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Effect of starvation on survival and biochemical profile of newborn juvenile lined seahorses, *Hippocampus erectus* (Perry, 1810)

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Abstract

This study assessed the effect of starvation on survival and nutritional status of newborn juveniles *H. erectus* (<10 days) to optimize rearing protocols, thereby helping to reduce wildlife exploitation. Maximum starvation time (MST) was estimated through the survival of juveniles continuously starved from birth. Resistance to starvation and the effect of food re-introduction after 1, 2, 4 and 6 days of starvation on survival and metabolite concentrations (total proteins, total lipids, acylglycerides, cholesterol, glucose) were also determined. Survival amongst continuously starved animals decreased from 6.6 ± 0.5 to 0% from days 9 to 10 of starvation. Seahorses under different starvation–refeeding treatments all had 100% survival up to day 5 of experiments. After 10 days, however, a 4-day starvation period followed by refeeding showed negative effects with <50% survival. During continuous starvation, lipids were the first energy reserve used to maintain basal metabolism, followed by proteins. Except for cholesterol, all metabolite concentrations differed between continuous starvations and feeding. Despite high seahorse survival after 5 days in the absence of food, the recovery of the metabolic status is possible after a starvation period of no more than 2 days, since irreversible physiological changes compromising the ultimate survival of the organisms take place after this time.

KEYWORDS
biochemical profile, *Hippocampus erectus*, juvenile seahorses, starvation, survival

1 | INTRODUCTION

The trade of ornamental marine species (fish and other invertebrates) to date is geographically broad, diverse and prosperous, involving economic investments of around 300 million dollars per year (Olivotto et al., 2017; Palmtag, 2017; Wabnitz, Taylor, Green, & Razak, 2003). In recent years, there has been significant growth in the seahorse (genus *Hippocampus*) trade (Foster, 2016; Kuo & Vincent, 2018). While most organisms are sold dry to satisfy the high demand of traditional Chinese medicine and, to a lesser extent, the curios markets (Chen, Wang, & Huang, 2015; Lourie, Foster, Cooper, & Vincent, 2004; Rosa, Defavari, Alves, & Oliveira, 2013), living organisms are mainly purchased by aquarists around the world (Lourie et al., 2004; Vincent, 1996; Vincent, Foster, & Koldewey, 2011). Unfortunately, most *Hippocampus* come from the wild (Foster, 2016), representing an economic source for developing countries (Monticini, 2010). To regulate this trade, all seahorse species have been included in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (CITES, 2004; Kuo & Vincent, 2018) and in the IUCN Red

List of Threatened Species (International Union for Conservation of Nature) (IUCN, 2017).

One of the four species inhabiting the Atlantic Ocean is *Hippocampus erectus* (Perry, 1,810). The species has a broad distribution from the coasts of Nova Scotia (Canada), throughout the Gulf of Mexico, and down to the coasts of Venezuela and the Caribbean Sea (Foster & Vincent, 2004) and, along with *H. kuda* and *H. reidi*, it is one of the most commercially important species (Evanson, Foster, Wiswedel, & Vincent, 2011).

Seahorse culture has been considered an alternative to reduce the exploitation of wild populations (Olivotto et al., 2008; Tlusty, 2002), and *H. erectus* is a candidate species for commercial production (Cohen, Valenti, Planas, & Calado, 2016; Engle, D'Abramo, Ponniah, & Slater, 2017). However, there are still difficulties associated with its reproduction in captivity and husbandry during the early stages of development that result in an overall low survival (Lin, Lin, & Zhang, 2008). Two main obstacles identified in rearing protocols are (a) the high mortality rates during the first stages of development (0–20 days after birth), and (b) the lack of adequate and economically sustainable food sources with nutritional profiles able to meet seahorse requirements (Alexandre, 2010). In the wild, seahorses have a varied diet of live prey (e.g., small crustaceans, invertebrates and fish larvae) (Foster & Vincent, 2004; Kitsos, Tzomos, Anagnostopoulou, & Koukouras, 2008), which are captured by means of a 'sit and wait' foraging behaviour (James & Heck, 1994). Moreover, prey need to be moving and of a suitable size in order to be detected and captured by seahorses (Leysen, Roos, & Adriaens, 2011; Roos et al., 2009; Roos, Van Wassenbergh, Herrel, Adriaens, & Aerts, 2010). For this reason, the lack of adequate nutrition during the first days of life has been considered one of the main causes of high mortality (Randazzo et al., 2018; Vite-Garcia, Lopez Jimenez, & Rangel Lopez, 2017).

The first study to evaluate the effects of starvation on fish larvae showed that, in the absence of food, larvae can experience severe alterations of the digestive organs (Piccinetti et al., 2015; Uriarte & Balbontín, 1987). After certain periods of starvation, re-fed individuals are capable to regain weight by increasing their growth rates through a mechanism known as compensatory growth (Delgadin et al., 2018; Piccinetti et al., 2015). However, when starvation is prolonged, fish are unable to regain weight and food re-introduction results in physical deterioration and ultimate death.

Adequate food and feeding strategies are key in culture protocols, because they constitute the single most costly factor ensuring survival, growth and the ability to successfully cope with the stressful events inherent to life in captivity (Dou, Masuda, Tanaka, & Tsukamoto, 2005; Koldewey & Martin-Smith, 2010; Sales & Janssens, 2003). Considered an intrinsic characteristic of each species (Lovell, 1998), resistance to starvation and the effect of subsequent food re-introduction acquire relevance in the context of aquaculture and need to be determined at the species level. Furthermore, knowledge of the conditions in which cultured ornamental species succeed in the absence of food is essential to ensure adequate animal welfare and compliance with the standards that preserve its commercial value (e.g., colour or behaviour; Wabnitz et al., 2003). When food availability is limited, adjustments in the metabolic routes to obtain energy take place, and biochemical mechanisms, such as using endogenous reserves, are triggered (McCue, 2010; Olivotto et al., 2011). Knowledge of such mechanisms is paramount to determine the organisms' capacity to tolerate starvation both in their natural habitats and under culture conditions, and information on the limits at which unfed animals will be able to recover is key for many culture and commercialization protocols (Yin & Blaxter, 1987).

