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

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Running head: Settlement patterns of gregarious ascidians
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 to larval behaviour, and we ask 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 six species of ascidians that form aggregations in the field, including four aliens with global distributions, and how they relate to adult habitat characteristics. In the laboratory, the larvae were held (1) 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, three settling preferentially on the bottom of experimental chambers, and one on the top. Offered a choice between dark and light, two species settled preferentially in the dark with no geotactic preferences and another two showed an interaction between light and geotaxis. For four of the species, the responses of settlers accorded with, and may contribute to, adult orientation patterns in the field. Adult extracts inhibited settlement of three species and failed to influence settlement of the other three, arguing against conspecific attraction being a cause of aggregation and an explanation of the propensity of ascidians to become invasive.

KEYWORDS: Ascidiacea, chemical cues, gregarious behaviour, invasive species, larval settlement, conspecific attraction.
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 fertilization (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 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).

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). Ascidians 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, Bennet & Marshall 2005). Svane & Young (1989) have 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 six solitary ascidians found along the South African coast (Ciona intestinalis, Ascidiella aspersa, Styela plicata, Microcosmus squamiger, Pyura herdmani and Pyura stolonifera), which belong to four different families from the two recognized 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, and personal observation). We chose these species to include four introduced species with global distributions (C. intestinalis, A. aspersa, S. plicata and M. squamiger) and two large native species (P. herdmani and P. stolonifera) that are not
known to be invasive, although congeners are recognized as invasive elsewhere (Castilla et al. 2004). All these species are important occupiers of hard substrata of coastal areas of South Africa (Branch et al. 2010). The larvae of four species had well developed statocytes and ocelli (Griffiths 1976, Niermann-Kerkenberg & Hofmann 1989, Jacobs et al. 2008, and personal observation) but *S. plicata* has a highly reduced ocellus (Ohtsuki 1990), and *M. squamiger* is unusual among Pyuridae in lacking an ocellus (personal observation, and see also Svane & Young 1991 for a closely related species). Thus, four species were expected to have both light and geotactic preferences, while the larvae of the remaining two 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 three specific 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 the species, and will contribute to the aggregated patterns of distribution of adults.

**MATERIAL AND METHODS**

**Field sites and surveys of adults**

Adult ascidians were surveyed and sampled at the locations characterized in Table 1. At each location we quantified adult distribution and associated circumstances. To standardise conditions, all sampling took place at 12:00 am on cloudless days in
October/November 2009 at depths of no more than 1 m. At each locality, 50x50 cm quadrats (n=10 per substratum orientation) were placed on horizontal hard substrata facing upwards (0-10°), downwards (170-180°), or on vertical substrata (80-100°). The number of individuals of any of the six 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 three random measurements within each quadrat using a photometer (Skye instruments Ltd, Scientific Associates) fitted with a sensor (Quantum Sensor, Wales).

**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: *P. stolonifera* and *M. squamiger* mature in spring and summer (Griffiths 1976, Rius et al. 2009a), *C. intestinalis* and *S. 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 two species indicated that they mature in spring.

**Fertilization 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 five hours. In the laboratory, specimens were housed in aerated seawater and maintained at room temperature (15ºC).

All manipulations and experiments were undertaken in filtered seawater obtained using vacuum filtration through 10-µm pore-size filters. For *C. intestinalis* and
A. aspersa, artificial fertilization 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 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 four individuals, preventing self-fertilization. Developing embryos were placed in an aerated beaker (containing 500 ml 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 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 three orientations. The containers were placed in a seawater tank for 24 hours 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 hours under the experimental conditions detailed below. The Perspex chamber was subsequently dismantled in seawater, so that any unattached larvae were washed away.

We performed three 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 fertilization event, we ran all the experiments described below in parallel.

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

In the second experiment, 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 were covered by black tape (reducing the light to 0.4 µmol m\(^{-2}\) s\(^{-1}\)), while the other half of these surfaces were exposed to the same artificial light (47 µmol m\(^{-2}\) s\(^{-1}\)).

