *E. megalocyathus* is classified as a specie with high levels

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of cannibalism (Ibañez and Keyl, 2010). External factors that trigger cannibalism have been studied in several aquatic species; where population density, and the quality, variability and amount of available food have been proposed as the main causes (Fox, 1975; Polis, 1981; Hecht and Pienaar, 1993; Abdussamad and Thampy, 1994). Physiological stress has also been pointed as a potential trigger of cannibalism in animal kingdom (Fox, 1975), and in the case of cephalopods (Ibañez and Keyl, 2010); however, this factor has not been directly assessed. To the best of our knowledge, only one study has evaluated potential triggering factors of cannibalism in the paralarvae of small octopus *Robsonella fontaniana*, under captivity conditions (Miranda et al., 2011). These authors observed that the paralarvae density (independent of food availability) was the main cause of cannibalism, being only in the lowest density where cannibalism was absent. In a previous study on cannibalism of *E. megalocyathus* paralarvae until 30 post-hatching (Espinoza, 2018) it was determined that rearing conditions of paralarvae density and food availability that decrease the paralarvae survival were 15 paralarvae L^{-1} and 0 and 2 preys paralarva^{-1} day^{-1} as shown in Fig. S1.

In the present study we compared the physiological stress levels, measuring 70 kD heat shock protein (*HSP70*) transcriptional levels, in paralarvae where starting signs of cannibalism were observed versus control where cannibalism was absent. Heat shock proteins (HSPs) are molecular chaperones that perform numerous functions in cells (Mosser et al., 2000; Bukau and Horwich, 1998). Because they protect the three-dimensional structure and function of other proteins, *HSP70* is usually induced under several environmental stress conditions. Looking for stress biomarkers, HSPs has been used this way in several mollusks (e.g., Jeno and Brokordt, 2014), including cephalopods (Trübenbach et al., 2013; Repolho et al., 2014; Capaz et al., 2017; Garrido et al., 2017). The evaluation of HSP70 at protein level usually do not discriminate between inducible and constitutive isoforms (Trübenbach et al., 2013), thus in the current study it was used the transcriptional level of an inducible *HSP70* as indicator of physiological stress. In the present study, we aimed to elucidate if physiological stress due to a food deprivation and/or to a high rearing density of paralarvae is a factor that triggers cannibalism in *E. megalocyathus*.

Nutritional deficiency has also been suggested as potential cause to onset cannibalism in carnivorous early juveniles of fish (Hecht and Pienaar, 1993) and in *Octopus bimaculoides* paralarvae (Solorzano et al., 2009). However, in the latter study this parameter was not directly quantified. Therefore, we further assessed the paralarvae nutritional condition from treatments where cannibalism was starting and where this was absent, in order to associate this status with the beginning of cannibalism in *E. megalocyathus* early paralarvae.

2. Materials and methods

2.1. Paralarvae rearing and cannibalism experiments

Enteroctopus megalocyathus females and males were captured in their natural environment at Hueihue, De Los Lagos region of Chile (42°S), and transferred to 200 L conditioning tanks in the Marine Invertebrate Hatchery at Universidad Austral de Chile (HIM-UACH). The methodology described by Uriarte et al. (2014) was followed for adult maintenance, reproduction, and egg brooding, until paralarvae hatching. Briefly, broodstock was conditioned during 4 months at 12 °C in 700 L tanks with sea water filtered at 1 μm and sterilized with ultraviolet light. Light intensity was maintained at levels below 50 Lux. Absolute fecundity occur between 1000 and 5000 eggs per female. In this work, the paralarvae come from a single brood.

Paralarvae rearing was performed at the HIM-UACH following Uriarte and Farías (2014). A factorial experiment (2 × 2) was carried out with newly hatched *E. megalocyathus* paralarvae, where the conditions that promoted cannibalism where; rearing densities of 10 and 15 paralarvae L^{-1}; and the food availabilities of 0 and 2 prey paralarvae^{-1} day^{-1}, giving four treatments: 1) 10 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1}; 2) 15 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1}; 3) 10 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1}; and 4) 15 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1}. Each treatment was replicated 3 times; each replica consisted of 3 L aquarium with 15 paralarvae each (45 paralarvae per treatment were assessed in total), where the volume was changed to attain the required density. Control condition without cannibalism consisted in 1 paralarvae per aquarium of 3 L fed with 30 prey paralarva^{-1} day^{-1}; replicated 36 times.

