

Larval settlement behaviour in six gregarious ascidians in relation to adult distribution

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ABSTRACT: Settlement influences the distribution and abundance of many marine organisms, although the relative roles of abiotic and biotic factors influencing settlement are poorly understood. Species that aggregate often owe this characteristic to larval behaviour, and we investigated whether this predisposes ascidians to becoming invasive, by increasing their capacity to maintain their populations. We explored the interactive effects of larval phototaxis and geotaxis and conspecific adult extracts on settlement rates of a representative suite of 6 species of ascidians that form aggregations in the field, including 4 aliens with global distributions, and how they relate to adult habitat characteristics. In the laboratory, the larvae were (1) held in light or dark, (2) offered the choice of settling in the light or dark, or (3) held in the presence or absence of adult extract. When confined in either light or dark conditions, all species settled equally in dark and light. Four showed strong geotaxis, 3 settling preferentially on the bottom of experimental chambers, and one on the top. Offered a choice between dark and light, 2 species settled preferentially in the dark with no geotactic preferences and another 2 showed an interaction between light and geotaxis. For 4 of the species, the responses of settlers accorded with, and may contribute to, adult orientation patterns in the field. Adult extracts inhibited settlement of 3 species and failed to influence settlement of the other 3, arguing against conspecific attraction being a cause of aggregation and an explanation of the propensity of ascidians to become invasive.

KEY WORDS: Ascidiacea · Chemical cues · Gregarious behaviour · Invasive species · Larval settlement · Conspecific attraction

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INTRODUCTION

Many aquatic organisms are free spawners, releasing enormous numbers of eggs and sperm into the environment (Yund 2000, Byrne et al. 2003), of which only a small portion will attain successful fertilisation (Underwood & Keough 2001). This situation parallels terrestrial plant systems, where the success of populations is greatly influenced by seed dispersal and conditions where the seeds land and germinate (Nathan & Muller-Landau 2000). Settlement patterns of dispersive propagules are therefore a major determinant of the distribution and abundance of adults. For example, some species avoid settlement in the presence of dominant competitors (Grosberg 1981), while others do not (Durante 1991, Bullard et al. 2004), and the production of bioactive substances by the adults of some species

can detrimentally affect the larvae of competitors (Koh & Sweatman 2000). Conversely, the presence of adults and associated chemical cues is normally regarded as an attractor for settlement alongside conspecific adults (Bryan et al. 1997, Ramsay et al. 1999, Hadfield & Paul 2001, Ward & Schlossberg 2004) or an inducer of metamorphosis (Svane et al. 1987, Tsukamoto 1999, Kopin et al. 2001, Dreanno et al. 2006), which may cause aggregation (Toonen & Pawlik 1994, Petersen & Svane 1995). In addition, phototactic and/or geotactic behaviour of the larvae can determine where settlement occurs (Svane & Young 1989, Svane & Dolmer 1995, Wendt & Woollacott 1999). For all of these reasons, settlement has the capacity to strongly influence habitat selection, determining adult distribution patterns of sessile and sedentary species (Keough & Downes 1986, Toonen & Pawlik 1994, Underwood & Keough 2001).

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Propagule pressure, defined as the combined effect of the number of individuals introduced and the number of introduction attempts, has been identified as an important predictor of invasiveness of non-native species (Colautti et al. 2006). Because conditions for propagule establishment and development often differ between the native and invaded ranges, most invasive species perform differently in localities to which they are introduced, where they are often more abundant (DeWalt et al. 2004, Kasper et al. 2008), larger (Ross & Auge 2008), comparatively free of predators (Wolfe 2002), less prone to parasitism (Calvo-Ugarteburu & McQuaid 1998) and have a higher reproductive output (Hinz & Schwarzlaender 2004). Moreover, invasive species generally show a strongly aggregated distribution (Kopin et al. 2001, Dulloo et al. 2002, Campbell & Donlan 2005, Dupont et al. 2006) and form large monospecific stands that can monopolise available habitat (Simberloff et al. 2005, Rius et al. 2009a). Consequently, species that are gregarious or aggregate may be pre-adapted to becoming alien invaders because they will more readily form groups that are sufficiently concentrated to be reproductively viable, whereas non-gregarious species will have more difficulty in reaching a viable density after arrival in a new environment. A possible mechanism for aggregated distribution might be gregarious settlement around conspecifics, which may help to secure alien species in their new environment. Despite their potential importance, both gregariousness and kinship concepts have scarcely been applied to the study of invasive species, although they could elucidate evolutionary processes behind biological invasions.

Marine ecosystems have experienced dramatic increases in the rate of introductions of non-indigenous species (Cohen & Carlton 1998, Whiteley & Bendell-Young 2007). Most of the species responsible for marine biological invasions are from lower trophic levels, with filter-feeding invertebrates making up 70% of invasions in coastal areas (Byrnes et al. 2007). Ascidiaceans are major contributors (Lambert 2005, 2007), and can severely modify the structure of coastal habitats by forming large aggregates (Lambert & Lambert 2003, Castilla et al. 2004, Rius et al. 2009a). Adults live attached to hard substrata (Monniot et al. 1991), and the only motile stage is their lecithotrophic larvae, which have very limited dispersal due to their short planktonic lifespans (Millar 1971, Svane & Young 1989). Some information is available regarding the distribution of adult ascidians in the field (e.g. Turon 1990, Mastrototaro et al. 2008), although the settlement patterns that may explain these adult distributions are well-understood for only a few species (e.g. Howes et al. 2007). Many factors can influence ascidian larval behaviour and settlement, including light, gravity, temperature, salinity, presence of adults

or competitors, biomechanical properties and energy limitations (Yamaguchi 1975, Svane et al. 1987, Svane & Young 1989, Young 1989, Vázquez & Young 1996, Thiagarajan & Qian 2003, McHenry & Patek 2004, Bennett & Marshall 2005). Svane & Young (1989) stated that the time required for settlement of aggregated solitary ascidians is inversely related to the concentration of adult extracts to which larvae are exposed. Other studies have considered the effects of abiotic conditions on settlement (e.g. Young & Chia 1985, Svane & Dolmer 1995). However, no attempt has been made to analyse in combination the relative roles of biotic and abiotic factors on settlement for a representative set of species and their implication for the success of invasive populations.