The third experiment 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 three 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-hour 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 orientation, with surface orientation as a fixed factor to compare the number of individuals per quadrat for each species. Tukey 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 pair-wise 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 (bottom, 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-Square 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 two factors
(i.e. the terms treatment*replicate, position*replicate, and treatment*position*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 two-way tables (treatment and position as factors), with higher
frequencies and fewer empty cells. The independence of these two factors was then examined by fitting a model that left out the interaction treatment*position. If the reduced model had a good fit to the observed frequencies, we then left out, one at a time, each of the two factors to test separately their contribution to the observed outcomes. If 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 pairwise 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 zeros (starting with the one with the highest standardized deviate from expectation), re-running the analyses and checking whether the significance of the factor position changed when omitting any given position.

For the first and third experiments we additionally analyzed the effects of respectively light intensity (light vs. dark) and tunic extract (extract vs. no extract) using t-tests on the proportions of settled larvae (arcsine square-root transformed). Position could not be analyzed 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 two levels were present in the same chamber and thus not independent.

All analyses were performed with SYSTAT v.12.02.00 (SYSTAT Inc., 2007).

RESULTS

Adult distribution

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

Orientation (see Fig. 1) had significant effects on the density of individuals only in the case of the three pyurid species (ANOVA, *M. squamiger*, $F_{2,7} = 5.351$, $p = 0.039$, Tukey test, $p < 0.05$, Upwards > Downward, both = Vertical; *P. herdmani*, $F_{2,7} = 17.052$, $p = 0.002$, Tukey test, $p < 0.01$, Downwards > other two 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 three species, we did not find significant differences among orientations (*C. intestinalis* $F_{2,7} = 0.503$, $p = 0.625$, *A. aspersa* $F_{2,7} = 0.672$, $p = 0.541$, and *S. 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 where the animals were collected, except for *P. stolonifera*, the only species collected in 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 the first experiment, results for *A. aspersa* and *S. plicata* were not analyzed 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 two factors were analyzed 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, *C. intestinalis* showed a clear preference for settlement on top surfaces, whereas
the three species belonging to the family Pyuridae (*M. squamiger*, *P. herdmani* and *P. stolonifera*) settled significantly more often on the bottom than elsewhere (Fig. 2, Table 2).

In the second experiment, 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 *A. aspersa* and *S. plicata*. For *C. intestinalis* and *M. squamiger*, no significant interaction was found between treatment and position. Contrary 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 two *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 it 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 four species that displayed significant geotactic patterns in the first experiment shifted to a more random pattern in the second experiment, with two (*C. intestinalis* and *M. squamiger*) now showing no geotactic preferences, and the other two species (*P. herdmani* and *P. stolonifera*) showing greater settlement on lateral and top surfaces than previously.

**Effect of tunic extracts**

Three species (*S. plicata*, *P. 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 three 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 *C. 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 the first experiment testing light/dark effects was maintained across all species in this third experiment, with the three pyurids *M. squamiger*, *P. herdmani* and *P. stolonifera* settling preferentially on the bottom (Fig. 4). For *C. intestinalis*, the highest number of settlers was again on top surfaces, although in the presence of adult extract there was no significant difference between top and bottom (Table 4). For *A. aspersa* there was no position effects, and for *S. 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 Fig. 1,5), a consistent pattern emerged: the more abundant a species was in a particular orientation, the more individuals there were per clump. When we analysed the number of individuals per clump across species, *M. squamiger* and *P. stolonifera* showed the highest numbers (Fig. 5), but significant differences existed only between *P. stolonifera* and two other species (ANOVA, *F*$_{5,54}$ = 4.207, *p* = 0.003, Tukey test, *P. stolonifera* > *S. 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 two species (ANOVA, *M. squamiger*, *F*$_{2,7}$ = 6.689, *p* = 0.024, Tukey test, Upwards greater than the other two orientations, *p* < 0.05; *P. herdmani*, *F*$_{2,7}$ = 38.068, *p* < 0.001, Tukey test, Downward greater than the other two orientations, *p* < 0.001).

For an overall perspective of the geotactic preference of each species, we pooled together all settlement data generated from the three 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 *A. aspersa* and *S. 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 three species (*M. squamiger; P. stolonifera* and *C. 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, one species (*C. intestinalis*) tended to settle preferentially on the top, whereas three (*M. squamiger, P. herdmani, P. stolonifera*) preferred settling on the bottom in experiment 1, with almost the same pattern emerging in experiment 3. In experiment 2 the geotactic responses evident in experiment 1 were either absent or altered. *A. aspersa* and *S. plicata* could be analyzed with respect to geotactic behaviour only in experiment 3, and neither showed any preference.