The prey used for each condition was *Artemia salina* enriched with *Nannochloropsis* according to Uriarte and Farías (2014). This diet was found to give one of the best growth and survival for *E. megalocyathus* paralarvae under captivity, and most important, attaining the settlement for the juvenile phase (Farías et al., 2016). To avoid prolonged fasting periods, *Artemia* was delivered in two daily pulses, 10 am in the morning, after cleaning the aquaria; and 5 pm in the afternoon.

Aquaria of 3 L capacity (25 lenght × 17 depth × 8 height cm) were connected to a recirculation system (composed of a biofilter, skimmer to eliminate organic material, and an additional 1 μm filter) with temperature control and an input of filtered seawater (5 μm) sterilized by UV. Salinity (30 ± 1‰), temperature (12 ± 1 °C) and dissolved oxygen (> 80% saturation) were measured daily. Photoperiod was set to 10 h light: 14 h dark cycle and light intensity was 30 Lux into the aquaria.

To determine the onset moment of cannibalism, all the aquaria were constantly monitored. Mortalities were recorded, dead or dying individuals were checked for any indication of cannibal attack, as well as body injuries including the losses of the arms, suckers or eyes, with a stereoscopic microscope (Stemi 2000C, Carl Zeiss). The disappearance of an individual was also considered cannibalism. Undamaged paralarvae were sampled to evaluate their nutritional status and stress level as soon as cannibalism was first observed. Control paralarvae were sampled on same treatments days that cannibalism was observed. The experiment continued until the day that last aquarium exhibited cannibalism.

2.2. Paralarvae nutritional analyses

Sampled paralarvae (n = 9 paralarvae per sample) were analyzed individually for protein and ash content. Dry mass of paralarvae were determined after freeze drying (Savant Brand lyophilizer model Novalyphe NL150). Lyophilized samples were maintained at −20 °C until they were analyzed in a CHN elemental microanalyzer (LECO CHN-900), and the nitrogen content was multiplied by 6.25 to obtain the protein content. The ash content was obtained after calcination at 500 °C for 6 h (Vulcan Muffle model A-550). Total lipid was estimated as the difference between protein and ash content per paralarvae, because carbohydrate represent < 1% of dry matter of paralarvae (Farías et al., 2016). The energy was calculated as the sum of protein and lipid multiplied by the constants 23.7 and 39.5 kJ g^{-1}, respectively, according to Guillaume et al. (2004). Then the nutritional status indicators of paralarvae were calculated in dry basis as:

i). Protein per paralarvae = mg protein paralarvae^{-1}.
ii). Energy per paralarvae = (g protein parlarvae^{-1} × 23.7 kJ g^{-1}) + (g lipid paralarvae^{-1} × 39.5 kJ g^{-1}) = kJ paralarvae^{-1}.
iii). Protein/energy ratio (P/E) per paralarvae = mg protein kJ total^{-1}.

2.3. HSP70 transcriptional analysis in paralarvae

To evaluate the presence of physiological stress during the onset of cannibalism, as soon as a paralarvae was observed attacking another one, undamaged paralarvae were sampled from the aquarium and at the same time an intact paralarvae was sampled from the control. They were quickly euthanized and submerged in RNALater for their

preservation and stored at −80 °C until total RNA extraction for *HSP70* gene expression evaluation. Samples were analyzed in the Immunology and stress of Aquatic Organisms Laboratory, Veterinary Sciences Faculty, Universidad Austral de Chile (UACH).

