

A hierarchy of settlement cues influences larval behaviour in a coral reef sponge

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ABSTRACT: For sessile marine invertebrates, processes contributing to larval release, dispersal, and settlement in favourable habitats are central to patterns of distribution and community structure. We quantified larval release patterns, phototactic behaviour, and settlement in response to environmental cues for the coral reef sponge *Luffariella variabilis*. Individual sponges released up to 830 larvae d⁻¹. Larvae displayed phototactic behaviours by swimming upwards after release for brief periods (40 min), after which time most larvae (>95%) exhibited negative phototaxis. Light played a role in determining numbers and rates of larval settlement. Light levels of 56 $\mu\text{mol s}^{-1} \text{m}^{-2}$ reduced the rate of settlement and inhibited larval settlement by 60% compared to dark controls. However, at lower light levels (0.7 to 0.34 $\mu\text{mol s}^{-1} \text{m}^{-2}$), both time to settlement and numbers of larvae settling were consistent with settlement in dark controls. Larval settlement increased in the presence of other larvae, with >95% of larvae settling when placed in treatments with 50 individuals, compared to 50% settlement for treatments containing only 1 individual. The gregarious settlement of *L. variabilis* larvae was associated with conspecific larval settlement cues. Settlement in 'conditioned' water from which 200 larvae had previously settled and subsequently been removed was 80%, compared to 20% in controls. Our study unequivocally demonstrates that a conspecific cue not related to adults or other biotic or abiotic factors induces settlement in larvae. Our observations, the preference of larvae to settle in response to low light levels and of settlement increased by gregariousness, correspond with the cryptic and clumped distribution of *L. variabilis* in the field.

KEY WORDS: Porifera · Light · Gregariousness · Larval settlement · Settlement cues

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INTRODUCTION

Most marine benthic invertebrates have complex life histories characterised by sessile and planktonic phases, and in most cases planktonic larvae are the primary mechanism of dispersal in sessile invertebrates (Caley et al. 1996). The length of the planktonic larval phase can vary from minutes to months and is usually predicated by the mode of development of the larva (lecithotrophic or planktotrophic) (Pawlik 1992, Hadfield & Paul 2001). The subsequent transition from a planktonic to benthic existence, where the future of metamorphosed larvae is dependent on an appropriate

habitat choice, is a crucial stage in the life history of sessile marine invertebrates (Keough & Raimondi 1995, Raimondi & Morse 2000).

Flow (Metaxas 2001), light (Maida et al. 1994) and gravity (Young 1995) all affect larval behaviour, settlement and metamorphosis at large scales (up to 100s km), while biotic and abiotic interactions predominantly influence larvae at smaller scales (mm to m) (Steinberg et al. 2001). These smaller scale factors include surface texture (Berntsson et al. 2000), chemical cues from biofilms (reviewed in Fusetani 2004), conspecifics (Dreanno et al. 2006a, Huggett et al. 2006), and other biological sources (Raimondi & Morse 2000, Swanson et al. 2006).

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In contrast to other organisms such as polychaetes (Butman et al. 1988, Minchinton 1997), bivalves (Butman et al. 1988), barnacles (Clare & Matsumura 2000, Dreanno et al. 2006b), oysters (Zimmerfaust & Tamburri 1994), ascidians (Stoner 1992) and bryozoans (Keough 1998), there is little information on the response of sponge larvae to environmental, biological and chemical stimuli (reviewed in Maldonado 2006). Of the environmental factors investigated for sponges, light plays a key role in influencing the release, behaviour, and settlement of larvae. For example, photoperiodicity determines larval release in *Calyspongia* sp. (Amano 1988), whilst *Halichondria panicea* releases larvae after being artificially shocked by intense illumination (Amano 1986, Maldonado & Young 1996). After release, the parenchymellae of demosponges can display photopositive (Mariani et al. 2005), photonegative (Maldonado et al. 1997, 2003, Leys & Degnan 2001) or photoneutral behaviour (Uriz et al. 1998). Photopositive behaviour is associated with dispersal by currents (reviewed in Maldonado 2006), whilst photonegative behaviour has been proposed to guide competent larvae to dark benthic microhabitats (Lindquist 1996, Maldonado et al. 1997).

Similarly, the selection of surfaces by sponge larvae on which to settle and metamorphose varies. Larvae of some species settle in the presence of geniculate coralline algae (Jackson et al. 2002), while others preferentially settle on biofilms (Woollacott & Hadfield 1996). In contrast, the larvae of some species indiscriminately select glass, basalt rock and porcelain (Bergquist & Sinclair 1968, Bergquist 1978). However, it is unclear from these studies how chemically mediated settlement is decoupled from surface textures or the presence of biofilms. Few studies link the range of cues that facilitate settlement processes, from larval release to metamorphosis, which are all critical for successful recruitment.

In this study, we elucidate a hierarchy of responses by the larvae of the Indo-Pacific dictyoceratid sponge *Luffariella variabilis* to physical, biological, and chemical cues, to determine the processes affecting their dispersal and habitat selection. We investigated (1) larval release by *L. variabilis*; (2) larval response to light after release from the parent sponge; (3) the influence of light on larval settlement and; (4) the responses of *L. variabilis* larvae to common invertebrate settlement cues, including the presence of newly settled and adult conspecifics.

MATERIALS AND METHODS

General. *Luffariella variabilis* is a cryptic, coral reef sponge distributed widely through the Indo-Pacific (Bergquist 1980, 1995). Adults are found in aggrega-

tions in areas of low illumination and occur in high abundance ($>1 \text{ m}^{-2}$, pers. obs.) on the Great Barrier Reef. All sponges were collected on a shallow coral reef slope (4 to 8 m) at Orpheus Island ($18^{\circ}35'37'' \text{ S}$, $146^{\circ}29'07'' \text{ E}$) in the Palm Islands group, Queensland, Australia. *L. variabilis* larvae are ciliated, hollow, and approximately $400 \mu\text{m} \times 200 \mu\text{m}$, with a band of cilia at the posterior pole which is typical of the larvae of the Subclass Ceractinomorpha, Order Dictyoceratida. The term 'settlement' used here describes the permanent attachment of larvae to the substratum by the anterior pole and the completion of metamorphosis. Metamorphosis involves the flattening of the posterior half of the larva to form a mauve coloured disc. The larvae from between 4 and 17 sponges were pooled for use in all experiments.