Resistance to starvation may vary depending on the species and metabolic pathways. Fish prioritize the use of lipids as the main source of energy (Paz, Pastrana, & Brandão, 2018). In consequence, the effect of starvation on survival rates and the profile of fatty acids have been pointed out as highly relevant in the culture of a variety of aquatic species (Chatzifotis, Papadaki, Despoti, Roufidou, & Antonopoulou, 2011; Echevarría, Martínez-Bebiá, & Zamora, 1997; Liu et al., 2019). Additionally, information on biochemical changes occurring in starved fish during early life could be used to maximize animal welfare during transfer from rearing areas to retail sites (Cohen, Planas, Valenti, Lillebø, & Calado, 2018). This in turn can help to ensure high survival, immunological status and other commercially important features such as colour, that are meant to increase their commercial value (Wabnitz et al., 2003).

Studies on the physiological response to starvation in species of genus *Hippocampus* are scarce. Recent research has determined the effects of starvation on growth, survival and first feeding for two species of seahorses: *H. kuda* and *H. trimaculatus* (Sheng, Lin, Chen, Shen, & Lu, 2007). Results showed that feeding efficiency increased with age in both fish species (Sheng et al., 2007). Given the commercial importance of *H. erectus* in North America, the aim of the present study was to determine the effect of different intervals of starvation on the survival percentage and nutritional profile of *H. erectus* newborn juveniles. This information provides a fundamental contribution to knowledge on the physiology of this species and promotes the optimization of rearing protocols.

2 | MATERIALS AND METHODS

This study followed the protocols for maintenance, manipulation and sacrifice of the experimental animals according to certified criteria established by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the Faculty of Chemistry, UNAM (OFFICE/CF/CICUAL/340/18). All efforts were made to minimize stress in experimental animals and meet standard levels of animal welfare (see below for conditions).

2.1 | Origin and maintenance of experimental animals

Juveniles used in the experiments came from four pregnant males captured at Laguna de Chelem, Yucatan, Mexico (21°17′N and 89°40′W) (SEMARNAT permit No. SGPA/DGVS/10959/15). Once

in the laboratory, males were placed in individual aquariums (30 cm width × 17 cm depth × 27 cm height; 14 L) until juveniles were born.

Experiments began on the first day of life of seahorses (day 0), and 7 cm width × 17 cm depth × 20 cm height glass aquaria with 3 L of capacity were used in all cases. Water was not recirculated, but constant aeration helped maintain oxygen levels near saturation. A 1 × 1 cm plastic mesh was placed at the bottom of each aquarium to serve as holdfast for seahorses. Water conditions were kept constant at 27 ± 1°C and 37‰ salinity. Photoperiod during experiments was kept constant at 8 hr light: 16 hr darkness, with gradual changes between these to resemble dusk and dawn. This was achieved using 12 watts lamps of white light (not leds; Tecnolite, Mexico). Seahorse health and general condition was monitored by daily visual inspection of experimental animals, making sure seahorses were active (not stationary in the bottom of the aquaria), with normal body posture (the head and snout in upright position), and regular opercular movements.

Newborn *H. erectus* were fed with *Artemia franciscana* nauplius enriched with a commercial product (Easy DHA SELCO); 180 nauplius/ml were offered 3 times a day (10, 14, 18 hr; Mascaró et al., 2016). Faeces and food residues were removed daily from each aquarium with a siphon, and 20% of water level was restored to keep salinity concentrations at predefined levels.

2.2 | Experiments

Three experiments with newborn *H. erectus* were carried out. Experiments 1 and 2 were conducted with newborn *H. erectus* born from each of two separate males. Experiment 3 was conducted with a pool of newborn seahorses born from the remaining two males. In each experiment, seahorses were starved for different periods after which food was re-introduced (Table 1).

2.2.1 | Maximum starvation time

The first experiment involved three treatments to define the maximum starvation time (MST: maximum period of starvation in which at least one individual survives) as a first step to characterize the resistance to starvation. Individuals were (a) continuously fed since birth (positive control: FC_1); (b) starved for 1 day and thereafter fed continuously ($S1_1$); starved continuously from birth (negative control: SC_1; Table 1). Ninety individuals were randomly and uniformly distributed in nine aquariums (n = 3 aquariums per treatment). The number of surviving seahorses in each aquarium was recorded daily, and the experiment ended when all individuals in treatment SC_1 died.

2.2.2 | Resistance to starvation

Once MST was established, a second experiment was designed to examine the effect of starvation and food re-introduction on seahorse survival through time. Treatments used in this experiment were (a) control treatment with individuals continuously fed since birth (FC_2); (b) 2 days of starvation followed by continuous feeding ($S2_2$); (c) 4 days of starvation followed by continuous feeding ($S4_2$); and (d) continuous starvation from birth (SC_2; Table 1). A total of 180 individuals were randomly and uniformly distributed into 20 aquariums (n = 5 aquariums with nine individuals per treatment). Survival

TABLE 1 Design of the three experiments carried out with early juvenile *Hippocampus erectus* to examine the effect of starvation and food re-introduction on survival and biochemical profile through time

	Days of experiment									
Treatments	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1. Maximum starvation time										
FC_1										
$S1_1$										
SC_1										
2. Resistance to starvation										
FC_2										
$S2_2$										
$S4_2$										
SC_2										
3. Variation of metabolites										
FC_3		X		X		X		X		
$S2_3$				X		X		X		
$S4_3$						X		X		
S63								X		
SC3		X		X		X		X		

Note: Feeding days are represented in light grey and starvation days in dark grey. Treatments were fed continuously (FC), starved continuously (SC) and different starvation intervals followed by refeeding (S1, S2, etc.). In the third experiment, sampling days for biochemical analysis are shown with an 'X'.

was again recorded daily in each aquarium, and the experiment ended on day 10.