Transcriptional level of gene encoding the HSP70 protein was used as an indicator of the stress response. To evaluate the mRNA levels of the *HSP70* from *E. megalocyathus* the following procedures were performed:

a) RNA extraction: Total RNA from paralarvae was extracted using Trizol® (Invitrogen) and the E.Z.N.A. RNA EXTRACTION KIT I according to the supplier's recommendations with minor modifications. Briefly, paralarvae samples frozen and stored in RNAlater were submitted to lysis under denaturing conditions which inactivate RNases. After the homogenization process, samples were applied to the HiBind® RNA mini columns which binds the total RNA, and then all cellular debris and other contaminants are effectively washed away after a few quick wash steps. Total RNA is eluted in DEPC water and its concentration was determined by measuring absorbance at 260 nm, using a MaestroGen spectrophotometer, Nano model. Integrity and quality of extracted RNA were assessed by visual inspection of rRNA bands in denaturing agarose gel electrophoresis. Subsequently, the extracted RNA was stored at −80 °C, and then the reverse transcription was performed.

b) Complementary DNA synthesis (cDNA) (Reverse transcription): Reverse transcription reaction was carried out using M-MVL Reverse Transcriptase (M-MVL-RT, Promega), according to the manufacturer's instructions, from 1 μg of total RNA. The synthesized cDNA was stored at −20 °C for subsequent quantification by real-time PCR (RT-qPCR).

c) *HSP70* isolation: In order to isolate *HSP70* specific primers were designed from cDNA sequences described for this gene in *Octopus vulgaris* (GenBank accession number KF195923.1). Primer sequences were as follows: *OvHSP70* forward, 5′- TTTGTCGAAGAATTCAAGCG-3′; reverse 5´-TGTTGATGCTCTTTCCTTGC-3′. PCR product from *E. megalocyathus* cDNA was amplified, purified and then sequenced by Austral Omics (from UACH, Chile). The obtained sequence of *E. megalocyathus* (GenBank accession number MK045854) showed a high homology (95%) with an inducible *HSP70* sequence of *O. vulgaris*, from which specific primers were designed for the quantification by RT-qPCR.

d) RT-qPCR for the quantification of *Hsp70* gene expression: Primers for RT-qPCR were designed using Primer Express v3.0 software (Applied Biosystems, CA, USA). α tubulin was used as endogenous control to normalize experimental results (Catalán, 2015) Primer sequences were as follows: *EpHSP70* forward, 5´-AACGAACGCTATCGA GCAGT-3′; reverse, 5´-AGTGGATCGGAAGAGGTCA-3′; α tubulin (forward, 5´-ACTGGTGTCCAACTGGCTTC-3′; reverse, 5´-TGCTTAACATGC ACACAGCA-3′). Each amplification reaction was performed using 2.4 μL of cDNA, 1.2 μL of 10 μM primers, 6 μL of the amplification mixture SYBR Green Master Mix PCR and 2.4 μL of nuclease-free water in a total volume of 12 μL. The reaction was carried out in a StepOne Real Time PCR System thermocycler (Applied Biosystem) for 48 samples. The program consisted of the following steps: one cycle of preincubation, an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles, each composed of denaturation at 95 °C for 15 s, pairing 60 °C for 60 s. After the PCR cycles, the purity of the PCR product was checked by the analysis of its melting curve; the thermal profile for melting curve analysis consisted of denaturation for 15 s at 95 °C, lowered to 55 °C for 15 s and then increased to 95 °C for 15 s with continuous fluorescence readings. During RT-qPCR, the efficiency of *HSP70* gene amplification was approximately equal to the housekeeping gene, and the comparative C_T method (also called $\Delta\Delta C_T$ method, Pfaffl, 2004) was applied for relative quantification.

2.4. Statistical analyses

Data were analyzed through a one-way ANOVA followed by the Tukey and Dunnett multiple comparisons test (Zar, 1999). Normality of the dependent variables (i.e., nutritional and stress associated parameters) were tested with the Shapiro-Wilks test (SAS Institute, 1999) and homogeneity of variances with the Levene test (Snedecor and Cochran, 1989) to verify that the data met model assumptions. The arcsine square root transformation was applied to normalize the data when necessary. Nonparametric data were analyzed using the Kruskall Wallis test, followed by the Mann-Whitney test to compare pairs of means when significant differences were found.