Larval release. To determine patterns of release and the number of larvae released in the field and in the aquarium, 20 gravid *Luffariella variabilis* were collected on 16 November 2005 and placed in flow-through aquaria until 19 December 2005. A further 11 gravid *L. variabilis* were marked in the field. Gravid sponges were identified by removing a $\sim 1 \text{ cm}^3$ piece of mesohyl and visually checking for the presence of white larvae ($\sim 400 \mu\text{m}$). Mesh traps were placed over gravid sponges to collect released larvae. Larvae swam up on release and were collected in an inverted container. To determine the time of spawning, traps on sponges in the aquarium were checked at dusk (18:15 h), midnight (00:00 h), dawn (05:00 h), morning (07:30 h), mid morning (10:00 h), midday (12:00 h) and late afternoon (16:00 h) for 5 sequential days. Larvae were also collected and counted for sponges in the field on the same days and at the same times except midnight. Larvae were only released during the day (see 'Results'). Subsequently, larvae were collected in the aquarium mid-morning and mid-afternoon. Larvae were also collected and counted at least once per day in the field to determine whether larval release patterns in the field were the same as those in the aquarium.

Behaviour of larvae on release. Larval release (commencing at $\sim 07:00 \text{ h}$) by *Luffariella variabilis* is cued by daylight (see 'Results'), and we determined the response of larvae to natural light directly on release. To determine the direction in which newly released larvae swam in response to light over time, larvae were obtained immediately on release from parent sponges in the aquarium and subjected to the light treatment experiments described below. To ensure only newly released larvae were used, traps were replaced on spawning sponges at 06:00 h. Low numbers of larvae were available directly on release and as such, experiments were repeated over 3 replicate days.

For each experiment, groups of 10 larvae were introduced into 1 l graduated cylinders containing $0.2 \mu\text{m}$ -

filtered sea water (hereafter FSW). To examine larval phototactic behaviours 4 treatments were tested, each offering different light exposures: (1) fully covered with black plastic, (2) top half covered, (3) lower half covered, or (4) uncovered. All experiments were conducted under natural light and photoperiod. The positions of larvae within the cylinders were recorded every 20 min for the first 120 min, and again at 240 min. To remove any bias associated with passive movement due to larval buoyancy, 10 newly released larvae were killed with a 5% formalin solution at this time to determine whether they were positively, negatively or neutrally buoyant.

Behaviour of larvae after 2 h (post-release behaviour). Only small numbers of larvae (single to tens) were available immediately on release, therefore, the response of larvae 2 to 4 h after release to light was quantified, resulting in the collection of hundreds to thousands of larvae and allowing for larger sample sizes. Two experiments were conducted that decoupled the effect of depth on larval behaviour in response to light.

In the first experiment, 3 replicate groups of 100 larvae were placed in 1 l graduated cylinders with the (1) top half covered, (2) lower half covered or, (3) fully uncovered. The position of swimming and settled larvae within the cylinders was recorded at 0.5, 1, 2, 3, 6, 8, 11 and 18 h. The experiment was conducted under natural light and photoperiod.

The second experiment was used to decouple the influence of depth on the phototactic response. In this experiment, 300 larvae were placed in a 40 × 20 cm aquarium half covered with black plastic and with 3 cm water depth. The cover created light levels equivalent to 449 $\mu\text{mol s}^{-1} \text{m}^{-2}$ outside the cover, and 36.2 $\mu\text{mol s}^{-1} \text{m}^{-2}$ under the cover. The experiment was conducted under natural light and photoperiod. The position of swimming and settled larvae was noted at 0.5, 1, 2, 3, 6, 8, 11 and 18 h and was replicated 3 times over 3 days.

Light levels and settlement. Given the behavioural response of larvae to light and that settlement only occurred in the dark (see 'Results'), we quantified the effect of light on settlement. Four replicate containers (20 ml plastic petri dishes containing 10 ml of 0.2 μm FSW), each holding 20 larvae, were placed at 5, 10, 15, 20, 25, 30 and 35 cm distance from an overhead cold light source (Leica CLS 150X). Larvae maintained in containers in the dark were used as controls. Experimental containers were configured to prevent shading. Light levels at each distance from the light source were measured using a LI-COR LI250 light meter and were 56.00 ± 2.78 $\mu\text{mol s}^{-1} \text{m}^{-2}$ (at 5 cm), 14.23 ± 0.88 (10 cm), 3.48 ± 0.12 (15 cm), 1.26 ± 0.03 (20 cm), 0.70 ± 0.01 (25 cm), 0.44 ± 0.01 (30 cm) and 0.34 ± 0.01 (35 cm). The proportion of larvae settled in each container was

measured at 0.5, 1, 2, 3, 6, 8, 11 and 18 h. Two replicate experiments were conducted over 2 consecutive days. A repeated measures general linear model (GLM) was run on arcsine square root transformed data with time as the within-subject factor. Distance and day were between-subject factors.

Settlement in the presence of conspecifics. Given the aggregated pattern of adult *Luffariella variabilis* in the field, the potential gregarious nature of larval settlement was quantified. Densities of 1, 2, 5, 10 and 50 larvae were placed in 20 ml plastic petri dishes containing 10 ml of 0.2 μm FSW, and the proportion of larvae settled was measured at 0.5, 1, 2, 3, 6, 8, 11 and 18 h. Five replicates for each density were set up in the dark and natural light at the beginning (mid-November 2005) of the spawning season. Settlement only occurred in darkness (see 'Results'), and experiments were repeated in the dark at the end of the spawning season (mid-December 2005), to determine whether larvae changed their behaviour over time. A repeated measures GLM with time as the within-subject factor and density as the between-subject factor was used to analyse the arcsine square root transformed settlement data. Single larvae were excluded from the analysis.

