

ARTICLE

Integrating of the seahorse *Hippocampus reidi* in multi-trophic organic farms of oysters and shrimp: Effects of density and diet

Integración del caballito de mar *Hippocampus reidi* en granjas de cultivo multitrófico orgánico de ostras y camarones: Efectos de la densidad y la dieta

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Resumen- El objetivo del estudio fue demostrar la viabilidad del cultivo del caballito de mar *Hippocampus reidi*, en un sistema orgánico de acuicultura multitrófica integrada, compuesta por cultivos de ostra *Crassostrea brasiliana* y camarón *Litopenaeus vannamei*. Se realizaron experimentos para evaluar los efectos de la densidad poblacional y la dieta (zooplancton natural, nauplii de *Artemia* sp., o ambos), en juveniles I, que corresponde de 1 a 15 días después del nacimiento. Se estudió el efecto de la densidad poblacional en el crecimiento y la supervivencia de juveniles II a partir de 30 días hasta el tamaño comercial en jaulas de malla. La supervivencia de la etapa juvenil I no fue afectada por densidades poblacional de 2 a 5 peces L⁻¹ y fue mayor al 70% en todos los tratamientos. El tamaño de los caballitos de mar, sin embargo, estuvo inversamente relacionado con la densidad poblacional. Ambos tratamientos, con zooplancton silvestre, promovieron una mayor supervivencia y crecimiento del caballito de mar que la dieta solo compuesta de *Artemia*, esto durante los primeros 15 días de vida. El crecimiento de juvenil II disminuyó con la densidad poblacional de 5 a 40 peces m⁻³ y la supervivencia no fue afectada. El beneficio total y la proporción de animales coloridos aumentaron significativamente con la densidad poblacional. Se confirma la viabilidad técnica de la producción de caballitos de mar multitróficos orgánicos en jaulas de malla, incluso en la densidad más alta probada en este estudio los caballitos de mar crecieron normalmente, lo que podría generar buenos rendimientos económicos.

Palabras clave: Caballito de mar, acuicultura multitrófica integrada, pez ornamental, producción animal

Abstract.- The goal of this study was to demonstrate the feasibility of culturing the seahorse *Hippocampus reidi* in an organic integrated multi-trophic aquaculture farm that produces the oyster *Crassostrea brasiliana* and the shrimp *Litopenaeus vannamei*. Experiments were conducted to evaluate the effects stocking density and food (natural zooplankton, *Artemia* nauplii, or both) for the juvenile I, which are fish from 1 to 15 days after birth. Stocking density effect was studied on growth and survival of juveniles II from 30 days to commercial size in net cages. Survival of the juvenile I was not affected by stocking densities from 2 to 5 ind L⁻¹ and was greater than 70% in all treatments. Seahorse length, however, was inversely related to stocking density. Both treatments with wild zooplankton promoted higher seahorse survival and growth than diet composed exclusively by *Artemia* sp. during the first 15 days of life. Juvenile II growth decreased with stocking density from 5 to 40 ind m⁻³ but survival was not affected by it. The total benefit and proportion of colored animals increased significantly with stocking density. The present study confirms the technical feasibility of organic multi-trophic seahorse production in net cages. Even at the highest density tested the seahorses grew well and could generate high profits.

Key words: Seahorse, integrated multi-trophic aquaculture, ornamental fish, husbandry

INTRODUCTION

Seahorses are small marine fish that have different morphology compared to other fish. Approximately 46 species belonging to the genus *Hippocampus* inhabit temperate and tropical seas, usually in coral reefs and along mangrove and seagrass beds (Lourie & Vincent 2004, Koldewey & Martin-Smith 2010). Seahorses are very popular in the ornamental fish market due to their beauty and peculiar reproductive behavior in which male incubates the

eggs (Bull & Shedd 2002). All seahorse species are included in Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES 2008)¹ and in the Red List of the International Union for Conservation of Nature (IUCN 2014) due to habitat threats, the dried seahorse market, and fisheries bycatch (Vincent

¹CITES. 2008. Convention on International Trade in Endangered Species of Wild Flora and Fauna. Appendices I, II e III. <<http://www.cites.org>>.

