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Franco, Sofia C; Aldred, Nick; Cruz, Teresa; Clare, Anthony S.

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Effects of culture conditions on larval growth and survival of stalked barnacles (*Pollicipes pollicipes*)

Sofia C. Franco[a,b], Nick Aldred[a], Teresa Cruz[b,c], Anthony S. Clare[a]

[a]School of Marine Science and Technology, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom

[b]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Évora , Portugal

[c]MARE – Marine and Environmental Sciences Centre, Laboratório de Ciências do Mar, Universidade de Évora, Portugal

E-mail: sofia.cota-franco@newcastle.ac.uk

Phone: +44(0)191 208 6653

Fax: +44(0)191 208 7891

Address: School of Marine Science and Technology, Newcastle University, Ridley Building, Claremont Rd, Newcastle upon Tyne, NE1 7RU, United Kingdom

Running title: Larval culture of stalked barnacles (*P. pollicipes*)

Abstract

Pollicipes pollicipes (Crustacea: Scalpelliformes) is a highly prized food in Portugal and Spain and consequently a species of considerable interest to aquaculture. Surprisingly, however, larval culture conditions for this barnacle have not been optimised. The present study investigated the effects of temperature, diet, photoperiod and salinity on the growth and survival of *P. pollicipes* larvae. Temperature had a significant effect on specific growth rate (2.6 to 5.9 % total width per day, from 11 to 24 ºC), reducing mean development time to the cyprid from 25 days at 11 ºC to 10 days at 24 ºC, though this was accompanied by a significant increase in mortality to over 90 % above 22 ºC. Mid-range temperatures (15 – 20 ºC) maximised total survival (19 to 31 %, respectively). Algal diets of *Tetraselmis suecica, T. suecica/Skeletonema marinoi* and *S. marinoi/ Isochrysis galbana* did not affect specific growth rate significantly, but survival (on average 39 % in 15 days) and the proportion of high-quality, healthy cyprids were significantly higher on the latter two diets (11 to 15 % of initial number of larvae). Photoperiod did not significantly affect the survival, although specific growth rate was significantly higher at 24:0 and 16:8 L:D. Salinity (20 – 40 gL^{-1} range) did not affect growth and survival significantly. The best growth and survival were accomplished using rearing temperatures of 15 – 20 ºC, daily feeding with *T. suecica/S. marinoi or I. galbana/S. marinoi* and a photoperiod of 24:0 L:D.

Keywords: larval development; temperature; diet; photoperiod; salinity; aquaculture.

1. Introduction

Pollicipes pollicipes (Gmelin, 1790) is one of the most commercially important barnacle species for human consumption (López *et al.*, 2010), being heavily exploited in Spain and Portugal (Borja et al. 2006, Sousa et al. 2013). In recent years, as concerns have grown over stock management, interest has risen in the aquaculture of this species. The few published investigations into culture conditions for *P. pollicipes* (e.g. Goldberg, 1984; Coelho, 1990, 1991; Molares *et al.*, 1994a, 1994b; Candeias, 2005; Cribeiro, 2007; Franco *et al.*, 2015) have often identified larval production and settlement as the main bottlenecks to production. Hatchery production is essential to ensure a regulated supply of larvae throughout the year with controlled timing and yield (Franco *et al.*, 2015), as well as to guarantee high larval quality. Furthermore, larval collection from the wild provides an unreliable solution for aquaculture, possibly presenting conservation issues and conflicts with stock management programmes. Larval culture and settlement of various barnacle species have long been investigated (e.g. Knight-Jones & Stevenson, 1950; Knight-Jones, 1953; Keough & Downes, 1982; Brown & Roughgarden, 1985; Gabbott & Larman, 1987; Maki *et al.*, 1988) and although protocols for species such as *Elminius modestus* (*Austrominius modestus)*, *Balanus improvisus* (*Amphibalanus improvisus*) and *Balanus amphitrite* (*Amphibalanus amphitrite*) are well established (after Moyse, 1963; Tighe-Ford *et al.*, 1970), protocols for others such as *P. pollicipes* are in their infancy.

Hatchery production of *P. pollicipes* larvae encompasses two key phases: the six stages of naupliar development to the cyprid and cyprid settlement, wherein a suitable surface is located on which to attach permanently. Gametogenesis and larval development of *P. pollicipes* have previously been described (Coelho, 1990; Molares *et al.*, 1994a, 1994b) and nauplii have been cultured to the cyprid in several studies despite difficulties in

obtaining high survival to this stage (Coelho, 1990; Molares, 1994; Molares *et al.*, 1994b). Nevertheless, knowledge of the effects of environmental factors and feeding on larval growth and survival of *P. pollicipes* remains poor. In fact, these essential steps are very rarely elaborated upon (e.g. Molares, *et al.*, 1994b), with most of the available information coming from grey literature sources (e.g. Coelho, 1990; Molares *et al.*, 2002; Candeias, 2005). Studies on the remaining Pollicipedidae, including *Capitulum mitella* (Qiu *et al.*, 1994a, 1994b; Lin *et al.*, 1994, 2002; Rao *et al.*, 2010; Zhang *et al.*, 2009) and *Pollicipes polymerus* (Lewis, 1975) are also scarce. In the absence of an integrated investigation of optimal larviculture for *P. pollicipes*, the preliminary works of several authors on larval production systems (Molares, *et al.*, 1994b; Molares *et al.*, 2002), rearing diets (Coelho, 1990, Candeias, 2005) and the effects of temperature and culture density (Coelho, 1990) can provide a basis from which to build. Coelho's (1990) preliminary experiments compared rearing at 15 and 22 ºC and showed that nauplii took longer to develop to stage VI at the lower temperature. The same study also suggested that diets of *Tetraselmis suecica* and *Isochrysis galbana* were required to ensure successful development to nauplius VI, although no data are presented. Candeias (2005), who conducted the most detailed study focusing on larval feeding behaviour, reported that satiation slows with development, varying with algal density and energetic value, and gave an indication of preferred diets, such as *I. galbana* and *Skeletonema costatum*. It was further suggested that nutritional requirements for early and later stages might differ as the larvae develop. Besides the studies developed by the previous authors on diet and temperature, no studies have investigated the effect of photoperiod and salinity on larval development and survival, with the requirements for larviculture of *P. pollicipes* remaining a research gap.

The present study aimed to establish the optimal culture conditions of temperature, diet, photoperiod and salinity for rearing *P. pollicipes* larvae to the cyprid stage. It is hypothesised that there is a range of environmental and dietary conditions that optimise culture performance, relating to natural values experienced in the wild during the reproductive season. Larvae of *P. pollicipes* were reared at a range of natural temperatures (11 to 24 ºC), with different monodiets and mixed diets (of *I. galbana*, *T. suecica* and *Skeletonema marinoi*), short to long-day photoperiod and a broad salinity range (20 to 40 gL^{-1}) and their growth, survival and quality were followed to the cyprid stage.

