

Early biotic interactions among introduced and native benthic species reveal cryptic predation and shifts in larval behaviour

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ABSTRACT: Recurrent introductions of non-indigenous species generate novel interactions that vary with local conditions and the composition of the receiving community. Most studies examine relationships of newcomers with native species, but interactions among introduced species could also affect community shifts. As early ontogenetic stages are particularly vulnerable to biotic interactions, we explored direct and indirect interactions across early life-history stages in space-dominating marine invertebrates. We used introduced ascidians and both native and introduced mussels. To increase generality, we ran our experiments in 2 distant locations, one in the northern and one in the southern hemisphere (Mediterranean and South Africa). We found no sperm interference between the ascidians, nor were there interspecific effects on settlement or metamorphosis success. However, larvae of the ascidian species reacted to each other by shifting from aggregated to random settlement. Juvenile mussels consumed large numbers of ascidian larvae, though larvae that avoided mussel predation showed higher settlement success. Mussel species in the southern hemisphere locality (native *Perna perna* and introduced *Mytilus galloprovincialis*) consumed more ascidian larvae than mussels in the northern locality (native *M. galloprovincialis*), with a tendency for ascidian larvae to avoid settling close to mussels in the northern locality. We conclude that larval consumption by mussels affects the establishment of ascidians, but that the magnitude of this effect is context dependant. These results emphasize the importance of the composition of the receiving community in determining its susceptibility to invasion. Whether the species comprising this community are native or introduced is, however, less important than what manner of species they are.

KEY WORDS: Competition · Settlement · Larviphagy · Invasive species · *Microcosmus squamiger* · *Styela plicata* · *Mytilus galloprovincialis* · *Perna perna* · Ascidians · Mussels

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INTRODUCTION

The introduction of species as a result of human activities is a major concern in the maintenance of biodiversity in marine systems (Carlton & Geller 1993, Ruiz

et al. 1997, Harris & Tyrrell 2001). Accordingly, the study of biological introductions often focuses on their negative effects on native species (e.g. Caro et al. 2011) and there has been the suggestion that interspecific facilitation among invasive species can result

in invasion meltdown (Simberloff & Von Holle 1999, Grosholz 2005). Although there is still no clear evidence of the widespread nature of this effect (Simberloff 2006), the intensity of recurrent introduction events often leads to an increase in community heterogeneity through the coexistence of diverse non-indigenous species that interact both among themselves and with the resident community (Byrnes & Stachowicz 2009, Dijkstra & Harris 2009). Indeed control of newcomers to a particular community can be exerted by already established introduced species rather than by native species. For example, habitats dominated by introduced species may be more resistant to further invasions than native communities (Osman & Whitlatch 2007). It is important, therefore, to understand interactions among dominant introduced species, which in marine systems often compete aggressively for space. This is a largely unexplored field, although the notion of complementarity (temporal, trophic or otherwise) among introduced species has been put forward as one factor that can modulate community function and dynamics (Byrnes & Stachowicz 2009). Nevertheless, few studies have analyzed the potential effects of biotic interactions among non-indigenous species colonizing a new area (e.g. Simberloff 2006, Rius et al. 2011), and our study aimed to contribute towards filling this gap.

Early ontogenetic stages of marine organisms (e.g. fertilization, larval settlement and survival) are particularly vulnerable to competitive interactions, which can strongly influence species fitness, and are key to the establishment of marine organisms (Grosberg 1981, Bingham & Walters 1989, Osman & Whitlatch 1995a,b, Lambert 2000, Porri et al. 2008). Despite this being a well-established idea, studies of interactions between introduced and resident species usually focus on adult interactions (e.g. Byers 2000, Grosholz et al. 2000, Nyström et al. 2001, Decottignies et al. 2007) or how adults affect new recruits (e.g. Osman & Whitlatch 1995a,b, Lohrer & Whitlatch 2002), with less attention to species interactions during early life-history stages (e.g. Rius et al. 2009a).

There is a range of potential direct and indirect competitive effects during the early life-history stages of marine benthic invertebrates. For closely related broadcast spawning species that share spawning periods, interference competition could happen at the fertilization stage owing to interspecific sperm competition (Lambert 2000). Once embryonic development is completed, some marine invertebrate larvae select their point of settlement taking into account future risks such as the presence of predators (Johnson & Strathmann 1989, Stoner 1994) or domi-

nant competitors (Grosberg 1981). Hence, the behaviour of larvae during settlement is a crucial determinant of successful recruitment (Young & Chia 1984, Young & Cameron 1989, Stoner 1994). Predation of larvae and juveniles can also play an important role in determining recruitment and population connectivity (Bingham & Walters 1989, Young & Cameron 1989, André et al. 1993) and may control the establishment of newcomers (Osman & Whitlatch 1998). Such biotic interactions can occur not only between introduced and resident species, but also among non-indigenous species in the same habitats.

The aim of the present work was to explore early ontogenetic interactions among introduced species using dominant introduced and native species. Ascidians and mussels are major ecosystem engineer species along the world's coastlines and have been widely introduced via human-mediated transport (e.g. Lambert 2007, McQuaid & Arenas 2009). They often colonize artificial substrata in harbours such as pilings, docks, floating pontoons, boat hulls and buoys (e.g. Bax et al. 2002). As these species are generally space dominating, coexistence on such substrata is likely to lead to either exploitation or interference competition. Co-occurring ascidians and mussels constitute a good study system for potential interactions as they are abundant and gregarious, coexist and can compete for substratum as a primary resource. Specifically, we wanted to (1) assess the effects of competition of conspecific and heterospecific sperm on fertilization success of ascidian species; (2) assess the effect of the presence of conspecific and heterospecific ascidian larvae on settlement success, settlement behaviour and metamorphosis; (3) assess the interaction between ascidian larvae and mussel juveniles; and (4) compare the outcome of this interaction in 2 locations, one in the northern and one in the southern hemisphere, allowing us to compare the effects of the same mussel species where it is native and where it is introduced. Our rationale was to evaluate interactions among benthic species that may take place when these dominant space competitors co-occur, and how propagules react to the presence of the same heterospecifics in different geographic regions.

