



## Effects of stocking-density in flow-through system on the mussel *Perna perna* larval survival

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**ABSTRACT.** This study evaluated the effects of larval densities, cultured in flow-through system, in the yield and quantity of larvae. The first experiment compared the larval yield of a flow-through system (80 larvae mL<sup>-1</sup>) and a batch system (8 larvae mL<sup>-1</sup>), which resulted in a higher yield and number of larvae in the flow-through system, after 23 days. The second experiment compared two larval densities (20 and 45 larvae mL<sup>-1</sup>) in flow-through system. The densities affected the yield after 15 and 23 days and the larvae final quantity after all tested times (8, 15, and 23 days). After 23 days of larviculture, the yield was highest for 20 larvae mL<sup>-1</sup> and the larval number for 45 larvae mL<sup>-1</sup>. The third experiment also compared two larval densities (100 and 150 larvae mL<sup>-1</sup>) in flow-through system. The larvae densities did not affect the yield and number of larvae for all tested times (8, 15, and 23 days). In general, the yield has reduced at higher larval densities. Therefore, considering the number of suitable larvae for settlement, the flow-through system was more efficient than the static system. However, in general, we observed reduced yield with high larval stocking-density in flow-through system.

**Keywords:** bivalve mollusc, yield, larviculture, density.

## Efeitos da densidade de estocagem em sistema contínuo na sobrevivência de larvas do mexilhão *Perna perna*

**RESUMO.** Este estudo avaliou os efeitos da densidade de larvas, no cultivo em sistema de fluxo contínuo, na recuperação e número de larvas. O primeiro experimento comparou o rendimento de larvas de um sistema de fluxo contínuo (80 larvas mL<sup>-1</sup>) e um sistema estático (8 larvas mL<sup>-1</sup>), resultando um rendimento e número de larvas mais elevado, após 23 dias, para o sistema de fluxo contínuo. O segundo experimento comparou duas densidades de larvas (20 e 45 larvas mL<sup>-1</sup>) em sistema de fluxo contínuo. As densidades afetaram o rendimento após 15 e 23 dias e a quantidade de larvas final nos tempos testados (8, 15 e 23 dias). Após 23 dias de larvicultura, o rendimento foi maior para 20 larvas.mL<sup>-1</sup> e o número de larvas para 45 larvas.mL<sup>-1</sup>. O terceiro experimento também comparou duas densidades larvais (100 e 150 larvas mL<sup>-1</sup>) em sistema de fluxo contínuo. As densidades de larvas não afetaram o rendimento e o número de larvas para todos os tempos testados (8, 15 e 23 dias). Assim, considerando o número de larvas capazes de assentar, o sistema de fluxo contínuo mostrou-se mais eficiente do que o sistema estático. Porém, em geral, observou-se diminuição do rendimento com alta densidade larval no sistema de fluxo contínuo.

**Palavras-chave:** moluscos bivalves, rendimento, larvicultura, densidade.

### Introduction

The development of techniques for mussel larval production, in laboratory, increases knowledge about the species, promotes the expansion of mussels farming and contributes to stability in seeds supply. Mussels seeds produced in hatchery can achieve greater survival rates and faster growth than that found in nature (NAIR; APPUKUTTAN, 2003). Furthermore, the study of native species reproduction for commercial interests could contribute to the preservation and maintenance of natural stocks and gene pool.

For bivalve mollusc seed production in laboratory, the use of closed recirculating system is not common, the most used methods in intensive culture are the batch (static) or flow-through (open) systems (CHRISTOPHERSEN et al., 2006).

Batch system uses large volumes of seawater, low larval densities and water exchange every 24 or 48h. This system is the method more commonly used in bivalve mollusc larviculture, such as for oyster, (ROBERT; GERARD, 1999) and scallop hatcheries (BOURNE et al., 1989; MILLICAN, 1997; WIDMAN JR. et al., 2001; TORKILDSEN;

MAGNESEN, 2004). The batch system is labor-intensive and time-consuming for technicians and laboratory staff, so it is necessary to develop efficient techniques for larval production.

In contrast, the flow-through system studied by Magnesen et al. (2006), Rico-Villa et al. (2009) and Ragg et al. (2010) can use small volumes and high larval densities. This system consists of constant exchange of water and food in larviculture tanks. The flow-through system is intended to maximize the production of seeds and reduce the daily handling. The development of technical systems for flow-through production of bivalve larvae is a strategy that reduces considerably the impact of high-intensity labor required by the batch system (SOUTHGATE; ITO, 1998; MAGNESEN et al., 2006). The smaller size of the flow-through system reduces the resources needed for the hatchery and minimizes the effort required to maintain the same (SOUTHGATE; ITO, 1998; SARKIS et al., 2006).

