Effect of food strategy and stocking density on larval performance of captively reared *Mytilus galloprovincialis*

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Abstract. Food availability is a crucial factor influencing the behavioral responses, development and physiology of bivalve's larvae. In this study, we investigated the effects of two feeding strategies (F_v: number of microalgae cells per volume and F_b: number of microalgae cells per biomass) on Mytilus galloprovincialis larvae reared at three different stocking densities (D_5: 5 larvae/ml, D_{15}: 15 larvae/ml and D_{30}: 30 larvae/ml). The results showed that larvae fed per volume (F_v) exhibited the highest survival rates across all tested densities (59%, 53% and 39% for D_5, $D_{15}$and D_{30} respectively), compared to larvae fed per biomass (F_b) (40%, 39% and 32% for D_{15}, D_{30} and D_5 respectively). Conversely, feeding per biomass led to a significant increase in cumulative shell length gain (D_5: 211.4 µm; D_{15}: 214.99 µm and D_{30}: 208.11µm) compared to feeding per volume (D_5: 201.12 µm; D_{15}: 166.23 µm and D_{30}:130.09 µm). Statistical analysis revealed that food availability significantly influenced survival only at low larvae density (D_5: $F=20.13$; $P<0.01$). However, it significantly affected cumulative gain only at high stocking densities D_{15} ($F=148.96$; $P<0.001$) and D_{30} ($F=318.74$; $P<0.001$). These findings emphasize the importance of feeding strategy and stocking density as crucial factors in regulating larval performance during captivity rearing.

1. Introduction

In recent years, there has been a growing recognition of restorative aquaculture due to its major environmental, biological, and social contributions [1]. Environmentally, this form of aquaculture provides different benefits, for instance, water quality improvement [2], habitat risks mitigation [3] as well as climate regulation [4]. On the other hand, one of the known restorative practices, is the implementation of shellfish and algal facilities. Bivalve hatcheries have become a widely adopted strategy, serving as an alternative to relying solely on natural spatfalls for the recovery of wild populations [5]. The overexploitation of natural habitats has disrupted ecosystem diversity and dynamics, leading to the depletion of environmental resources and the degradation of natural landscapes [6, 7, 8]. Bivalve husbandry shows great potential in promoting a sustainable shellfish supply, as evidenced by the presence of hatchery-originated juveniles in restorative sites, shellfish reef restoration, and the positive impact of larvae produced in hatcheries on wild settlement [1, 9, 10]. Therefore, improving hatchery practices will not only enhance stock production but also aim towards achieving sustainable outcomes in natural ecosystems.

The success of mass production in captivity largely depends on the quality of larval rearing, since an optimized performance of the latter will consequently increase the number of the produced batches and therefore, maximize the utility of hatchery facilities. [11]. This phase of production is influenced by several external factors, including temperature for instance, it was demonstrated that filtration activity of suspension-feeding bivalves is directly controlled by temperature fluctuations [12]. As well as, for salinity, stocking density, and diet, they are considered of a significance importance that influences physiological and behavioral responses of the reared species [13, 14, 15]. Consequently, optimizing larval nutrition and feeding behavior is crucial during this critical life stage, as food availability and capture directly impact larval development [16]. Microalgae are the primary source of nutriment in the aquatic food chain [17]. Their nutritional profile, highly rich in essential fatty acids [18] emphasized their production and usage in both commercial and experimental fish or shellfish hatcheries. On the other hand, for bivalves' microalgae is considered a key supplement that enhances larval and post-larval growth and performance [19]. For instance, it was shown that *M. galloprovincialis* larvae performance was greater when fed a trispecific microalgal diet due to its richness with high levels of polysaturated fatty acids (PUFA) [15]. As well, oysters' growth was directly related to carbohydrate and PUFA levels within microalgae [20]. The success of hatchery productivity became directly related to the composition richness and availability of food during the rearing process.

Furthermore, the feeding behavior of veliger larvae is a continuous process consisting of four distinct phases: generation of a flow field, particle capture, particle retention, and ingestion [21]. There is also evidence of a link between the functional behavior of molluscs' larvae and the dispersal of microalgae cells [22]. The capture efficiency of larvae is proportional to moderate levels of food concentration, beyond which the rate of capture becomes limited [23]. Previous studies have shown that high food concentrations can result in poor larval growth because the energy required to reject excess cells becomes a limiting factor that decreases the ingestion rate [24, 25]. Thus, the aim of this study is to investigate the effect of spatial and temporal food availability on larval performance in terms of viability and quality.