1996, Planas *et al.* 2008). Therefore, the trade of naturally collected Brazilian longsnout seahorse *Hippocampus reidi* Ginsburg, 1933 is regulated by the Ministry of Environment of Brazil (IBAMA)².

Commercial breeding and grow-out of approximately 13 seahorse species are conducted to supply the ornamental market (Koldewey & Martin-Smith 2010). Production usually occurs in indoor recirculating water systems using plastic or glass tanks. However, some studies suggest it is possible to grow them in cages, which would offer benefits such a minimum labor costs and use of naturally occurring food (Carlos *et al.* 2009, Garcia & Hilomen-Garcia 2009, Garcia *et al.* 2012).

Recently, Fonseca *et al.* (2017) have demonstrated that the introduction of seahorse culture in *Litopenaeus vannamei* and *Crassostrea brasiliana* integrated multi-trophic aquaculture (IMTA) system in Brazilian estuarine ponds is technically feasible, profitable, resilient and showed high liquidity in the economic point of view. IMTA systems are based on the use of natural live food and an equilibrated culture environment that usually is associated with a poly-culture system (Barrington *et al.* 2009).

Stocking density is among the most important characteristics of a production system, because a high number of fish per area or volume negatively influences the production of total biomass, fish body size, and survival (Mokoro *et al.* 2014, Shoko *et al.* 2016). It also induces aggressive behavior of the fish (Miki *et al.* 2011). Another fundamental parameter of an aquaculture system is feeding management, especially for newborn pelagic larvae, in which the type and quality of live food used affect the production. As the IMTA is based on naturally occurred food, the maximum number of fishes to be produced per volume and the quality of live food are important information in all production stages.

Therefore, the goal of this study was to evaluate the use of different live food and stocking density effect on growth and survival of seahorse juveniles produced in an organic IMTA system with the oyster *Crassostrea brasiliana* and the shrimp *Litopenaeus vannamei*.

MATERIALS AND METHODS

This study was conducted at Primar Organic Aquaculture Farm in Rio Grande do Norte State, Brazil (6°13'17.22"S; 35°8'19.56"W). This farm was the first Brazilian certified organic farm using IMTA to produce white shrimp *Litopenaeus vannamei* and the mangrove oyster *Crassostrea brasiliana*. The total productive area is 42.4 ha mainly made by estuarine ponds that are filled by tide variation and pumps through a main supply channel. Shrimp and oyster were culture in association without the use of commercial food and chemical products. The seahorse culture, developed in a pilot scale at 402 m², was described by Fonseca *et al.* (2017) and involved three phases: broodstock's maintenance, nursery phase of growing of pelagic juveniles (juveniles I), and grow-out phase of rearing benthic juveniles (juveniles II) until commercial size.

BROODSTOCK MAINTENANCE

Breeders were collected in the estuary in which the farm is located (Guarairas Bay, 6.19°S, 35.11°W) and was authorized by the Brazilian Institute of Natural Resources and Environment (IBAMA, authorization number 23875). Adult specimens were acclimatized and released into a 2,000 L fiberglass tank with constant aeration. The surface of this tank was protected by a 70% shade net to minimize temperature changes. The daily management consisted of partial water changes and feeding seahorses with small crustaceans (*e.g.*, amphipods and *Alpheus* shrimps) and polychaetes collected daily in oyster bags. The oyster bags were washed with pond water and the live food was collected with a 200 µm net and concentrated in feeders constructed using the bottom of PET bottles. After two weeks in these tanks, the seahorses instantly approached the feeders at feeding time. The tank was inspected every morning to check for the presence of newborn juveniles; if present, they were collected and transferred to juvenile I culture tanks.

NURSERY PHASE

This phase is the rearing of newborn until 15 days after born, classified here as juvenile I phase. In this phase the juveniles are essentially pelagic and represent the most sensible phase of *Hippocampus reidi* rearing (Hora & Joyeux 2009). Usually, this rearing phase is conducted in 200 L polyethylene barrels, but for the experiments of this study 20 L white plastic tanks were used. Two experiments were conducted at this developmental phase to evaluate the effect of stocking density (trial 1) and the use of plankton collected in ponds and/or *Artemia* sp. nauplii as live food (trial 2).