Robert and Gerard (1999) report that to improve techniques of mollusc larviculture, it is important to develop systems with a continuous supply of food in the cultivation of larvae and post larvae, as this is one of the most promising methods. Sarkis et al. (2006) emphasize the efficiency of the flow-through system for the scallop *Argopecten gibbus* larviculture, highlighting the profitability of this system over the batch system.

In flow-through systems, larvae can receive a constant and optimal amount of algae, which does not occur in a batch system where there is higher mortality of microalgae, thus favoring bacteria growth in larviculture tanks (ANDERSEN et al., 2000; TORKILDSEN; MAGNESEN, 2004; MAGNESEN et al., 2006).

Aiming to contribute to larval mussel production, this study evaluated the yield and number of seeds cultured in flow-through system testing different larval densities.

## Material and methods

### Location, broodstock and D-larval rearing

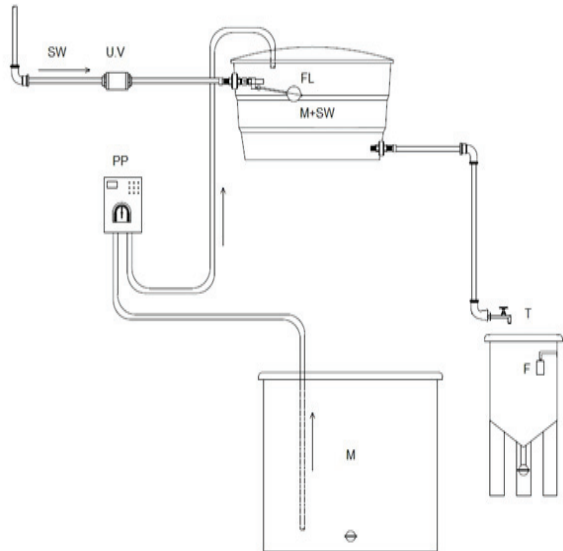
The larvae used in the experiments were obtained from mussel broodstocks cultured in the experimental area of the Laboratory of Marine Molluscs (LMM), located at Sambaqui beach, Florianópolis, Santa Catarina State, Brazil (27°35'S and 48°32'W). Mature broodstocks were cleaned to remove fouling and then transferred to plastic boxes without water to the hatchery of the LMM, located at Barra da Lagoa (23°37'S and 48°27'W).

In laboratory, 300 animals were allowed to spawn in tanks (200 L) containing seawater treated with UV. To stimulate the gametes release, seawater temperature was gradually increased (2°C at each 20 min.) from 22 to 28°C. Between each temperature increase, animals were exposed to air for 10 min. When the animals started to spawn, males and females were separated and transferred to 5 L containers to continue releasing gametes. After spawn, female gametes were screened (65 µm) to remove impurities and retained on a 18 µm mesh and the volume adjusted to 20 L. For the fertilization, male gametes were added four times (once every 20 minutes) to the 20 L of female gametes in a proportion of 10:1 (male: female). After fertilization, larvae were kept for 24 hours in a tank (15,000 L) with seawater until D-larval phase. Larvae quantification was performed in a Sedgewick-Rafter counting chamber under light microscope.

### Flow-through system for larviculture

The flow-through system consisted of six cylinder-conical tanks (250 L), called larviculture tanks, a tank (850 L) for concentrated microalgae storage, called storage tank, a tank (200 L) for microalgae dilution, called dilution tank, a UV and a peristaltic pump (Figure 1). Each larviculture tank had a central bottom drain, aeration system near to the drain and a banjo (PVC section of 5 cm with meshes on both sides) with mesh size of 35, 55 or 80 µm (banjo mesh sizes was adjusted according to larval size). To maintain the same diluted microalgae level in the dilution tank, we installed a level valve for the inflow of sterilized seawater and a peristaltic pump (Provitec AWG 9000L) for microalgae inflow (0.55 L min.<sup>-1</sup>) from the storage tank. The larviculture tank was fed by gravity with a flow of 1.73 L min.<sup>-1</sup> (daily exchange rate of 10 times); seawater temperature and salinity was maintained at 24°C and 35, respectively. The dead larvae and those exhibiting slow growth were removed every 72h, using nylon mesh screens.

