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

93 conditions for propagule establishment and development often differ between the native
94 and invaded ranges, most invasive species perform differently in localities to which they
95 are introduced, where they are often more abundant (DeWalt et al. 2004, Kasper et al.
96 2008), larger (Ross & Auge 2008), comparatively free of predators (Wolfe 2002), less
97 prone to parasitism (Calvo-Ugarteburu & McQuaid 1998), and have a higher
98 reproductive output (Hinz & Schwarzlaender 2004). Moreover, invasive species
99 generally show a strongly aggregated distribution (Kopin et al. 2001, Dulloo et al. 2002,
100 Campbell & Donlan 2005, Dupont et al. 2006) and form large monospecific stands that
101 can monopolise available habitat (Simberloff et al. 2005, Rius et al. 2009a).
102 Consequently, species that are gregarious or aggregate may be pre-adapted to becoming
103 alien invaders because they will more readily form groups that are sufficiently
104 concentrated to be reproductively viable, whereas non-gregarious species will have
105 more difficulty in reaching a viable density after arrival in a new environment. A
106 possible mechanism for aggregated distribution might be gregarious settlement around
107 conspecifics, which may help to secure alien species in their new environment. Despite
108 their potential importance, both gregariousness and kinship concepts have scarcely been
109 applied to the study of invasive species, although they could elucidate evolutionary
110 processes behind biological invasions.

111 Marine ecosystems have experienced dramatic increases in the rate of
112 introductions of non-indigenous species (Cohen & Carlton 1998, Whiteley & Bendell-
113 Young 2007). Most of the species responsible for marine biological invasions are from
114 lower trophic levels, with filter-feeding invertebrates making up 70% of invasions in
115 coastal areas (Byrnes et al. 2007). Ascidians are major contributors (Lambert 2005,
116 2007), and can severely modify the structure of coastal habitats by forming large
117 aggregates (Lambert & Lambert 2003, Castilla et al. 2004, Rius et al. 2009a). Adults

118 live attached to hard substrata (Monniot et al. 1991) and the only motile stage is their
119 lecithotrophic larvae, which have very limited dispersal due to their short planktonic
120 lifespans (Millar 1971, Svane & Young 1989). Some information is available regarding
121 the distribution of adult ascidians in the field (e.g. Turon 1990, Mastrototaro et al.
122 2008), although the settlement patterns that may explain these adult distributions are
123 well-understood for only a few species (e.g. Howes et al. 2007). Many factors can
124 influence ascidian larval behaviour and settlement, including light, gravity, temperature,
125 salinity, presence of adults or competitors, biomechanical properties and energy
126 limitations (Yamaguchi 1975, Svane et al. 1987, Svane & Young 1989, Young 1989,
127 Vázquez & Young 1996, Thiyagarajan & Qian 2003, McHenry & Patek 2004, Bennet
128 & Marshall 2005). Svane & Young (1989) have stated that the time required for
129 settlement of aggregated solitary ascidians is inversely related to the concentration of
130 adult extracts to which larvae are exposed. Other studies have considered the effects of
131 abiotic conditions on settlement (e.g. Young & Chia 1985, Svane & Dolmer 1995).
132 However, no attempt has been made to analyse in combination the relative roles of
133 biotic and abiotic factors on settlement for a representative set of species and their
134 implication for the success of invasive populations.

135 We investigated the settlement patterns of larvae of six solitary ascidians found
136 along the South African coast (*Ciona intestinalis*, *Ascidiella aspersa*, *Styela plicata*,
137 *Microcosmus squamiger*, *Pyura herdmani* and *Pyura stolonifera*), which belong to four
138 different families from the two recognized Orders of Ascidiacea (Kott 1985) and are all
139 commonly found aggregated in the field (Petersen & Svane 1995, Rius et al. 2009a,
140 Branch et al. 2010, and personal observation). We chose these species to include four
141 introduced species with global distributions (*C. intestinalis*, *A. aspersa*, *S. plicata* and
142 *M. squamiger*) and two large native species (*P. herdmani* and *P. stolonifera*) that are not

143 known to be invasive, although congeners are recognized as invasive elsewhere
144 (Castilla et al. 2004). All these species are important occupiers of hard substrata of
145 coastal areas of South Africa (Branch et al. 2010). The larvae of four species had well
146 developed statocytes and ocelli (Griffiths 1976, Niermann-Kerkenberg & Hofmann
147 1989, Jacobs et al. 2008, and personal observation) but *S. plicata* has a highly reduced
148 ocellus (Ohtsuki 1990), and *M. squamiger* is unusual among Pyuridae in lacking an
149 ocellus (personal observation, and see also Svane & Young 1991 for a closely related
150 species). Thus, four species were expected to have both light and geotactic preferences,
151 while the larvae of the remaining two species were expected to respond to geotactic
152 stimuli alone.

153 We examined how larval behaviour determines settlement patterns in different
154 phototactic and geotactic conditions and in the presence or absence of conspecific
155 extracts. The larval responses were compared with patterns of adult distribution in the
156 field. *A priori*, we advanced three specific hypotheses. (1) Light will influence
157 settlement, with dark being preferred over light in species that are found in dark
158 habitats, and the opposite for those that occur in well-lit habitats. (2) Geotactic
159 behaviour will be important in those species that have adults with clear orientation
160 preferences. (3) Adult extracts will have a positive effect on settlement on all the
161 species, and will contribute to the aggregated patterns of distribution of adults.
162

163 **MATERIAL AND METHODS**

164 **Field sites and surveys of adults**

165 Adult ascidians were surveyed and sampled at the locations characterized in
166 Table 1. At each location we quantified adult distribution and associated circumstances.
167 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

193 *A. aspersa*, artificial fertilization followed the methods of Young & Chia (1985), which
194 involved dissection and collection of gametes from the oviduct and sperm duct. For the
195 remaining species we followed the methods of Marshall et al. (2000), modified from
196 those of Svane & Young (1991): gametes were extracted by dissection of the ripe
197 gonads and a mix of eggs and sperm poured through a 100-µm filter with seawater into
198 a small beaker, so the eggs were retained by the filter, but the excess sperm and
199 seawater passed through into the beaker. For all species we crossed the gametes of four
200 individuals, preventing self-fertilization. Developing embryos were placed in an aerated
201 beaker (containing 500 ml seawater) in a constant-temperature cabinet at 20°C and
202 complete darkness. In all species, motile larvae hatched within 14 h of fertilisation.

