ONTOGENY OF DIGESTIVE ENZYMATIC CAPACITIES IN JUVENILE SEAHORSES
***Hippocampus guttulatus* FED ON DIFFERENT LIVE DIETS**

Blanco Andreu*, Planas Miquel¹, Moyano Francisco Javier²

¹Instituto de Investigaciones Marinas (IIM-CSIC), Eduardo Cabello 6. 36208 Vigo (Spain)

²Dpto de Biología y Geología, Campus de Excelencia Internacional del Mar (CEI-MAR), Universidad de Almería, Ctra de la Cañada s/n. 04120 Almería (Spain)

*andreublanco@iim.csic.es

Abstract

Differences in survival and growth rates in seahorse *Hippocampus guttulatus* juveniles feeding on *Artemia* sp. or copepods have been related to specific digestive capacities of seahorse newborn, which are capable of actively forage on available prey from the first days of live. Other seahorse species, such as *H. abdominalis* and *H. hippocampus*, show high success feeding on *Artemia* nauplii suggesting species-specific differences in the digestibility of prey among seahorses. In the present study, the profiles of digestive enzyme activity during the initial 15 Days After Release (DAR) were very low for trypsin, chitinase and α -amylase. In contrast, higher activities towards any of the assayed substrates for lipase (butyrate, octanoate and oleate) were evident from 0 DAR onwards. From 15 DAR onwards, the effect of diet composition became evident in juveniles previously fed on a mixed diet (*Artemia* + copepods), which showed a clear increase in all the assayed enzymes when compared to juveniles fed on *Artemia* as a sole prey. As a practical applicability of this study, a feeding schedule ensuring an adequate digestibility of the prey is proposed based on ontogenetic enzymatic activities of seahorse juveniles fed on different prey.

Keywords: enzyme activity, *Hippocampus guttulatus*, digestibility, ontogeny, *Artemia*, copepods.

27 Introduction

28 Seahorses (*Hippocampus* spp.) are fishes of the Family Syngnathidae with particular anatomical and
29 physiological features such as head shape, snout and mouth morphology, male pregnancy and feeding
30 behavior (Blanco, 2014). The increased knowledge on seahorse biology has permitted significant
31 advances in rearing techniques for some species (Olivotto et al. 2011b). Despite seahorse newborn are
32 almost fully developed and capable to feed on live prey it seems that some species have a limited ability
33 to digest *Artemia* (Koldewey & Martin-Smith 2010; Olivotto et al. 2011b; Blanco, 2014). Although the
34 effects of different feeding schedules have been studied and higher survival has been reported in captivity
35 (Blanco, 2014), only few studies have evaluated their digestive enzyme activity (Wardley 2006; Álvarez
36 et al. 2009; Quintas et al. 2010). Hence, great differences in survival and growth rates have been reported
37 when feeding seahorses on either *Artemia* or copepods, although it seems that the combination of both
38 types of live prey may result in a greater rearing success in some seahorse species (Payne & Rippingale
39 2000; Woods 2000; Job et al. 2002; Sheng et al. 2006; Planas et al. 2009; Olivotto et al. 2008). However,
40 feeding and nutrient assimilation still remains as a main factor of mortalities during seahorse ontogeny
41 (Blanco et al. 2011).

42 Mortality during early stages of development is a main bottleneck in the production of many species of
43 marine fish (Kim et al. 2001; Pérez-Casanova et al. 2006; Olivotto et al. 2011b), being survival closely
44 related to the availability of suitable food items adapted to the nutritional requirements of the species and
45 to physiological changes with growth (Bolasina et al. 2006; Lazo et al. 2007). Critical periods in the early
46 development of most marine fish generally occurs after the shifting from endogenous to exogenous
47 feeding and the development of the digestive tract (Ribeiro et al. 1999; Kim et al. 2001). These events are
48 closely related to the ontogeny of the digestive system and activity of digestive enzymes has been used as
49 a reliable indicator of the ability of the species to digest different feed substrates as well as of the degree
50 of maturation of the digestive tract (Perez-Casanova et al. 2006, Kamarudin et al. 2011, Sanz et al. 2011,
51 Srichanun et al. 2011, Gisbert et al. 2013).

52 Considering the aforementioned, the aim of the present study was a) to assess the ontogeny of some of the
53 main digestive pancreatic enzymes (trypsin, α -amylase, lipase and chitinase) during the early
54 developmental stages of the long-snout seahorse *Hippocampus guttulatus* and b) to evaluate changes in
55 their enzymatic profile when fed on different prey types (*Artemia* or copepods). That knowledge would

contribute to adjust accordingly the most appropriate feeding schedule for the rearing of this threatened species and hence to improve the fulfillment of their nutritional requirements.

Material and Methods

Broodstock

Adult *Hippocampus guttulatus* were collected by scuba diving from 2010 to 2011 offshore in Galicia (NW Spain) and transported to the facilities at the Institute of Marine Research (CSIC) in Vigo (Spain). After their arrival to the laboratory, the fish were acclimated and transferred to 630 l aquaria units (Planas *et al.* 2008). Seahorse broodstock was fed *ad libitum* three times per day on adult enriched *Artemia* supplemented with captured Mysidacea (*Leptomysis* sp. and *Siriella* sp.). The *Artemia* enrichment was made on a mixture of the microalgae *Phaeodactylum tricornutum* (1.6×10^7 cells ml^{-1}) and *Isochrysis galbana* (10^7 cells ml^{-1}) and one daily dose (0.1 g l^{-1}) of Red Pepper (Bernaqua, Belgium) for at least 5 days. Feces and uneaten food were siphoned out before feeding. Water quality was checked periodically for NO_2 , NO_3 and NH_4/NH_3 content (0 mg l^{-1}) by using Sera Test Kits. Salinity and pH levels were 38 ± 1 ppt and 8.1 ± 0.1 , respectively

Three pregnant seahorses were transferred from the broodstock aquaria to 30 l aquaria (18°C and 12 L : 12 D light regime) and maintained isolated for a few days until release of the newborn ($n = 218, 299$ and 598 , respectively). Three batches of newborn (one per male) were used in the experiments. Each batch was distributed into aquaria and submitted to feeding or starving conditions.