In the flow-through system, microalgae in the storage tank were replenished daily to desired concentration. Banjos were cleaned daily, and every 72h all components of the flow-through system were cleaned with lime solution. The lime juice was prepared according to Carvalho et al. (2013); briefly, two Tahiti lime (*Citrus aurantifolia*) were ground in a blender with 1 L freshwater [lime (n): water (L)– 2:1], and diluted in 3 L freshwater to be ready to use.



**Figure 1.** Flow-through system design. SW = Seawater, UV = ultraviolet filter, FL = float; PP = peristaltic pump, M = Microalgae; M + SW = Microalgae + Seawater, T = cylinder-conical larviculture tank, F = Banjo.

### Batch system for larviculture

The batch system used the same larviculture tanks of the flow-through system, but without the banjo. The seawater temperature and salinity in the tanks was maintained at 24°C and 35, respectively. Every 24 hours, seawater was completely exchanged, all components of the system cleaned with lime juice (2:1). The dead larvae and those exhibiting slow growth were removed every 72 hours, using nylon mesh screens. After the seawater exchange, microalgae were added to the desired concentration.

### Larval feeding and sampling

For both systems, flow-through and batch, we used a diet composed of the microalgae *Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros muelleri*, in the proportions of 1:1:2, respectively. The final microalgae diet concentration in larviculture tanks on the first experimental day was  $0.5 \times 10^4$  cells  $\text{mL}^{-1}$  (T0) increasing to  $10 \times 10^4$  cells  $\text{mL}^{-1}$  on the last day (T23).

The larviculture experimental period was fixed to 23 days. For each sampling (8 days: T8; 15 days: T15; and 23 days: T23), we collected 3 samples from each larviculture tank for analysis of yield and number of larvae. For larvae sampling at T8, we used a net with 70  $\mu\text{m}$  mesh for the experiment 2 and with 65  $\mu\text{m}$  mesh, for the experiment 3; at T15, 120  $\mu\text{m}$  mesh for both experiments (2 and 3); and at T23, 210  $\mu\text{m}$  mesh for all experiments (1, 2, and 3).

The larvae yield was calculated, as follow:

$$\text{Larval yield} = \frac{\text{total number of larvae}}{\text{number of larvae larger than } 210 \mu\text{m}}$$

### Experiments

All experiments were conducted in a completely randomized design with two treatments and three replications.

Experiment 1 - The experiment 1 evaluated the effect of flow-through and batch system on larval survival and yield after 23 days. The flow-through system used a larval density of 80 larvae  $\text{mL}^{-1}$  and the batch system, 8 larvae  $\text{mL}^{-1}$ .

Experiment 2 - The experiment 2, performed in a flow-through system, evaluated the effects of two larviculture densities (20 larvae  $\text{mL}^{-1}$ , treatment called D20 and 45 larvae  $\text{mL}^{-1}$ , called D45) on larval survival and yield.

Experiment 3 - The experiment 3, also performed in a flow-through system, evaluated the effects of two larviculture densities (100 larvae  $\text{mL}^{-1}$ , treatment called D100 and 150 larvae  $\text{mL}^{-1}$ , called D150) on larval survival and yield.

### Statistical analysis

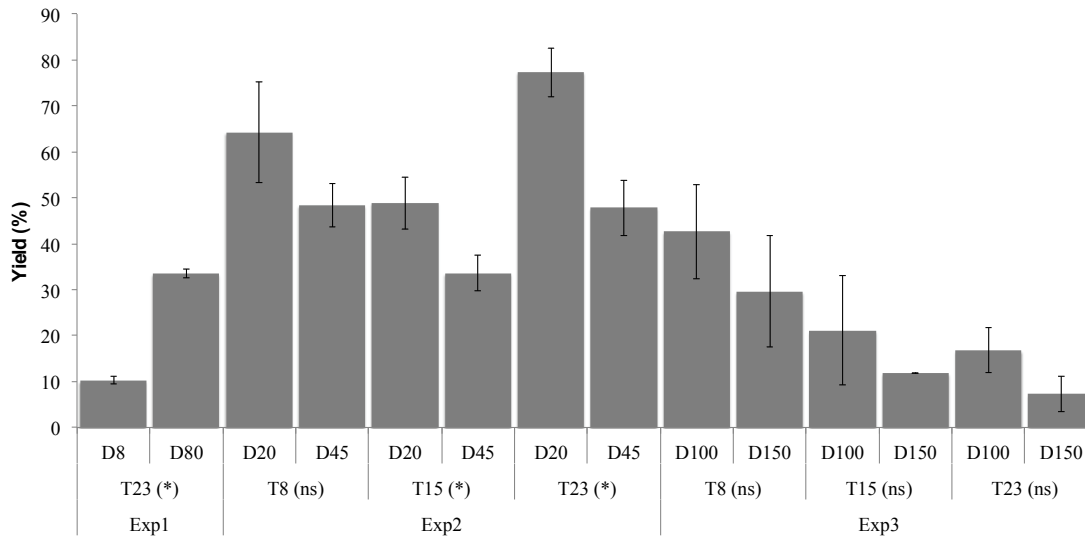
The comparison between densities (yields and total larvae number) in each experiment was performed by one-way analysis of variance (ANOVA) in the sampling times T23 (experiment 1) and T8, T15, and T23 (experiments 2 and 3) using the SAS® program (SAS, 2003). The significant level was determined with F values.