We investigated the settlement patterns of larvae of 6 solitary ascidians found along the South African coast (*Ciona intestinalis*, *Asciidiella aspersa*, *Styela plicata*, *Microcosmus squamiger*, *Pyura herdmani* and *P. stolonifera*), which belong to 4 different families from the 2 recognised orders of Ascidiacea (Kott 1985) and are all commonly found aggregated in the field (Petersen & Svane 1995, Rius et al. 2009a, Branch et al. 2010). We chose these species to include 4 introduced species with global distributions (*C. intestinalis*, *A. aspersa*, *S. plicata* and *M. squamiger*) and 2 large native species (*P. herdmani* and *P. stolonifera*) that are not known to be invasive, although congeners are recognised as invasive elsewhere (Castilla et al. 2004). These species are all important occupiers of hard substrata of coastal areas of South Africa (Branch et al. 2010). The larvae of 4 species have well-developed statocytes and ocelli (Griffiths 1976, Niermann-Kerkenberg & Hofmann 1989, Jacobs et al. 2008, authors pers. obs.) but *S. plicata* has a highly reduced ocellus (Ohtsuki 1990), and *M. squamiger* is unusual among Pyuridae in lacking an ocellus (authors pers. obs.; see also Svane & Young 1991 for a closely related species). Thus, 4 species were expected to have both light and geotactic preferences, while the larvae of the remaining 2 species were expected to respond to geotactic stimuli alone.

We examined how larval behaviour determines settlement patterns in different phototactic and geotactic conditions and in the presence or absence of conspecific extracts. The larval responses were compared with patterns of adult distribution in the field. *A priori*, we advanced 3 hypotheses: (1) Light will influence settlement, with dark being preferred over light in species that are found in dark habitats, and the opposite for those that occur in well-lit habitats. (2) Geotactic behaviour will be important in those species that have adults with clear orientation preferences. (3) Adult extracts will have a positive effect on settlement on all tested species, and will contribute to the aggregated patterns of distribution of adults.

MATERIALS AND METHODS

Field sites and surveys of adults. Adult ascidians were surveyed and sampled at the locations characterised in Table 1. At each location, we quantified adult distribution and associated circumstances. To standardise conditions, all sampling took place mid-day at 12:00 h on cloudless days in October/November 2009 at depths of no more than 1 m. At each locality, 50 × 50 cm quadrats (n = 10 per substratum orientation) were placed on horizontal hard substrata facing upwards (0 to 10°), downwards (170 to 180°), or on vertical substrata (80 to 100°). The number of individuals of any of the 6 species present and the number of individuals per clump were counted. Due to the aggregating nature of ascidians and because they were often covered by algae or other fouling organisms, we removed clumps and brought them to the laboratory where they could be cleaned and sorted to count the number of individuals precisely. Light intensity was recorded at each sampling point by taking 3 random measurements within each quadrat using a photometer (Skye Instruments, Scientific Associates) fitted with a sensor (Quantum Sensor).

Timing of laboratory experiments. All laboratory experiments were conducted during the early spring of 2007 (end of August to early September) to coincide with the timing of reproductive maturity for all species: *Pyura stolonifera* and *Microcosmus squamiger* mature in spring and summer (Griffiths 1976, Rius et al. 2009a), *Ciona intestinalis* and *Styela plicata* in spring, summer and winter (Yamaguchi 1975, Rius et al. 2009b), and previous observations undertaken in South Africa (M. Rius unpublished data) on the remaining 2 species indicated that they mature in spring.

Fertilisation methods. About 10 adults of each species were collected from each of the locations specified in Table 1 and transported in insulated containers with 20 l seawater to the laboratory within 5 h. In the labo-

ratory, specimens were housed in aerated seawater and maintained at room temperature (15°C).

All manipulations and experiments were undertaken in filtered seawater obtained using vacuum filtration through 10 µm pore size filters. For *Ciona intestinalis* and *Asciidiella aspersa*, artificial fertilisation followed the methods of Young & Chia (1985), which involved dissection and collection of gametes from the oviduct and sperm duct. For the remaining species, we followed the methods of Marshall et al. (2000), modified from those of Svane & Young (1991): gametes were extracted by dissection of the ripe gonads, and a mix of eggs and sperm was poured through a 100 µm filter with seawater into a small beaker, so the eggs were retained by the filter, but the excess sperm and seawater passed through into the beaker. For all species, we crossed the gametes of 4 individuals, preventing self-fertilisation. Developing embryos were placed in an aerated beaker (containing 500 ml of seawater) in a constant-temperature cabinet at 20°C and complete darkness. In all species, motile larvae hatched within 14 h of fertilisation.

Experiments. Our experimental units were transparent cylindrical Perspex containers, sealed at the top and bottom with Perspex sheets and held together with an elastic band. The cylinders were 11 mm tall and 44 mm in diameter, with exactly the same surface area (15.205 cm²) on the top, bottom and lateral surfaces, thus offering equivalent surface areas for larval settlement in each of these 3 orientations. The containers were placed in a seawater tank for 24 h prior to introduction of larvae, to create a biofilm, which is known to enhance settlement (Keough & Raimondi 1995). Once motile larvae of a given species were formed, we pipetted out and placed 20 larvae per container filled with seawater (final volume 16.72 cm³), and immersed the containers in seawater in a 200 ml beaker at 20°C for 24 h under the experimental conditions detailed below. The Perspex chamber was subsequently dismantled in seawater, so that any unattached larvae were washed away.

Table 1. Characteristics of the sites where each species was collected. The numbers of replicates used for each experimental trial and species are also indicated. Experiments—1: light vs. dark; 2: half light vs. half dark; 3: tunic extracts. For details see 'Materials and methods: Experiments'

Species	Field sites				No. of replicates per experiment		
	Location	Latitude/longitude	Wave exposure	Substrata	1	2	3
<i>Ciona intestinalis</i>	Cape Town harbour	34°54'22" S, 18°25'37" E	Sheltered	Artificial	7	5	5
<i>Microcosmus squamiger</i>	Port Alfred marina	33°35'41" S, 26°53'32" E	Sheltered	Artificial	5	5	5
<i>Pyura herdmani</i>	Langebaan marina	33°01'07" S, 17°56'48" E	Moderately exposed	Artificial	8	10	8
<i>Pyura stolonifera</i>	St. James	34°07'14" S, 18°27'31" E	Highly exposed	Natural	3	3	3
<i>Asciidiella aspersa</i>	Cape Town harbour	34°54'22" S, 18°25'37" E	Sheltered	Artificial	6	4	4
<i>Styela plicata</i>	Knysna marina	34°03'17" S, 23°03'46" E	Sheltered	Artificial	5	5	6

We performed 3 experiments. The number of replicates (i.e. experimental units with 20 larvae each) per treatment and experiment varied from 3 to 10 due to variability in the number of larvae obtained (see Table 1). Once we obtained enough larvae in a given fertilisation event, we ran all experiments described below in parallel.

