Self-fertilization as an alternative mode of reproduction in the solitary tunicate *Pyura chilensis*

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ABSTRACT: The hermaphroditic broadcasting tunicate *Pyura chilensis* Molina, 1782 is a sessile filter-feeder organism that occurs in intertidal and subtidal habitats along the Chilean and Peruvian coast. In natural populations, *P. chilensis* form dense aggregations, small patches or occur as isolated individuals. This suggests that self-fertilization could be a potential insurance against adverse conditions for cross-fertilization. In this study, *P. chilensis* were reared in the laboratory as isolated and paired individuals, to assess occurrence and success of fertilization, settlement and metamorphosis. Occurrence of self-fertilization was also compared between specimens forced to cross-fertilize and specimens maintained in reproductive isolation for different periods. We also manipulated cross- and self-fertilization using strip-spawned gametes. Our study shows that *P. chilensis* is a hermaphroditic species with adolescent gonochorism; that is, individuals first developed male function and then later male and female functions simultaneously as specimens increased in size (protandrous hermaphroditism). The results also show more frequent fertilization in paired specimens and in manipulated fertilization involving cross sperm. Moreover, no perceptible differences in fertilization, settlement, and metamorphosis success among self and outcross progeny were found. Prolonged periods of reproductive isolation resulted in more frequent self-fertilization. Occurrence of selfing and highly successful settlement and metamorphosis of progeny originating from reproductively-isolated specimens suggest that even though outcrossing predominates, selfing is an advantageous alternative when sources of allosperm are scarce.

KEY WORDS: Hermaphroditism · Broadcast spawner · Self-fertilization · Settlement · Metamorphosis · Inbreeding · Reproductive success

INTRODUCTION

One of the primary goals of benthic ecology has been the determination of the factors limiting populations. Missing from such analyses are the processes that directly or indirectly affect the number of larvae that settle and metamorphose, such as reproductive or mating strategies, gamete characteristics and fertilization success. In sessile marine benthic invertebrates, the encounter between mating partners may be limited when broadcast gametes are exposed to wastage (Yund 1990, 2000, Yund & McCartney 1994). In broad-cast spawning species in which sperm and eggs are released into the seawater, the rates of fertilization commonly vary between 0 and 100% (Levitan 1995, Pemberton et al. 2003). Since fertilization success and mating distance in these species depend on the chance of intersection between sperm and eggs, the species should spawn synchronously to assure fertilization and to avoid the loss of sperm through dilution (e.g. Levitan & Petersen 1995, Watson et al. 2003, Soong et al. 2005). Outcross mating has been regarded as the predominant mode of successful fertilization for many simultaneous hermaphroditic animals (Maynard Smith 1978,

Ghiselin 1987). Selfing, however, is another potential means of assuring fertilization in broadcast spawners. Generally, contact between gametes originating from the same individual has been associated with fertilization failure or impaired post-fertilization performance. Inbreeding depression, the main deleterious effect of self-fertilization, may appear at different stages of the life cycle in the form of sperm inactivation (Kawamura et al. 1987, Dorken & Husband 1999), larval abnormality (Beaumont & Budd 1983, McCune et al. 2002, 2004) or small brood size (Hunter & Hughes 1993, Kranz et al. 2002). In contrast to the traditional perspective, theoretical predictions and available evidence in sessile organisms, such as plants and invertebrates, show that inbreeding depression is not ubiquitous (Shields 1993, Cheptou et al. 2000, Culley 2000).

Simultaneous hermaphroditism is common in several invertebrate groups (reviewed by Ghiselin 1969, Michiels 1998), including ascidians (Sahade et al. 2004), bryozoans (Hughes et al. 2002a), crustaceans (Bauer 2000), polychaete worms (Sella et al. 1997, Watson et al. 2003), and gastropods (Greeff & Michiels 1999, Winkler & Estévez 2003) among others. This substantial temporal overlap between male and female functions provides the potential for self-fertilization. However, the extent to which selfing actually occurs may reflect the balance of costs and benefits of self-fertilization in a particular ecological setting. Many organisms partition reproductive output into male and female components. Simultaneous or synchronous and sequential or successive hermaphrodites may vary resource allocation to male and female gamete production (Charnov 1982, Hughes et al. 2002b, 2003, Guitian et al. 2004). Therefore, simultaneous hermaphrodites offer a unique opportunity to directly assess optimal investment of resources into male and female reproduction.

Worldwide, there are few species of ascidians that are fished commercially, but in Chile, *Pyura chilensis* supports an important small-scale fishery activity (Davis 1995, SERNAP 2002). This solitary ascidian occurs from the low intertidal down to the subtidal habitat along the Chilean and Peruvian coast (Vásquez 1983, Davis 1995). In rocky intertidal habitats, this ascidian is found as isolated individuals or forming patches of matrices of barrel-like individuals with hard outer tunic cemented together on the substratum. However, subtidally the species forms broad, 3-dimensional matrices containing thousands of individuals. *P. chilensis* is a digonic hermaphrodite with separate male and female gonads that free-spawn externally to fertilize gametes, which leads to the development of swimming tadpole larvae (Cea 1969). Moreover, recent information suggests that brooding is also possible in *P. chilensis* (Díaz 1999). The simultaneous occurrence

of mature female and male gonads in *P. chilensis* (Cea 1969) and the presence of isolated specimens suggest that self-fertilization could be a potential method of fertilization under unfavorable conditions for cross-fertilization. The inevitable loss of sperm fertility a few hours after spawning should promote fertilization in the vicinity of broadcasting specimens. This, together with a short pelagic larval period and settlement concentrated on conspecifics (Cea 1969, P. H. Manríquez & J. C. Castilla pers. obs.) should be important aspects modulating the spatial distribution of this species. However, so far, self-fertilization in *P. chilensis* has not been investigated. Although this species is described as a simultaneous hermaphrodite, the ontogenetic manifestation of sexual maturity is unknown. Sessile hermaphroditic organisms with limited dispersal exhibit opportunities for inbreeding and are useful for studies in the evolution of reproductive systems. Since successful fertilization of eggs is a key element of maximizing fitness in animals, we aimed to investigate the size of sexual maturity and the likelihood and consequences of selfing in *P. chilensis*. In particular, we investigated whether progeny originating through self-fertilization or outcrossing differ in terms of settlement and early post metamorphosis success. Moreover, we aimed to investigate the potential for self-fertilization depending on the duration of reproductive isolation, and by using manipulated fertilization, to compare fertilization success among self- and outcross-fertilization.