Settlement cues. The effect of common invertebrate settlement cues on larval settlement was determined. Experiments were conducted early in the spawning season (mid-November 2005) and late in the spawning season (mid-December 2005), to determine whether larvae altered their response to settlement cues over time.

In the first experiment (mid-November 2005), 3 replicate groups of 10 larvae were introduced into 20 ml plastic petri dishes containing 10 ml of 0.2 μm FSW plus a settlement cue, and time to settlement was measured at 0.5, 1, 2, 5, 8, 11 and 18 h. As larvae do not settle in the light (see 'Results'), the experiment was conducted in both light and dark to determine whether light in combination with settlement cues induced settlement. Treatments contained (1) a biofilm on a polyethylene container left in flowing unfiltered seawater for 24 h, after which the water was removed and replaced with FSW; (2) 20 settled and metamorphosed live larvae settled on the base of a polyethylene container with the water removed and replaced with FSW; (3) a 0.5 mm^2 piece of mixed crustose coralline algae collected at Orpheus Island; (4) 20 μl of crustose coralline extract (Harrington et al. 2004), for which 100 g of the surface of *Neogoniolithon fosliei* was extracted twice in 300 ml of methanol and the extracts dried under rotary evaporation and nitrogen. The extract was then redissolved in dimethyl sulfoxide (DMSO) and made up in methanol to give 5 g extract l^{-1} methanol in 10% DMSO, equivalent to 0.01 mg ml^{-1} DMSO; (5) 20 μl of a 10% DMSO blank control (equivalent to 0.01 mg ml^{-1} DMSO); (6) a sterile container

with FSW as a control. A repeated measures GLM, with time as the within-subject factor and cue as the between-subjects factor, was used to analyse the arcsine square root transformed settlement data. The experiment was repeated each day for 3 days.

In the second experiment (mid-December 2005), a modified design incorporating the same cues as above was repeated in the dark, as larvae did not settle in the light (see 'Results'). A single experiment was run with 3 replicates of each of the treatments above and time to settlement measured again at 0.5, 1, 2, 5, 8, 11 and 18 h. Two additional treatments using a 1 mm² piece of sponge skeleton, or a 1 mm² piece of fresh *Luffariella variabilis* pinacoderm/mesohyl, were also incorporated to determine the effects of the presence of adults on larval settlement. A repeated measures GLM was run with time as the within-subject factor and cue as the between-subject factor to analyse the arcsine square root transformed settlement data.

In a final experiment, the effects of the cues described above on settlement of single larvae were also tested in mid-December 2005 with 3 replicate treatments for each cue, each with a single larva. This was to determine the response of single larvae to settlement cues. These data were not formally analysed.

Conspecific settlement cues. To determine whether there was a settlement cue associated with conspecifics, groups of 200 larvae were settled overnight in 60 ml aliquots of 0.2 µm FSW to produce 'conditioned' water. This 'conditioned' water was subsequently filtered over 0.2 µm. 10 ml aliquots of the water were then placed in 20 ml petri dishes, and individual larvae ($n = 85$) or groups of 10 larvae ($n = 17$) were added. Larvae placed in 1 d old aerated FSW were treated as controls. All experiments were conducted in the dark and the proportion of larvae settled was measured at 0.5, 1, 2, 5, 8, 11 and 18 h. A repeated measures GLM with time as the within-subject factor and 'conditioned water' as the between-subject factor was used to analyse the arcsine square-root transformed settlement data. Single larvae were excluded from this analysis.

Statistical analyses. Hypotheses were tested using either repeated measures or univariate analyses of variance (ANOVA). Assumption of normality and homogeneity of variance was checked graphically for each dataset by plotting residuals, and data were transformed where necessary (Quinn & Keough 2002). Any experiments with single larvae were excluded from formal analyses. Variance-covariance sphericity of the data used in any repeated measures GLM was estimated using the Greenhouse-Geisser epsilon, and significances of within-subjects F ratios were adjusted accordingly. Tukey's post-hoc tests were used to determine experimental groupings. Gaines-Howell post-

hoc tests were used on repeated measures data if unequal variances were encountered (e.g. from proportional settlement data). All analyses were done using SPSS (v.12).

RESULTS

Larval release

The larval release period for *Luffariella variabilis* was between 07:00 and 16:00 h in the field and in the aquarium throughout the study period (~5 wk), with the maximum release of larvae (up to 500 larvae sponge⁻¹) occurring mid-morning. No larvae were found in traps emptied at dusk (18:15 h), midnight (00:00 h) and at dawn (05:00 h) in the aquarium, demonstrating that there was no larval release after 16:00 h. Similarly, no larvae were released in the field between 18:15 and 05:00 h. The maximum release by an individual sponge was 830 larvae sponge⁻¹ d⁻¹, with the 30 sponges releasing a total of 45 283 larvae over the spawning period.

The number of larvae released by individual sponges was not consistent over time, with some sponges releasing small numbers of less than 100 larvae sponge⁻¹ d⁻¹ and occasional large pulses (>400 larvae sponge⁻¹ d⁻¹) (Fig. 1). In contrast, others released large pulses of larvae (> 400 larvae sponge⁻¹ d⁻¹) on most days (Fig. 1). However, sponges in the aquarium had the same release patterns as those in the field, and larval release almost ceased in the aquarium and in the field during a period of rough, overcast weather. Spawning ended abruptly in all sponges in mid-December (Fig. 1).

Behaviour of larvae on release

This experiment determined the directional response to light of larvae at the time of release from parent sponges. Formalin fixed (i.e. dead) larvae sank, confirming that larvae are negatively buoyant on release and therefore actively maintained their position within the water column.

All larvae swam upward after release, regardless of treatment (Fig. 2). However, there was a marked reversal of behaviour after 20 to 40 min, when larvae exhibited a negative phototaxis and moved towards the darkest parts of all treatments, regardless of orientation (Fig. 2a,b). In the uncovered treatment, half the larvae were found at the surface (4.3 ± 1.4) and the other half (5.3 ± 1.2) at the bottom (Fig. 2c). Larvae did not move from the top of the fully covered (all dark) cylinder (Fig. 2d).

