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Research Article

Short-tailed pipefish (*Microphis brachyurus*) juvenile culture: effect of stocking density on growth, survival and condition factor

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ABSTRACT. The present study aimed to test the effect of three stocking densities: 100, 200, and 300 ind m^{-3} (D100, D200, and D300, respectively) on survival, growth (weight and total length), and condition factor of *Microphis brachyurus*. At the end of the six-week trial, there were no significant differences in the fish's survival, growth, and condition. The results suggest that this species presents high adaptability under culture conditions. A suboptimal stocking density generates a suboptimal use of infrastructure and decreases the production system's profitability. Based on the present study, a stocking density of 300 ind m^{-3} is recommended to increase the aquaculture infrastructure's profitability for ornamental or conservation purposes.

Keywords: *Microphis brachyurus*; syngnathids; aquaria; juvenile; *Artemia*; aquaculture

INTRODUCTION

According to Nelson et al. (2016), about 37 pipefish species inhabit freshwater and saltwater systems in the American continent. Currently, *Microphis brachyurus* in Mexico is marketed as an aquarium fish. Due to unregulated fishing, natural populations of *M. brachyurus* are pressured, such as other syngnathid species used for the aquarium trade (Rosa et al. 2011). Improving the cultivation techniques of *M. brachyurus* could help diversify Mexican aquaculture and alleviate unregulated captures, which gradually deteriorate wild populations (Waples et al. 2007). Studies focused on the innovation of fish culture techniques are a useful tool to increase production and protect native species (Pérez-Sánchez & Páramo-Delgadillo 2008, Martinez-Cardenas & Purser 2011).

Several variables are used to measure fish performance, such as growth, survival, and condition factor. As with other culture factors, a suboptimal stocking density can produce poor fish growth and survival, mainly due to sub-optimal space and food availability that can cause inadequate food ingestion because of stress (Ellis et al. 2002, Diana et al. 2004, Ashley 2007, Abdel-Tawwab 2012, Akinwole et al. 2014). Stocking density is a major factor in fish culture to be considered when preparing facilities for this activity that can impact fish performance (Oppedal et al.

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2011, Riche et al. 2013). Based on the adopted system/practice, its effects can be negative or positive depending on the species and its life stage (Tolussi et al. 2010, Tibile et al. 2016).

Pipefish morphology is peculiar as their weight is smaller about their length when compared with other teleost fish. Due to the small amount of tissue in pipefish, specific techniques have been used to estimate the physiological response of animals to the factors tested. This scarcity in tissue made it impossible to conduct conventional techniques such as proximate analyses of protein and lipids used to estimate the nutritional response of fish; therefore, the carbon and nitrogen ratio (C:N) analyses were used, since it is an accurate indicator of the condition of fish as the protein has a C:N ratio close to 3 in a fish in good nutritional health (Westernhagen et al. 1998). Another tool to measure the condition of the fish is the moisture content analysis. An elevated moisture level is associated with a poor condition in fish, as nutritionally stressed fish replace body protein with water to maintain homeostasis (Shackley et al. 1993).

In some research studies, a high stocking density can cause stress due to an excessive number of fish cultured in a space unit, which might cause the development of hierarchical interactions (Bolasina et al. 2006). Most research on fish stocking density have reported positive results while using low stocking densities, and those studies were focused mainly on freshwater fish species with economic importance, such as tilapia (Osofero et al. 2009, Yuan et al. 2010, Mensah et al. 2013, Daudpota et al. 2014, Ronald et al. 2014), and African catfish *Clarias gariepinus* (Van de Nieuwegiessen et al. 2009, Dasuki et al. 2013). Research also has been conducted using a relatively semi-intensive culture system (ponds and floating cages). Chakraborty & Banerjee (2010), Shubha & Reddi (2011), and Aragón-Flores et al. (2014), are some of the few studies in which a low stocking density causes a negative performance, the latter reporting an acute case of aggressiveness. The detection of an adequate stocking density helps optimize production systems, generating quality products, resulting in good profitability for the producer. Therefore, the present study aimed to examine the effect on growth (wet weight, total length, and specific growth rate [SGR]), condition (moisture content, C:N ratio, and Fulton's K), and survival of *M. brachyurus* cultured in 100 (D4), 200 (D8), and 300 (D12) ind m^{-3}.