Seahorse rearing system

Newborn batches were raised separately in 30 l Kreisel-type aquaria ($5 \text{ juveniles l}^{-1}$) connected to a semi-opened recirculation system which included a degasifying column and two 50 l biofilters with mechanical (up to $20 \mu\text{m}$) and biological filters, aerators and skimmers. The seawater was pumped from the biofilters to 36w UV light units and after to 50 l reservoir tanks. Water temperature was maintained constant until the end of the experiments at $19 \pm 1^\circ\text{C}$ (Planas *et al.* 2012) by using an inlet heating system (HC300/500 A, HAILEA). The total volume of the rearing system was renovated twice per hour by means of an external inflow (24 l h^{-1}) of $20 \mu\text{m}$ filtered and UV- treated seawater. A photoperiod of 16L : 8D was established, being lighting supplied by 20 w fluorescent lamps (Power Glo) placed laterally at 20 cm of the aquaria. Opaque black plastic films covered the half upper sides of the aquaria back-walls. Aeration was gently provided at 15 cm deep from the water surface near the outlet window of the aquaria and

seawater inflow was located above the water surface in the opposite corner of the aeration inflow (Blanco *et al.* 2014).

Live prey production

Copepods were cultivated on mixtures of the microalgae *Isochysis galbana* (10^7 cells ml^{-1}) and *Rhodomonas lens* (1.6×10^7 cells ml^{-1}) in 250 l and 500 l tanks. *Artemia* cysts (EG; Iberfrost, Spain) were incubated daily at 28°C for 24 h and the freshly hatched nauplii were transferred to 5 l buckets (100 *Artemia* ml^{-1}). *Artemia* metanauplii enrichment was carried out for 24 h at 100 *Artemia* ml^{-1} on a 1:1 mixture of *I. galbana* (10^7 cells ml^{-1}) and *Phaeodactylum tricornutum* (1.6×10^7 cells ml^{-1}). Prior to their use, all preys were washed and sieved on different mesh sizes depending on the species (125µm for *Artemia*, and 180µm for copepods), and then counted before feeding the seahorse juveniles.

Feeding conditions

Two batches of newborn ($n = 282 \pm 57$) were fed on different diets differing in the type of prey supplied during the first 10 days of life. The prey composition of the diets was as follows: (Fig. 1):

- Diet A (*Artemia* diet): Three daily doses (09.00, 12.00, and 15.00 h) of GSL *Artemia* nauplii (1 *Artemia* ml^{-1} dose $^{-1}$) from 0 to 10 DAR (Days After Release).
- Diet M (Mixed diet): A single daily dose (09.00 h) of cultivated copepods (about 87% *Acartia tonsa* and 13% *Tisbe* sp.) at a density of 0.7 copepods ml^{-1} from 0 to 5 DAR; and a daily dose of GSL *Artemia* nauplii (10.00 h; *Artemia* ml^{-1}) and copepods (18.00 h; 0.7 copepods ml^{-1}) from 6 to 10 DAR.

From 11 DAR until the end of the experiment at 30 DAR, both experimental groups received three daily doses of *Artemia* nauplii + 24 h enriched *Artemia* metanauplii (1:1; 1 *Artemia* ml^{-1} dose $^{-1}$). The aquaria were provided with a 500µm mesh during daytime to allow the exit of the remaining prey between feeding times and a 250 µm mesh at night to avoid prey from leaving the aquaria while allowing water circulation (Blanco *et al.* 2014).

Growth and development

Samples of seahorses ($n=10$) were taken at 0, 5, 10, 15 and ($n= 5$) 30 DAR in fed groups and at 0, 1, 2, 3 and 4 DAR in the starvation group. Prior to sampling, all seahorses were starved overnight for complete gut emptiness. Seahorses were sampled in the morning (09.00), anaesthetized with MS222 (0.1 g l^{-1}),

114 washed with tap water, transferred individually to Petri dishes and photographed for standard length (SL)
 115 measurements to the nearest 1µm. SL was measured as head + trunk + tail length (curved measurement),
 116 as reported by (Lourie *et al.* 1999). Measurements were made on digital images using image-processing
 117 software (NIS, Nikon). Then, the excess of water was removed and the seahorses were individually
 118 weighted on a Sartorius microbalance MC210P (± 0.01 mg). After this, the fish were frozen at -80°C ,
 119 freeze-dried (Ilshin Lab Co., Ltd.) and stored at -80°C until determination of enzyme activities.

120 Effective day-degrees (D°_{eff}) (Kamler, 1992) as a model for the quantification of the relationship between
 121 ontogenetic rate and temperature has been recommended for temperature independence studies (Kamler,
 122 1992; Cunha & Planas, 1997), which has been demonstrated to describe ontogenetic development in
 123 seahorses (Planas *et al.* 2012). Therefore, D°_{eff} have been used as a temperature independent time-scale in
 124 order to standardize the data and make it comparable to other fish larvae studies. The index was
 125 calculated as follows:

$$126 \quad D^{\circ}_{\text{eff}} = \Delta t \cdot T_{\text{eff}} = \Delta t \cdot (T - T_0)$$

127 where T_{eff} is the biologically effective temperature ($T_{\text{eff}} = T - T_0$), T is the temperature in $^{\circ}\text{C}$ and T_0 is the
 128 species-specific threshold temperature. However, days after release (DAR) will be used for clarity in the
 129 seahorse ageing.

130 Calculations involving development were performed according to the formulations described in Otterlei
 131 *et al.* (1999). Daily weight-specific growth rates (G ; $\%\text{day}^{-1}$) in juveniles were calculated as:

$$132 \quad G = 100 * (e^g - 1)$$

133 being g the instantaneous growth coefficient obtained by the following equation:

$$134 \quad g = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

135 where W_2 and W_1 are the average dry weights (mg) of fish on days t_2 and t_1 , respectively.

136 Fulton's condition factor increment (ΔK_F) was calculated to avoid batch bias between treatments (Planas
 137 *et al.* 2012) as:

$$138 \quad \Delta K_F = K_{Ft} - K_{F0}$$

139 where K_{Ft} and K_{F0} are the Fulton's condition factor at a time t and in newborn seahorses, respectively.
 140 K_F s were calculated as:

$$141 \quad K_F = (DW/SL^a) * 1000$$

142 with DW dry weight, SL standard length and α the exponential relationship between DW and SL for each
143 treatment.

144 *Evaluation of enzyme activities*

145 Enzyme extracts were prepared by mechanical homogenization (Ultra Turrax; Ika, Germany) of each
146 single seahorse juvenile (0, 5, 10, 15, and 30 DAR) and their prey (copepods, *Artemia* nauplii and
147 *Artemia* metanauplii) in distilled water followed by sonication using three short pulses of 3s (Vibra-cell,
148 Sonics, USA). The homogenates were then centrifuged for 15 min at $11,000 \times g$ at 4°C and the extracts
149 aliquoted and stored until being analyzed. Enzymatic activities were analyzed using fluorescent substrates
150 in a Fluoroskan reader (ThermoFisher Scientific; U.S.A.) using 96-well CLINIPLATE black flat bottom
151 microplates (Thermo Scientific). The substrates and conditions in each assay were:

152 *Trypsin*: Boc-Glan-Ala-Arg-methylcoumarin hydrochloride (SIGMA B4153) was diluted in dimethyl
153 sulfoxide (DMSO) to a final concentration of $20 \mu\text{M}$. Five μl of the substrate were mixed with 195 μl of
154 50 mM Tris-HCl, 10 mM CaCl_2 buffer, and 10 μl of the diluted homogenate were added to the
155 microplate. Fluorescence was measured at 380/440 nm EX/EM (Rotllant *et al.* 2008).