MATERIALS AND METHODS

Study species and regions

Both *Microcosmus squamiger* Michaelsen, 1927 and *Styela plicata* (Lesueur, 1823) are solitary ascidians that have been widely introduced to harbours

and marinas of warm and temperate oceans (Pineda et al. 2011, Rius et al. 2012). *S. plicata* is probably native to the NW Pacific Ocean (Pineda et al. 2011 and references therein), although stochastic and recurrent introductions over a long period of time make its source difficult to ascertain (Pineda et al. 2011). *M. squamiger* is native to Australia (Rius et al. 2012 and references therein). Both species are highly tolerant of abiotic stressors, such as pollution and changes in temperature and salinity, particularly *S. plicata* (Naranjo et al. 1996, Pineda et al. 2012). Although *S. plicata* has a wider distribution around the world than *M. squamiger*, both species often coexist at high densities. In addition, *M. squamiger* has a noticeable capacity for regional dispersal, which allows it to spread once it has reached a new area (Ordóñez et al. 2013). This species colonizes natural substrata in the western Mediterranean (Rius et al. 2009b, Ordóñez et al. 2013), where it coexists with the native mussel *Mytilus galloprovincialis* (Lamarck, 1819) (authors' pers. obs.).

The Mediterranean mussel *Mytilus galloprovincialis* is a key space occupier in shallow sublittoral and intertidal parts of the Mediterranean Sea (Bacchiocchi & Airoldi 2003, Rius & Zabala 2008), but is also found in many regions around the world (Apte et al. 2000). This mussel has become an invasive species in many temperate zones of the world (Branch & Steffani 2004), including the south coast of South Africa (McQuaid & Phillips 2000) where the mussel *Perna perna* (Linnaeus, 1758) is native (Siddall 1980). Both mussels co-occur in natural intertidal beds and can be found together with the ascidians *Styela plicata* and *Microcosmus squamiger* in harbours and marinas of the region (authors' pers. obs.).

Gamete collection and sperm interactions experiment

Microcosmus squamiger and *Styela plicata* were collected from ropes and floating pontoons in the SW Indian Ocean at the marina of Port Elizabeth, South Africa (33° 58' 01.38" S, 25° 38' 02.47" E) (Fig. 1A) during the austral spring of 2010. Both species are reproductively active during this time of the year elsewhere (Yamaguchi 1975, Rius et al. 2009b, Pineda et al. 2013). Samples were stored in seawater and transported (2 h) to the laboratory in insu-

lated containers. Once in the lab, ascidians were kept in aerated tanks at 19 to 20°C. We used constant light conditions to prevent light-induced spawning (West & Lambert 1976).

Individuals of both ascidian species (see details below) were dissected to separate the gonads as described in Marshall et al. (2000). Ascidians are hermaphroditic (Lambert 2005), thus separation of eggs from sperm was necessary. Gonads from each individual were smashed separately in Petri dishes and filtered using an upper filter of 500 µm mesh and a lower filter of 100 µm mesh. The upper filter retained the tissue remains and the lower retained the eggs. Sperm passed with water through both filters and was collected in a beaker. Eggs retained in the 100 µm filter were rinsed with seawater and collected in a separate beaker. Thus, we obtained the sperm and eggs from the same individual separately. Filtered seawater was used at every step. This protocol was repeated for all experiments.

A total of 20 individuals of each species were used to perform this experiment. To prevent possible self-fertilization, the sperm of 10 individuals were pooled and used to fertilize the eggs of the 10 remaining individuals. Sperm concentration of the sperm homogenate was calculated using a Neubauer hemocytometer as spermatozooids ml⁻¹. For each species, 1 ml of egg homogenate (~300 eggs ml⁻¹) was added to

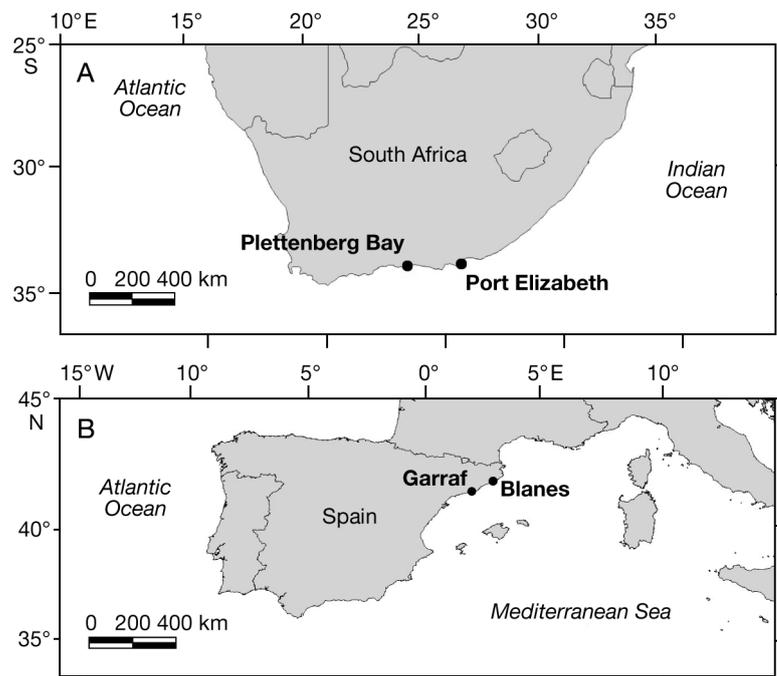


Fig. 1. Sampling sites in the 2 regions. (A) Southern hemisphere—south coast of South Africa (SW Indian Ocean). (B) Northern hemisphere—NE coast of Spain (NW Mediterranean Sea)

Table 1. Sperm concentrations (spermatozooids ml⁻¹) of each ascidian species (*Microcosmus squamiger* or *Styela plicata*) used for the fertilization of conspecific eggs: eggs fertilized in the presence of conspecific sperm only (C1 and C2); treatments using mixed sperm of both species at different concentrations (M1, M2 and M3)

	Non-mixed treatments		Mixed treatments		
	C1	C2	M1	M2	M3
Conspecific sperm	1 × 10 ⁷	2 × 10 ⁷	1 × 10 ⁷	0.5 × 10 ⁷	1.5 × 10 ⁷
Heterospecific sperm	–	–	1 × 10 ⁷	1.5 × 10 ⁷	0.5 × 10 ⁷

a Petri dish (65 mm in diameter) with 15 ml of filtered seawater. Then, the appropriate volume of sperm was added to the Petri dish to obtain the desired final concentration for each treatment (see details in Table 1). C1 and C2 were Petri dishes with eggs exposed to 2 different conspecific sperm concentrations to test for intraspecific interactions. M1, M2 and M3 were 'mixed' Petri dishes with eggs of each ascidian exposed to sperm of *M. squamiger* and *S. plicata* together in different relative proportions. For each treatment and species, 10 replicates (i.e. 10 Petri dishes) were run. Petri dishes were stored at 19 to 20°C under constant light. After 14 h, when embryonic development was already completed, each Petri dish was examined under a binocular microscope to obtain a measure of fertilization success as follows:

$$\% \text{ fertilization} = \frac{\text{embryos}}{\text{embryos} + \text{unfertilized eggs}} \times 100$$

Larval interactions

Individuals of both ascidian species were sampled at the marina of Port Elizabeth, South Africa (Fig. 1A) as indicated above. As hundreds of larvae were required, 30 individuals of each species were dissected to obtain the gametes for cross-fertilization. Reciprocal crosses were carried out using eggs of half of the individuals of each species and sperm from the remaining individuals. Gametes were kept together for fertilization for 50 min, and eggs were filtered afterwards with a 100 µm mesh filter to wash off the sperm. Eggs were collected in a 1 l beaker with filtered seawater, oxygenated by an aquarium pump and stored at 19 to 20°C. This was done separately for each species. After ca. 14 h the larvae started hatching and were carefully pipetted into 65 mm Petri dishes with 15 ml of filtered seawater. 'Low density' (LD) and 'high density' (HD) treatments were single species treatments with 20 and 40 larvae, respectively, to test for intraspecific interactions.