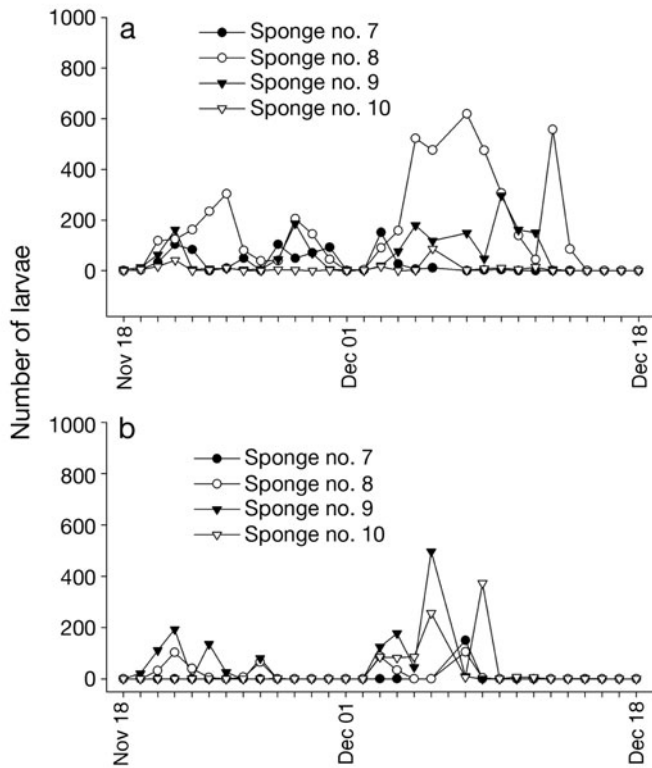


Fig. 1. *Luffariella variabilis*. Larval release by a subset of 4 adult sponges (nos. 7, 8, 9, 10) (a) in aquaria, (b) in the field

Behaviour of larvae between 2 and 4 h old

The experiment was intended to demonstrate the directional response of larvae to light 2 to 4 h following release. At this time, larvae maintained the negative phototaxis first exhibited after 20 min and moved directly to the darkest areas of all treatments. After 30 min, an average of 95.0 ± 5.0 larvae could be found at the top of the cylinders covered at the top (Fig. 3a), while 98.3 ± 1.7 were located at the bottom of the cylinders covered at the bottom (Fig. 3b). Settlement occurred almost exclusively in the dark. Settlement of 50% of the larvae occurred after ~5 h in the cylinders covered at the top and after ~9 h in the cylinders covered at the bottom. In natural light (the uncovered cylinders), larvae were more broadly distributed. After 30 min, 64.0 ± 32.0 larvae were found on the bottom and 34.0 ± 32.0 at the surface (Fig. 3c). After 2 h, 80.0 ± 9.0 larvae were on the bottom and 16.0 ± 9.0 at the surface. Larvae did not begin to settle until natural light ceased after 6 h (Fig. 3c).

In the second experiment to determine the effect of light on 2 to 4 h old larvae without the potential bias of depth inherent in the use of measuring cylinders for incubations, all larvae rapidly moved to the dark area of the aquarium within 30 min. No larvae moved away from the dark area, and all larvae settled when natural light ceased (~6 h).

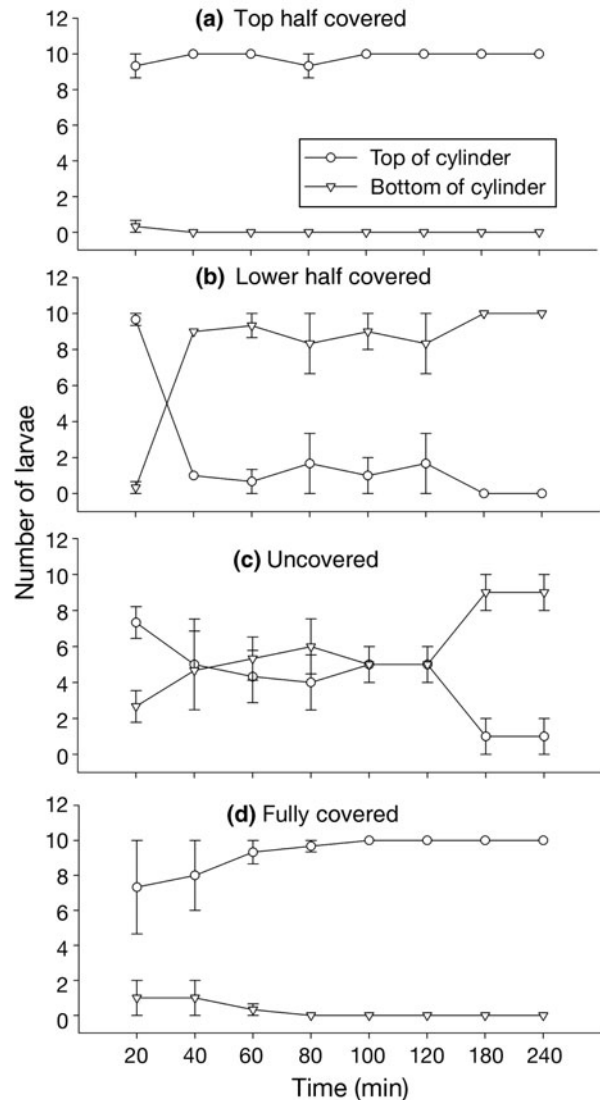


Fig. 2. *Luffariella variabilis*. Settlement positions of newly released larvae in tanks in response to light levels (mean \pm SE, $n = 3$, 10 ind. per replicate); (a) top half covered cylinder, (b) lower half covered cylinder, (c) uncovered cylinder, (d) fully covered cylinder

