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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.

Keywords: *Ostrea edulis*, larvae, temperature, feeding regime, flow-through, DEB parameters

Introduction

The European flat or 'Native' oyster, *Ostrea edulis* (Linnaeus, 1758), 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 24 000 t in the late 1960s to present levels of 1000–1500 t (Buestel, Ropert, Prou & Goulletquer 2009; Robert, Luis-Sanchez, Pérez-Parallé, Ponis, Kamermans & O'Mahoney 2013). The extension of such disease across Europe led to the collapse of this industry at the European level (Baud, Gerard & Naciri-Graven 1997 and references therein; da Silva, Fuentes & Villalba 2005 and references therein) with most of the works since devoted to a better knowledge of both illness in natural surroundings (e.g. van Banning 1990; Montes, Villalba, Lopez, Carballal & Mourelle 1991; Culloty & Mulcahy 1996; Berthe,

Le Roux, Adlard & Figueras 2004; Flannery, Lynch, Carlsson, Cross & Culloty 2014) or under controlled conditions (da Silva & Villalba 2004; Lallias, Arzul, Heurtebise, Ferrand, Chollet, Robert, Beaumont, Boudry, Morga & Lapègue 2008; Arzul, Gagnaire, Bond, Chollet, Morga, Ferrand, Robert & Renault 2009; Prado-Alvarez, Lynch, Kane, Darmody, Pardo, Martínez, Cotterill, Wontner-Smith & Culloty 2015). Different strategies have been tried to minimize the effect of these diseases, such as modified husbandry (e.g. Le Bec, Mazurié, Cochennec & le Coguic 1991; Robert, Borel, Pichot & Trut 1991), introduction of exotic flat oysters (e.g. *Ostrea puelchana*: Pascual, Martin, Zampatti, Coatanéa, Deffosez & Robert 1991), genetic improvement (Naciri-Graven, Martin, Baud, Renault & Gérard 1998; Montes, Ferro-Soto, Conchas & Guerra 2003) or oyster bed restoration (Laing, Walker & Areal 2005; Lallias, Boudry, Lapègue, King & Beaumont 2010; Sawusdee, Jensen, Collins & Hauton 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 programme provides a possibility to enhance flat oyster farming as demonstrated earlier for *Crassostrea virginica* (Ford, Figueras & Haskin 1990) or *Crassostrea gigas* (Ward, English, McGoldrick, Maguire, Nell & Thompson 2000), and progress has been made in breeding for diseases resistance (Lynch, Flannery, Hugh-Jones, Hugh Jones & Culloty 2014) including the use of new genetic tools for improving selection (Lallias, Gomez-Raya, Haley, Arzul, Heurtebise, Beaumont, Boudry & Lapègue 2009; Morga, Arzul, Faury & Renault 2010; Martín-Gómez, Villalba, Carballal & Abollo 2012; Morga, Renault & Arzul 2012; Harrang, Heurtebise, Faury, Robert, Arzul & Lapègue 2015; Vera, Bello, Àlavarez-Dios, Pardo, Sánchez, Carlsson, Bartolomé, Maside & Martinez 2015). 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 in *O. edulis* production led to the introduction in 1971 of the Pacific oyster, *C. gigas* which successfully became the major species farmed in France comprising 98% of oyster production (Grizel & Héral 1991; Robert *et al.* 2013). Since 2008, however, massive losses in the French shellfish industry due to summer mortalities of *C. gigas* associated with the OsHV-1 (e.g. Renault 2011; Pernet, Barret, Le Gall, Corporeau, Degremont, Lagarde, Pépin & Keck 2012) 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 & Spencer 1991), *Ruditapes philippinarum* (Helm & Pellizzato 1990) or *Mercenaria mercenaria* (Castagna & 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. Through a European project called 'SETTLE', we addressed efforts to fill this gap. Because it is the first step to master in hatchery, to allow production of larvae of good initial quality (Berntsson, Jonsson, Wängberg & Carlsson 1997; Helm, Bourne & Lovatelli 2004), we focused, on the one hand, on broodstock conditioning (González-Araya, Quéau, Quéré, Moal & Robert 2011; González-Araya, Lebrun, Quéré & Robert 2012; González-Araya, Quillien & Robert 2013) and, on the other hand, on larval development (González-Araya, Mingant, Petton & Robert 2012). 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.

Growth environment in terms of water quality is believed to have major impact on larval performance as well as the rearing technique.

The water environment can be controlled by improved hygiene (Elston 1984) and/or by alternative rearing methods such as flow-through systems (vs. static seawater renewed each 2nd day). The present work relies on advances in the development of continuous flow-through systems for larvae that have shown to be efficient for *Pecten maximus* (Magnesen, Bergh & Christophersen 2006) or *C. gigas* (Rico-Villa, Wöerther, Mingant, Lepiver, Pouvreau, Hamon & Robert 2008). Thus, unexplained flat oyster larval mortalities have

often been reported in hatchery on day 8 (ICES, 2004; Laing *et al.* 2005). We hypothesized that 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* sp. (Jeffries 1983; Prado, Romalde, Montes & Barja 2005; Elston, Häse, Hasegawa, Humphrey & Polyak 2008).