The first experiment (Expt 1) involved exposing the chambers with larvae to either artificial light ($47 \mu\text{mol m}^{-2} \text{s}^{-1}$) or complete darkness ($0 \mu\text{mol m}^{-2} \text{s}^{-1}$).

In the second experiment (Expt 2), which was modified from the approach of Jiang et al. (2005), we placed larvae in chambers in which half of the top, bottom and lateral surfaces was covered by black tape (reducing the light to $0.4 \mu\text{mol m}^{-2} \text{s}^{-1}$), while the other half of these surfaces was exposed to the same artificial light ($47 \mu\text{mol m}^{-2} \text{s}^{-1}$).

The third experiment (Expt 3) tested the effect of adult extracts on larval settlement, and for this we followed the general method of Svane et al. (1987), which involved dissolving tunic extracts in seawater. An initial concentration of 0.5 g (wet weight) of tunic, previously homogenised using a blender and filtered to eliminate the biggest fragments, was diluted in seawater to obtain a final concentration of 5% in the experimental chambers. Settlement of larvae in seawater with or without tunic extracts (control treatment) was then compared in complete darkness.

In all 3 experiments, a stereomicroscope was used to count the numbers of settlers and score their orientation (top, bottom or lateral sides of the containers) after a 24 h period.

Data analysis. For the field data on adult distributions, a 1-way analysis of variance (ANOVA) on square-root transformed data was used to test for differences in adult orientations (upwards, downwards or vertical), with surface orientation as a fixed factor to compare the number of individuals per quadrat for each species. Tukey's Honestly Significant Difference (HSD) post hoc tests were subsequently performed to assess significant differences among different orientations. To evaluate among the different species the level of gregariousness found in the field, we compared the number of individuals per clump found for each species using a 1-way ANOVA, with Species as a fixed factor. To test for differences in adult orientation, we used surface orientation as a fixed factor and compared the number of individuals per clump for each species using 1-way ANOVA. The data were 4th-root transformed, and significant differences were tested using pairwise comparisons with Tukey HSD post hoc tests.

For the laboratory experiments, we tabulated the number of settlers in 3-way frequency tables incorporating replicates (experimental chambers), treatments (light–dark, extract–control) and position of the settlers (bot-

tom, lateral, top), and used log-linear models for formal statistical testing of the significance of these factors and their interactions (Knoke & Burke 1991). Full models (including all factors and their interactions) were compared to reduced models which omitted the interactions or individual factors. The expected value for each cell in the table under the reduced model was computed by an iterative Newton-Raphson algorithm. The goodness of fit of the table of expected values to the observed table was then evaluated by the likelihood ratio test (Quinn & Keough 2002), using the chi-squared distribution to assess levels of significance. A poor fit indicated that the factor or interaction omitted contributed significantly to explaining the observed values.

First, we tested the effect of the different replicates by fitting to the 3-way tables a model that excluded all interactions of the factor replicate with the other 2 factors (i.e. the terms Treatment \times Replicate, Position \times Replicate, and Treatment \times Position \times Replicate). This tested whether settlement levels in the different replicates were independent of the other factors. As these reduced models had a good fit to the observed values in all cases ($p > 0.05$ in the likelihood ratio test), the different replicates were pooled and the analyses continued with 2-way tables (treatment and position as factors), with higher frequencies and fewer empty cells. The independence of these 2 factors was then examined by fitting a model that left out the interaction Treatment \times Position. If the reduced model had a good fit to the observed frequencies, we then left out, one at a time, each of the 2 factors to test separately their contribution to the observed outcomes. If the interaction was significant (i.e. the model without interaction had a poor fit), separate log-linear analyses were run for each factor at each level of the other factor.

In all cases where the factor 'position' proved significant, post hoc-like comparisons were used to test which particular position deviated significantly from expectation. This was done by setting the cells corresponding to the different positions as structural 0s (starting with the one with the highest standardised deviate from expectation), re-running the analyses and checking whether the significance of the factor position changed when omitting any given position.

In Expts 1 and 3, we additionally analysed the effects of respectively light intensity (light versus dark) and tunic extract (extract versus no extract) using *t*-tests on the proportions of settled larvae (arcsine square-root transformed). Position could not be analysed in these tests, as the different positions in chambers were not independent. The same constraint applied to the light/dark factor in the second experiment, as the 2 levels were present in the same chamber and thus not independent. All analyses were performed with SYSTAT v.12.02.00.

RESULTS

Adult distribution

Each of the species examined exhibited differences in habitat orientation in the field (Fig. 1). *Ciona intestinalis*, *Microcosmus squamiger* and *Pyura herdmani* were most abundant on poorly lit surfaces, while *P. stolonifera* preferred well-lit surfaces. The 2 remaining species showed no obvious patterns with respect to light.

Orientation (Fig. 1) had significant effects on the density of individuals only in the case of the 3 pyurid species (ANOVA: *Microcosmus squamiger*, $F_{2,7} = 5.351$, $p = 0.039$, Tukey test, $p < 0.05$, Upwards > Downwards, both = Vertical; *Pyura herdmani*, $F_{2,7} =$

17.052, $p = 0.002$, Tukey test, $p < 0.01$, Downwards > other 2 categories; *P. stolonifera*, $F_{2,7} = 5.097$, $p = 0.043$, Tukey test, $p < 0.05$, Upwards > Downwards, both = Vertical). In the case of the other 3 species, we did not find significant differences among orientations (*Ciona intestinalis* $F_{2,7} = 0.503$, $p = 0.625$; *Ascidella aspersa* $F_{2,7} = 0.672$, $p = 0.541$; *Styela plicata* $F_{2,7} = 2.641$, $p = 0.140$), although *C. intestinalis* was most abundant on downward-facing surfaces, and both *A. aspersa* and *S. plicata* were more abundant on downward and vertical surfaces.

Light intensities were usually highest on vertical surfaces (Fig. 1) due to the characteristics of the floating pontoons from which all animals were collected, except *Pyura stolonifera*, which was collected from natural rocky shore. Low light intensities on upward-facing surfaces for the remaining species reflected the fact that they grew on artificial substrata that were poorly illuminated due to other structures that screened them.