Light levels and settlement

Light had a significant negative effect on the settlement of *Luffariella variabilis* larvae. Settlement rate and proportion decreased with increasing light intensity (Fig. 4). Light levels of 56.00 ± 2.78 and $14.23 \pm 0.88 \mu\text{mol s}^{-1} \text{m}^{-2}$ slowed the settlement rate of larvae and inhibited overall settlement after 18 h by ~60 and 35%, respectively, compared to controls (>95% settlement). Light levels of $3.49 \pm 0.11 \mu\text{mol s}^{-1} \text{m}^{-2}$ to $1.26 \pm 0.03 \mu\text{mol s}^{-1} \text{m}^{-2}$ slowed settlement rates, but resulted in the same overall settlement compared to controls (Fig. 4). Settlement of larvae in all other light treat-

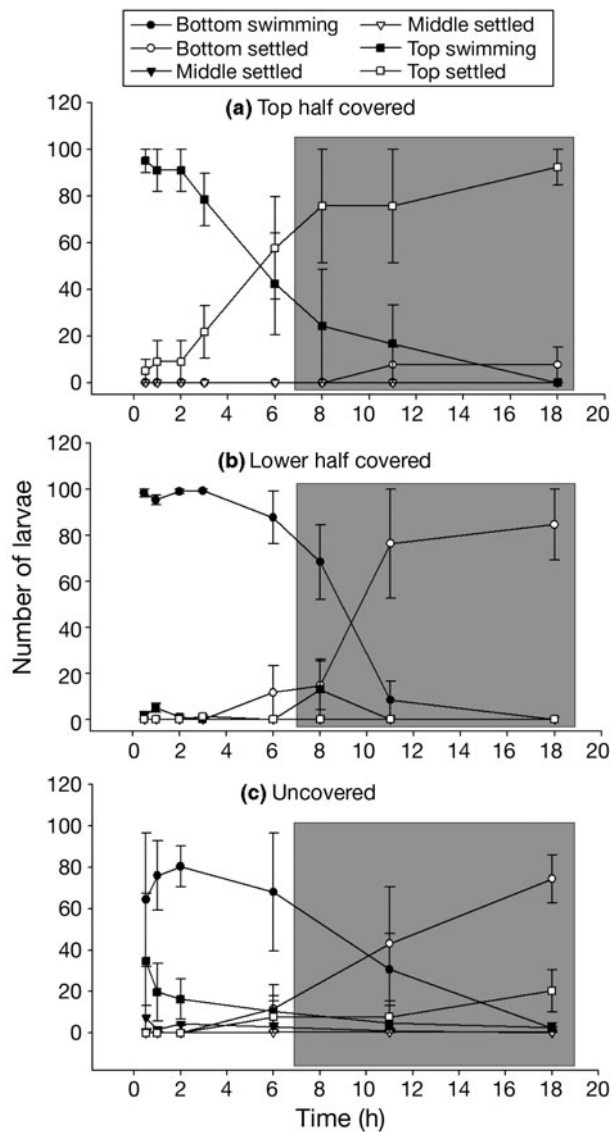


Fig. 3. *Luffariella variabilis*. Settlement positions of 2 to 4 h old larvae in aquaria in response to light levels (mean ± SE, n = 3, 100 ind. per replicate); (a) top half covered cylinder, (b) lower half covered cylinder, (c) uncovered cylinder. Grey boxes represent end of daylight

ments did not differ from that of the controls. Therefore, larvae were able to settle in similar overall proportions compared to dark controls when subjected to light levels less than $3.49 \mu\text{mol s}^{-1} \text{m}^{-2}$ (15 cm from the light source) (Fig. 4).

A repeated measures GLM on arcsine square root transformed data found no significant effect of replicate days on which the experiment was run. All replicates were thus combined, and the analysis re-run with time and distance as within-subject and distance as the between-subject factors (Table 1). The variation in total settlement between treatments was indicated

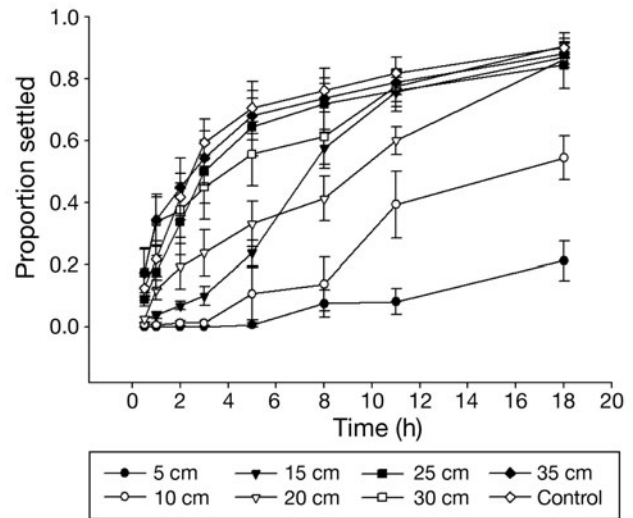


Fig. 4. *Luffariella variabilis*. Proportion of larvae settled at different distances from a cold light source (mean ± SE, n = 4, 20 ind. per replicate)

by a significant time term ($F_{4,203} = 176.51$; $p < 0.001$). Furthermore, the differing overall settlement rates were indicated by a significant time × distance term ($F_{25, 203} = 3.54$; $p < 0.001$). A Games-Howell post-hoc test ($p = 0.05$) on distance determined 4 groups of treatments: 5 and 10 cm (Group a); 15 cm (Group bc); 20 cm, 25 cm, 30 cm (Group cd); 35 cm and control (dark) (Group d).