MATERIALS AND METHODS

Study organism. We collected specimens of *Pyura chilensis* in rocky intertidal habitats in central Chile, which were transported to the laboratory in the Estación Costera de Investigaciones Marinas (ECIM) at Las Cruces (32° 43′ S, 71° 38′ W). Specimens were collected from the following 4 localities: Santo Domingo (33° 21′ S, 71° 21′ W), Pichilemu (33° 14′ S, 72° 01′ W), Matanzas (33° 32′ S, 71° 29′ W), and Constitución (35° 21′ S, 72° 27′ W). As a fifth population, we sampled specimens from floats of suspended scallop cultures at Isla Santa Maria (23° 24′ S, 70° 35′ W) in northern Chile. Since larger specimens are not common in intertidal habitats, we included subtidal specimens collected at Pichilemu from a depth of approximately 4 m. In the laboratory, specimens were maintained in aquaria with running seawater and fed with the microalgae *Isochrysis galbana* and *Chaetoceros calcitrans*. In general terms, the morphology of *P. chilensis* is pyriform; one surface is attached to the substrate and the opposite bears the buccal and atrial siphon. In the field, the siphons are often obscured by

the presence of epibionts, and accurate measurements of the distance between the siphons are difficult to assess. Therefore, in the field we chose to measure the maximum linear and upper diameter (hereafter MLD; Astorga et al. 2002). Each sampling included specimens from ~1 cm (minimum perceivable and handled size) to ~4 cm MLD. However, for specimens reared in the laboratory and involved in gamete spawn experiments (outlined below), we measured the distance between the centers of both siphons (hereafter siphonal distance; Astorga et al. 2002). All measurements were taken from live specimens, and since *P. chilensis* have a strong outer coat, the state of contraction was not considered during measurement. In the laboratory, animals were maintained in a through-flow seawater aquarium at ambient salinity and temperature. To avoid any potential source of unwanted sperm from different species, all visible encrusting organisms were removed from the tunic of the experimental specimens.

Size at sexual maturity. To determine the minimum size at which *Pyura chilensis* reached sexual maturity, we chose 30 specimens from natural populations from each of the 5 size classes according to MLD (see Fig. 1). We dissected the specimens, and the corresponding gonoducts were visually assigned to gonad stages proposed by Cea (1973), in which Stages III and IV designate sexual maturity. Moreover, gonadic tissues were dissected to confirm microscopically the presence of mature eggs and sperm.

Fertilization in natural spawning (isolated specimens). Sexually mature and immature *Pyura chilensis* are visually indistinguishable, and so in this experiment we used specimens corresponding to the size at which mature individuals had been found to predominate (as discussed above). To test for the capability of self-fertilization within each population, we placed 30 specimens in separate 1.8 l plastic bottles (above) and left them in reproductive isolation for 90 d. This procedure was applied to specimens from Pichilemu, Matanzas, Santo Domingo, Constitución, and Santa Maria. The water was continuously aerated, and to prevent accidental transmission of allosperm by aerosol, we placed a plastic lid on top of each bottle. All the experiments were conducted in a room maintained at 16 ± 2°C, with seawater of ambient salinity (33 PSU). Lighting was provided by a 40 W fluorescent tube operating with a 12:12 h light:dark cycle photoperiodicity. A combination of *Isochrysis galbana* and *Chaetoceros calcitrans* in its exponential growth phase (3 to 6×10^6 cells ml^{-1} for *I. galbana*; 4 to 7×10^6 cells ml^{-1} for *C. calcitrans*) was added daily as food. In the laboratory, *P. chilensis* initiate spawning once the light is activated (P. H. Manríquez pers. obs.). To assess the presence of gametes and larvae, every day between 09:00 and 11:00 h, we filtered the water from each bottle through a 140 µm mesh sieve. The filtered seawater was placed in a glass beaker and we used a subsample of 50 ml for egg counting. Then, we filled the bottles with 1.3 l of 2 d old, 1 µm UV-sterile-filtered seawater of 33 PSU (hereafter FSW). To estimate total sperm emitted, samples of 100 ml were removed and filtered through a 25 mm diameter, 0.45 µm pore-size cellulose-nitrate membrane and treated with a drop of 0.1 mg ml^{-1} suspension of Hoechst 33342 stain (bis-benzimide trihydrochloride, Sigma®) as described by Bishop (1988). We placed the membrane on a glass slide mounted with a cover slip and sealed with nail varnish. The sperm were visualized in an Eclipse E400 Nikon epifluorescence microscope and were counted along 8 transects. The number of sperm counted along transects was used to estimate the total number of sperm in the membrane area and then used to project the total number of sperm in the bottles. For a single sperm-spawning episode, we used the number of sperm recorded 24 h after the water in the rearing bottles had been changed. Using this protocol, the relationship between a spawner's size and the number of released eggs and sperm was assessed for each spawning episode.

Fertilization in natural spawning (isolated and paired specimens). In another set of experiments, we used specimens from 2 localities (Table 1), with a total of 16 specimens per locality. In the control treatment, 3 types of *Pyura chilensis* breeding pairs were used according to locality source: 4 pairs from each population, and 4 mixed pairs, with specimens from both populations. In the alternative treatment, we maintained 8 specimens from each population in sexual isolation (Table 1). We ran all the experiments for 60 d, using experimental conditions as described above. Prior to the experiment, all specimens were maintained in the laboratory under reproductive isolation for 45 d. During this period, gamete release was not monitored.

To assess fertilization success in the set of experiments described in the above paragraph, we removed 120 undamaged eggs from each rearing bottle and assigned them to glass Petri dishes with a small volume of seawater. In line with other studies on *Pyura chilensis* (Cea 1969, 1973), fertilization success was evaluated as the proportion of eggs that developed into embryos and resulted in tadpole larvae. As a criterion to assess settlement success, we used the cessation of larval swimming accompanied by attachment to the substrata, and absorption of the tail. We used circular, adult-conditioned acetate sheets placed in the bottom of flat, plastic multiwells as experimental settlement units. Metamorphosis success was evaluated as the proportion of settlers able to survive for 1 mo under laboratory conditions as described above.

