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Influence of feeding regime and temperature on development and settlement of oyster *Ostrea edulis* (Linnaeus, 1758) larvae

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Abstract :

Under controlled conditions of food density and temperature, larval performances (ingestion, growth, survival and settlement success) of the flat oyster, *Ostrea edulis*, were investigated using a flow-through rearing system. In the first experiment, oyster larvae were reared at five different phytoplankton densities (70, 500, 1500, 2500 and 3500 μm^3 μL^{-1}: ≈1, 8, 25, 42 and 58 cells μL^{-1} equivalent TCg), and in the second, larvae were grown at four different temperatures (15, 20, 25 and 30°C). Overall, larvae survived a wide range of food density and temperature, with high survival recorded at the end of the experiments. Microalgae concentration and temperature both impacted significantly larval development and settlement success. A mixed diet of *Chaetoceros neogracile* and *Tisochrysis lutea* (1:1 cell volume) maintained throughout the whole larval life at a concentration of 1500 μm^3 μL^{-1} allowed the best larval development of *O. edulis* at 25°C with high survival (98%), good growth (16 μm day^{-1}) and high settlement success (68%). In addition, optimum larval development (survival ≥97%; growth ≥17 μm day^{-1}) and settlement (≥78%) were achieved at 25 and 30°C, at microalgae concentrations of 1500 μm^3 μL^{-1}. In contrast, temperature of 20°C led to lower development (≤10 μm day^{-1}) and weaker settlement (≤27%), whereas at 15°C, no settlement occurred. The design experiments allowed the estimation of the maximum surface-area-specific ingestion rate $\{\dot{j}_{Xm}\}$ = 120 ± 4 μm^3 day^{-1} μm^{-2}, the half saturation coefficient $\{X_K\}$ = 537 ± 142 μm^3 μL^{-1} and the Arrhenius temperature T_A = 8355 K. This contribution put a tangible basis for a future *O. edulis* Dynamic Energy Budget (DEB) larval growth model.

1. Introduction

The European flat or "Native" oyster, *Ostrea edulis*, propagates along European shores, from the coasts of Norway to Morocco, including the Mediterranean Sea (Harry, 1985; Shpigel, 1989). At the end of the 1970s, two successive outbreaks associated with the parasites *Marteilia refringens* and *Bonamia ostreae* dramatically impacted *O. edulis* populations. French production (mainly located in Brittany) dropped from 24000 t in the late 1960s to present levels of 1000-1500 t (Buestel et al., 2009; Robert et al., 2013). The extension of such disease across Europe led to the collapse of this industry at the European level (Baud et al., 1997 and references therein; da Silva et al., 2005 and references therein) with most of the works since devoted to a better knowledge of both illness in natural surroundings (e.g. : Berthe et al., 2004; Culloty and Mulcahy 1996; Flanery et al, 2014; Montes et al., 1991; van Banning 1991) or under controlled conditions (Arzul et al., 2009; da Silva et Villalba, 2004; Lallias et al., 2008, Prado-Alvarez et al., 2015). Different strategies have been tried to minimize the effect of these diseases, such as modified husbandry (e.g. Le Bec et al., 1991; Robert et al., 1991), introduction of exotic flat oysters (e.g. *Ostrea puelchana*: Pascual et al., 1991), genetic improvement (Montes et al., 2003; Naciri-Graven et al., 1998) or oyster bed restoration (Laing et al., 2005; Lallias et al., 2010; Sawusdee et al., 2015). The situation was similar for most countries in Europe after the disease spread and flat oyster populations have never recovered. In this context, except for some limited disease-free areas (e.g. Scotland, North of Ireland, Norway, Sweden, Denmark and Croatia), flat oyster farming consists of improving oyster growth before the fateful limit of 3 years old or equivalent size and, accordingly, *O. edulis* production in Europe is constrained. Currently, a selective breeding program provides a possibility to enhance flat oyster farming as demonstrated earlier for *Crassostrea virginica* (Ford et al., 1990) or *C. gigas* (Ward et al., 2000) and progress has been made in breeding for diseases resistance (Lynch et al., 2014) including the use of new genetic tools for improving selection (Harrang et al., 2015; Lallias et al., 2009; Martin-Gomez et al., 2012; Morga et al., 2010, 2012). Such targeted genetic improvement, however, will not be feasible until the lack of reliable methods in hatchery production for this species is overcome.

In France, the decrease of *O. edulis* production led to the introduction in 1971 of the Pacific oyster, *Crassostrea gigas* which successfully became the major species farmed in France comprising 98% of oyster production (Grizel and Héral, 1991; Robert et al., 2013). Since

2008 however, massive losses in the French shellfish industry due to summer mortalities of *C. gigas* associated to the OsHV-1 (e.g.: Pernet et al., 2012; Renault, 2011) contributed to the reconsideration of *O. edulis* as an alternative production in a context of diversification. Both conditions were filled to give back interest to *O. edulis* production under controlled conditions.

Hatchery techniques are now relatively well-known for many bivalve species such as *C. gigas* (Utting and Spencer, 1991), *Ruditapes philippinarum* (Helm and Pellizzato, 1990) or *Mercenaria mercenaria* (Castagna and Kraeuter, 1981). Although great knowledge has been acquired by pioneer works such as Walne (1965, 1966, 1979), the state of the art in hatchery culture of *O. edulis* has remained insufficient to support a reliable seed production, probably due to a lack of updated and detailed knowledge of the biology of this species. The life cycle of marine bivalves is strongly related to environmental parameters such as temperature and food availability as well as to cycles of storage and utilization of biochemical substrates (Burke et al., 2008; Fabioux et al., 2005; Rico-Villa et al., 2010). Through a European project called "SETTLE" we addressed efforts to fill this gap. Firstly, we focused on broodstock conditioning by studying the effects of food on *O. edulis* reproduction, because it is the first step to master in hatchery to allow production of larvae of good initial quality. This point is particularly crucial for *O. edulis* that is a larviparous species with high maternal effects that have been shown to affect larval growth and survival (Berntsson et al., 1997). Based on ingestion and assimilation of different microalgae species combined to fatty acid and sterol transfer from diet to gonad, we selected an appropriate diet for *O. edulis* broodstock conditioning based on a mixed diet of *Rhodomonas salina* and *Chaetoceros neogracile* (Gonzalez-Araya et al., 2011, 2012a, 2013). Thereafter, we focused on the effects of food assemblage (quality aspects) on *O. edulis* larval development that confirmed the efficiency of a bispecific diet *vs* single diet for larvae regardless to broodstock diet (Gonzalez-Araya et al., 2012 b).