### Results

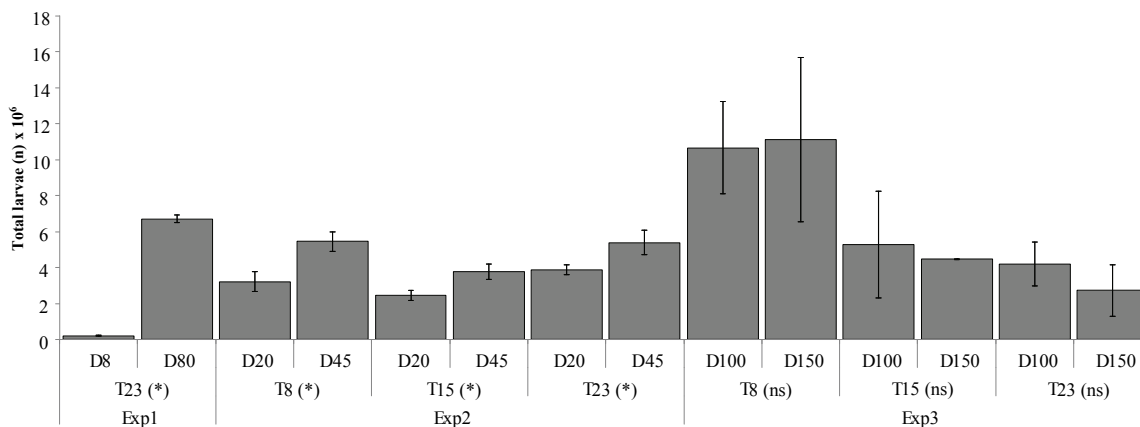
In the experiment 1, a significant difference ( $p < 0.05$ ) was detected between culture systems for yield, with the highest yield (Figure 2) for the flow-through system ( $33.5 \pm 1.03\%$ ). Also, the larval number at T23 was significantly ( $p < 0.05$ ) higher for the flow through system (Figure 3).

In the experiment 2, we observed a significant difference ( $p < 0.05$ ) between culture densities for yield. The density D20 was significantly ( $p < 0.05$ ) higher at T15 ( $48.89 \pm 5.67\%$ ) and T23 ( $77.33 \pm 1.03\%$ ) (Figure 2). However, when evaluated the number of larvae, the density D45 was significantly ( $p < 0.05$ ) higher at T8 ( $5.44 \pm 0.54$  million), T15 ( $3.77 \pm 0.43$  million) and T23 ( $5.37 \pm 0.68$  million) (Figure 3).

In experiment 3, no difference was found between the densities for yield (Figure 2) at all times sampling (T8, T15, and T23), as well as the number larvae was not affected by the densities.



**Figure 2.** Mean and standard deviation of larval yield (%) in the experiment 1 (treatment D80) at 23 days (T23) of larviculture, experiments 2 (treatments D20 and D45) and 3 (D100 and D150) at 8 (T8), 15 (T15) and 23 (T23) days of larviculture, all three experiments in a flow-through system. The densities were analyzed by ANOVA in each sampling time. Where: (\*) =  $p < 0.05$ ; (ns) = non-significant.



**Figure 3.** Mean and standard deviation of total number of larvae in the experiment 1 (treatment D80) at 23 days (T23) of larviculture, experiments 2 (treatments D20 and D45) and 3 (D100 and D150) at 8 (T8), 15 (T15) and 23 (T23) days of larviculture, all three experiments in a flow-through system. The densities were analyzed by ANOVA in each sampling time. Where: (\*) =  $p < 0.05$ ; (ns) = non-significant.