'Mixed' was a treatment in which 20 larvae of *Microcosmus squamiger* were mixed with 20 larvae of *Styela plicata* (added at the same time) to assess inter-specific interactions using LD and HD treatments as controls for comparison. For each treatment, 11 replicates (i.e. Petri dishes) were performed (a total of 880 larvae for each species). Petri dishes had been previously submerged in seawater for 24 h to create a biofilm, which favours the settlement of ascidian larvae (Keough & Raimondi 1995, Wicczorek & Todd 1997). After 48 h, all larvae were either settled or dead, and their numbers were counted under a microscope. After 72 h the number of completely metamorphosed settlers (hereafter 'post-metamorphs') was also counted. Settler positions were marked on the Petri dish lid using a felt-tip pen. Results were recorded as percentages as follows:

$$\% \text{ settlement} = \frac{\text{settlers}}{\text{larvae added}} \times 100$$

$$\% \text{ metamorphosis} = \frac{\text{post-metamorphs}}{\text{settlers}} \times 100$$

Pictures of each marked dish lid were taken using a digital camera (Canon PowerShot G11) and the distances between settlers on each dish (see 'Image treatment and data analyses') were analysed by the Laboratory of Image Analysis and Treatment of the CCiTUB (Scientific and Technological Centre of the University of Barcelona, see details below) using the proprietary program IMAT.

Ascidian–mussel interactions

Fertilizations were carried out to obtain larvae from both ascidian species as above. To obtain juvenile mussels, we collected adults directly from natural intertidal populations at low tide and carefully removed the juveniles found among byssus threads of adults under a binocular microscope.

In the southern hemisphere (SW Indian Ocean), *Microcosmus squamiger* and *Styela plicata* were collected at the Port Elizabeth marina, South Africa (Fig. 1A) as above. The 2 species of mussels, *Perna perna* and *Mytilus galloprovincialis*, were sampled at the same time as the ascidians from the shore of Port Elizabeth (33° 58' 47.27" S, 25° 39' 27.61" E) and the shore of Plettenberg Bay, South Africa (34° 03' 35.44" S, 23° 22' 48.75" E), respectively (Fig. 1A). Both species of mussels were stored in seawater and transported

to the laboratory in insulated containers (~2 and 6 h journeys, respectively). In the lab, mussels were kept in tanks with water aeration at 19 to 20°C, while juveniles were removed from adult byssus threads. Juvenile mussels were kept in aerated containers at the same temperature and with seawater from the place where they had been collected until they were used for the experiments.

The ascidian–mussel interactions study was repeated in the northern hemisphere (NW Mediterranean Sea). Adults of both *Microcosmus squamiger* and *Mytilus galloprovincialis* were sampled by snorkelling at the outer edge of the breakwater of the marina in Garraf, Spain (41° 14' 08.50" N, 1° 54' 08.63" E), while *Styela plicata* was collected at the marina in Blanes, Spain (41° 40' 30.57" N, 2° 47' 58.43" E) by pulling up ropes (Fig. 1B). All of the collections in Spain were carried out during early summer 2011. Both ascidians and mussels were stored in seawater, transported to the lab (~1 h) and processed as in the previous experiment.

In both geographic regions, we randomly selected 25 juvenile mussels of 3 to 9 mm shell length, and placed them haphazardly in a 65 mm Petri dish with 25 ml of filtered seawater. Mussels released byssal threads for adhesion soon after their deployment. Subsequently, 40 ascidian larvae were carefully released in the Petri dish using a micropipette. Dishes with 40 ascidian larvae alone were used as controls simultaneously to the mixed dish experiments. All Petri dishes had been previously submerged in seawater for 24 h as above. For Spain, the different treatments and numbers of replicates were: mixed *Microcosmus squamiger*–*Mytilus galloprovincialis*, n = 12; mixed *Styela plicata*–*M. galloprovincialis*, n = 10; controls, n = 10 for each ascidian species. For South Africa, the treatments and number of replicates were: mixed *M. squamiger*–*M. galloprovincialis*, n = 6; mixed *S. plicata*–*M. galloprovincialis*, n = 6; mixed *M. squamiger*–*Perna perna*, n = 8; mixed *S. plicata*–*P. perna*, n = 8; controls, n = 14 for each ascidian species.

To calculate mortality due to mussel consumption (see 'Results'), the number of settlers and the number of dead and unattached larvae was recorded after 48 h, and a consumption percentage was calculated as:

$$\% \text{ consumption} = [(\text{added larvae} - \text{settlers} - \text{dead larvae}) / \text{added larvae}] \times 100$$

All non-consumed larvae were either settled or dead after 48 h in all treatments. Mussels were carefully scrutinized to look for settled or hidden larvae,

and none were found. Settlement percentage was also recorded among the non-consumed larvae as:

$$\% \text{ settlement} = [\text{settlers} / (\text{settlers} + \text{dead larvae})] \times 100$$

Mussels and ascidian settlers of all dishes were also outlined on the lids with a felt-tip pen to take pictures, which were treated with the program IMAT as above.

Image treatment and data analyses

Every picture (taken in the larval interactions and ascidian–mussel interactions experiments) was manually checked to ensure that the program IMAT correctly identified the marks of settlers and/or mussels. We chose to measure the shortest distance between a given settler and the closest conspecific and/or heterospecific individual. These were distances between ascidians for the larval interaction experiments. For mussel–ascidian interaction experiments, distances were measured between ascidian settlers, between mussels, and between ascidians and mussels. For the distance between ascidians and mussels, we recorded the distance between the settler and the nearest edge of the closest mussel or mussel aggregate. The distribution of nearest-neighbour distances is a good descriptor of spatial patterns (Clark & Evans 1954), as they tend to be smaller than expected (under random settlement) for aggregated settlement, and larger when there is settler avoidance (leading to regular distribution).

