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The influence of temperature and light on larval pre-settlement metamorphosis: a study of the effects of environmental factors on pre-settlement metamorphosis of the solitary ascidian *Styela canopus*

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This study investigated the effects of two environmental factors, temperature and light, on larval settlement and metamorphosis in the solitary ascidian *Styela canopus*. The results revealed that larval settlement rates decreased with increasing temperature in the range 12–30°C. We also demonstrated for the first time that pre-settlement metamorphosis of ascidian larvae can occur as a function of temperature. We suggest this could be an adaptation to avoid the greater energetic cost of active larval swimming, presumably resulting from the increasing temperature. They are able to metamorphose into passive drifting post-larvae and to continue planktonic life. This finding has implications for larval dispersal, especially under conditions of ocean warming. In addition, the effect of light intensity on larval settlement and metamorphosis was significantly different between photoperiods of 24L:0D and 12L:12D. These results provide some insight into the complex cues affecting settlement and metamorphosis of ascidian larvae and ascidian distribution in nature.

Keywords: ascidian; larva; *Styela canopus*; behavior; settlement; metamorphosis; pre-settlement; temperature; light

Introduction

At the end of their free-swimming phase, the larvae of most benthic marine organisms settle to the substratum and metamorphose. In the natural environment, these complex processes of larval settlement and metamorphosis are determined by the interaction of biotic and abiotic factors which operate at different temporal and spatial scales. Abiotic factors include physical and chemical cues. Important among these are temperature, salinity, the contour of the settlement substratum surface, water flow velocity, and light intensity (Rodriguez et al. 1993; Bates 2005).

Ascidians are not only important members of the marine benthos, but also contribute to the economic and technical problems of marine biofouling because of their growth on the surfaces of industrial objects, such as ships, buoys, and fishing nets. Studies on larval settlement and metamorphosis of ascidians have been restricted to the roles of chemical factors (Glaser and Anslow 1949; Whittaker 1964;

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Patricolo et al. 1981; Svane et al. 1987; Berking and Herrmann 1990; Holmstrom et al. 1992; Degnan et al. 1997; Wieczorek and Todd 1997; Degnan and Johnson 1999; Eri et al. 1999; Tsukamoto et al. 1999; Green et al. 2002; Kimura et al. 2003; Woods et al. 2004) and physical cues have not received as much attention. Up to the present, only a few physical factors have been investigated for their effects on settlement and metamorphosis of ascidian larvae. Usually, light is regarded as an important settlement cue for the tadpole larvae of most ascidian species (Young and Chia 1984; Svane 1987; Svane and Young 1989). Larvae of many ascidians are known to be photonegative at settlement, which is believed to play an important role in preferential settlement of ascidians in cryptic habitats (Young and Chia 1984; Svane and Dolmer 1995; McHenry and Strother 2003; Manriquez and Castilla 2007). Water temperature is also believed to be an important environmental variable in determining ascidian distribution (Lambert 2005). However, Thiyagarajan and Qian (2003) reported that temperatures of 18°C, 22°C, 26°C, and 30°C have no significant influence on larval settlement and metamorphosis of *Styela plicata*. GropPELLI et al. (2003), who observed the effects of different lithological substrata on the settlement of the ascidian *Phallusia mammillata* larvae, suggested that the mineral composition of the substratum can contribute to the regulation of the spatial distribution of tunicate communities. Flores and Faulkes (2008) also suggested that ascidian tadpole larvae are capable of finer tactile discrimination than merely detecting a hard surface.

