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*Research Article*

# Effect of stocking density on growth and survival of fine flounder *Paralichthys adspersus* (Steindachner, 1867) larvae

**Angélica Castro[1], Melissa Montes[1], Lucas Orihuela[1], Joel Linares[1], Noemí Cota[1] Lili Carrera[1], Pedro Toledo[2] & Juan Pablo Lazo[3]**
[1]Instituto del Mar del Perú (IMARPE), Chucuito, Callao, Perú
[2]Departamento de Acuicultura, Facultad de Ciencias del Mar
Universidad Católica del Norte, Coquimbo, Chile
[3]Fish Nutrition Laboratory, Department of Aquaculture
Center for Scientific Research and Higher Education of Ensenada, Baja California, México
Corresponding author: Angélica Castro (acastro@imarpe.gob.pe)

**ABSTRACT.** Larviculture is a critical stage during the production of marine fish species and it is related to highest mortalities. The production of juveniles depends on a successful larval rearing, consequently the importance to investigate all aspects related to this culture stage, such as the stocking-density which is the extreme importance to improve culture conditions and reduce stress and mortality. Therefore, the objective of this study was to evaluate the effect of three stocking densities (10, 20 and 30 larvae $L^{-1}$) on growth and survival of the fine flounder larvae, an important commercial species, under laboratory conditions. The number of larvae for each treatment was distributed randomly in 12-141 L fiberglass-tanks in a static-batch system. We used one day post-hatching larvae (DPH) and first feeding began two DPH with enriched rotifers (0.5 to 4 rotifers $mL^{-1}$). The standard length (mm) was determined at 1, 5, 10 and 20 DPH, and the dry weight (mg) at the beginning and end of the experimental trial. Besides, the specific growth rate (SGR) and the coefficient of variation (CV) were calculated. Our results exposed that the stocking density of 10 larvae $L^{-1}$ had significantly higher growth (8.14 mm and 0.90 mg), SGR (20.71 ± 1.48% $d^{-1}$) and survival (42.38%) compared to the 20 and 30 larvae $L^{-1}$ stocking densities. Additionally, treatment stocked with 10 larvae $L^{-1}$ resulted in the highest final weight (0.90 ± 0.25 mg) and the lowest variation in sizes (CV = 5.86 ± 1.56). In conclusion, there is a significant effect of the stocking density on the growth and survival during the larval rearing of *P. adspersus*, and according to the results obtained in this study, the best stocking density for this species was that of 10 larvae $L^{-1}$. More research is necessary to evaluate other parameters to improve growth and survival during the larval rearing of this species.

**Keywords:** *Paralichthys adspersus*; larval rearing; stock density; growth; survival

## INTRODUCTION

Aquaculture in Peru has grown 20% per year; based mainly on the production of three species, Peruvian scallop (*Argopecten purpuratus*), white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*). In order to diversify Peruvian aquaculture and making it more sustainable over time, it is essential to develop culture technology of other commercially important species such as the fine flounder, *Paralichthys adspersus* (Mendoza, 2013). The fine flounder is naturally caught by artisanal fisheries and has a high demand in Peru, reaching prices in the coastal fishing ports from US$6 to US$12 per kg (INFOMAR-IMARPE, 2016). Recently, Peruvian gastronomy has included flounder as one of the essential dishes, increasing prices in the supermarkets as high as US$30 per kg of fillet (Villa Maria del Triunfo, Lima, Perú; Fierro, 2015). This species inhabits gulfs and bays with a sandy bottom, ranging from Paita, Peru to Lota and Juan Fernández Island, Chile (Acuña & Cid, 1995; Sielfeld *et al.*, 2003). Recently, Chile has developed research with commercial and repopulation purposes with relative success (IFOP, 2012). Nonetheless, to achieve industrial production of this species, sustainable and constant output of juveniles is needed,

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where the larviculture stage is still the most critical phase in the productive cycle, due to high mortalities (Qin, 2008).