Effects of light and orientation on larval settlement

In Expt 1, results for *Ascidella aspersa* and *Styela plicata* were not analysed due to the low number of settlers. For the remaining species, there was no significant interaction of the light treatment with the position of the settlers (Table 2). When the 2 factors were analysed separately, no effect of the light/dark treatment was found (Fig. 2, Table 2 and *t*-tests on proportion of settlers: all $p > 0.05$). For the position factor, *Ciona intestinalis* showed a clear preference for settlement on top surfaces, whereas the 3 species belonging to the family Pyuridae (*Microcosmus squamiger*, *Pyura herdmani* and *P. stolonifera*) settled significantly more often on the bottom than elsewhere (Fig. 2, Table 2).

In Expt 2, in which the larvae had the option of settling on light or dark surfaces in the same chamber, a different picture emerged (Table 3, Fig. 3). Again, the low number of settlers prevented analyses of *Ascidella aspersa* and *Styela plicata*. For *Ciona intestinalis* and *Microcosmus squamiger*, no significant interaction was found between treatment and position. Con-

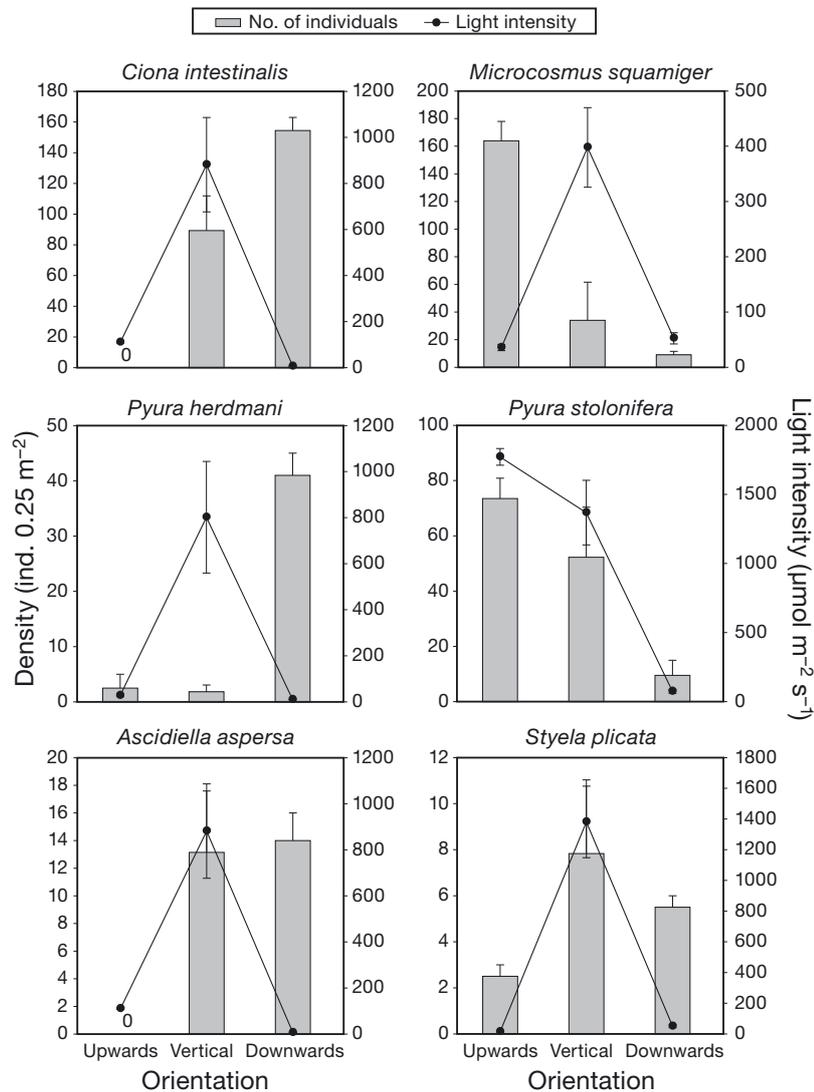


Fig. 1. Adult distribution in the field, indicated as the mean density of individuals, and mean light intensity in relation to surface orientation. Lines connecting levels of light intensity are inserted for guidance only. Error bars denote + 1 SE. Note differences in scales of y-axes

Table 2. Log-linear analyses of the outcomes of Expt 1. Post hoc-like comparisons were performed when appropriate. LR: likelihood ratio; df: degrees of freedom. Significant values are indicated in **bold**

	Log-likelihood	LR χ^2	df	p	Pairwise comparisons
<i>Ciona intestinalis</i>					
Light \times Position	-15.320	0.594	2	0.743	
Light	-15.691	1.340	3	0.720	
Position	-73.624	117.200	4	<0.001	Top > Lateral = Bottom
<i>Microcosmus squamiger</i>					
Light \times Position	-11.064	4.955	2	0.084	
Light	-11.284	5.390	3	0.145	
Position	-53.378	89.580	4	<0.001	Bottom > Lateral = Top
<i>Pyura herdmani</i>					
Light \times Position	-6.246	1.778	3	0.619	
Light	-6.555	2.400	4	0.663	
Position	-30.570	89.580	4	<0.001	Bottom > Lateral = Top
<i>Pyura stolonifera</i>					
Light \times Position	-11.249	1.447	2	0.485	
Light	-11.261	1.470	3	0.689	
Position	-21.709	22.370	4	<0.001	Bottom > Lateral = Top

trary to the previous experiment, both species showed a marked preference for dark surfaces, and no significant preference for any orientation (Table 3). In the case of the 2 *Pyura* species, *P. herdmani* and *P. stolonifera*, a significant interaction existed (Table 3). *P. herdmani* continued to prefer bottom surfaces in the light but selected both bottom and top in the dark. *P. stolonifera* changed light preferences depending on the surface considered, but overall more larvae settled in light (Fig. 3) and preferred lateral surfaces in the lit part of the chambers. These results are generally in accordance with what we found in the field for adults of *C. intestinalis*, *M. squamiger* and *P. herdmani* (see Fig. 1), all of which settled in the dark, and also for *P. stolonifera*, which (largely) settled in the light.

The 4 species that displayed significant geotactic patterns in Expt 1 shifted to a more random pattern in Expt 2, with 2 species (*Ciona intestinalis* and *Microcosmus squamiger*) now showing no geotactic preferences, and the other 2 species (*Pyura herdmani* and *P. stolonifera*) showing greater settlement on lateral and top surfaces than previously.