MATERIALS AND METHODS

System design and general methods

A total of 72 juveniles (mean ± standard deviation, SD) 9.18 ± 0.14 cm in total length (distance between the tip of the mouth to the tip of the tail) and 0.23 ± 0.004 g in wet weight, were stocked in a holding 100 L plastic tank at a water temperature of 26°C and water salinity of 0. The experiment was conducted in a closed temperature-controlled room (26°C), where nine 40-L glass tanks were used; each tank had biological and mechanical filtration provided by a platform filter (with 3 mm gravel particles above as filtration substrate) activated by an aeration elevator using an aeration pump (Whisper, Tetra®; Blacksburg, VA, USA) and air stone diffusion. A thermostat-controlled heater was set to maintain the desired water temperature in each tank. A 12 h light:12 h dark photoperiod was provided (lights on at 08:00 h, lights off at 20:00 h) by a timer-controlled cool white light 35 W (General Electric Company; Fairfield, CT, USA) producing an intensity of 4.8 μE m^{-2} s^{-1} at the water surface. Water quality for the experiment was maintained as follows: average pH 7.4 (range 7.0-7.9), dissolved oxygen 6.48 ppm, total ammonia nitrogen (TAN) < 0.5 mg L^{-1}, nitrite < 0.25 mg L^{-1}, nitrate < 5 mg L^{-1}. A colorimetric saltwater liquid test kit (Aquarium Pharmaceuticals Inc; Chalfont, PA, USA) was used to determine pH, TAN, nitrite, and nitrate and recorded every 48 h during the experiment. The temperature was monitored every 24 h, feces were disposed of, and any mortalities were inspected every day.

Experimental design

Three culture densities were tested for this study. Treatment D100 consisted of a stocking density of 4 individuals per 40 L tank (100 ind m^{-3}), 8 individuals per 40 L tank (200 ind m^{-3}) were seed in treatment D200, and in treatment D300, 12 individuals per 40 L tank (300 ind m^{-3}) were located. During the experiment, the food provided was live *Artemia nauplii* (enriched with Super Selco® for 24 h at 26°C) maintaining a ration rate of 14% body weight per day (dry weight *Artemia*: wet weight fish), divided into two equal-sized meals (09:00 and 16:00 h). Feeding was adjusted based on the daily mortality (assigned the previously recorded mean weight) per tank (the rations corresponding to mortalities were not fed to the fish remaining on the tank). *Artemia* fed at 16:00 h were from the same batch as the morning feed but were enriched for seven hours.

The total length of pipefish was measured by placing the fish on a 1 mm scaled sheet covered with plastic. Wet weight was measured on an electronic scale and recorded to the nearest 0.001 g. Fish were deprived of food for 24 h before each weighing. The total length and wet weight of individual pipefish were recorded at the start of the trial. Weekly growth was also recorded from bulk measures of wet weight. Fish were not fed for 24 h prior to each weighing. After six weeks, the surviving fish were counted, and their wet weight and total length were measured individually; with these data, the following parameters were calculated:

Daily total length gain (cm d^{-1}) = final total length - initial total length / experiment days

Daily weight gain (g d^{-1}) = final weight - initial weight / experiment days

Final biomass (g m^{-3}) = biomass gain × stocking density m^3, where biomass gain is: initial biomass (g) - final biomass (g)

Fulton's K = (W / L^3) × 100, where W: wet weight (g) and L: total length (cm)

Specific growth rate (SGR) was calculated as SGR% increase in body weight per day = [(lnWf - lnWi) / t] × 100, where Wf: final weight (g), Wi: initial wet weight (g), and t: time (d).

The coefficient of variation (CV) (100 × SD / mean) of final fish body weight (BW) was calculated following Kestemont et al. (2003), followed by size heterogeneity = CVwf / CVwi; where wf: final weight; wi: initial wet weight.