2. Materials and Methods

2.1 Collection of broodstock and egg lamellae

Clusters of stalked barnacles (*P. pollicipes*) were collected from the south-west coast of Portugal (Cabo Sardão, Portugal, 37°36'24.70", -8°49'2.00") and transported to the rearing facilities at the School of Marine Science of Technology (Newcastle University, UK) within 24h of collection. Barnacles were collected from the same area multiple times during the breeding cycle (July to September) in consecutive years (2011 to 2013). Mature egg lamellae ($n \geq 40$) were immediately dissected from adult barnacles ($n \geq 60$; $\geq$ 12.5 mm rostro-carinal distance, RC), separated and cut into pieces to assist naupliar release. Healthy nauplii were separated from dead nauplii, unhatched embryos and lamella membranes, by attraction to a point light source and then transferred to culture.

2.2 Culture conditions

The newly hatched nauplii were sampled and counted to determine larval numbers and divided into experimental groups, each with three independent replicates per treatment.

Each replicate culture consisted of a 500-ml conical aquarium (JBL Artemio ®) of 0.22 µm filtered natural seawater (NSW), provided with weak bottom aeration. The effect of stocking density was not tested and therefore initial stock densities were kept intentionally low (5 larvae ml^{-1}; approximately 2500 larvae per aquarium). As it was difficult to acquire sufficient numbers of larvae from the same batch to run all experiments simultaneously or allow for a multifactorial design, experiments were run separately with distinct batches of larvae. Overlap in the conditions tested allowed for standardisation between experiments, although each experiment tested a different environmental factor. Four experiments were conducted, testing temperature (experiment 1), diet (experiment 2), photoperiod (experiment 3) and salinity (experiment 4). Standard culture conditions included a temperature of 20 ± 1 ºC (except in experiment 1), light intensity of 2187 ± 102 lux, photoperiod of 16:8 L:D (except in experiment 3), salinity of 33 ± 1 gL^{-1} (except in experiment 4) and antibiotics (0.0232 g l^{-1} penicillin G and 0.0369 g l^{-1} streptomycin). Larvae were fed every 2 days with a mixed diet of *T. suecica* and *I. galbana* (100 000 cells mL^{-1} 1:1) (except in experiment 2) after seawater changes.

2.3 Experimental design

Experiment 1 tested the effect of temperature (11, 15, 20, 22 and 24 ºC) on larval growth and survival. This experiment was divided in two, due to limited larval numbers at any one time, with larvae collected from two distinct batches of adults. The first part tested temperatures of 11 ± 1 °C, 15 ± 1 °C and 20 ± 1 °C, while the second part tested temperatures of 20 ± 1 °C, 22 ± 1 °C and 24 ± 1 °C. Each treatment was kept in separate temperature-controlled incubators (LabHeat© and LabCold© RLCH0400 Incubator Units, UK). Cultures were sampled daily (as described in section 2.4) until over 50 % of

larvae had reached the cyprid stage in all replicates, at which point the treatment was terminated.

Experiment 2 tested the effect of diet on larval growth and survival by using various monodiets and mixed diets of *I. galbana*, *T. suecica* and *S. marinoi*. Diets were as follows: (a) *I. galbana*; (b) *T. suecica*; (c) *S. marinoi*; (d) *I. galbana* and *S. marinoi*; (e) *I. galbana* and *T. suecica*; and (f) *T. suecica* and *S. marinoi*. Cultures were provided with 100 000 cells ml^{-1}, which were in a 1:1 ratio for mixed diets (50 000 cells ml^{-1} of each food item). Algae were cultured in 10-L carboys in autoclaved NSW, 20 ± 1 °C, 37 ± 1 gL^{-1}, 16:8 L:D photoperiod, 2022 ± 58 lux (RS-01 Light Meter©, UK), grown with F/2 medium (Guillard & Ryther, 1962; Guillard, 1975).

Experiment 3 tested the effect of photoperiod on larval growth and survival. The larvae were reared under the following photoperiods: (a) 24:0 L:D; (b) 16:8 L:D; (c) 8:16 L:D; and (d) 0:24 L:D. Treatments were kept in temperature- and photoperiod-controlled incubators (LabHeat© RLCH0400 Incubator Unit, UK), with light intensities of 2245 ± 256 lux (lamps Osram Fluora, L 36W/77, UK).

Experiment 4 examined the effects of salinity (20, 30 and 40 gL^{-1}) on larval growth and survival. Salinity was checked daily with a refractometer (Hand Held Refractometer B+S©) and adjusted accordingly, to account for variations caused by feeding and water evaporation.

2.4 Data collection and analysis

Cultures were sampled (n=3) every other day, before feeding. A given volume was filtered (80 µm mesh) from each culture and the larvae were preserved with Lugol's iodine solution, until analysis. Due to limiting larval numbers and in order to minimise

sampling impact, the volume to be sampled was calculated at every sampling event and adjusted for the changing culture density to ensure that a minimum of 30 nauplii per sample were taken from each replicate. Samples were imaged using a stereo microscope and the total number of larvae counted. Each larva was classified according to developmental stage (following Molares, 1994; Kugele & Yule, 1996), photographed (Olympus© E-410) and measured using the software Image J© (v1.49).

Total width for nauplii (TW) and carapace width for cyprids (CW) were used to estimate the larval greatest width (LGW). Stage progression index (SPI, #), calculated using the total number of larvae per each developmental stage at any one time, was used as a measure of larval development through the culture period. This was calculated by [$SPI=(\sum^{n}_{i=1}(n_i \times s_i))/n$], where n is total number of larvae, n_i is number of individuals at development stage i, and s_i is development stage i (with i=1, 2, 3, 4, 5, 6 and 7, respectively for nauplii I, II, III, IV, V, VI and cyprids) at a given time. Larval specific growth rate (SGR, %TW d^{-1}), which factored nauplius TW and total time of development until nauplius VI dominance, was used to assess naupliar growth. This was calculated as [$SGR=(ln((avTW_f)/avTW_i)/(t_f-t_i)\times 100)$], where SGR is specific growth rate as a percentage of total width increase per day, $avTW$ is the average naupliar total width per replicate, t_i is initial time or day 0 when 50 % of larvae are nauplius I and t_f is final time when 50 % of larvae reached nauplius VI. Median culture development time from nauplius I to the cyprid (MDT, days or d) was calculated at the time when sampling ended by [$MDT=t_f-t_i$)], where t_i is initial time or day 0 when 50 % of larvae are at nauplius I and t_f is final time or day when ≥ 50 % larvae are cyprids.