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 (Fabioux, Huvet, Le Souchu, Le Pennec & Pouvreau 2005; Burke, Batailler, Miron, Ouellette & Tremblay 2008; Rico-Villa, Bernard, Robert & Pouvreau 2010). Nutrition is the major factor influencing larval bivalve survival and growth, as reviewed comprehensively by Marshall, McKinley and Pearce (2010). After feeding, temperature is another main factor controlling the development of mollusc larvae, and tolerance to temperature is species-specific. Most of the works related to temperature and larval feeding requirements of *O. edulis* are ancient, and accordingly, there is a need to update such information in the context of new rearing methods. Because bivalve growth is clearly temperature and food dependent, energetic models have been developed the last fifteen years to determine the part of biotic and abiotic parameters on growth (e.g. Powell, Bochenek, Klinck & Hofmann 2002; Hofmann, Powell, Bochenek & Klinck 2004; Pouvreau, Bourles, Lefebvre, Gangnery & Alunno-Bruscia 2006). These last authors used a more mechanistic energetic model based on the Dynamic Energy Budget (DEB) theory (Kooijman 2000), and such model has been successfully applied to *C. gigas* larvae based on the determination of 21 different parameters (Rico-Villa *et al.* 2010). It was not realistic to develop such a model for *O. edulis* larvae within the lifetime of SETTLE project, but feeding and temperature experiments have been set up to have the potential to estimate three essential parameters, two related to nutrition, the maximum surface-area-specific ingestion rate $\{\dot{J}_{Xm}\}$ and the half saturation coefficient X_K, and one linked to temperature, the Arrhenius temperature (T_A).

The aim of this study was to determine the optimal phytoplankton concentration and temperature for the hatchery culture of *O. edulis* larvae, based on survival, larval growth, ingestion and metamorphosis with experiments that were carried out to achieve data that could be used for estimating relationships between physiological rate (ingestion) and temperature, sensitive parameters needed to establish a forthcoming DEB larval growth model.

Material and methods

Broodstock conditioning

A total of 250 four-year-old, *Bonamia* and *Marteilia*-free, flat oysters (*O. edulis*) originating from western Norway (Agapollen, Bømlo county) in March 2009 were maintained under controlled conditions, at the Ifremer Argenton hatchery (North-west Brittany, France). They were conditioned during spring 2009 to allow SETTLE experiments on the effects of diet assemblage on larval development (González-Araya, Mingant *et al.* 2012). From the end of this first hatchery spring spawning period, the same flat oyster broodstock was progressively exposed from 19 to 9°C (1°C per week) from mid-July to mid-November 2009 (Fig. 1). Held in shadow light, in 700-L flow-through tanks supplied with 10-μm-filtered, UV-sterilized seawater, they were fed *Tisochrysis lutea* (CCAP 927/14: Ti) plus *Chaetoceros neogracile* (UTEX LB2658: Cg) at 3% dry weight microalgae (mg) per oyster meat (g). This mixed diet is currently used in our hatchery for the maintenance of adults and rearing of *C. gigas* early stages. From mid-November onward, flat oysters were progressively acclimated to 19°C (1°C per week increase: Fig. 1), with permanent light, in 1-μm-filtered cotton bag seawater, UV-treated, continuously enriched with *Rhodomonas salina* (CCAP 978/24: Rs) plus *Chaetoceros neogracile* that has been shown to be the most efficient diet for flat oyster conditioning (González-Araya *et al.* 2011; González-Araya, Lebrun *et al.* 2012). When temperature reached 19°C, broodstock received a daily ration of 6% dry weight microalgae (mg) per oyster meat (g) vs. 3% at lower temperatures. During the whole pre-conditioning and conditioning process, tanks were drained and daily cleaned, and treated once a week with a bactericide/fungicide/virucide solution (25.5% $C_2H_4O_2$ + 15% H_2O_2 + 59.5% H_2O) by pulverization.

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.

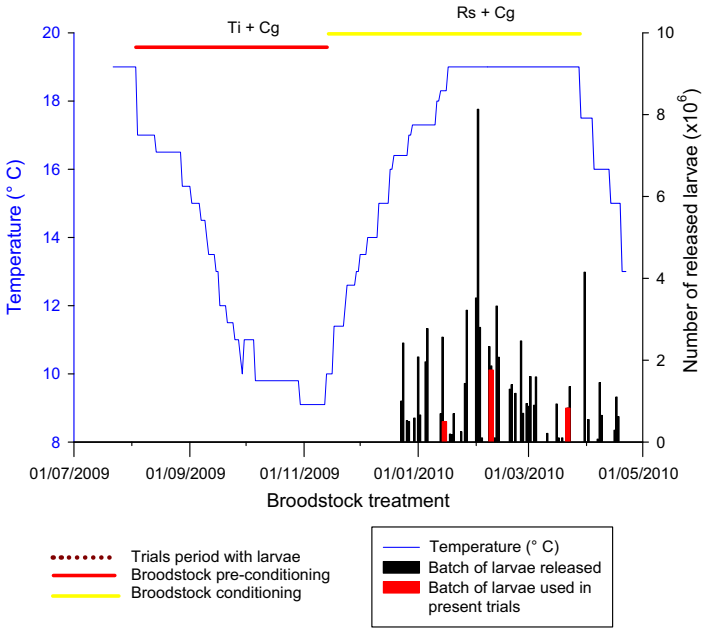

Figure 1 Temperature and food conditions applied during pre-conditioning and conditioning periods (autumn 2009) and number of batch of larvae released during conditioning period (winter and spring 2010): Ti, *Tisochrysis lutea*; Cg, *Chaetoceros neogracile*; *Rs, Rhodomonas salina*. [Colour figure can be viewed at wileyonlinelibrary.com]

At each release, larvae were counted (Fig. 1) and the number of *Vibrio* sp. per larvae determined. To do so, samples of 1000 larvae was crushed by a Thomas potter and density of bacteria *Vibrionaceae* was estimated by the plate counting method, using thiosulfate citrate sucrose medium (TCBS) in sterile atmosphere. The batch of larvae was kept when *Vibrio* load was <1 per larva. In this condition and when a sufficient number of larvae was 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 temperatures (see Section Temperature experiment) and ambient salinity (34 PSU), at a renewal rate of 100% h^{-1}, that is 87 mL min^{-1}. Oyster larvae were fed a bispecific algal diet consisting of *Tisochrysis lutea* (Ti ≈ 40 μm^3 volume diameter) plus *Chaetoceros neogracile* (Cg ≈ 80 μm^3) (1:1: equivalent volume) continuously maintained by peristaltic pumps at different experimental concentration (see Section Feeding ratio experiment). 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. Three different batches of larvae were used for the experiments (Fig. 1).