2. Materials and methods

2.1. Spawning and fertilization

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Mytilus galloprovincialis broodstock (74.71 mm ± 5.23) was collected from suspended ropes on longlines systems in AMSA Bay 35°31'59.5"N 5°13'29.0"W, Tétouan, Morocco, on May 2022. Prior to spawning induction, the broodstock was air dried for 1 hour following the protocol of bivalves' production in hatchery [14]. Thermal stimulation was then performed, mussel broods were placed in fiberglass tanks and put alternatively in cold temperature (14 ± 1.5 °C) for 30 min, then hot temperature (26 ± 1 °C) for 1 hour. This process was repeated for three cycles until the release of gametes. The spawned oocytes and spermatozoids were gently separated and sieved through 20 µm nylon mesh sieve. After the examination of the quality and quantity of eggs batches, fertilization took place using a ratio of 120 sperm per oocyte.

2.2. Larval culture

Larvae were reared in 20-liter polycarbonate tanks using 1 µm filtered and UV-treated seawater at a temperature of 21°C, for 16 days. Every 48 hours until the end of the experiment, larvae were sieved to allow for water exchange and samples were taken for performance (survival and growth) evaluation. To assess larvae growth, more than 15 larvae were randomly sampled for length and width measurements using profile projector. Then, growth rate was estimated using the following formula: $\mathbf{G_r = (M1 - M0) / Nd}$ with:

G_r: Growth rate

M0: Initial measure (length and width) of larvae

M1: Final measure (length and width) of larvae

Nd: Number of days

As for the viability, larvae were counted in three aliquots (25 µl) that were randomly taken of the sieved larvae culture. Then, survival rate was estimated using the following formula: $\mathbf{SR = (N0 - N1)/N0}$ with:

SR: survival rate

N0: Initial number of larvae

N1: Final number of larvae

2.3. Larval density and feeding

For each feeding strategy (feeding/volume and feeding/biomass), three rearing densities (D5: 5 larvae/mL; D15: 15 larvae/mL; and D30: 30 larvae/mL) were implemented during larval rearing in triplicates. Based on previous studies on mussel larvae, 5 to 10 larvae/mL is a commonly used as an optimum rearing density. Hence through this study we aimed to study larval performance at low density (D5), as well as at high densities (D15 and D30). The same microalgae diet, consisting of a combination of three strains *(Isochrysis galbana, Tetraselmis suecica,* and *Chaetoceros calcitrans),* was provided to the larvae in each treatment. The microalgae cultures were produced and maintained at phytoplankton units (100 Liters) in AMSA hatchery. Where the concentrations vary from 2 to 7 million cells/ml for *I. galbana* and *C. calcitrans*, as for *T. suecica*, it differs from 2 to 3 million cells/ml. In the feeding/volume strategy, microalgae cells were provided to the larvae based on the volume of seawater (cells/µl), while in the feeding/biomass strategy, microalgae cells were given per larvae (cells/larvae) (Table 1). Correspondingly, for the two treatments diet was provided to larvae regularly once a day.

Table 1. Number of microalgae cells provided to larvae in each of the two feeding strategies (Feeding/volume: cell/µl) and (Feeding/biomass: cell/larvae).

Days of rearing	Feeding/volume (cell/µl/day)	Feeding/biomass (cell/larvae/day)
1	20	1000
3	40	1500
5	55	2500
7	100	4000
9	100	4500
12	100	5000
16	100	5000

2.4. Statistical analysis

To determine the effect of the feeding strategy and stocking density on larval performance, a one-way ANOVA (Fisher test) was conducted at a significance level of $P \leq 0.05$, corrected with the Welch test. The selected test was used to analyze differences in growth and survival data between the three tested densities, as well as among the two feeding strategies. All statistical analyses were performed using R software with the Rcmdr package, version 2.7.2, and RStudio version 2022.12.0+353 [26].