²IBAMA. 2004. Instrução Normativa n° 56, 23 de novembro de 2004. Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis-IBAMA, Ministério do Meio Ambiente, Brasília. <<https://sogi8.sogi.com.br/Arquivo/Modulo113.MRID109/Registro61002/documento%201.pdf>>

Physical-chemical water parameters were monitored every day. Dissolved oxygen concentration, pH, temperature, and salinity mean values for trial 1 and trial 2 were 6.0 and 7.9 mg L⁻¹; 7.6 and 8.0; 24 and 29 °C; and 25 and 32, respectively. Both trials lasted 15 days and at the end the seahorses were collected, counted, and measured as indicated by Lourie *et al.* (1999) to evaluate mean total length and survival.

TRIAL 1

The effect of stocking densities, 2, 3, 4, and 5 newborn juveniles L⁻¹, on survival and length was evaluated. This experiment followed an entirely randomized design with 3 replicates per density and a total of 12 experimental units. Each experimental unit consisted of a 20 L white plastic tank with no air stone and weak airlift to maintain water and food circulation. Fifty to 100% water exchanges were performed every day via bottom siphoning to remove the waste. The water used was pumped from supply channel of ponds and filtered through a 100 µm mesh.

Juvenile seahorses were fed twice per day with zooplankton collected in a farm pond with a 120 µm net. The collected zooplankton were concentrated in pond water and homogenized in order to supply the same quantity of food (*ca.*, 70 mL of plankton suspension) to all experimental units to maintain a concentration of at least 400 organisms L⁻¹ during the experiment. On average this zooplankton consisted of 73% copepods, 19% nauplii, 5% decapod larvae, and 3% amphipods, nematodes, and polychaetes.

TRIAL 2

The effect of three diets: zooplankton collected in ponds, newly hatched *Artemia* sp. nauplii, and the mixture of both at 50% proportion, on survival and length of seahorse juvenile were tested in this trial. The experiment consisted of 4 replicates of each treatment resulting in 12 experimental units. The experimental units used were the same as those in trial 1, and the stocking density was 1 newborn juvenile L⁻¹. The water exchanges were performed as described for trial 1. The live food was provided two times per day to maintain a concentration of at least 400 organisms L⁻¹. Zooplankton collection and mean composition were similar to that described for trial 1. Newly hatched *Artemia* sp. nauplii were prepared as described by Brown *et al.* (1990).

GROW-OUT PHASE (TRIAL 3)

This phase represents the rearing of juveniles II (more than 15 days after born), when they start to become benthic, until the commercial size (7 to 10 cm). The juveniles II used for this experiment were produced in 200 L polyethylene barrels at 1.5 newborn juvenile L⁻¹ and fed only on zooplankton collected in ponds. The experiment at this phase evaluated the effect of stocking density on growth, survival and color of seahorses in net cages. Juveniles II of 30 days old were

stocked at net cages (2.0 x 1.0 x 1.2 m deep, 2 m³ of useful volume) constructed of an iron frame painted with marine coat, mosquito netting (1 mm mesh), and four 20 L plastic gallon jugs that acted as floats to keep the cage bottom at least 20 cm above the pond bottom at all times regardless of changes in water level due to tidal variation. The top of the cage was covered with mosquito netting and secured with Velcro to keep predators and invaders out of the cage. Sixteen net cages were installed in an oyster grow-out pond and stocked with 5, 10, 20, or 40 seahorses m⁻³. The experiment followed an entirely randomized design with 4 replicates per treatment and lasted 106 days. Each cage contained four net substrates that had been in the water for 15 days prior to the start of the experiment; these substrates acted as holdfasts for seahorse and were colonized by benthos, representing another food source for seahorses in cages as well as plankton present in ponds.

The management in the first 30 days consisted of daily cleaning of the mosquito net and weekly addition of supplementary food consisting of amphipods and *Alpheus* sp. shrimps sampled from oyster bags. From 30 days onwards, nets were cleaned once a week and no supplementary food was provided. The net substrates were not changed until the end of the experiment.