Table 1. *Pyura chilensis*. Proportion of *P. chilensis* from 5 localities (P: Pichilemu; M: Matanzas; C: Constitución; SD: Santo Domingo; SM: Santa Maria) spawning sperm, eggs, and fertilized eggs under 2 contrasting reproductive scenarios (paired and isolated specimens) over a 60 d period of observation in 4 different experiments. Before the experiments, all specimens were maintained in the laboratory under reproductive isolation for 45 d. Values in parentheses represent the average spawning frequency (±SD) measured as the number of events and the number of days between successive spawning events throughout the experiments. Fertilized eggs correspond to events in which sperm and eggs were released simultaneously. In the experiment, 4 paired specimens were used for each combination of localities and 8 single specimens were used for each locality

Locality	Only sperm (events/elapsed days)	Only eggs (events/elapsed days)	Fertilized eggs (events/elapsed days)
P–P	4/4 (2.0 ± 0.0–25.0 ± 9.4)	2/4 (0.5 ± 0.6–23.0 ± 1.4)	4/4 (1.3 ± 0.5–20.6 ± 6.9)
P–M	4/4 (1.8 ± 0.5–26.4 ± 9.8)	0/4	3/4 (1.0 ± 0.6–28.3 ± 5.5)
M–M	4/4 (1.8 ± 0.5–26.3 ± 7.5)	1/4 (0.3 ± 0.5–27.0 ± 0.0)	3/4 (1.3 ± 0.6–24.8 ± 5.7)
P	8/8 (2.0 ± 0.0–21.3 ± 7.8)	2/8 (0.3 ± 0.0–24.5 ± 3.5)	2/8 (0.3 ± 0.5–29.5 ± 9.2)
M	8/8 (2.0 ± 0.5–23.8 ± 8.5)	3/8 (0.4 ± 0.5–29.3 ± 6.0)	3/8 (0.3 ± 0.5–30.0 ± 6.1)
C–C	4/4 (1.8 ± 0.5–25.9 ± 6.3)	1/4 (0.3 ± 0.5–35.0 ± 0.0)	4/4 (1.0 ± 0.0–35.5 ± 5.0)
C–P	4/4 (2.0 ± 0.0–24.1 ± 7.3)	0/4	2/4 (0.7 ± 0.6–29.0 ± 4.2)
P–P	4/4 (2.0 ± 0.0–22.9 ± 7.5)	0/4	3/4 (1.0 ± 0.0–34.0 ± 7.5)
P	8/8 (2.1 ± 0.4–22.9 ± 6.6)	2/8 (0.0 ± 0.5–31.0 ± 2.8)	3/8 (0.3 ± 0.5–32.7 ± 8.1)
C	8/8 (1.9 ± 0.4–22.4 ± 9.5)	2/8 (0.3 ± 0.5–34.4 ± 2.1)	1/8 (0.1 ± 0.4–32.0 ± 0.0)
SD–SD	4/4 (1.5 ± 0.6–21.8 ± 5.9)	2/4 (0.5 ± 0.6–32.0 ± 2.8)	4/4 (1.0 ± 0.0–33.0 ± 6.3)
SD–P	4/4 (2.0 ± 0.6–21.0 ± 10.4)	3/4 (0.8 ± 0.5–32.7 ± 8.0)	3/4 (1.0 ± 0.0–34.3 ± 7.0)
P–P	4/4 (2.0 ± 0.8–21.3 ± 7.5)	0/4	3/4 (1.0 ± 0.0–31.0 ± 6.2)
P	8/8 (2.1 ± 0.4–18.5 ± 10.1)	0/8	0/8
SD	8/8 (1.5 ± 0.5–27.1 ± 5.4)	1/8 (0.1 ± 0.4–41.0 ± 0.0)	2/8 (0.3 ± 0.5–28.5 ± 6.4)
SM–SM	4/4 (2.0 ± 0.0–20.6 ± 9.7)	3/4 (0.5 ± 0.6–33.0 ± 7.0)	2/4 (0.5 ± 0.6–28.5 ± 2.1)
SM–P	4/4 (2.3 ± 0.5–17.3 ± 6.7)	1/4 (0.3 ± 0.5–32.0 ± 0.0)	4/4 (1.0 ± 0.0–31.8 ± 6.6)
P–P	4/4 (2.5 ± 0.6–16.3 ± 11.3)	1/4 (0.3 ± 0.5–27.0 ± 0.0)	2/4 (0.7 ± 0.6–33.5 ± 0.7)
P	8/8 (2.1 ± 0.6–18.2 ± 10.7)	1/8 (0.1 ± 0.4–34.0 ± 0.0)	1/8 (0.1 ± 0.4–40.0 ± 0.0)
SM	8/8 (2.3 ± 0.5–19.6 ± 12.0)	3/8 (0.4 ± 0.5–27.0 ± 4.6)	2/8 (0.3 ± 0.5–34.0 ± 1.4)

Extended reproductive isolation and self-fertilization. In a different experiment, we investigated self-fertilization ability as a function of the duration of reproductive isolation. All specimens were collected from Pichilemu and moved to the lab where they were maintained in a mass culture with running seawater in which cross-fertilization was potentially favored. From this mass culture, groups of 5 sexually mature specimens were assigned to sexual isolation for different periods as follows: 0, 1, 2, 4, 8, 12 and 16 mo (isolated culture). During these periods of reproductive isolation, production of progeny was not evaluated. The number of settlers generated over the 45 d following the corresponding periods of reproductive isolation was compared with 5 control specimens removed at similar periods from the mass culture of *Pyura chilensis* (non-isolated culture) and assigned to reproductive isolation in similar rearing conditions. New settlers in the rearing bottles were counted daily, and to avoid recounting, their positions in the bottles were marked with the aid of a waterproof pen. During the 45 d period of the experiment, the specimens from the isolated and non-isolated treatments were reared in reproductive isolation and in conditions similar to those described in the previous experiments. However, unlike in the previous experiments, in this section, gamete release was not monitored.

Manipulated fertilization. We manipulated self-fertilization using gametes removed from 12 specimens in each of whose gonoducts we had detected simultaneously late-developing eggs and sperm. We used eggs mixed with sperm removed from a different father as a control for cross-fertilization. We obtained sperm and eggs by dissecting the outer tunic and tissues to expose the gonoducts. Eggs from each individual were divided into 3 batches of 100 per Petri dish. Each batch was exposed to selfsperm and allosperm at a concentration not limiting for fertilization (ranging from 10^6 to 10^7 sperm ml^{-1}; authors' unpubl. data). We exposed the third batch of eggs to a mix of similar concentrations of self- and allosperm. We incubated the solutions at $14 \pm 2°C$ for 1 h, after which time the excess of sperm solution was rinsed off with FSW. Then we transferred the eggs to FSW to assess fertilization, settlement, and metamorphosis success as described above.

RESULTS

Morphometric measurements

Siphonal distance proved to be an accurate estimator of *Pyura chilensis* size, as demonstrated by the signifi-

cant relationships found among siphonal distance (*S*) and the maximum linear and upper diameter (MLD) (MLD = 2.024*S* + 0.3285, r^2 = 0.87, p < 0.01).

Size at sexual maturity

During the months in which *Pyura chilensis* were sampled, individuals with sexually mature gonads were recorded in all sampled localities (Fig. 1).