2.2.3 | Variations in metabolites through time

A third experiment with juveniles was undertaken to examine the effect of starvation and food re-introduction in whole body metabolite composition (total proteins, cholesterol, acylglycerides, glucose and total lipids (mg/g)) through time. Treatments were as follows: control with individuals continuously fed since birth (FC_3); 2 days of starvation followed by feeding ($S2_3$); 4 days of starvation followed by feeding ($S4_3$); 6 days of starvation followed by feeding ($S6_3$); and continuous starvation since birth (SC_3; Table 1). In this experiment, 10 individuals were placed in 12, 9, 6, 4 and 12 aquariums, corresponding to treatments FC_3, $S2_3$, $S4_3$, $S6_3$ and SC_3 respectively. Whole body samples for biochemical analysis were taken on days 2, 4, 6, and 8, and the experiment ended after day 8 (Table 1). In order to keep the number of sacrificed individuals at a minimum, samples of treatment SC_3 on day 2 represented the nutritional status of seahorses from any given treatment after 2 days of starvation. Samples from newborn seahorses previously collected were used to establish the initial nutritional status.

Metabolite concentrations were determined using pools of whole organisms (from three to five seahorses, depending on their weight) from aquaria corresponding to each treatment. A sample of 25 mg was homogenized with pyrogen-free water (1:3 ratio). This dilution factor was established considering the sensitivity of measurement techniques. After two 5 min 15,294 *g* centrifugation cycles at 4°C, the final 75 µl supernatant volume was obtained (minimum quantity necessary to perform the analysis).

Quantification of total lipids was obtained through the colorimetric analysis technique (Frings, Fendley, Dunn, & Queen, 1972). The homogenate was incubated in a double boiler at 90°C for 10 min with concentrated sulphuric acid (9681-02-BAKER ANALYZED® A.C.S. Reagent, Avantor Performance Materials, USA). Subsequently, 20 µl of homogenate was transferred to a micro-plate and incubated with 200 µl of phospho-vanillin reagent (vanillin [z4250, *ReagentPlus*® 99%, Sigma-Aldrich®, USA], pyrogen-free water and 85% phosphoric acid [P 5811-BioReagent 85%, Sigma-Aldrich®, USA]) for 40 min at room temperature, in the dark. After this period, sample absorbance was determined with a micro-plate reader at 540 nm. Glucose, acylglycerides and cholesterol were quantified by colorimetric analysis in micro-plate using clinical diagnosis commercial reagents (ELITech Group, France) based on chemical reactions with appropriate enzymes, whereas total proteins were determined following the Bradford method (1976). Analyses used a sample volume of 10 µl with 200 µl of reagent. Absorbance of glucose, acylglycerides and cholesterol was measured at 500 nm, whereas total protein absorbance was measured at 595 nm wavelength. The reference value for each analysis was based on the standard absorbance curve. Most metabolite standard curves were built using standard solutions, and olive oil was used for total lipids. Concentration (mg/ml) was estimated using the absorbance values of the samples, the values of the linear regression analysis of the standard curve (intercept and slope) and the value of the dilution factor:

$$\text{Concentration (mg/ml)} = \left[\left(\text{Sample absorbance} - \text{intercept}\right)/\text{slope}\right] * \text{dilution factor}$$

Metabolite concentration was expressed as mg/g of wet weight and was calculated with the weight of the sample (g) and the volume of pyrogen-free water (ml) used for the homogenation:

$$\text{Concentration (mg/g)} = \frac{\left[\text{Concentration (mg/ml)} * \text{volume of pyrongen - free water(ml)}\right]}{\text{wet weight(g)}}$$

2.3 | Data analysis

Survival results (%) for experiments 1 and 2 were averaged for each treatment and presented in tabular form along with the inter-quartile range as a measure of dispersion associated with the mean. The variation in the concentration of metabolites was analysed separately in two groups of descriptors: (a) glucose, acylglycerides, cholesterol, and (b) total lipids and proteins. Both groups were examined through time, in each of the four sampling moments in experiment 3 (days 2, 4, 6 and 8) using PCA (principal component analysis) of previously transformed ($\log(x + 1)$) and standardized data. In all cases, samples from newborn individuals were included as the reference point at time 0. Finally, a two-way ANOVA was used to analyse variations in metabolites over time (factor with four levels) considering only the NC_3 and PC_3 treatments in the third experiment (factor with two levels). Analysis of residual was used to ensure that all general linear model requirements were met (Zuur, Ieno, & Smith, 2007). Variations in time were plotted for each metabolite in both treatments, and the corresponding newborn seahorse values in each case were included for comparative purposes.

3 | RESULTS

3.1 | Maximum starvation time

The experiment to determine MST showed that all *H. erectus* in treatments FC_1 and $S1_1$ survived until the end of the experiment (10 days; Table 2). Two critical moments were evidenced in treatment SC_1 with 76 and 36% survival on days 3 and 7 respectively. On day 9, <10% of the seahorses in this treatment remained alive, but animals had all died by day 10 (Table 2). The MST for *H. erectus* reared at 27°C was thus set at 9 days.