156 α -Amylase: Ultra α -amylase Assay Kit (E33651) from Molecular Probes was used for the analysis. 100 μl
157 of the substrate solution were added to 10 μl of fish extracts, being fluorescence measured at 485/538 nm
158 EX/EM.

159 *Lipases*: Non-specific esterases were evaluated following a method modified from Vaneechoutte *et al.*
160 (1988) using substrates of a different chain length: 4-methylumbelliferyl butyrate (MUB; Fluka 19362),
161 6,8-difluoro-4-methylumbelliferyl octanoate (MUOc; Invitrogen D-12200) and 4-methylumbelliferyl
162 oleate (MUO; Sigma 75164). The stock solutions were prepared by dissolving 0.39 mg, 0.54 or 0.69 mg
163 of MUB, MUOc or MUO in 1 ml DMSO, to which 100 μl Triton X-100 was added. These stock solutions
164 were then diluted in phosphate buffer pH 7.0 to a final concentration of $30 \mu\text{M}$. Ten μl of the diluted
165 homogenate were added to the microplate and mixed with 190 μl of each fluorogenic substrate.
166 Fluorescence was measured at 355/460 nm EX/EM.

167 *Chitinase*: The activity was analysed using an Assay Kit (CS1030; Sigma) which provides three different
168 substrates for the detection of the various types of chitinolytic activity: 4-Methylumbelliferyl N,N'-
169 diacetyl- β -D-chitobioside (chitobiosidase activity), 4-Methylumbelliferyl N-acetyl- β -D-glucosaminide (β -
170 N-acetylglucosaminidase activity), and 4-Methylumbelliferyl β -D-N,N',N''-triacylchitotriose (substrate
171 suitable for endochitinase activity). A previous trial on those substrates showed that only endochitinases

were detected in the seahorses (data not shown), consequently, only the substrate 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotriose was used in the present study. The substrate was diluted (1:100) in Assay Buffer to obtain the substrate solution. 90 μ l of substrate solution were placed into each well of the microplate and mixed with 10 μ l of the homogenates. Fluorescence was measured at 360-450 EX/EM. All the assays with the exception of those of chitinase, were performed at room temperature, this latter required heating of samples at 40°C. Enzyme activities were expressed in relation to dry weight and also in relation to soluble protein. In all the cases, units were calculated as μ mol of methylumbelliferone released per min, using a standard curve as a reference.

Statistical analysis

The ontogenetic effects of the diet on DW, SL and enzyme activities in seahorses were analysed applying MANOVA test with sequential Bonferroni adjustment. Robustness of MANOVA allows validating the results even when violating the assumptions of the test (Bray & Maxwell 1985; Camacho Rosales 1995; Walters & Coen 2006). Univariate responses were examined when multivariate effects were significant. Pair wise effects were also analysed among the tested variables both for enzyme activity and biometric responses.

Ethical statement

All procedures involving animals in the present study were conducted in accordance with the Spanish laws on animal experimentation and were approved by the Bioethics Committee of CSIC. Seahorses sampled to study the ontogenetic effects of the diet on growth and digestive enzyme activities were sacrificed using an anaesthetic overdose of MS222 (0.1 g l⁻¹)

Results

Growth and survival during the early development of the long-snouted seahorse *Hippocampus guttulatus* are detailed in Table 1. The effect of the type of prey supplied at first feeding on survival was noticeable in both survival and growth. Survival rates achieved at 10 DAR with diets A and M were 77% and 98%, respectively, whereas final survival rates at the end of the experiment were 58% in fish fed on diet A and 86% in those fed on diet M. Survival of starved juveniles was 0% by 5DAR, when all seahorses were dead or sampled. Growth both in weight and SL was significantly higher (MANOVA, $P < 0.05$) from day

201 5 onwards in seahorse juveniles fed on diet M (1.34 ± 0.35 mg and 18.89 ± 1.53 mm, respectively), when
202 compared to those fed on diet A (1.01 ± 0.28 mg and 17.59 ± 1.21 mm, respectively) and under starvation
203 (0.52 ± 0.02 mg and 14.39 ± 0.29 mm, respectively). Significantly higher DW and SL was obtained by
204 the end of the experiment (MANOVA, $P = 0.000$) in seahorses from diet M, whose final dry weight
205 (17.73 ± 4.23 mg) was almost 4-folds higher than in fish from diet A (4.85 ± 1.18 mg). Final SLs were
206 also significantly different (MANOVA, $P < 0.000$), with lengths of 28.4 ± 1.8 and 42 ± 4.8 mm for fish
207 fed on diets A and M, respectively.

208 The pattern of change in K_F values (K_F increment at different times since 0 DAR) during the experimental
209 period was rather similar in both diets, being characterized by a sharp decrease in values from 0 to 5 DAR
210 followed by a continuous increase until 30DAR (Figure 1). However, according to ΔK_F values, juveniles
211 fed on diet M showed a higher performance for the whole experimental period than those fed on diet A.

212 Changes with age in enzyme activities expressed either in relation to dry weight or to soluble protein are
213 summarized in Figures 2 and 3, respectively. Changes in relation to dry weight were selected to allow a
214 better comparison between treatments, reducing so far the effect of the above-mentioned great differences
215 in size between treatments. A similar pattern in the digestive activity in relation to dry weight was
216 observed in both treatments for trypsin, α -amylase and chitinase during seahorse ontogeny and, in
217 general, low values of activity were measured in seahorses, particularly for α -amylase and chitinase.
218 Those levels were particularly lower than those in the live food offered (*Artemia* nauplii/metanauplii and
219 copepods) (Figure 2).

220 Significant differences between dietary treatments were detected only at 10 and 30 DAR (MANOVA, $P <$
221 0.001 and $P = 0.000$, respectively), when higher enzymatic activities both related to dry weight and
222 soluble protein were recorded in juveniles fed on diet M, especially regarding trypsin, chitinase and α -
223 amylase. In contrast, all lipase activities measured in relation to soluble protein increased significantly at
224 30 DAR in both dietary treatments (except for butyrate in seahorse juveniles fed on diet A), even though
225 values measured in juveniles fed on diet A were significantly lower than in those from diet M
226 (MANOVA, $P < 0.05$). It is noteworthy that the activities of lipase measured towards octanoate were 2-
227 folds higher than those measured to either butyrate or oleate.