Each culture was followed until the majority comprised cyprids (≥ 50 %), at which time the experiments were terminated by filtering the culture through a 60 µm mesh to

collect all larvae. The larvae were then counted, measured and classified according to stage. These final samples were used to determine the total number of cyprids produced and to visually estimate the proportion of high-quality larvae. For the visual estimation, all larvae (nauplii and cyprids) were compared for activity (static or active), appearance of appendages (damaged/protruded or normal) and fouling of the larvae (fouled or unfouled), with cyprids further observed for the presence of lipid droplets (absent or present). Larvae were classified as high-quality larvae (H-Q larvae, %) if they satisfied the majority of the above-mentioned criteria for healthy larvae (i.e. larvae that were active, had healthy appendages, were unfouled and had visible lipidic droplets when at the cyprid stage). The percentage of high-quality larvae H-Q larvae (HQ_l, %) was calculated by [$HQ_l=nHQ_l/n_f$], where nHQ_l is the number of high-quality larvae collected at the end of the experiment and n_f is the total number of collected larvae. The percentage of cyprids (C, %) was calculated by [$C=nC/n_f$], where nC is the number of cyprids collected at the end of the experiment and n_f is the total number of larvae collected. Both the percentage of H-Q larvae and cyprids were calculated in relation to the total number of larvae collected in each replicate at the end of the experiment. The total percentage of high-quality cyprids (H-Q cyprids, %) was calculated with regard to the number of larvae initially stocked per replicate and the number of high-quality cyprids counted at the end of the experiment, as [$HQ_c=nHQ_c/n_i$], where nHQ_c is the number of high-quality cyprids collected at the end of the experiment and n_i is the total number of larvae initially stocked at day 0.

Percentage survival (tS, %) was calculated at the end of the experiment using [$S=n_f/n_i$], where n_f is the total number of larvae at the time point where sampling was terminated and n_i is the total number of larvae initially stocked at day 0. The survival rate (dS, % d^{-1}) and mortality rate (dM, % d^{-1}) were calculated on a daily basis. Although sampling

effort was adjusted to minimise impact, sampling was nevertheless extractive. The removal of larvae was not accounted for in the calculations (values presented are not-corrected), but the maximum impact of sampling was estimated, considering the numbers of extracted larvae per replicate, and implications for survival will be discussed later.

All variables were calculated per replicate and results were analysed using Statistica ® (v.7.0.61.0, StatSoft Inc.) at a significance level of 0.05. Analyses were carried out to determine homogeneity of variance (Levene's test) and normality (Kolmogorov-Smirnov test), while significant differences were detected using one-way ANOVA or ANCOVA with time as a covariate where appropriate. Post-hoc investigation used Tukey's HSD test when relevant. Data in percentage format were arcsine transformed prior to analysis. All results are presented as mean ± standard error, unless stated otherwise.

3. Results

3.1 Temperature

Specific growth rate (SGR) was significantly influenced by both the low (ANOVA, $F=57.50$, $p<0.01$) and high (ANOVA, $F=48.47$, $p<0.01$) temperatures. When both experiments were analysed concurrently, the data for the overlapping temperature (20 ºC) did not differ significantly between experiments (ANOVA, $F=57.5$, $p<0.01$; Tukey Test $p=0.89$) implying that data collected from the experiment at higher temperatures could reasonably be used to extrapolate from the end of the low-temperature experiment. Higher temperatures led to higher SGR, varying from 5.93 ± 0.35 % TW d^{-1} at 24 ºC to 2.60 ± 0.08 % TW d^{-1} at 11 ºC (Table 1). Differences were significant amongst all of the temperatures (Tukey Tests, $p<0.01$) with the exception of both values

recorded at 20 ºC. Accordingly, median development time (MDT) showed an identical pattern, varying significantly (ANOVA, $F=86.03$, $p<0.01$) from 25 days at 11 °C to 10 days at 24 °C, with no differences at 20 °C (Tukey Test, $p=0.97$).

Larvae increased in size with time (Fig. 1) despite a different SGR for each temperature (Table 1), to a stabilisation point that coincided with the appearance of cyprids. Once cyprids comprised >50% of the culture, average larval greatest width (LGW) decreased slightly due to the smaller size of cyprids compared to nauplius VI. No significant differences were detected in cyprid carapace width (CW) for cultures reared at different temperatures (low temperatures: ANOVA, $F=0.31$, $p=0.73$; high temperatures; ANOVA, $F=0.25$, $p=0.81$; and overlapping temperature of 20 °C, ANOVA, $F=0.27$; $p=0.79$). CW averaged 209.03 ± 1.92 µm. Larval stage progression index (SPI) gave a clearer view of stage dominance (Fig. 2), particularly when the majority of the larvae were cyprids. At this point, high mortality was observed if no appropriate settlement substrata were provided. Asynchronous development was more noticeable in cultures raised at lower temperatures (e.g. 11–15 ºC).

Larval survival decreased steadily with time at all temperatures (Fig. 3). At low temperatures, daily mortality rate did not vary significantly according to temperature (ANCOVA, $F=2.01$, $p=0.14$), however overall survival was significantly different (ANOVA, $F=59.10$, $p<0.01$) due to differences in MDT. However, temperatures ≥ 20 ºC had a significant effect on daily mortality rate (ANCOVA, $F=5.43$, $p<0.01$) in addition to the expected effect on survival (ANOVA, $F=64.55$, $p<0.01$). When the survival results from both temperature experiments were analysed together, significant differences were evident (ANOVA, $F=1.59$, $p<0.03$), while both 20 °C treatments produced similar results (Tukey Test, $p=0.99$). Survival was significantly lower (Tukey Test, $p<0.01$) at 11, 22 and 24 °C (not significantly different; Tukey Test, $p\geq0.89$)

compared to 15 and 20 °C (not significantly different; Tukey Test, $p \geq 0.99$; see Table 1). Survival increased significantly with increasing temperature until optimal conditions were achieved (Table 1), from 10.81 ± 5.26 % at 11 °C to 26.99 ± 4.56 % at 20 °C (average). Temperatures of 22 and 24 °C led to mortalities above 90 %, equivalent to less than 250 surviving larvae per replicate. It is noted that the sampling process could have decreased the estimate of total survival by up to 15 % in the various treatments, depending on the median development time and respective sampling pressure.

In a similar way to the difference in survival, there were significant differences in the percentage of high-quality cyprids produced at different temperatures (H-Q cyprids; ANOVA, $F=16.69$, $p<0.01$). This was significantly higher (Tukey Test, $p<0.03$) at 15 °C and both 20 °C treatments (not significantly different, Tukey Test, $p \geq 0.57$; average of 13.46 ± 0.89 % of initial larvae) in comparison to larvae reared at 11, 22 and 24 °C (not significantly different, Tukey Tests, $p \geq 0.99$; average of 5.07 ± 0.59 % of initial larvae). There were no differences in the percentage of high-quality larvae collected (H-Q larvae; ANOVA, $F=2.84$, $p=0.06$), on average 62.33 ± 0.94 %, or cyprids (ANOVA, $F=1.62$, $p=0.23$), on average 89.22 ± 1.20 %.

3.2 Diet

There were no significant differences in SGR (ANOVA, $F=1.87$, $p=0.17$) according to diet (Table 2). LGW and SPI showed similar patterns of change over time between treatments (data not shown). Similarly, CW was the same irrespective of larval diet (ANOVA, $F=0.41$, $p=0.65$; Table 2). Average SGR varied between 3.17 and 3.78 % TW d^{-1} (Table 3). The differences observed had only a minor influence on MDT, which varied between 15 and 16 days (Table 3) and were not significantly different between diets (ANOVA, $F=0.23$, $p=0.88$).