However, specimens with only the developed male function were recorded solely in specimens with an MLD of <1 cm (Fig. 1), corresponding to a siphonal distance of 0.3 to 0.5 cm. Individuals showing sperm and mature oocytes simultaneously were only found in specimens with an MLD >1 cm (Fig. 1), indicating that larger *P. chilensis* are simultaneous hermaphrodites. Over the entire sampling period, brooded larvae were never recorded from dissections. Larger specimens liberated more gametes than smaller spec-
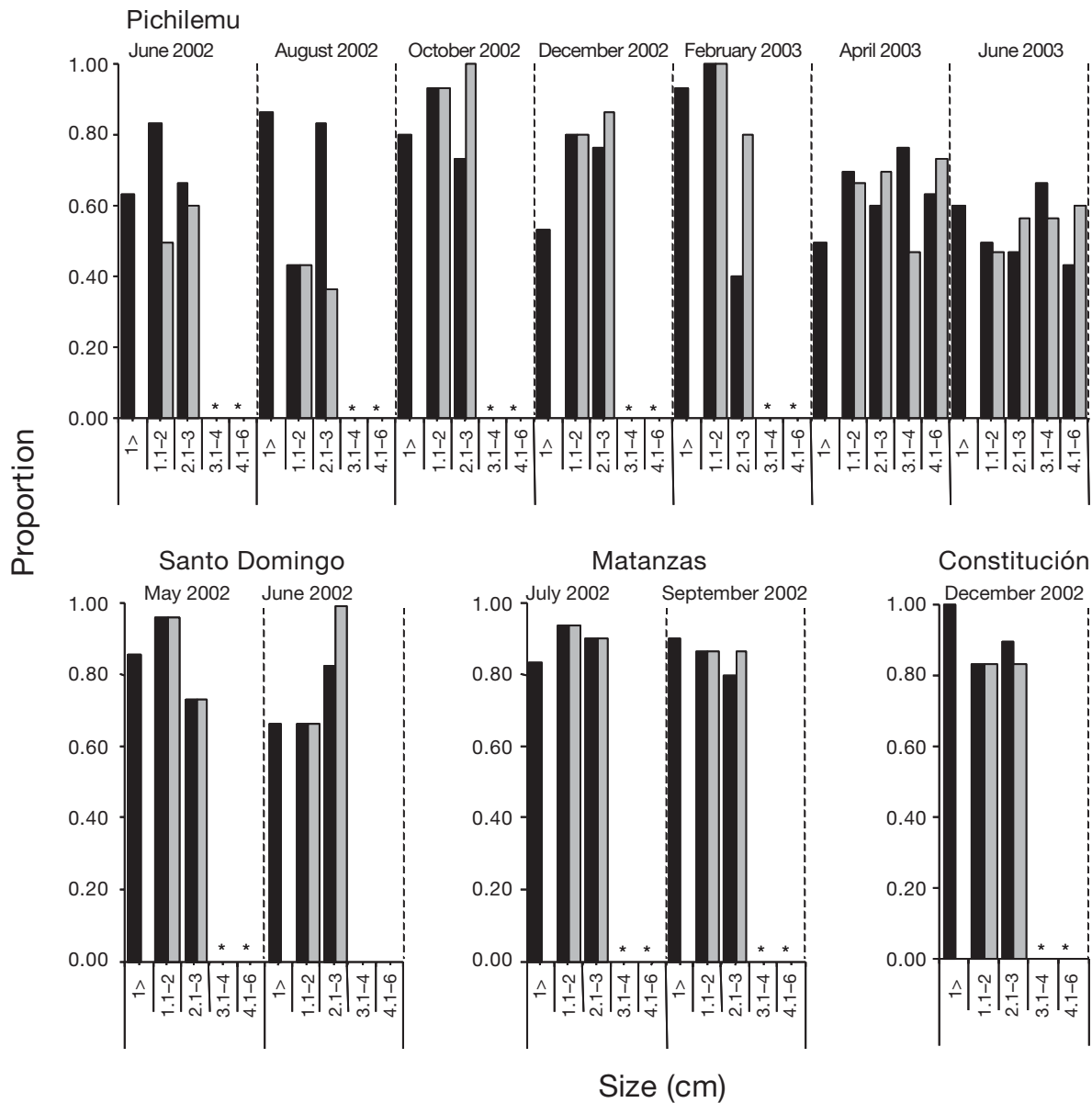
Fig. 1. *Pyura chilensis*. Relationships between the size of *P. chilensis* and the proportion of sexually mature specimens (n = 30 specimens per class size) from 4 distinct localities. Sexually mature specimens were considered specimens in gonadic Stages III and IV (Cea 1973), denoting availability of gametes capable of fertilization. Specimens were assigned to 5 class sizes according to the maximum linear dimension for the dorsal-ventral axis: (1) <1 cm, (2) 1–2 cm, (3) 2.1–3 cm, (4) 3.1–4 cm, and (5) 4.1–6 cm. Black bars: male gonads; grey bars: female gonads; *: absence of data

imens. Total sperm emitted by single specimens ranged from 4×10^{14} in a specimen with a siphonal distance of ~0.5 cm to between 5×10^{15} and 6×10^{17} per episode in larger specimens. On average, 1.6×10^{17} (SE = 2.2×10^{16}) sperm were released per individual per spawning episode. The high variation in sperm emitted by a single specimen of the larger specimens did not allow us to fit a single regression to predict sperm numbers from animal size. Egg numbers ranged from 5 to 600 thousand eggs per individual per spawning episode (eggs = $105.07S - 64.68$, $r^2 = 0.5$, $p < 0.01$, $n = 80$).

Fertilization in natural spawning (isolated specimens)

Independent of the sampling locality, sperm were detected in all isolated specimens throughout the entire experimental period. An average of 3 sperm-spawning events were detected over the 90 d experiment, with an average period of about 4 wk between events. Egg-spawning events in the absence of sperm were nil or rare (Table 2). Fertilized eggs were recorded in no more than 4 out of 30 specimens. In these specimens, the frequency of spawning events was low, with an average period of about 3 to 4 wk between events (Table 2). Fertilization, settlement, and metamorphosis success of sexually-isolated specimens are summarized in Fig. 2. Regardless of the sampling locality, fertilization success always exceeded 0.65. One-way ANOVA on arcsine-transformed results of fertilization success showed that localities were not significantly different ($F_{3,10} = 0.69$, $p > 0.05$, Fig. 2a). Settlement success ranged from 0.7 to 0.8, and no sig-nificant differences were detected in 1-way ANOVA on arcsine-transformed results of settlement success ($F_{3,10} = 0.69$, $p > 0.05$, Fig. 2b). Metamorphosis success ranged from 0.7 to 0.95, and no significant differences were detected in 1-way ANOVA on arcsine-transformed results of metamorphosis success ($F_{3,10} = 1.21$, $p > 0.05$, Fig. 2c).