Different factors affect the success of the larval culture period, among them, stocking density of the larvae is extremely important. Some investigations has been performed evaluating the effect of stocking density, on larval growth and survival in different species of marine fish such as *Paralabrax maculatofasciatus* (Álvarez-González *et al*., 2001), *Dicentrarchus labrax* (Hatziathanasiou *et al.*, 2002), *Seriola lalandi* and *Atractoscion nobilis* (Stuart *et al.*, 2013). More specifically of the genus *Paralichthys*, research has been performed on species such as *P. olivaceus* (Dou *et al.*, 2003; Bolasina *et al.*, 2006), *P. lethostigma* (Daniels *et al.*, 1996), *P. dentatus* (King *et al.*, 2000). The results of these studies were reported high mortality associated with high stocking densities, but some high densities (*i.e.*, up to 300 larvae $L^{-1}$) have resulted in a reduction in social dominance and antagonistic behavior among individuals. These high densities led to a rise in survival but were generally associated with a decrease in growth. Therefore, this study aimed to investigate the effect of three stocking densities in *P. adspersus* from 1 to 20 DPH and its relationship to growth, size heterogeneity, and survival.

## MATERIALS AND METHODS

### Broodstock maintenance and spawning

Broodstock was conditioned to spawn in two fiberglass tanks of 2 $m^3$ with a water volume of 1.6 $m^3$, adapted to a seawater recirculation system (Carrera *et al.*, 2013). The experiment was performed in the Laboratory of Fish Culture of the Instituto del Mar del Perú (IMARPE) - Callao. The broodstock was kept with a photoperiod of 12L:12D, and there was daily monitoring for physical and chemical parameters of water quality, such as temperature (16.66 ± 0.75°C), pH (8.26 ± 0.07) and dissolved oxygen (8.50 ± 1.14 mg $L^{-1}$) under laboratory condition. Also, total ammonia nitrogen (0.27 ± 0.14 mg $L^{-1}$), nitrite (1.32 ± 1.14 mg $L^{-1}$) and nitrate (14.67 ± 2.17 mg $L^{-1}$); and carbon dioxide (5.33 ± 1.03 mg $L^{-1}$) were measured weekly to ensure adequate water quality. Feeding was based on pieces of anchovy *Engraulis ringens*, offered three times per week at a rate of 2.0% of the total biomass of each culture tank.

Broodstock biometrics and reproductive stage were assessed monthly, recording weight (g), total length (cm) and oocyte development. Briefly, fishes were anesthetized in a seawater solution with clove oil (81 mg $L^{-1}$). Gonadal maturity was monitored to select the specimens suitable for hormonal induction. For females, a sample of oocytes was obtained by ovarian biopsy or cannulation (Mylonas *et al.*, 2010). The sample was analyzed in an optical microscope (LEICA®) with a digital camera and the image program from Leica Application Suite Version 4.3. The diameter of 30-50 oocytes was measured to determine gonadal maturation stage. Maturation was classified into three stages taking into account the diameter, characteristics, and proportion of each type of oocyte (*i.e.*, previtellogenic, vitellogenic and mature) (Perea *et al.*, 2015); stage I or "sedentary stage" is characterized by the highest proportion of previtellogenic oocytes (average diameter of oocytes <200 μm); stage II or "in-maturation stage" has the higher proportion of vitellogenic oocytes (diameter of 200 to 350 μm); and stage III or "mature stage" has the highest proportion of mature oocytes (diameter >350 μm). In males, a semen sample was collected by abdominal pressure according to the methodology described by Lanes *et al.* (2010). Sperm quality was evaluated by measuring sperm concentration and sperm motility.

For hormonal induction, a mature female (stage III) with oocytes (diameter 541.48 ± 2.11 μm) and two males with sperm motility greater than 50% and an average sperm concentration of 0.84 ± 0.09×$10^{10}$ spermatozoa $mL^{-1}$ were selected. The female was administered with an intraperitoneal injection, using a synthetic analog of the gonadotropin-releasing hormone (GnRH, buserelin acetate {Pyr-His-Trp-Ser-Tyr-D-Ser- (But) -Leu-Arg-Pro). -NHEt Conceptase® with a dose of 0.84 μg per kg of fish (0.1 mL $kg^{-1}$). In the case of males, the same analog was injected via intramuscular at a dose of 0.84 μg per fish (0.1 mL $fish^{-1}$).

At 48 h post-hormone induction, fish were anesthetized, and gametes collected by abdominal pressure or "stripping." Eggs and sperm were placed in a beaker and mixed by the "dry method" (Rottmann *et al*., 1991; Silva & Oliva, 2010). A total of 78,900 eggs were obtained. Percent fertilization was 96.64 ± 1.67%. The eggs were incubated in a 225 $L^{-1}$ conical tank at a temperature of 18 ± 0.63°C in a static system. After 24 h, 50% of the water was exchanged, and dead eggs siphoned out. Hatching occurred 48 h after spawning with a hatching rate of 53.33 ± 12.86%.