The present work, which is in the continuity of the "SETTLE" project, will be addressed to the effects of temperature and food (quantity aspect) on *O. edulis* larval development. Such work relies on recent advances in the development of continuous flow-through systems for larvae that have shown to be efficient for *Pecten maximus* (Magnesen et al., 2006) or *C. gigas* (Rico-Villa et al., 2008). These improvements in rearing methods using flow-through systems can be applied to *O. edulis* culture, thus reducing the constraints involved in the generally used "static system", such as tank cleaning, frequent water renewal or stress related to larval handling (Rico-Villa et al., 2008). Indeed, unexplained flat oyster larval mortalities have often

been reported in hatchery on day 8 and at post-settlement (anonymous, 2004; Laing et al., 2005). Moreover, these optimized flow-through units consisting of relatively small volume containers (5 l), stocked at high larval densities and supplied by a constant flow of phytoplankton-enriched seawater, can allow greater replication and a better control of larval rearing parameters. Flow-through systems may help to overcome larval mortalities of *O. edulis* in hatchery, which have been often associated with bacterial contamination such as Vibrio (Jeffries, 1983; Prado et al., 2005; Elston et al., 2008).

The aim of the present study was to determine the optimal temperature and phytoplankton concentration for the hatchery culture of *Ostrea edulis* larvae, based on survival, larval growth, ingestion and metamorphosis.

2. Material and methods

2.1 Broodstock conditioning

A total of 250 four-year-old, *Bonamia* and *Marteilia*-free, flat oysters (*O. edulis*) originating from Norway, were maintained under controlled conditions, at the Ifremer Argenton hatchery from the end of the hatchery spawning period. From mid-July to December, oyster broodstock were progressively decreased from 19°C to 10°C (1°C per week) and held in shadow light, in three 700-l flow-through tanks supplied with 10-µm filtered, UV-sterilized seawater. Oysters were continuously fed *Tisochrysis lutea* (CCAP 927/14: T) plus *Chaetoceros neogracile* (UTEX LB2658: Cg) by means of a peristaltic pump. From mid-December onward, they were progressively acclimated to 19°C (1°C per week increase), with permanent light, in 1µm-cotton bag filtered seawater, UV treated, and continuously enriched with *Rhodomonas salina* (CCAP 978/24) plus *C. neogracile*. When temperature reached 19°C broodstock received a daily ration of 6% dry weight microalgae (mg) per oyster meat (g). Tanks were drained and daily cleaned, and treated once a week with a bactericide/fungicide/virucide solution by pulverization.

2.2 Larval rearing

Following 6 weeks of conditioning and onwards, expelled larvae were collected from the water surface by means of sieves placed under the outflow. At each release, larvae were counted and the number of *Vibrio sp.* per larvae was determined. The batch of larvae was kept when Vibrio load was <1. In this condition and when a sufficient number of larvae were

obtained to allow the set-up of 15 experimental conditions, larvae were distributed in 5-l translucent methacrylate cylinders in triplicate per experimental condition, and reared in flow-through with 1-µm cartridge filtered and UV-treated seawater (FSW). After degasification in a column to avoid bubble disease, FSW was maintained at different experimental temperature (see 2.3) and ambient salinity (34 PSU), at a renewal rate of 100% h^{-1}, i.e. 87 ml min^{-1}. Oyster larvae were fed a bispecific algal diet consisting of *Tisochrysis lutea* (≈ 40 μm^3 volume diameter) and *Chaetoceros neogracile* (≈ 80 μm^3) continuously maintained by peristaltic pumps at different experimental concentration (see 2.4). Food remained suspended in the water column by means of air, bubbling from the base of the cone (30 ml min^{-1}). Air was previously filtered at 0.2 µm.

2.3 Temperature experiment

2.3.1 System design

Following release from the broodstock, larvae were distributed in triplicate in 5-l translucent methacrylate cylinders at four different temperatures: 15, 20, 25 and 30°C. Larvae were held at a density of 50 larvae ml^{-1} in FSW and fed a bispecific diet (*T. lutea* plus *C. neogracile*) continuously maintained at 1500 μm^3 μl^{-1}, previously determined as the best phytoplankton concentration for *Crassostrea gigas* (Rico-Villa et al., 2009) and confirmed here for *O. edulis* larval development (see 2.4). To allow for the maintenance of four temperatures without any disturbance, two larval rooms were used (Fig 1). Seawater at the entry of the first room was continuously maintained at 15°C by means of a thermo-regulated flow gate; whereas, the FSW at the entry of the second room was continuously thermo-regulated at 30°C. Both thermo-regulated filtered-seawater (FSW) flows were either delivered directly to the rearing cylinders (*via* 2 columns of degasification) or mixed in 2 tanks to allow the acquisition of the 2 other temperatures, 20 and 25°C. Seawater was distributed from these tanks to the cylinders by means of pumps.

2.3.2 Sampling procedure

Few larvae were lost during larval rearing and accordingly, larval performances reported here relate to the whole population. According to Rico-Villa et al (2009), larval growth and metamorphosis of *C. gigas* are closely associated to temperature. For this reason, we used the daily cumulated temperature (degree-days or °days) to allow comparison of different conditions in a similar scale, by adding all temperatures every day. To assess larval growth, 15 to 20 ml of FSW containing larvae ($n \geq 175$) were sampled from each cylinder. Larval size

was estimated every second day under the microscope using image analysis techniques (WinImager 2.0 and Imaq Vision Builder 6.0 software for image capture and treatment respectively). Larval survival was estimated at the end of the larval period at ≈ 180°days (days 6 to 13 at temperature ranging from 15°C to 30°C) by direct counting of empty shells (dead larvae). Survival was also estimated at mid-metamorphosis ≈ 270°days (days 9 to 16) and at the end of the metamorphosis ≈ 270°days (days 12 to 18). Rearing cylinders were drained and the whole larval population was transferred in 100-ml test tubes to be precisely sampled (6 x 200 µl to 6 x 500 µl). The percentage of eyed larvae (*i.e.* competent) was recorded at ≈ 180°days before transferring the whole population to similar larval cylinders without any cultch nor epinephrine for another week of rearing to allow metamorphosis. Settlement success (in %) was indirectly estimated 3 and 6 days later (days 9 to 18 from release) by determining the number of remaining larvae (absence of dissoconch). Complementary trials were run at 20 and 25°C to verify the effects on survival, growth and metamorphosis. Seawater temperature was daily controlled at the inlet of each larval rearing.