Due to the lack of differences in MDT, sampling had a similar impact across treatments, where approximately 9 % of larvae were sampled. Survival varied significantly with diet (ANOVA, $F=9.93$, $p<0.01$) (Table 2). The significant differences in total survival were also reflected in similar differences in daily mortality. Larvae grown on diets of *T. suecica*, *T. suecica/S. marinoi* and *I. galbana/S. marinoi* had significantly higher survival (Tukey, $p\leq0.01$); although the mixed diets did not differ significantly from each other in supporting successful development of larvae (Tukey, $p\geq0.99$). The lowest survival (Tukey, $p\leq0.01$) was recorded with diets of *I. galbana,* followed by *T. suecica/ I. galbana* and *S. marinoi*, which were not significantly different (Tukey, $p\geq0.99$; Table 2). Mortalities were noted in the first day of culture on the diet of *I. galbana*, compared to more gradual mortality for the other diets, with survival decreasing steadily with time. Independently, none of the diets tested was toxic to the larvae and all were capable of sustaining development beyond nauplius stage II (the first feeding stage), in spite of different degrees of success.

Interestingly, when larvae were assessed for quality (Table 3), there were significant differences between treatments (ANOVA, $F=89.92$, $p<0.01$). Diets of *S. marinoi* and *I. galbana/ S. marinoi* led to a higher percentage of H-Q larvae (69.67 ± 7.09 and 63.67 ± 6.51 %, respectively; not significantly different; Tukey, $p=0.74$), followed by *T. suecica/ S. marinoi* (not different from *I. galbana/S. marinoi*; Tukey, $p=0.17$), and *T. suecica* and *T. suecica/ I. galbana* (not different from each other; Tukey, $p=0.95$), with *I. galbana* leading to the lowest larval quality (Tukey, $p<0.01$), with no H-Q larvae observed. Similarly, the percentage of larvae that developed to the cyprid stage varied significantly with diet (ANOVA; $F=179.36$; $p<0.01$). This was higher for mixed diets of *T. suecica* and *I. galbana* with *S. marinoi* (90.33 ± 4.00 %; inter-diet comparison not significantly different; Tukey, $p=1.00$), followed by monodiets of *S. marinoi* (49.67 ±

311 1.45 %; Tukey, p<0.01; Table 3) and *T. suecica/ I. galbana* (32.67 ± 3.48 %; Tukey,
312 p<0.01). The lowest percentage of cyprids was observed in cultures reared on
313 monodiets of *T. suecica* (9.67 ± 2.03 %), with no cyprids observed in cultures reared on
314 *I. galbana* (Table 3). Most importantly, the final number of H-Q cyprids, obtained from
315 the initial larvae, was significantly different (ANOVA, F=24.39, p<0.01) for cultures
316 reared with mixed diets containing *S. marinoi*, either provided together with *I. galbana*
317 or *T. suecica*, where respectively 15.15 ± 2.62 % and 11.32 ± 4.66 % of initial nauplii
318 became H-Q cyprids. The remaining diets produced, on average, less than 2 % of
319 cyprids from the initial stock (Table 3).

320 3.3 Photoperiod

321 Photoperiod affected larval SGR significantly (ANOVA, F=223.47, p<0.01) and
322 consequently MDT to the cyprid (ANOVA, F=17.18, p<0.01). SGR varied on average
323 between 4.03 and 5.27 % TW d^{-1} (Table 4). Larvae grown on a 24:0 L:D cycle had the
324 highest SGR, though not significantly different from a 16:8 L:D (Tukey, p=0.79),
325 followed by 8:16 L:D, while the 0:24 L:D treatment had the lowest growth rate (all
326 significantly different; Tukey, p<0.01). The increase in LGW with time is noticeable in
327 Figure 4a and correlates with differences in the MDT to the cyprid (Table 4) and SPI
328 (Fig. 4b). There was, however, no significant difference in CW (ANOVA, F=0.35,
329 p=0.71). Furthermore, larvae grown in full-day photoperiod developed into cyprids in
330 14 days, significantly faster than other photoperiods (Tukey Test; p≤0.03). Results from
331 MDT for long and short-day photoperiods were not significantly different from each
332 other (Tukey Test; p=0.96) and the longest development time (17 days) was in full-
333 darkness. The early appearance of nauplius VI and cyprids in these cultures (clearly
334 visible after day 11, approximately 4 days in advance of the other photoperiods (Fig. 4
335 a, b) was also consistent with the shorter development time in full-illumination.

Nevertheless, though survival varied between treatments, these differences were not significant (ANOVA, F=2.39, p=0.08), in spite of the markedly different development times (with sampling removing 9–10 % of larva). Cultures grown at 0:24 L:D, 16:8 L:D, 24:0 L:D and 8:16 L:D had survival of 21.94 ± 5.45 %, 24.44 ± 4.12 %, 27.00 ± 3.01 % and 37.33 ± 4.42 %, respectively (Table 4).

There were no significant differences between cultures maintained under different photoperiods with respect to the percentage of H-Q larvae (ANOVA, F=1.57, p=0.27), percentage of cyprids (ANOVA, F=3.02, p=0.09) and percentage of H-Q cyprids (ANOVA, F=2.52, p=0.13), which were 60.00 ± 1.90 %, 87.25 ± 1.69 % and 14.82 ± 0.84 % of the initial number of larvae, respectively.

3.4 Salinity

No effects of salinity were observed (20, 30 and 40 gL^{-1}) on larval SGR (ANOVA, F=0.66, p=0.52), survival (ANOVA, F=0.87, p=0.42), MDT to the cyprid (ANOVA, F=0.13, p=0.98), or CW (ANOVA, F=0.22, p=0.91), with approximately 8 % of larvae sampled in total per replicate. The results are summarised in Table 5. SGR, total survival and MDT averaged 4.18 ± 0.05 % d^{-1}, 24.26 ± 3.89 %, and 15 days, respectively. LGW and SPI showed similar variation with time, independent of treatment (data not shown). There were no significant effects of salinity on the percentage of H-Q larvae (ANOVA, F=1.29, p=0.34), percentage of cyprids (ANOVA, F=2.81, p=0.14) or percentage of H-Q cyprids (ANOVA, F=0.25, p=0.79), which were similar to the other experiments investigating photoperiod and temperature (data not shown).

