FEATURE ARTICLE

Physiological condition of *Balanus amphitrite* cyprid larvae determines habitat selection success

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ABSTRACT: A field experiment was used to assess the relationship between the physiological condition of barnacle larvae and habitat selectivity at settlement. Three experiments were carried out on methacrylate (Plexiglas) disks precolonized with biofilms of different ages (0, 7, 14 and 21 d) that were arranged within 3 blocks and placed at 3 intertidal positions corresponding to the upper limit, mid zone and lower limit of the adult *Balanus amphitrite* (Darwin) habitat. To limit the effect of post-settlement mortality, each experiment was conducted over 2 full tidal cycles only. During these periods, larvae in the water column were sampled and abundance estimated at each high tide at the depths corresponding to the 3 intertidal positions. Larval physiological condition was assessed using lipid classes analyzed by liquid chromatography. Ratios of total triacylglycerols to sterol content (TAG/ST) were used to quantify the energy status of larvae. Our results indicate that the cyprids’ TAG levels reflect the level of settlement success independent of cyprid abundance in the water column. Intertidal position and biofilm characteristics were significant factors determining the settlement success with respect to cyprid energy content. The number and the energy content of settling cyprids were maximal on clean surfaces and decreased gradually with the degree of biofilm precolonization. Our study shows (1) the importance of lipid levels, particularly TAG, in habitat discrimination at small temporal scales in *B. amphitrite* cyprids and (2) that larval energy content is a critical variable for understanding benthic–pelagic coupling.

KEY WORDS: Larvae · Settlement success · Physiological state · Lipid class · Biofilm · Habitat discrimination

INTRODUCTION

Over 70% of benthic invertebrate species in temperate and tropical regions have a life cycle involving a planktonic dispersal phase (Thorson 1950). Larval drifting periods lasting from weeks to months promote the formation of large, homogeneous, randomly mating populations. This planktonic dispersion exposes larvae to high risks leading to mortalities well over 90% (Morgan 1995). Pelagic mortality has numerous causes, including physiological stress induced by environmental conditions (e.g. temperature, salinity, oxygen, UV; Pechenik et al. 1990), larval advection (Incze et al. 2000), predation (Navarrete & Wieters 2000) and starvation (McEdward & Qian 2001). In coastal waters, natural food resources are often thought to be too low to support larval growth and development (Paulay et
This study, we analyzed the energy status of cyprids in controlling the habitat selection behaviour of larval physiologic condition of these cyprids, representing their energy content and age, would be an interesting opportunity to demonstrate the role of larval physiology in controlling the habitat selection behaviour leading to permanent attachment in the field. Thus, in this study, we analyzed the energy status of cyprids collected during this earlier experiment (Olivier et al. 2000) to test the hypothesis that habitat selectivity and settlement success relies more on the physiological condition of the larvae rather than on the supply of larvae. The physiological condition of the larvae was examined in relation to the abundance of both non-settled and settled larvae, substrata of different quality and intertidal position.

MATERIALS AND METHODS

Experimental design. Balanus amphitrite cyprids, which were analyzed for lipid class content as a measure of their energy status, were collected during an experiment conducted by Olivier et al. (2000). The Olivier et al. (2000) study was carried out under the Duke University Marine Laboratory (DUML) dock on Pivers Island (Beaufort, North Carolina, USA; 34°43′03″N, 76°40′18″W) in April 1998. This site is characterized by the presence of several barnacle species, but in April only B. amphitrite recruit (Holm 1990, Roberts et al. 1991). Three experiments taking place over 6 d assessed the relative roles of larval supply, biofilm composition (intertidal position and age) and tidal height during cyprid settlement. As presented in Fig. 1 of Olivier et al. (2000), 3 sets of 3 Plexiglas sheets (1.22 × 1.22 m), named ‘block’ in this design, were secured under the DUML dock 1 d before the beginning of an experiment at 3 intertidal positions, high (H), medium (M) and low (L). Each experimental block had been sandblasted (40 µm grit) to create homogeneous rugosity suitable for barnacle settlement and 12 holes were cut by laser beam (Mazak laser 2000 W) providing 12 disks of 20.32 cm diameter (area 324.3 cm²). Each disk was considered a cell in the experimental design. Prior to the start of the experiment, 3 sets of fouling community films of 7, 14, 21 d were obtained by placing 9 sets of 27 disks at the 3 predetermined intertidal positions (3 sets/intertidal position). At the beginning of each experiment, precolonized disks corresponding to the 3 intertidal origins (O_H, O_M, O_L) and 4 levels of biofilm colonization 0, 7, 14 and 21 d were randomly arranged within the blocks so that each block included 12 disks corresponding to the 3 intertidal positions (O_H, O_M, O_L) and 4 ages of biofilm (0, 7, 14, 21 d). Three blocks were installed at each intertidal position. Cyprid habitat selection was studied using disks precolonized at various intertidal positions for different durations to obtain a biofilm abundance gradient. Before starting experiments, all biofilm surfaces were closely examined and no settlers were observed. See Olivier et al. (2000) for more details.