2.5. Ethics statement

Animal maintenance and experimental manipulations in this study were carried out in strict accordance with the recommendations in the CCAC (Canadian Council on Animal Care) guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing. All efforts were made to minimize suffering during animal manipulations and surgery. There are no aspects of the experiments that would cause aggravated or unnecessary harm or stress to the paralarvae involved. Sampled paralarvae were euthanized with a freezing shock of −80 °C (enough to anesthetize the specimens).

3. Results and discussion

Regardless of the density and food availability under which *Enteroctopus megalocyathus* paralarvae were maintained, in general, cannibalism started early during the first 6 DAH (days after hatching) in 7 of the 12 total aquaria. There were no significant differences in mortality [Kruskall-Wallis H (3, $N = 12$) = 1.91, $P = .5903$] and cannibalism onset [Kruskall-Wallis H (3, N = 12) = 4.75, $P = .1904$] between treatments. The most delayed cannibalism onset was observed on the treatment of 10 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1}; and the earliest in the the treatment of 10 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1} (Table 1). These results agree with previous observations made in *E. megalocyathus* by Uriarte et al. (2013), where the onset of cannibalism in paralarvae maintained under different conditions was observed also during the first 2 weeks after hatching. In other octopus species such as *Robsonella fontaniana* cannibalism has been observed to start 7 days post-hatching; and this behavior was also present in fed or not fed paralarvae (Miranda et al., 2011).

It is interesting to note a trend of lower mortality in the treatments with food deprivation. This was also observed by Espinoza et al. (2017) in *R. fontaniana* paralarvae, where the highest mortality existing in

Table 1
Mortality, cannibalism onset and weight increment (± SE) of cannibalism in *E. megalocyathus* paralarvae, in days after hatching (DAH), under different paralarvae conditions of densities and prey availability (P is paralarvae, A is Artemia). Each treatment was replicated 3 times; each replicate consisted of a 3 L aquarium with 15 paralarvae.

Treatment	10P 0A	10P 2A	15P 0A	15P 2A
Mortality (%)	8.9 ± 8.9	13.3 ± 7.7	4.4 ± 2.2	15.5 ± 4.5
Cannibalism onset (DAH)	9.3 ± 3.5	3.0 ± 1.0	8.0 ± 2.0	7.7 ± 1.2
Weight increment (mg per paralarvae)	$30.7 \pm 6.4^{B,X}$	$45.1 \pm 7.6^{B;Y}$	$18.6 \pm 4.4^{A,X}$	$30.2 \pm 5.2^{A;Y}$

Different letters A, B indicate statistical differences between densities of paralarvae at $P < .05$ level. Different letters X, Y indicate statistical differences between prey availabilities at $P < .05$ level. DAH = days after hatching.