### Larval culture

A total of 24,000 larvae (standard length 3.13 mm and dry weight 0.017 mg) were used for the experiment. The larvae were transferred at one-day post-hatching (DPH) to the experimental units by using 2 L plastic containers (jars) to avoid any stress due to handling. The appropriate number of larvae for each treatment was seeded in 12,141 L (0.60 cm diameter and 0.50 cm

height) fiberglass tanks with a volume of 100 L. Culture seawater (salinity 35) was previously filtered and sterilized by ultraviolet radiation (UV). Experimental tanks were provided with constant aeration using air diffusers. It was used white light between 905 and 1,245 lux. During the first ten days of culture a photoperiod of 24L:0D was used, after that, the photoperiod was changed to 12L:12D until the end of the experiment. There was daily monitoring for physicochemical parameters of water quality, such as temperature (20.55 ± 0.87°C), dissolved oxygen (7.28 ± 0.65 mg $L^{-1}$) and pH (7.83 ± 0.29) using a YSI Pro 1020 multiparameter.

Larvae were cultured using a combined static-batch system. Briefly, initial volume was set to 60 L, and 10 L of UV sterilized seawater were added per day for the next four days, to reach a volume of 100 L. 6 DPH, 40 L of water in the tanks were siphoned out using a 500 µm sieve to reach 60 L and then the process restarted. This method was used to avoid larval stress and to siphon-out any dead larvae. 10 to 20% of the water was exchanged daily, beginning on 11th-day post-hatching, until the end of the experiment (20 DPH). The "green water" technique was used (Skiftesvik *et al.*, 2003; Sanaye *et al.*, 2014), adding the microalgae *Isochrysis galbana* and *Nanochloropsis oculata* to the culture tanks. Algae concentration in the culture tanks was targeted at 635,800 cells $mL^{-1}$ and 8,065,000 cells $mL^{-1}$, respectively. Exogenous feeding of the larvae began 2 DPH using rotifers *Brachionus plicatilis* enriched with Easy Selco®. Feeding protocol is described in Table 1. Three times a day, counts of rotifer density in the culture tanks were made to keep desired rotifer density in-check and to add freshly enriched rotifer to the experimental tanks.

**Experimental design**

The experiment was designed to evaluate the effect of three stocking densities; SD10 (10 larvae $L^{-1}$), SD20 (20 larvae $L^{-1}$) and SD30 (30 larvae $L^{-1}$) with four replications per treatment. The standard length (mm) was monitored by measuring the larvae on days 1, 5, 10, 15 and 20 DPH and the dry weight (mg) determined at the beginning and end of the experiment (Pepin, 1995). Survival was determined at the end of the experiment (20 DPH). The specific growth rate (SGR) was calculated in relation to standard length and dry weight for each treatment, with the following equation (De Oliveira *et al.*, 2012; Lugert *et al.*, 2014):

$$\mathrm{SGR} = \frac{\ln(Wt) - \ln(Wi)}{\text{final time} - \text{initial time}} \times 100 \qquad (1)$$

where: ln: natural logarithm, Wt: final length/weight, Wi: inicial length/weight.

Size heterogeneity of the larvae among treatments was assessed using the coefficient of variation (CV) and was calculated according to the equation described by Merino *et al.* (2007):

$$\mathrm{CV} = \frac{\text{Standard desviation}}{\text{Mean length}} \times 100 \qquad (2)$$

**Statistical analysis**

The differences between group mean of the parameters for water quality, standard length, CV, dry weight, SGR, and survival were analyzed with the non-parametric test Kruskal-Wallis ($\alpha = 0.05$), because variables did not show a normal distribution (Shapiro-Wilk test), nor homogeneity of variances (Levene test). When significant differences were observed, the Dunn post hoc test ($\alpha = 0.05$) was applied. The statistical analysis was performed using the statistical program R (R Core Team, 2016).

## RESULTS

Water quality parameters by treatment during the experiment are presented in Table 2.