Moisture and nitrogen/carbon content

The biomass of the pipefish did not meet the minimum amount of tissue necessary to conduct conventional proximate analyses. Instead, the moisture, nitrogen, and carbon content were quantified in the carcass to determine if pipefish cultured in one of the stocking densities tested presented a better condition than the rest of the treatments. At the end of the trial, one pipefish per tank (randomly selected) was euthanized with an overdose of benzocaine (400 mg L^{-1}), blotted dried, and weight and total length were recorded. Each pipefish was subjected to a dehydration process by lyophilization using the Thermo Savant Modulyo D-115 for 72 h at -49°C and a vacuum pressure of 36×10^{-3} mbar. In addition, as low moisture content has been associated with a good condition in fish (Shackley et al. 1993), those dried samples obtained were used for moisture content calculation by determining the difference from the wet weight. Then pipefish were individually ground with a mortar and pestle to analyze nitrogen and carbon by oxidation/infrared detection, using a CHNS auto-analyzer (Dhaliwal et al. 2014).

Statistical analysis

A one-way ANOVA was used to compare the average among treatments of survival (previously transformed arcsine square root), growth in weight among treatments per week of bioassay (g), weight growth per treatment among bioassay weeks (g) initial and final total length (cm), initial and final wet weight (g), weight gain and total length gain, coefficient of variation (fish body weight g), size heterogeneity (fish body weight g), Fulton's K (K), final biomass per m^3 (g m^{-3}), final biomass coefficient of variation and SGR (% d^{-1}). A two-way ANOVA was used to determine if there were significant differences in growth as a function of time, taking treatments and weeks of growth as factors. A significance level of $P < 0.05$ was used. Levene's test and residual plots were used to test homogeneity of variance, and the Kolmogorov-Smirnov test was used to corroborate normality. Tukey's HSD *post-hoc* test was used to identify differences among treatment means (SPSS 17.0).

RESULTS

There were no significant differences in either juvenile total length ($F_{2,9} = 0.317$, $P = 0.740$) or wet weight ($F_{2,9} = 0.288$, $P = 0.760$) among treatments at the start of the trial. After six weeks no significant differences were found between treatments in survival (89.35 ± 7.13%; $F_{2,9} = 0.457$, $P = 0.653$), final total length (10.72 ± 0.02 cm; $F_{2,9} = 0.006$, $P = 0.994$), weight gain (0.33 ± 0.03 g; $F_{2,9} = 0.305$, $P = 0.748$), total length gain (1.54 ± 0.09 cm; $F_{2,9} = 0.174$, $P = 0.845$), final wet weight (0.56 ± 0.03 g; $F_{2,9} = 0.286$, $P = 0.761$), coefficient of variation ($F_{2,9} = 0.771$, $P = 0.504$), size heterogeneity ($F_{2,9} = 4.055$, $P = 0.077$) and Fulton´s K (0.045 ± 0.002; $F_{2,9} = 1.096$, $P = 0.393$), carbon:nitrogen ratio ($F_{2,9} = 0.254$, $P = 0.784$), moisture content ($F_{2,9} = 2.556$, $P = 0.157$) and specific growth rate (2.07 ± 0.12; $F_{2,9} = 0.322$, $P = 0.736$). There were significant differences in the final possible biomass produced by m^3 ($P < 0.05$) being greater for D300 (100.77 ± 35.64 g) which was similar to D200 (70.40 ± 13.59 g) and different from D100 (29.92 ± 5.38 g); which in turn was similar to D200. A final biomass coefficient of variation of more than 35% was observed in D300, while for D200 and D100 it was between 18 and 19% (Table 1). No statistical differences in growth were observed among treatments for each of the seven measuring days ($P > 0.05$), but differences were observed among the seven measuring days in growth among treatments ($P < 0.05$), indicating the change in growth of the three treatments over time (Fig. 1).

DISCUSSION

The absence of significant differences in *Microphis brachyurus* survival indicates that the stocking densities tested did not affect this factor in the present study. The overall survival of 80% was higher compared to the only two experimental studies on this species in which similar conditions to the present study were used, a stocking density of 10 fish per tank, a

Table 1. Effect of three stocking densities: 100, 200, and 300 ind m^{-3} (D100, D200, and D300, respectively) in wet weight and total length (initial and final), change in weight and the total length, specific growth rate (SGR), size heterogeneity, coefficient of variation, Fulton's K, C:N ratio, final biomass per m^3, and final biomass coefficient of variation (mean ± 1 standard deviation of three replicates per treatment) in *Microphis brachyurus* cultured for six weeks. [1]Means with different superscripts within a row are significantly different (one-way analysis of variance, $P < 0.05$); CV of final biomass does not present superscript as shows direct data.