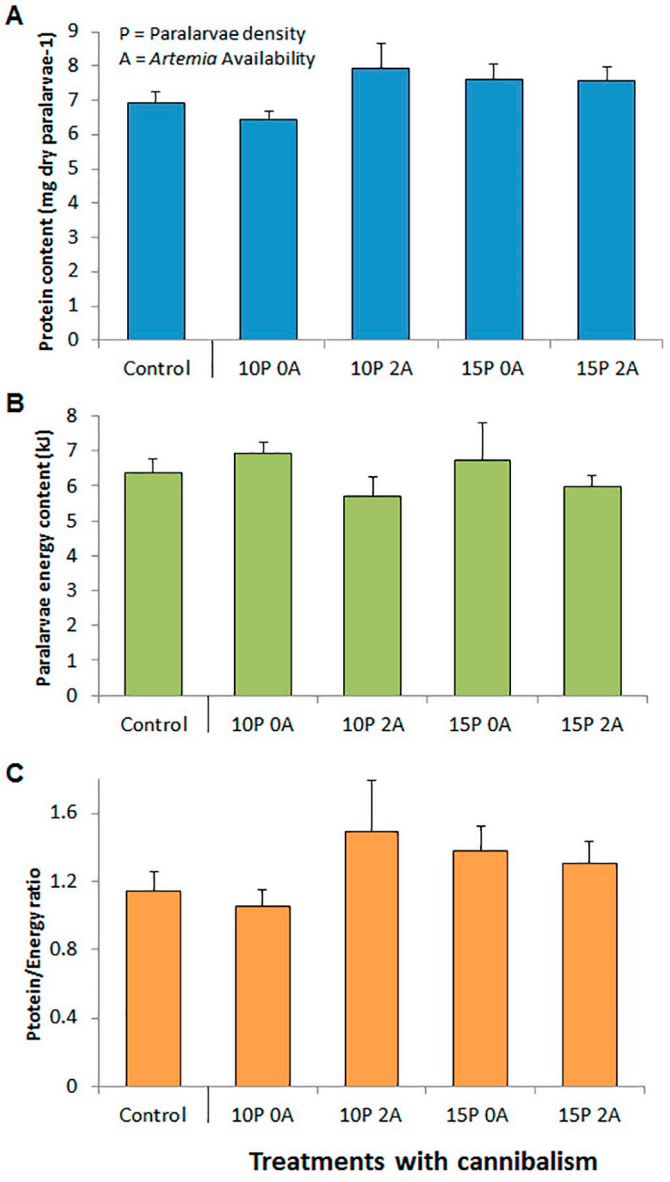

Fig. 1. Nutritional contents in *Enteroctopus megalocyathus* paralarvae reared under different conditions that promoted cannibalism as 1) 10 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1} (10P 0A); 2) 15 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1} (15P 0A); 3) 10 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1} (10P 2A); and 4) 15 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1} (15P 2A). Each combination replicated 3 times. Control without cannibalism was 1 paralarvae per tank fed with 30 prey paralarvae^{-1} day^{-1}. A: Total protein content per dry paralarvae; B: Total energy content in dry paralarvae; C: Protein, energy ratio (mg protein/kJ). Bars represent means ± standard error.

paralarvae fed, with respect to those subjected to starvation, was explained because Artemia was unable to provide enough nutrients and energy for the active metabolism of fed paralarvae. Whereas the energy used for basal metabolism in starved paralarvae was lower and allowed extended survival times.

Absolute protein content (Fig. 1A) did not differ between control (without cannibalism) and the paralarvae belonging to aquaria where cannibalism was observed [Kruskall-Wallis H (4, $N = 42$) =7.48, $P = .113$]. Also, no differences were observed in absolute energy content (Fig. 1B), nor in protein/energy ratio (Fig. 1C) between control paralarvae and those that showed cannibalism [Kruskall-Wallis H (4, $N = 37$) =1.08, $P = .772$; H (4, N = 37) =4.44, $P = .349$, respectively]. According to Domingues et al. (2004), cephalopods have a protein metabolism that is very different from fish and crustaceans; they have a high requirement of amino acids to produce high amounts of proteins, making the latter their main source of energy. Failing in these nutritional requirements would lead to cannibalism according to Solorzano et al. (2009). However, our results with *E. megalocyathus* paralarvae suggest that nutritional status in terms of total protein and energy contents would not be associated to cannibalism manifestation.

Interestingly, our findings show that the absence or presence of food, did not affected the nutritional condition of *E. megalocyathus* paralarvae (Fig. 1). These results may be associated to nutritional reserves from maternal origin to support basal energetic requirements in *E. megalocyathus* early paralarvae, according to Farías et al. (2016). These reserves would allow the paralarvae to recover from starvation if the time is not extended for > 1 week, as have been observed in *R. fontaniana* (Espinoza et al., 2017). In accord with Lamarre et al. (2012); Lamarre et al. (2016) and Speers-Roesch et al. (2016) the first stage under food deprivation of juvenile cuttlefish is the decrease of protein synthesis plus fatty acid mobilization, followed by a second stage of lipid reserves depletion in the digestive gland; and then a third and final stage where body proteins are used as primary fuel for metabolism. In agreement with Espinoza et al. (2017), during the first days of food deprivation in early paralarvae of *R. fontaniana*, they show amino acids mobilization with decreases on methionine, valine and arginine and increments of taurine, where taurine could be fulfilling an important role as osmoregulator (taurine being produced from methionine); whereas arginine would be used as phosphagen. In *R. fontaniana* paralarvae the structural changes occur only after 8 days post-hatching (DAH) with food deprivation, when a decrease in the ratio arm length/mantle length is observed together with atrophy of digestive gland, that becomes irreversible at 12 DAH (Espinoza et al., 2017). Similarly, in *Sepia officinalis* juvenile lipids depletion occurs in the digestive gland after a prolonged state of food deprivation for 12 days (Speers-Roesch et al., 2016). We propose that in the early *E. megalocyathus* paralarvae the first stage in the metabolic response to starvation or malnutrition is amino acids mobilization, similar to the early paralarvae of *R. fontaniana*, and takes longer than in juveniles' cuttlefish or *R. fontaniana*. The second stage are lipid-based fuels, similar to *S. officinalis* juveniles (Speers-Roesch et al., 2016), and occurs after 12 DAH. Thus, the firsts 12 DAH in the paralarvae stage, depletion of lipids or the use of body proteins has not started yet. This hypothesis should be tested in the future for *E. megalocyathus*.