Water parameters monitored every day were as follows: dissolved oxygen concentration > 6.0 mg L⁻¹; pH 6.4-8.8; temperature 24-29 °C; and salinity 20-32. At the end of the trial, seahorses were collected, counted, measured, and weighed as indicated by Lourie *et al.* (1999). Final survival and seahorse size were assessed. Seahorse color was also evaluated. Total benefit was estimated as well, considering black seahorses price as USD 5.00 and colored (yellow or orange) seahorses as USD 15.00.

Other crustaceans and fish species present in the cages were counted and identified. At the end of trial, substrate samples were collected and fixed in 4% formalin for identification of the benthic organisms present in the cages that could be used as food by seahorses. Species were identified under a stereoscopic microscopic, and density was presented as numbers of organisms per area. The amount of mud in the bottom of the cages was also quantified to determine if the position of the cage in the pond influenced the amount of mud in the cage. A 250 mL sample of the mud from each cage was frozen and stored for organic matter content analysis (EMBRAPA 2006).

STATISTICAL ANALYSIS

After checking for homoscedasticity and normality of the data, a regression analysis was performed for growth and survival in trial 1 and trial 3 and for total benefit and color proportion in trial 3. Data from trial 2 and 3 were analyzed using one-way analysis of variance and Tukey's test to compare means between treatments. The significant level considered was 0.05.

RESULTS

TRIAL 1

The survival of seahorse juveniles I was not significantly affected by the stocking densities (from 2 to 5 juveniles L⁻¹), and it was greater than 70% in all treatments (Fig. 1). However, seahorse length was significantly inversely related with stocking density (Fig. 1).

TRIAL 2

Seahorses fed on wild zooplankton and a mix of diet (wild zooplankton and *Artemia* sp. nauplii) had a significantly higher body length and higher survival than seahorses fed only on *Artemia* sp. nauplii during the first 15 days of life (Table 1).

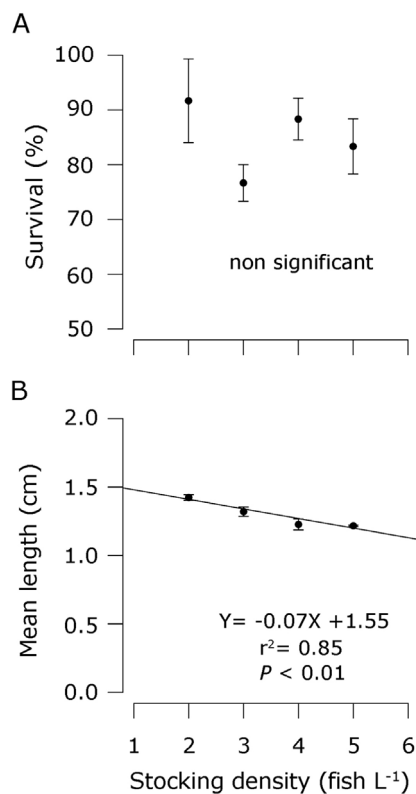


Figure 1. Effect of stocking density on *Hippocampus reidi* juvenile I produced in trial 1. **A)** survival and **B)** mean length / Efecto de la densidad en el juvenil I de *Hippocampus reidi* en el ensayo 1. **A)** supervivencia y **B)** tamaño promedio

Table 1. Growth and survival of *Hippocampus reidi* fed *Artemia* sp. nauplii, zooplankton, or both (trial 2) / Crecimiento y supervivencia de *Hippocampus reidi* alimentado con larvas nauplii de *Artemia* sp., zooplankton o ambos (ensayo 2)

Diet	Length (cm)	Survival (%)
<i>Artemia</i> nauplii	1.24 ± 0.09 ^b	25.0 ± 7.1 ^b
Wild zooplankton	1.70 ± 0.04 ^a	95.0 ± 5.8 ^a
Both	1.75 ± 0.06 ^a	92.5 ± 9.6 ^a
P-value	< 0.001	< 0.001

Values are means (± standard error) of 4 tanks, except for the *Artemia* nauplii treatment for which only 2 tanks remained at the end. Different letters in columns denote significant differences between experimental systems at $\alpha = 0.05$ determined by Tukey's test

TRIAL 3

Seahorse growth significantly decreased with increasing stocking density. Survival was not significantly affected by stocking density. The total benefit significantly increased with stocking density. Stocking density also significantly affected the color proportion, as the frequency of black animal decreases as the density increased (Fig. 2, Table 2).