Three successive experiments were carried out to assess the variability in Balanus amphitrite settlement density.
according to (1) variations in larval supply (larval density in the water column) and (2) differences in the energy levels of the larval population. By conducting 3 successive experiments over a 6 d period (23 to 28 April), we expected the cyprid larvae in the water column to be in different physiological conditions and to have different responses to the biofouled surfaces. At the end of each barnacle settlement experiment, each lasting 2 tidal cycles (2 immersion periods) to limit the effect of post-settlement mortality, all disks were quickly and carefully removed from the holders and transferred to running seawater aquaria at DUML. In the laboratory, the settled larvae were collected using a binocular microscope, counted and then stored at −80°C prior to lipid analyses.

**Larval supply.** During the 3 experiments, plankton samples were collected at high slack tide through an 80 µm mesh Nitex net for 2 min using a self-priming centrifugal pump (Monarch BSGF-8, Type A). Three replicate water samples of 923 l each were filtered from the northern side of the pier for each depth, corresponding to the high, medium and low experimental disk intertidal position. Each sample was immediately sorted to collect 20 live cyprids that were immediately placed at −80°C for physiological condition determinations.

**Physiological conditions.** Neutral lipids were extracted based on protocols described in Ouellet & Taggart (1992) in the first 6 mo after collection. Individual cyprids were homogenized with a glass pestle in 500 µl of chloroform–methanol (2:1, v/v) on ice and, dependent on availability, 6 to 15 individuals were analysed by treatment. The homogenate was rinsed with KCl solution (0.88%) to obtain CH₂Cl₂–CH₂OH–KCl (2:1:0.6, by vol.; Folch et al. 1957). The homogenates were mixed and centrifuged twice at 4000 rpm (1500 × g) for 2 min to cause phase separation. The lipid fraction (lower phase) was removed after each wash and transferred to a clean tube. We added 10 µg of hexadecanone, an ethyl ketone, as an internal standard (Parrish & Ackman 1985). The solvent was evaporated under nitrogen and lipids were resuspended in 20 µl of chloroform. The entire sample was spotted onto S-III Chromarods (Iatron Laboratories) using a Hamilton syringe. The rods were initially developed for 30 min in hexane–ethyl ether–formic acid (55:29.7:0.075, by vol.) for separation of the TAG, free fatty acids (FFA) and free sterols (ST). Between each development, Chromarods were secondarily developed for 30 min in hexane–ethyl ether–formic acid (82:2.5:0.045, by vol.), allowing separation of the ketone. Each rod was secondarily developed for 30 min in hexane–ethyl ether–formic acid (55:29.7:0.075, by vol.) for separation of the TAG, free fatty acids (FFA) and free sterols (ST). Between each development, Chromarods were scanned by the flame ionization detection system (FID) of the Iatroscan Mark-V analyzer (Iatron Laboratories). Lipid classes were identified and quantified using the standard calibration curves obtained for each lipid class (Parrish & Ackman 1985). Within each set of rods, we included one lipid standard and one extraction blank. TAG content can be related to larval physiological condition if TAG content is corrected for larval size. As there is a positive correlation between sterol content and size (Fraser 1989), we used the TAG/ST ratio to correct for size and to quantify larval energy stores. This correction factor has been previously applied to shrimp (Ouellet & Taggart 1992) and bivalve (Pernet et al. 2003) larvae.