Feeding ratio experiment

System design

Following release, larvae were counted, distributed in triplicate in 5-L translucent methacrylate cylinders and maintained at 25°C, temperature successfully applied in our hatchery for *C. gigas* larval development (Rico-Villa, Pouvreau & Robert 2009) and routinely used since for that species (da Costa, Petton, Mingant, Bougaran, Rouxel, Quéré, Wikfors, Soudant & Robert 2015). 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 five different phytoplankton concentrations: 70 (no microalgae added); 500; 1500; 2500; 3500 μm^3 μL^{-1} (≈1, 8, 25, 42 and 58 cells μL^{-1} equivalent TCg).

Sampling procedure

Larval growth was assessed, every second day, by sampling 15–20 mL of FSW containing larvae ($n \geq 175$) from each cylinder, and larval shell length was estimated under the microscope using image analysis techniques. 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 × 200 μL to 6 × 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. Relative settlement success (in %), that took place on the wall of the rearing unit, was estimated 4 and 7 days later (days 10–13 from release) by determining the number of remaining larvae (absence of dissoconch) and divided by the number of larvae initially stocked.

Ingestion rates

Enriched FSW was sampled twice a day at the inlet and outlet of each larval rearing cylinder (Fig. 2) 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 $\mu m^3\ day^{-1}\ larvae^{-1}$ 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 ($\mu L\ day^{-1}$), recorded twice a day; and N, number of larvae in each tank.

Temperature experiment

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 *T. lutea* plus *C. neogracile* continuously maintained at 1500 $\mu m^3\ \mu L^{-1}$, determined in the first trial, as the best phytoplankton concentration for *O. edulis* larval development (see Table 1 and Fig. 3). To allow for the maintenance of four temperatures without any disturbance, two larval rooms were used (Fig. 2). 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 two columns of degasification) or mixed in two 150-L tanks to allow the acquisition of the two other temperatures, 20 and 25°C. Seawater was distributed from these tanks to the cylinders by means of pumps.

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 with 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, 15–20 mL of FSW containing larvae ($n \geq 175$) was 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: National Instrument, Austin, TX, USA). Larval survival was estimated at the end of the larval period at ≈180° days (days 6–13 at temperature ranging from 15 to 30°C) by direct counting of empty shells (dead larvae). Survival was also estimated at mid-metamorphosis ≈ 270° days (days 9–16) and at the end of the metamorphosis ≈ 270° days (days 12–18). Rearing cylinders were drained, and the whole larval population was transferred in 100-mL test tubes to be precisely sampled (6 × 200 μL to 6 × 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 and settlement that took place on the wall. Relative settlement success (%) was indirectly estimated 3 and 6 days later (days 9–18 from release) by determining the number of remaining swimming larvae (absence of dissoconch) and compared to the number of larvae initially stocked. Seawater temperature was daily controlled at the inlet of each larval rearing. Larval ingestion was estimated daily by means of a particle coulter counter (Multisizer 3) and the food ratio adjusted.

Complementary trials were run at 20 and 25°C to confirm the effects on survival, growth and metamorphosis. Indeed, in French natural surroundings, *O. edulis* larval

Table 1 Effects of food concentration on larval survival, competence (day 6) and settlement (day 13) of *Ostrea edulis*

Phytoplankton density ($\mu m^3\ \mu L^{-1}$)	Larval survival (%)			Competence (%)	Settlement (%)
	Day 6	Day 10	Day 13	Day 6	Day 13
70	89.1 (5.3)[a]	44.4 (8.9)[a]	0.0 (0.0)	0.0 (0.0)[a]	0.0 (0.0)[a]
500	96.8 (1.6)[a]	69.4 (16.4)[a]	0.2 (0.3)	16.8 (26.7)[b]	22.9 (20.3)[b]
1500	98.0 (1.4)[a]	19.3 (9.3)[b]	0.1 (0.2)	16.5 (5.7)[b]	68.1 (3.6)[c]
2500	95.7 (1.8)[a]	19.6 (4.1)[b]	0.0 (0.0)	10.2 (4.8)[b]	54.2 (7.8)[c]
3500	96.8 (1.6)[a]	28.5 (8.6)[b]	0.3 (0.3)	8.2 (2.9)[b]	52.1 (4.6)[c]

70 $\mu m^3\ \mu L^{-1}$ correspond to unfed larvae which only received continuously 1-µm-filtered UV-treated seawater. Values with same letters in the same row are not significantly different at $P > 0.05$, whereas those in bracket correspond to SD.