3.2 | Resistance to starvation

Here again, the totality of the seahorses from treatment FC_2 survived (Figure 1, Table 2), confirming high survival when seahorses are continuously fed. From day 1 to day 5, no decrease in survival was observed in any experimental treatment. In the 2-day starvation treatment ($S2_2$), a constant decrease in survival rate was observed from day 6 onwards, with a final survival of 89% on day 10 (Figure 1, Table 2). In the 4-day starvation treatment ($S4_2$), survival started to

TABLE 2 Survival (%) of *Hippocampus erectus* juveniles throughout experiments to determine the maximum starvation time and the effect of starvation and food re-introduction on survival

		Days of experiment									
n.	Treatments	1	2	3	4	5	6	7	8	9	10
	1. Maximum starvation time										
3	FC_1	100	100	100	100	100	100	100	100	100	100
3	$S1_1$	100	100	100	100	100	100	100	100	100	100
3	SC_1	100	100	76.6 ± 5	73.3 ± 5	73.3 ± 5	73.3 ± 5	36.6 ± 5	36.6 ± 5	6.6 ± 5	0
	2. Resistance to starvation										
5	FC_2	100	100	100	100	100	100	100	100	100	100
5	$S2_2$	100	100	100	100	100	95.5 ± 11.1	93.3 ± 11.1	93.3 ± 11.1	91.1 ± 11.1	88.89
5	$S4_2$	100	100	100	100	100	100	86.6	68.8 ± 66.6	60.0 ± 44.4	44 ± 33.3
5	SC_2	100	100	100	100	100	100	100	95.5 ± 11.1	66.7 ± 22.2	6.6

Note: Feeding days are represented in light grey and starvation days in dark grey. Values are means ± inter-quartile range; *n* is the number of true replicate aquaria; 10 and 9 seahorses per aquaria were used in experiment 1 and 2 respectively.

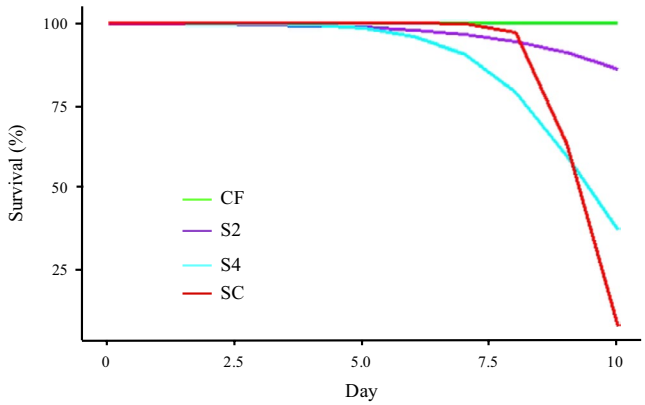

FIGURE 1 Proportion of newborn *Hippocampus erectus* that survived through time (days) during experiments to determine maximum starvation time and resistance to starvation and food re-introduction. Seahorses were subject to different combinations of starvation and refeeding (Table 1): CF (continuously fed), S2 and S4 (starvation for 2 and 4 days, respectively, followed by continuous feeding), SC (starved continuously)

decrease on day 7, reaching a final value of 44% on day 10. Besides exhibiting the lowest survival values, mortality in this treatment was higher than in $S2_2$ (Figure 1; Table 2). In the continuous starvation treatment (SC_2), survival began to decrease on day 8, with a final value of only 6% on day 10 (Figure 1; Table 2).

3.3 | Variations in metabolites through time

Overall, PCAs on metabolite concentrations in seahorses at days 2, 4, 6 and 8 showed that the first two components combined accounted for 90%–93% of data variation in each ordination plot (Figure 2: glucose, acylglycerides, cholesterol; Figure 3: lipids and proteins). Samples characterized by low variable values were concentrated to the left side of the four ordination plots, while those with high values were concentrated to the right (Figures 2 and 3). Samples of individuals under continuous starvation treatment (SC_3) consistently coincided with those of newborn individuals and exhibited low concentrations of all metabolites (particularly lipids and glucose) throughout the entire experiment (Figures 2a–d; 3a–d). By contrast, samples from continuously fed (FC_3) seahorses always had high concentrations of acylglycerides, proteins and glucose (Figures 2a–d; 3a–d).

Ordinations corresponding to samples from day 4 (Figures 2b, 3b) showed that the metabolic profile of organisms subject to 2 days of starvation ($S2_3$) was similar to the profile of those continuously fed (FC_3), indicating recovery had taken place just 2 days after food re-introduction. Samples from day 6 (Figures 2c, 3c) showed that metabolite concentrations in $S2_3$ were similar to those from the positive control (continuously fed). In seahorses subject to 4 days of starvation ($S4_3$), metabolite concentration had not recovered after 2 days of food re-introduction (day 6), and the profile was similar to those continuously starved (SC_3) (Figures 2c, 3c). However, samples from day 8 (Figures 2d, 3d) clearly showed that organisms had recovered from treatment $S4_3$ with metabolite concentrations closer to those of continuously fed seahorses (FC_3). Samples from seahorses subject to 6 days of starvation ($S6_3$) taken after 2 days of food re-introduction (day 8: Figures 2d, 3d) showed marked variation, with profiles spreading in both directions: similar to organisms in other starvation treatments ($S2_3$, $S4_3$) as well as those in the control treatment (FC_3).

Concentrations of total lipids, total proteins, acylglycerides and glucose of organisms in continuous starvation (SC_3) were consistently lower than those of the individuals fed (FC_3) from day 2 to the end of the experiment (Figure 4a–d). However, cholesterol values were similar in both treatments at all moments sampled, showing an increase on days 2 and 4, and a subsequent decrease near the end of the experiment (Figure 4e). In addition, a constant decrease in the concentration of total proteins and total lipids was observed over time in seahorses subject to continuous starvation. The