Settlement in the presence of conspecifics

Larvae of *Luffariella variabilis* exhibited gregarious settlement with increasing densities of individuals (Fig. 5). Groups of 50 and 25 larvae settled the fastest and achieved a higher total settlement compared with groups of 10, 5, 2 larvae and single individuals. Single larvae showed the slowest and lowest overall settlement. In mid-December, settlement proceeded in the

Table 1. *Luffariella variabilis*. Settlement at different distances from a cold light source (repeated measures ANOVA on arcsine square root transformed data)

Source	df	MS	F	p
Within subjects				
Time	3.62	8.35	176.51	<0.001
Time × Distance	25.38	0.17	3.54	<0.001
Error (Time)	203.10	0.05		
Between subjects				
Distance	7	4.08	22.41	<0.001
Error	56	0.18		

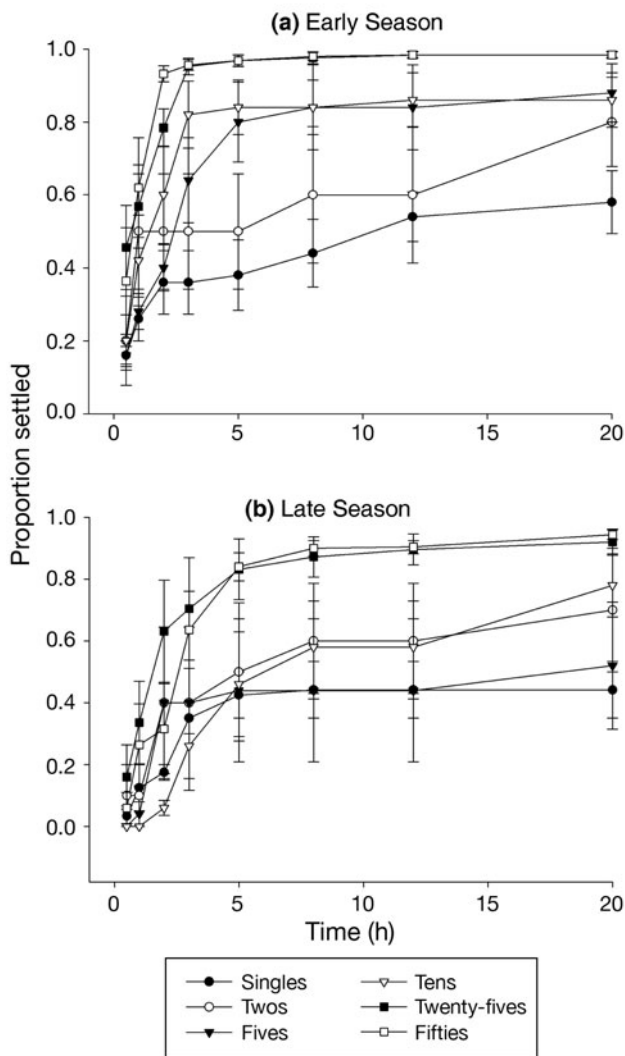


Fig. 5. *Luffariella variabilis*. Proportion of larvae settled at different densities (a) in mid-November and (b) in mid-December in the dark (mean \pm SE, $n = 5$)

same overall pattern, but more slowly, with groups of 50 and 25 larvae reaching maximum settlement at 5 to 8 h, compared to 3 h in November. Overall settlement was ~95% at both times.

A repeated measures 3-factor GLM was run on arcsine transformed data with time, density and season as within-subject factors and density and season as between-subject factors. Significant within-subject factors were only found for density ($F_{4,40} = 3.94$; $p < 0.009$) and season ($F_{1,40} = 12.37$; $p < 0.001$), demonstrating the overall difference between the densities of individuals in the treatments and the timing of the experiments (Table 2).

A 2-factor GLM on combined settlement data run at $t = 5$ h, where settlement began to plateau, detected significant density ($F_{4,40} = 3.46$; $p < 0.016$) and season

Table 2. *Luffariella variabilis*. Influence of different larval densities at the beginning and end of the spawning season (repeated measures ANOVA on arcsine square root transformed data)

Source	df	MS	<i>F</i>	<i>p</i>
Within subjects				
Time	2.94	6.81	80.96	<0.001
Time \times Season	2.94	0.14	1.77	0.158
Time \times Density	11.75	0.07	0.89	0.589
Time \times Season \times Density	11.75	0.12	1.50	0.136
Error (Time)	117.52	0.09		
Between subjects				
Season	1	4.97	12.37	0.001
Density	4	1.58	3.93	0.009
Season \times Density	4	0.17	0.43	0.785
Error	40	0.40		

($F_{1,40} = 5.39$; $p < 0.025$) terms (both fixed). Density \times season was not significant ($F_{4,40} = 0.72$; $p = 0.585$). A Tukey's post-hoc test for density (i.e. both seasons combined) at $p < 0.05$ resulted in 3 groups of 2 larvae (Group a), 5 and 10 larvae (Group ab), 25 and 50 larvae (Group b).

Settlement cues

This experiment determined whether common invertebrate settlement cues affected the settlement of *Luffariella variabilis* larvae and whether any effects changed throughout the spawning period. No cue had any significant effect on settlement at any time or with any larval density, although larvae settling in the presence of a piece of live adult *L. variabilis* displayed a faster settlement rate (Figs. 6 & 7, Table 3). The variation in total settlement between treatments was indicated by significant time terms for both the early ($F_{3,113} = 124$; $p < 0.001$) and late seasons ($F_{2,35} = 64.53$; $p < 0.001$). However, differing overall rates of settlement between days and cues were only found in the early season, as indicated by significant time \times day ($F_{6,113} = 3.03$; $p = 0.008$) and time \times cue ($F_{15,113} = 2.12$; $p = 0.012$) interactions.