203 **Experiments**

204 Our experimental units were transparent cylindrical Perspex containers, sealed at
205 the top and bottom with Perspex sheets and held together with an elastic band. The
206 cylinders were 11 mm tall and 44 mm diameter with exactly the same surface area
207 (15.205 cm^2) on the top, bottom, and lateral surfaces, thus offering equivalent surface
208 areas for larval settlement in each of these three orientations. The containers were
209 placed in a seawater tank for 24 hours prior to introduction of larvae, to create a biofilm,
210 which is known to enhance settlement (Keough & Raimondi 1995). Once motile larvae
211 of a given species were formed, we pipetted out and placed 20 larvae per container
212 filled with seawater (final volume 16.72 cm^3), and immersed the containers in seawater
213 in a 200 mℓ beaker at 20ºC for 24 hours under the experimental conditions detailed
214 below. The Perspex chamber was subsequently dismantled in seawater, so that any
215 unattached larvae were washed away.

216 We performed three experiments. The number of replicates (i.e. experimental
217 units with 20 larvae each) per treatment and experiment varied from 3 to 10 due to

218 variability in the number of larvae obtained (see Table 1). Once we obtained enough
219 larvae in a given fertilization event, we ran all the experiments described below in
220 parallel.

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

223 In the second experiment, which was modified from the approach of Jiang et al.
224 (2005), we placed larvae in chambers in which half of the top, bottom and lateral
225 surfaces were covered by black tape (reducing the light to 0.4 μmol m^{-2} s^{-1}), while the
226 other half of these surfaces were exposed to the same artificial light (47 μmol m^{-2} s^{-1}).

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

234 In all three experiments, a stereomicroscope was used to count the numbers of
235 settlers and score their orientation (top, bottom or lateral sides of the containers) after a
236 24-hour period.

237 **Data analysis**

238 For the field data on adult distributions, a 1-way analysis of variance (ANOVA)
239 on square-root transformed data was used to test for differences in adult orientation,
240 with surface orientation as a fixed factor to compare the number of individuals per
241 quadrat for each species. Tukey HSD post-hoc tests were subsequently performed to
242 assess significant differences among different orientations. To evaluate among the

243 different species the level of gregariousness found in the field, we compared the number
244 of individuals per clump found for each species using a 1-way ANOVA, with Species as
245 a fixed factor. To test for differences in adult orientation, we used surface orientation as
246 a fixed factor and compared the number of individuals per clump for each species using
247 1-way ANOVA. The data were 4^{th} root transformed, and significant differences were
248 tested using pair-wise comparisons with Tukey HSD post-hoc tests.

249 For the laboratory experiments, we tabulated the number of settlers in 3-way
250 frequency tables incorporating replicates (experimental chambers), treatments (light-
251 dark, extract-control) and position of the settlers (bottom, lateral, top), and used log-
252 linear models for formal statistical testing of the significance of these factors and their
253 interactions (Knoke & Burke 1991). Full models (including all factors and their
254 interactions) were compared to reduced models which omitted the interactions or
255 individual factors. The expected value for each cell in the table under the reduced model
256 was computed by an iterative Newton-Raphson algorithm. The goodness of fit of the
257 table of expected values to the observed table was then evaluated by the likelihood ratio
258 test (Quinn & Keough 2002), using the Chi-Square distribution to assess levels of
259 significance. A poor fit indicated that the factor or interaction omitted contributed
260 significantly to explaining the observed values.

261 First, we tested the effect of the different replicates by fitting to the 3-way tables
262 a model that excluded all interactions of the factor replicate with the other two factors
263 (i.e. the terms treatment*replicate, position*replicate, and treatment*position*replicate).
264 This tested whether settlement levels in the different replicates were independent of the
265 other factors. As these reduced models had a good fit to the observed values in all cases
266 ($p > 0.05$ in the likelihood ratio test), the different replicates were pooled and the
267 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 pair-wise 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

293 poorly lit surfaces, while *P. stolonifera* preferred well-lit surfaces. The two remaining
294 species showed no obvious patterns with respect to light.

295 Orientation (see Fig. 1) had significant effects on the density of individuals only
296 in the case of the three pyurid species (ANOVA, *M. squamiger*, $F_{2,7} = 5.351$, $p = 0.039$,
297 Tukey test, $p < 0.05$, Upwards > Downward, both = Vertical; *P. herdmani*, $F_{2,7} =$
298 17.052, $p = 0.002$, Tukey test, $p < 0.01$, Downwards > other two categories; *P.*
299 *stolonifera*, $F_{2,7} = 5.097$, $p = 0.043$, Tukey test, $p < 0.05$, Upwards > Downwards, both
300 = Vertical). In the case of the other three species, we did not find significant differences
301 among orientations (*C. intestinalis* $F_{2,7} = 0.503$, $p = 0.625$, *A. aspersa* $F_{2,7} = 0.672$, $p =$
302 0.541, and *S. plicata* $F_{2,7} = 2.641$, $p = 0.140$), although *C. intestinalis* was most
303 abundant on downward-facing surfaces, and both *A. aspersa* and *S. plicata* were more
304 abundant on downward and vertical surfaces.

305 Light intensities were usually highest on vertical surfaces (Fig. 1) due to the
306 characteristics of the floating pontoons from where the animals were collected, except
307 for *P. stolonifera*, the only species collected in natural rocky shore. Low light intensities
308 on upward-facing surfaces for the remaining species reflected the fact that they grew on
309 artificial substrata that were poorly illuminated due to other structures that screened
310 them.