The weight gain respect to the initial weight of paralarvae (98.6 ± 5.1 mg) showed differences between densities ($F_{1, 30} = 4.96$, $P = .03$) and between prey availability ($F_{1, 30} = 4.56$, $P = .04$) (Table 1). There was no interaction between both factors. Increased density causing more between-paralarvae interactions could explain the lower weight gain under this condition, especially when no food was provided. Paralarvae should have the best condition without undue high interaction or competition with their own congeners. Although paralarvae under all conditions increased their masses, those provided with 2 prey per day showed higher weight gains than paralarvae without preys. Farías et al. (2016) documented that paralarvae that used only their inner yolk reserves had the lowest weight gain, however they showed better condition than paralarvae without food.

Cannibalism in cephalopods is not an abnormal behavior, is common in stressed populations and is considered as an energy storage strategy under both adverse and favorable food availabilities (Ibañez and Keyl, 2010). Several authors have mentioned that overpopulation combined with food limitation causes stress in organisms, which in turn induces cannibalism (Miranda et al., 2011; Boal et al., 1999; Hecht and Pienaar, 1993; Polis, 1981; Fox, 1975). We evaluated if physiological stress, measured through *HSP70* transcriptional induction, would be a trigger of cannibalism during early development of *E. megalocyathus* paralarvae. It has been demonstrated that inducible HSP70 chaperone protein, responsible for protein folding and cellular apoptosis

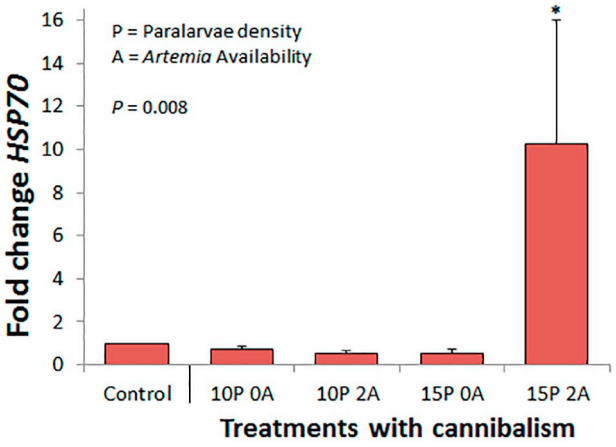

Fig. 2. Relative transcriptional levels of *HSP70* from *Enteroctopus megalocyathus* paralarvae reared under different conditions that promoted cannibalism: 1) 10 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1} (10P 0A); 2) 15 paralarvae L^{-1} with 0 prey paralarvae^{-1} day^{-1} (15P 0A); 3) 10 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1} (10P 2A); and 4) 15 paralarvae L^{-1} with 2 prey paralarvae^{-1} day^{-1} (15P 2A). Each combination was replicated 3 times. Control without cannibalism was 1 paralarvae per tank fed with 30 prey paralarvae^{-1} day^{-1}. Bars represent means ± standard error.