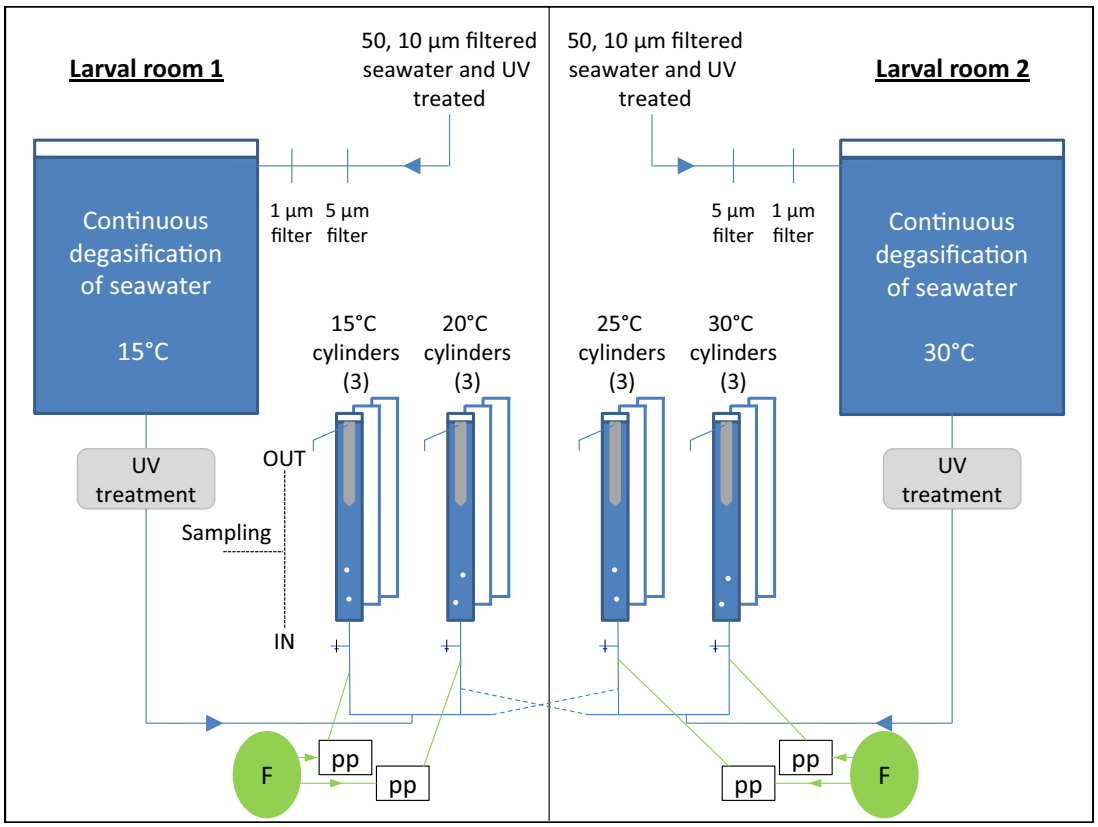

Figure 2 Experimental design used to allow the continuous thermoregulation of the four temperatures, in flow-through larval rearing system, from 15 to 30°C: PP, peristaltic pump; F, Food. [Colour figure can be viewed at wileyonlinelibrary.com]

development and settlement occurred at temperatures ranging from 20 to 25°C (Robert, His & Dinet 1988). Confirming the first set of results for both temperatures will be helpful for flat oyster natural reproduction and spatfall intensity knowledge.

Statistical analysis

Statistical analyses were performed using JMP software (version 9.0: SAS, Cary, NC, USA). Depending on the studied factors (food ration, temperature), one-way analysis of variances

(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.

Concept and DEB model formulation for the larval stage

Concept and DEB model formulation for the larval stage have been previously detailed (Rico-Villa *et al.* 2010), so only a brief outline will be given here. In DEB theory, an organism is partitioned into three main body components: (1) structural biovolume or somatic tissue; (2) stored energy reserve; and (3) gonads and/or stored energy reserves allocated to maturity and reproduction (Kooijman 2000). Moreover, in the theory, three life stages are distinguished: (1) embryos, which neither feed nor reproduce; (2) juveniles, which feed but do not reproduce; and (3) adults which both feed and reproduce. DEB theory describes a larva as a juvenile because larvae feed and their resources are not yet allocated to reproduction, but to other developmental processes. Therefore, energy from food is stored directly as reserves and is then directed towards growth, development and their maintenance.

According to DEB theory, a larva can be described by three state variables: the energy in the reserve, E; the structural body volume, V; and the amount of energy invested into development to reach juvenile stage, E_R. The ingestion rate, $\dot{J}_x$ (μm^3 day^{-1}), is proportional to the biosurface of a larva, $V^{2/3}$ (μm^2), and relies on available food density, X (phytoplankton expressed in μm^3 μL^{-1}), in the environment: $\dot{J}_x = \{\dot{J}_{Xm}\} f V^{2/3}$, where $\{\dot{J}_{Xm}\}$ is the maximum ingestion rate per unit of biosurface (expressed in μm^3 day^{-1} μm^{-2}), X_K is the half saturation coefficient (μm^3 μL^{-1}), and f is the feeding functional response, which can vary between 0 and 1 (dimensionless).

Physiological rates, such as ingestion and maintenance, depend on body temperature. This relation is usually well described by an Arrhenius-type equation within a species-specific tolerance range between upper and lower temperature boundaries (Kooijman 2000). According to the Arrhenius relationship, this dependency can be expressed as: $\dot{k}(T) = \dot{k}_1 \exp\{T_A/T_1 - T_A/T\}$ where $\dot{k}(T)$ is the value of the physiological rate that depends upon an ambient temperature T (in Kelvin), $\dot{k}_1$ is the value of the physiological rate at a reference temperature T_1 of 298 K (25°C), and T_A is the so-called Arrhenius temperature (in Kelvin). Growth rate data were accordingly standardized to a value of 1 for 298 K and estimation of the Arrhenius parameter achieved on a Newton–Raphsen algorithm written in Matlab®. *Ostrea edulis* data were plot on a similar data model previously developed for *C. gigas* larvae using as first approximation similar boundaries of the temperature tolerance range as regards to close larval performances of both species (Rico-Villa *et al.* 2009 and present contribution) when exposed to a close range of temperature (17–32°C for *C. gigas* vs. 15–30°C for *O. edulis*).

The feeding experimental design allowed us to determine two primaries parameters: the maximum surface-area-specific ingestion rate $\{\dot{J}_{Xm}\}$ and the half saturation coefficient X_K, whereas the temperature experiment led to the acquisition of the Arrhenius temperature T_A.