To generate the expected nearest-neighbour distances between ascidians and/or mussels in the different replicates, a simulation program was written ad hoc in Turbo Pascal. The program generates a virtual arena of the same circular area as the Petri dish and places ascidian settlers and/or mussels into this arena at random to match the actual numbers in every experimental replicate. To match the experimental setting, for the larval interaction experiment, 'settlers' assigned to the 2 species were added alternately to the virtual arena, whereas for the mussel–ascidian interaction experiment, 'mussels' were allowed to settle first and 'ascidian settlers' were added afterwards. The virtual ascidian settlers were given an area equivalent to the mean area recorded for the actual settlers (as variation in this parameter was low). Virtual mussel settlers were given exactly the same area as observed in the mussels of the corresponding replicate (as mussel areas changed considerably because they often grouped in clumps). The program avoided placing a new virtual settler on top of existing ones or mussels. To compensate for potential edge effects

(Sinclair 1985), virtual mussels or ascidians were allowed to settle at the margins of the virtual Petri dish even if the whole area of the settler did not fit inside it. The same distance parameter recorded for the actual replicate was calculated from the simulated Petri dish, and the procedure was repeated 1000 times to generate a mean distance value under random settlement. The actual values recorded were transformed to percentage deviation (positive or negative) from the generated mean values.

Mean and SE of the percentage deviation for each experiment were obtained by averaging all replicates, and significant departures from randomness were recorded whenever the confidence interval of the mean did not include zero. Significant positive deviations (i.e. nearest-neighbour distances larger than expected) indicate avoidance behaviour, and significant negative deviations indicate aggregative behaviour.

ANOVA was performed to assess the effects of the different treatments. The variables analysed (fertilization, settlement, metamorphosis and consumption) did not comply in general with the assumptions of parametric analyses, as assessed by the Kolmogorov-Smirnov test (normality) and Levene median test (homogeneity of variances) even after trying several transformations, including some recommended for percentage data, such as arcsine or logit (Warton & Hui 2011). We finally adopted the rank-transformation method (Conover & Iman 1981, Potvin & Roff 1993), in which data are transformed to ranks and parametric models are then fitted to the ranked data (Quinn & Keough 2002). Rank-transformed data in all cases met the assumptions of parametric tests. Post-hoc tests were made using the Student-Newman-Keuls (SNK) test. Significant interactions were interpreted by examining plots of effects and performing SNK comparisons for levels of one factor at each level of the other factor using the common error mean square (Quinn & Keough 2002). The programs Statistica v. 8 (StatSoft) and SigmaStat v. 3.11 (Systat Software) were used to run the analyses.

RESULTS

Sperm interactions

Overall, fertilization success was low for all experiments even though they were done during the reproductive season. There were no significant differences

Table 2. ANOVA results of the ascidian sperm interactions experiment. Treatment levels as in Table 1

	df	SS	MS	F	p
Fertilization success					
Species	1	10197.79	10197.79	14.98	<0.001
Treatment	4	1966.59	491.65	0.72	0.579
Species × Treatment	4	6553.26	1638.32	2.41	0.055
Residual	88	59909.09	680.78		

in the fertilization success among treatments, nor was there a significant Species × Treatment interaction (ANOVA, Table 2). There was a significant effect of species due to higher success in *Styela plicata* (mean % ± SE, 3.51 ± 0.20) than in *Microcosmus squamiger* (2.62 ± 0.12). Thus, at the concentrations tested here, there was no effect of increasing conspecific sperm concentration (C1 vs. C2), or of the presence of different proportions of heterospecific sperm (M1 to M3) (Fig. 2).

Larval interactions

No significant differences were found for the variables analysed (% settlement, % metamorphosis) as a result of intraspecific competition (2 densities, HD and LD) or interspecific competition (mixed treatment compared to HD) (Fig. 3, Table 3), nor was there a significant Treatment × Species interaction. Overall, there was a marked species effect, reflecting the fact that *Styela plicata* had better success than

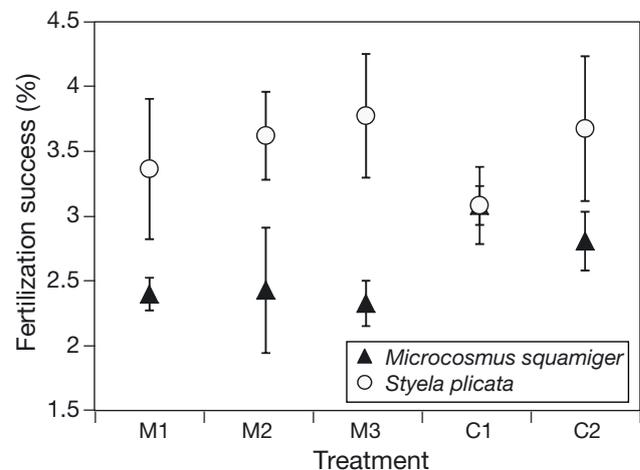


Fig. 2. *Microcosmus squamiger* and *Styela plicata*. Mean (±SE) fertilization success (%) of both species' eggs in each experimental condition. C1 and C2: eggs fertilized in the presence of conspecific sperm only; M1, M2 and M3: treatments using mixed sperm of both species at different concentrations

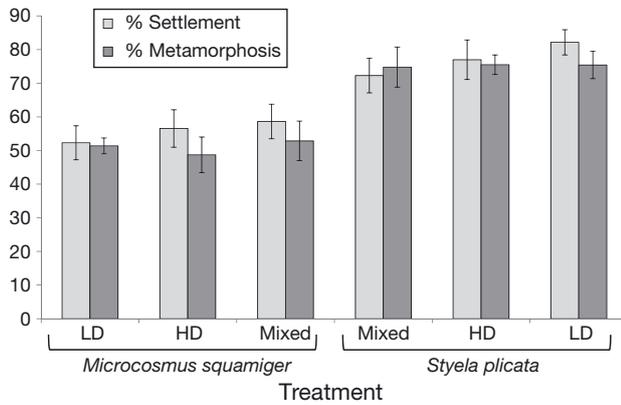


Fig. 3. *Microcosmus squamiger* and *Styela plicata*. Mean (\pm SE) % settlement and % metamorphosis in each treatment for both ascidian species. LD (low density): 20 larvae; HD (high density): 40 larvae; mixed: 20 larvae of each species. The % settlement is relative to the number of larvae added, while the % metamorphosis is relative to the number of settled larvae; differences from 100% reflect larval and settler mortality, respectively

Microcosmus squamiger in terms of % settlement of larvae (mean \pm SE, 77.13 ± 2.89 vs. 55.82 ± 2.97 , respectively) and % metamorphosis of settlers (mean \pm SE, 75.23 ± 2.50 vs. 50.98 ± 2.68 , respectively).