In general, the larvae of most benthic marine organisms metamorphose only after settlement on an appropriate substratum, but settlement is not always essential for metamorphosis in ascidians (Millar 1971). Cloney (1982) suggested that when the papillae of ascidian tadpole larvae do not evert but simply secrete adhesive, the events of metamorphosis follow in a sequence characteristic of the species. This follows even if the larvae are not settled. The phenomenon in which a proportion of larvae metamorphose without actual settlement has been found in many ascidian species, such as *Herdmania momus* (Degnan et al. 1997; Jacobs et al. 2006), *Herdmania curvata* (Degnan and Johnson 1999), *Boltenia villosa* (Cloney 1961), *Eudistoma ritteri* and *Diplosoma listerianum* (Levine 1945), *Ciona intestinalis* (Carlisle 1961) and *S. plicata* (Thiyagarajan and Qian 2003). Interestingly, it has been suggested that planktonic metamorphosis is probably usual for a proportion of ascidian larvae (Millar 1971). In this work, we use “pre-settlement metamorphosis” synonymously with “planktonic metamorphosis” or “metamorphosis without settlement” or any other such terms describing this phenomenon. Research on this interesting phenomenon may provide some insight into the mechanisms of and relationship between settlement and metamorphosis of ascidian tadpole larvae and hence the regulation of larval recruitment and distribution patterns of ascidian populations. However, little work has been done on the factors influencing the pre-settlement metamorphosis of ascidian larvae.

This article reports on the responses of larval settlement and metamorphosis, especially, pre-settlement metamorphosis of the solitary ascidian *Styela canopus*, to various temperatures and various light intensities under two photoperiods. Seawater temperature is generally recognized as one of the most important environmental factors influencing the life of marine organisms. Since we studied *S. canopus* samples collected from Xiamen, China, where annual seawater temperatures vary between 12.7°C and 27.9°C (Gu et al. 1999), temperatures from 12°C to 30°C were tested to establish whether they have any effect on larval settlement and metamorphosis of

this species. Due to the worldwide distribution of *S. canopus* in temperate and tropical areas (Lambert 2001), temperatures from 12°C to 30°C are probably experienced by these larvae in the field. As far as the effect of light is concerned, there are many relevant papers on the responses of ascidian larvae to light intensity at settlement (Svane 1987; Svane and Young 1989; Svane and Dolmer 1995; McHenry and Strother 2003; Manriquez and Castilla 2007). However, nothing is known about the influence of photoperiod, another important characteristic of light and also one of the main environmental challenges that every organism has to cope with in order to survive in nature (Villamizar et al. 2009). It is of interest to note that the length of the larval period of *S. canopus* has been found to vary approximately from 9 to 105 h, and some other ascidian species, such as *Phallusia nigra* and *Polyandrocarpa tinctoria* also have larval periods with a variation range greater than 24 h (Grave 1944), which indicates that, in the field, larvae of these ascidian species may be subjected to natural light cycles, and there is a probability that photoperiod may influence the response of settling larvae to light. To examine this possibility, we observed larval settlement and metamorphosis in different light intensities under the extreme photoperiod of 24 L (light):0 D (dark) and the 12 L:12 D photoperiod, which is closer to the natural photoperiodic regime.

Materials and methods

Animals and larval culture

Adult *S. canopus* were collected from submerged rafts in Xiamen (24°26'N, 118°04'E) and kept in laboratory aquaria. After 1–3 days acclimation, the adult *S. canopus* were put into a glass beaker with fresh 0.22 µm filtered seawater. Three or four air stones were then placed into the glass beaker and adjusted to produce diffuse vigorous bubbling in the seawater. After 0.5 h, the air stones were taken out and after a few minutes, eggs and sperms of *S. canopus* were released. After fertilization, embryos were collected, washed several times with the aid of a mesh and cultured in filtered seawater at 25°C (room temperature) and under natural light. Larvae hatched after 10 h. The 3 h post-hatching larvae, which attained competence to settle and metamorphose (Huang et al. 2003), were collected and immediately used for subsequent tests.