**Data analysis.** As described in Olivier et al. (2000), 2-way ANOVA was used to examine the effects of intertidal position and the experimental date on physiological conditions (TAG and ST contents, TAG/ST ratio) and on cyprid abundance in the water column (larval supply) over the 6 d experimental period (n = 54). All physiological results are new in this study and never presented in Olivier et al. (2000). Data for abundance and TAG/ST ratio were normalized using log transformations. Least-square means tests (LS means, SAS 1988) were adopted for post-hoc comparisons corrected for the total number of comparisons. Two-way ANOVA was used to examine the variability of settled larvae among experiments in relation to intertidal position following post-hoc LS means comparisons. Finally, we used split-split-plot factorial ANOVAs to analyze the effect of the biofilm origin and degree of periphyton colonization on the physiological condition and density of newly settled cyprids at the 3 intertidal positions for each settlement experiment. All the experimental data sets had a factor origin (Oₖ, Oₘ and Oₗ) and a factor age of biofilm (0, 7, 14 and 21 d), thereby generating 12 different treatments (3 origins × 4 ages).

**RESULTS**

**Larval supply and physiological condition**

As described in Olivier et al. (2000), cyprid concentration in the water column varied significantly during the experimental period (F = 10.65, p < 0.001), reaching a maximum during Expt 3; there was no significant difference between Expts 1 and 2. Furthermore, no significant variation in cyprid abundance was observed vertically in the water column except in Expt 3 between the high and low positions (Fig. 1a). The physiological condition of cyprids from the water column, measured on 15 individuals per treatment, varied among the 3 experiments (F = 28.29, p < 0.0001), with the highest TAG/ST ratios found during Expt 1. Cyprids from Expts 2 and 3 had similar TAG/ST ratios (Fig. 1b). The significant differences in the TAG/ST ratios were related to variability in the TAG content (F = 21.66, p < 0.0001; Fig. 1c). No significant differences were observed for ST content (F = 1.14, p = 0.38;
Fig. 1. *Balanus amphitrite*. Abundance and quality of cyprid larvae in the water column: mean (+SE) (a) abundance, (b) triacylglycerol (TAG) to sterol (ST) ratio, (c) TAG and (d) ST content. Cyprid larvae were sampled at the high tide slack water at the 3 intertidal depths (high, medium, low) for each of the 3 experiments. Different letters indicate significant differences. (a) is identical to Fig. 5 in Olivier et al. (2000)

Fig. 1d). FFA made up less than 5% of the total lipids and showed no significant variability among treatments ($F = 0.71, p = 0.77$). The physiological conditions of cyprid larvae did not vary significantly according to the larva’s vertical distribution in the water column (TAG/ST ratio: $F = 0.26, p = 0.78$; TAG: $F = 1.85, p = 0.16$).

**Settlement experiments**

One-way ANOVA showed that the number of post-larvae varied during the experimental period (Fig. 2; $F = 14.916, p < 0.001$), with the highest value found in Expt 1 ($p < 0.001$). Overall larval settlement was reduced by a factor of 4 in Expts 2 and 3 (Fig. 2). The physiological conditions of post-larvae were characterized only for Expt 1; the scarcity of settled larvae in Expts 2 and 3, particularly in old biofilm (14 and 21 d) and high tidal level precluded statistical analyses. Thus, we focused on cyprids from Expt 1 to examine the relationship between physiological conditions and substrate selectivity. As described in Olivier et al. (2000), the number of larvae settling over 2 tidal cycles was highest on the clean surfaces and decreased gradually with the degree of periphyton precolonization. The number of settled larvae was also influenced by intertidal position (Fig. 3a; $F = 80.85, p < 0.0001$). For the 0 d and 7 d biofilms, larval abundance was significantly lower at the H than at the M and L intertidal position. No differences were observed for the number of larvae settled on the 14 d and the 21 d biofilms. Settlement larvae were most abundant on the 0 d disks at all intertidal positions. Settlement was not related to the intertidal origin of the biofilm ($F = 2.21, p = 0.119$).