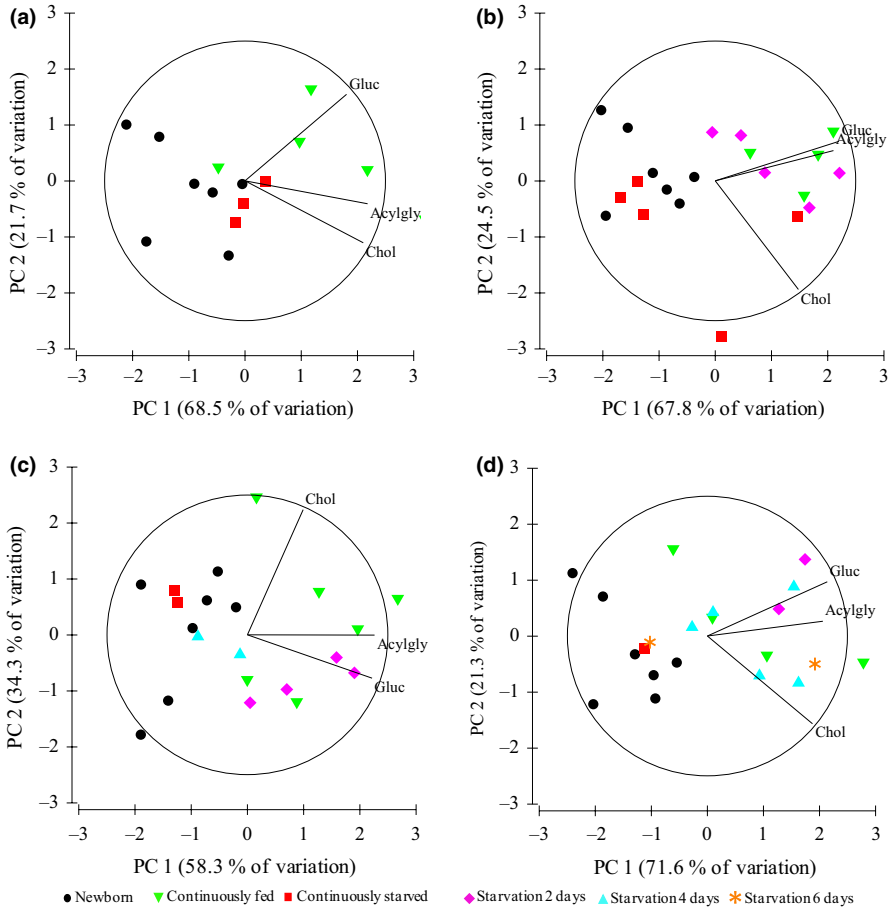

FIGURE 2 Relations amongst cholesterol, acylglycerides and glucose concentrations measured in whole body samples of newborn *Hippocampus erectus* in treatments (FC, SC, S2, S4, S6). Analyses were performed at four different moments over the course of the experiment: days 2 (a), 4 (b), 6 (c) and 8 (d). Metabolite values were also measured in newborn (day 0) to establish the metabolic condition before the experiment and were used as reference values

concentrations of these metabolites also decreased compared to those of newborn seahorses (Figure 4a, b). By contrast, concentrations of all metabolites in fed seahorses increased towards day 4 and were higher than those in newborn individuals (Figure 4a–e). Finally, continuously fed seahorses showed an increase in lipids, proteins, acylglycerides and glucose on day 4 of the experiment (Figure 4a–d). Notwithstanding the patterns described, statistical analysis of data only showed significant differences in the concentration of total proteins, acylglycerides, lipids and glucose between seahorses subjected to continuous starvation and those constantly fed (Table 3), probably because of the small and uneven number of replicas.

4 | DISCUSSION

Results of the present work showed that *H. erectus* exposed to prolonged starvation had low mortality (below 25%) during the first 5 days after birth. From then onwards, mortality rapidly increased reaching almost 100% after 10 days without food (treatments SC1 y SC2). The maximum survival period in unfed juvenile *H. erectus* was 9–10 days at 27°C. Such time interval is longer than that reported for newborn *H. reidi* (3 days) and *H. trimaculatus* (3–7 days) (Sheng et al., 2007; Willadino et al., 2012). This could be related to the difference in the duration of the planktonic stage immediately after birth, during which newborns must attach to a substrate. While *H. erectus* shows a short planktonic phase, lasting <24 hr after birth (López Hidalgo, 2014), *H. reidi* and *H. trimaculatus* may remain in this phase for longer (Sheng et al., 2007).

In the context of aquaculture, an extended tolerance to starvation could imply a potential reduction in the economic loss normally associated with transportation during the distribution of organisms to their final retail destinations. When both shipping and acclimation time at the final retail destinations are taken into account (Correia & Rodrigues, 2017), periods in which animals need to go by without food may be considerably long. In addition, starvation prior to and during transportation has been used as an efficient technique to reduce the accumulation of end products of the metabolism of proteins (Cohen et al., 2018).

An outstanding feature of the present study was that survival was consistently 100% from day 1 to day 5 amongst all treatments where food was re-introduced, showing a great tolerance to starvation in *H. erectus* (Table 2), particularly when compared to *H. hippocampus* (Otero-Ferrer et al., 2010), *H. abdominalis* (Woods, 2000) and *H. barbouri* (Ambas, 2009). Furthermore, delaying the first feeding for 2 days ($S2_2$) resulted in a small decrease in survival (88% seahorse remained alive on day 10). This indicates that the effect of a 2-day starvation period on the nutritional status was reversible at this age and that seahorses are born with enough energy reserves to survive without food for short periods of time (Table 2). In contrast, after an interval of 4 days of starvation ($S4_2$), survival decreased to 44% on