3. Results

3.1. Effect of food availability on larval survival

The impact of food availability on larval survival at different rearing densities is depicted in Figure 1. The results indicate that larval survival is influenced by food availability across all stocking densities. This effect is particularly pronounced at low density and becomes less important when increasing stocking density of larvae. Among the tested densities (D5, D15, and D30), higher survival rates were observed for larvae fed based on volume (cells/µl) compared to that fed based on biomass (cells/larvae). At the conclusion of the experiment, larvae reared at the low density (D5) and fed per volume exhibited a higher survival rate (0.59 ± 0.10) than those fed per biomass (0.32 ± 0.02). Conversely, the impact of food availability on larval survival was less pronounced at the medium density (D15) (0.53 ± 0.18 and 0.41 ± 0.05 for feeding per volume and feeding per biomass, respectively). Furthermore, no effect of food availability on larval performance was detected at high density (D30) (0.39 ± 0.04 and 0.39 ± 0.01 for both feeding protocols, respectively).

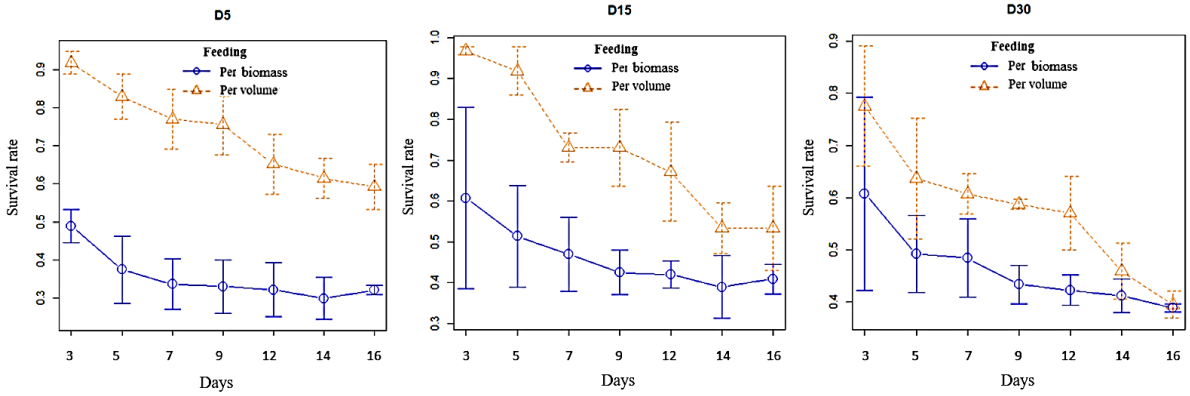

Fig. 1. Evolution of *M. galloprovincialis* larvae survival in response to feeding strategy (feeding/volume and feeding/biomass) at different rearing densities (D5: 5 larvae.ml^{-1}; D_{15}: 15 larvae.ml^{-1} and D_{30}: 30 larvae.ml^{-1})

Statistical analysis indicated that food availability significantly affected larval performance only at low density (D5: F = 20.134, P < 0.05) (Table 2). However, no significant difference in survival associated with food availability was observed when the rearing density was increased (D15: F = 1.31, P = 0.35; D30: F = 0.04, P = 0.84).

Table 2. Statistical analysis (ANOVA) of the effect of food availability on larvae survival reared at different densities, F= Fisher test; df=degree of freedom, * significant at $p < 0.05$

Stocking density	Feeding strategy	Mean ± Sd	Df	*F*	*p*
D_5	cell/µl	0.592 ± 0.102	1	20.134	0.039*
	cell/larvae	0.320 ± 0.017			
D_{15}	cell/µl	0.533 ± 0.177	1	1.310	0.351
	cell/larvae	0.408 ± 0.051			
D_{30}	cell/µl	0.395 ± 0.045	1	0.046	0.846
	cell/larvae	0.389 ± 0.013			

3.2. Effect of food availability on larval growth

Contrary to survival, larval growth (cumulative gain in shell length) was more influenced by food availability when rearing density increased (Figure 2). During early larval rearing, there was no difference in growth between larvae with respect to feeding process. However, during late larval rearing, larvae fed based on biomass showed better growth than those fed based on volume, especially at high densities D15 and D30. No difference in growth was observed between feeding strategies at low rearing density. At the end of the experiment, larvae fed based on biomass had a final gain in shell length of 211.4 µm ± 7.30, 214.99 µm ± 5.81, and 208.11 µm ± 7.19, which was greater than larvae fed based on volume, with measurements of 201.12 µm ± 1.52, 166.23 µm ± 3.75, and 130.09 µm ± 2.35 (for D5, D15, and D30 respectively).