Experiments

All experiments were conducted in 6 cm diameter Petri dishes, with 27.0 salinity millipore-filtered (0.22 µm) seawater. In the temperature experiment, four treatments (12°C, 18°C, 22°C, and 30°C) were used. Each treatment was placed into a separate temperature-controlled incubator with the light intensity in all being 0 lx. In the light experiment, six levels of light intensity (0, 2000, 4000, 6000, 8000, and 10000 lx) were designed under two photoperiods (12 L:12 D and 24 L:0 D). Each treatment was in a separate illumination incubator at one of the six levels of light intensity, one of the two photoperiods, and a temperature fixed at 25°C. The light intensities inside the illumination incubators were measured just above the dishes using a digital lux meter LX-1330B (Shuang Xu Electronic Company, Shanghai, China) and were taken as the light intensities in which the larvae were cultured. Each of the two experiments was run using a single batch of larvae on a single occasion. For each treatment in

each experiment, there were three replicates. In each replicate, 30–80 competent larvae of *S. canopus* were added into each Petri dish together with 10 ml of 0.22 µm filtered seawater, and among treatments, the actual numbers of larvae in each dish were randomly assorted. Larval settlement and metamorphosis of *S. canopus* are not influenced at densities lower than 10 larvae per ml (Huang et al. 2005), and so the different densities in the experiment would not affect the result. After 24 and 48 h, the number of larvae that had settled, or that had completed metamorphosis, or that had completed metamorphosis without settlement was counted under a dissecting microscope.

The biological indices

Based on the description of Svane and Young (1989) and the biological characteristics of *S. canopus* tadpole larvae, the biological indices applied in this work were as follows: larval settlement confirmed by adhesive papillae of tadpole larvae adhering permanently to substratum; metamorphosis confirmed by the whole tail being resorbed; and pre-settlement metamorphosis confirmed by larvae floating on the surface of the seawater with the whole tail being resorbed, i.e. larvae that completed metamorphosis without settlement.

Statistical analysis

The number of larvae that had settled, or that had completed metamorphosis, or that had completed metamorphosis without settlement was converted into percentages. Prior to statistical analysis, these percentage values were arcsine transformed. The differences in larval settlement or metamorphosis or pre-settlement metamorphosis among treatments in each experiment were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was set at $P < 0.05$. The data presented in the figures are not transformed.

Results

Responses of larval settlement and metamorphosis to temperature

Styela canopus larvae were found to tolerate a wide range of temperatures within the limits selected for the experiment. Furthermore, they were able to spontaneously settle and metamorphose at all levels of experimental temperature. As shown in Figure 1a, in the range 12–30°C, larval settlement rates decreased with increasing temperature. At 48 h, the settlement rates of *S. canopus* larvae significantly decreased from 67.5% at 12°C to 31.6% at 30°C. On the other hand, no significant differences in metamorphosis rates were observed among the four temperature treatments at either 24 or 48 h (Figure 1b and Table 1).

As far as pre-settlement metamorphosis of *S. canopus* larvae was concerned, the effect of temperature was highly significant with the rates of pre-settlement metamorphosis increasing from 1.1% to 15.4% over the temperature range used at 24 h (Figure 1c and Table 1). At 48 h, larval pre-settlement metamorphosis showed a similar response. Larvae at higher temperatures (24°C and 30°C) metamorphosed without settlement at significantly higher rates compared with those at lower temperatures (12°C and 18°C). Overall, as shown in Figure 1c, increase in

