

Crepidula Slipper Limpets Alter Sex Change in Response to Physical Contact with Conspecifics

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Abstract. Chemical signaling, especially signaling with waterborne cues, is an important mode of communication between conspecifics of aquatic organisms. Although conspecific associations play an important role in sex allocation of sequential hermaphroditic slipper limpets, the mode of signaling is unknown. We tested the hypothesis that the effects of conspecifics on animal size and time of sex change in the tropical slipper limpet *Crepidula* cf. *marginalis* are mediated by waterborne cues. In our experiment, pairs of snails (one small and one large) were kept in cups, either together or partitioned off with fine or coarse mesh, or partitioned, but switched from side to side to allow contact with the cup mate's pedal mucus. The larger snails that were allowed contact with the smaller companions grew faster, and generally changed sex sooner, than did the larger snails in the barrier treatments, which allowed no physical contact. The smaller snails that were allowed contact with the larger cup mate delayed sex change compared to those separated from their cup mates. We were, therefore, able to reject the hypothesis that waterborne cues mediate communication between these snails. Our results suggest that the cue that affects size and time to sex change requires some kind of physical interaction that is lost when the snails are separated. Furthermore, contact with another snail's pedal mucus does not compensate for the loss of physical contact. Since males often attach to the shell of larger females, direct contact may mediate this kind of physical interaction *via* positional information, physical stimulation, or contact-based chemical communication. Whatever the cue, contact with conspecifics influences both partners, resulting in, surprisingly, a higher growth rate in the larger animal and delayed sex change in the smaller animal.

Introduction

In the vast and dilute marine environment, organisms employ diverse strategies to overcome the challenges of communication between, and recognition of, conspecifics (Hay, 2009; Webster and Weissburg, 2009). Waterborne compounds that are produced, released, and received in the sea may seem to be the most obvious way to establish interactions (Atema, 1995). Such waterborne chemical cues seem especially likely to play an important role for animals with reduced motility and poorly developed eyes, such as many sessile suspension-feeders or slow-moving benthic invertebrates. In marine molluscs, chemical cues have been shown to play a role in numerous types of intra- and interspecific interactions, including alarm responses and escape from predators in adults (Atema and Stenzler, 1977; Sleeper *et al.*, 1980; Nolen *et al.*, 1995; Jacobsen and Stabell, 2004), and in early developmental stages (Miner *et al.*, 2010); choice of site for egg deposition (Rittschof *et al.*, 2002); larval settlement and metamorphosis in response to cues from prey organisms (Lambert and Todd, 1994; Krug and Manzi, 1999; Hadfield and Paul, 2001); attraction of conspecifics; and social recognition (Croll, 1983; Boal and Marsh, 1998).

Chemical cues are particularly important in intraspecific interactions, as they facilitate mating encounters and gregarious settlement in a number of taxa (Zimmer and Butman, 2000). For instance, swimming larvae of some polychaetes preferentially settle and metamorphose when exposed to cues from conspecific adult tubes (Jensen and Morse, 1984); other species respond to cues from adult bodies, but not to tubes (Toonen and Pawlik, 1996). Settlement that is cued by conspecific adults has been documented across a variety of marine invertebrate taxa (Burke, 1986). In some species of *Crepidula*, increased larval settlement occurs in response to adult-conditioned seawater

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compared to filtered seawater controls (McGee and Targett, 1989; Zhao and Qian, 2002).

Less well documented than conspecific cues for settlement are those conspecific cues that affect reproduction. Sex allocation in both simultaneous and sequential hermaphrodites is often influenced by the presence of conspecifics (Collin *et al.*, 2005; Velando *et al.*, 2008; Schleicherová *et al.*, 2010). Sex allocation theory predicts that the optimal size to change sex in sequential hermaphrodites and the optimal allocation to male and female function in simultaneous hermaphrodites depend on the composition of an individual's mating group or local population (Charnov, 1982, and references therein). Field studies using diverse sex-changing taxa have largely borne out these predictions (Hoagland, 1978; Charnov and Anderson, 1989; Collin, 1995; Koeller *et al.*, 2000; Morrey *et al.*, 2002; Baldwin and Bauer, 2003; Schleicherová *et al.*, 2006; Avise and Mank, 2009; Hoch and Cahill, 2012). Intraspecific communication must be involved in these responses to local conspecifics, but the nature of the cues or signals involved, and the mechanisms producing these patterns of optimal sex allocation have not been closely examined in many species.

The effects of conspecifics on sex allocation can be mediated by direct behavioral interactions and by waterborne cues (Shapiro, 1983; Cole and Shapiro, 1995; Morrey *et al.*, 2002; Lorenzi *et al.*, 2006; Schleicherová *et al.*, 2006). In the benthic goby *Coryphopterus glaucofraenum*, waterborne chemicals produced by conspecific females increase the probability of sex change in visually isolated females; yet visual cues also play a role in sex change (Cole and Shapiro, 1995). Likewise, the simultaneously hermaphroditic worm *Ophryotrocha diadema* responds to increased concentration of waterborne cues from conspecifics by decreasing allocation to egg production (Schleicherová *et al.*, 2006). Despite being one of the most well-studied systems of protandrous invertebrates, the nature of the intraspecific cues involved in mediating the effect of conspecifics on sex change in *Crepidula* slipper limpets, the subject of this study, are unknown to date (Collin, 2013).