228 Similar reduction in the activity of trypsin to either starved or fed (both with diet A and M) juveniles was
229 evidenced. During the first five days of life similar reductions of the other enzymes (lipases assayed with

the different substrates) were obtained when juveniles were starved or fed on diet A compared to those of juveniles fed on diet M (Table 2).

Discussion

The evaluation of the activity of digestive enzymes during the initial stages of development in marine fish is a common tool to assess maturation of their digestive capacities, providing important information on the more suitable moment to introduce changes in the feeding schedule as well as on the suitability of diet composition (Ribeiro *et al.* 1999; Bolasina *et al.* 2006; Kamarudin *et al.* 2011). In contrast to most marine fish larvae, which are dependent on endogenous reserves during the first days of live, seahorse newborn are completely developed and physiologically functional at the moment of male's pouch release, being very active hunters on available prey (Yin & Blaxter 1987; Sheng *et al.* 2006). In spite of this, relatively low success for most seahorse species has been reported in rearings (Olivotto *et al.* 2011b), being the match between prey nutritional quality and seahorse nutritional requirements one of the limiting factors. In addition, there is a lack of information regarding the development of the digestive tract, as well as on digestive capacity in some species of interest; like the long-snouted seahorse *Hippocampus guttulatus*, being results in this work the first ones obtained on this species.

The nutritional status and energetic condition of juveniles are very likely related to their digestive capabilities. Fulton's condition factor (K_F) has been used as a criterion to assess fish condition in advanced stages of development (Ferron & Leggett 1994), but it is not adequate to describe fish condition of early developmental stages at the onset of exogenous feeding (Neilson *et al.* 1986). However, it has been successfully applied in *H. guttulatus* juveniles submitted to different temperature regimes and feeding conditions, in which low K_F -values at 5 DAR were consistent with survival, growth and behaviour (Planas *et al.* 2012). In the present study, K_F -values from 0DAR to 5DAR also dropped steadily, though a progressive recovery occurred from day 10 in both diets. At day 30, K_F -values in juveniles fed on diet A ($\Delta K_F = 0.03$) approached to those of newborn and increased on those fed on diet M ($\Delta K_F = 0.22$). The decrease of K_F -values in juveniles from male's pouch release up to 5DAR was independent of diet type and would reflect an adaptation of young seahorses to feeding and a low digestion/assimilation efficiency, as suggested previously by Planas *et al.* (2012).

The profiles of digestive enzyme activity during the initial 15 DAR reflected a slow development of secretory organs and hence the presence of limited digestive capabilities at early developmental stages of

260 *H. gutturalis*. In contrast to what described for other marine fish larvae, the activity of trypsin was not
261 increased with development (Alvarez-González et al, 2008, Uscanga et al, 2011). The low activity of this
262 enzyme, which plays a major role in the digestive bioavailability of nitrogen, suggests a reduced ability of
263 this species in obtaining this nutrient under the form of complex macromolecules. This points to a greater
264 dependence on the intake of nitrogen compounds under the form of simple molecules like peptides or free
265 amino acids, at least during the initial stages of development, as described for several other larvae
266 (Rønnestad et al. 2003). This should be supported by the higher growth obtained in the seahorses fed on
267 the mixed diet containing copepods, a live prey which presents a higher contents in simple forms of N
268 than the *Artemia* nauplii (Van der Meeren et al. 2007).

269 Chitinase and α -amylase activities followed a pattern similar to that described for trypsin. Chitin
270 represents the main carbohydrate in marine environments and this activity has been suggested as essential
271 in some fish species in order to digest exoskeletons of their preys (Gutowska et al. 2004, Ikeda et al.
272 2009). Chitinolytic activity is due to a two-component enzyme system in which chitin is firstly
273 hydrolyzed by chitinases into oligosaccharides that will be subsequently degraded to monomers by β -N-
274 acetylglucosaminases (Molinari et al. 2007). The results in the present study show that chitinase activity
275 was present in young seahorses just after male's pouch release, but only increased significantly from 15
276 DAR onwards. This activity has been also measured in the gut of other marine and freshwater fish larvae
277 (Kim et al. 2001; Meetei et al. 2014). Different origins have been proposed by chitinase activity measured
278 in fish; since the intake of chitinous exoskeletons in the live food has been linked to increased levels of
279 activity when compared to other feeding regimes (Meetei et al. 2014) being suggested that it may be
280 either contained in the live food or produced by hosted bacteria (Ray et al. 2012). Also the possibility of
281 internal secretion can be considered, as demonstrated in larvae of *Labrus bergylta*, a marine species that
282 produces pancreatic chitinase (Hansen et al. 2013). It seems that pancreatic chitinase is not only important
283 in degrading the chitinous exoskeleton of live prey, but also in the digestion of carbohydrates towards
284 metamorphosis and later in life (Ikeda et al. 2009). A similar role in larvae of seahorses can be
285 considered.

286 Amylase activity has been detected in larval stages of most marine fish (Zambonino & Cahu 2001;
287 Kamarudin et al. 2011) including the pot-bellied seahorse *H. abdominalis* (Wardley 2006). In the present
288 study, α -amylase activity was almost undetectable during the initial days after release, but increased
289 significantly in 30 DAR juveniles, especially in those fed on diet M. In fact, low carbohydrate