We observed variability in the TAG content and the TAG/ST ratio. As was the case with the abundance of newly settled cyprids, the TAG/ST ratios were higher in individuals sampled on disks with no periphyton colonization (Table 1, Fig. 3b). The TAG/ST ratios of settled larvae were higher at 0 d compared to all other biofilm ages (LS means, $p < 0.05$). No effect of intertidal position, origin or block was observed. The variability of the TAG/ST ratio was related to the TAG content (Table 1, Fig. 3c), with higher values of TAG content found on the 0 d biofilms (LS means, $p < 0.05$). For ST, no significant variability of factor effects was observed (Table 1). For FFA, the levels observed were low below the detection limits for many samples, preventing statistical analyses.
DISCUSSION

Our integrative study confirms the hypotheses that physiological conditions of Balanus amphitrite cyprid larvae significantly influence settlement success. As in the study of Jarrett & Pechenik (1997), we observed, in field conditions, variability of physiological conditions and energy content between different cyprid cohorts. However, to our knowledge, our results are the first to show clearly that physiological condition controlled habitat selection behaviour.

Larval supply and settlement

Olivier et al. (2000) observed no correlation between larval supply and the intensity of settlement at the short-term scale of 1 wk. As discussed by those authors, the flux of larvae to the substratum is related to the flow velocities and care was taken in the present study to conduct repeated experiments. Current velocity patterns were comparable among experiments and were strongly related to tidal cycle, as presented in Olivier et al. (2000). Larval concentrations in the water column did not vary during the first 2 experiments, whereas the densities of newly settled cyprids decreased significantly and drastically. Larval supply was maximal during the third experiment, but settlement was identical to the previous 2 experiments (Olivier et al. 2000). Since the physical variables measured during the experimental period were stable, we propose that the weak correlation observed between larval supply and larval settlement could be related to a shift in the cyprid’s overall capacity to discriminate surface quality.
The metamorphosis of cyprid barnacles to juveniles is an energetically expensive process, requiring up to 30% of the organic carbon reserves (Lucas et al. 1979). A storage protein known as Cyprid Major Protein (CMP), which is biochemically and immunologically similar to vitellin, has been identified in Balanus amphitrite (Shimizu et al. 1996). These authors observed that CMP was most abundant in cyprids and abruptly decreased in quantity following metamorphosis to the juvenile stage. Satuito et al. (1996) suggested that cyprids use this storage protein (CMP) mainly for the production of juvenile protein structures during metamorphosis. Thus, the decrease in settlement success may occur when CMP levels have been depleted to the point where the production of juvenile protein structures required during metamorphosis cannot be supported. Following this hypothesis, a delay in the metamorphosis of cyprid larvae could result in poorer settlement and metamorphosis success due to a reduction in biochemical components, such as CMP (Satuito et al. 1997) and lipids (Lucas et al. 1979, Rittschof et al. 1984), to quantities insufficient to produce cellular structures essential to metamorphosis. Our results are consistent with this theory. Thus, the cyprid larvae in Expt 1, which showed highest settlement, had the most abundant lipid reserves, as indicated by the high TAG level content and high TAG/ST ratio. Neutral lipids, particularly TAG, are the main energetic reserves used by marine invertebrate larvae (Pernet et al. 2003). In the other 2 experiments, which were characterized by low settlement, the TAG content and TAG/ST ratio decreased significantly. Thus, in B. amphitrite, the TAG level in cyprids appears to be reflected in the level of settlement success independent of the density of cyprids in the water column. Jarrett (2003) obtained similar results in both laboratory and field studies. He observed a threshold energetic level of approximately 13 µg total carbon (including lipids, proteins and carbohydrates) in Semibalanus balanoides cyprids above which metamorphosis was not compromised. Below this value, however, settlement and metamorphosis success were reduced. However, in our study we could not establish a relationship with metamorphosis success, as many of the settlers collected were cemented to the surface, but had not yet metamorphosed.

In Expt 3, the larval supply was higher than during the other 2 experiments but the TAG content was low, possibly too low to generate a high settlement success. These results could be related to the advection of a new cohort of larvae. The periodic transport of large numbers of larvae by water flow is a well-documented phenomenon (Drouin et al. 2002). Jarrett & Pechenik (1997) have already observed important differences in the physiological quality of barnacle post-larvae from different cohorts. These physiological variations observed among cohorts may have resulted from the influence of several factors, such as variability of nutritional conditions during larval ontogeny (Hentschel & Emlet 2000), effects of delayed settlement on cyprid energy content (Lucas et al. 1979) and/or have a genetic basis (recruitment from different geographic populations or larvae produced from genetically different adults within a single population).