Fertilization in natural spawning (isolated and paired specimens)

Similar to the results of the previously discussed experiment, solitary sperm-spawning without con-current release of eggs was detected throughout the entire experiment involving the paired and isolated specimens (Table 1). In the 4 experiments, sperm was recorded twice, on average, in all of the bottles containing isolated and paired specimens over the 60 d period (Table 1). Solitary egg-spawning without the presence of sperm was nil or rarely detected in either the isolated or paired specimens. The frequency of egg spawning was on average about 0.1 to 0.8 events over the 60 d period, with an average temporal distance of about 3 to 5 wk between events (Table 1). With the exception of 1 group of isolated specimens from Pichilemu, fertilized eggs were recorded in all paired and isolated specimens (Table 1). However, fertilized eggs were mainly recorded in paired specimens. In isolated specimens, the production of fertilized eggs was on average 3 times less frequent than in paired specimens (Table 1). Results of fertilization, settlement, and metamorphosis success in the contrasting conditions are summarized in Figs. 3 & 4. In all of the experiments, when spontaneous fertilization took place in

Table 2. *Pyura chilensis*. Proportion of sexually mature sized *P. chilensis* (n = 30 per population) from 5 geographically distinct localities in which gamete spawning and self-fertilization (sperm and eggs released simultaneously) were detected under repro-ductive isolation during an experimental period of 90 d. Values in parentheses represent the average spawning frequency (±SD) measured as the number of spawning events and the number of days between successive spawn events. Fertilized eggs correspond to events in which sperm and eggs were released simultaneously

Locality	Only sperm release (events/elapsed days)	Only egg release (events/elapsed days)	Fertilized eggs (events/elapsed days)
Pichilemu	30/30 ($3.1 \pm 0.7 - 24.0 \pm 9.3$)	2/30 ($0.1 \pm 0.4 - 32.3 \pm 16.0$)	4/30 ($0.4 \pm 1.0 - 25.5 \pm 15.9$)
Matanzas	30/30 ($3.2 \pm 0.6 - 23.9 \pm 10.0$)	0/30	3/30 ($0.3 \pm 0.9 - 25.1 \pm 8.6$)
Santo Domingo	30/30 ($2.81 \pm 0.5 - 26.3 \pm 10.6$)	0/30	1/30 ($0.1 \pm 0.6 - 21.0 \pm 9.0$)
Constitución	30/30 ($2.5 \pm 0.6 - 29.1 \pm 13.6$)	1/30 ($0.03 \pm 0.2 - 23.0 \pm 0.0$)	4/30 ($0.4 \pm 1.0 - 26.5 \pm 10.5$)
Santa Maria	30/30 ($3.6 \pm 0.7 - 28.1 \pm 12.2$)	1/30 ($0.0 \pm 0.2 - 26.0 \pm 0.0$)	3/30 ($0.3 \pm 0.9 - 27.2 \pm 10.2$)

reproductive isolation, or in paired specimens, fertilization success always exceeded 0.75. Regardless of the locations, 1-way ANOVAs on arcsine-transformed data showed no significant differences in fertilization success among isolated and paired specimens: Pichilemu and Matanzas ($F_{3,11} = 0.28$, p > 0.05), Constitución ($F_{2,9} = 0.28$, p > 0.05), Santo Domingo
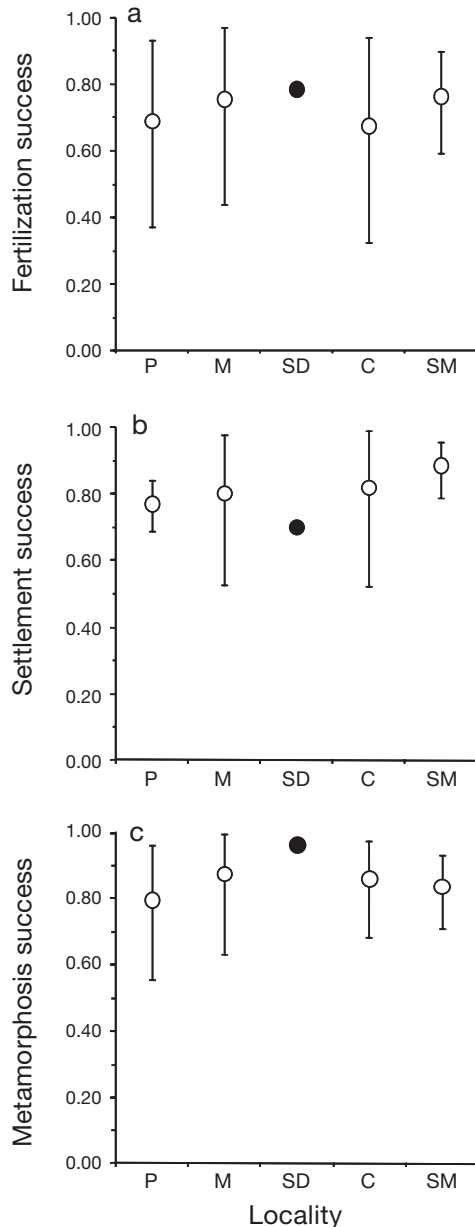

Fig. 2. *Pyura chilensis*. (a) Fertilization, (b) settlement, and (c) metamorphosis success of sexually mature *P. chilensis* collected from different localities (P: Pichilemu; M: Matanzas; C: Constitución; SD: Santo Domingo; SM: Santa Maria) held under laboratory conditions in isolated reproduction for 45 d. ●: single gamete spawn not included in the analysis. Error bars indicate ± SD of the mean

($F_{2,9} = 0.14$, p > 0.05), and Santa Maria ($F_{2,9} = 0.75$, p > 0.05). Settlement and metamorphosis success always exceeded 0.85. One-way ANOVAs of arcsine-transformed results of settlement success did not find significant differences among isolated and paired specimens: Pichilemu and Matanzas ($F_{3,11} = 0.42$, p > 0.05), Constitución ($F_{2,9} = 0.78$, p > 0.05), Santo Domingo ($F_{2,9} = 0.32$, p > 0.05), and Santa Maria ($F_{2,9} = 0.77$, p > 0.05). Similarly, 1-way ANOVAs of arcsine-transformed results of metamorphosis success found no significant differences among isolated and paired specimens: Pichilemu and Matanzas ($F_{3,11} = 1.34$, p > 0.05), Constitución ($F_{2,9} = 6.35$, p > 0.05), Santo Domingo ($F_{2,9} = 0.05$, p > 0.05), and Santa Maria ($F_{2,9} = 0.88$, p > 0.05).