Significant differences were observed in the standard length of the larvae between the different treatments beginning on day 10 DPH (Fig. 1). At the end of the experimental trial, larvae in the SD10 treatment were significantly ($P < 0.05$) larger than the other two densities (SD20 and SD30). No significant differences were observed ($P > 0.05$) between the larvae grown at 20 and 30 larvae $L^{-1}$ (Fig. 1).

Significant differences ($P < 0.05$) regarding the dry weight of the fine flounder larvae reared at different stocking densities were observed at the end of the experiment (Table 3). Larvae of SD10 treatment were significantly heavier compared to the larvae reared in the SD20 and SD30 treatments. Similarly, to the standard length, there were no significant differences ($P > 0.05$) in final dry weight between the SD20 and SD30 treatments. Consequently, highest values for SGR in length and weight were observed in the larvae culture at the lowest stocking density (10 larvae $L^{-1}$), which were significantly higher ($P < 0.05$) compared to the other two densities of 20 and 30 larvae $L^{-1}$ (Table 3).

Coefficients of variation for standard length, dry weight and SGR were significantly different between treatments. The coefficient of variation (CV) was significantly lower for the SD10 treatment compared to the other two treatments, while no significant differences were found between the SD20 and SD30 CV.

**Table 1.** Fine flounder *Paralichthys adspersus* larvae culture protocol under laboratory conditions. Iso: *Isochrysis galbana*, Np: *Nanochloropsis oculata*.

| DPH | Rotifers ($mL^{-1}$) | Water exchange (% $d^{-1}$) | Actual water volume (L) | Microalgae Iso-Np (L) |
|---|---|---|---|---|
| 1 | 0.5 | 0 | 60 | 0.4-1.6 |
| 2 | 1 | 0 | 70 | 0.4-1.6 |
| 3 | 1 | 0 | 80 | 0.2-0.8 |
| 4 | 1 | 0 | 90 | 0.4-1.6 |
| 5 | 2 | 0 | 100 | 0.2-0.8 |
| 6 | 2 | 50 | 60 | 0.8-3.2 |
| 7 | 2 | 0 | 70 | 0.8-3.2 |
| 8 | 3 | 0 | 80 | 0.8-3.2 |
| 9 | 4 | 0 | 90 | 0.4-1.6 |
| 10 | 4 | 0 | 100 | 0.4-1.6 |
| 11 | 4 | 10 | 100 | 0.8-3.2 |
| 12-14 | 3 | 15 | 100 | 1.6-6.4 |
| 15-16 | 3 | 20 | 100 | 1.4-5.6 |
| 17-20 | 3 | 20 | 100 | 1.2-4.8 |

**Table 2.** Physical and chemical parameters during the larval culture of *Paralichthys adspersus* at different stocking densities (values expressed as the average ± SD), n = 56.

| | Density | | |
|---|---|---|---|
| | SD10 | SD20 | SD30 |
| Temperature (°C) | 20.56 ± 0.87 | 20.56 ± 0.87 | 20.51 ± 0.89 |
| Oxygen (mg $L^{-1}$) | 7.21 ± 0.69 | 7.31 ± 0.61 | 7.32 ± 0.65 |
| pH | 7.83 ± 0.30 | 7.83 ± 0.27 | 7.82 ± 0.31 |

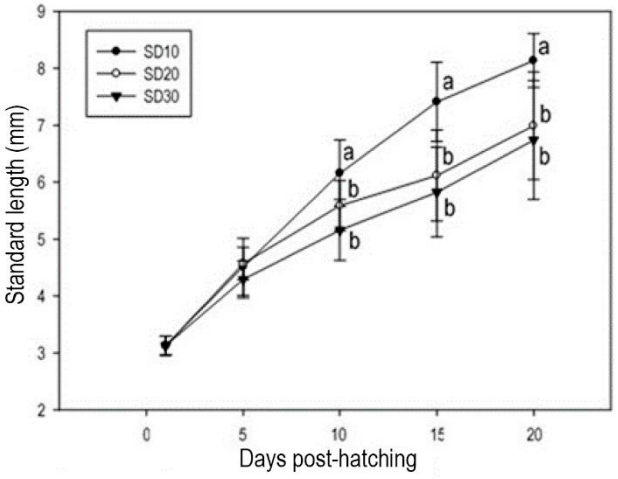


**Figure 1.** Mean standard length of fine flounder *Paralichthys adspersus* larvae (n = 40) at different stocking densities. The different letters on each day after hatching indicate significant differences ($P < 0.05$).