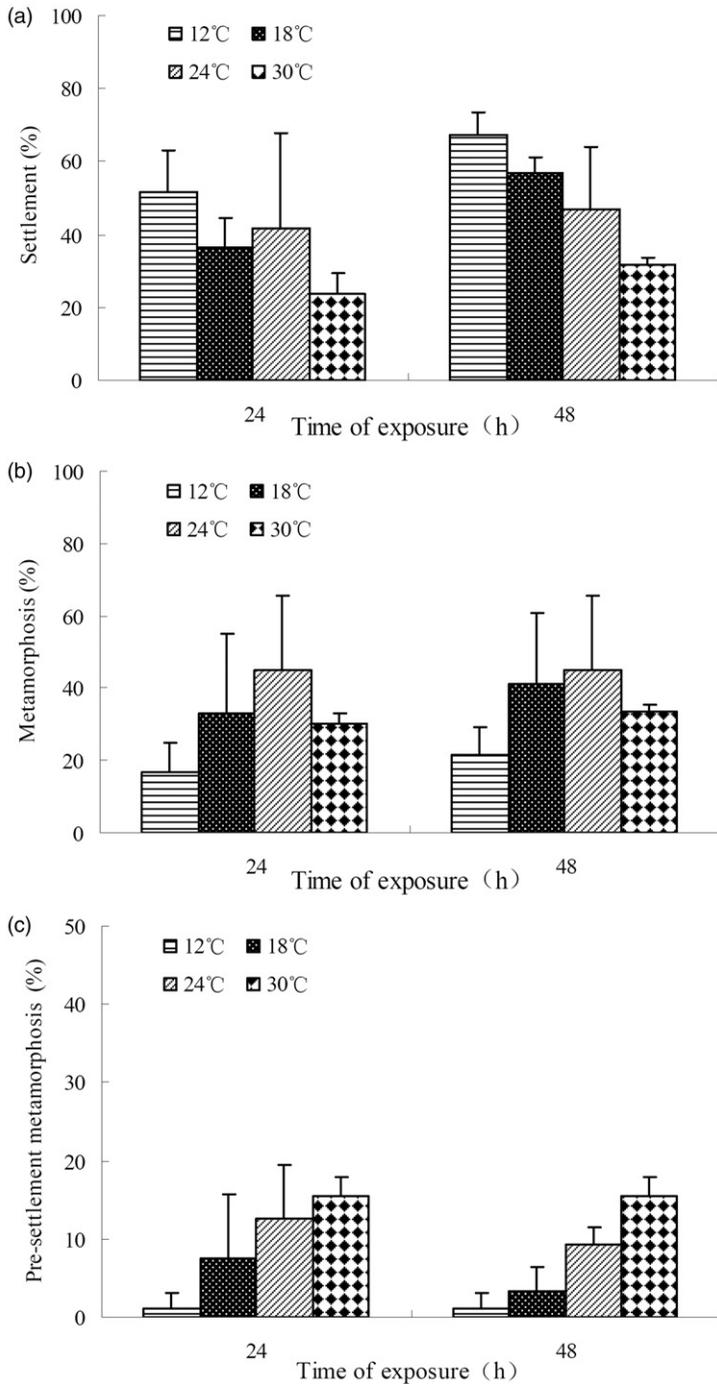


Figure 1. Percentages of (a) settlement, (b) metamorphosis, and (c) pre-settlement metamorphosis in *S. canopus* larvae after exposure to various temperatures for 24 and 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars.

Table 1. ANOVA and Tukey's test showing the effects of temperature and light intensity under two photoperiods on larval settlement and metamorphosis of the solitary ascidian *S. canopus*.

Physical factors	Larval responses	Time (h)	ANOVA results		Tukey's test				
			df	F-value	P-value				
Temperature (°C)	Settlement	24	11	1.508	0.285	12 a	18 a	24 a	30 a
		48	11	7.450	0.011*	12 a	18 a	24 a b	30 b
	Metamorphosis	24	11	1.865	0.214	12 a	18 a	24 a	30 a
Pre-settlement metamorphosis		48	11	1.538	0.278	12 a	18 a	24 a	30 a
		24	11	4.143	0.048*	12 a	18 a b	24 a b	30 b
Light intensity (lx, under the 12L:12D photoperiod)	Settlement	48	11	8.491	0.007*	12 a	18 a b	24 b c	30 c
		24	17	1.666	0.217	0 a	2000 a	4000 a	6000 a
Metamorphosis		48	17	1.342	0.312	0 a	2000 a	4000 a	6000 a
		24	17	0.349	0.874	0 a	2000 a	4000 a	6000 a
		48	17	0.804	0.568	0 a	2000 a	4000 a	6000 a
Pre-settlement metamorphosis		24	17	4.150	0.020*	0 a b	2000 a	4000 b	6000 a b
		48	17	2.048	0.143	0 a	2000 a	4000 a	6000 a
Light intensity (lx, under the 24L:0D photoperiod)	Settlement	24	17	5.937	0.005*	0 a	2000 a b	4000 a b	6000 a b
		48	17	7.316	0.002*	0 a	2000 a b	4000 a b	6000 b
Metamorphosis		24	17	7.793	0.002*	0 a	2000 a b	4000 b	6000 b
		48	17	12.411	0.000*	0 a	2000 b	4000 b c	6000 b c
Pre-settlement metamorphosis		24	17	1.051	0.433	0 a	2000 a	4000 a	6000 a
		48	17	1.365	0.304	0 a	2000 a	4000 a	6000 a