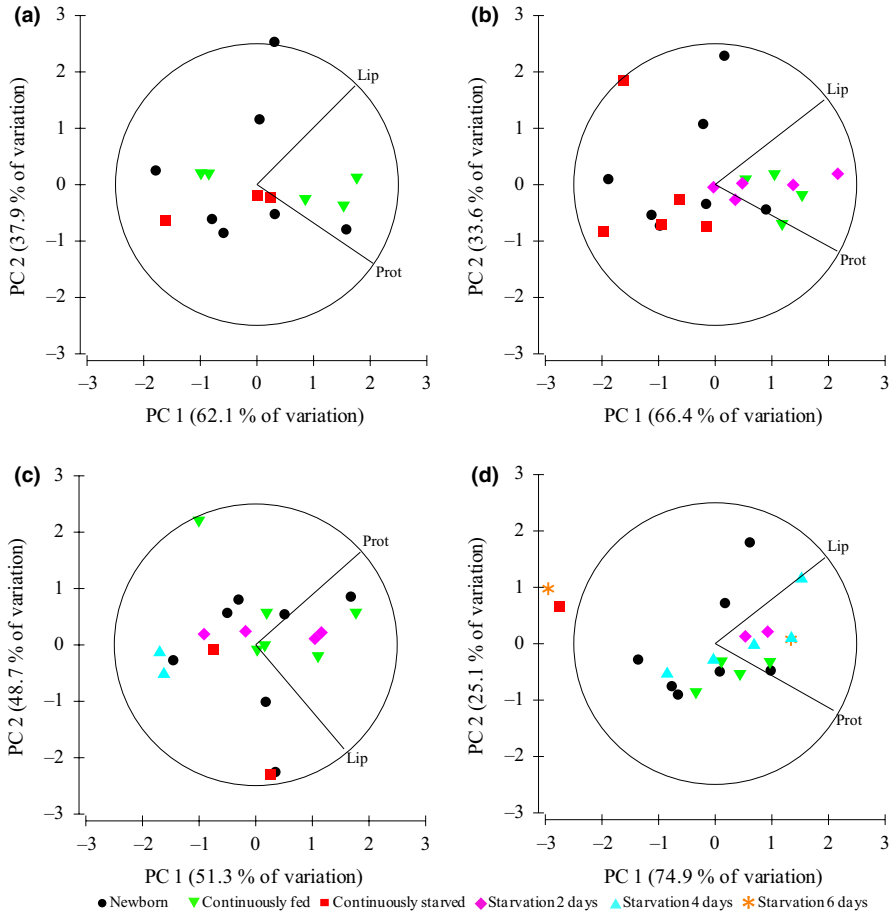

FIGURE 3 Relations amongst total proteins and total lipids concentrations measured in whole body samples of newborn *Hippocampus erectus* in treatments (FC, SC, S2, S4, S6). Analyses were performed at four different moments over the course of the experiment: days 2 (a), 4 (b), 6 (c) and 8 (d). Metabolite values were also measured in newborn (day 0) to establish the metabolic condition before the experiment and were used as reference values

day 10, showing a lack of recovery even after feeding was resumed (Table 2 and Figure 1). Consequently, a 4-day starvation period causes irreversible disruptions, even if food is later re-introduced.

Results in the present study differ from those reported for *H. trimaculatus* and *H. kuda* subjected to various starvation intervals followed by refeeding (Sheng et al., 2007). When subjected to 2 days of starvation, survival of *H. trimaulatus* and *H. kuda* on day 7 was 32% and 66.7% respectively. However, when subjected to 4 days of starvation, all individuals of both species had perished by day 6 (Sheng et al., 2007). Dissimilarities like these have been explained by interspecific differences in the size at birth and the amount of energy invested in searching and capturing prey between large and small individuals (Koldewey & Martin-Smith, 2010; Lourie et al., 2004; Zhang, Yin, & Lin, 2011). The mechanism seahorses use to capture prey is based on the snout's suction force and the size of the mouth; hence, larger individuals can feed on prey more heterogeneous in size, reducing the proportion of energy spent in feeding when compared to smaller individuals (Leysen et al., 2011; Roos et al., 2010; Vargas-Abúndez, Simões, & Mascaró, 2018). In addition, Sheng et al. (2007) hypothesized that larger individuals, with better swimming abilities, spend less energy searching for food and therefore have greater tolerance to starvation than smaller individual. Considering that newborn *H. erectus* have a larger size than *H. kuda* and *H. trimaculatus* (11, 7 and 6 mm, respectively) (Lourie et al., 2004), our results constitute evidence in favour of this idea. In fact, both overall survival and starvation time for *H. erectus* were higher than in *H. kuda* and *H. trimaculatus*. This result suggests that 4–5 days of starvation may cause an irreversible disruption of certain physiological processes that compromises the ability to recover, even if food is re-introduced.

Juveniles constantly fed during the first 10 days of life showed 100% survival (treatments FC_1 y FC_2). This result is higher than those of *H. reidi* (13% at 14 days after birth) and *H. trimaculatus* (39.2% at 7 days after birth) (Garcia-Manchón et al., 2013; Sheng et al., 2007; Willadino et al., 2012). Although the present study did not compare other types of food, results suggest that feeding based on enriched *A. franciscana* nauplius is adequate for the species in this phase of its life cycle. This diet has also been successful in culture protocols for other species of seahorses, such as *H. abdominalis* (Shapawi & Purser, 2003; Woods, 2003), *H. reidi* (Willadino et al., 2012) and *H. hippocampus* (Otero-Ferrer et al., 2010).

The evaluation of metabolite concentrations in whole seahorses offered an integral vision of energy use during the first 8 days of life of *H. erectus* under different feeding conditions. These results confirm that proteins and lipids are essential in the first stages of development, (Farrell, 2011; Navarro & Gutiérrez, 1995; Tocher, 2003). With the nutritional status at birth as reference (day 0), results showed that seahorses that were continuously fed during the first 4 days increased their metabolite concentration because of effective food breakdown (Figure 4a–d). In particular, a gradual increase in acylglycerides was observed (Figure 4c), confirming the important