The distribution of settlers in the experimental dishes, as assessed by nearest-neighbour distances, showed that *Microcosmus squamiger* settled at random at low densities, but significantly aggregated at high densities, while *Styela plicata* had an aggregated settlement distribution at both densities (Fig. 4). When the 2 species were together (mixed treatment), the behaviour of the larvae changed. Although there was a tendency towards aggregation both for intra- and interspecies comparisons (negative deviations), there was considerable variability and the mean values were not significantly different from what would be expected if settlement was random.

Table 3. ANOVA results of the ascidian larval interactions experiment

	df	SS	MS	F	p
Settlement					
Species	1	6740.74	6740.74	24.66	<0.001
Treatment	2	41.20	20.60	0.08	0.927
Species \times Treatment	2	700.46	350.23	1.28	0.285
Residual	60	16403.09	273.38		
Metamorphosis					
Species	1	10792.97	10792.97	50.37	<0.001
Treatment	2	57.02	28.51	0.13	0.876
Species \times Treatment	2	214.59	107.29	0.50	0.609
Residual	60	12857.41	214.29		

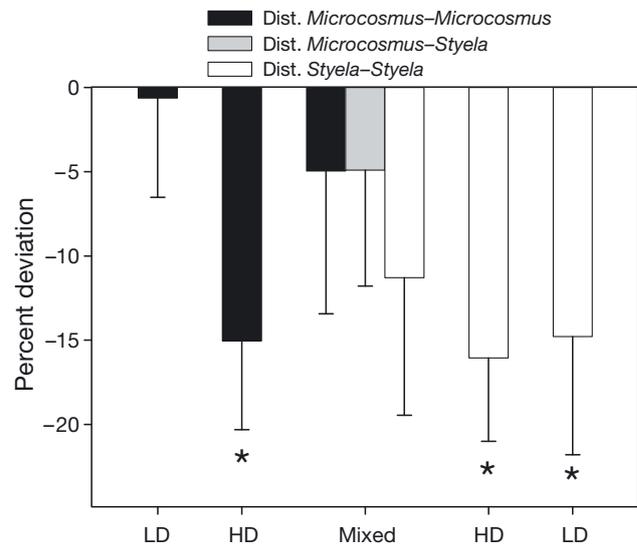


Fig. 4. Mean (\pm SE) percent deviations [(observed – expected)/expected \times 100] of the shortest distances between ascidian settlers in the larval interaction experiment. Expected values obtained through simulation. Asterisks: significant departures from zero (i.e. non-random distribution)

Ascidian–mussel interactions

Some of the 25 initial mussels attached separately to the Petri dish using their byssal threads, but most showed gregarious behaviour and tended to group in clumps. *Perna perna* had a higher tendency to form clumps and, as a result, formed fewer aggregates with higher numbers of individuals in each aggregate. On average, *P. perna* formed 7.06 ± 0.40 (mean \pm SE) aggregates per Petri dish, while *Mytilus galloprovincialis* formed 8.54 ± 0.30 aggregates, the difference being significant (t -test: $t = 2.82$; $df = 97$; $p = 0.006$).

There was strong larval consumption by mussels in all trials (Fig. 5). When we compared consumption by *Mytilus galloprovincialis* between South Africa and Spain, the factor region was significant (Table 4A), with consumption being less marked in Spain than in South Africa. The overall consumption percentage, pooling both ascidians in the mussel's native Mediterranean, was smaller than in its introduced range of South Africa (mean % \pm SE, 73.75 ± 1.52 and 91.25 ± 1.52 , respectively). A significant species effect (Table 4A) reflected the higher consumption of *Styela plicata* larvae (Fig. 5). The differences were coherent across geographical regions (non-significant interaction term).

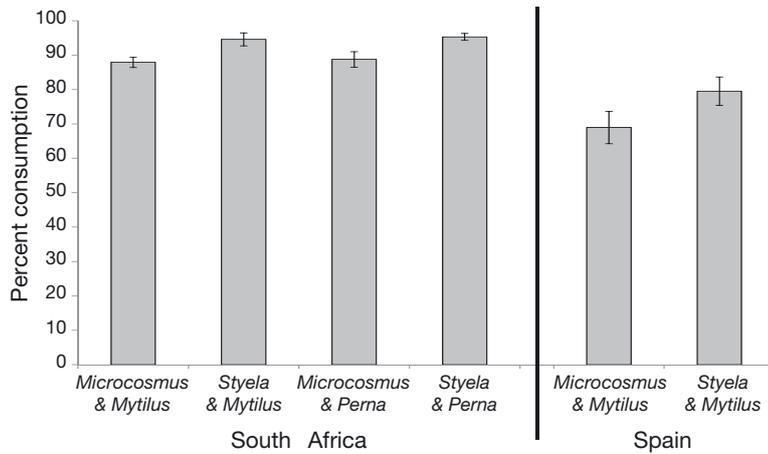


Fig. 5. Mean (\pm SE) consumption of ascidian larvae by mussels in South Africa and Spain in mixed treatments (ANOVA results in Table 4)

In South Africa, the 2 mussel species consumed larvae similarly (non-significant species effect), but the response of the 2 ascidians was different (Table 4B). The overall consumption percentage (both mussel species pooled) was lower for *Microcosmus squamiger* (mean % \pm SE, 88.40 \pm 0.01) than for *Styela plicata* (95.00 \pm 0.01). The interaction between ascidian species and mussel species was non-significant (Table 4B).

Non-consumed larvae showed significantly higher settlement success in the presence of mussels in both geographic regions and for both mussel species (Fig. 6). The comparison between Spain and South Africa showed significant effects of the main factors: region (higher settlement in the Mediterranean), ascidian species (higher settlement in *Styela plicata*) and treatment (higher settlement of surviving larvae

in the presence of mussels). However, the Ascidian \times Treatment interaction was significant, as was the 3-way interaction (Table 5A), which could hinder interpretation of the main effects. Comparisons at fixed levels of region showed that the Ascidian \times Treatment interaction was significant in South Africa but not in Spain, and examination of plots of effects and post-hoc SNK tests indicated that this significant interaction was due to a more marked response for *S. plicata* than *Microcosmus squamiger* to the presence of mussels in South Africa. The interactions were due, therefore, to a difference in the magnitude, not in the direction of the response (an overall increase in settlement success

of both ascidian species when in the presence of mussels, with the effect being stronger for *S. plicata*).

In the comparison of settlement percentage between the controls and the 2 mussel species, *Mytilus galloprovincialis* and *Perna perna*, in South Africa, the factors ascidian species and treatment were highly significant (Table 5B), while no significant interaction was found. Settlement success was higher for *Styela plicata* than *Microcosmus squamiger*, and a SNK test on treatment effects revealed no differences associated to the 2 mussel species, with both mussel treatments featuring significantly higher settlement than the controls (Fig. 6).