Significant differences were observed in the final survival of larvae reared at different stocking densities. The highest survival was recorded for larvae in the SD10 (42.37 ± 6.64%) treatment. However, no significant differences were observed for the SD20 (8.69 ± 5.50%) and SD30 (14.8 ± 3.95%) treatments (Fig. 2).

## DISCUSSION

The evaluation of the real effect of stocking density in the larval culture of *Paralichthys adpersus* allows defining one of the most important parameters for adequate growth and survival in this critical stage of fish culture. In the present study, a growth regarding standard length and weight were significantly higher for larvae reared at 10 larvae $L^{-1}$. Our results showed an inverse relationship between stocking density and dry weight, in other words at lower stocking densities, the higher the dry weight of larvae. A similar relationship has been reported for the Japanese flounder *P. olivaceus* larvae at 20 DPH (Bolasina *et al*., 2006).

Final survival was higher in the SD10 treatment. Both response variables; growth and survival were dependent on stocking density, and a strong inverse relationship was observed where the higher the stocking density, the lower the growth and survival obtained. Similar results were reported for Japanese flounder *Paralichthys olivaceus* larvae stocked at densities of 5, 10 and 15 larvae $L^{-1}$ (Dou *et al*., 2003),

**Table 3.** Standard length, coefficient of variation (CV), dry weight and specific growth rate (SGR) of the fine flounder *Paralichthys adspersus* larvae at different stocking densities (expressed as average values ± SD, n = 4). The different letters in a row indicate significant differences ($P < 0.05$). DPH: day post-hatching.

| | Stocking density (larvae $L^{-1}$) | | |
|---|---|---|---|
| | 10 | 20 | 30 |
| Standard length (mm) | | | |
| 1 DPH | 3.130 ± 0.165 | 3.130 ± 0.165 | 3.130 ± 0.165 |
| 20 DPH | 8.136 ± 0.517[a] | 6.991 ± 0.240[b] | 6.739 ± 0.242[b] |
| | (n = 40) | (n = 40) | (n = 40) |
| CV (%) | | | |
| 1 DPH | 11.378 ± 7.840 | 6.252 ± 1.350 | 7.559 ± 1.485 |
| | (n = 4) | (n = 4) | (n = 4) |
| 20 DPH | 5.855 ± 1.557[a] | 13.526 ± 0.494[b] | 15.490 ± 2.396[b] |
| | (n = 4) | (n = 4) | (n = 4) |
| Dry weight (mg) | | | |
| 1 DPH | 0.017 ± 0.001 | 0.017 ± 0.001 | 0.017 ± 0.001 |
| 20 DPH | 0.902 ± 0.253[a] | 0.412 ± 0.070[b] | 0.360 ± 0.070[b] |
| | (n = 40) | (n = 40) | (n = 40) |
| SGR (% $day^{-1}$) | | | |
| Standard length | 5.019 ± 0.333[a] | 4.227 ± 0.179[b] | 4.034 ± 0.190[b] |
| | (n = 4) | (n = 4) | (n = 4) |
| Weight dry | 20.707 ± 1.483[a] | 16.678 ± 0.895[b] | 15.963 ± 0.939[b] |
| | (n = 80) | (n = 80) | (n = 80) |

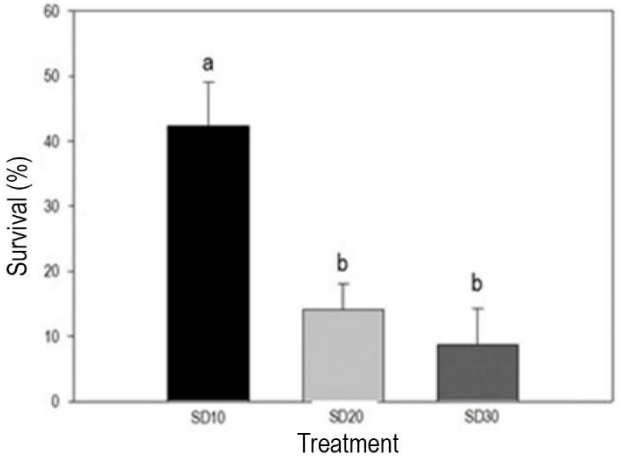


**Figure 2.** Survival (%) of the fine flounder *Paralichthys adspersus* larvae reared at different stocking densities. Different densities indicate significant differences ($P < 0.05$).

this study showed that at lower stocking densities, faster growth and higher survival were obtained. Although the specific cause of this relationship is unknown (*i.e.*, might be multifactorial), some authors have suggested factors such as the reduction of the number of prey, the stress of the larvae and the influence of social interactions such as cannibalism among others (King *et al*., 2000; Álvarez-González *et al*., 2001; Dou *et al*., 2003; Zarski *et al*., 2011). In the present study, the number of prey available for larvae was evaluated three times per day; this was adjusted according to the laboratory protocol mentioned in materials and methods; maintaining the food densities according to the volume of the larval tanks.