Settlement assays using conditioned water

While there was no effect of common invertebrate settlement cues on settlement of larvae, both single individuals and groups of 10 placed in 'conditioned' water reached 55% settlement after only 20 min, in contrast to <10% settlement in controls. Moreover, single larvae never reached more than 20% settlement in

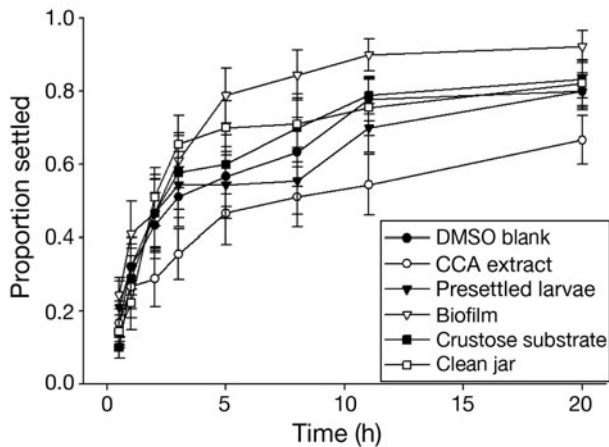


Fig. 6. *Luffariella variabilis*. Proportion of larvae settled in response to common invertebrate larval settlement cues in mid-November (mean ± SE, n = 3, 10 ind. per replicate) DMSO = dimethyl sulfoxide. CCA = crustose coralline algae

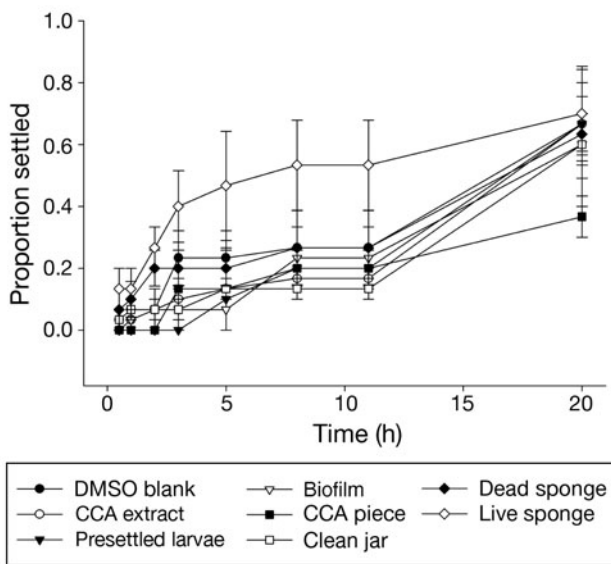


Fig. 7. *Luffariella variabilis*. Proportion of larvae settled in response to common invertebrate settlement cues in mid-December (mean ± SE, n = 3, 10 ind. per replicate). DMSO = dimethyl sulfoxide. CCA = crustose coralline algae.

controls, while settlement after 18 h was 80 % for single larvae in 'conditioned' water (Fig. 8a). In contrast, groups of 10 larvae reached ~80% settlement for both treatments and controls; however, settlement was more rapid for larvae in 'conditioned' water (Fig. 8b). A repeated measures GLM run on untransformed data for 10 larvae found no significant effect of replicate days in the experiment. All replicates were combined and the analysis re-run with time as the within-subject and conditioned water as the between-subject factor.

Table 3. *Luffariella variabilis*. Influence of common invertebrate settlement cues early and late in the spawning season (repeated measures ANOVA on arcsine square root transformed data)

Source	df	MS	F	p
<i>Early in the spawning season</i>				
Within subjects				
Time	3.16	11.01	124.00	<0.001
Time × Day	6.32	0.270	3.03	0.008
Time × Cue	15.80	0.19	2.12	0.012
Time × Day × Cue	31.59	0.08	0.87	0.661
Error (Time)	113.71	0.09		
Between subjects				
Day	2	5.22	8.35	0.001
Cue	5	1.34	2.14	0.083
Day × Cue	10	0.76	1.22	0.314
Error	36	0.62		
<i>Late in the spawning season</i>				
Within subjects				
Time	2.18	2.63	64.53	<0.001
Time × Cue	15.24	0.40	0.86	0.610
Error (Time)	34.83	0.04		
Between-Subjects				
Cue	7	0.18	2.59	0.055
Error	16	0.07		

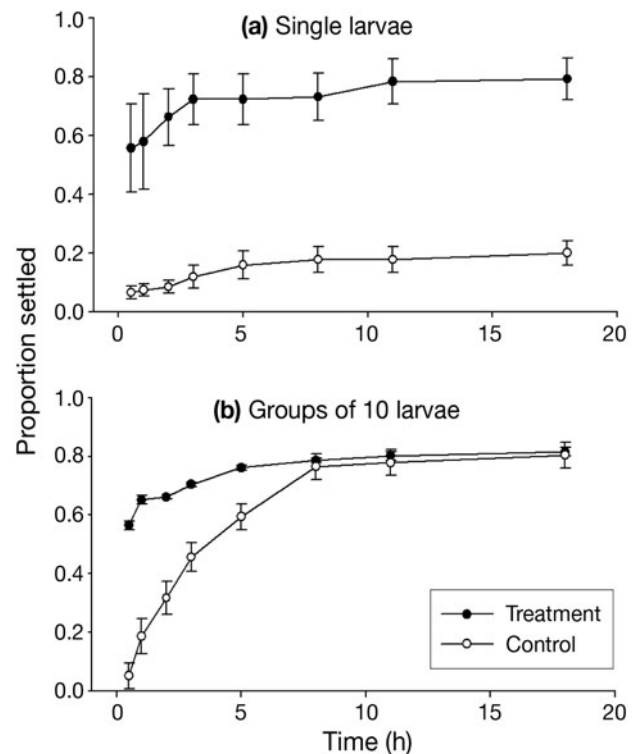


Fig. 8. *Luffariella variabilis*. (a) Proportion of single larvae settled in 'conditioned' water and in controls (mean ± SE, n = 85), (b) Proportion of larvae settled in the presence of conspecifics in 'conditioned' water and in controls (mean ± SE, n = 17, 10 ind. per replicate)

Table 4. *Luffariella variabilis*. Settlement in the presence of a conspecific settlement cue (repeated measures ANOVA on arcsine square root transformed data)

Source	df	MS	F	p
Within subjects				
Time	3.78	2.70	81.40	<0.001
Time × Condwater	3.78	1.19	35.75	<0.001
Error (Time)	121.04	0.03		
Between subjects				
Condwater	1	4.71	29.46	<0.001
Error	32	0.16		

The more rapid rate of settlement of those larvae in conditioned water was demonstrated by a significant time × conditioned water term ($F_{4,121} = 35.75$; $p < 0.001$) (Table 4).