4. Discussion

4.1 Temperature

Temperature had a significant effect on specific growth rate (SGR), as had been reported previously for larvae of *P. polymerus* (Lewis, 1975), *C. mitella* (Zhang *et al.*, 2009; Rao *et al.*, 2010) and *P. pollicipes* (Coelho, 1990). The differences in SGR can be explained by the effects of increasing temperature on metabolism. The literature suggests a minimum development time for *P. pollicipes* from hatching to the cyprid of 23 to 28 days at 20 ºC (Molares *et al.*, 1994b), 14 days at 17.5 ºC (to nauplii VI; Candeias, 2005) and 11 to 24 days (average 15 days) at 15 to 24 ºC (Kugele & Yule, 1996). In the present study, medium development time (MDT) ranged from 10 to 25 days between 24 °C and 11 °C, and was 15 to 16 days at 20 °C. These results are in line with the previous reports, except for the study by Molares *et al.* (1994b), where development time was longer. This discrepancy was most likely due to considerable differences in feeding quantity and quality between the two studies. The differences in SGR with temperature, reflected in lower development times at higher temperatures, are in accordance with Coelho (1990); to our knowledge the only study to date on the effect of temperature on *P. pollicipes* larval growth. Coelho (1990) reported that the duration of development from nauplius stage I to nauplius VI decreased from 20 days at 15 ºC to 9 days at 22 ºC, but no details were provided of specific growth rate or survival. Rather, the time of appearance of each stage was reported, which provides limited information about culture performance. Similarly, for *C. mitella* with its longer larval phase (Qiu *et al.*, 1994a), higher temperatures have been reported to decrease development time from 11 days (at 24 ºC) to 7 days (at 30 to 31 ºC) (Zhang *et al.*, 2009; Rao *et al.*, 2010).

There was a direct relation between SGR and survival at the lower temperature range, as the higher the SGR and SPI, the lower the overall mortality. However, at higher temperatures (>20 ºC), daily mortality rate increased with temperature, creating an opposing trend. This is in accordance with Lewis (1975) for *P. polymerus*, who

suggested that higher temperatures can increase the growth rate, while fitness and survival. Likewise, Coelho (1990) reported mass mortality at 22 ºC. The question therefore remains, what would be the highest temperature at which the decrease in development time does not correlate with an increase in mortality? Overall survival varied between 19 to 31 % at an optimal temperature range of 15 to 20 ºC, to values below 11 % at 11 ºC and above 22 ºC. Temperatures between 14 to 20 °C are within the range normally experienced by this species in its natural habitat, from May to September (Sines, Portugal; Instituto Hidrográfico 2015), during the breeding season (Cruz 2000). The fact that *P. pollicipes* survival dropped when reared above 20 ºC and virtually no cyprids developed at 24 ºC, raises concerns over using temperatures above 20 ºC. Lower survival at higher temperatures could also be ascribed to a putative decrease in water quality at these temperatures or eventual proliferation of contaminating species, as reported by Candeias (2005). From our experience, most of the culture problems resulted directly from the long development time (a minimum of 15 days at 20 ºC). This is much longer than commonly cultured barnacles, such as *B. amphitrite* and *B. improvisus*, whose cyprids can be obtained in 4 to 5 days (28 °C; Franco, pers. obs.). In fact, several authors (Molares, 1994; Candeias, 2005; Coelho, 1990) have reported very high mortality (≥ 85 %) during the first two weeks of rearing, which highlights the importance of decreasing the rearing period for this species and improving culture conditions. Furthermore, the effect of sampling in the present study also impacted the measure of survival leading to a slight underestimation. Although each replicate would ideally have been stocked initially with sufficient larvae for the impact of sampling to be negligible, available methods for larval collection were limiting. A very large number of wild-collected adults would also have been required, as these in turn often

showed high variability in maturity and contained egg lamellae at different stages of development.

While a clear indication of best rearing temperature was obtained, survival was poor compared to *C. mitella*, for which total survival to the cyprid ranged from 90 to 99 % and metamorphosis from 73 to 81 % at the optimal temperature of 27 °C (Rao *et al.* 2010). For *P. pollicipes*, close monitoring of cultures is also required so that they can be filtered when there are >50 % cyprids, as high mortality can occur and cyprid quality can decrease abruptly at this point. Results for other species (e.g. *B. amphitrite*; Costlow & Bookhout, 1959) also suggest that the transition from the last nauplius stage to the cyprid is a particularly vulnerable point in the life cycle, with increased mortality occurring prior to the cyprid stage.

4.2 Diet

Feeding and nutritional requirements are of paramount importance to the successful larval culture of *Pollicipes* spp. (Moyse, 1963; Lewis, 1975). The only studies to investigate larval diet were conducted by Lewis (1975) for *P. polymerus*, and by Coelho (1990) and Candeias (2005) for *P. pollicipes*. For *P. pollicipes*, consumption rate varied according to algal species provided as food (Candeias, 2005). From the diets tested in the present study, no significant differences were detected for SGR and MDT, although monodiets of *T. suecica* gave the highest growth rates. Diet did not significantly influence growth and development time, but it did significantly affect survival, which ranged from 13 to 40 %. Unlike the results from previous studies (Coelho, 1990), all the diets tested allowed progression through the naupliar stages, though not necessarily to the cyprid and the production of high-quality cyprid larvae (H-Q cyprids). Inadequacy of diet may be due to poor nutritional profile and algal bioavailability, with inadequate feeding causing mechanical interference with swimming (e.g. live preys become trapped

in the larval appendages), the accumulation of lethal metabolites, deficient development and precocious death (Franco, pers. obs.; Moyse, 1963; Lewis, 1975).

Highest survival was attained with diets of *T. suecica* alone, and mixed diets of *T. suecica* or *I. galbana*, with *S. marinoi*. Furthermore, the larvae fed with the *S. marinoi* monodiet and mixed diets containing this species had the highest percentage of high-quality larvae (H-Q larvae) and survival to the cyprid. Thus, while *S. marinoi* alone proved sub-optimal for larval development, when it was used in conjunction with *T. suecica* or *I. galbana* it gave good results with respect to larval growth and survival to the cyprid. Candeias (2005) concluded that all *P. pollicipes* larval stages show a preference for ingesting *I. galbana* and *S. costatum* and, to a lesser extent, *T. suecica*. Additionally, mixed diets and flagellate-based diets (*Rhinomonas reticulata*) produced higher survival than monodiets of diatoms, namely *S. marinoi* (Candeias, 2005), as also observed in the present study. Despite the general preference of temperate species for flagellates (Moyse, 1963; Stone, 1988), Candeias (2005) suggested that *P. pollicipes* might have developed a propensity for feeding on diatoms due to to seasonal upwelling.

In spite of the poor survival results obtained in this experiment with mixed diets of *T. suecica*/*I. galbana*, results from the other experiments undertaken in similar conditions where the same diet was used (e.g. experiments 1 and 3, for treatments cultured in similar conditions) recorded an average higher survival (20 to 30 % survival) for this diet. Independently, the poor survival results here observed with *I. galbana* are in accordance with results from previous experiments.

Coelho (1990) also concluded that from monodiets of *S. costatum*, *T. suecica*, *Thalasiosira pseudonana*, *I. galbana*, *Chaetoceros gracilis* and *Chorella* sp., only *T. suecica* and *I. galbana* supported larval development to the cyprid, with remaining

458 monodiets not sustaining development beyond nauplius stages II and III. This
459 contradicts the results reported here, where monodiets of *S. marinoi* also sustained
460 development to the cyprid stage. However, none of the diets used in this study resulted
461 in more than 15% of nauplii surviving to H-Q cyprids so there is considerable scope for
462 improvement.