Second, in terms of light/dark responses, none of the four species analyzed showed any statistical preferences in experiment 1, where the larvae were held either in light or dark. However, in experiment 2, when they had a choice between dark and light, three species (*C. intestinalis, M. squamiger* and *P. 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 the third experiment, three species showed no response, while settlement of the other three (*C. intestinalis, M. squamiger* and *A. 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. *C. intestinalis* is a common fouling species in sheltered 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 1998, this study). Correlated with this, its larvae showed preferences for dark conditions and settlement on beneath the upper surface of the experimental chambers. *P. 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. *M. squamiger* and *P. herdmani* adults displayed clear preferences for dark surfaces, and accordingly their larvae preferred dark conditions and upward-facing surfaces. Both *A. aspersa* and *S. plicata* exhibited no habitat preference in the field and no preferential geotactic or phototactic larval responses. Overall, the first two 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 four of the six 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 two 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 shaded and light conditions, three 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 four species that could be statistically analyzed (C. intestinalis, M. squamiger, P. 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 S. plicata and A. aspersa are common introduced species in South Africa (M. Rius, C.L. Griffiths and X. Turon, in preparation) 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 two 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 six other solitary ascidian species that were exposed to different light regimes. In our study we found strong patterns in four species out of six, 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. *C. intestinalis*, *P. herdmani* and *P. stolonifera*, all of which have well-developed ocelli, showed significant phototactic behaviour, while *S. plicata*, with a much reduced ocellus, displayed no phototaxis. However, *A. aspersa*, which has well-developed sensory organs, showed no response to different light conditions, and the larvae of *M. 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 distribution 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 colonization. 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; but other authors using adult extracts have found 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 are 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 hydrodynamic conditions may coincidentally be associated with the presence of adults, or even created by adults, leading indirectly to aggregations. For instance, a baffle effect of 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 learnt 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, *C. intestinalis* is widespread in dark, sheltered conditions in harbours and successfully colonizes the culture ropes of mussel farms in South Africa, with important economic impacts (Robinson et al. 2005), as also in northeast American coastal waters (Ramsay et al. 2008).
Overall, because each of the six 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 it 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 to 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.
ACKNOWLEDGEMENTS

We are grateful to J. Murray for assistance in the field and continuous stimulating discussions. Two anonymous reviewers contributed valuable discussions and comments. We thank G. du Plessis (Zoology Department, University of Cape Town) for constructing the equipment. MR was supported by a travel grant from the Spanish ‘‘Ministerio de Educación y Ciencia’’ during his stay at the University of Cape Town and by projects CTM2007-66635 and CSIC-PIE 2007-301026 of the Spanish Government. This project was funded by a grant to CLG from the DST-NRF Centre of Excellence for Invasion Biology and an Andrew Mellon Foundation Grant to GMB. The work was carried out under permit and in accordance with the laws of South Africa.
LITERATURE CITED


Young CM (1989) Selection of predator-free settlement sites by larval ascidians. Ophelia 30:131-140


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<sup>st</sup>: Light vs Dark, 2<sup>nd</sup>: Half light vs half dark, 3<sup>rd</sup>: Tunic extracts.