DISCUSSION

A hierarchy of cues drives dispersal and habitat choice in *Luffariella variabilis* larvae. Light cues the release of larvae by adult sponges and larvae swim upwards at the time of release. Subsequently, larvae become strongly photonegative and settle at the same rate as dark controls when subjected to light levels lower than $\sim 3 \mu\text{mol s}^{-1} \text{m}^{-2}$. There are strong gregarious settlement effects with increasing densities of larvae, leading to higher overall settlement. Our study demonstrates that a cue released by settling larvae significantly increases the rate of settlement, which is most apparent when single larvae are exposed to this cue. Common invertebrate settlement cues have no effect on the settlement of *L. variabilis* larvae.

This study demonstrates that light cues the release of brooding larvae of *Luffariella variabilis* and determines an entire season's larval release from a large sample size of sponges. The dynamics of larval release appear similar to other brooding demosponges with 1 or 2 annual peaks lasting for weeks or months, usually during summer (Maldonado & Young 1996, Lindquist et al. 1997, Mariani et al. 2005). However, some brooding demosponges also release small amounts of larvae throughout the year in addition to large outputs once or twice a year (Zea 1993, Lindquist et al. 1997), while others release larvae all year round (Leys & Degnan 2002). The rate of larval release by sponges ranges from several larvae per individual over a few hours to the release of the entire brood at one time (reviewed in Maldonado 2006), and the release rate of 10s to 1000s larvae d^{-1} for *L. variabilis* corresponded with that of other demosponges (Meroz & Ilan 1995, Lindquist et al. 1997).

Newly released *Luffariella variabilis* larvae initially swim upwards, indicating either a positive phototaxis or negative geotaxis. This upward movement may facilitate dispersal (Bergquist & Sinclair 1968, Wapstra & Van Soest 1987, Maldonado et al. 1997). Subsequently, larvae became negatively phototactic after 40 min. This change towards negative phototaxis was confirmed by the observation of 2 to 4 h old larvae, which actively swam either up, down or sideways to access the darkest areas of the vessels in which they were held. A light cue for larval release is proposed to ensure the daytime release of some demosponge larvae (Amano 1986, 1988). The change in phototaxis of sponge larvae from positive to negative is suggested to facilitate dispersal and increase the chance of intercepting settlement cues (sensu Wapstra & Van Soest 1987, Harrison & Wallace 1990, Raimondi & Morse 2000).

Light-dependent settlement of *Luffariella variabilis* larvae is corroborated by the distribution of adult *L. variabilis* in the field, which are almost always found in areas of low irradiance (i.e. crevices, caves and between rubble). Other demosponge larvae show strong responses to light. For example, the parenchymellae of *Halichondria caerulea* stop swimming at a given distance from a light source, suggesting that a photonegative response is only displayed below a given irradiance level (Maldonado et al. 1997). In our study, *L. variabilis* larvae were released during the day, but even very low amounts of light delayed their settlement, suggesting that daytime release is probably required to provide a light gradient guiding larvae to dark microhabitats. The selection of dark habitats potentially provides protection against grazers, silt and ultraviolet radiation, or mitigates competition with photo-autotrophs (Lindquist & Hay 1996, Maldonado & Uriz 1998). Accordingly, dispersal potential of *L. variabilis* larvae is likely to be restricted, due to the short time they swim upwards and the rapid onset of a negative phototaxis. This may contribute to genetically structured local populations (Goffredo et al. 2004).

While light cues the release of larvae and guides their behaviour and settlement at large scales, smaller scale environmental variables, such as chemical cues and surfaces, may explain some local sponge settlement patterns. *Luffariella variabilis* larvae did not respond to a variety of common invertebrate settlement cues. While no settlement was observed in response to settled, attached and metamorphosed conspecifics motile *L. variabilis* larvae settled gregariously. This also corresponds with the aggregated distribution of *L. variabilis* adults *in situ*. Gregarious settlement by other invertebrate larvae has been observed to lead to aggregations of conspecific adults (Burke 1986, Gotelli 1990). Gregarious settlement is thought to increase protection from predation (Sebens 1983, Keough 1984), enhance competitive

abilities (Maldonado & Uriz 1998), and reduce juvenile and adult mortality (Osman & Whitlatch 1995).

In the case of *Luffariella variabilis*, gregarious settlement is associated with conspecific larvae rather than adults, although there was some settlement response to adults. When larvae were placed in conditioned water, initial settlement rates were 6 times faster compared to controls, indicating that larvae may release a waterborne settlement cue. Furthermore, the effect was highest on single larvae with a 4-fold increase in overall settlement. This is one of the few studies to unequivocally demonstrate that a conspecific cue not related to adult conspecifics or other biotic or abiotic factors induces settlement in larvae. A similar model is found in barnacles, where gregarious settlement is mediated by larval pheromones (Matsumura et al. 1998, Dreanno et al. 2006a,b), which are either waterborne or surface-bound in larval footprints (Clare et al. 1994). There is a likelihood that the efficiency of any conspecific cues would be reduced with dilution, particularly if water currents are considered. Nevertheless, whilst our study was undertaken in still water, the aggregated distribution of adult *L. variabilis*, coupled with the findings of gregarious settlement, suggests that conspecific cues contribute to larval settlement. Further experiments to determine the ecological benefits of these effects (especially in natural flow situations) would contribute to an understanding of how gregarious behaviour guides larvae to find appropriate habitats in the restricted time they have for dispersal, and how cues affect post-larval distribution and survival of this species.

In conclusion, a hierarchy of cues influences the settlement of *Luffariella variabilis* larvae. Light cues the release of larvae, and they swim upwards immediately after release. Subsequently, larvae become strongly photonegative and only settle in areas with low light levels. At smaller scales, there are strong gregarious settlement effects, and a waterborne cue released by settling larvae significantly increases the number of settled larvae and the settlement rate, which corresponds to the clumped distribution of adults in dark habitats in the field. Our study is one of the few of a marine invertebrate species that integrates factors affecting larvae from initial release through to settlement and metamorphosis.

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