463 Optimising the diet of *P. pollicipes* larvae is essential to close the life cycle of this
464 species in culture as settlement may otherwise be compromised. Settlement of *B.*
465 *amphitrite* cyprids was lower, for example, when reared on *Dunaliella tertiolecta*
466 compared to *S. costatum*. (Clare *et al.* 1994). From the current results, mixed diets
467 containing *S. marinoi* produced maximal survival, H-Q larvae and percentage of
468 cyprids. Therefore, future studies could investigate effects of diet on cyprid settlement,
469 providing that suitable settlement surfaces can be identified; *P. pollicipes* being averse
470 to settling on artificial surfaces in captivity (e.g. Coelho, 1991; Kugele & Yule, 1996).

471 4.3 Photoperiod

472 Although there have been no studies into the effects of light exposure on *P. pollicipes*
473 larval growth and survival, there have been studies of how light affects behaviour
474 (Molares *et al.*, 2002). *P. pollicipes* larvae are phototactic and Molares (1994)
475 established that most of the time this will manifest as positive phototaxis. This
476 characteristic has long been used for selection of the healthiest larvae, by attraction to
477 light. The prevailing choice in previous studies has been to use natural photoperiod (e.g.
478 Coelho, 1990) or full day photoperiod (Molares 1994; Molares *et al.*, 1994b), due to the
479 lack of supporting evidence for any other photoperiod being preferable. In the present
480 study, larval growth was affected by photoperiod but there was no significant effect on
481 survival. Larvae grown in 24:0 L:D, short and long day photoperiod had the highest
482 SGR. Longer-day photoperiods can directly promote algal proliferation, allowing for

greater food availability for an extended period of time, which may contribute to faster growth in the full-light photoperiod. In the Crustacea, there is high variability in response to photoperiod during larval rearing (e.g. *Strombus pugilis,* Andrés *et al.*, 2010; *Portunus pelagicus,* Bermudes & Ritar, 2008; *Jasus edwaedsii*, Manzano *et al.*, 1998) and therefore species-specific protocols are required. The present results support the use of longer-day photoperiods, which is in accordance to what has been used to date by previous authors. Furthermore, it is interesting to note that during the peak of *P. pollicipes*' breeding season (June to August) temperature normally approximates 20 °C and photoperiod ranges between 14 – 15 hours of light, agreeing with the present results for best larval performance.

4.4 Salinity

The effect of salinity on the growth and survival of marine invertebrate larvae has received considerable attention. Unnatural regimes have proven beneficial in species of bivalves, crustaceans and fish, and several authors working with larvae (e.g. *Penaeus semisulcatus*, Innes & Haley, 1977; *Mytilus edulis*, Fonds, 1979; *Solea solea.* Kumlu *et al.*, 2000) have shown that higher salinities, within a tolerance range, tend to enhance growth. Lower salinities, on the contrary, often improve survival. In the case of *P. pollicipes* larval culture, salinities reported in the literature have usually been within the natural range of salinities 33 to 34 gL^{-1} (Coelho, 1990; Molares, 1994; Molares *et al.*, 1994a). The effect of salinity on a species will depend largely on the ability to osmoregulate at early stages of development (e.g. Charmantier, 1998) and whether or not there are effects on the biochemical composition of the larvae such as lipid reserves (e.g. Torres *et al.*, 2002). In the present study, there was no effect of salinity on larval survival, SGR, MDT and CW, in the range 20 to 40 gL^{-1}, indicating that *P. pollicipes* is highly tolerant within this range, in spite of the salinities tested spanning beyond the

ones found in the natural habitat. In the wild, *P. pollicipes* adults are normally found at salinities of 33 to 37, although salinity in the intertidal environment can vary significantly due to evaporation in the hours of air exposure and rainfall. In spite of the limitations of this work, where the interactions of factors (e.g. temperature *vs*. salinity) were not studied due to insufficient larval numbers, results suggest that *P. pollicipes* can survive throughout this range.

5. Conclusions

Based on the present results, improved growth and survival can be accomplished using rearing temperatures of 15 to 20 ºC, daily feeding with *T. suecica/S. marinoi or I. galbana/S. marinoi* and a photoperiod of 24:0 L:D. The use of higher temperatures (22 and 24 ºC) significantly increased mortality and reduced the number of cyprids, while lower temperatures (11 to 20 ºC) extended the growth period, which led to higher mortality. In practical terms, the decision as to which temperature to use within this range (15 to 20 ºC) should rely upon the relative benefits of having a longer or shorter growth period in any given situation. In spite of no differences in specific growth rate with regard to diet, *T. suecica/S. marinoi* or *I. galbana/S. marinoi* assured the best results in terms of the percentage of high-quality cyprids produced, therefore maximising rearing efficiency. Photoperiod, on the other hand, affected growth with better results in 24:0 and 16:8 L:D photoperiods, but with median development time reduced by full-day photoperiods. A 24-h period of darkness should be avoided. No effects were observed with regard to salinity, suggesting that *P. pollicipes* larvae tolerate a wide range of salinities. In order to improve water quality and reduce maintenance costs, while decreasing handling of larvae, further research could focus on the use of recirculating systems for larviculture. As there are currently no viable protocols for ensuring settlement of *P. pollicipes* larvae in the laboratory (see Kugele &

533 Yule, 1996), it would also be prudent to build upon the present results and further
534 investigate the implications of larval culture conditions towards competence to settle
535 and ability to complete metamorphosis.

536

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672

673 **Figure legends**

674 Fig. 1 Average larval greatest width (µm) according to culture time (days), for larvae
675 grown at: a) low temperatures (11, 15, 20 °C) and b) high temperatures (20, 22 and 24
676 °C). Values are presented as mean ± SE.

677

678 Fig. 2 Larval stage progression (#) according to culture time (days), for larvae grown at:
679 a) low temperatures (11, 15, 20 °C) and b) high temperatures (20, 22 and 24 °C). Stages
680 were assigned as follows: 1) nauplii I, 2) nauplii II, 3) nauplii III, 4) nauplii IV, 5)
681 nauplii V, 6) nauplii VI, and 7) cyprids. Values are presented as mean ± SE.

682

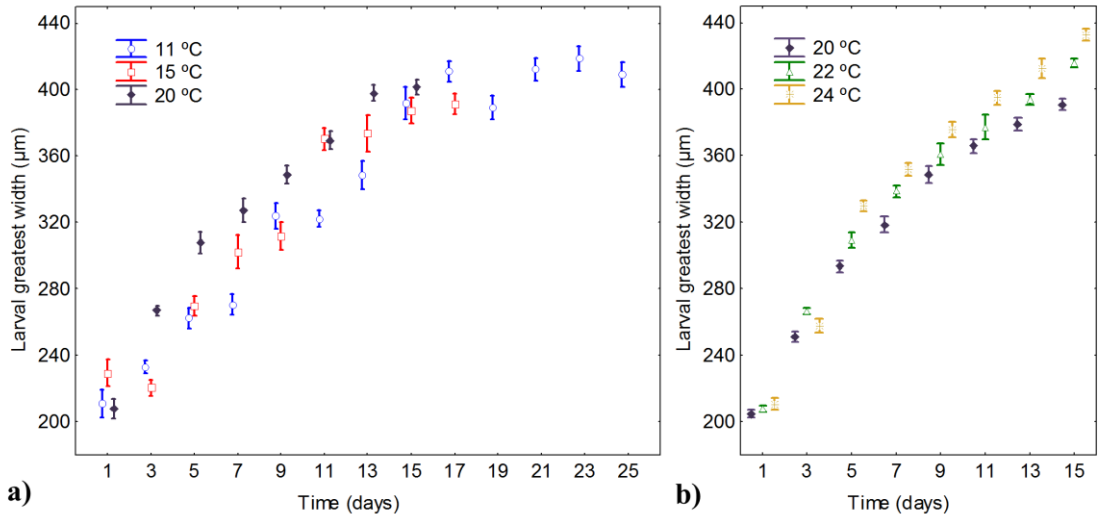
683 Fig. 3 Survival (%) according to culture time (days), for larvae grown at: a) low
684 temperatures (11, 15, 20 °C) and b) high temperatures (20, 22 and 24 °C). Values are
685 presented as mean ± SE.