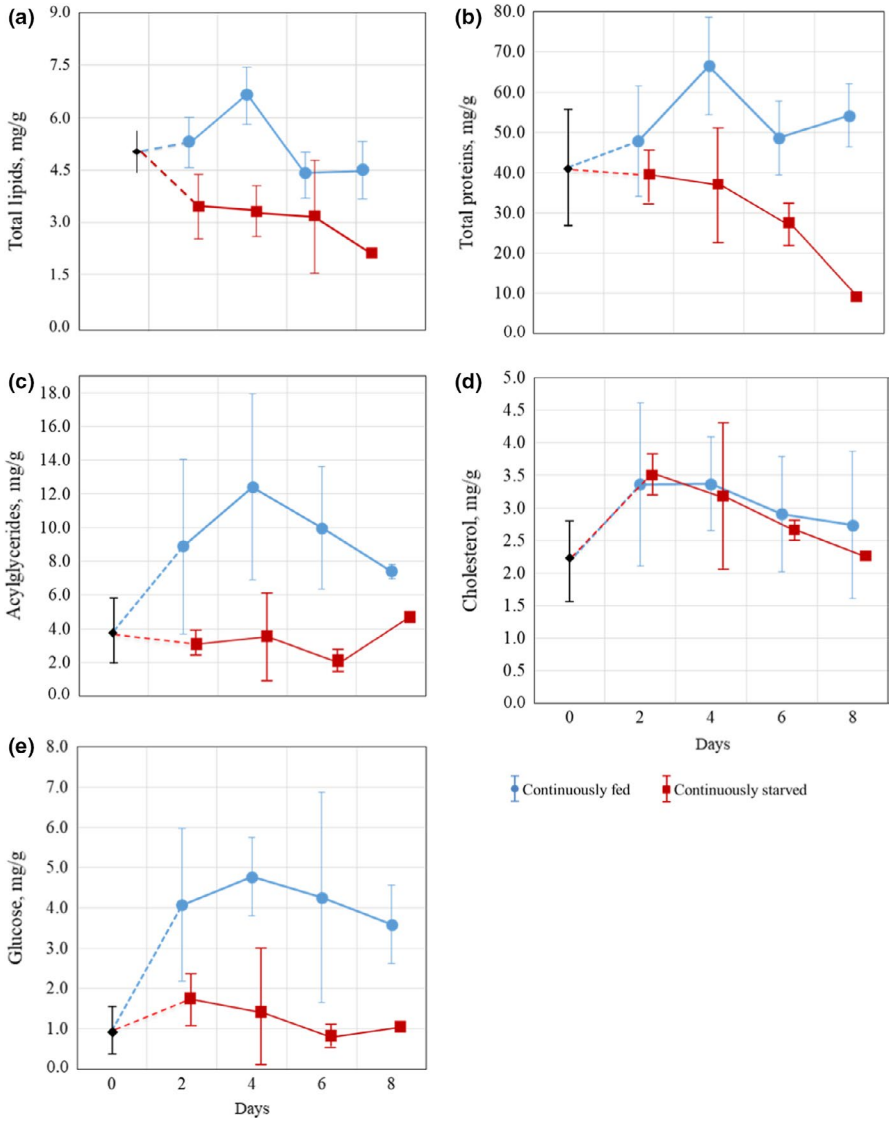

FIGURE 4 Variations in metabolite concentrations over time (days 2, 4, 6 and 8) in continuously starved (SC_3) and continuously fed (FC_3) newborn *Hippocampus erectus*. Metabolite concentrations were measured in whole body samples of newborns (day 0) before the experiment and were used as reference values. The lines between sampling points were inserted as visual aids to explain results and do not represent dependency relationships

TABLE 3 Results of factorial ANOVA performed on metabolites (total proteins, total lipids, cholesterol, acylglycerides and glucose) in whole body samples of newborn *Hippocampus erectus* subject to continuous starvation or feeding treatments (factor 1) and quantified on days 2, 4, 6 and 8 (factor 2)

	Proteins		Acylglycerides		Cholesterol		Glucose		Lipids	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Treatment (*T*)	18.36	<.05	10.88	<.05	0.42	.52	18.77	<.05	8.46	<.05
Sampling time (*D*)	2.55	.08	1.21	.32	1.00	.41	0.27	.84	1.03	.39
T × *D*	2.36	.09	0.24	.86	0.23	.86	0.17	.91	0.45	.71

Note: p-values were considered significant at <.05.

role that stored acylglycerides play in the growth of this and other marine species (Ozkizlick & Chu, 1994; Willmer, Stone, & Johnston, 2005). Such is the case of the digestive system of newborn *H. guttulatus*, where lipid metabolism plays a preponderant role during the first days of life (Ofelio, Cohen, Adriaens, Radaelli, & Díaz, 2019).

Histological and histochemical studies on both *H. reidi* (Novelli et al., 2015) and *H. gutullatus* (Ofelio, Díaz, Radaelli, & Planas, 2018) showed the presence of yolk remains in newborn seahorses of both species, suggesting a complementary endogenous source of energy until roughly 48 hr after birth. Despite the lack of similar studies in *H. erectus*, the observed decrease in metabolites in continuously starved individuals (Figure 4a–d) could be due to a partial depletion of food reserves remaining in the yolk sac (Kamler, 1992b). Cholesterol levels in *H. erectus* under continuous starvation,

however, did not change (Figure 4d) probably because, as in other marine fish, cholesterol is not used as a source of metabolic energy (Chatzifotis et al., 2011), but as a structural component of cell membranes (Tocher, 2003). By contrast, a marked decrease in lipids and, to a lesser extent, in total proteins was observed from the first 2 days of starvation (Figure 4a, b).

A gradual breakdown of proteins during the first 4 days of starvation occurs because this metabolite is a last-resource fuel source, and the physiological change from lipid catabolism to protein catabolism takes place when lipid levels reach a critical threshold (McCue, 2010). The use of both metabolites (lipids and proteins) is associated with oxidation processes that ensure obtaining the necessary energy to sustain basal metabolism during starvation (Caloin, 2004; McCue, 2010). The higher calorific coefficient and efficiency of lipids compared to proteins as a source of metabolic energy (38, and 23.6 kJ/g, respectively; Jobling, 1994) further explain the sequential and matching patterns of variation in these metabolites through time. Energy loss in hungry organisms could be considered a cause of physiological impairment (swimming, growth, efficiency of the immune system) and ultimate death (Caloin, 2004; Lall, 2000; Sheng et al., 2007). Most surely, the mortality observed in organisms subject to continuous starvation was caused by the depletion of these nutrients and the metabolic imbalance resulting from the absence of energy to sustain vital biological functions (McCue, 2010, 2012). Comparisons at an interspecific level have revealed a similar metabolic dynamic in juvenile *Dicentrarchus labrax* (Govoni, Boehlert, & Watanabe, 1986), establishing a connection with the metabolic pathways of the tricarboxylic cycle or gluconeogenesis where amino acids are used to produce energy (Murray et al., 2009; Sargent, Tocher, & Bell, 2003).