311 **Effects of light and orientation on larval settlement**

312 In the first experiment, results for *A. aspersa* and *S. plicata* were not analyzed
313 due to the low number of settlers. For the remaining species, there was no significant
314 interaction of the light treatment with the position of the settlers (Table 2). When the
315 two factors were analyzed separately, no effect of the light/dark treatment was found
316 (Fig. 2, Table 2 and *t*-tests on proportion of settlers: all $p > 0.05$). For the position
317 factor, *C. intestinalis* showed a clear preference for settlement on top surfaces, whereas

318 the three species belonging to the family Pyuridae (*M. squamiger*, *P. herdmani* and *P.*
319 *stolonifera*) settled significantly more often on the bottom than elsewhere (Fig. 2, Table
320 2).

321 In the second experiment, in which the larvae had the option of settling on light
322 or dark surfaces in the same chamber, a different picture emerged (Table 3, Fig. 3).
323 Again, the low number of settlers prevented analyses of *A. aspersa* and *S. plicata*. For
324 *C. intestinalis* and *M. squamiger*, no significant interaction was found between
325 treatment and position. Contrary to the previous experiment, both species showed a
326 marked preference for dark surfaces, and no significant preference for any orientation
327 (Table 3). In the case of the two *Pyura* species, *P. herdmani* and *P. stolonifera*, a
328 significant interaction existed (Table 3). *P. herdmani* continued to prefer bottom
329 surfaces in the light but selected both bottom and top in the dark. *P. stolonifera* changed
330 light preferences depending on the surface considered, but overall more larvae settled in
331 light (Fig. 3), and it preferred lateral surfaces in the lit part of the chambers. These
332 results are generally in accordance with what we found in the field for adults of *C.*
333 *intestinalis*, *M. squamiger* and *P. herdmani* (see Fig. 1), all of which settled in the dark,
334 and also for *P. stolonifera*, which (largely) settled in the light.

335 The four species that displayed significant geotactic patterns in the first
336 experiment shifted to a more random pattern in the second experiment, with two (*C.*
337 *intestinalis* and *M. squamiger*) now showing no geotactic preferences, and the other two
338 species (*P. herdmani* and *P. stolonifera*) showing greater settlement on lateral and top
339 surfaces than previously.

340 **Effect of tunic extracts**

341 Three species (*S. plicata*, *P. herdmani* and *P. stolonifera*) showed no effect of
342 tunic extracts in the water (Fig. 4, Table 4 and *t*-tests, $p > 0.05$). The other three showed

343 a significant inhibition of settlement in the presence of tunic extracts (Fig. 4, Table 4,
344 and *t*-tests, all $p < 0.05$), although in *C. intestinalis* the log-linear analysis revealed a
345 significant interaction, with the extract inhibition being significant for the lateral and top
346 surfaces only (Table 4).

347 The geotactic behaviour found in the first experiment testing light/dark effects
348 was maintained across all species in this third experiment, with the three pyurids *M.*
349 *squamiger*, *P. herdmani* and *P. stolonifera* settling preferentially on the bottom (Fig. 4).
350 For *C. intestinalis*, the highest number of settlers was again on top surfaces, although in
351 the presence of adult extract there was no significant difference between top and bottom
352 (Table 4). For *A. aspersa* there was no position effects, and for *S. plicata* there was no
353 effect of either extract or position on settlement in the chambers.

354 **Integrating field and laboratory data**

355 Comparing the level of aggregation and the overall abundance of individuals in
356 the field (see Fig. 1,5), a consistent pattern emerged: the more abundant a species was in
357 a particular orientation, the more individuals there were per clump. When we analysed
358 the number of individuals per clump across species, *M. squamiger* and *P. stolonifera*
359 showed the highest numbers (Fig. 5), but significant differences existed only between *P.*
360 *stolonifera* and two other species (ANOVA, $F_{5,54} = 4.207$, $p = 0.003$, Tukey test, *P.*
361 *stolonifera* > *S. plicata* = *P. herdmani*, $p < 0.05$). In terms of the numbers of individuals
362 per clump in relation to orientation in the field (Fig. 5), significant differences emerged
363 for two species (ANOVA, *M. squamiger*, $F_{2,7} = 6.689$, $p = 0.024$, Tukey test, Upwards
364 greater than the other two orientations, $p < 0.05$; *P. herdmani*, $F_{2,7} = 38.068$, $p < 0.001$,
365 Tukey test, Downward greater than the other two orientations, $p < 0.001$).

366 For an overall perspective of the geotactic preference of each species, we pooled
367 together all settlement data generated from the three laboratory experiments, on the

368 assumption that in terms of geotactic behaviour, larvae in the field would encounter a
369 combination of both phototactic stimuli and adult extracts. Setting aside *A. aspersa* and
370 *S. plicata* on the grounds that their settlement rates were too low for consideration, the
371 mean percentage of settlers on each surface showed the same trend as the number of
372 individuals per clump for three species (*M. squamiger*; *P. stolonifera* and *C.*
373 *intestinalis*), whereas *P. herdmani* showed no correlation (Fig. 5).
374
375 Three trends emerged from the laboratory data (as summarised in Table 5). First,
376 in relation to orientation, one species (*C. intestinalis*) tended to settle preferentially on
377 the top, whereas three (*M. squamiger, P. herdmani, P. stolonifera*) preferred settling on
378 the bottom in experiment 1, with almost the same pattern emerging in experiment 3. In
379 experiment 2 the geotactic responses evident in experiment 1 were either absent or
380 altered. *A. aspersa* and *S. plicata* could be analyzed with respect to geotactic behaviour
381 only in experiment 3, and neither showed any preference.
382 Second, in terms of light/dark responses, none of the four species analyzed
383 showed any statistical preferences in experiment 1, where the larvae were held either in
384 light or dark. However, in experiment 2, when they had a choice between dark and light,
385 three species (*C. intestinalis*, *M. squamiger* and *P. herdmani*) displayed preference for
386 settling in the dark, and a fourth (*P. stolonifera*) settled most often in the light, although
387 this preference changed on bottom surfaces, leading to an interaction between the
388 factors.
389 Third, in relation to the presence or absence of adult tunic extracts in the third
390 experiment, three species showed no response, while settlement of the other three (*C.*
391 *intestinalis, M. squamiger* and *A. aspersa*) was inhibited in the presence of tunic
392 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