Results

Effects of food density

On survival and settlement

Regardless of food concentration from 70 to 3500 μm^3 μL^{-1}, larval survival was high, that is ≥89%, on day 6 at 25°C (150° days: Table 1) with no significant differences between conditions. During metamorphosis, larval survival on day 10 ranged from 44% to 70% at the lowest food concentrations and from 19% to 29% for food concentration ≥1500 μm^3 μL^{-1}, with significant differences between both groups ($P < 0.01$, Table 1). On day 13, most of the remaining larvae were dead regardless of food concentration.

On day 6, competence was nil at a phytoplankton concentration of 70 μm^3 μL^{-1}, and low at all other food concentrations, that is <20% (Table 1) with no significant differences between conditions, from 500 to 3500 μm^3 μL^{-1} (Table 1). A similar trend occurred on day 13 with no settlement at 70 μm^3 μL^{-1} and low value at 500 μm^3 μL^{-1} (23%: Table 1). In contrast, high settlement

occurred from 1500 to 3500 μm^3 μL^{-1} (≈50–70%, Table 1) with significant differences at $P < 0.01$ between both groups (Table 1).

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 μL^{-1}, maximum consumption was thus obtained on day 5 with 61 000 cells per larvae, whereas at 500 μm^3 μL^{-1}, the highest consumption was 26 000 cells per larvae on day 4 (Fig. 3).

A microalgae uptake decrease, corresponding to metamorphosis, was thereafter observed, followed, from day 10, by an active re-initiation of consumption for phytoplankton densities ≥1500 μm^3 μL^{-1}, when sufficient settlement was reached (Fig. 3). At 500 μm^3 μL^{-1}, a gradual decrease was observed from day 7, corresponding to the beginning of larval mortality (Fig. 3 and Table 1).

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 at $P < 0.05$ (Fig. 4). Growth ranged from 10 to 17 $\mu m\ day^{-1}$ before larval growth decreased on day 10 due to the initiation of metamorphosis. Indeed, the largest larvae had settled first, whereas the smallest remained in the water column, meaning that such apparent decrease was an artefact (Fig. 4).

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 (Fig. 4).

When *O. edulis* ingestion rate was expressed as a function of larval biosurface and food density, larval growth occurred up to a phytoplankton density of 1500 μm^3 μL^{-1} (25 cells μL^{-1} equivalent TCg). Above, at higher phytoplankton concentration, larval growth was not significantly improved and a plateau was reached (Fig. 5).

Effects of temperature

On survival and settlement

At the end of the larval rearing, which lasted from days 6 to 13 after release (related to temperature), survival was high regardless to temperatures (≥79%: Table 2). At mid-metamorphosis (i.e. from days 9 to 16), larval survival ranged from 0% to 43%, whilst all unsettled larvae were dead from day 13 (Table 2).

Larval competence was low at 15°C (11.7%) and increased significantly ($P < 0.01$) with temperature to reach 96% at 30°C at the end of the larval rearing (Table 3). No settlement occurred at 15°C and was low at 20°C (≈28%). Best significant performances ($P < 0.01$) were recorded above 25°C with metamorphosis ranging from 78% to 92% (Table 2).

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 (Fig. 6). At 30°C, the

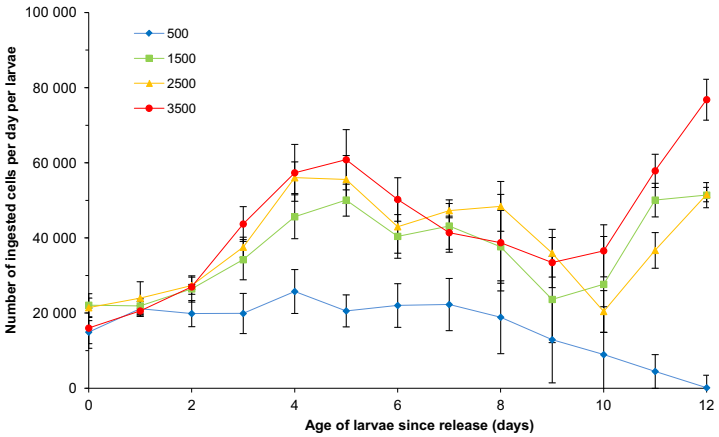

Figure 3 Effects of phytoplankton concentrations (500, 1500, 2500 and 3500 μm^3 μL^{-1}) on microalgae consumption in *Ostrea edulis* larvae. Values are mean ($n = 3$) ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

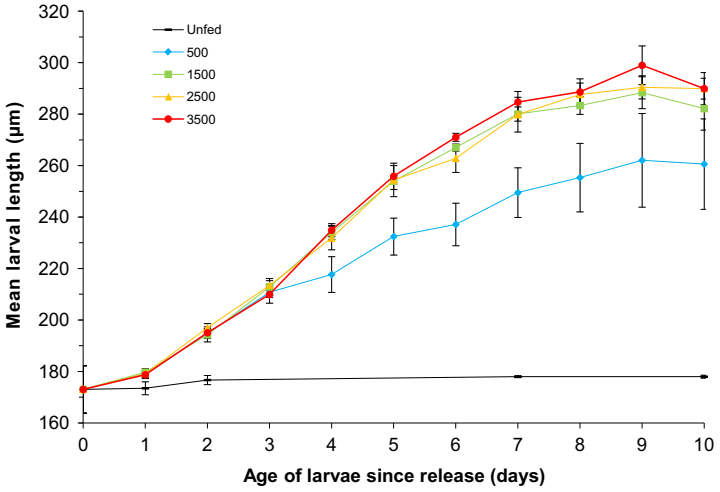

Figure 4 Effects of phytoplankton concentrations (500, 1500, 2500 and 3500 $\mu m^3\ \mu L^{-1}$) on *Ostrea edulis* larval growth. Values are mean ($n = 3$) ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