concentrations (including glycogen) and a consequent poor utilization of this substrate as energy source have been previously reported in starving *H. guttulatus* juveniles at early stages (Blanco *et al.* 2011). Accordingly, a low α -amylase activity could be expected in this species. In older juveniles, when prey intake increases and the nutritional condition is improved, amylase activities increased moderately in juveniles fed on both diets A and M, which is in agreement with Munilla-Morán and Saborido-Rey (1996). Conversely, results reported for *H. abdominalis* showed α -amylase activity just after the release of juveniles and maintained constant during the first month of life (Wardley 2006). Amylase ontogeny profile has been correlated with the secondary development of the gastrointestinal tract (Kim *et al.* 2001). Nevertheless, in many other marine fish, α -amylase activity was detected a few days after hatching but reduced or almost disappeared during the following days until that juveniles reached a certain age. That fact has been related to the absence of a suitable substrate for the activity of the enzyme in the live food routinely used for rearing of most larvae (Ribeiro *et al.* 1999; Zambonino Infante & Cahu 2001). Since lipids are the most important energy source during the early ontogeny in many species of marine fishes it is expected that they should possess a great ability to digest that type of compounds (Olivotto *et al.* 2006; Olivotto *et al.* 2011a). It is admitted that lipid catabolism is performed primarily in fish larvae by esterase action hydrolyzing fatty acids as energy source, while true lipase is dependent on colipase and bile salts, acting over phospholipids and triacylglycerols (Van Tilbeurgh *et al.* 1992); this enzyme is responsible for releasing highly polyunsaturated fatty acids and other more complex compounds, generally observed when the maturation of the digestive system is completed. In the present study a progressive increase with no fluctuations in the activity related to the change in diet composition were observed, although the activity towards butyrate was reduced in older seahorses fed solely on *Artemia*. Activities towards any of the assayed substrates for lipase were evidenced from 0 DAR onwards and, in contrast to what was observed for other enzymes, high activities towards butyrate or octanoate were measured at the initial stages, being the greater activity observed towards this latter substrate. Differences in the ability to hydrolyse lipids of different chain length have been reported in some other species (Caballero *et al.* 2002; Morais *et al.* 2007). In this sense, some of the observed differences in the activities between dietary treatments during the initial days of life could reflect differences in the lipid profile of live prey (i.e. a greater contents in short chain fatty acids in copepods compared to medium chain fatty acids in enriched *Artemia*).

319 It is worthwhile to point the great variability observed in the values of enzyme activities within samples
320 of the same age. This may reflect a great variation in the feeding status of the different individuals, in
321 spite of being sampled at the same moment and after previous starving. This heterogeneity is not a strange
322 feature in many other physiological parameters measured in groups of young larvae, although it is not
323 usually evidenced since most studies present results obtained from pooled data samples instead of from
324 individually sampled larvae.

325 The aforementioned supports the effect of the type of live food on the initial development and digestive
326 enzyme activities in young *H. guttulatus* observed in the present study. Although seahorse juveniles fed
327 on *Artemia* nauplii and doubled their dry weight from 0 to 15 DAR the presence of intact nauplii in faeces
328 (Blanco, 2014) and the low levels of enzymatic activity (at least from 0 until 5 DAR, depending on the
329 type of prey), suggests that the digestion was inefficient at very early stages. Additionally, seahorses fed
330 on diet M showed higher digestive activity than those fed on diet A and, therefore, much higher weight
331 gain during the first 15 days of life. Previous reports suggest that *Artemia* is hardly digested by early
332 developmental stages in this species (Olivotto et al. 2011b, Planas *et al.* 2012), even being excreted alive,
333 as well as in others such as Atlantic herring (Rosenthal & Hempel 1970), anchovy (Chitty 1981) or
334 Atlantic halibut (Luizi *et al.* 1999). In contrast, the good nutritional value of copepods for early stages of
335 marine fish is supported by several studies (Støttrup *et al.* 1986; Rajkumar & Kumaraguru 2006, Schipp
336 2006). It follows that an increase in the period of feeding on mixed diets might improve the nutritional
337 condition and hence the success in larval rearing of *H. guttulatus*. On the other hand, although a better
338 knowledge on the digestive system and its functional capabilities may contribute to understand
339 ontogenetic changes and to progress in the development and improvement of rearing techniques, a more
340 accurate evaluation of the nutritional status and digestive capabilities in early developing seahorses
341 should require a combination of morphological, histological and physiological information.

342

343 Acknowledgments

344 The study was financed by the Spanish Government (Plan Nacional, Project CGL2009-08386) and the
345 Regional Government of Galicia (Xunta de Galicia, Project 09MDS022402PR). We are grateful to the
346 Xunta de Galicia for providing permission in the capture of wild seahorses. A. Blanco was supported by
347 PhD JAE-Pre grants (Junta para la Ampliación de Estudios Program) from the Spanish National Research
348 Council (CSIC), co-financed by the European Social Fund. We really like to thank A. Chamorro for his

349 help in the maintenance of the seahorse rearing system and C. Peake for his help with the statistical
350 analysis.
351
352

For Review Only

353 **References**

- 354 Alliot E., Pastoreaud A. & Trelu J. (1980) Evolution des activités enzymatiques dans le tractus digestif
 355 au course de la vie larvaire de la Sole. Variations des protéinogrammes et des zymogrammes.
 356 *Biochemical Systematics and Ecology* **8**, 441–445.
- 357 Alvarez-González C.A, Moyano F.J., Civera R., Carrasco V., Ortiz J.L, & Dumas S. (2008)
 358 Development of digestive enzyme activity in larvae of spotted sand bass *Paralabrax*
 359 *maculatofasciatus*. Biochemical analysis. *Fish Physiol Biochem* **34**(4); 373-384
- 360 Andrés M., Gisbert E., Díaz M., Moyano F.J., Estévez A. & Rotllant G. (2010) Ontogenetic changes in
 361 digestive enzymatic capacities of the spider crab, *Maja brachydactyla* (Decapoda: Majidae).
 362 *Journal of Experimental Marine Biology and Ecology* **389**, 75-84.
- 363 Blanco A. (2014). Rearing of the seahorse *Hippocampus guttulauts*: Key factors involved in growth and
 364 survival. PhD Thesis, University of Balearic Islands (Spain).
- 365 Blanco A., Chamorro A. & Planas M. (2014) Implications of physical key factors in the early rearing of
 366 the long-snouted seahorse *Hippocampus guttulatus*, *Aquaculture* **433**, 214-222.
- 367 Blanco A., Quintas P. & Planas M. (2011) Catabolic sources in the early development of the long-snouted
 368 seahorse *Hippocampus guttulatus* under starving conditions. 5th International Husbandry
 369 Symposium - The Husbandry, Management and Conservation of Syngnathids, 2–4 Nov 2011,
 370 Chicago (USA), p. 31
- 371 Bolasina S., Pérez A. & Yamashita Y. (2006) Digestive enzymes activity during ontogenetic development
 372 and effect of starvation in Japanese flounder, *Paralichthys olivaceus*. *Aquaculture* **252**, 503-515.
- 373 Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of
 374 protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- 375 Bray J.H. & Maxwell S.E. (1985) Multivariate Analysis of Variance. Multivariate Analysis of Variance.
 376 Sage University Paper series on Quantitative Research Methods, Newbury Park
- 377 Caballero M.J., Obach A., Rosenlund G., Montero D., Gisvold M. & Izquierdo M.S. (2002) Impact of
 378 different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and
 379 histology of rainbow trout, *Oncorhynchus mykiss*, *Aquaculture* **214**, 253-271.
- 380 Camacho Rosales J. (1996) Análisis multivariado con SPSS/PC+. Ediciones Universitarias de Barcelona,
 381 Barcelona