<table>
<thead>
<tr>
<th>Species</th>
<th>Field sites</th>
<th>Number of replicates per experiment</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt;</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ciona intestinalis</strong></td>
<td>Cape Town harbour 34º 54′ 22″ S, 18º 25′ 37″ E</td>
<td>Sheltered</td>
<td>Artificial</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td><strong>Microcosmus squamiger</strong></td>
<td>Port Alfred marina 33º 35′ 41″ S, 26º 53′ 32″ E</td>
<td>Sheltered</td>
<td>Artificial</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Pyura herdmani</strong></td>
<td>Langebaan marina 33º 01′ 07″ S, 17º 56′ 48″ E</td>
<td>Moderately exposed</td>
<td>Artificial</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td><strong>Pyura stolonifera</strong></td>
<td>St. James 34º 07′ 14″ S, 18º 27′ 31″ E</td>
<td>Highly exposed</td>
<td>Natural</td>
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<td>3</td>
</tr>
<tr>
<td><strong>Ascidia aspersa</strong></td>
<td>Cape Town harbour 34º 54′ 22″ S, 18º 25′ 37″ E</td>
<td>Sheltered</td>
<td>Artificial</td>
<td>6</td>
<td>4</td>
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<tr>
<td><strong>Styela plicata</strong></td>
<td>Knysna marina 34º 03′ 17″ S, 23º 03′ 46″ E</td>
<td>Sheltered</td>
<td>Artificial</td>
<td>5</td>
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Table 2. Log-linear analyses of the outcomes of the 1st experiment. Post-hoc-like pair-wise comparisons were done when appropriate. LR: likelihood ratio; df, degrees of freedom; \( p \): probability value. Significant values are indicated in bold.

<table>
<thead>
<tr>
<th></th>
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<th>LR Chi-Square</th>
<th>df</th>
<th>( p )</th>
<th>Pair-wise comparisons</th>
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<td></td>
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</tr>
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<td>1.447</td>
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<td></td>
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<tr>
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<td>0.689</td>
<td></td>
</tr>
<tr>
<td>Position</td>
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<td>22.370</td>
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<td>&lt;0.001</td>
<td>Bottom &gt; Lateral = Top</td>
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Table 3. Log-linear analyses of the outcomes of the 2nd experiment. Interaction was tested first and, if significant, each factor was tested at fixed levels of the other factor. Post-hoc-like pair-wise comparisons were done when appropriate; LR: likelihood ratio; df, degrees of freedom; \( p \): probability value. Significant values are indicated in bold.

<table>
<thead>
<tr>
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<th>Log-likelihood</th>
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<th>df</th>
<th>( p )</th>
<th>Pair-wise comparisons</th>
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<tbody>
<tr>
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<td>0.092</td>
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<td><strong>0.001</strong></td>
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<td>4</td>
<td>0.326</td>
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<td><strong>Pyura herdmani</strong></td>
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<td></td>
</tr>
<tr>
<td>Light * Position</td>
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<td>17.661</td>
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<tr>
<td>Light (Bottom)</td>
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<td>1.093</td>
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<td>0.296</td>
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</tr>
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<td>Light (Lateral)</td>
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<tr>
<td>Position (with light)</td>
<td>-15.918</td>
<td>21.449</td>
<td>2</td>
<td><strong>&lt;0.001</strong></td>
<td>Bottom &gt; Lateral = Top</td>
</tr>
<tr>
<td>Position (with darkness)</td>
<td>-29.885</td>
<td>47.161</td>
<td>2</td>
<td><strong>&lt;0.001</strong></td>
<td>Bottom = Top &gt; Lateral</td>
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<tr>
<td><strong>Pyura stolonifera</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Light * Position</td>
<td>-15.085</td>
<td>17.082</td>
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<tr>
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<td>Light (Top)</td>
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<td>1</td>
<td><strong>0.019</strong></td>
<td>Light &gt; Dark</td>
</tr>
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<tr>
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<td>5.982</td>
<td>2</td>
<td>0.050</td>
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Table 4. Log-linear analyses of the outcomes of the 3rd experiment. Interaction was tested first and, if significant, each factor was tested at fixed levels of the other factor. Post-hoc-like pair-wise comparisons were done when appropriate. LR: likelihood ratio; df, degrees of freedom; $p$: probability value. Significant values are indicated in bold.