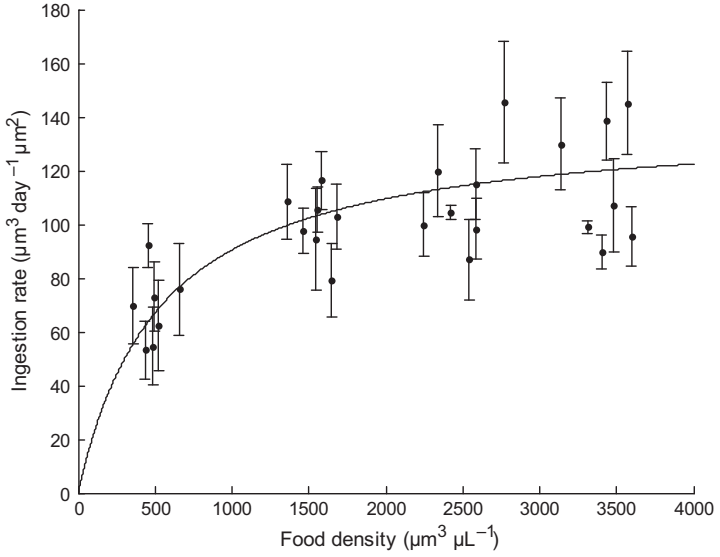

Figure 5 Ingestion rate, $\dot{J}_x$, of *Ostrea edulis* larvae, reared at 25°C, as a function of larval bio-surface and food density: feeding functional response.

Table 2 Effects of four seawater temperatures on mean larval survival, competence and settlement rates of *Ostrea edulis* (±SD) during pelagic and benthic phase (trial 1)

	Larval survival (%)			Larval 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)^{a}	42.9 (44.6)^{a}	0.0 (0.0)^{a}	11.7 (3.2)^{a}	0.0 (0.0)^{a}
20	92.7 (3.3)^{ab}	0.0 (0.0)^{b}	–	51.7 (1.5)^{b}	26.7 (1.1)^{b}
25	99.4 (0.2)^{b}	10.8 (6.0)^{c}	0.3 (0.4)^{a}	89.0 (2.0)^{c}	78.3 (4.7)^{c}
30	97.2 (1.0)^{ab}	7.8 (3.3)^{c}	0.5 (0.3)^{a}	96.0 (1.0)^{d}	91.7 (1.1)^{d}

° d, ° days or degree-days; D, day.

Values with same letters in the same row are not significantly different at $P > 0.05$, whereas those in bracket correspond to SD.

Table 3 Effects of two seawater temperatures on mean (±SD) larval survival and growth from day 9 and settlement from day 18 in *Ostrea edulis* (trial 2)

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

° d, ° days or degree-days.
Values with same letters in the same row are not significantly different at $P > 0.05$, whereas those in bracket correspond to SD.

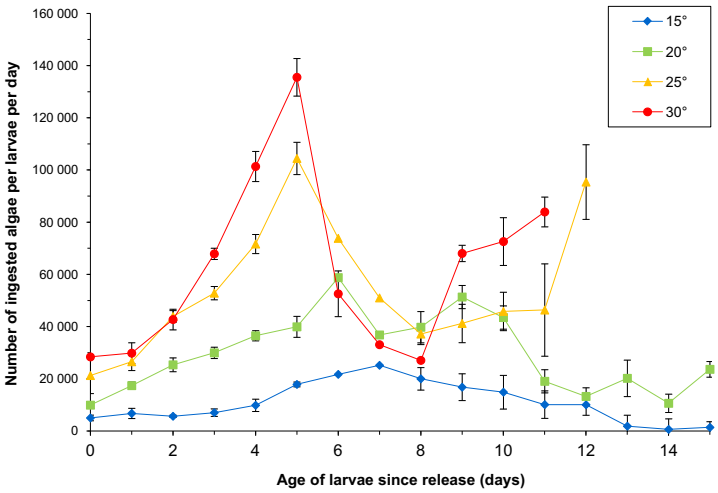

Figure 6 Effects of four seawater temperatures (15, 20, 25 and 30°C) on microalgae consumption in *Ostrea edulis* larvae (trial 1) during larval and benthic phases. Values are mean ($n = 3$) ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

maximum consumption was obtained on day 5 with 135 000 cells ingested per larvae but did not exceed 25 000 cells at 15°C on day 7 (Fig. 6). A sharp decrease was thereafter observed (from days 5 to 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 ≥25°C (Fig. 6).

On larval growth

Growth clearly relied on phytoplankton consumption (Fig. 6) with low growth observed at 15°C from day 2 and high growth at 30°C (Fig. 7). Daily growth, from the day of release to the beginning of metamorphosis, ranged from 4.3 to 21 µm day^{-1}, and the pelagic larval period lasted 6–13 days (175°–195° days). On day 6, significant differences in larval length ($P < 0.001$) were observed at all temperatures; thereafter, at temperatures ≥25°C, such differences collapsed due to settlement initiation at 30°C introducing a larval size bias (slowing growth). 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 ≈64 µm for larvae whose initial mean length was 183.3 ± 9.5 µm (Fig. 7).

Complementary experiment

In the second 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 3) with no significant differences ($P = 0.05$). In contrast, temperature has a highly significant effect ($P < 0.001$) on growth with ≈50% additional daily growth value recorded at 25°C (Table 3).