- 382 Celino F.T., Hilomen-Garcia G.V. & del Norte-Campos A.G.C. (2012) Feeding selectivity of the
383 seahorse, *Hippocampus kuda* (Bleeker), juveniles under laboratory conditions. *Aquaculture*
384 *Research* **43**, 1804-1815.
- 385 Chitty N. (1981) Behavioral observations of feeding larvae of bay anchovy, *Anchoa mitchilli*, and bigeye
386 anchovy, *Anchoa lamprotaenia*. Rapports et Proces-verbaux des Réunions. Conseil International
387 pour l'Exploration de la Mer, 178pp.
- 388 Cunha, I. & Planas, M. (1997) Temperature does not affect the fatty acid utilization in unfed turbot
389 (*Scophthalmus maximus* L.) larvae. Third International Symposium on Research for
390 Aquaculture: Research and Applied Aspects, 24–27 August. Barcelona.
- 391 Faulk C.K., Benninghoff A.D. & Holt G.J. (2007) Ontogeny of the gastrointestinal tract and selected
392 digestive enzymes in cobia *Rachycentron canadum* (L.). *Journal of Fish Biology* **70**, 567-583.
- 393 Ferron A. & Leggett W.C. (1994) An appraisal of condition measures for marine fish larvae. In: Blaxter
394 JHS, Southward AJ (ed) *Advances in Marine Biology*. Academic Press, pp 217-303.
- 395 Foster S.J. & Vincent A.C.J. (2004) Life history and ecology of seahorses: implications for conservation
396 and management. *Journal of Fish Biology* **65**, 1-61.
- 397 Gisbert E, Morais S and Moyano FJ 2013. Feeding and digestion. In: Kim JG (ed). *Larval fish*
398 *aquaculture*. Nova Publishers, New York, pp 73–123.
- 399 Gisbert E., Piedrahita R.H. & Conklin D.E. (2004) Ontogenetic development of the digestive system in
400 California halibut (*Paralichthys californicus*) with notes on feeding practices. *Aquaculture* **232**,
401 455-470.
- 402 Govoni J., Boehlert G. & Watanabe Y. (1986) The physiology of digestion in fish larvae. *Environmental*
403 *Biology of Fishes* **16**, 59-77.
- 404 Gutowska M.A., Drazen J.C. & Robison B.H. (2004) Digestive chitinolytic activity in marine fishes of
405 Monterey Bay, California. *Comparative Biochemistry and Physiology: Part A* **139**, 351-358.
- 406 Hansen T.W., Folkvord A., Grøtan E. & Sæle Ø. (2013) Genetic ontogeny of pancreatic enzymes in
407 *Labrus bergylta* larvae and the effect of feed type on enzyme activities and gene expression.
408 *Comparative Biochemistry and Physiology: Part B* **164**, 176-184.
- 409 Ikeda M., Miyauchi K., Mochizuki A. & Matsumiya M. (2009) Purification and characterization of
410 chitinase from the stomach of silver croaker *Pennahia argentatus*. *Protein Expression and*
411 *Purification* **65**, 214-222.

- IUCN (2012). The IUCN Red List of Threatened Species. Version 2012.2. .
- Job S.D., Do H.H., Meeuwig J.J. & Hall H.J. (2002) Culturing the oceanic seahorse, *Hippocampus kuda*. *Aquaculture* **214**, 333-341.
- Kamarudin M.S., Otoi S. & Saad C.R. (2011) Changes in growth, survival and digestive enzyme activities of Asian redbelly catfish, *Mystus nemurus*, larvae fed on different diets. *African Journal of Biotechnology* **10**, 4484-4493.
- Kamler, E. (1992) Early life history of fish: An energetics approach. Chapman & Hall, London, 267 pp.
- Kim B. & Brown C. (1994) Hormonal manipulation of digestive enzyme ontogeny in marine larval fishes-effects on digestive enzymes. *UNJR Technical Reports* **28**, 47-55.
- Kim B., Divakaran S., Brown C. & Ostrowski A. (2001) Comparative digestive enzyme ontogeny in two marine larval fishes: Pacific threadfin (*Polydactylus sexfilis*) and bluefin trevally (*Caranx melampygus*). *Fish Physiology and Biochemistry* **24**, 225-241.
- Koldewey H.J. & Martin-Smith K.M. (2010) A global review of seahorse aquaculture. *Aquaculture* **302**, 131-152.
- 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.
- Lemieux H., Blier P. & Dutil J.D. (1999) Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? *Fish Physiology and Biochemistry* **20**, 293-303.
- Lourie S.A., Pritchard J.C., Casey S.P., Truong S.K., Hall H.J. & Vincent A.C.J. (1999) The taxonomy of Vietnam's exploited seahorses (family Syngnathidae). *Biological Journal of the Linnean Society* **66**, 231-256.
- Luizi F.S., Gara B., Shields R.J. & Bromage N.R. (1999) Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia* prey. *Aquaculture* **176**, 101-116.
- Martinez-Cardenas L. & Purser G.J. (2007) Effect of tank colour on *Artemia* ingestion, growth and survival in cultured early juvenile pot-bellied seahorses (*Hippocampus abdominalis*). *Aquaculture* **264**, 92-100.