Four of the nine fish species collected from the cages are potential seahorse predators (Table 3). Three crustacean species were also collected inside the cages and can be seahorse prey depending on their size (Table 3). The benthic fauna at substrates (Table 4) also presented potential seahorse preys. The total mud volume collected varied from 35 to 100 L and did not depend on the position of the cage or on the treatment. The organic matter content of the mud varied randomly from 22 to 38% of dry matter.

DISCUSSION

Seahorses are most often reared in artificial glass, concrete, fiberglass, or plastic tanks equipped with flow-through or recirculation systems and are fed live or frozen food items collected in nature or produced in the farm (Koldewey & Martin-Smith 2010). Examples of seahorse culture in net cages in the field, as in the present work, are scarce. *H. kuda* and *H. barbouri* have been reared in net cages in the Philippines (Garcia & Hilomen-Garcia 2009, Garcia *et al.* 2012), and *H. reidi* was reared in an organic shrimp/oyster farm in Brazil (Carlos *et al.* 2009, Fonseca *et al.* 2017).

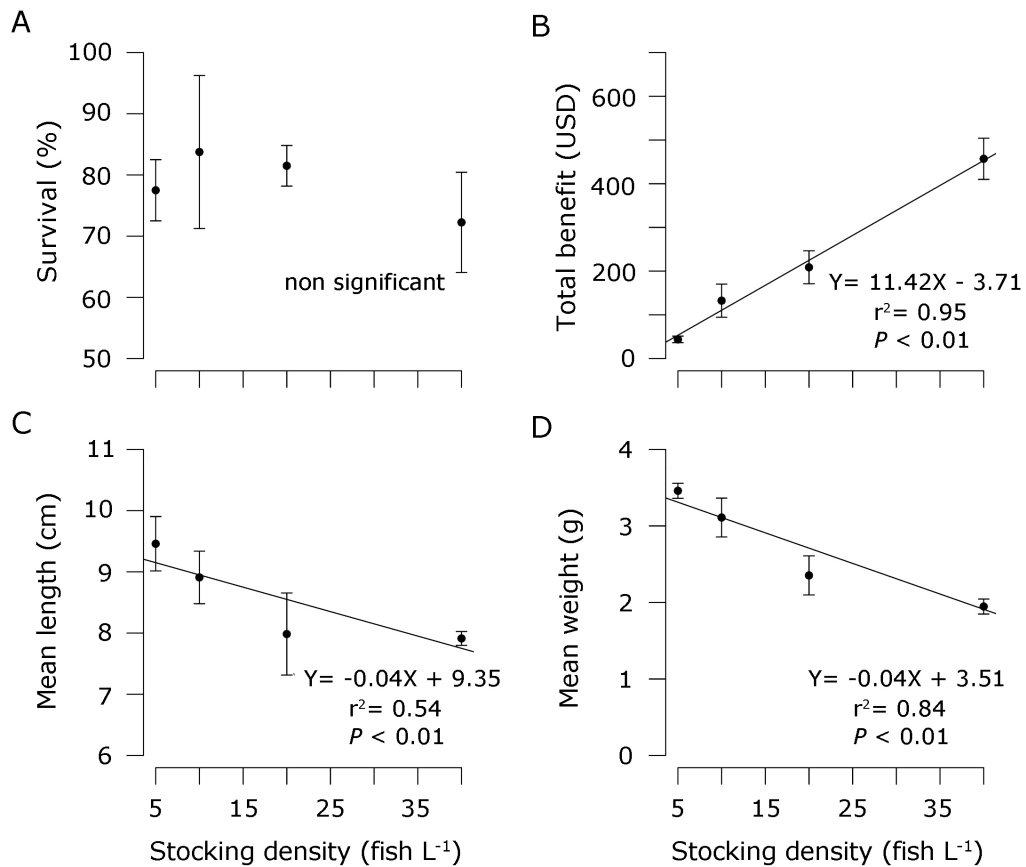


Figure 2. Effect of stocking density on *Hippocampus reidi* juvenile II produced in trial 3 and cages. **A)** survival, **B)** total benefit, **C)** mean length, and **D)** mean weight / Efecto de la densidad en el juvenil II de *Hippocampus reidi* en el ensayo 3 y en jaulas. **A)** supervivencia, **B)** beneficio total, **C)** el tamaño promedio, y **D)** peso promedio