Stocking density	D100	D200	D300
Final observed survival (%)	83.33 ± 28.86^a	87.66 ± 12.50^a	97.33 ± 4.61^a
Initial individual weight (g)	0.23 ± 0.004^a	0.23 ± 0.016^a	0.23 ± 0.001^a
Final individual weight (g)	0.53 ± 0.05^a	0.58 ± 0.08^a	0.57 ± 0.12^a
Daily weigth gain (g d^{-1})	0.30 ± 0.05^a	0.35 ± 0.07^a	0.34 ± 0.12^a
Daily total length gain (cm d^{-1})	1.62 ± 0.25^a	1.57 ± 0.10^a	1.43 ± 0.62^a
Size heterogeneity (body weight g)	0.52 ± 0.27^a	0.70 ± 0.16^a	0.97 ± 0.12^a
Coefficient of variation (final body weight g)	23.60 ± 12.14^a	18.58 ± 5.22^a	26.33 ± 2.36^a
Initial total length (cm)	9.13 ± 0.13^a	9.15 ± 0.38^a	9.28 ± 0.22^a
Final total length (cm)	10.74 ± 0.14^a	10.71 ± 0.29^a	10.72 ± 0.50^a
Fulton's K	0.042 ± 0.002^a	0.047 ± 0.004^a	0.046 ± 0.003^a
C:N ratio	4.10 ± 0.12^a	4.47 ± 0.85^a	4.17 ± 0.75^a
Moisture (%)	78.06 ± 5.07^a	72.38 ± 3.17^a	78.72 ± 2.63^a
SGR (% d^{-1})	1.96 ± 0.26^a	2.19 ± 0.21^a	2.07 ± 0.51^a
Final biomass per m^3 (g m^{-3})	29.92 ± 5.38^b	70.40 ± 13.59^{ab}	100.77 ± 35.64^a
Final biomass coefficient of variation[*]	17.97	19.31	35.37

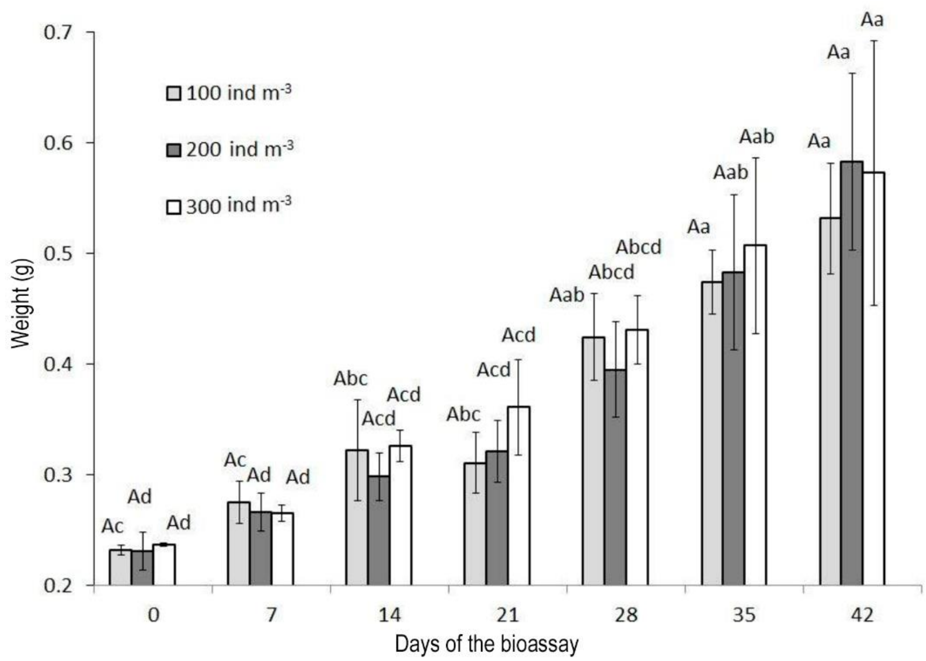

Figure 1. In a growth trial, the wet weight of juvenile *Microphis brachyurus* was cultured at three different densities (100, 200 and 300 individuals per m^3; ind m^{-3}). Fish were fed at a 14% body weight per day adjusted daily based on growth and mortality. The different capital letters on the bars show statistical differences among the treatments in each of the seven measuring days and the different lowercase letters on the bars show the statistical differences among the seven measuring days per treatment ($P < 0.05$). All values represent the mean of three replicates per treatment ± 1 standard deviation.