Effect of tunic extracts

Three species (*Styela plicata*, *Pyura herdmani* and *P. stolonifera*) showed no effect of tunic extracts in the water (Fig. 4, Table 4 and *t*-tests, $p > 0.05$). The other 3 showed a significant inhibition of settlement in the presence of tunic extracts (Fig. 4, Table 4, and *t*-tests, all $p < 0.05$), although in *Ciona intestinalis* the log-linear analysis revealed a significant interaction, with the extract inhibition being significant for the lateral and top surfaces only (Table 4).

The geotactic behaviour found in Expt 1 testing light vs. dark effects was maintained across all species in Expt 3, with the 3 pyurids *Microcosmus squamiger*, *Pyura herdmani* and *P. stolonifera* settling preferentially on the bottom (Fig. 4). For *Ciona intestinalis*, the highest number of settlers was again on top surfaces, although in the

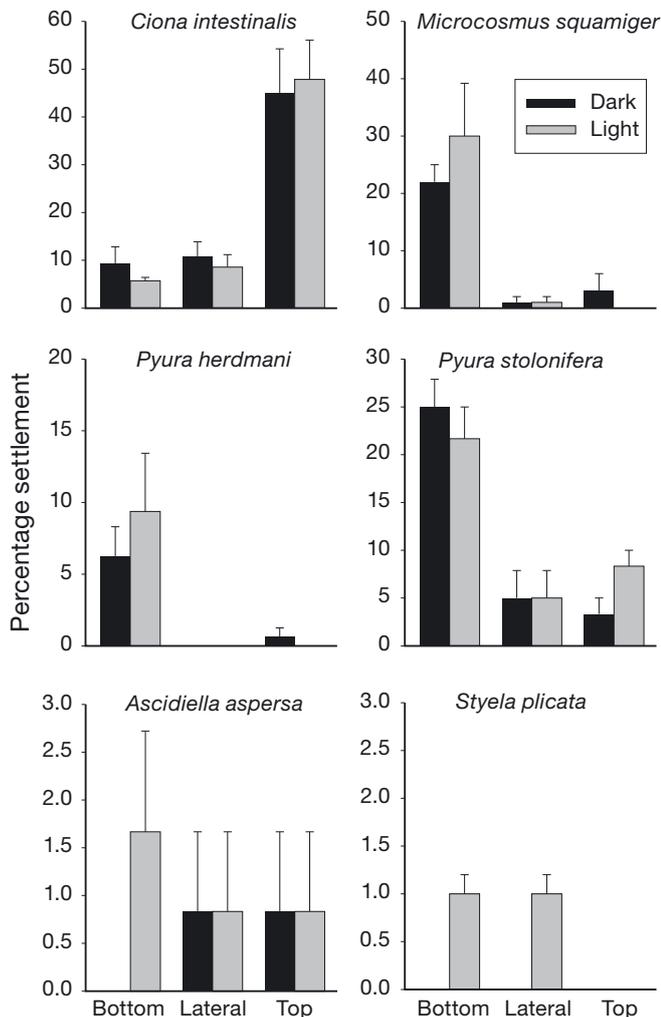


Fig. 2. Mean percentage settlement in relation to position (bottom, lateral or top) and treatment (light: grey bars, dark: black bars) in Expt 1, in which larvae were held either in the dark or in the light. Error bars denote +1 SE. Note differences in scales of y-axes

Table 3. Log-linear analyses of the outcomes of Expt 2. Interaction was tested first and, if significant, each factor was tested at fixed levels of the other factor. Post hoc-like comparisons were performed when appropriate. LR: likelihood ratio; df: degrees of freedom. Significant values are indicated in **bold**

	Log-likelihood	LR χ^2	df	p	Pairwise comparisons
<i>Ciona intestinalis</i>					
Light \times Position	-11.252	3.138	2	0.208	
Light	-18.480	17.593	3	<0.001	Dark > Light
Position	-13.683	7.999	4	0.092	
<i>Microcosmus squamiger</i>					
Light \times Position	-6.792	2.047	2	0.359	
Light	-12.277	13.018	3	0.001	Dark > Light
Position	-7.914	4.290	4	0.326	
<i>Pyura herdmani</i>					
Light \times Position	-20.333	17.661	2	<0.001	
Light (Bottom)	-5.493	1.093	1	0.296	
Light (Lateral)	-2.477	0.340	1	0.560	
Light (Top)	-22.554	36.610	1	<0.001	Dark > Light
Position (with light)	-15.918	21.449	2	<0.001	Bottom > Lateral = Top
Position (with darkness)	-29.885	47.161	2	<0.001	Bottom = Top > Lateral
<i>Pyura stolonifera</i>					
Light \times Position	-15.085	17.082	2	<0.001	
Light (Bottom)	-4.405	5.545	1	0.019	Dark > Light
Light (Lateral)	-10.628	14.699	1	<0.001	Light > Dark
Light (Top)	-4.405	5.545	1	0.019	Light > Dark
Position (with light)	-15.007	22.190	2	<0.001	Lateral > Top > Bottom
Position (with darkness)	-5.624	5.982	2	0.050	

presence of adult extract there was no significant difference between top and bottom (Table 4). For *Ascidella aspersa* there were no position effects, and for *Styela plicata* there was no effect of either extract or position on settlement in the chambers.

Integrating field and laboratory data

Comparing the level of aggregation and the overall abundance of individuals in the field (see Figs. 1 & 5), a consistent pattern emerged: the more abundant a species was in a particular orientation, the more individuals there were per clump. *Microcosmus squamiger* and *Pyura stolonifera* showed the highest numbers of individuals per clump (Fig. 5), but significant differences existed only between *P. stolonifera* and 2 other species (ANOVA: $F_{5,54} = 4.207$; $p = 0.003$, Tukey test, $P. stolonifera > Styela plicata = P. herdmani$, $p < 0.05$). In terms of the numbers of individuals per clump in relation to orientation in the field (Fig. 5), significant differences emerged for 2 species (ANOVA: *M. squamiger*, $F_{2,7} = 6.689$, $p = 0.024$, Tukey test: Upwards greater than the other 2 orientations, $p < 0.05$; *P. herdmani*, $F_{2,7} = 38.068$, $p < 0.001$, Tukey test: Downward greater than the other 2 orientations, $p < 0.001$).

For an overall perspective of the geotactic preference of each species, we pooled together all settlement data generated from the 3 laboratory experiments, on the assumption that in terms of geotactic behaviour, larvae in the field would encounter a combination of both phototactic stimuli and adult extracts. Setting aside *Ascidella aspersa* and *Styela plicata* on the grounds that their settlement rates were too low for consideration, the mean percentage of settlers on each surface showed the same trend as the number of individuals per clump for 3 species (*Microcosmus squamiger*, *Pyura stolonifera* and *Ciona intestinalis*), whereas *P. herdmani* showed no correlation (Fig. 5).