regulation, is overexpressed in cephalopods when animals are under stress, like thermal stress in the octopus *O. vulgaris* (Repolho et al., 2014), or hypoxia stress in the Jumbo squid *Dosidicus gigas* (Trübenbach et al., 2013). By contrast, in the cuttlefish *Sepia officinalis* HSP70 protein level was not induced after a short exposure to low dissolved oxygen saturation (DO) of 50% (Capaz et al., 2017). However, this result suggests that this level of DO did not induce cellular stress in cuttlefish as indicated by low levels polyubiquinated proteins (Capaz et al., 2017). Our results showed significant differences in the relative transcriptional level of the *HSP70* gene between the control without cannibalism and the treatment where cannibalism was started (ANOVA, $F_{(3, 15)} = 5.086$, $P = .0077$). However, this difference was only due to the high relative *HSP70* mRNA levels observed in the treatment with 15 paralarvae L^{-1} with availability of 2 prey paralarvae^{-1}, which exceeded the results of other treatments including control, in ~10 folds (Fig. 2).

Therefore, these results suggest that among the conditions that promote cannibalism, only the highest density (15 paralarvae L^{-1}) combined with a deficient prey availability (2 prey paralarvae^{-1} day^{-1}) may induced physiological stress in *E. megalocyathus* paralarvae. It was expected that in all treatments, or at least in those with high paralarvae densities where cannibalism was initiated, there would be an overexpression of the *HSP70* transcripts with respect to the control; however, it was not the case. Two potential explanations arise from this result, the first would be associated with the need of food supplementation to support *HSP70* overexpression; and the second with a probable food competition among the paralarvae under suboptimal prey availability conditions. The first hypothesis is supported by evidences obtained from other mollusks, which indicate that the limitation to induce HSP70 under stress have been associated with the availability of fatty acids, and specifically polyunsaturated fatty acids (PUFAs) under highly nutritional demanding conditions. In fact, Perez et al. (2016) showed that a diet rich in PUFAs increased the ability to induce *HSP70* mRNA levels against stress factors, in adults and larvae of the bivalve mollusk *Argopecten purpuratus*. Besides, the HSP70 synthesis demanded high energy costs and it could not occur despite the existence of physiological stress if the animal has limited energy availability (Sørensen, 2010; Morris et al., 2013; Brokordt et al., 2015). That situation has been documented for mature and spawned scallops and for starved *Concholepas concholepas* gastropod under thermal and hypoxia stressors (Jeno and Brokordt, 2014; Brokordt et al., 2015). In *E. megalocyathus* no differences were observed in the energy levels and protein status of the paralarvae between different treatments, even when the paralarvae were limited in energy as under the food deprivation. However, paralarvae maintain under the highest density with no food showed the lower weight gain, suggesting a lower physiological status. Because of the main important function of HSPs is to protect cells, the tissues and the whole organism from a stress injury, these present results enlighten the relevance of having the necessary molecular and energetic support to induce these cellular chaperons during paralarvae development. The second possible explanation for the higher *HSP70* mRNA observed in the highest density with deficient food availability (i.e., 15 paralarvae L^{-1} with 2 prey paralarvae^{-1}), could be associated to an increased stress due to the competition for food by paralarvae. This condition would not be present in the treatment with the same density but without food. However, these hypotheses should be tested in future studies. On the other hand, we can not rule out the possibility that most of the tested culture conditions did not generate physiological stress, or that the isoform of HSP70 evaluated was not the best suited to detect the stress generated by each condition.

In conclusion, our results suggest that: 1) Physiological stress, due to food deprivation and/or high rearing density of paralarvae, was detected only by the *HSP70* indicator in the most critical condition of 15 paralarvae L^{-1} and the food availability of 2 artemia paralarvae^{-1} day^{-1}; 2) cannibalism was observed in all treatments but mortality tends to be greater with more prey available. This reinforces the idea that competition between paralarvae for suboptimal food is an activity that demands more nutrients or energy that are not available and therefore increases mortality and triggers cannibalism; and 3) transcriptional level of an inducible *HSP70* was a sensitive way to detect critical stress, but future studies are necessary to confirm the suitability of this stress biomarker, or look for the effects of limited energy or specific nutrients affecting the expression of this biomarker.

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