For *Microcosmus squamiger*, the spatial distribution of the ascidians and mussels in the experimental plates showed that larvae significantly avoided settling close to mussels or mussel clumps in Spain (Fig. 7), while the pattern of distribution of settlers relative to mussels was not different from random in South Africa for either mussel species. The ascidian settlers themselves tended to adopt a regular distribution when mussels were present (significant only in South Africa). The mussels (individuals or clumps) showed in all cases a significant tendency towards avoiding other clumps.

In the case of *Styela plicata* (Fig. 7), again an avoidance reaction was detected in the presence of native *Mytilus galloprovincialis* in Spain, while in South Africa the distribution of settlers was not different from random with respect to mussels. In this case, the ascidian settlers showed no significant spatial pattern, with mean positive deviations of ex-

Table 4. ANOVA results for consumption percentage of the ascidian–mussel interactions experiment. (A) Testing differences in the effect of *Mytilus galloprovincialis* on both ascidian species (Ascidian factor, *Microcosmus squamiger* and *Styela plicata*) in the 2 different geographical regions (Region factor, South Africa and Spain). (B) Testing differences in the effect of *Perna perna* and *M. galloprovincialis* (Mussel factor) on both ascidians (Ascidian) in South Africa

	df	SS	MS	F	p
(A) Comparison between regions					
Ascidian	1	358.86	358.86	6.04	0.020
Region	1	1043.25	1043.25	17.55	<0.001
Ascidian \times Region	1	18.01	18.01	0.3	0.586
Residual	30	1783.35	59.44		
(B) Comparison between mussel species					
Ascidian	1	613.44	613.44	13.37	0.001
Mussel	1	42.15	42.15	0.92	0.347
Ascidian \times Mussel	1	0.29	0.29	0.01	0.936
Residual	24	1100.77	45.86		

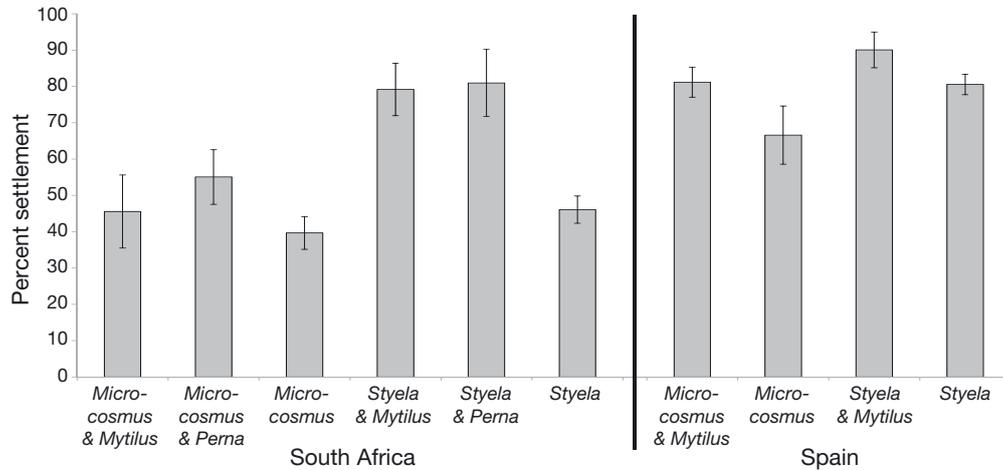


Fig. 6. Mean (\pm SE) ascidian larval settlement success (%) for the experiment testing ascidian–mussel interactions in mixed and control Petri dishes. Note that values were obtained by considering only non-consumed larvae (ANOVA results in Table 5)

pected distances in Spain, and negative in South Africa, albeit with large standard errors. The mussels and mussel aggregates showed the same significant pattern of avoidance as before.

DISCUSSION

We found interactions ranging from subtle to very strong, including both direct and indirect effects. The studied ascidian species interacted in quite mild ways. There was a strong difference in fertilization success between the 2 species, but no effect of either conspecific sperm concentration or the presence of heterospecific sperm. The same was true for larval

interactions; settlement success differed between species with no effects of the presence of heterospecific larvae on settlement success, though this did alter spatial patterns of settlement. On the other hand, the effects of mussels on ascidians were much more dramatic. Cryptic predation of ascidian larvae by juvenile mussels was observed, with similar high levels of larviphagy by the 2 studied mussel species. However, when we compared consumption by *Mytilus galloprovincialis*, we found greater values within its introduced range than in its home region. Thus the presence of mussels stimulated 2 indirect responses in ascidians: (1) larval settlement was higher and (2) larvae avoided settling near their predators. Taken together, the results indicate differ-

Table 5. ANOVA results for settlement percentage of the ascidian–mussel interactions experiment. (A) Testing differences in the effect of *Mytilus galloprovincialis* on both ascidian species in a 3-way ANOVA: Ascidian factor, *Microcosmus squamiger* and *Styela plicata*; Region factor, South Africa and Spain; Treatment factor, control and mixed (with *M. galloprovincialis* only). (B) Testing differences in the effect of both mussel species on both ascidians in South Africa in a 2-way ANOVA: Ascidian factor, as above; Treatment factor: control (without mussels), mixed with *M. galloprovincialis*, mixed with *Perna perna*

	df	SS	MS	F	p
(A) Comparison between regions					
Ascidian	1	1294.88	1294.88	7.03	0.010
Region	1	5946.21	5946.21	32.29	<0.001
Treatment	1	2054.88	2054.88	11.16	0.001
Ascidian \times Treatment	1	778.75	778.75	4.23	0.044
Ascidian \times Region	1	0.24	0.24	0.00	0.971
Treatment \times Region	1	24.24	24.24	0.13	0.718
Ascidian \times Treatment \times Region	1	854.79	854.79	4.64	0.036
Residual	56	10313.25	184.16		
(B) Comparison between mussel species					
Ascidian	1	7515.59	7515.59	19.65	<0.001
Treatment	2	12114.59	6057.30	15.83	<0.001
Ascidian \times Treatment	2	587.37	293.69	0.77	0.468
Residual	78	29839.36	383.56		