temperature of 26°C, and a salinity of 0 (Martinez-Cardenas et al. 2013, 2014). As reported in most studies, in high stocking densities, the intraspecific competition increases, which generates stress that results in poor condition of the attacked fish (Barcellos et al. 1999, Grant et al. 2002, Lin et al. 2009, Osofero et al. 2009, Van de Nieuwegiessen et al. 2009, Zhang et al. 2010, Narejo et al. 2010, Castillo-Vargasmachuca et al. 2012, Chattopadhyay et al. 2012, De Oliveira et al. 2012, Luo et al. 2012). High stocking densities can lead to poor food intake and increased energy expenditure due to competition for space and food (Diana et al. 2004), leading to anorexia or deformities due to nutritional deficiencies (Garcia et al. 2013). Similar to this study, Webb et al. (2007) and Gonçalves de Oliveira et al. (2012), on *Rachycentron canadum* and *Arapaima gigas*, respectively, found no significant differences in survival, reporting approximately 97%. However, in the present study, the lack of significant differences in most variables measured could be explained by the trophic physiology of this species, which shows a non-fast swimming behavior in accordance with its habitat of slow waters and its stealthily sneaking predatory strategy (Miller et al. 2005, Nelson et al. 2016).

The results of the present study indicate that *M. brachyurus* could be cultured in the highest stocking density tested without presenting problems similar to the findings with other syngnathid species: Carvalho et al. (2019) found no effect of different stocking densities (2, 3, 4, and 5 newborn juveniles L^{-1}) in *Hippocampus reidi* survival; however, seahorse length, was inversely related to stocking density. Wong & Benzie (2003) also reported that stocking density (1.0 and 0.5 ind L^{-1}) did not influence *Hippocampus whitei* growth, but there was an indication that reproduction might be inhibited at stocking densities greater than one ind L^{-1}. Er et al. (2020) found that there was no significant difference ($P > 0.05$) in the growth of *H. barbouri* juveniles cultured under different stocking densities (0.3, 0.4, and 0.5 juveniles L^{-1}). The authors suggested that their result may be because the stocking densities employed in that study had not reached their maximum limit. This suggestion may apply to the results situation of the present study due to the limited number of densities tested. In contrast, some research has found marked differences in responses between population densities, such as the study by Lin (2009) on subadult *Hippocampus erectus* seahorses, where the growth rate decreased with increasing population density (0.25, 0.5, 1 and 1.5 ind L^{-1}, respectively). Also, in early juveniles of the same species, Lin (2010) found a positive trend in growth and survival at the highest densities tested (0.5, 1, 1.5, 2, 3, 4, and 6 ind L^{-1}, respectively), which suggests that syngnathid response to population densities varies by species and life stage.

The present study's findings are similar to the results of various studies in other commercial teleosts, such as the effect of stocking density on *Symphysodon aequifasciatus* (Tibile et al. 2016). However, the authors reported that the lack of statistical differences in condition index and size in that species were limited to the reduced sample numbers. Another species that does not present an evident damaging effect is the rainbow trout (*Oncorhynchus mykiss*), as reported in a review article by Ellis et al. (2002). Similarly, Riche et al. (2013) concluded that stocking density does have a clear influence on the condition index of market size cobia. Osofero et al. (2009) tested in juveniles of *Oreochromis niloticus* in stocking densities of 50, 100, 150, and 200 ind m^{-3}. The authors reported no significant differences among treatments after 90 days; therefore, they recommended the treatment of 150 ind m^{-3}. This recommendation arises because since there are no significant differences between the treatments, the higher cultivation density allows the optimization.