Three trends emerged from the laboratory data (as summarised in Table 5). First, in relation to orientation, 1 species (*Ciona intestinalis*) tended to settle preferentially on the top, whereas 3 (*Microcosmus squamiger*, *Pyura herdmani*, *P. stolonifera*) preferred settling on the bottom in Expt 1, with almost the same pattern emerging in Expt 3. In Expt 2, the geotactic responses evident in Expt 1 were either absent or altered.

Ascidella aspersa and *Styela plicata* could be analysed with respect to geotactic behaviour only in Expt 3, and neither showed any preference.

Second, in terms of light/dark responses, none of the 4 species analysed showed any statistical preferences in Expt 1, where the larvae were held either in light or dark. However, in Expt 2, when they had a choice between dark and light, 3 species (*Ciona intestinalis*, *Microcosmus squamiger* and *Pyura herdmani*) displayed preference for settling in the dark, and a fourth (*P. stolonifera*) settled most often in the light, although this preference changed on bottom surfaces, leading to an interaction between the factors.

Third, in relation to the presence or absence of adult tunic extracts in Expt 3, 3 species showed no response, while settlement of the other 3 (*Ciona intestinalis*, *Microcosmus squamiger* and *Ascidella aspersa*) was inhibited in the presence of tunic extracts.

DISCUSSION

To a large extent, the range of conditions where adults of each species occurred in the field correlated well with the behaviour of the larvae in the laboratory. *Ciona intestinalis* is a common fouling species in shel-

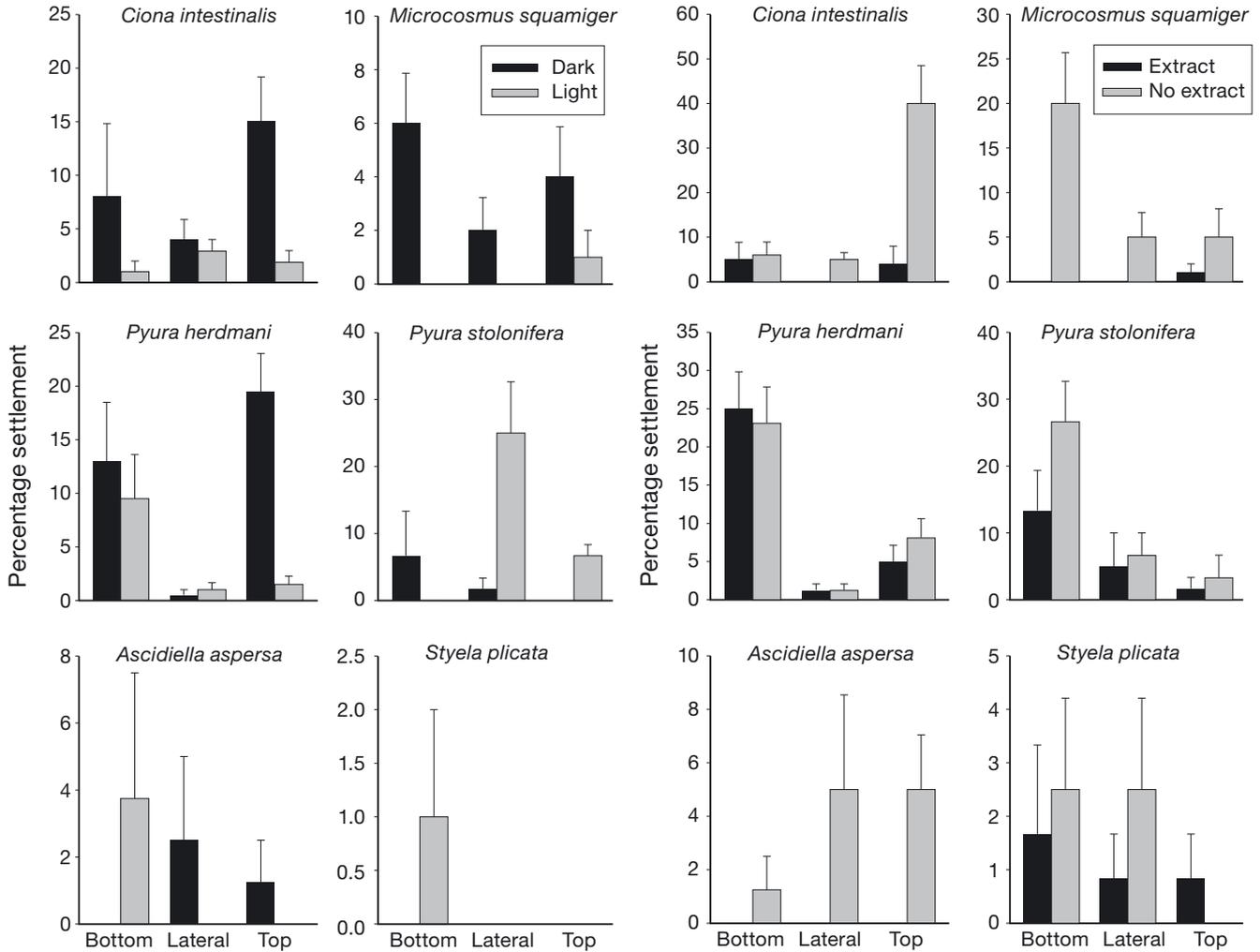


Fig. 3. Mean percentage settlement in relation to position (bottom, lateral and top) and treatment (light: grey bars, dark: black bars) in Expt 2, in which larvae had the choice of settling in light or dark portions of the same chamber. Error bars denote +1 SE. Note differences in the scales of y-axes

Fig. 4. Mean percentage settlement with respect to position (bottom, lateral and top) and treatment (control: grey bars, tunic extract: black bars) in Expt 3, in which larvae were held in chambers either with or without adult extract. Error bars denote +1 SE. Note differences in the scales of y-axes

tered marinas and harbours (Monniot et al. 2001, Lambert & Lambert 2003), where it is found in relatively dark places on the lower surfaces of substrata (Branch & Branch 1981, this study). Correlated with this, its larvae showed preferences for dark conditions and settlement beneath the upper surface of the experimental chambers. *Pyura stolonifera* lives on well-lit upper or lateral surfaces, and its larvae settled on the bottoms or sides of chambers and preferred light conditions when settling on the sides. *Microcosmus squamiger* and *P. herdmani* adults displayed clear preferences for dark surfaces, and accordingly their larvae preferred dark conditions and upward-facing surfaces. Both *Ascidiella aspersa* and *Styela plicata* exhibited no habitat preference in the field and no preferential geotactic or phototactic larval responses. Overall, the first 2 of our

initial hypotheses (phototactic preference for dark places and geotactic behaviour in those species with clear orientation preference) were supported, emphasising the importance of settlement in determining adult distribution patterns, with 4 of the 6 species displaying larval behaviour that was in agreement with field observations. In addition, we showed how the biotic factor examined (presence or absence of tunic extracts) and the 2 abiotic factors (phototaxis and geotaxis) can play an integrated role in determining settlement patterns, providing insight into how such factors may influence adult distribution in the field.