Notes: In Tukey's test results, values significantly different are followed by different letters in a row.
*P < 0.05.

temperature resulted in a gradual increase in the rates of pre-settlement metamorphosis and the effect of temperature appeared to be nearly linear. This suggests, for the first time, that pre-settlement metamorphosis is affected by temperature.

Responses of larval settlement and metamorphosis to light intensity and photoperiod

Under the 12 L : 12 D photoperiod

As shown in Figure 2 and Table 1, neither settlement rate nor metamorphosis rate was significantly different among the six light intensity treatments under the 12L:12D photoperiod. Furthermore, no significant effect of light intensity on pre-settlement metamorphosis was found under the 12 L : 12 D photoperiod, except for the significant difference in pre-settlement metamorphosis between the 2000 and 4000 lx treatments at 24 h.

Under the 24 L : 0 D photoperiod

Under the 24L:0D photoperiod, light intensity had a significant effect on the settlement and metamorphosis of *S. canopus* larvae (Figure 3 and Table 1). There appeared to be a general trend of decreasing settlement rate and metamorphosis rate with increasing light intensity. On the other hand, no significant difference in the rates of pre-settlement metamorphosis was observed among the light intensity treatments under the 24 L : 0 D photoperiod.

Discussion

In sessile marine tunicates, the role of the larval stage is to disperse and select a suitable site to settle. The settlement behavior of their larvae is believed to have an important influence on the distribution and abundance of adults, and also on community structure and dynamics (Svane and Young 1989; Davis et al. 1991). Most solitary ascidians produce non-feeding, free-swimming tadpole larvae that may respond to environmental factors in ways that promote, delay, or inhibit larval settlement and metamorphosis. Our results showed that larvae of *S. canopus*, an introduced species in many places (Lambert 2001; Lambert and Lambert 2003; Salgado-Barragán and Toledano-Granados 2004; da Rocha and Kremer 2005), were tolerant to temperatures from 12°C to 30°C and successfully settled and metamorphosed at temperatures as low as 12°C. This is consistent with the generalization that species that transfer readily are those that are tolerant of a wide range of environmental conditions (Naranjo et al. 1996; Nomaguchi et al. 1997; Lambert 2001).

One interesting new finding of this investigation was that *S. canopus* larvae underwent increasing levels of pre-settlement metamorphosis as a function of temperature. It is suggested that there might be a relatively simple mechanism whereby temperature works on the activation of the morphogenetic pathway to trigger pre-settlement metamorphosis in the ascidians. Important to interpreting this result and understanding the possible underlying mechanism is the report that the swimming speed in ascidian tadpole larvae increases linearly with rising temperature (Batty et al. 1991). Like all ascidian larvae, the *S. canopus* tadpole has a muscular tail for locomotion (Grave 1944; Huang et al. 2003). It is recognized that larval