An adequate stocking density serves to indicate the concentration of the fish to be cultured per space unit, gaining relevance for the best use of the system, production performance, and water use, which aids to maximize food and space use with less stress, minimum energy investment and better growth of fish (Chakraborty & Banerjee 2010). On the contrary, M'balaka et al. (2012), who tested three different densities (5, 7, and 9 ind m^{-3}) in a 10 weeks trial in three strains of tilapia *Oreochromis shiranus* with an initial weight of 6.7 g, did not find significant differences between the lowest densities; however, a poor growth was observed at the highest density, choosing as the adequate density 5 ind m^{-3}. Also, Chakraborty & Banerjee (2010), in a Nile tilapia study conducted in production systems, tested densities of 0.5, 1, 1.5, 2, 2.5, and 3 ind m^{-3}, and the authors reported significant differences in growth, with a reversed trend between growth and density and proposed as an adequate density 2 ind m^{-3}.

The present study found differences in the final biomass obtained, with no difference in the growth parameters, which may be related to the organisms' growth time and growth stage. Regarding this, Reátegui-Acosta et al. (2017), in a growth period of 30 days, found that, although there were no statistical differences in growth and hematological parameters in juveniles of *Piaractus brachypomus*. Contrary, Gonçalves de Oliveira et al. (2012), carrying out a bioeconomic comparison, found that profitability, with *Arapaima gigas* juveniles, is better at the lowest density. Also, Garcia et al. (2013) in an experiment with tilapia *O. niloticus*, reported that the best yields occur at the lowest densities. However, these observations are given in long-term cultures where the result may be due to interaction and ethology during growth where at lower densities, there is less probability of injuries and energy expenditure and a better intake.

However, low stocking densities do not contribute to the system's final profitability.

The overall absence of significant differences in most of the variables recorded in the present trial could also be related to the provided food ratio of 14%, which was enough to prevent nutritional stress in the fish. The 14% food ratio used in this study aimed to ensure adequate feed intake by *M. brachyurus,* as in a previous syngnathid study (Martinez-Cardenas & Purser 2007, 2011). This feed ratio was found to be in excess for experimental seahorse rearing; moreover, in other studies on teleosts, a 5% feeding ratio has already been considered an excessive amount (El-Sayed & Garling Jr. 1988, Jabeen et al. 2004, Abid & Ahmed 2009, Adewolu et al. 2010, Barron et al. 2012, Lee et al. 2014). Whole-body samples were collected at the end of the experiment to robust the observations on *M. brachyurus* growth as a C:N ratio of 3.0 is considered an indicator of good condition in fish (Harris et al. 1986). The ratio observed in juveniles cultured in the three stocking densities was not significantly different and exceeded 3.0 (over 4 in all treatments), suggesting that the fish were not nutritionally stressed. The lack of significant differences on the other condition variables recorded (Fulton's K and moisture content) were consistent with the C:N ratio results, confirming that the fish were not nutritionally stressed.

It has been reported in previous research on *M. brachyurus* (Martinez-Cardenas et al. 2013, 2014) that this species accepts enriched *Artemia* nauplii as a good food source in captivity. The similarity of the pipefish response in the present study may also explain the homogeneous distribution of the nauplii throughout the tank. Contrarily to the observations in the study by Martinez-Cardenas & Purser (2011), due to the unidirectional light source, the nauplii tended to congregate near the tank's surface, following their phototactic behavior, preventing the fish to prey in the water column. Also, a digestive physiology study has recently confirmed the adaptability of *M. brachyurus* to enriched *Artemia* (Martinez-Cardenas et al. 2020). This species presents good digestive capacity since it can degrade many proteins due to their efficient proteolytic activity. The effort to improve the knowledge on *M. brachyurus* culture techniques focuses on alleviating the current threats the species is experiencing, such as unregulated fishing and anthropogenic alteration of their habitat.

From the results of the present study, it can be concluded that under the experimental conditions described, the low mortality rates, overall good condition, and the similar growth of *M. brachyurus* cultured in any of the stocking densities tested can be considered an attribute for aquarium market aquaculture as well as for the conservation culture of this species as the higher the stocking density used, the greater the number of fish kept safely. Both perspectives aim to optimize the use of infrastructure by culturing fish at high stocking densities for at least a certain stage of the life cycle without showing negative effects. However, future research is needed to find the optimal stocking density to improve this species' culture.

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