Figure 5 summarizes the four possible physiological conditions in response to various intervals of starvation and feeding. During continuous starvation (Figure 5a), organisms use the energy reserves they were born with to sustain basal metabolism. The resulting decrease in the concentration of metabolites reduces the probability of survival (Navarro & Gutiérrez, 1995). Depending on the time of starvation, to resume feeding may lead to two physiological conditions with varying metabolite concentration and chances of survival. One possible outcome is that food metabolism does not translate into increased metabolite concentrations, so individuals do not recover their previous physiological state and die (Figure 5b). This has been associated with the decline of digestive organs and/or physiological processes (Gisbert, Conklin, & Piedrahita, 2004), a decrease in foraging behaviour (Sheng et al., 2007) or in the need of additional energy to metabolize food in an already compromised physiological condition (Kamler, 1992a). Another possible outcome is the recovery of the previous physiological state, through the metabolization of food that would result in high metabolite concentration and starvation having limited effects on survival (Figure 5c). This last condition has similar features to those of continuous feeding, where metabolite concentrations and survival are high (Figure 5d).

Results in the present study demonstrate that under continuous starvation, total lipids are the first metabolic source of energy used by seahorses to sustain basal physiology and that proteins are metabolized only in a second instance. Under such circumstances, survival reaches very low values (0%–10%). A starvation interval of 4 days or more compromises the physiological status of organisms in such a way that the increased energy demand to metabolize the food that could be re-introduced further impairs the already frail metabolic state, undermining probabilities of survival. By contrast, with starvation periods of <2 days, followed by a feeding protocol aimed at covering the basic energy requirements of newborn seahorses (i.e., *Artemia* nauplius enriched with fatty acids), a robust metabolic

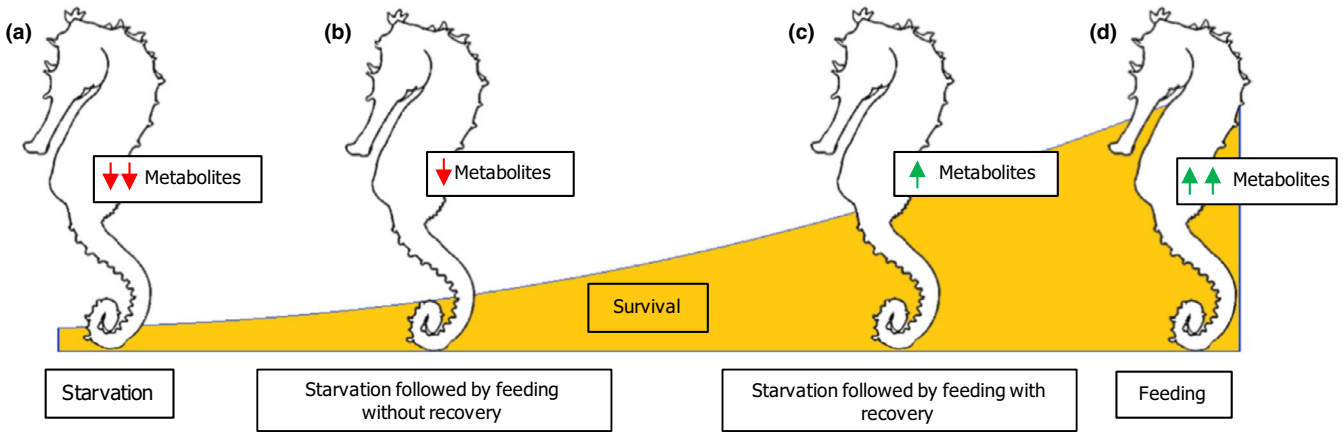

FIGURE 5 Conceptual diagram representing the four physiological conditions that result from the following feeding schemes: I Continuous starvation determines low survival rates and low metabolite concentrations. II Starvation followed by feeding without recovery: Although food is provided after a starving interval, a compromised physiological condition does not allow nutrient metabolization, metabolite concentrations do not increase, and survival decreases. III Starvation followed by food re-introduction does not compromise physiological processes irreversibly and allows food to be metabolized, with the consequent increase in metabolite concentrations and a small impact on survival. IV Continuous feeding and metabolization of food increase metabolite concentrations and do not affect survival

status can be recovered, and metabolite concentrations increased, reaching values similar to those observed in individuals fed continuously since the first day after hatching. Results suggest that the cost of energy investment in recovery has a slight impact on survival (88%), but indicate that this starvation interval does not cause irreversible damage to homeostasis and allows seahorses to continue with most biological processes.

The characterization of juvenile *H. erectus* metabolites is the first contribution to the understanding of the nutritional status of the early phases of this species and allows a better understanding of the use of energy reserves by individuals in starving conditions. Finally, this information is relevant for the manipulation of juvenile *H. erectus* during transportation in order to ensure maximum survival and well-being of the organisms destined for trade. In addition, our results demonstrate the great potential of this species as a candidate for aquaculture insofar as it meets commercial demands of the ornamental trade, avoiding the illegal exploitation of wild populations.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

The original idea and experimental design was provided by FOF, NS and MM. GV carried out the experiments, laboratory work and data analysis. Biochemical analyses were coordinated and interpreted by CP and statistical analyses were so by MM. CR and NS contributed to the general interpretation of results. GV and MM wrote the first draft of the manuscript and CR, CP, FOF and NS contributed substantially to revisions.

DATA AVAILABILITY STATEMENT

Original data from this study are shared at https://zenodo.org/record/2628847 https://doi.org/10.5281/zenodo.2628846.

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