418 accordance with the general statement that shading facilitates the dominance of hard
419 substrata by sessile invertebrates while well-lit surfaces lead to algal-dominated
420 communities (Miller & Etter 2008). For those species settling in the dark, this might
421 incidentally lead to settlement among adult conspecifics, where light is reduced in the
422 shade of adults, ultimately contributing to a gregarious distribution. An interesting result
423 of the second experiment was that the four species that could be statistically analyzed
424 (*C. intestinalis*, *M. squamiger*, *P. herdmani*, *P. stolonifera*) all altered their geotactic
425 behaviour from that displayed in the first experiment, showing a more haphazard
426 geotactic settlement distribution or alteration of preferences in the second experiment.
427 These results contrast with what has previously been found for the tadpole larvae of
428 another solitary ascidian (*Ascidia mentula*) and for the planulae of a scyphozoan, in
429 which the larvae did not alter their negative geotactic behaviour across a range of light
430 conditions (Svane & Dolmer 1995). Our results suggest that during settlement, time of
431 day and weather conditions (which can alter light conditions) may greatly influence
432 larval behaviour.

433 Both *S. plicata* and *A. aspersa* are common introduced species in South Africa
434 (M. Rius, C.L. Griffiths and X. Turon, in preparation) and have succeeded in
435 establishing populations worldwide (Carlton 1996, Lambert & Lambert 2003, Barros et
436 al. 2009). The fact that there were no settlement preferences in either of these species
437 may indicate that they can successfully settle under a range of conditions and on a range
438 of surfaces, increasing the likelihood of their colonising new localities. However, the
439 proportions of settlement found for these two species were the lowest of all studied
440 species and therefore any interpretation of their settlement preferences must be cautious.
441 Young & Braithwaite (1980) have shown that *Styela montereyensis*, like *S. plicata* and
442 *A. aspersa*, shows no discrimination with respect to light or substratum type. Similarly,

443 Young & Chia (1985) failed to find any settlement preferences in six other solitary
444 ascidian species that were exposed to different light regimes. In our study we found
445 strong patterns in four species out of six, with light intensity being an important factor
446 modulating larval geotactic behaviour.

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

457 Both conspecific attraction and gregarious behaviour have been identified as
458 driving forces for the distribution of many organisms (Alonso et al. 2004, Budke et al.
459 2004, Gautier et al. 2006). In contrast to the third of our initial hypotheses, our results
460 point to either an absence of response of larvae to cues from extracts of the adults, or
461 strong inhibition by tunic extract. Similar to our findings, the percentage of
462 metamorphosis of the solitary ascidian *Molgula citrina* decreases when its larvae are
463 exposed to conspecific tunic homogenate (Durante 1991). This has implications for
464 understanding how prior invasions might affect further colonization. Our study showed
465 that settlement was not promoted by the presence of adult extracts. However, it is
466 possible that the adult extracts we employed acted as a repellent because they signalled
467 damaged tissues of a conspecific; but other authors using adult extracts have found that

468 the presence of extracts induced metamorphosis (Svane et al. 1987), so we consider it
469 unlikely that the extracts signal damaged tissues. Our findings indicate that the
470 gregarious distribution of adults observed in the field are unlikely to be explained by
471 larval attraction to adult cues, but may be the result of settlement being concentrated in
472 habitats characterised by particular physical conditions. For many other marine species,
473 physical factors seem to be stronger cues for settlement than chemical attraction by
474 conspecific adults (Berntsson et al. 2004). Sometimes these preferred physical
475 conditions such as light intensity and hydrodynamic conditions may coincidentally be
476 associated with the presence of adults, or even created by adults, leading indirectly to
477 aggregations. For instance, a baffle effect of created by aggregations of adults (see
478 Eckman 1983) may enhance the settlement of new larvae and protect the juveniles,
479 thereby increasing their survival. However, more needs to be learnt concerning the
480 mechanisms driving the effect of conspecific adult attraction and further experiments
481 using gregarious ascidians have the potential to provide important insights.

482 In confined environments, such as harbours and marinas, where invasive
483 ascidians are highly successful, the specific biological features of each species such as
484 larval movement and offspring retention (Petersen & Svane 1995), the particular
485 hydrodynamics of the location (Havenhand & Svane 1991) and adequate conditions for
486 settlement (as shown in our study) may play important roles in influencing species
487 distributions and the success of introduced populations. For example, *C. intestinalis* is
488 widespread in dark, sheltered conditions in harbours and successfully colonizes the
489 culture ropes of mussel farms in South Africa, with important economic impacts
490 (Robinson et al. 2005), as also in northeast American coastal waters (Ramsay et al.
491 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.

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TABLES

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: 1st: Light vs Dark, 2nd: Half light vs half dark, 3rd: Tunic extracts.

Species	Field sites				Number of replicates per experiment		
	Name	Latitude/Longitude	Wave exposure	Substrata	1st	2nd	3rd
Ciona intestinalis	Cape Town harbour	34º 54′ 22″ S, 18º 25′ 37″ E	Sheltered	Artificial	7	5	5
Microcosmus squamiger	Port Alfred marina	33º 35′ 41″ S, 26º 53′ 32″ E	Sheltered	Artificial	5	5	5
Pyura herdmani	Langebaan marina	33° 01′ 07″ S, 17° 56′ 48″ E	Moderately exposed	Artificial	8	10	8
Pyura stolonifera	St. James	34° 07′ 14″ S, 18° 27′ 31″ E	Highly exposed	Natural	3	3	3
Ascidiella aspersa	Cape Town harbour	34º 54′ 22″ S, 18º 25′ 37″ E	Sheltered	Artificial	6	4	4
Styela plicata	Knysna marina	34º 03′ 17″ S, 23º 03′ 46″ E	Sheltered	Artificial	5	5	6