Extended reproductive isolation and self-fertilization

The occurrence of the first progeny was detected in *Pyura chilensis* specimens maintained in reproductive isolation for 4 mo. From this period of 4 mo of sexual reproductive isolation to the maximum extended isolation, occurrence of progeny was always detected with an average of about 0.02 reproductive events per day (Fig. 5a). In the contrasting condition, with animals maintained under favorable cross-fertilization conditions during the pre-experimental period, progeny were only detected in specimens after 12 mo of rearing with an average of 0.005 reproductive events per day (Fig. 5a). On average, 7 to 12×10^3 settlers were produced by specimens removed from reproductive isolation (Fig. 5b), almost 4 times more settlers than in the only record of progeny in the contrasting treatment.

Manipulated fertilization

All manipulated fertilizations with allosperm produced developing embryos, from which larvae hatched successfully. However, developing embryos were only recorded in 7 of the 12 self-fertilization trials. Regardless of the sperm source, average fertilization success was always higher than 0.6 (Fig. 6a). A Kruskal-Wallis test on fertilization success found significant differences among sperm treatments (p < 0.01). Higher fertilization success was found when allosperm was used. Average settlement and metamorphosis success were close to 0.9. No significant differences were found among treatments for settlement success (ANOVA on arsine-transformed data, $F_{2,90} = 1.99$, p > 0.05, Fig 6b) or for metamorphosis success (ANOVA on arsine-transformed data, $F_{2,90} = 1.68$; p > 0.05; Fig 6c).

DISCUSSION

The presence of sexually mature specimens of *Pyura chilensis* year-round is in agreement with other studies (Cea 1973, Cancino et al. 1998). We suggest that *P. chilensis* changes from male to cosexual with a short delay between maturation of sperm and eggs. Male

function begins at sizes of S = ~0.5 cm, corresponding to specimens of about 1 cm maximum linear dimension for the dorsal-ventral axis. This change has also been reported in other ascidians (Millar 1952) and has been suggested to represent a true protandrous sex change (Policansky 1978). Cosexuality in larger *P. chilensis* illustrates that expression of both genders may represent costly resource allocation only possible in larger specimens. These results also suggest that the change from male to cosexual in *P. chilensis* represents a true sex change. The significance of this adolescent gonochorism in *P. chilensis* (sensu Ghiselin 1974) may reveal a trade-off between growth and female investment that has been described in other hermaphroditic invertebrates such as pond snails (Koene & Maat 2004) and bryozoans (Hughes et al. 2002b). Sexual maturity at sizes recorded in the present study is in agreement with similar findings in southern populations of *P. chilensis* (Davis 1995, Cancino et al. 1998). Our estimates of gamete output are in agreement with other marine invertebrates in which sperm are more numerous than eggs (Levitan 1995), which can be interpreted as an adaptation to increase the probability of sperm-egg collisions and to avoid the inevitable consequences of gamete dilution in the sea (Bishop 1988). Gamete spawning in single specimens of *P. chilensis* occurred synchronously with other specimens kept under the same laboratory conditions, always within the first hour after the laboratory lighting had been switched on. This adaptation is in agreement with gamete spawning in response to light following a dark adaptation described for solitary ascidians (Strathmann 1987). This suggests that light can induce spawning, and is possibly advantageous to avoid the loss of gametes and therefore low fertilization rates due to gamete dispersal in free spawning organisms (Levitan & Petersen 1995). Synchronous sperm release has evolved in many marine organisms (Giese & Kanatani 1987), and has been reported in both laboratory reared *P. chilensis* (Díaz 2000) and *in situ* observations of subtidal *P. chilensis* (H. Carrillo pers. comm.). Similarly, simultaneous gamete spawning has been observed in intertidal populations of *P. praeputialis* (P. H. Manríquez pers. obs.) and *P. stolonifera* (Marshall 2002). In a different way,
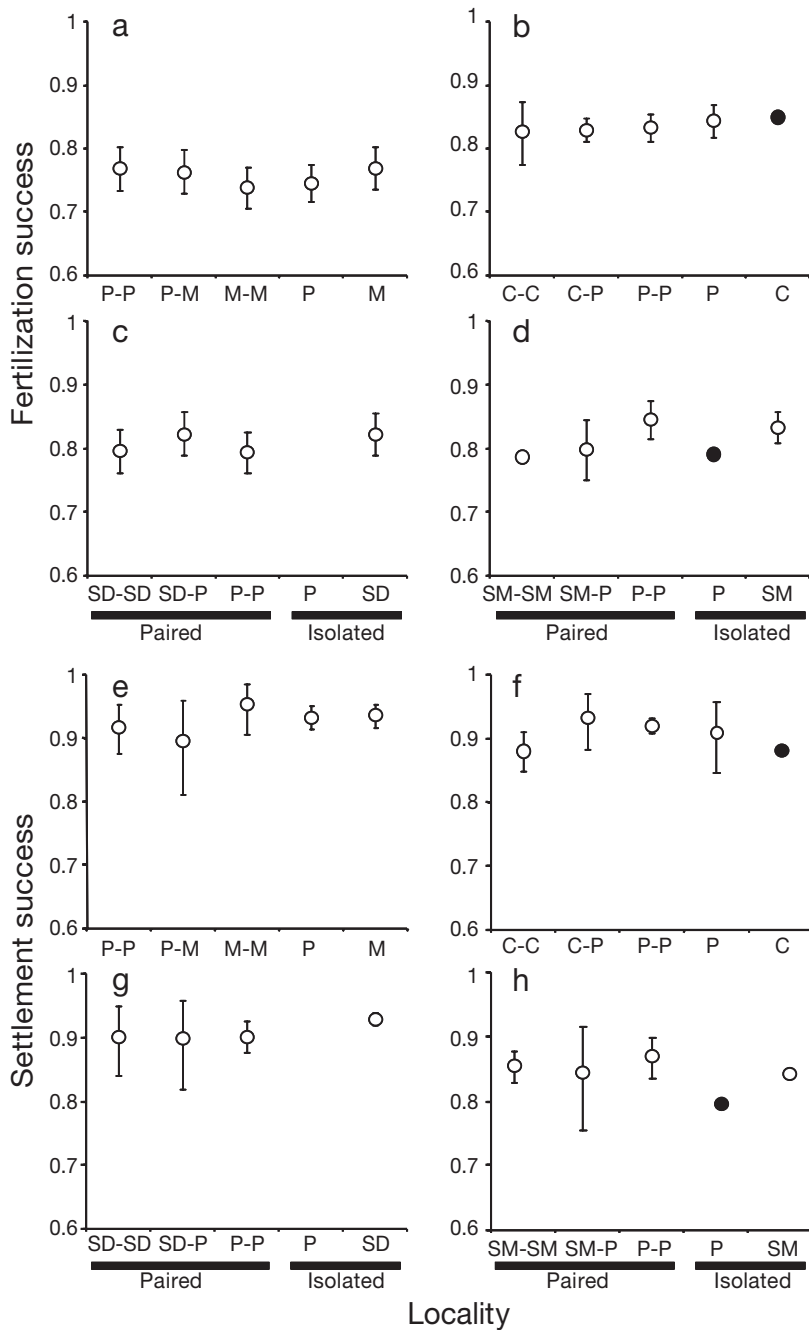