Table 2. Effect of stocking density (fish m⁻³) on survival (%), total benefit (USD), mean length (cm), mean weight (g) and frequency of black seahorses of *Hippocampus reidi* juvenile produced in cages / Efecto de la densidad (número de peces m⁻³) en la supervivencia (%), beneficio total (USD), tamaño promedio (cm), peso promedio (g) y frecuencia de caballitos de mar negros del juvenil *Hippocampus reidi* producidos en jaulas

Stocking density (fish m ⁻³)	Survival (%)	Total benefit (USD)	Mean length (cm)	Mean weigh (g)	Frequency of black seahorses (%)
5	77,5 ± 5.0	43.75 ± 8 ^d	9.5 ± 0.4 ^a	3.46 ± 0.1 ^a	72.5 ± 5.0 ^a
10	83.7 ± 12.5	132.5 ± 38 ^c	8.9 ± 0.4 ^{ab}	3.11 ± 0.2 ^a	65.0 ± 17.8 ^a
20	81.5 ± 3.3	208.8 ± 38 ^b	8.0 ± 0.7 ^{bc}	2.35 ± 0.2 ^b	52.5 ± 2.0 ^{ab}
40	72.2 ± 8.2	457 ± 47 ^a	7.9 ± 0.1 ^c	1.95 ± 0.1 ^c	32.8 ± 14.3 ^b
	<i>P</i> > 0.05	<i>P</i> < 0.01	<i>P</i> < 0.01	<i>P</i> < 0.01	<i>P</i> < 0.01

Values are means (± standard error) of four cages. Different letters in columns denote significant differences among stocking densities at $\alpha = 0.05$ determined by Tukey's test

Table 3. Fish and crustacean species collected in the water and on the substrate of cages / Especies de peces y crustáceos recolectados en el agua y sustrato de las jaulas

Species	Potential predator	% (n) of cages
Fish		
<i>Achirus lineatus</i>	Yes	6,25 (1)
<i>Atherinella brasiliensis</i>	No	100,00 (16)
<i>Bathygobius soporator</i>	No	31,25 (5)
<i>Centropomus pectinatus</i>	Yes	6,25 (1)
<i>Dominator maculatus</i>	No	50,00 (8)
<i>Guavina guavina</i>	No	18,75 (3)
<i>Larimus breviceps</i>	Yes	25,00 (4)
<i>Lycengraulis grossidens</i>	Yes	18,75 (3)
<i>Poecilia vivipara</i>	No	31,25 (5)
Crustaceans		
<i>Alpheus</i> sp.	No	25,00 (4)
<i>Litopenaeus vannamei</i>	No	50,00 (8)
<i>Panopeus</i> sp.	No	100,00 (16)

The present study illustrates new aspects of the technical feasibility of organic IMTA seahorse production as demonstrated by Fonseca *et al.* (2017). After 106 days of culture in trial 3, the survival rate was higher than 70%. This value is very high compared to those of other seahorse culture systems (usually ~50% survival) (Koldewey & Martin-Smith 2010). In all stages of the culture, seahorses fed on the plankton and benthos collected from the organic estuarine ponds. Even at the highest density tested (40 fish m⁻³), the seahorses grew well and likely could generate high profits. The system proposed here is a simple and efficient way to produce seahorses at a lower cost compared to traditional systems used to breed and grow marine ornamental fish species (Planas *et al.* 2008, Hora & Joyeux 2009, Murugan *et al.* 2009).

In a pilot trial, not detailed here, open net cages with minimal management proved to be ineffective, as all seahorses died during the experiment. Thus, some management is needed for cage production of seahorses. As shown in trial 3, cleaning and covering of the cage resulted in good production. The cages used in trial 3 had a small mesh size (1 mm) that did not allow the entry of large predators but did not eliminate their early stages. Although fish and shrimp larvae could enter the cage and grow inside it together with the seahorses, the grow-out period was not long enough for potential seahorse predators to reach a size that could compromise seahorse production.