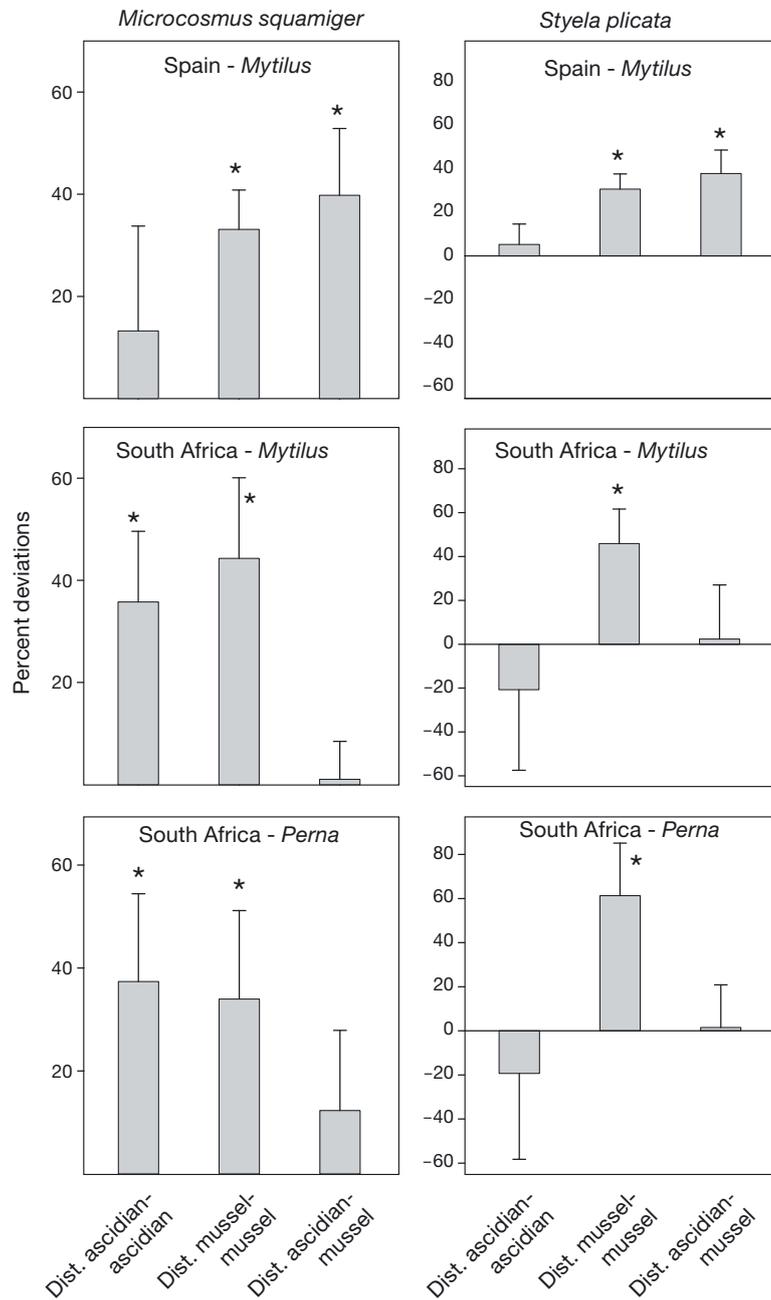


Fig. 7. Mean (\pm SE) percent deviations [(observed – expected)/expected \times 100] of the shortest distances between ascidian settlers, between mussel juveniles (or aggregates), and between ascidians and mussels for the mixed ascidian–mussel experimental plates (in Spain and in South Africa). Expected values obtained through simulation. Asterisks: significant departures from zero (i.e. non-random distribution)

ent interactions among introduced and native species at early life-history stages, which can combine to produce both direct and indirect effects that can fundamentally influence invasion success and community assembly.

In our experiments, *Styela plicata* had higher success in fertilization, settlement and metamorphosis than *Microcosmus squamiger*, and the only interspecies interaction found at the early-life history stages tested concerned spatial patterns at settlement. There was no effect of heterospecific sperm on the fertilization process, nor any density-dependent effect of homologous sperm at the concentrations assayed. In ascidians, besides intraspecific sperm competition (Yund 1998), interspecies sperm competition can occur (Lambert 2000). Even though fertilization is species-specific in most ascidians, in solitary species heterologous sperm can trigger mechanisms that block polyspermy, hindering subsequent fertilization by homologous sperm (Lambert 2001). Follicle cells enveloping the eggs are involved in the fertilization process and in the establishment of this primary block to polyspermy in response to conspecific and heterospecific sperm (Lambert 2009). This process did not seem to act in the present work. In previous experiments with the same species used here, Rius et al. (2009a) first exposed eggs of *M. squamiger* in its native range (Australia) to the sperm of introduced *S. plicata*, then the eggs were washed, and finally exposed to conspecific sperm. That study found no effects of the heterologous sperm on the fertilization process. In our study, we exposed oocytes of each ascidian species to conspecific and heterospecific sperm at the same time and at different concentrations with similar results. The species studied here are space dominant and sympatric; they are both free spawners and largely overlap in the timing of their reproduction (Yamaguchi 1975, Rius et al. 2009b, Pineda et al. 2013). Thus, it seems likely that, in nature, oocytes of the 2 species are often exposed to sperm of both species, especially as ascidian sperm is able to fertilize eggs 12 h or more after dilution (Bolton & Havenhand 1996). In this sense, lack of interaction with heterologous sperm would be beneficial for these invaders. However, even though we found no direct effect of one species on the other, the sperm environment plays an important role in

gamete phenotype and quality in at least one of the studied species (*S. plicata*; Crean & Marshall 2008), so we cannot exclude the possibility of more subtle intraspecific and interspecific effects that could not be detected with our approach.

There is evidence that the resident community can influence the larvae of marine invertebrates (e.g. Grosberg 1981, Johnson & Strathmann 1989, Osman et al. 1989, Bingham & Young 1991, Stoner 1994) including adult ascidians (Svane & Young 1989), which suggests important interactions affecting the settlement process. We found no evidence of species interference when looking at the effect of the larvae of one species on the larvae of the second species in terms of settlement or metamorphosis success. The only effect observed was on the spatial arrangement of the settlers. Larvae of both species settled gregariously (*Microcosmus squamiger* only so at the highest concentration tested), and showed more random settlement in the presence of larvae of the other species. In contrast to our results, Rius et al. (2009a) found that the settlement success of *M. squamiger* in its native Australian range was negatively affected by previously settled recruits of the introduced *Styela plicata* and vice versa. We expected some kind of interference effect as both species form dense aggregations in nature on artificial human-made substrata, so that competition for space is likely to be strong between them. We hypothesized that such competition would be detectable already at the larval stage, but in our experimental setup no effect was found when larvae of both ascidian species were placed together. Interference effects, therefore, seem to occur when a larva faces already established settlers rather than larvae of the other species. Although larval encounters are likely in the studied environments, finding already settled competitors may be a more common situation, and indeed recruitment timing can play an important role in introduction success (Stachowicz et al. 2002).