2.3.3 Ingestion rates

Enriched FSW was sampled twice a day at the inlet and outlet of each larval rearing cylinder to control larval ingestion by means of a particle coulter counter (Multisizer 3) and to adjust the food input in case of deviation from the theoretical value. In a flow-through culture system, larval ingestion rate (IR) expressed in $\mu m^3\ d^{-1}\ larvae^{-1}$ corresponds to:

$$IR = ((C_i - C_o)*S_F)/N$$

where C_i = food concentration of the incoming seawater and C_o = food concentration of the outcoming seawater ($\mu m^3\ \mu l^{-1}$); S_F = seawater flow ($\mu l\ d^{-1}$), recorded twice a day; and N = number of larvae in each tank.

2.4 Feeding ratio experiment

2.4.1 System design

Following release, larvae were counted and distributed in triplicate in 5-l translucent methacrylate cylinders. Larvae were held at a density of 50 larvae ml^{-1} in FSW and fed continuously, by means of four peristaltic pumps, a bispecific diet (1:1) consisting of *T. lutea* plus *C. neogracile* at different phytoplankton concentrations: 70 $\mu m^3\ \mu l^{-1}$ (no microalgae added); 500 $\mu m^3\ \mu l^{-1}$; 1500 $\mu m^3\ \mu l^{-1}$; 2500 $\mu m^3\ \mu l^{-1}$; 3500 $\mu m^3\ \mu l^{-1}$.

2.4.2 Sampling procedure

Larval growth was assessed every second day by sampling 15 to 20 ml of FSW containing larvae ($n \geq 175$) from each cylinder, and larval shell length was estimated under the microscope using image analysis techniques. Larval survival at the end of the larval period was estimated at ≈ 150°days (or day 6), at mid-metamorphosis (250°days or day 9) and at the end of the metamorphosis period (325°days or day 12). Cylinders were drained and the whole larval population was transferred in 100-ml test tubes to be precisely sampled (6 x 200 µl to 6 x 500 µl). The percentage of eyed larvae (i.e. competent) was recorded on day 6 before transferring the whole population to similar larval cylinders without any cultch nor epinephrine for another week of rearing to allow metamorphosis and settlement. Settlement success (in %), that took place on the wall of the rearing unit, was estimated 4 and 7 days later (days 10 to 13 from release) by determining the number of remaining larvae (absence of dissoconch). Larval ingestion was estimated daily by means of a particle coulter counter (Multisizer 3) and the food ratio adjusted.

2.5 Statistical analysis

Statistical analyses were performed using STATISTICA software (version 8.0). Depending on the studied factors (temperature, food ration), one way analyses of variance (ANOVA) were performed on survival rates and settlement success data. Before ANOVA analysis, all percentage data were arcsine-square root transformed to improve normality. When significant effects of factors were found ($p \leq 0.05$), a posteriori multiple comparison of the means was performed (Tukey's test). Normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were checked. When normality requirement was not met, a non-parametric test (Kruskal-Wallis test) was used for multiple comparisons of mean ingestion rate and shell length. To compare means of larval shell length at release (T_0) between the two successive experiments, a student t-test for independent samples was used ($p<0.05$).

3. Results

3.1 Effects of temperature on survival and settlement

At the end of the larval rearing, which lasted from day 6 to day 13 after release, survival was high (≥ 92%), whereas at 15°C, larval survival was lower (79%: Table 1). At mid

metamorphosis (i.e. from days 9 to 16), larval survival ranged from 0 to 43%, while all unsettled larvae were dead from day 13.

Larval competence was low at 15°C (11.7%), and increased with temperature to reach 96% at 30°C at the end of the larval rearing, (Table 1). No settlement occurred at 15°C and was low at 20°C (≈ 30%). Best performances were recorded above 25°C with metamorphosis ranging from 78% to 90%

3.2 Effects of temperature on larval ingestion

Food ingestion relied on temperature, and could be generally divided in three phases. A first phase of increase in food consumption was observed with an intensity and/or duration which were closely related to temperature. At 30°C, the maximum consumption was obtained on day 5 with 135000 cells ingested per larvae but did not exceed 25000 cells at 15°C on day 7 (Fig. 2). A sharp decrease was thereafter observed (from day 5 to day 7) corresponding to metamorphosis. When sufficient settlement had occurred, an active re-initiation of consumption could be observed from days 8 to 11 at temperatures above 25°C.

3.3 Effects of temperature on larval growth

Growth clearly relied on phytoplankton consumption (Fig. 2) with lower growth observed at 15°C from day 2 and higher growth at 30°C (Fig. 3). Daily growth from the day of release to the beginning of metamorphosis ranged from 4.3 µm d^{-1} to 21 µm d^{-1}, and the pelagic larval period lasted 6 to 13 days (175°days to 195°days). It is important to note that the effects of temperature on larval growth occurred swiftly. Indeed four days after release, the difference in length recorded between the two extreme temperatures (15 and 30°C) was 60 µm for larvae whose initial mean length was 183.1 ± 9.5 µm (Fig. 3).

3.4 Complementary experiment

In the second consecutive experiment, the initial quality of the larvae (initial size 164 ± 0.5 µm) was poorer because at release, 25% of dead larvae were already recorded despite no *Vibrio* detected. Accordingly, at the end of larval rearing (estimated at 225°days), survival ranged from 42 to 53% (Table 2).

At 25°C daily larval length increments were similar between both trials (15.5 µm d^{-1} *vs* 17.5 µm d^{-1} in the first trial); whereas at 20°C similar growth (9.1 µm d^{-1} *vs* 10 µm d^{-1} in the first trial) and settlement (25% *vs* 27%) were recorded (Tables 1 and 2).

3.5 Effects of food density on survival and settlement

Regardless of food concentration from 70 to 3500 μm^3, larval survival was high, i.e. ≥ 89%, on day 6 at 25°C (150°days: Table 3). During metamorphosis, larval survival on day 10 ranged from 44 to 70% at the lowest food concentration, and from 19 to 29% for food concentration ≥1500 μm^3. On day 13, all larvae were dead regardless of food concentration.

On day 6, competence was nil at a phytoplankton concentration of 70 μm^3 and low at all other food concentrations, i.e. <20% (Table 3). No settlement took place at 70 μm^3. It was low at 500 μm^3 but exhibited best performances at 1500 μm^3 μl^{-1} with ≈ 70% (Table 3).

3.6 Effects of food density on larval ingestion

Ingestion was highly dependent on food concentration and could generally be described as a three phase process. A first phase of increased consumption was observed with an intensity and/or duration closely related to phytoplankton density. At 3500 μm^3, maximum consumption was thus obtained on day 5 with 61000 cells per larvae, whereas at 500 μm^3 the highest consumption was 26000 cells per larvae on day 4.