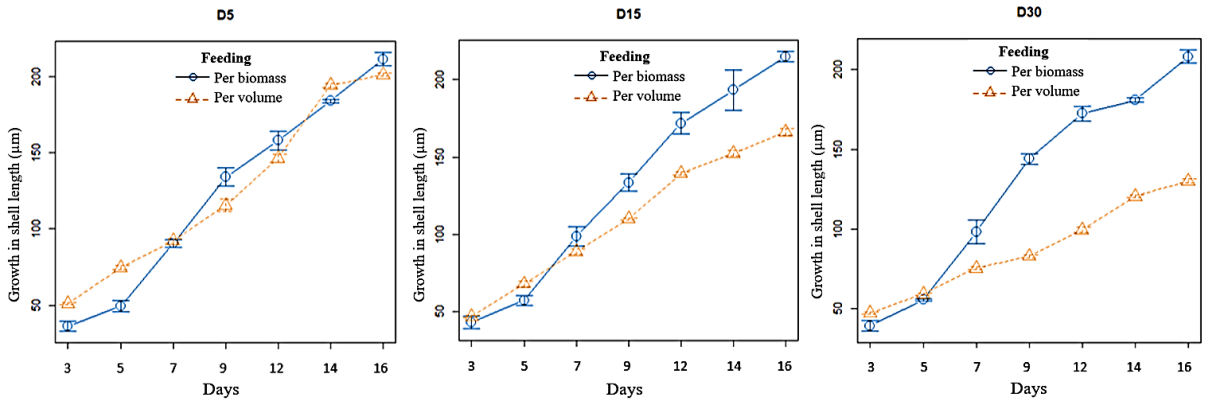

Fig. 2. Evolution of *M. galloprovincialis* larvae growth in response to feeding strategy at different rearing densities (D_5: 5 larvae.ml^{-1} D_{15}: 15 larvae.ml^{-1} and D_{30}: 30 larvae.ml^{-1})

In Table 3, statistical results revealed that the effect of food availability on larval growth was highly significant for medium and high densities (F= 148.96; P <0.001 and F= 318.74; P< 0.001) while no significance was revealed for larvae reared at the low density (F= 5.736; P=0.128). It is noteworthy, that the standard deviation in the three densities of larvae fed per volume, were lower than of larvae fed per biomass, due to the degree of heterogeneity of the batches.

Table 3. Statistical analysis (ANOVA) of the effect of food availability on larvae growth reared at different densities, *F*= Fisher test; df=degree of freedom, *** significant at $p < 0.001$

Density	Feeding strategy	Mean ± Sd	Df	*F*	*p*
D_5	cell/µl	201.12 ± 1.52	1	5.73	0.128
	cell/larvae	211.43 ± 7.30			
D_{15}	cell/µl	166.23 ± 3.75	1	148.96	0.00061***
	cell/larvae	214.99 ± 5.81			
D_{30}	cell/µl	130.09 ± 2.35	1	318.74	0.00124***
	cell/larvae	208.11 ± 7.19			

4. Discussion

Suspension-feeding of bivalves during larval stages has been extensively studied due to its significant impact on the success of larval culture in hatcheries [27, 28, 29, 30]. However, previous research in this field has primarily focused on the composition of the feed (quality of microalgae) [31, 32, 15] rather than the feeding pattern. In this study, we aimed to investigate the effect of food availability on larvae survival and growth. Our findings clearly demonstrate that feeding larvae per volume (cell/µl) ensures high food availability. For example, (at the low density) during the early larvae phase (day 1 to 6), larvae were provided with a daily intake of 10000 cells/larvae (1.13 µg/larvae). In the late larvae phase (day 7 to 16), the feeding rate increased to 20000 cells/larvae (3.1 µg/larvae). Conversely, when feeding per biomass, the number of microalgae cells was constrained by the existing larvae population, resulting in lower food availability. Consequently, during the early larvae phase, larvae were fed with 1900 cells/larvae (0.23 µg/larvae), while in the late larvae phase, they only received 4625 cells/larvae (0.75 µg/larvae).