Fig. 3. *Pyura chilensis*. (a–d) Fertilization and (e–h) settlement success of sexually mature sized *P. chilensis* collected from different localities and held in 2 contrasting conditions: reproductively isolated and paired specimens. ●: single gamete spawn not included in the analysis. See Fig. 2 for more details and abbreviations of locations. Error bars indicate ± SD of the mean
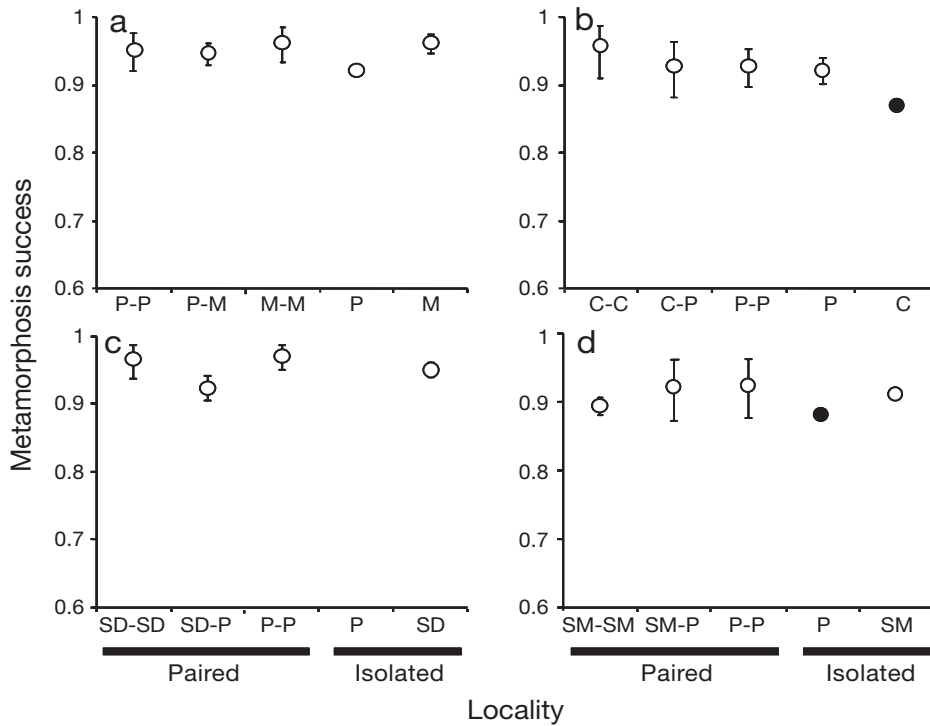
Fig. 4. *Pyura chilensis*. (a–d) Metamorphosis success of sexually mature *P. chilensis* collected from different localities and held in 2 contrasting conditions: reproductively isolated and paired specimens. ●: single gamete spawn not included in the analysis. See Fig. 2 for more details and abbreviations of locations. Error bars indicate ± SD of the mean

selfing is prevented in the compound ascidian *Botryllus schlosseri* by the asynchronous release of eggs and sperm within the colony (Sabbadin 1971). We propose that synchronous spawning in *P. chilensis* can be interpreted as a mechanism evolved to ensure outbreeding and reduce the negative effects of sperm dilution. However, the simultaneous release of sperm and eggs in the same breeding episode certainly should also

promote chances for self-fertilization in *P. chilensis*. Since spawning in *P. chilensis* can be simultaneous among individuals, there was a high chance of jeopardizing the interpretation of fertilization success in our rearing bottles because of unavoidable polyspermy. Similar fertilization success obtained throughout our semi-natural spawnings in comparison with manipulated fertilization suggests that experimental
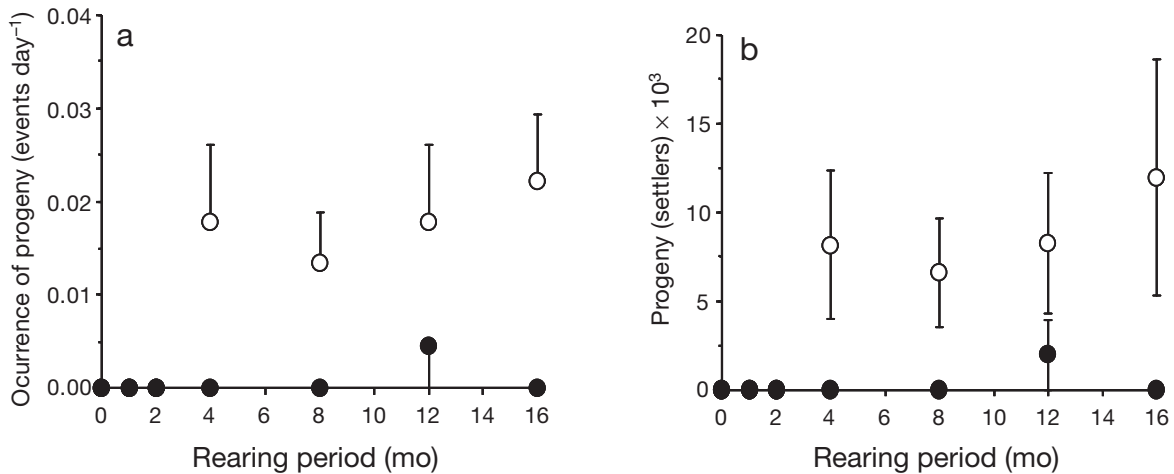