A decrease was thereafter observed corresponding to metamorphosis and when sufficient settlement was reached, an active re-initiation of consumption was observed for phytoplankton densities above 1500 μm^3 from days 9-10 (Fig. 4). At 500 μm^3 a gradual decrease was observed from day 7, corresponding to the beginning of larval mortality, while dispersion was higher (CV = 26%; Table 3).

3.7 Effects of food density on larval growth

Lower growth was recorded at 500 μm^3 μl^{-1} from day 4, and higher growth was recorded at food concentration ≥ 1500 μm^3 μl^{-1}. There were no significant differences between the three upper values (Fig. 5). Growth ranged from 10 µm d^{-1} to 17 µm d^{-1} before larval growth decreased due to the initiation of metamorphosis (the largest larvae being settled whereas the smallest remaining in the water column, meaning that such apparent decrease was an artefact), and total larval life lasted 8 to 9 days.

It is noteworthy that, regardless of food density, growth was similar during the first three days after release. However, compared to unfed larvae that exhibited a weak length increment during the first two days, additional food played a major role on growth since release.

4. Discussion

The present study aimed to define the optimal temperature and phytoplankton concentration for the hatchery culture of the larvae of *Ostrea edulis*, a species with a regained interest in the French oyster industry. Under controlled conditions using a novel rearing method, the flow-through system, this study demonstrated that larval performances such as growth rate, survival or settlement rate were highly dependent of temperature condition and feeding regime.

First of all, our study showed the relatively high temperature tolerance of *O. edulis* larvae (20 – 30°C) with the best performances obtained ≥ 25°C. The highest temperatures tested, i.e. 25 and 30°C, resulted in the best larval performances, with high survival (≥ 97%), high growth rates (≥ 15.5 µm d^{-1}) and high settlement success (≥ 78%). This is in agreement with Rico-Villa et al. (2009) who, using a flow-through system, found that optimum larval development and settlement of *C gigas* occurred at 27°C. Early works showed that *O. edulis* larvae could be reared at a wide variety of temperature (Walne, 1965, 1966; Davis and Calabrese, 1969). However, some results obtained in the current work are contradicting with these pioneering studies and clearly show the need to update and improve the knowledge in larval rearing of *O. edulis*. According to Walne (1965), best larval results were obtained at about 22°C whereas Davis and Calabrese (1969) demonstrated that the ideal temperature range for satisfactory survival (i.e. ≥ 70%) of *O. edulis* larvae was 12.5 to 27.5°C, with poor survival occurring at 30°C. This variability in results highlights the importance of larval rearing techniques used between studies (i.e. static *vs* flow-through systems).

More specifically, increased ingestion was recorded as temperature increased throughout larval development. This increase of ingestion activity with temperature may be related to an increase of the ciliary activity of the larvae, in order to regulate metabolism (Strathmann, 1978; Baldwin and Newell, 1991). Our study also confirmed that *O. edulis* larval growth increased markedly with increasing temperature as previously shown in the literature (Walne, 1965; Robert et al., 1988). Maximum growth rate of the larvae was observed at 30°C but was not significantly different from 25°C. These results are similar to Rico-Villa et al. (2009) which demonstrated that optimal temperature range for *C. gigas* larvae was between 27 and 32°C, with no significant differences. Moreover, low mortalities (≤ 5%) were recorded at 30°C at the end of the larval stage, suggesting that 30°C is not the upper thermal limit for *O. edulis* larvae. However, additional experiments should be accordingly carried out to determine

upper thermal limits above 30°C for *O. edulis* larvae. For instance, previous studies on the larvae of *O. edulis* (Davis and Calabrese, 1969), *C. gigas* (Helm and Millican, 1977) and *C. virginica* (Davis and Calabrese, 1964) reported high mortalities at temperature ≥ 30°C, 32°C and 35°C respectively.

A rearing temperature of 30°C proved to have beneficial effects on metamorphosis leading to the highest settlement success of larvae (about 92%). This result is in agreement with Rico-Villa et al. (2009) who showed a positive relationship between metamorphosis of *C. gigas* and increasing temperatures. The lowest temperature tested (15°C) did not significantly impact survival, with rates remaining relatively high (79%). However, as shown by Rico-Villa et al. (2009) with *C. gigas*, the lowest temperature negatively affected ingestion rates, therefore reducing larval growth. Such impairment of larval growth may lengthen the larval period and increase the potential risks of predation, disease or dispersion (Davis and Hidu, 1969). As reported by Bayne (1983), extended larval period and delays in metamorphosis were observed (18 days) following exposure to low temperature, which directly impaired settlement success as shown by our data (0%). This result demonstrates that 15°C should be considered as an unsuitable temperature for commercial hatchery culture of *O. edulis* from an economic approach. On the contrary, growing larvae at 25 or 30°C significantly improved survival, shortened the larval rearing period (i.e. 8 days) and clearly improved settlement success (≥ 78%). Consequently, these rearing temperatures are recommended for *Ostrea edulis* hatchery production. Nevertheless, great care must be taken when rearing larvae at 30°C, especially in terms of feeding requirements and limitation of bacterial proliferation (Prado et al., 2005). For that reason and from an economic standpoint, a temperature of 25°C is recommended for larval production of flat oysters in hatchery.

Lastly, results from the present study suggest an ability of *O. edulis* larvae to adjust physiological processes in response to environmental temperature changes. In addition, this broad tolerance range of temperature may explain the extensive distribution of *O. edulis* along the European coast, from the cold waters of Norway or Scotland to the warm waters of the Mediterranean lagoons (Harry, 1985; Hidu and Lavoie, 1991; Shpigel, 1989).

In the current work, food was provided in a reliable and continuous way thanks to an efficient flow-through rearing system. As a result, larvae exhibited an adequate and sustained response in ingestion activity within a wide range of algae concentration. This study showed that larval development and metamorphosis were closely associated with feeding regime, which

remained within a broad range of microalgae supply throughout larval rearing, from 70 to 3500 μm^3 μl^{-1}.