Additionally, to the effect of food availability, stocking density is a crucial factor that controls larval development. As it was shown that at high larval densities, competition for food becomes a limiting factor for larval performance [23]. Therefore, in this study, the adapted feeding strategies were applied at low (5 larvae/ml), medium (15 larvae/ml), and high (30 larvae/ml) larval densities. At low density, the survival rate of larvae fed per biomass was significantly lower (32%) than those fed by volume (59%). This result can be explained by the fact that at low rearing density, when feeding per biomass -low algal availability- , larvae tend to expend more energy to search for food. This larvae behavior, requires a level of energy that can be backed up at early stages (mixotrophic phase) due to the reserved energy [15]. However, at latter stages (exotrophic phase), were larvae viability and development rely mainly on external fueling, the low food availability coupled with the intensive larval activity may cause the decrease in their development. In contrast, at medium and high densities, no significant difference was observed between the two feeding strategies in terms of survival. However, for both densities (D15 and D30) larval growth was significantly greater for larvae fed per biomass, with a shell length of 214.99 µm and 208.10 µm after 16 days of rearing at D15 and D30 densities, respectively. On the other hand, feeding per volume resulted in a growth of 166.23 µm and 130.09 µm for larvae reared at D15 and D30 densities, respectively. The dispersion rate of microalgae may be one of the reasons for the observed deviations in growth. When feeding per volume, the spatial dispersion of feeding particles increased during larvae development but remained constant regardless of the studied densities. However, when feeding per biomass, the spatial dispersion also increased during larvae development, taking into account the rearing densities. The spatial dispersion of microalgal cells affects the feeding

behavior of larvae [33]. At low food concentrations, the encounter probability of feeding particles by larvae is low, as these particles exist outside the capture zone of larvae. Consequently, the larvae devote more energy to reach the particles within their capture zone. Conversely, at high food concentrations, larvae are forced to actively reject excessive food particles, which requires high energy expenditure and directly affects ingestion rates and larval growth [23, 34].

The excellent performance of larvae at high densities when fed per biomass encourages the adoption of this feeding strategy. However, it is essential to consider the expected consequences of using high larval densities, such as deterioration of water quality due to fecal matter and metabolic waste accumulation, as well as decreased ingestion rates, oxygen consumption, and growth efficiency [34, 35, 36, 37]. Numerous studies have demonstrated the importance of stocking density, favoring lower densities for optimal larval performance [33, 39, 40]. On the other hand, many studies on bivalve larval performance have adopted the strategy of feeding per volume, which has shown optimized results [31, 15, 41]. However, no studies were conducted with the aim of comparing the different feeding strategies used in hatchery production. Hence, comes the originality of the current study that highlights the significance of optimizing both feeding strategy and stocking density for enhanced larval culture. Additionally, further research is warranted to explore the importance of feeding strategy and its effect on physiological pattern, nutritional behavior, biochemical composition of bivalves' larvae to refine the understanding in this field.

5. Conclusion

In conclusion, this study has demonstrated that the efficiency of the adopted feeding strategy has a significant impact on larval performances, including survival and growth, as well as the mass production of seed in hatcheries. The findings highlight the importance of considering feeding particles dispersal for both production capacity and yield quality of the produced spat, particularly in terms of absorption efficiency. Furthermore, the observed differences in larval survival and growth between the tested strategies emphasize the need for additional studies on the effect of algal densities on larval culture. Additionally, it is crucial to assess the correlation between stocking density and food availability in order to understand the physiological and behavioral responses of bivalve larvae.

To achieve optimized mussel seed production in captivity and ultimately contribute to mussel stock recovery in natural habitats, further investigations are warranted. Future studies should focus on specific areas such as determining optimal algal densities, evaluating the relationship between stocking density and food availability, and exploring other factors that may impact larval culture success. By addressing these research gaps, we can enhance our understanding and improve the outcomes of mussel seed production and restoration efforts.

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