807 **Table 2**. Log-linear analyses of the outcomes of the 1st experiment. Post-hoc-
808 like pair-wise comparisons were done when appropriate. LR: likelihood ratio; df,
809 degrees of freedom; *p*: probability value. Significant values are indicated in bold.
810

	Log-likelihood	LR Chi-Square	df	*p*	Pair-wise comparisons
Ciona intestinalis					
Light * 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
Microcosmus squamiger					
Light * 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
Pyura herdmani					
Light * 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
Pyura stolonifera					
Light * 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

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824 **Table 3**. Log-linear analyses of the outcomes of the 2nd experiment. Interaction
825 was tested first and, if significant, each factor was tested at fixed levels of the other
826 factor. Post-hoc-like pair-wise comparisons were done when appropriate; LR:
827 likelihood ratio; df, degrees of freedom; *p*: probability value. Significant values are
828 indicated in bold.
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	Log-likelihood	LR Chi-Square	df	*p*	Pair-wise comparisons
Ciona intestinalis					
Light * 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	
Microcosmus squamiger					
Light * 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	
Pyura herdmani					
Light * 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
Pyura stolonifera					
Light * 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	

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837 **Table 4.** Log-linear analyses of the outcomes of the 3rd experiment. Interaction
838 was tested first and, if significant, each factor was tested at fixed levels of the other
839 factor. Post-hoc-like pair-wise comparisons were done when appropriate. LR: likelihood
840 ratio; df, degrees of freedom; *p*: probability value. Significant values are indicated in
841 bold.
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	Log-likelihood	LR Chi-Square	df	*p*	Pair-wise comparisons
Ciona intestinalis					
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
Microcosmus squamiger					
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
Pyura herdmani					
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
Pyura stolonifera					
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
Ascidiella aspersa					
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	
Styela plicata					
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	

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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.

	1st experiment		2nd experiment		3rd experiment	
	Position	*Light vs. Dark*	*Position*	*Light / Dark*	*Position*	*Tunic*
Ciona intestinalis	Top	─	─	Dark	Bottom & Top *	Inhib
Microcosmus squamiger	Bottom	─	─	Dark	Bottom	Inhil
Pyura herdmani	Bottom	─	Bottom & Top*	Dark*	Bottom	-
Pyura stolonifera	Bottom	─	Lateral*	Light*	Bottom	-
Ascidiella aspersa	nt	nt	nt	nt	─	Inhil
Styela plicata	nt	nt	nt	nt	─	-

885 **FIGURE LEGENDS**

886 **Fig. 1**. Adult distribution in the field, indicated as the mean density of
887 individuals, and mean light intensity (μmol m^{-2} s^{-1}) in relation to surface orientation.
888 Lines connecting levels of light intensity are inserted for guidance only. Error bars
889 denote + 1 SE. Note differences in scales of y-axes.

890 **Fig. 2**. Mean percentage settlement in relation to orientation (bottom, lateral or
891 top) and treatment (light - white bars, dark - dark bars) in the 1st experiment, in which
892 larvae were held either in the dark or in the light. Error bars denote + 1 SE. Note
893 differences in scales of y-axes.

894 **Fig. 3**. Mean percentage settlement in relation to orientation (bottom, lateral and
895 top) and treatment (light - white bars, dark - dark bars) in the 2nd experiment, in which
896 larvae had the choice of settling in light or dark portions of the same chamber. Error
897 bars denote + 1 SE. Note differences in the scales of y-axes.

898 **Fig. 4**. Mean percentage settlement with respect to orientation (bottom, lateral
899 and top) and treatment (control - white bars, tunic extract - dark bars) in the 3rd
900 experiment, in which larvae were held in chambers either with or without adult extract.
901 Error bars denote + 1 SE. Note differences in the scales of y-axes.

902 **Fig. 5**. Mean numbers of individuals per clump in the field, and mean percentage
903 of settlers from all the experiments pooled, in relation to orientation. Error bars denote +
904 1 SE. Note differences in scales of y-axes.

905

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908

909

FIGURES

Figure 1.