686

687 Fig. 4 (a) Larval greatest width (µm), and (b) stage progression index (#) according to
688 culture time (days), for larvae grown at four different photoperiods, namely full dark
689 photoperiod (0:24 L:D), 16 h of light and 8 h of darkness (16:8 L:D), 8 h of light and 16
690 h of darkness (8:16 L:D), and full light photoperiod (24:0 L:D). Values are presented as
691 mean ± SE.

692

693 **Figures**



694
695 Fig. 1
696
697

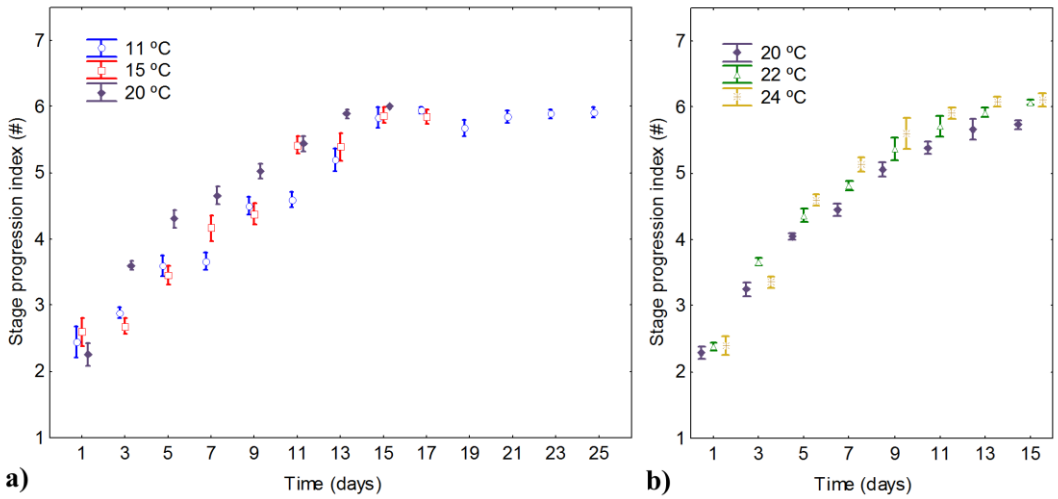

11 °C
15 °C
20 °C
Stage progression index (#)
Time (days)
a)
20 °C
22 °C
24 °C
Stage progression index (#)
Time (days)
b)

Fig. 2

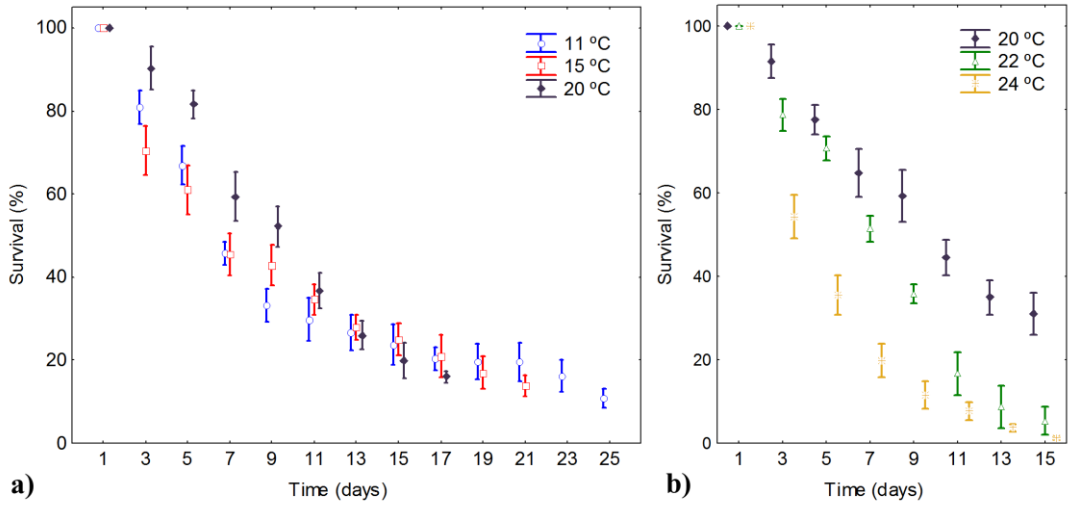
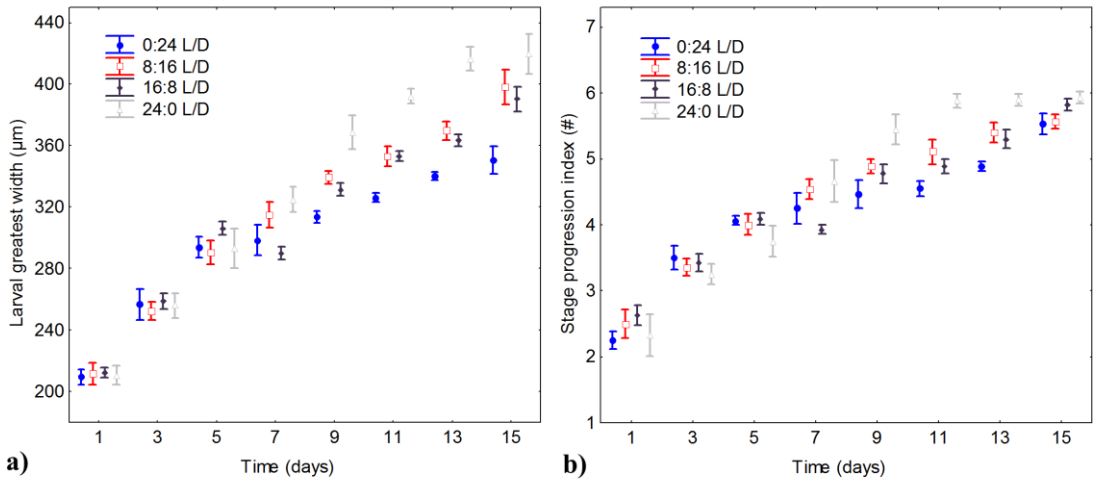

Survival (%)
Time (days)
11 °C
15 °C
20 °C
a)
Survival (%)
Time (days)
20 °C
22 °C
24 °C
b)

Fig. 3


0:24 L/D
8:16 L/D
16:8 L/D
24:0 L/D
Larval greatest width (µm)
Time (days)
a)
Stage progression index (#)
Time (days)
b)

705
706 Fig. 4

Tables

Table 1. Specific growth rate (SGR, % d^{-1}), total survival to cyprid (tS, %), median development time to cyprids (MDT, days) and cyprid width (CW, µm), for larvae grown at low temperatures (11, 15, 20 °C) and high temperatures (20, 22 and 24 °C). Values are presented as mean ± SE. Different superscripts indicate significant differences, for each parameter, following ANOVA and Tukey's HSD tests.