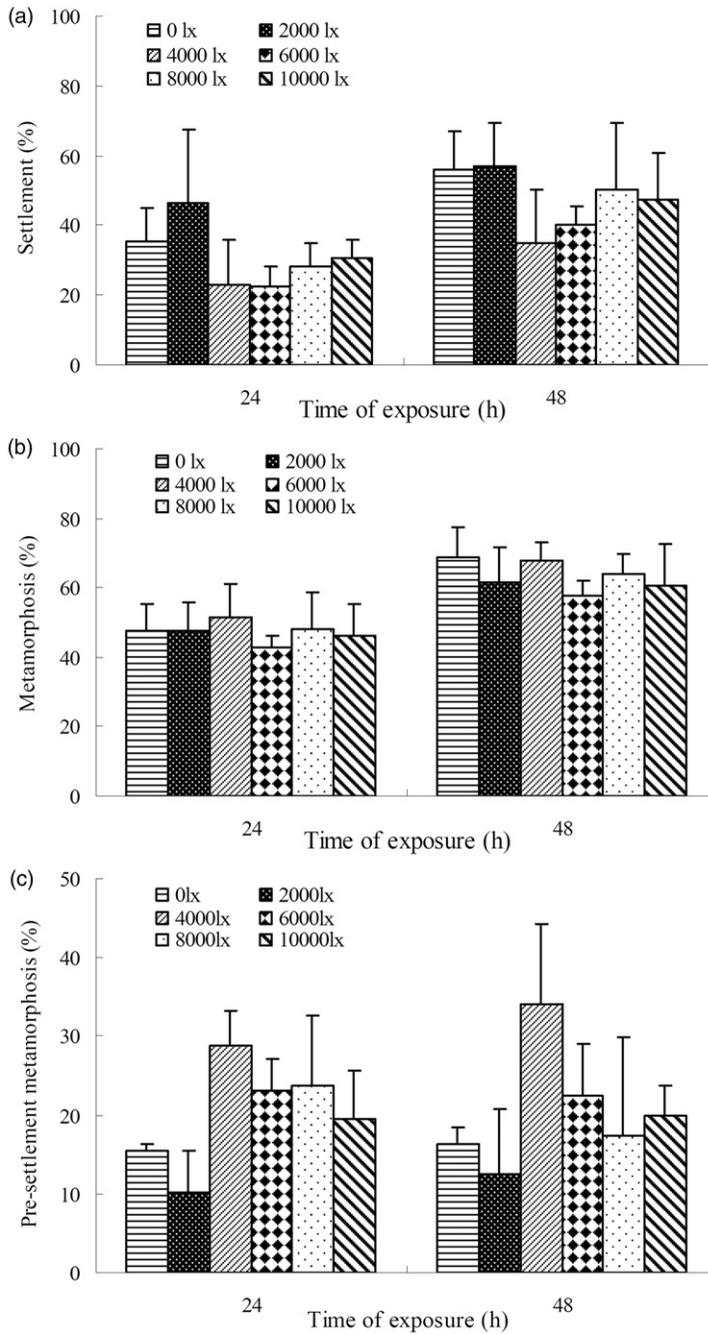


Figure 2. Percentages of (a) settlement, (b) metamorphosis, and (c) pre-settlement metamorphosis in *S. canopus* larvae after exposure to various light intensities under the 12 L:12 D photoperiod for 24 and 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars.