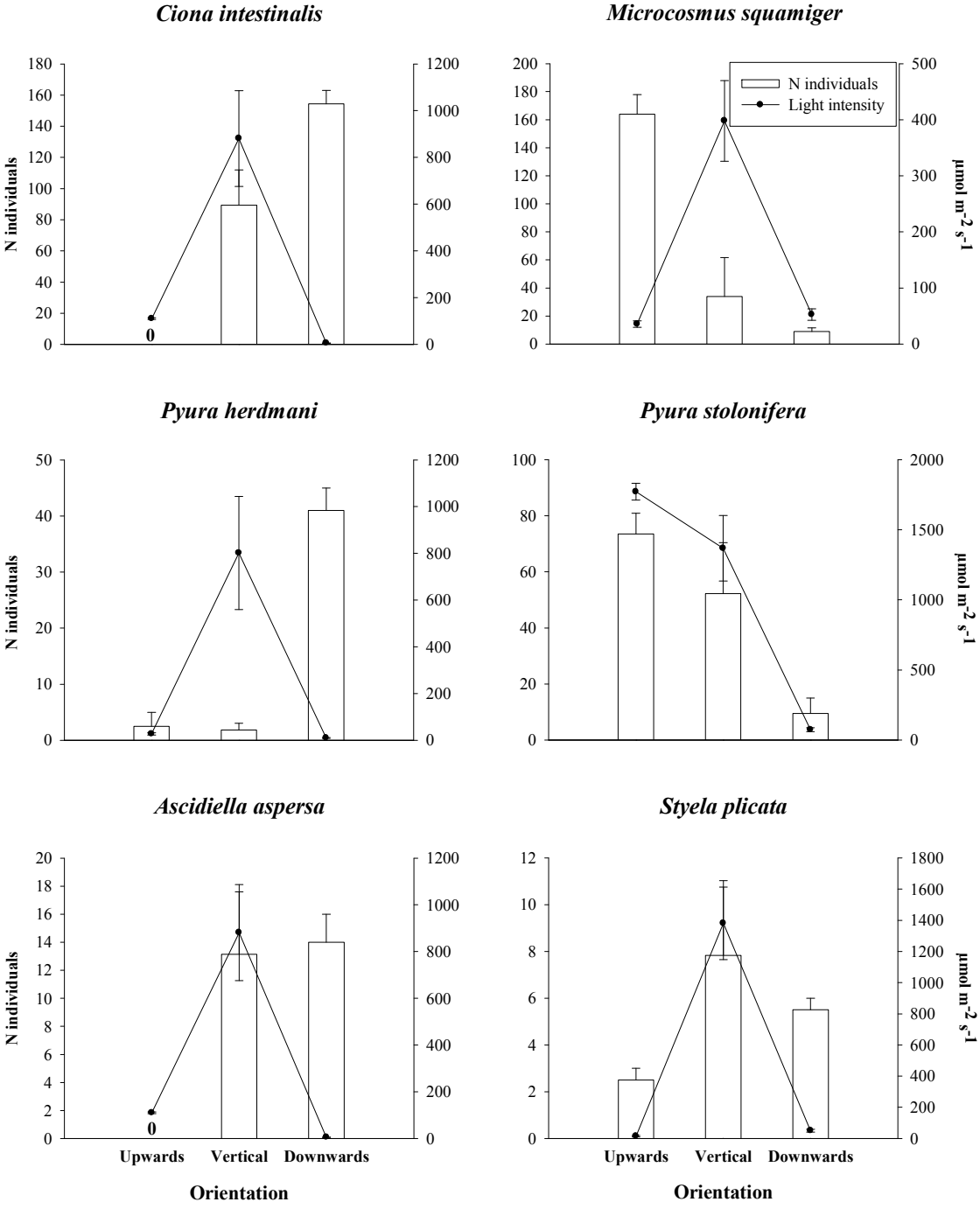


Figure 2.

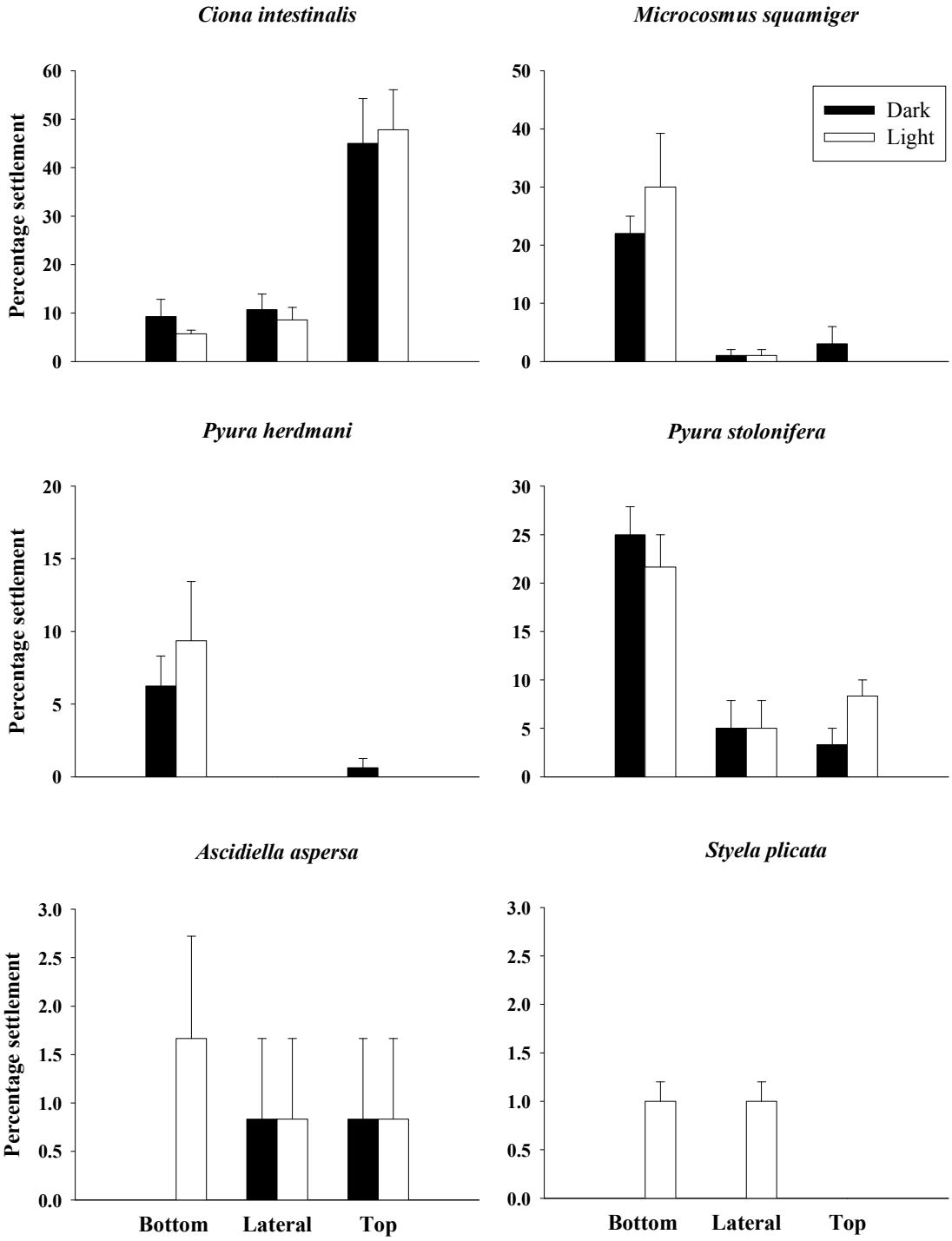

Ciona intestinalis
Microcosmus squamiger
Dark
Light
Pyura herdmani
Pyura stolonifera
Ascidiella aspersa
Styela plicata
Percentage settlement
Bottom
Lateral
Top

Figure 3.