Experiment	T (ºC)	SGR (% d^{-1})	tS (%)	MDT (d)	CW (µm)
Low temp.	11	2.60 ± 0.08^{a}	10.81 ± 5.26^{a}	24.83 ± 0.29^{a}	200.00 ± 7.96^{a}
	15	2.97 ± 0.10^{b}	19.08 ± 2.83^{b}	17.67 ± 0.58^{b}	209.65 ± 3.52^{a}
	20	4.57 ± 0.15^{c}	22.87 ± 3.86^{b}	15.23 ± 0.68^{c}	211.78 ± 2.34^{a}
High temp.	20	4.50 ± 0.14^{c}	31.11 ± 5.26^{b}	16.06 ± 0.39^{c}	205.12 ± 4.52^{a}
	22	5.31 ± 0.47^{d}	8.69 ± 5.38^{a}	13.45 ± 0.51^{d}	208.24 ± 3.83^{a}
	24	5.93 ± 0.35^{e}	7.67 ± 2.29^{a}	9.81 ± 0.25^{e}	203.03 ± 2.54^{a}

715 Table 2. Specific growth rate (SGR, % d^{-1}), total survival to cyprid (tS, %), median development time to
716 cyprids (MDT, days) and cyprid width (CW, µm), for larvae cultured using various diets, of *Tetraselmis*
717 *suecica*, *Isochrysis galbana* and *Skeletonema marinoi*, presented as both mono- and mixed-diets. In
718 treatments where cyprids were not observed, median development time was classified as *n.a.* - not-
719 applicable. Values are presented as mean ± SE. Different superscripts indicate significant differences, for
720 each parameter, following ANOVA and Tukey's HSD tests.

Diet	SGR (% d^{-1})	tS (%)	MDT (d)	CW (µm)
T. suecica	3.78 ± 0.28 ^{a}	38.30 ± 6.65 ^{a}	14.99 ± 2.54 ^{a}	212.35 ± 3.62 ^{a}
I. galbana	3.17 ± 0.38 ^{a}	13.03 ± 3.03 ^{b}	n.a.	n.a.
S. marinoi	3.40 ± 0.30 ^{a}	16.89 ± 2.26 ^{b}	15.30 ± 2.88 ^{a}	209.45 ± 2.99 ^{a}
T. suecica /I. galbana	3.39 ± 0.28 ^{a}	14.60 ± 3.93 ^{b}	15.32 ± 3.55 ^{a}	210.98 ± 3.17 ^{a}
I. galbana /S. marinoi	3.26 ± 0.24 ^{a}	38.72 ± 8.60 ^{a}	16.05 ± 3.18 ^{a}	207.15 ± 4.03 ^{a}
T. suecica /S. marinoi	3.43 ± 0.17 ^{a}	40.82 ± 2.37 ^{a}	15.27 ± 2.54 ^{a}	202.86 ± 3.88 ^{a}

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Table 3. Percentage of high-quality larvae (H-Q larvae, %), cyprid larvae (Cyprids, %) and high-quality cyprids (H-Q cyprids, %) obtained after rearing and larvae collection, for larvae cultured using various diets, of *Tetraselmis suecica*, *Isochrysis galbana* and *Skeletonema marinoi*, presented as both mono- and mixed-diets. Values are presented as mean ± SE. Different superscripts indicate significant differences, for each parameter, following ANOVA and Tukey's HSD tests.

Diet	H-Q larvae (%)	Cyprids (%)	H-Q cyprids (% n)
T. suecica	14.33 ± 4.04 ^{a}	9.67 ± 2.03 ^{a}	0.21 ± 018 ^{a}
I. galbana	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
S. marinoi	69.67 ± 7.09 ^{c}	49.67 ± 1.45 ^{b}	1.27 ± 2.19 ^{a}
T. suecica /I. galbana	18.00 ± 5.57 ^{a}	32.67 ± 3.48 ^{b}	0.20 ± 0.35 ^{a}
I. galbana /S.marinoi	63.67 ± 6.51 ^{cd}	90.33 ± 3.76 ^{c}	15.15 ± 2.62 ^{b}
T. suecica /S. marinoi	52.33 ± 5.51 ^{d}	90.33 ± 4.26 ^{c}	11.32 ± 4.66 ^{b}

Table 4. Specific growth rate (SGR, % d^{-1}), total survival to cyprid (tS, %), median development time to cyprids (MDT, days) and cyprid width (CW, µm), for larvae cultured at various photoperiods. Values are presented as mean ± SE. Different superscripts indicate significant differences, for each parameter, following ANOVA and Tukey's HSD tests.

Photoperiod	SGR (% d^{-1})	tS (%)	MDT (d)	CW (µm)
0:24 L:D	4.03 ± 0.05 ^{a}	21.94 ± 5.45 ^{a}	16.67 ± 0.33 ^{a}	206.76 ± 2.76 ^{a}
8:16 L:D	4.87 ± 0.07 ^{b}	37.33 ± 4.42 ^{a}	15.33 ± 0.31 ^{b}	208.87 ± 3.55 ^{a}
16:8 L:D	5.22 ± 0.08 ^{c}	24.44 ± 4.12 ^{a}	15.13 ± 0.11 ^{b}	204.45 ± 3.71 ^{a}
24:0 L:D	5.27 ± 0.07 ^{c}	27.00 ± 3.01 ^{a}	13.67 ± 0.29 ^{c}	211.27 ± 3.82 ^{a}

739 Table 5. Specific growth rate (SGR, % d^{-1}), total survival to cyprid (tS, %), median development time to
740 cyprids (MDT, days) and cyprid width (CW, µm), for larvae cultured at various salinities (20, 30 and 40
741 psu). Values are presented as mean ± SE. Different superscripts indicate significant differences, for each
742 parameter, following ANOVA and Tukey's HSD tests.

Salinity	SGR (% d^{-1})	tS (%)	MDT (d)	CW (µm)
20 psu	4.20 ± 0.07 [a]	28.61 ± 4.51 [a]	14.35 ± 1.99 [a]	210.33 ± 2.59 [a]
30 psu	4.18 ± 0.06 [a]	23.06 ± 3.81 [a]	14.45 ± 2.08 [a]	212.18 ± 3.62 [a]
40 psu	4.14 ± 0.07 [a]	21.11 ± 4.15 [a]	14.78 ± 1.87 [a]	205.88 ± 4.11 [a]

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