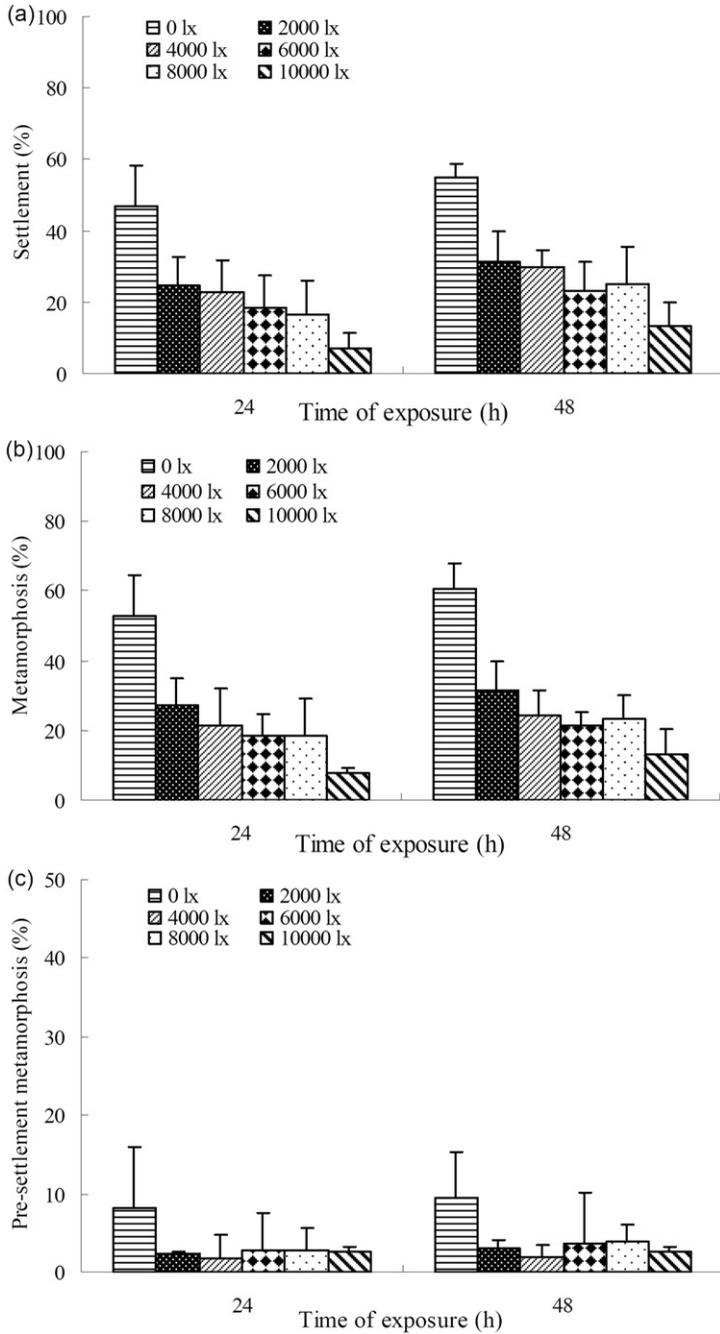


Figure 3. Percentages of (a) settlement, (b) metamorphosis, and (c) pre-settlement metamorphosis in *S. canopus* larvae after exposure to various light intensities under the 24 L:0 D photoperiod for 24 and 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars.

swimming with a muscular tail in ascidians has an energetically high cost (Marshall et al. 2003; Thiyagarajan and Qian 2003; Bennett and Marshall 2005; Elkin and Marshall 2007). Bennett and Marshall (2005) demonstrated that larval swimming consumes 4.5–10% of total energy reserves per hour in the ascidian *D. listerianum*. Marshall et al. (2003) suggested that for ascidian larvae, the energy for larval maintenance, swimming, and metamorphosis comes from a common pool, rather than being strongly partitioned. Lecithotrophic larvae have limited energetic reserves to support their development during the planktonic phase and through metamorphosis, so it is reasonable to assume that an increase in swimming speed could decrease the energy reserves they have for metamorphosis. We suggest that the increasing tendency for *S. canopus* larvae to undergo pre-settlement metamorphosis with increasing temperature may be adaptive. The energetic cost of active swimming by a tadpole can be reduced if it metamorphoses into the passive drifting post-larva. This could increase the survival of larvae in danger of depleting their energy stores below the level necessary for completing metamorphosis. This has potentially important implications for larval dispersal by permitting the post-larvae to continue planktonic life to encounter suitable habitats. Furthermore, the functional siphons of the pelagic post-larvae were observed to be open and filter feeding which suggests that they could be replenishing their energy reserves. Our result that pre-settlement metamorphosis is a function of temperature in ascidian larvae might provide some support for the “desperate larva hypothesis” in marine invertebrates (Toonen and Pawlik 2001; Marshall and Keough 2003; Bishop et al. 2006; Elkin and Marshall 2007; Swanson et al. 2007) by indirectly verifying its tenet that a decrease in energetic reserves can be transduced into a morphogenetic output.