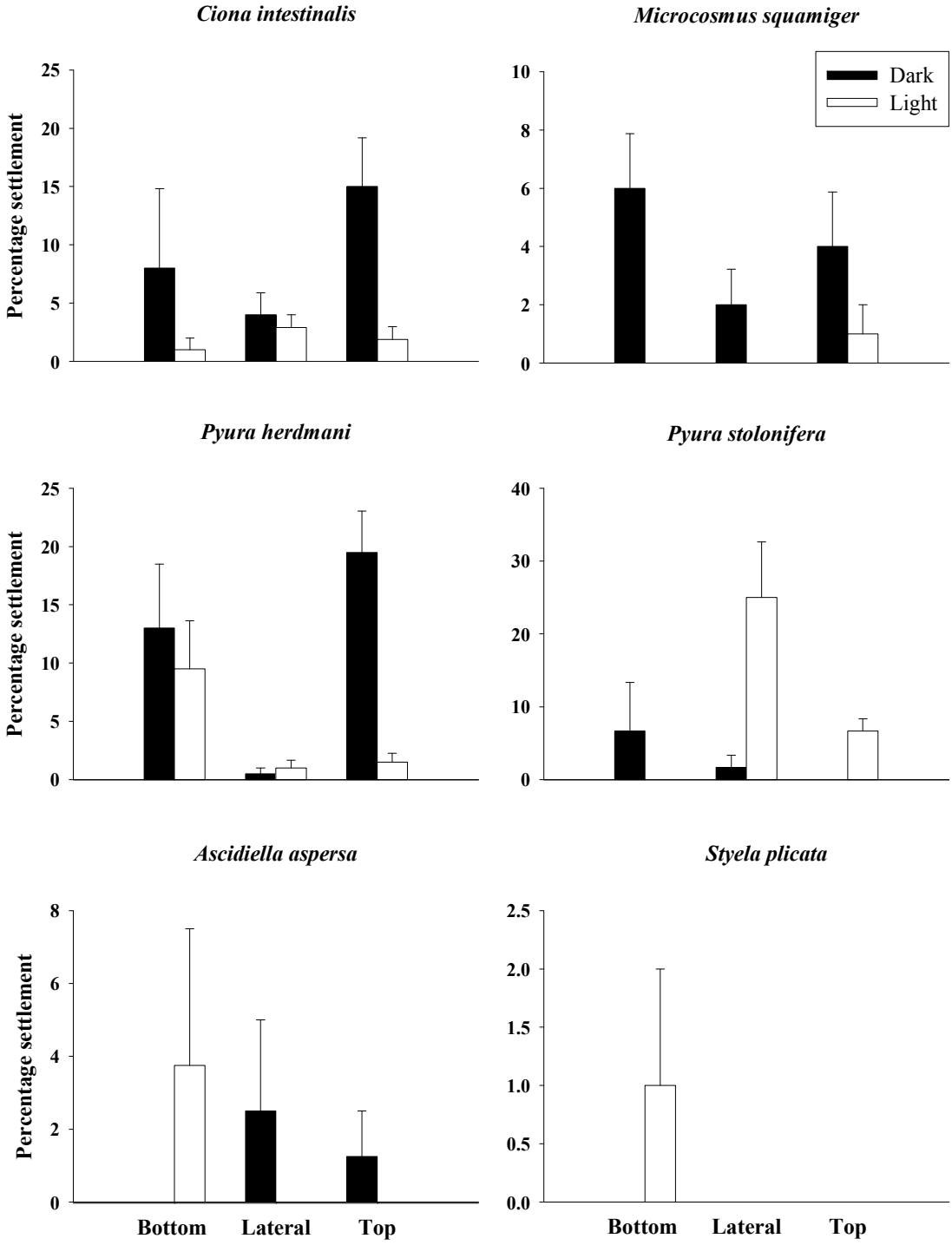

Ciona intestinalis
Microcosmus squamiger
Dark
Light
Percentage settlement
Pyura herdmani
Pyura stolonifera
Ascidiella aspersa
Styela plicata
Bottom
Lateral
Top

Figure 4.