<table>
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<tr>
<th></th>
<th>Log-likelihood</th>
<th>LR Chi-Square</th>
<th>df</th>
<th>$p$</th>
<th>Pair-wise comparisons</th>
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<tbody>
<tr>
<td><strong>Ciona intestinalis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract * Position</td>
<td>-14.087</td>
<td>8.759</td>
<td>2</td>
<td><strong>0.013</strong></td>
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<tr>
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<td>-3.615</td>
<td>0.091</td>
<td>1</td>
<td>0.763</td>
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<tr>
<td>Extract (Lateral)</td>
<td>-5.206</td>
<td>6.931</td>
<td>1</td>
<td><strong>0.008</strong></td>
<td>No Extract &gt; Extract</td>
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<tr>
<td>Extract (Top)</td>
<td>-21.493</td>
<td>34.189</td>
<td>1</td>
<td><strong>&lt;0.001</strong></td>
<td>No Extract &gt; Extract</td>
</tr>
<tr>
<td>Position (with Extract)</td>
<td>-7.078</td>
<td>7.410</td>
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<td><strong>0.025</strong></td>
<td>Bottom = Top &gt; Lateral</td>
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<tr>
<td>Position (Control)</td>
<td>-28.194</td>
<td>43.718</td>
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<td><strong>&lt;0.001</strong></td>
<td>Top &gt; Lateral = Bottom</td>
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<tr>
<td><strong>Microcosmus squamiger</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract * Position</td>
<td>-8.616</td>
<td>3.429</td>
<td>2</td>
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<tr>
<td>Extract</td>
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<td>Position</td>
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<td><strong>0.003</strong></td>
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<tr>
<td>Extract * Position</td>
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<td>Extract</td>
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<tr>
<td><strong>Pyura stolonifera</strong></td>
<td></td>
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<td>Extract * Position</td>
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Table 5. Summary of significant outcomes of the three experiments for each factor and species. Dashes indicate an absence of any significant preference; nt = not tested statistically; – indicates no significant effect, * indicates a significant interaction between the effects of position and treatment, and therefore results may apply only to particular levels of each factor.

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<tr>
<th>Species</th>
<th>1st experiment</th>
<th>2nd experiment</th>
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<tr>
<td>Microcosmus squamiger</td>
<td>Bottom</td>
<td>─</td>
<td>─</td>
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<tr>
<td>Pyura herdmani</td>
<td>Bottom</td>
<td>─</td>
<td>Bottom &amp; Top</td>
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<tr>
<td>Pyura stolonifera</td>
<td>Bottom</td>
<td>─</td>
<td>Lateral*</td>
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<td>Asciidiella aspersa</td>
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<tr>
<td>Styela plicata</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
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</tbody>
</table>


FIGURE LEGENDS

**Fig. 1.** Adult distribution in the field, indicated as the mean density of individuals, and mean light intensity (µmol m$^{-2}$ s$^{-1}$) 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.

**Fig. 2.** Mean percentage settlement in relation to orientation (bottom, lateral or top) and treatment (light - white bars, dark - dark bars) in the 1st experiment, 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.

**Fig. 3.** Mean percentage settlement in relation to orientation (bottom, lateral and top) and treatment (light - white bars, dark - dark bars) in the 2nd experiment, 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 orientation (bottom, lateral and top) and treatment (control - white bars, tunic extract - dark bars) in the 3rd experiment, 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.

**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.
Figure 1.

**Ciona intestinalis**

**Pyura herdmani**

**Ascidiella aspersa**

**Microcosmus squamiger**

**Pyura stolonifera**

**Styela plicata**

**Ciona intestinalis**

**Microcosmus squamiger**

**Pyura herdmani**

**Pyura stolonifera**

**Ascidiella aspersa**

**Styela plicata**
Figure 2.

- *Ciona intestinalis*
- *Microcosmus squamiger*
- *Pyura herdmani*
- *Pyura stolonifera*
- *Asciella aspersa*
- *Styela plicata*
Figure 3.

**Ciona intestinalis**

**Microcosmus squamiger**

**Pyura herdmani**

**Pyura stolonifera**

**Asciella aspersa**

**Styela plicata**

Percentage settlement

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<tr>
<th></th>
<th>Bottom</th>
<th>Lateral</th>
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<tr>
<td>Microcosmus</td>
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<td></td>
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<tr>
<td>Pyura</td>
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<tr>
<td>Asciella</td>
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<td>Styela</td>
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</tbody>
</table>
Figure 4.

**Ciona intestinalis**

**Microcosmus squamiger**

**Pyura herdmani**

**Pyura stolonifera**

**Ascidiella aspersa**

**Styela plicata**
Figure 5.

Ciona intestinalis

Pyura herdmani

Pyura stolonifera

Ascidiella aspersa

Styela plicata

Microcosmus squamiger