Table 4. Abundance of live organisms collected in net substrate samples in the cages / Abundancia de organismos vivos recolectados en muestras de sustrato de la red de las jaulas

Group	Number of org. per 100 cm ² (relative proportion)
Copepoda	12 (6.5)
Nauplius	1 (0.5)
Kinorhyncha	1 (0.5)
Nematoda	140 (76.1)
Turbellaria	1 (0.5)
Amphipoda	23 (12.5)
Polychaeta	5 (2.7)
Isopoda	1 (0.5)
Total	184 (100)

After the 106 days of trial 3, the seahorses in every treatment had reached commercial size and could be sold at market for 5.00 USD (black) to 15.00 USD (yellow/orange). *H. reidi* changes its color depending on the environment, and the higher proportion of colored fish in the higher density compared to the lower densities could be caused by a stimulus from other seahorses. For example, the presence of one colored fish could stimulate the others to change color. Other factors that are known to influence color are the color of the tank and seahorse lineage. Thus, the use of natural colored parental seahorses or the transfer of specimens from the cages to a colored tank with clean water could increase the proportion of colored fish, thereby increasing profits.

A clear advantage of the system proposed herein is the use of naturally available food. Seahorses usually do not accept an artificial diet and must be acclimatized to eat frozen food (*e.g.*, shrimp, mysids) to be maintained in the home aquarium (Olivotto *et al.* 2008). The commonly used production systems are limited by the need to culture or collect live food in the natural environment. In all steps of the organic seahorse aquaculture system described here, the food is either present at the production site or collected from the farm ponds. This availability eliminates the need for zooplankton/benthos culture or collection in natural environment, avoiding the risk of introducing disease and the uncertainty of food availability. Although previous adaptation to frozen food will be necessary before selling the produced seahorses to ornamental market.

The inverse relationship between stocking density and growth of juvenile seahorses was previously described for *H. abdominalis* (Woods 2003) and *H. erectus* (Lin *et al.* 2009, Zhang *et al.* 2010) but not for *H. whitei* (Wong & Benzie 2003). The stocking density usually used to rear

seahorses in the first days of life has varied between 0.5 to 5 juveniles L⁻¹ (Olivotto *et al.* 2008, Hora & Joyeux 2009, Lin *et al.* 2009, Murugan *et al.* 2009, Zhang *et al.* 2010, Celino *et al.* 2012, Willadino *et al.* 2012, Pham & Lin 2013, Mélo *et al.* 2016, Sales *et al.* 2016). Martinez-Cardenas & Purser (2011) studied the effect of a higher stocking density ranging from 1.6 to 15 seahorses L⁻¹ on newborn *H. abdominalis* and found that growth and survival were independent of stocking density. More recently Hora *et al.* (2019) also studied the effect of a higher stocking density ranging from 1 to 15 seahorses L⁻¹ on newborn *H. reidi* in aquaria and found that growth and survival were independent of stocking density. In our study the density of 5 juveniles L⁻¹ was effective, as it did not significantly reduce juvenile survival compared to lower densities. The decrease of size with density observed here should not represent a great problem at this culture phase.

As seahorses grow, the stocking density must be decreased (Hora & Joyeux 2009) to achieve one adult breeding seahorse per 20-50 L in an aquarium system (Otero-Ferrer *et al.* 2012, Planas *et al.* 2013). Garcia & Hilomen-Garcia (2009) stocked 20 or 60 seahorses m⁻³, depending on their age. More recently, Garcia *et al.* (2012) used only 4 to 7 seahorses m⁻³ in cages. In the present study, the density of 40 seahorses m⁻³ generate very good results in terms of size, survival, skin color and total benefit.

In conclusion, this study demonstrated the technical feasibility of integrating all stages of *H. reidi* culture in an organic IMTA *C. brasiliensis* and *L. vannamei* farm. Future studies should focus on evaluating the effect of net cage mesh size, the need of supplementary food in the cages, improving breeding performance and developing a protocol to acclimatize the organically produced seahorses to feed on frozen food.

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