- 441 Meetei L.I., Ninawe A.S. & Chakrabarti R. (2014) Influence of feeding regimes on the digestive enzyme
 442 profile and ultrastructure of digestive tract of first feeding *Catla catla* (Hamilton) larvae. *Journal*
 443 *of Aquaculture Research & Development* **5**, 243.
- 444 Molinari L.M., Pedroso R.B., Scoaris D.d.O., Ueda-Nakamura T., Nakamura C.V. & Dias Filho B.P.
 445 (2007) Identification and partial characterisation of a chitinase from Nile tilapia, *Oreochromis*
 446 *niloticus*. *Comparative Biochemistry and Physiology: Part B* **146**, 81-87.
- 447 Moraes S., Conceição L.E.C., Rønnestad I., Koven W., Cahu C., Zambonino Infante J.L. & Dinis M.T.
 448 (2007) Dietary neutral lipid level and source in marine fish larvae: Effects on digestive
 449 physiology and food intake. *Aquaculture* **268**, 106-122.
- 450 Munilla-Morán R. & Saborido-Rey F. (1996) Digestive enzymes in marine species. II. α -amylase
 451 activities in gut from seabream (*Sparus aurata*), turbot (*Scophthalmus maximus*) and redfish
 452 (*Sebastes mentella*). *Comparative Biochemistry and Physiology: Part B* **113**, 827-834.
- 453 Neilson J., Perry R., Valerio P. & Waiwood K. (1986) Condition of Atlantic cod *Gadus morhua* larvae
 454 after the transition to exogenous feeding: morphometrics, buoyancy and predator avoidance.
 455 *Marine Ecology Progress Series* **32**, 229-235.
- 456 O'Brien-MacDonald K., Brown J.A. & Parrish C.C. (2006) Growth, behaviour, and digestive enzyme
 457 activity in larval Atlantic cod (*Gadus morhua*) in relation to rotifer lipid. *ICES Journal of*
 458 *Marine Science* **63**, 275-284.
- 459 Olivotto I., Rollo A., Sulpizio R., Avella M., Tosti L. & Carnevali O. (2006). Breeding and rearing the
 460 Sunrise Dottyback *Pseudochromis flavivertex*: the importance of live prey enrichment during
 461 larval development. *Aquaculture*, **255**: 480-487.
- 462 Olivotto I., Avella M.A., Sampaolesi G., Piccinetti C.C., Navarro Ruiz P. & Carnevali O. (2008)
 463 Breeding and rearing the longsnout seahorse *Hippocampus reidi*: Rearing and feeding studies.
 464 *Aquaculture* **283**, 92-96.
- 465 Olivotto I., Di Stefano M., Rosetti S., Cossignani L., Pugnali A., Giantomassi F., Carnevali O.
 466 (2011a). Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in False
 467 percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larval development and
 468 metamorphosis: molecular and biochemical implications. *Comparative biochemistry and*
 469 *physiology. Part A* **159**, 207- 218.

- 470 Olivotto I., Planas M., Simões N., Holt G.J., Avella M.A. & Calado R. (2011b) Advances in breeding and
471 rearing marine ornamentals. *Journal of the World Aquaculture Society* **42**, 135-166.
- 472 Otero-Ferrer F., Molina L., Socorro J., Herrera R., Fernández-Palacios H. & Soledad Izquierdo M. (2010)
473 Live prey first feeding regimes for short-snouted seahorse *Hippocampus hippocampus*
474 (Linnaeus, 1758) juveniles. *Aquaculture Research* **41**, e8-e19.
- 475 Papadakis I.E., Kentouri M., Divanach P. & Mylonas C.C. (2013) Ontogeny of the digestive system of
476 meagre *Argyrosomus regius* reared in a mesocosm, and quantitative changes of lipids in the liver
477 from hatching to juvenile. *Aquaculture* **388–391**, 76-88.
- 478 Payne M.F. & Rippingale R.J. (2000) Rearing West Australian seahorse, *Hippocampus subelongatus*,
479 juveniles on copepod nauplii and enriched *Artemia*. *Aquaculture* **188**, 353-361.
- 480 Pérez-Casanova J.C., Murray H.M., Gallant J.W., Ross N.W., Douglas S.E. & Johnson S.C. (2006)
481 Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and
482 Atlantic cod (*Gadus morhua*). *Aquaculture* **251**, 377-401.
- 483 Planas M., Blanco A., Chamorro A., Valladares S. & Pintado J. (2012) Temperature-induced changes of
484 growth and survival in the early development of the seahorse *Hippocampus guttulatus*. *Journal*
485 *of Experimental Marine Biology and Ecology* **438**, 154-162.
- 486 Planas M., Chamorro A., Quintas P. & Vilar A. (2008) Establishment and maintenance of threatened
487 long-snouted seahorse, *Hippocampus guttulatus*, broodstock in captivity. *Aquaculture* **283**, 19-
488 28.
- 489 Planas M., Quintas P., Chamorro A. & Balcazar J.L. (2009) Husbandry and rearing of the seahorse
490 *Hippocampus guttulatus* (Project Hippocampus). World Aquaculture Society, Veracruz
491 (México).
- 492 Rajkumar M. & Kumaraguru K.P. (2006) Suitability of the copepod, *Acartia clausi* as a live feed for
493 Seabass larvae (*Lates calcarifer* Bloch): Compared to traditional live-food organisms with
494 special emphasis on the nutritional value. *Aquaculture* **261**, 649-658.
- 495 Ray A.K., Ghosh K. & Ringø E. (2012) Enzyme-producing bacteria isolated from fish gut: a review.
496 *Aquaculture Nutrition* **18**, 1365-2095.
- 497 Ribeiro L., Zambonino-Infante J.L., Cahu C. & Dinis M.T. (1999) Development of digestive enzymes in
498 larvae of *Solea senegalensis*, Kaup 1858. *Aquaculture* **179**, 465-473

- 499 Rosenthal H. & Hempel G. (1970) Experimental studies in feeding and food requirements of herring
500 larvae (*Clupea harengus* L.). In: Steele JH (ed) Marine Food Chains. Oliver and Boys,
501 Edinburgh, pp 344-364
- 502 Rotllant G., Moyano F.J., Andrés M., Díaz M., Estévez A. & Gisbert E. (2008) Evaluation of fluorogenic
503 substrates in the assessment of digestive enzymes in a decapod crustacean *Maja brachydactyla*
504 larvae. *Aquaculture* **282**, 90–96.
- 505 Sanz A., Llorente J.I., Furné M., Ostos-Garrido M.V., Carmona R., Domezain A. & Hidalgo M.C. (2011)
506 Digestive enzymes during ontogeny of the sturgeon *Acipenser naccarii*: intestine and pancreas
507 development. *Journal of Applied Ichthyology* **27**, 1139-1146.
- 508 Sarasquete M.C., Polo A. & Yúfera M. (1995) Histology and histochemistry of the development of the
509 digestive system of larval gilthead seabream, *Sparus aurata* L. *Aquaculture* **130**, 79-92.
- 510 Schipp G. (2006) The use of calanoid copepods in semi-intensive, tropical marine fish larviculture. In:
511 Cruz Suárez L, Marie D, Salazar M, Nieto López M, Villarreal Cavazos D (eds) Avances en
512 nutrición acuicola VIII. Universidad Autónoma de Nuevo León, Monterrey, pp 84-94.
- 513 Sheng J., Lin Q., Chen Q., Gao Y., Shen L. & Lu J. (2006) Effects of food, temperature and light
514 intensity on the feeding behavior of three-spot juvenile seahorses, *Hippocampus trimaculatus*
515 Leach. *Aquaculture* **256**, 596-607.
- 516 Srichanun M., Tantikitti C., Vatanakul V. & Musikarune P. (2012) Digestive enzyme activity during
517 ontogenetic development and effect of live feed in green catfish larvae (*Mystus nemurus* Cuv.
518 and Val.). *Songklanakarin Journal of Science and Technology* **34**, 247-254.
- 519 Støttrup J.G., Richardson K., Kirkegaard E. & Pihl N..J (1986) The cultivation of *Acartia tonsa* Dana for
520 use as a live food source for marine fish larvae. *Aquaculture* **52**, 87-96.
- 521 Uscanga U., Perales N, Álvarez C., Moyano F.J., Tovar D, Gisbert E., Márquez G., Contreras W., Arias
522 L. & Indy J. (2011) Changes in digestive enzyme activity during initial ontogeny of bay snook
523 *Petenia splendida*. *Fish Physiol Biochem* **37**(3):667-680
- 524 van Tilbeurgh H., Sarda L., Verger R. & Cambillau C. (1992) Structure of the pancreatic lipase-
525 procolipase complex. *Nature* **359**, 159–162.
- 526 van der Meeren T., Olsen R.E., Hamre K. & Fyhn H.J. (2007) Biochemical composition of copepods for
527 evaluation of feed quality in production of juvenile marine fish, *Aquaculture* **274**, 375-397.