## Discussion

Results obtained by Torkildsen and Magnesen (2004) and by Magnesen et al. (2006) with flow-through system principle indicated that flow-through cultures can replace batch cultures. A great advantage of the flow-through system is that it requires less handling than batch systems. Therefore, the costs of larval production can be reduced, decreasing man-hours required by larviculture (MAGNESEN et al., 2006). Southgate and Ito (1998) reported that the partial flow system has simplified the cultivation of *P. margaritifera* larvae, thus reducing the labor demands. The handling of the larvae was reduced,

and the improvement in water quality was obtained due to the higher rate of water exchange. Also, the flow-through culture system can generate a stable environment with a continuous addition of microalgae. Magnesen et al. (2006) reported in their study that a smaller quantity of algae was used for larval rearing in flow-through culture.

However, several factors need to be improved in the flow-through system, including flow regulation. The flow may change during the day due to clogging by the accumulation of microalgae. Additional care must be taken with the filter in this system, which must be properly cleaned to prevent clogging and

carefully examined not to contain holes that would allow the escape of larvae.

Stocking-density of bivalve larvae is an important factor for larviculture success and has been reported for different bivalve species under hatchery condition.

For the cockle *Clinocardium nuttalli*, Liu et al. (2010) observed that lower densities (2 and 4 larvae mL<sup>-1</sup>) with an algae density of 5.0 x 10<sup>4</sup> cells mL<sup>-1</sup> shown to be optimal conditions for the survival of larvae of this species. Nevertheless, for the clam *Meretrix meretrix*, Liu et al. (2006) observed that the tested stocking density did not influence the larval survival rate. Magnesen et al. (2006) found a decline in larval survival for the scallop *Pecten maximus* at densities greater than 6 larvae mL<sup>-1</sup>. For another pectinidae, *Argopecten gibbus*, Sarkis et al. (2006) observed no difference in survival at high density, 8 - 24 larvae mL<sup>-1</sup>. For the Pacific oyster, Rico-Villa et al. (2008) showed no significant difference in growth and survival when reared at higher densities (50 and 100 larvae mL<sup>-1</sup>), and also, survival was lower when larvae were reared at 5 larvae mL<sup>-1</sup>. These authors (RICO-VILLA et al., 2008) also showed that the density of 20 larvae mL<sup>-1</sup> resulted in 94% survival while the density of 50 larvae mL<sup>-1</sup> were 38% after 11 days of culture. Subhash and Lipton (2010) tested four stocking densities (100, 1000, 2000, and 3000 larvae mL<sup>-1</sup>) of *Pinctata fucata* and observed higher survival at low densities (100 and 1000 larvae mL<sup>-1</sup>).

In the present study, the density D20, after 23 days of larviculture, showed the highest yield in flow-through system. The low yield observed at higher densities (D100 and D150) can be related to the limited water volume, which can affect the feeding behavior of animals, as also do metabolic waste and food availability. The most important metabolic waste from molluscs is ammonia, ammonium and nitrite. These compounds can affect bivalve larvae in different ways, for example reducing the pH of seawater. Meantime, additional studies need to be developed to identify the effects of different densities on these factors.

Magnesen et al. (2006) pointed a negative correlation between survival and algal concentration, indicating that food concentration can be a major factor for the success of flow-through systems in hatcheries. The combination of high larval density, high water exchange and a feeding regime requires a detailed understanding of larval dietary requirements (RAGG et al., 2010).

Although the lower yield observed in the present study for D45 and D80, the final larval number

(after 23 days) was higher, which can be an important aspect for commercial hatcheries. High number of larvae per volume can considerably improve the overall system production in a commercial hatchery, reducing water usage and, consequently, heating and water treatment costs, among others. In our study, the rate of return ranged from 7.25% at high density (150 larvae mL<sup>-1</sup>) to 77.33% at low density (20 larvae mL<sup>-1</sup>). However, the larvae number suitable for settlement at the density of 80 larvae mL<sup>-1</sup> led to an increased production of larvae (6.7 million). Considering that the tested flow-through system had six cultivation tanks, with these densities, it could be produced more than 30 million larvae suitable for settlement at the end of each larval rearing. In this sense, the flow-through system is more efficient compared with the batch system.

Considering the variation in survival rates between larvicultures, different factors can interfere with larval growth. In this sense, more studies should be carried out with flow-through system. Different cultivation densities, diets, flow rates, aeration intensity, light and color of tanks should all be tested.

## Conclusion

In conclusion, the yield of the mussel *Perna perna* larvae has decreased at higher stocking-densities. Therefore, when considered the number of larvae suitable for settlement, the flow-through system, at densities of 45 and 80 larvae mL<sup>-1</sup>, is more efficient for commercial production. It should be noted that the culture density at 80 larvae mL<sup>-1</sup> in a volume of 250 L allows the production of 6.7 million larvae for settlement.

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