In the first experiment, when larvae were held under either light or dark conditions, geotactic preferences drove larval behaviour. However, in the second experiment, when larvae had the option of choosing between

Table 4. Log-linear analyses of the outcomes of Expt 3. Interaction was tested first and, if significant, each factor was tested at fixed levels of the other factor. Post hoc-like comparisons were performed when appropriate. LR: likelihood ratio; df: degrees of freedom. Significant values are indicated in **bold**

	Log-likelihood	LR χ^2	df	p	Pairwise comparisons
<i>Ciona intestinalis</i>					
Extract × Position	-14.087	8.759	2	0.013	
Extract (Bottom)	-3.615	0.091	1	0.763	
Extract (Lateral)	-5.206	6.931	1	0.008	No Extract > Extract
Extract (Top)	-21.493	34.189	1	<0.001	No Extract > Extract
Position (with Extract)	-7.078	7.410	2	0.025	Bottom = Top > Lateral
Position (Control)	-28.194	43.718	2	<0.001	Top > Lateral = Bottom
<i>Microcosmus squamiger</i>					
Extract × Position	-8.616	3.429	2	0.180	
Extract	-25.686	37.568	3	<0.001	No Extract > Extract
Position	-14.932	16.060	4	0.003	Bottom > Lateral = Top
<i>Pyura herdmani</i>					
Extract × Position	-12.923	1.280	2	0.527	
Extract	-12.942	1.319	3	0.725	
Position	-57.187	89.808	4	<0.001	Bottom > Lateral = Top
<i>Pyura stolonifera</i>					
Extract × Position	-9.823	0.216	2	0.897	
Extract	-11.316	3.202	3	0.362	
Position	-20.470	21.511	4	<0.001	Bottom > Lateral = Top
<i>Ascidella aspersa</i>					
Extract × Position	-4.268	0.004	3	0.999	
Extract	-10.504	12.477	3	0.006	No Extract > Extract
Position	-5.470	2.409	5	0.301	
<i>Styela plicata</i>					
Extract × Position	-7.414	2.231	2	0.328	
Extract	-7.616	2.634	3	0.526	
Position	-8.967	5.337	4	0.254	

shaded and light conditions, 3 species clearly preferred to settle on dark surfaces. Our results are in accordance with the general statement that shading facilitates the dominance of hard substrata by sessile invertebrates, while well-lit surfaces lead to algal-dominated communities (Miller & Etter 2008). For those species settling in the dark, this might incidentally lead to settlement among adult conspecifics, where light is reduced in the shade of adults, ultimately contributing to a gregarious distribution. An interesting result of the second experiment was that the 4 species that could be statistically analysed (*Ciona intestinalis*, *Microcosmus squamiger*, *Pyura herdmani*, *P. stolonifera*) all altered their geotactic behaviour from that displayed in the first experiment, showing a more haphazard geotactic settlement distribution or alteration of preferences in the second experiment. These results contrast with what has previously been found for the tadpole larvae of another solitary ascidian (*Ascidia mentula*) and for the planulae of a scyphozoan, in which the larvae did not alter their

negative geotactic behaviour across a range of light conditions (Svane & Dolmer 1995). Our results suggest that during settlement, time of day and weather conditions (which can alter light conditions) may greatly influence larval behaviour.

Both *Styela plicata* and *Ascidella aspersa* are common introduced species in South Africa (M. Rius, C. Griffiths, X. Turon unpublished) and have succeeded in establishing populations worldwide (Carlton 1996, Lambert & Lambert 2003, Barros et al. 2009). The fact that there were no settlement preferences in either of these species may indicate that they can successfully settle under a range of conditions and on a range of surfaces, increasing the likelihood of their colonising new localities. However, the proportions of settlement found for these 2 species were the lowest of all studied species, and therefore any interpretation of their settlement preferences must be cautious. Young & Braithwaite (1980) have shown that *Styela montereyensis*, like *S. plicata* and *A. aspersa*, shows no discrimination with respect to light or substratum type. Similarly, Young & Chia (1985) failed to find any settlement preferences in 6 other solitary ascidian species that were exposed to different light regimes. In our study we found strong patterns in 4 species out

of 6, with light intensity being an important factor modulating larval geotactic behaviour.

We found that the presence or absence of photoreceptors (ocelli) was only a moderate predictor of the behaviour of the larvae. *Ciona intestinalis*, *Pyura herdmani* and *P. stolonifera*, all of which have well-developed ocelli, showed significant phototactic behaviour, while *Styela plicata*, with a much reduced ocellus, displayed no phototaxis. However, *Ascidella aspersa*, which has well-developed sensory organs, showed no response to different light conditions, and the larvae of *Microcosmus squamiger*, a species with no ocelli, showed a strong preference for settlement in the dark in the second experiment. This contrasts with the behaviour of the larvae of a closely related species that also lacks photoreceptors, *M. exasperatus*, which displays no light sensitivity or preferences (Svane & Young 1991).