Fig. 5. *Pyura chilensis*. (a) Occurrence of progeny and (b) number of progeny generated over the 45 d period of observation after extended rearing periods under (O) conditions of reproductive isolation and (●) favorable conditions for cross-fertilization. Error bars indicate ± SD of the mean
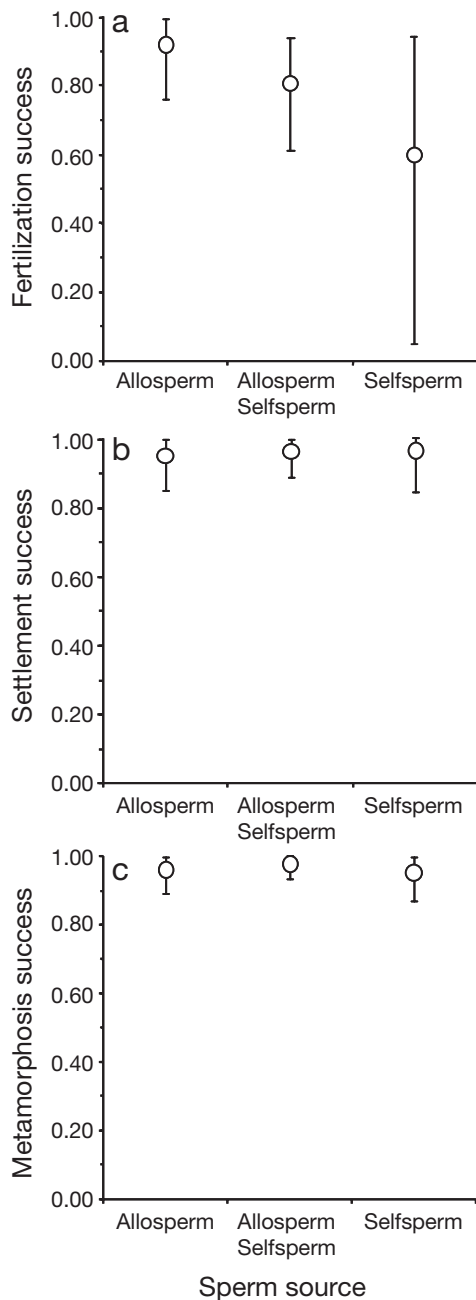
Fig. 6. *Pyura chilensis*. (a) Fertilization, (b) settlement, and (c) metamorphosis success of manipulated fertilization using self-, allosperm and a mix of self- and allosperm of *P. chilensis.* Error bars indicate ± SD of the mean

polyspermy can be ruled as a potential force generating deleterious effects on fertilization. Similarly, in our experiments, sperm concentration was always several times higher than the minimum required in order to avoid sperm limitation.

Although several species of large ascidians can self-fertilize, the practice of this phenomenon has not been well documented in the existing literature (Ryland &

Bishop 1990). We suggest that fertilization failure in *Pyura chilensis* can be attributed to fertilization blocking when gametes of the same individual come into contact, although the mechanisms involved in blocking self-fertilization are still unknown. In ascidians, the site that discriminates between self- and non-self has been found to reside in the vitelline coat of the egg (Morgan 1923, Rosati & De Santis 1978, Fuke & Numakunai 1996). In laboratory experiments, it has been observed that the solitary ascidian *Halocynthia roretzi* induces self-sterility just before spawning by a trypsin-like protease in the ovary. However, in nature the establishment of self-sterility begins about 20 h after spawning (Fuke & Numakunai 1996). Moreover, in *Ciona intestinalis* self-fertilization can be induced artificially (Kawamura et al. 1987). These results suggest that the barrier to self-fertilization in ascidians, like *P. chilensis*, is flexible. Since self-fertilization was more frequent in specimens reared in a more extended period of reproductive isolation, we suggest that the blocking of self-fertilization could be regulated by previous mating history. Given that more events of natural egg spawning followed by successful settlement and metamorphosis were recorded in our paired specimens and in our manipulated cross trials (allosperm only or mixed with selfsperm), it appears that cross-fertilization predominates in this species. The more frequent occurrence of progeny through self-fertilization in more extended periods of reproductive isolation also suggests that blocking of self-fertilization can be modulated by external signals such as previous conditions allowing cross-fertilization. The contrast between the presence of selfed progeny in the reproductively-isolated specimens in the 2 natural spawning experiments and the absence of selfed progeny in the isolated specimens after a previous experimental condition in which cross-fertilization was allowed for up to 2 mo in the laboratory, suggests an apparent inconsistency. We suggest that the failure to produce selfed progeny may be the result of eggs and sperm being released at different times. Since gamete release was not monitored in this experiment, this possibility cannot be ruled out and should be investigated in future studies. Since almost no progeny were produced under conditions favorable for cross-fertilization in the prolonged rearing experiment, it appears that extended periods of laboratory rearing had a negative effect on the ability to self- and cross-fertilize. However, given that progeny were produced in the contrasting condition of reproductive isolation, we suggest that extended periods of laboratory rearing had a negative effect on the ability to cross-fertilize.

Inbreeding has been recorded in *Corella inflata*, a solitary ascidian that mostly outcrosses and self-fertilizes when potential mates are limited (Cohen

1996). Although the absence of inbreeding depression could be misleading in an experiment conducted with 1 inbred population (Charlesworth & Charlesworth 1987), our results are validated because we also conducted outbreeding experiments. In *Pyura chilensis*, settlement concentrated on conspecifics or self-facilitation indicates that breeding with closely related specimens is possible in the field. Nevertheless, larval dispersal can permit outbreeding when levels of homozygosity are deleterious. Self-fertilization, the highest level of inbreeding, traditionally has been regarded as a viable option for simultaneously hermaphroditic sessile organisms that happen to be isolated from conspecifics. While selfing is well known in plants (e.g. Waser 1993), the relatively small amount of information available shows that the traditional assumptions may not hold true for most sessile marine invertebrates (Ryland & Bishop 1993, Bishop et al. 1996, Hughes et al. 2002a). Observations of larval release in *P. chilensis* have been interpreted as evidence of brooding (Díaz 1999), which is inconsistent with our observations. Absence of larvae within dissected *P. chilensis* discards the possibility of brooding in this species. Furthermore, in agreement with published information on ascidians (Young 1988), our observations of *P. chilensis* and *Polyzoa reticulata* tadpoles found in the gut and faecal string of *P. chilensis* suggest that cannibalism of embryos and larvae could be common in the field.

Given that a greater number of events of fertilized eggs were observed in paired specimens, we suggest that *Pyura chilensis* can reproduce through inbreeding and outbreeding according to mating opportunities. Both mating strategies are followed by high fertilization success with no evident deleterious effect on settlement and metamorphosis success. Declines in species fitness due to inbreeding have long been known (Darwin 1859, 1868, Jiménez et al. 1994). However, in agreement with the low or nonexistent cost for inbreeding recorded by other studies in solitary ascidians (Havenhand 1991), our study showed no apparent consequences of self-fertilization on early post metamorphosis performance (1 mo). However, deleterious effects of inbreeding arising late in the ontogeny cannot be disregarded. We suggest that *P. chilensis* is not a strict self-sterile ascidian in which self-fertilization would emerge in scenarios with a low chance of receiving allosperm, a characteristic of small breeding populations. However, outbreeding might be favored in large breeding populations, where allosperm is abundant. Taken together, our results highlight the importance of understanding how ecological circumstances could affect the degree to which individual fitness in natural populations of hermaphrodites is maintained through self- or cross-fertilization.

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