Our results showed however that during the first 2 days, whilst shell length was increasing, larval ingestion was relatively low and independent from the phytoplankton concentrations. This result suggests that newly released *O. edulis* larvae rely not only on exogenous source of food but also on maternal reserves (Labarta et al., 1999). This is in agreement with Gonzalez-Araya et al. (2012) who showed that diet assemblage during broodstock conditioning was influencing greatly initial larval quality. This mixotrophic phase could therefore explain the low ingestion activity observed. Following day 2, with the exception of the 500 μm^3 μl^{-1} concentration, ingestion was highly dependent on microalgae concentration, and could be described as a three phase process. When larvae were surrounded by algal densities from 1500 to 3500 μm^3 μl^{-1}, ingestion increased significantly by 2 to 3 fold from day 0 to day 5 (phase 1). Microalgae ingestion then decreased, corresponding to the initiation of metamorphosis around day 9-10 (phase 2), until a sudden increase when the metamorphosis was completed and the newly settled spat started to feed again (phase 3). During larval development, the amount of food consumed becomes an essential factor in successful settlement as larvae must accumulate sufficient reserves to meet the energy demands required during the metamorphosis (Holland and Spencer, 1973). For that reason, ingestion activity increases very rapidly during phase 1. The progressive decrease in food consumption observed during phase 2 can be explained by behavioural and morphological changes undergone by the pediveliger larvae. Indeed, when reaching competency for metamorphosis, late pediveliger larvae enhance their crawling behaviour using their foot to find a suitable substrate to settle on, hence reducing their filtration activity (Cole, 1937). Movement and feeding are also inhibited because the velum is absorbed and replaced by the gills (Cole, 1938b). These drastic anatomic changes may explain the decrease in ingestion rates observed at the end of the larval life, and the increase of ingestion observed during the post-metamorphic/benthic stage (phase 3).

Our results showed that the ingestion activity of *O. edulis* increased in relation to food density up to a threshold level above which ingestion remains fairly constant. This relationship was described for *C. virginica* (Baldwin and Newell, 1995) or *Mercenaria mercenaria* (Gallager, 1988) as a hyperbolic function which increased to a plateau. In mollusc larvae, ingestion capability is directly linked to the velar ciliary tract (Strathmann, 1978). To avoid impairing the digestive system function at a high concentration of particles, mollusc larvae have the ability to regulate their ingestion rates (Crisp et al., 1985) by cessation of the beating of the ciliary band (Strathmann and Leise, 1979) or by controlling the rate of ingestion or rejection

of particles at the mouth (Gallager, 1988). Such a process has been called the saturation level and differs between species (Baldwin and Newell, 1995). For instance, Crisp et al. (1985) demonstrated that *O. edulis* larvae could reach a saturation level of ingestion rate at concentrations of 200 to 250 cells μl^{-1} using *Pavlova lutheri* as food supply. Based on cell volumes of a bispecific diet of *C. neogracile* and *T. lutea* (at 1:1) equivalent to ≈ 60 μm^3 (Gonzalez-Araya et al., 2012) our data could be converted into cell equivalents, resulting in a saturation level of 50 to 70 cells μl^{-1} TCg equivalent diameter. This concentration is much lower than that found by Crisp et al. (1985) but higher than those reported by Rico-Villa et al. (2009) for *C. gigas* and by Baldwin and Newell (1995) for *C. virginica*, reaching 20 cells μl^{-1} and 10-40 cells μl^{-1} of *T. lutea* respectively.

In addition, our results showed that growth was directly correlated with the food available to the larvae. At phytoplankton densities higher than 1500 μm^3 μl^{-1}, larval growth was not significantly improved and a plateau was reached. On the other hand, metamorphosis success, which was attained after 13 days, was significantly higher at phytoplankton densities of 1500 μm^3 μl^{-1}. These results suggest that larvae surrounded by 1500 μm^3 μl^{-1} (equivalent to ≈ 25 cells μl^{-1} of TCg equivalent diameter) of a bispecific diet of microalgae throughout larval rearing achieved the best performances in terms of growth and metamorphosis. Consequently, this background concentration should be maintained throughout larval rearing of *O. edulis* in commercial hatcheries. Lastly, it is noteworthy to mention that when no algae was provided, larval survival was relatively high after 6 days (89%) but started to be significantly affected from day 10 (44%) to day 13 (0%). Moreover, no competence or metamorphosis was recorded in starved larvae. These results clearly suggest that during their pelagic life, the amount of food consumed by the larvae is essential as they must store sufficient reserves to meet the energy demands required during the metamorphosis (Holland and Spencer, 1973) and to ensure their capacity for survival (Haws et al., 1993).

Conclusion

Using a flow-through rearing system, the present study determined the optimal temperature and feeding regime for the hatchery culture of *O. edulis*. At 30°C, a mixed diet of *T. lutea* + *C. neogracile* maintained throughout the whole larval life at the concentration of 1500 μm^3 μl^{-1} allowed the best larval development of *O. edulis* with high survival (97%), high growth (21µm d^{-1}) and high settlement (92%). At 25°C larval performances were quite similar and accordingly this temperature is recommended for *O. edulis* larval rearing. In contrast

temperature ≤ 20°C led to low development and weak settlement (≤ 27%). At the temperature of 25°C, a bispecific algal diet of *C. neogracile* + *T. lutea* maintained throughout the whole larval life at a concentration of 1500 μm^3 μl^{-1} allowed the best larval development of *O. edulis* with high survival (99%), good growth (15 µm d^{-1}) and high settlement success (68%). However, at higher concentrations, consumption was not significantly different, yielding similar larval performances. As a result, a constant residual concentration of 1500 μm^3 μl^{-1} (or 25 cells μl^{-1} equivalent TCg diameter) of a bispecific diet is recommended for *O. edulis* larval rearing.

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Legend of tables

Table 1. Effects of four seawater temperatures on mean larval survival, competence and settlement rates of *O. edulis* (± SD) during pelagic and benthic phase (trial 1). °d: °days or degree-days; D: day.

Table 2. Effects of two seawater temperatures on mean (± SD) larval survival and growth from day 9 and settlement from day 18 in *O. edulis* (trial 2). °d: °days or degree-days.

Table 3. Effects of food concentration on larval survival, competence (day 6) and settlement (day 13) of *O. edulis*. 70 µm3 μl^{-1} correspond to unfed larvae which only received continuously 1µm-filtered UV treated seawater.

Legend of figures

Figure 1. Experimental design used to allow the continuous thermoregulation of the four temperatures in flow through from 15 to 30°C.

Figure 2. Effects of four seawater temperatures on microalgae consumption in *O. edulis* larvae (trial 1) during larval and benthic phases.

Figure 3. Effects of four seawater temperatures on O. edulis larval growth (trial 1).

Figure 4. Effects of phytoplankton concentration on microalgae consumption in *O. edulis* larvae.

Figure 5. Effects of phytoplankton concentration on *O. edulis* larval growth.

Table 1.