### Intertidal position and biofilm

In our study, intertidal position and biofilm characteristics were significant factors in determining the vertical patterns of the newly settled cyprids during the first experiment. At the cyprid stage, a barnacle is able to settle and metamorphose and above all, to explore the substratum to select the best habitat for juvenile and adult development in response to environmental cues (Rittschof et al. 1984, Roberts et al. 1991, Walters et al. 1999). The local hydrodynamic conditions are significant factors in the transfer of larvae from the planktonic phase to initial adhesion on the surface (Jonsson et al. 2004) and for the post-settlement growth and survival (Larsson & Jonsson 2006). In the intertidal zone, the exploration of substratum by cyprids is correlated with height on the shore, a variable directly related to the duration of immersion (Miron et al. 1995). Thus, at medium and low intertidal positions, cyprids had more time to explore the substratum, which would allow the larvae in better physiological condition to find an appropriate substratum and habitat. Thus, the significant relation between the short-term vertical patterns of larvae in the water column and the short-term vertical patterns of settlement are in agreement with the supply-side theory (Underwood & Fairweather 1989, Olivier et al. 2000). In contrast, the longer-term (i.e. over the 3 experiments) results do not support the supply-side theory. Indeed, even when the density of cyprids in the water column increased (e.g. Expt 3), the settlement success remained low. Over the 6 d experimental period, the physiological conditions of cyprids in the water column better explained the settlement pattern than did larval supply.

The relative importance of the physical and biological factors in governing settlement processes has been widely debated. Many cues have been identified, including salinity, temperature, light, water flow, substrate characteristics, pheromones, juvenile hormones and the presence of conspecific adults (Satuito et al. 1996, Miron et al. 2000). Our study clearly shows that in Balanus amphitrite, biological cues related to the presence of natural biofilms play an important role in determining the suitability of a particular settlement...
site. To assess the role of biofilm colonization on the settlement of cyprids in the field, we conducted experiments limiting the interaction of this factor with others. Three experiments were conducted over a short period (6 d) to obtain comparable fluctuations of temperature, salinity, water velocity and light as described in Olivier et al. (2000). The number and physiological condition, as characterized by the TAG level, of larvae that settled during the 2 tidal cycles was maximal on the clean surfaces and decreased gradually with the degree of periphyton precolonization. These results show that B. amphitrite cyprid larvae with high energetic reserves preferred clean surfaces. However, when TAG lipid reserves decreased, cyprids seemed to risk settlement in poorer quality sites rather than postpone settling. Thus, when TAG levels and TAG/ST ratios decline, both settlement intensity and settlement choice decline, suggesting that our results confirm the ‘desperate larva hypothesis’ (Toonen & Pavlik 1994), where cyprids lose their settlement selectivity with age. The origin of the biofouled surfaces (H, M, L) did not affect the density or the physiological condition of the newly settled B. amphitrite cyprids, suggesting a lack of discrimination for a specific intertidal position in contrast to Semibalanus balanoides cyprids (Miron et al. 1995).

For cyprids to settle when their energy content is high is not only a means to increase metamorphosis success but also to increase juvenile growth potential and thus juvenile performance (Jarrett 2003, Thiagarajan et al. 2005, Emlet & Sadro 2006). The effect of cyprid energetic reserves on juvenile growth has been detected up to 12 d after settlement (Jarrett & Pechenik 1997, Thiagarajan et al. 2003). Since the first few days of post-metamorphosis are generally considered to be critical, the physiological condition of larvae before settlement is likely significant for recruitment (Gosselin & Qian 1997).

**CONCLUSIONS**

Our study shows that the energetic reserves in cyprid larvae vary considerably among cohorts. Specifically, we show that the success of Balanus amphitrite settlement is related not only to larval supply, but also to the physiological state of larvae. Thus, our results emphasize the importance of larval physiology in controlling habitat selection behaviour leading to permanent attachment.

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