At 25°C, daily larval length increments were quite close between both trials (15.5 µm day^{-1} vs. 17.5 µm day^{-1} in the first trial), whereas at 20°C, similar growth (9.1 µm day^{-1} vs. 10 µm day^{-1} in the first trial) and settlement (25% vs. 27%) were recorded (Tables 2 and 3).

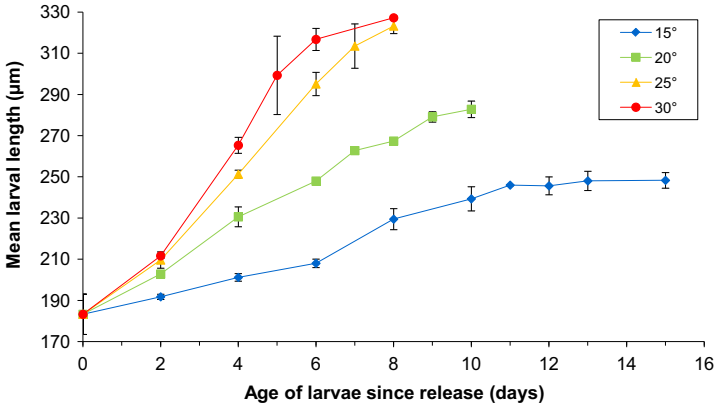

Figure 7 Effects of four seawater temperatures (15, 20, 25 and 30°C) on *Ostrea edulis* larval growth (trial 1). Values are mean (n = 3) ± SD. [Colour figure can be viewed at wileyonlinelibrary.com]

Parameters estimates

Maximum surface-area-specific ingestion rate $\dot{J}_{Xm}$ and half saturation coefficient X_K

The feeding functional response, *f*, for *O. edulis* larvae at different phytoplankton density levels, shown on Fig. 5, allowed a first estimation of two ingestion parameters for larvae reared at 25°C: $\{\dot{J}_{Xm}\}$ = 120 ± 4 µm^3 day^{-1} µm^{-2} and $\{X_K\}$ = 537 ± 142 µm^3 µL^{-1}.

Arrhenius temperature T_A

The lower, T_L, and upper, T_H, boundaries of the temperature tolerance range were fixed at 285 and 303 K, respectively (Fig. 8). The Arrhenius temperature, T_A, which corresponds to the slope of the increase with this adjustment, was estimated at 8355 K for *O. edulis* larvae (Fig. 9).

Discussion

The present study aimed to define the optimal phytoplankton concentration and temperature for the hatchery culture of the larvae of *O. 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, survival or settlement were highly dependent of feeding regime and temperature condition.

In the current work, food was provided in a reliable and continuous way thanks to an efficient

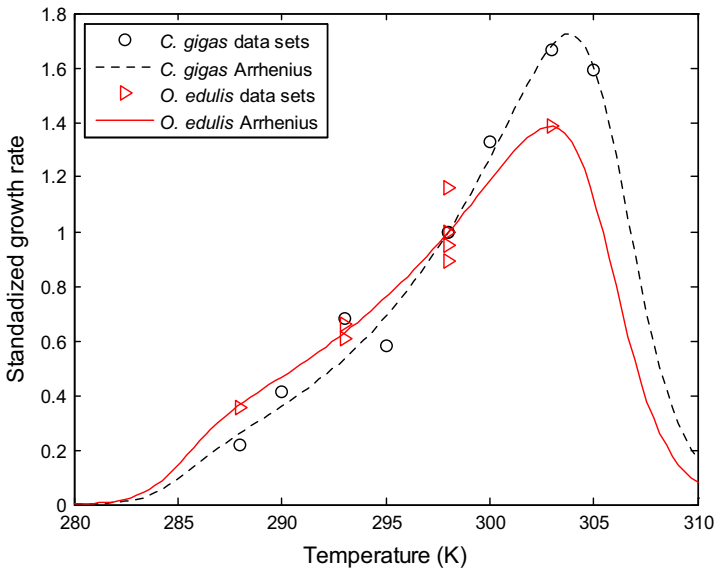

Figure 8 Effect of temperature on standardized growth rate of *Ostrea edulis* larvae (present work) and *Crassostrea gigas* larvae (Rico-Villa *et al.* 2010). The continuous curve shows the fitting of Arrhenius equation to own experimental *O. edulis* data sets represented by triangle, whereas the dotted line and circles are related to *C. gigas*. [Colour figure can be viewed at wileyonlinelibrary.com]

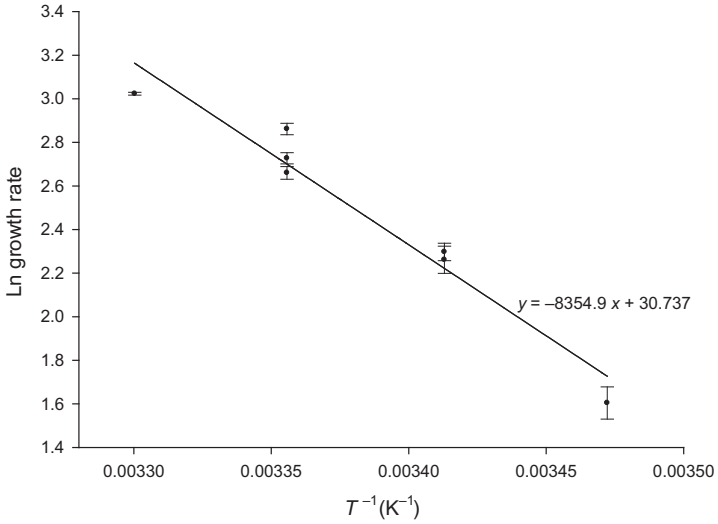

Figure 9 Regression line (R^2 = 0.966) of *Ostrea edulis* growth rate against temperature (K) allowing the estimation of Arrhenius temperature (T_A) corresponding to the slope of the line. Values are mean (n = 3) ± SD.

flow-through rearing system. As a result, larvae exhibited an adequate and sustained response in ingestion activity within a wide range of algae concentration.