	Larval survival (%)			Competence (%)	Settlement (%)
Temperature (°C)	End of larval rearing period 175-195°d (D6-D13)	Mid metamorphosis period 240-270°d (D9-D16)	End of metamorphosis period 270-360°d (D13-D18)	End of larval rearing period 175-195°d (D6-D13)	End of metamorphosis period 270-360°d (D13- D18)
15	79.0 (16.7)	42.9 (44.6)	0.0 (0.0)	11.7 (3.2)	0.0 (0.0)
20	92.7 (3.3)	0.0 (0.0)	----	51.7 (1.5)	26.7 (1.1)
25	99.4 (0.2)	10.8 (6.0)	0.3 (0.4)	89.0 (2.0)	78.3 (4.7)
30	97.2 (1.0)	7.8 (3.3)	0.5 (0.3)	96.0 (1.0)	91.7 (1.1)

Table 2

Temperature (°C)	Period of larval rearing (days) 225°d	Survival (%)	Growth (µm d^{-1})	Settlement (%)
20	11	42.2 (12.5)	9.1 (4.8)	25.0 (3.6)
25	9	52.7 (9.3)	15.5 (6.8)	No data (overflow)

Table 3

	Larval survival (%)			Competence (%)	Settlement (%)
Phytoplankton density ($\mu m^3\ \mu l^{-1}$)	Day 6	Day 10	Day 13	Day 6	Day 13
70	89.1 (5.3)	44.4 (8.9)	0	0	0
500	96.8 (1.6)	69.4 (16.4)	0.2 (0.3)	16.8 (26.7)	22.9 (20.3)
1500	98.0 (1.4)	19.3 (9.3)	0.1 (0.2)	16.5 (5.7)	68.1 (3.6)
2500	95.7 (1.8)	19.6 (4.1)	0	10.2 (4.8)	54.2 (7.8)
3500	96.8 (1.6)	28.5 (8.6)	0.3 (0.3)	8.2 (2.9)	52.1 (4.6)

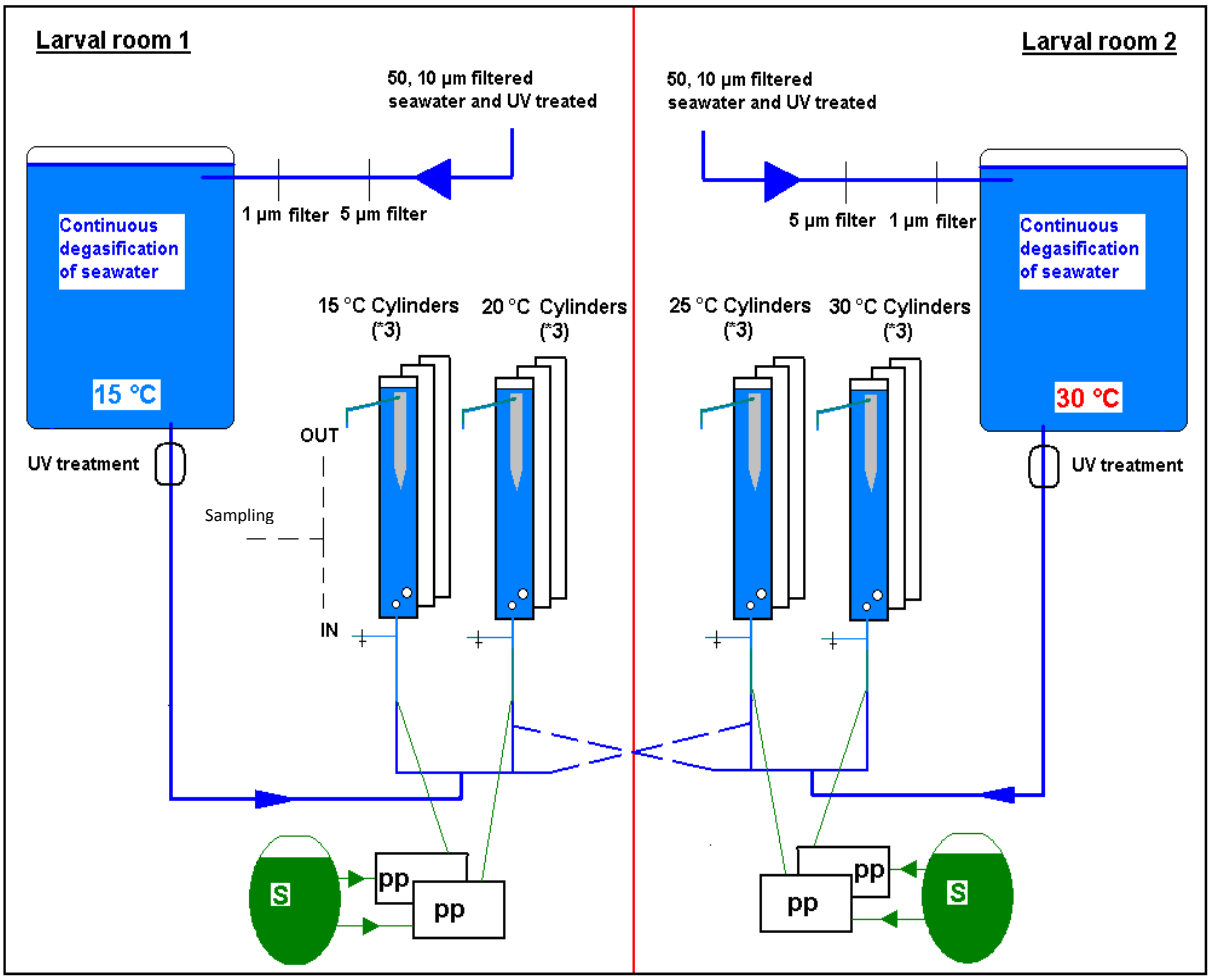
Larval room 1
Larval room 2
50, 10 µm filtered
seawater and UV treated
50, 10 µm filtered
seawater and UV treated
1 µm filter
5 µm filter
5 µm filter
1 µm filter
Continuous
degasification
of seawater
Continuous
degasification
of seawater
15 °C
30 °C
15 °C Cylinders
(*3)
20 °C Cylinders
(*3)
25 °C Cylinders
(*3)
30 °C Cylinders
(*3)
OUT
IN
Sampling
UV treatment
UV treatment
S
pp
pp
pp
pp
S