Additionally, some authors have suggested that poorer physicochemical characteristics of water of the higher stocking densities are responsible for the poorer performance of the higher stocking densities (King *et al*., 2000; Dou *et al*., 2003; Sánchez *et al*., 2010). Likewise, in the present trial, water quality parameters were maintained at optimal levels for this species by making the appropriate water changes every day. Based on these results, a likely factor to consider is the stress caused to the larvae on the high stocking densities due to social interactions and cannibalism. Although we did not directly observe any cannibalism, the higher CV of the larvae stocked at higher densities is indicative of a possible "adverse" social interaction at higher densities. Some authors have observed a direct relationship between stocking density and CV for length (Lambert & Dutil, 2001; Merino *et al*., 2007). The increase of the CV is considered an indication of the establishment of hierarchies in fish within a population (Lambert & Dutil, 2001) associated with competition for food, space and other resources (Sánchez *et al*., 2010). In this sense, the significant higher CV observed at the end of the experiment at the higher densities of the 20 larvae $L^{-1}$ (13.53 ± 0.49%) and 30 larvae $L^{-1}$ (15.49 ± 2.40%) treatment compared to the 10 larvae $L^{-1}$ (5.86 ± 1.56%), reveals the effect of the density on the heterogeneity of

the size, which coincided with aforementioned interpretations.

It is interesting to note that the differences in growth regarding the standard length of *P. adspersus* were evident as early as 10 DPH, where the highest growth was recorded for the lower stocking density (10 larvae $L^{-1}$). This observation suggests that the effect of stocking densities and the establishment of hierarchies is a relatively fast process affecting the larval population early in the culture process and should be considered when defining densities for larval culture. Similar results were observed in *P. olivaceus*, where after only 15 DPH, larvae in the lower density group grew larger compared to the high-density groups (Bolasina *et al.*, 2006). However, this effect has not been observed in all studied species of flatfish. For instance, there were no significant differences in growth in *Paralichthys lethostigma* larvae stocked up to densities of 80 larvae $L^{-1}$ at 21 DPH (Daniels *et al.*, 1996). Similar results were observed for *Paralichthys dentatus*, where the researchers found no significant differences in larvae stocked at 10, 20, 30 and 40 larvae $L^{-1}$ up to 39 DPH (King *et al.*, 2000).

Percent survival of *P. adspersus* in the present study was 42.38% at 20 DPH in the lower density (10 larvae $L^{-1}$). Survival is affected by the susceptibility of the larvae to pathogens in culture systems, stress and deterioration of water quality, affecting the welfare of organisms (King *et al*., 2000; Sánchez *et al.*, 2010). Similar results were reported for *P. dentatus* at 20 DPH, where survival of the larvae stocked at 10 larvae $L^{-1}$ was significantly higher (60%) than the density of 60 larvae $L^{-1}$ (54%) (King *et al.*, 2000). Similar results were observed in *P. olivaceus* at 35 DPH, where percent survival was 77% for the lower density (10 larvae $L^{-1}$) compared to the highest density (50 larvae $L^{-1}$), which was 25% (Bolasina *et al.*, 2006).

In conclusion, stocking density had a significant effect on growth and survival for larvae of the fine flounder *P. adspersus*. Larvae stocked at a density of 10 larvae $L^{-1}$ resulted in the highest growth and survival compared to 20 and 30 larvae $L^{-1}$. An inverse relationship was observed between stocking density and growth and survival. Based on our results and the higher CV observed in, the higher stocking densities it is likely that the social interactions and/or cannibalism at these densities could be responsible for the lower growth and survival observed. Finally, this experiment allowed us to improve the larviculture protocol of this laboratory, resulting in a 20% increase in final survival and consequently the production of juveniles then should be considered for future studies and commercial projects.

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