The effects of marine predators on the success of biological introductions have been extensively studied (e.g. Osman & Whitlatch 1998, Rilov et al. 2002, Epelbaum et al. 2009, Dumont et al. 2011). Such studies often concentrate on the role of small, vagile predators preying on new recruits. However, suspension-feeding organisms processing large volumes of water can potentially exert considerable predation pressure on free planktonic larvae (Porri et al. 2008), thus preventing successful recruitment (Bingham & Walters 1989, Young & Cameron 1989). Mussels are highly efficient filter feeders (Norén et al. 1999, Porri et al. 2008) and, although traditionally thought to be basically consumers of small phytoplankton, there is compelling evidence that they can also efficiently ingest mesozooplankton species (e.g. Davenport et al. 2000, Lehane & Davenport 2002, 2006) and thus have an omnivorous diet (Maloy et al. 2012). *Mytilus*

species have been suggested to be major determinants of mesozooplankton abundance through grazing (Lehane & Davenport 2006). In a study focusing on cannibalism, the 2 mussel species studied in South Africa ingested mussel larvae and settlers of up to 400 µm in length (Porri et al. 2008). Our ascidian larvae measured around 500 µm total length (Pineda et al. 2012). This fits nicely in the range of zooplankton sizes found in the stomach contents of mussels, which is generally about 400 to 600 µm (Lehane & Davenport 2002), but can reach several millimetres (Davenport et al. 2000, Lehane & Davenport 2006). These studies are biased towards detection of prey with hard parts (molluscs, crustaceans), and the rarity of soft-bodied prey may be an artefact. Indeed, ascidian larvae are the right size and, lacking appendages or cilia, are unable to avoid being trapped in the mucus on the filtering apparatus of mussels.

In our experiments, we found a marked effect of consumption by mussels of ascidian larvae with 70 to >90% of total larvae being consumed. This was despite the small size of the juvenile mussels. Consumption by adult mussels would presumably be dramatically higher. Previous experimental studies on mussel consumption of zooplankton showed consumption even at the smallest size classes tested (1.5 to 2.0 cm; Lehane & Davenport 2002). According to our results, the ability to capture larvae is already established at earlier stages of mussel development (sizes smaller than 1 cm). We can safely conclude that the effect of a well-developed mussel bed on larvae swimming by would be far stronger. Bivalves are able to select food before ingestion and reject unwanted particles as pseudofaeces (e.g. Foster-Smith 1975, Kiorboe & Mohlenberg 1981, Bougrier et al. 1997) within a few hours after ingestion (Foster-Smith 1975). We did not find production of pseudofaeces by our juvenile mussels after 48 h of larval addition, coherent with reports of zooplankton in the stomachs rather than the pseudofaeces of mussels (Davenport et al. 2000). Moreover, mussels and mussel aggregates were scrutinized for hidden larvae or settlers and none were found, so the only explanation for the disappearance of larvae is that mussel juveniles ingested them. Among non-consumed larvae, a general response was a higher settlement success, which could be due to an acceleration of the settlement process as an adaptive response to minimize risks of being captured by mussels.

The effects of *Mytilus galloprovincialis* on ascidian larvae were stronger in the introduced range of the mussel (South Africa), where consumption was

higher. The increase in settlement response of ascidians to the presence of *M. galloprovincialis* was stronger where the mussel was native than in its introduced range, which could explain in part the lower consumption. Changes in consumption rates in *M. galloprovincialis* could be due to an adaptation to the invasion of new areas (Lee 2002), but other explanations can be put forward as well. *M. galloprovincialis* comprises several genetic groups (Sanjuan et al. 1997, Daguin & Borsa 2000). In particular, the South African introduced populations are related to the European Atlantic stock, while the Mediterranean populations belong to a different genetic clade (Daguin & Borsa 2000). Thus, we have used *M. galloprovincialis* individuals from 2 genetically differentiated sources, which could explain the different behaviours observed. Alternatively, and considering that the consumption by the native *Perna perna* in South Africa was of the same level as that by *M. galloprovincialis* there, environmental factors could be driving an increased filtering activity in South Africa. Temperature changes the metabolic activity of mussels (Anestis et al. 2007). The mean annual surface water temperature for the past 30 yr in the 2 regions where we performed the experiments are significantly different (Mann-Whitney *U*-test, $T = 200.00$; $p = 0.004$) with a mean temperature (\pm SE) of $17.14 \pm 0.90^\circ\text{C}$ in the Mediterranean Sea (www.meteoestartit.cat), and $20.86 \pm 0.36^\circ\text{C}$ off the south coast of South Africa (M. Rius unpubl. data). Thus, although the laboratory temperature was the same in both experiments, adaptation to water temperature in each area could play a role in determining filtration activity of the mussels used in the present study. Assessing the ultimate causes of the observed differences in ascidian larval consumption would require further studies beyond the scope of the present work.

When comparing predatory effects between mussel species in South Africa, no differences in consumption or settlement percentages were found between native and introduced mussels. In all cases, *Styela plicata* experienced significantly greater losses through consumption by mussels than *Microcosmus squamiger*. Accordingly, *S. plicata* also had a stronger response in terms of settlement, which was higher than in *M. squamiger* in the presence of mussels. The greater change in settlement for *S. plicata* explains the significant interactions found in these analyses.

Both *Styela plicata* and *Microcosmus squamiger* avoided settling close to *Mytilus galloprovincialis* in its native area (Mediterranean Sea), as shown by ascidian–mussel distances being larger than expected. In South Africa, however, the pattern of

ascidian settlement was not different from random for either mussel species tested. The settlement of *M. squamiger* changed from aggregated (as seen in the settlement experiment) to a regular pattern in the presence of mussels (particularly in South Africa), while that of *S. plicata* changed from aggregated to random. Overall, then, there was an increase in the spacing among ascidian settlers in the presence of mussels, allowing the testing of as many different places as possible in an environment where competition for space with mussels will be predictably high. However, laboratory experiments are simplified systems and thus caution is needed when interpreting these results.

In summary, we found weak competitive interactions between the studied ascidian species and strong predatory effects of juvenile mussels on ascidian larvae. This dramatic reduction of the larval pool of ascidians suggests that propagule pressure must be intense to allow ascidians to colonize space in habitats where mussel beds are well developed, such as the fouling communities or rocky shores. We found evidence that ascidian larval behaviour shifted in the presence of predators, by increasing settlement success and avoidance of mussels. A general lack of interaction between gametes or larvae of 2 dominant introduced ascidians was found, with only slight changes in spatial distribution of settlers. As both ascidian species can form dense populations that monopolize space (Rius et al. 2009b, Pineda et al. 2013), our results suggest that interference competition occurs at later, post-metamorphic stages. We showed that co-occurring introduced species can interact intensely at early ontogenetic stages, especially in terms of predatory interference among broadcast spawners. Our results reinforce the importance of the composition of the target community in determining the susceptibility to invasion success. Whether the species comprising the target community are native or introduced seems to be less important than what manner of species they are.

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