Figure 1

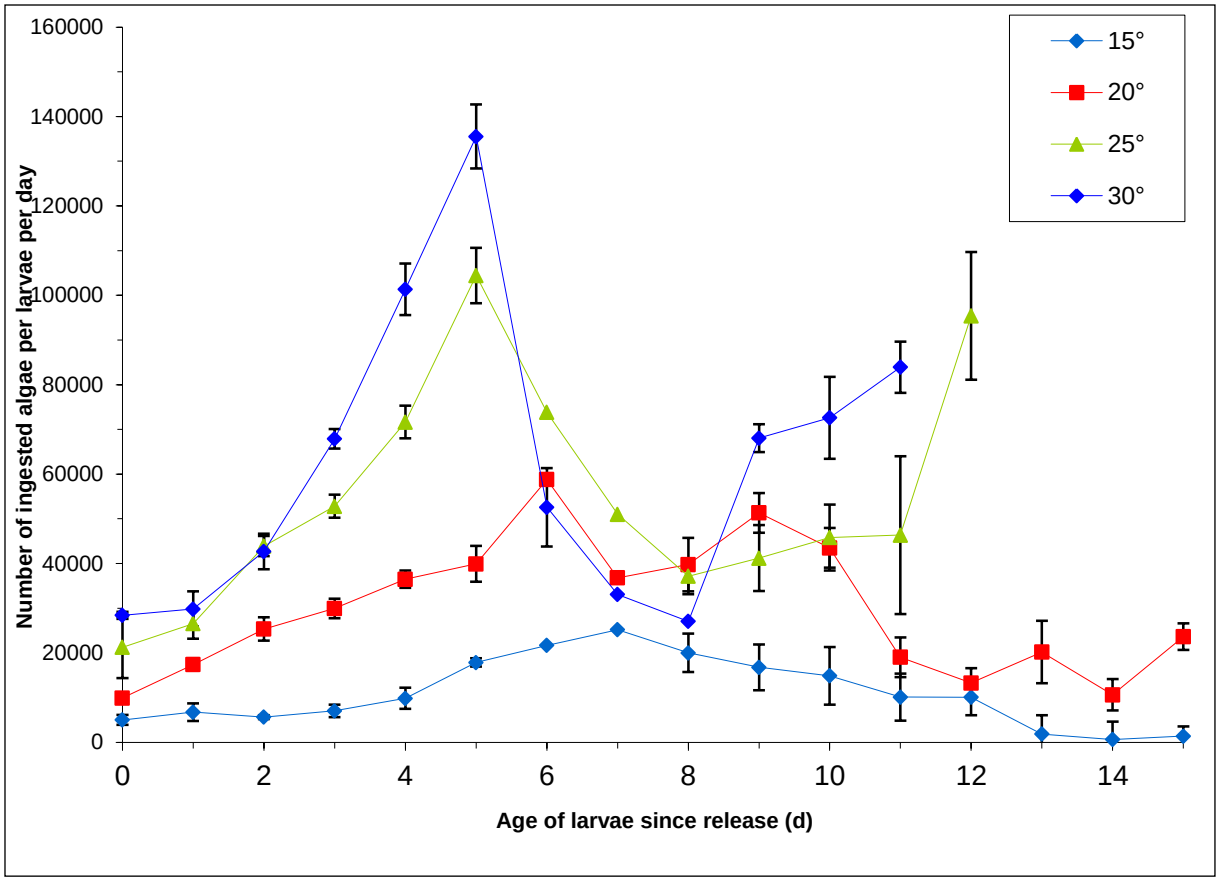
160000
140000
120000
100000
80000
60000
40000
20000
0
Number of ingested algae per larvae per day
0
2
4
6
8
10
12
14
Age of larvae since release (d)
15°
20°
25°
30°

Figure 2

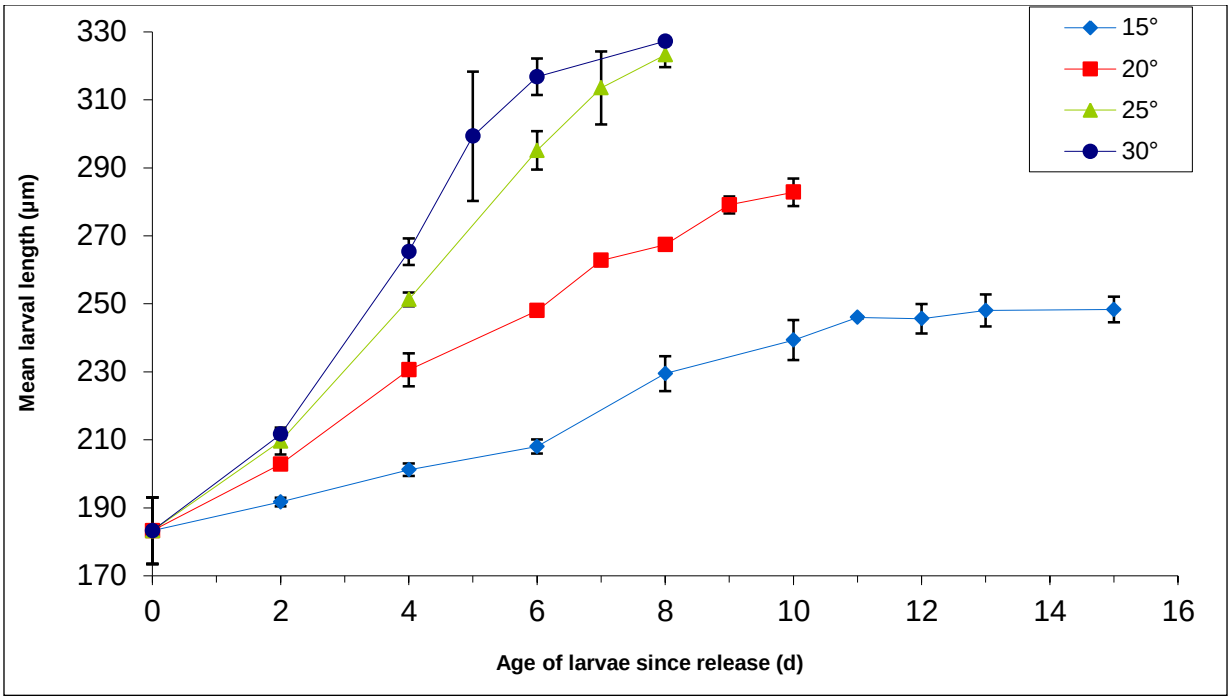
Mean larval length (µm)
330
310
290
270
250
230
210
190
170
0
2
4
6
8
10
12
14
16
Age of larvae since release (d)
15°
20°
25°
30°

Figure 3.