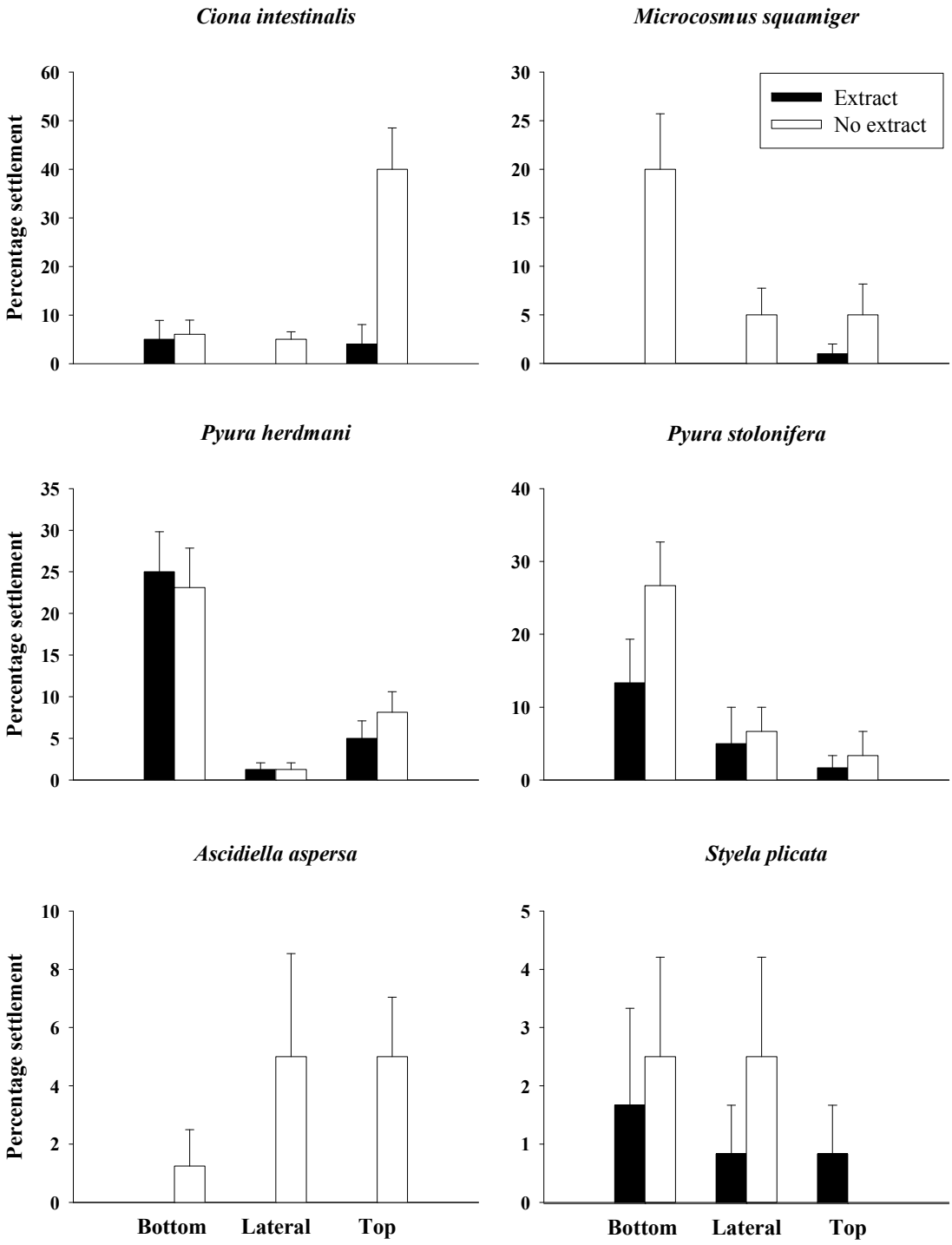
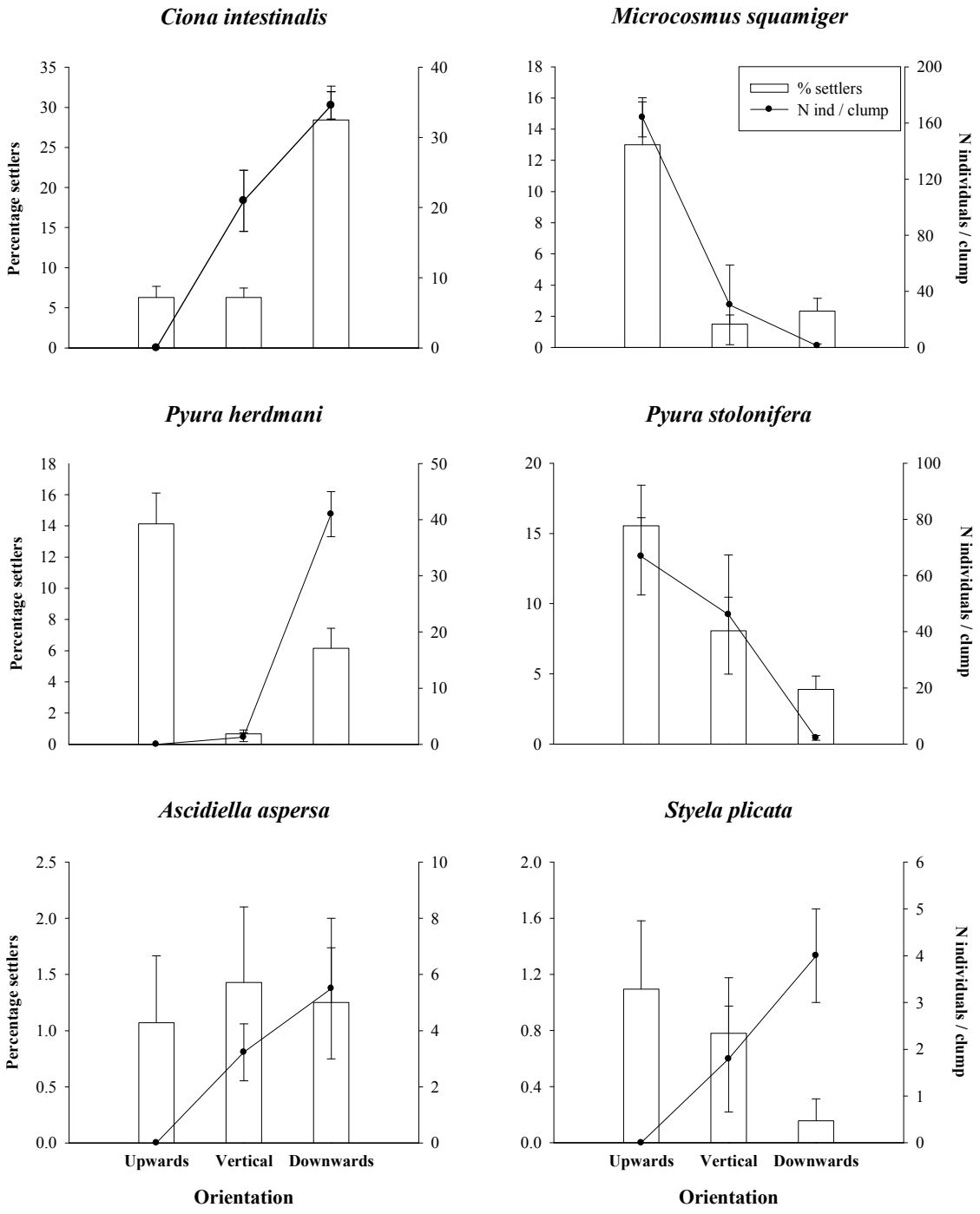

Ciona intestinalis
Microcosmus squamiger
Pyura herdmani
Pyura stolonifera
Ascidiella aspersa
Styela plicata
Extract
No extract
Percentage settlement
Bottom
Lateral
Top

Figure 5.


Ciona intestinalis
Microcosmus squamiger
% settlers
N ind / clump
Pyura herdmani
Pyura stolonifera
Ascidiella aspersa
Styela plicata
Percentage settlers
N individuals / clump
Upwards
Vertical
Downwards
Orientation