Millar (1971) suggested that ascidian larvae which undergo metamorphosis while still planktonic can subsequently settle. In this study, we observed that a proportion of *S. canopus* larvae that completed metamorphosis without settlement would later transit from a planktonic life to a periphytic life by settlement indicating that they still have the ability to settle. The adhesive papillae are the primary adhesive organs of most ascidian larvae and generally degenerate during metamorphosis. If they had not completely retracted, later settlement using the adhesive papillae might occur. The epidermal ampullae and the sticky surface of the tunic of the pelagic post-larvae could also provide a post-metamorphic settlement mechanism (Lane 1973; Vázquez and Young 2000).

In the past 20–40 years, the dispersal of nonindigenous ascidian species such as *S. canopus* has increased remarkably throughout the world. This is generally believed to be due to human activities, such as the ever-increasing volume of shipping and aquaculture (Lambert 2001, 2005). Stachowicz et al. (2002) suggested a link between climate change and invasions by ascidian species. He suggested that increasing ocean temperatures may facilitate the invasion of nonindigenous ascidians by giving exotic species an earlier start and by increasing their growth and recruitment relative to natives. There is evidence of pre-settlement metamorphosis in ascidian larvae in both culture (Degnan et al. 1997; Degnan and Johnson 1999; Thiyagarajan and Qian 2003) and in nature, where juveniles have been found in the plankton (Jacobs et al. 2006). Our description of the inductive effect of high temperature on the pre-settlement metamorphosis of ascidian larvae suggests that warming might facilitate the introduction of nonindigenous ascidian species by increasing the percentage of larvae that survive a long-distance pelagic life and successfully settle. This provides a

new perspective on the effects of global warming on nonindigenous invasions of marine invertebrates.

The light intensity experiments performed under two photoperiods here had different outcomes. Larval settlement and metamorphosis of *S. canopus* were not significantly affected by light intensity under the 12 L:12 D photoperiod, but light intensity significantly affected them under the 24 L:0 D photoperiod regime. The rates of larval settlement and metamorphosis decreased with increased light intensity under the extreme light regime. This was not unexpected since Grave (1944) showed that *S. canopus* tadpoles, like the larvae of most ascidian species, exhibit a negative response to light at the time of settlement. The reason there were no significant differences in the rates of larval settlement and metamorphosis among treatments of different light intensities under the photoperiod that is closer to the natural photoperiodic regime is likely to be that most larvae settled and metamorphosed during the 12-h period of darkness. It seems reasonable to assume that under natural conditions, tadpole larvae of most ascidian species would prefer to settle in cryptic habitats during daytime, whereas during nighttime, the larvae would settle on various sites, including cryptic and exposed habitats. Compared with the larvae which settled in cryptic habitats, those larvae located in exposed habitats are probably more subject to potential sources of mortality, such as lethal ultraviolet radiation, increased predation, sedimentation, strong currents, and algal overgrowth (Young and Chia 1984; Svane and Young 1989; Bingham and Reyns 1999) and to suffer higher post-settlement mortalities. In the field, most ascidians occur in cryptic habitats, such as cracks and crevices and on shaded substrata. This is generally attributed to their settlement behavior (Young and Chia 1984; Olson 1985; Hurlbut 1993; Svane and Dolmer 1995; Vázquez and Young 1998; McHenry and Strother 2003; Manriquez and Castilla 2007). The results here suggest that ascidian larvae may settle on exposed habitats during the nighttime and that high post-settlement mortality may contribute to the regulation of the field distribution pattern of ascidians. Future field studies are needed to investigate this suggestion.

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