- 528 Walford J. & Lam T.J. (1993) Development of digestive tract and proteolytic enzyme activity in seabass
529 (*Lates calcarifer*) larvae and juveniles. *Aquaculture* **109**, 187-205.
- 530 Walters K. & Coen L.D. (2006) A comparison of statistical approaches to analyzing community
531 convergence between natural and constructed oyster reefs. *Journal of Experimental Marine*
532 *Biology and Ecology* **330**, 81-95.
- 533 Wardley R.T. (2006) A study on the feeding of the Pot-bellied seahorse (*Hippocampus abdominalis*)-
534 Reducing the reliance on Brine shrimp (*Artemia*). PhD Thesis, University of Tasmania.
- 535 Willadino L., Souza-Santos L.P., Mélo R.C.S., Brito A.P., Barros N.C.S., Araújo-Castro C.M.V., Galvão
536 D.B., Gouveia A., Regis C.G. & Cavalli R.O. (2012) Ingestion rate, survival and growth of
537 newly released seahorse *Hippocampus reidi* fed exclusively on cultured live food items.
538 *Aquaculture* **360-361**, 10-16.
- 539 Woods C.M.C. (2000) Improving initial survival in cultured seahorses, *Hippocampus abdominalis*
540 Leeson, 1827 (Teleostei: Syngnathidae). *Aquaculture* **190**, 377-388.
- 541 Yin M.C. & Blaxter J.H.S. (1987) Feeding ability and survival during starvation of marine fish larvae
542 reared in the laboratory. *Journal of Experimental Marine Biology and Ecology* **105**, 73-83.
- 543 Yúfera M. & Darias M.J. (2007) The onset of exogenous feeding in marine fish larvae. *Aquaculture* **268**,
544 53-63.
- 545 Zambonino Infante J.L. & Cahu C.L. (2001) Ontogeny of the gastrointestinal tract of marine fish larvae.
546 *Comparative Biochemistry and Physiology: Part C* **130**, 477-487.
- 547 Zhang D., Zhang Y., Lin J. & Lin Q. (2010) Growth and survival of juvenile lined seahorse,
548 *Hippocampus erectus* (Perry), at different stocking densities. *Aquaculture Research* **42**, 9-13.

549 **Figures**

550 Figure 1. Changes with growth in Fulton’s condition index increment (ΔK_F) in seahorse juveniles fed on diets A and
551 M.

552
553 Figure 2. Enzymatic activities (mU mg DW^{-1}) in seahorse juveniles fed on different diets. Black rhombus: Seahorses
554 fed on diet A.; Gray squares: Seahorses fed on diet M. White triangles corresponded to enzymatic activity of prey,
555 where C: Copepods, N: *Artemia* nauplii and M: *Artemia* metanauplii.

556
557 Figure 3. Specific enzymatic activities by soluble protein (U mg Prot^{-1}) in seahorse juveniles fed on different diets.
558 Black rhombus: Seahorses fed on diet A; Gray squares: Seahorses fed on diet M.

559

239x150mm (96 x 96 DPI)

135x147mm (96 x 96 DPI)

135x147mm (96 x 96 DPI)

Table 1. Summary on survival, dry weight (DW), standard length (SL) and Fulton’s condition index (K_F) in *Hippocampus guttulatus* juveniles fed on diets A and M. D°_{eff} : Effective day-degrees. Sampling size from 0 to 15 DAR, n=10; and at 30 DAR, n = 5.

Day	D°_{eff}	Survival (%)		DW (mg)		SL (mm)		K_F	
		Diet A	Diet M	Diet A	Diet M	Diet A	Diet M	Diet A	Diet M
0	0	100	100	0.84 ± 0.05	0.87 ± 0.07	15.6 ± 0.5	15.5 ± 0.7	2.93	2.59
5	25	93	99	1.01 ± 0.27	1.34 ± 0.35	17.6 ± 1.2	18.9 ± 1.5	2.49	2.25
10	49	67	98	1.27 ± 0.47	2.95 ± 0.76	18.7 ± 2.1	24.6 ± 1.6	2.63	2.33
15	74	64	98	1.87 ± 0.71	5.51 ± 2.49	21.2 ± 2.5	30.0 ± 3.6	2.68	2.51
30	147	58	85	4.85 ± 1.18	17.73 ± 4.23	28.4 ± 1.8	42.0 ± 3.8	2.97	2.81

Table 2. Enzymatic activity of *Hippocampus guttulatus* juveniles under starvation treatment (mU DW^{-1}) and enzymatic activity shifting slope from 0 to 5DAR in fed seahorses and 0-4DAR in starved seahorses.

DAR	D _{eff}	Diet	Trypsin (mU DW^{-1})	Butirate (mU DW^{-1})	Octanoate (mU DW^{-1})	Oleate (mU DW^{-1})
0	0	Starvation	55,2 ± 16,5	57,3 ± 18,5	74,5 ± 8,7	8,4 ± 4,0
1	4,9	Starvation	44,0 ± 17,2	53,1 ± 15,8	70,5 ± 18,5	6,4 ± 1,6
2	9,8	Starvation	37,5 ± 13,3	54,5 ± 16,2	64,3 ± 16,1	7,1 ± 4,4
3	14,7	Starvation	20,7 ± 8,4	40,7 ± 12,0	65,9 ± 20,1	6,1 ± 1,7
4	19,6	Starvation	15,4 ± 6,4	37,6 ± 7,1	53,3 ± 6,8	7,0 ± 1,0
Slope D.0-D.4		Starvation	-10,3	-5,2	-4,7	-0,3
Slope D.0-D.5		<i>Artemia</i>	-13,9	-4,5	-3,2	-0,3
Slope D.0-D.5		Mixed	-14,3	-2,3	-0,7	0,4