Both conspecific attraction and gregarious behaviour have been identified as driving forces for the distribu-

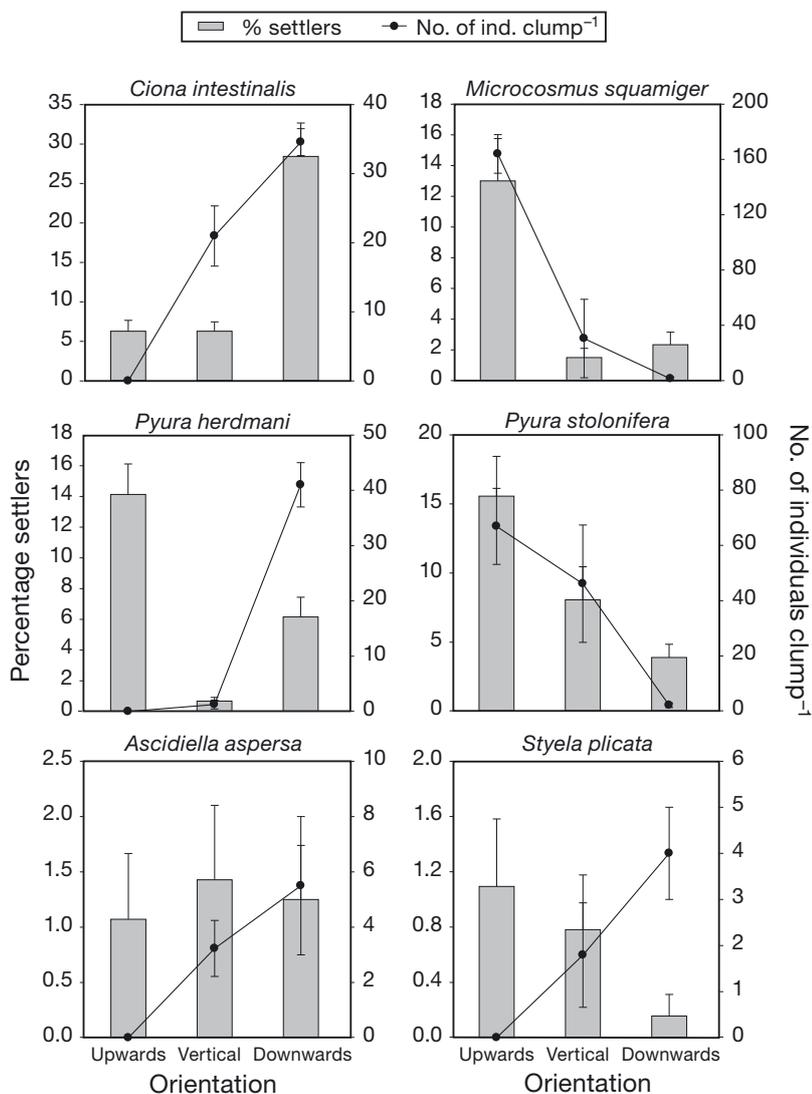


Fig. 5. Mean numbers of individuals per clump in the field, and mean percentage of settlers from all the experiments pooled, in relation to orientation. Error bars denote +1 SE. Note differences in scales of y-axes

tion of many organisms (Alonso et al. 2004, Budke et al. 2004, Gautier et al. 2006). In contrast to the third of our initial hypotheses, our results point to either an absence of response of larvae to cues from extracts of the adults, or strong inhibition by tunic extract. Similar to our findings, the percentage of metamorphosis of the solitary ascidian *Molgula citrina* decreases when its larvae are exposed to conspecific tunic homogenate (Durante 1991). This has implications for understanding how prior invasions might affect further colonisation. Our study showed that settlement was not promoted by the presence of adult extracts. However, it is possible that the adult extracts we employed acted as a repellent because they signalled damaged tissues of a conspecific. Other authors using adult extracts have found, however, that the presence of extracts induced metamorphosis (Svane et al. 1987), so we consider it unlikely that the extracts signal damaged tissues. Our findings indicate that the gregarious distribution of adults observed in the field is unlikely to be explained by larval attraction to adult cues, but may be the result of settlement being concentrated in habitats characterised by particular physical conditions. For many other marine species, physical factors seem to be stronger cues for settlement than chemical attraction by conspecific adults (Berntsson et al. 2004). Sometimes these preferred physical conditions such as light intensity and hydrody-

Table 5. Summary of significant outcomes of the 3 experiments for each factor and species showing preferential settlement position or treatment. Dashes indicate an absence of any significant preference. nt: not tested statistically; * indicates a significant interaction between the effects of position and treatment, and therefore results may apply only to particular levels of each factor

Species	Expt 1		Expt 2		Expt 3	
	Position	Light vs. Dark	Position	Light/Dark	Position	Tunic extract
<i>Ciona intestinalis</i>	Top	–	–	Dark	Bottom & Top*	Inhibition*
<i>Microcosmus squamiger</i>	Bottom	–	–	Dark	Bottom	Inhibition
<i>Pyura herdmani</i>	Bottom	–	Bottom & Top*	Dark*	Bottom	–
<i>Pyura stolonifera</i>	Bottom	–	Lateral*	Light*	Bottom	–
<i>Ascidiella aspersa</i>	nt	nt	nt	nt	–	Inhibition
<i>Styela plicata</i>	nt	nt	nt	nt	–	–

dynamic conditions may coincidentally be associated with the presence of adults, or even created by adults, leading indirectly to aggregations. For instance, a baffles effect created by aggregations of adults (see Eckman 1983) may enhance the settlement of new larvae and protect the juveniles, thereby increasing their survival. However, more needs to be learned concerning the mechanisms driving the effect of conspecific adult attraction, and further experiments using gregarious ascidians have the potential to provide important insights.

In confined environments, such as harbours and marinas, where invasive ascidians are highly successful, the specific biological features of each species such as larval movement and offspring retention (Petersen & Svane 1995), the particular hydrodynamics of the location (Havenhand & Svane 1991) and adequate conditions for settlement (as shown in our study) may play important roles in influencing species distributions and the success of introduced populations. For example, *Ciona intestinalis* is widespread in dark, sheltered conditions in harbours and successfully colonises the culture ropes of mussel farms in South Africa, with important economic impacts (Robinson et al. 2005), as has also been reported in northeast American coastal waters (Ramsay et al. 2008).

Overall, because each of the 6 species we examined responded uniquely to the variables explored, it is not possible to generalise ascidian settlement behaviour. Biotic factors and chemical cues, other than those arising from conspecific adults, may determine aggregated settlement of ascidians in the field (Davis 1996, Hadfield & Paul 2001). However, our results favour the view that the aggregated distribution of the solitary ascidians considered reflects responses to abiotic rather than biotic factors, although there is always the possibility that complex biotic interactions, such as competition or facilitation, occur during juvenile and adult stages, as has been demonstrated in other gregarious organisms (Rius & McQuaid 2009). There is a need to further study the mechanisms that determine gregarious distribution in invasive species. Comparisons of species performance and biology across both introduced and native ranges could be enlightening (see Bossdorf et al. 2005). Concepts such as conspecific and kinship attraction, and gregarious behaviour should be incorporated in the study of the distribution of invasive species, as they might be key features for our understanding of the viability and success of these populations.

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