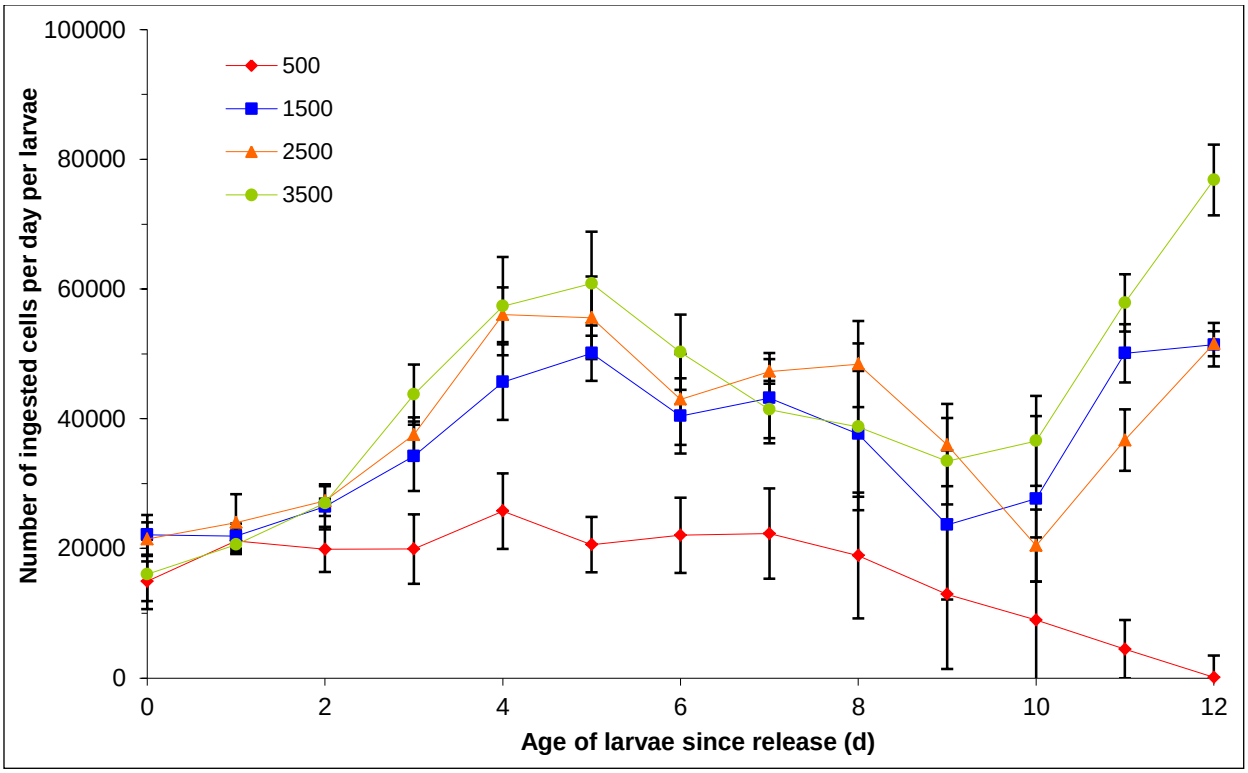
100000
80000
60000
40000
20000
0
Number of ingested cells per day per larvae
500
1500
2500
3500
0
2
4
6
8
10
12
Age of larvae since release (d)

Figure 4.

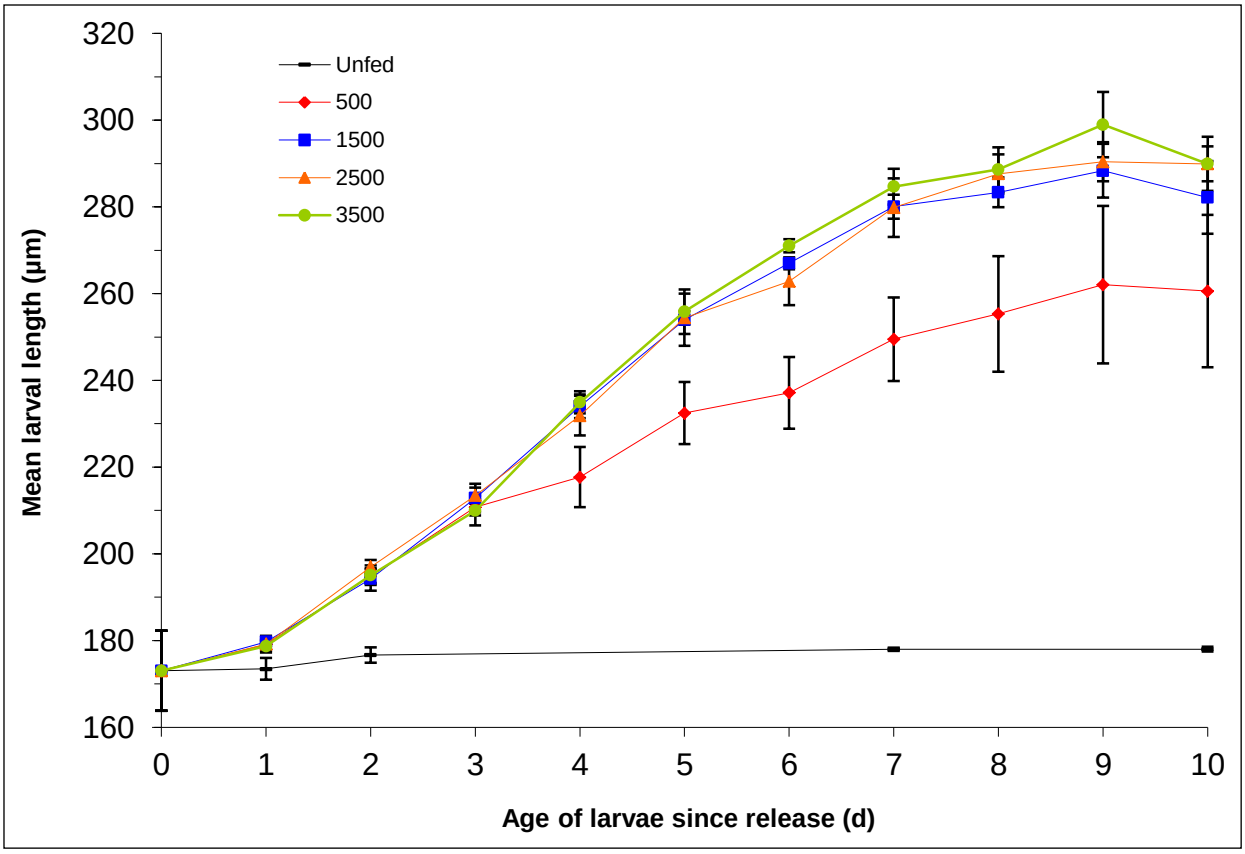
Unfed
500
1500
2500
3500
Mean larval length (µm)
320
300
280
260
240
220
200
180
160
0
1
2
3
4
5
6
7
8
9
10
Age of larvae since release (d)

Figure 5.