First of all, 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}. However, 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, Fernández-Reiriz & Pérez-Camacho 1999). This is in agreement with González-Araya, Lebrun *et al.* (2012) and González-Araya, Mingant *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 twofold to threefold 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 & 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 competence 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 1938). These drastic anatomic changes may explain the decrease in ingestion rates observed at the end of the larval life, and the increase in 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 also described for *C. virginica* (Baldwin & Newell 1995) or *M. 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, Yule

& White 1985) by cessation of the beating of the ciliary band (Strathmann & 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 & Newell 1995). For instance, Crisp *et al.* (1985) demonstrated that *O. edulis* larvae could reach a saturation level of ingestion rate at concentrations of 200–250 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 $\approx$60 μm^3 (González-Araya, Lebrun *et al.* 2012; González-Araya, Mingant *et al.* 2012), our data could be converted into cell equivalents, resulting in a saturation level of 20–30 cells μL^{-1} TCg equivalent diameter. This concentration is much lower than that found by Crisp *et al.* (1985) but close to that 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, 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. This limitation has been clearly pointed out when representing *O. edulis* ingestion rate as a function of larval biosurface and food density with such limitation occurring beyond $\approx$1400 μm^3 μL^{-1} at 25°C. Such feeding functional response led to a first estimation of two ingestion parameters for *O. edulis* larvae, $\dot{J}_{Xm}$ (120 ± 4 μm^3 day^{-1} μm^{-2}) and X_K (537 ± 142 μm^3 μL^{-1}) whose values are close to those reported for *C. gigas* (137 ± 4 μm^3 day^{-1} μm^{-2} and 600 ± 50 μm^3 μL^{-1}, respectively; Rico-Villa *et al.* 2010) meaning that their feeding behaviour is quite similar at least in our controlled conditions.

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 $\approx$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 & Spencer 1973) and to ensure their capacity for survival (Haws, DiMichele & Hand 1993).

Secondly, 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, that is 25 and 30°C, resulted in the best larval performances, with high survival (≥97%), high growth rates (≥15.5 μm day^{-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 & 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 12.5–27.5°C, with poor survival occurring at 30°C. The variability in results that can also be partially originated from differences between locally adapted strains 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 augmentation of ingestion activity with temperature may be related to an increase in the ciliary activity of the larvae, to regulate metabolism (Strathmann 1978; Baldwin & Newell 1991). Our study also confirmed that *O. edulis* larval growth rose markedly with increasing temperature as previously shown in the literature (Walne 1965; Robert *et al.* 1988). Maximum growth of the larvae was observed at 30°C and was significantly different from 25°C. Thus, the

Arrhenius temperature was estimated at 8355 K for *O. edulis* larvae. Arrhenius temperature is typically high for species that naturally experience small temperature changes, such as those that occur in pelagic species or in pelagic life stages (e.g. planktotrophic larvae, like oysters). Nevertheless, this value is lower than that reported for *C. gigas* (11 400 K: Rico-Villa *et al.* 2010), meaning that *O. edulis* is more tolerant to this parameter change than *C. gigas*. 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 & Calabrese 1969), *C. gigas* (Helm & Millican 1977) and *C. virginica* (Davis & Calabrese 1964) reported high mortalities at temperature ≥30, 32 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, in the natural surroundings, the potential risks of predation, disease or dispersion (Davis & 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. In contrast, growing larvae at 25 or 30°C significantly improved survival, shortened the larval rearing period (i.e. 6–8 days) and clearly improved settlement success (≥78%). Consequently, these rearing temperatures are recommended for *O. 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).

Overall, 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; Shpigel 1989; Hidu & Lavoie 1991).

By estimating three essential larval parameter values, the maximum surface-area-specific ingestion rate $\{\dot{J}_{Xm}\}$, the half saturation coefficient X_K and the Arrhenius temperature T_A, this contribution put a tangible basis for a future *O. edulis* Dynamic Energy Budget (DEB) growth model which needed however additional experiments to define, beyond the most important parameters, the Shape coefficients, the egg energy content, the maintenance rate coefficient and the volume-specific costs for structural growth.

Conclusion

Using a flow-through rearing system, the present study determined the optimal feeding regime and temperature for the hatchery culture of *O. edulis*. 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 $\mu m^3\ \mu L^{-1}$ (25 cells μL^{-1} equivalent TCg) allowed the best larval development of *O. edulis* with high survival (99%), good growth (15 μm day^{-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 $\mu m^3\ \mu L^{-1}$ (or 25 cells μL^{-1} equivalent TCg diameter) of a bispecific diet is recommended for *O. edulis* larval rearing.

At 30°C, a mixed diet of *T. lutea* + *C. neogracile* maintained throughout the whole larval life at the concentration of 1500 $\mu m^3\ \mu L^{-1}$ allowed the best larval development of *O. edulis* with high survival (97%), high growth (21 μm day^{-1}) and high settlement (92%). Nevertheless, great care must be taken when rearing larvae at 30°C, especially in terms of feeding requirements and limitation of bacterial proliferation. For that reason and from an economic standpoint, a temperature of 25°C is

recommended for larval production of flat oysters in hatchery.

Lastly, the estimation of the maximum surface-area-specific ingestion rate $\{\dot{J}_{Xm}\} = 120 \pm 4$ μm^3 day^{-1} μm^{-2}, the half saturation coefficient $\{X_K\} = 537 \pm 142$ μm^3 μL^{-1} and the Arrhenius temperature $T_A = 8355$ K put a tangible basis for a future Dynamic Energy Budget (DEB) larval growth model of *O. edulis*.

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