Calyptraeid gastropods (*e.g.*, slipper limpets and allies) are well-known protandrous (*i.e.*, male to female), sequential hermaphrodites (Hoagland, 1978; Collin, 2003). Within the Calyptreaeidae, species of *Crepidula* are the most well-studied animals with respect to sex change (Coe, 1938; Gould, 1952; Coe, 1953; Collin, 1995, 2013). These sedentary filter feeders vary widely in gregariousness; some species form stacks of up to 20 individuals or more, others form small stacks of two or three, and still others do not stack (Hoagland, 1978; Warner *et al.*, 1996; Collin, 2006). The duration of these stacks and the rate of movement between stacks are not known. Likewise, the population sex ratio can vary from heavily male-biased to heavily female-biased (Collin, 2006).

Group size is thought to play a role in calyptraeid sex change, as in other sex-changing animals (Collin, 1995; Warner *et al.*, 1996; Hoch and Cahill, 2012). In laboratory experiments, males of several *Crepidula* species that were raised alone changed sex earlier and at a smaller size than did similar males that were paired with a female (Coe, 1938; Hoagland, 1978; Collin *et al.*, 2005). This result is consistent with the influence of females or larger males in suppressing sex change. However, those studies were confounded by the possibility that competition for food in the paired treatments resulted in reduced growth of the smaller animals from pairs compared to those raised alone.

Calyptraeids copulate, making close physical contact necessary for successful reproduction, even in those species that do not live permanently in aggregations. Therefore, it is possible that waterborne or tactile cues, or both, play a role in mediating the influence of conspecifics on sex change. We tested the hypothesis that waterborne cues alone induce responses similar to those that are induced by the physical presence of conspecifics. Specifically, we aimed to determine if growth rate, size at sex change, and timing of sex change in *Crepidula cf. marginalis* is influenced by waterborne chemical cues, chemical cues in benthic mucus trails, or by direct contact with another individual.

Materials and Methods

Snails were collected with permission from Autoridad de Recursos Acuáticos de Panamá (ARAP), and in compliance with local laws and regulations. Small males of *Crepidula cf. marginalis* were collected by hand at Playa Chumical (8.88 N, 79.64 W), near Veracruz, on the Pacific coast of Panama. At this site, these animals are abundant under rocks that are slightly submerged in intertidal pools; they usually occur either individually attached to rocks or in pairs of one male and one female. The average size at sex change, as assessed by loss of the penis, ranges from 10.2 to 12.2 mm in field-collected and lab-reared animals (Mérot and Collin, 2012a). To ensure that neither animal of a pair had begun to change sex prior to the start of the experiment, we collected snails below this size. In the laboratory, animals were raised in pairs in 350-ml cups and maintained at 23 to 25 °C. They were fed a microalgae diet (*Isochrysis galbana*) of approximately 38×10^6 cells/d, and their water was changed three times a week (following Mérot and Collin 2012a). The snails were assigned to treatments 3 d after they were collected in June 2012 for a preliminary experiment (Experiment 1), and again, in April 2013, for a more detailed experiment (Experiment 2).

Experiment 1

To determine if the effects of conspecifics are mediated *via* waterborne cues, two treatments were applied. For each treatment, 30 small (6.3 ± 0.2 mm; mean \pm SE) males were

paired with a larger (8.6 ± 0.2 mm; mean \pm SE) male. In the Separated treatment, each pair was maintained in a plastic, food-quality cup in which a fine mesh (350- μ m Nitex mesh; Dynamic Aqua-Supply, Ltd., Surrey, BC, Canada) barrier was hot-glued across the middle. Animals in the Separated treatment were placed on opposite sides of the barrier, and each was maintained on the assigned side throughout the experiment. In the Together treatment, pairs were placed in a cup with no barrier. As no mesh or glue was used in the Together treatment, this treatment was comparable to previous tests with paired *Crepidula* (Collin *et al.*, 2005), but did not control for possible confounding effects of these materials. In this experiment, the larger male of each pair was expected to become female first (Collin *et al.*, 2005).

Experiment 2

Four different treatments each were applied to 38 replicate pairs of snails. The average sizes of the small and large animals at the start of the experiment were 5.9 ± 0.1 mm and 8.4 ± 0.1 mm, respectively. In the Separated treatment, pairs were separated by a 350- μ m Nitex mesh barrier glued across the middle of the cup, as in the Separate treatment of Experiment 1. In the Switched treatment, pairs were partitioned by the same 350- μ m mesh, but switched from side to side each week to allow each snail to have contact with the pedal mucus deposited by its partner on the cup walls. Pairs of males in the Wide treatment were kept apart by a 1-mm mesh barrier placed across the middle of the cup. This larger mesh size allowed greater water exchange and the possibility of more contact between the males than the denser 350- μ m mesh. In the Together treatment, pairs of males were kept together in a cup with hot-glued samples of the 1-mm and 350- μ m mesh affixed to the inside walls. This configuration enabled the group to serve as a control for the mesh and glue used in the other treatments (Fig. 1).

In both Experiments 1 and 2, shell length was measured weekly to the nearest 0.1 mm using calipers. The presence of a penis and/or female genital papilla was documented each week. Males of *Crepidula cf. marginalis* have a long penis behind the right tentacle, which gradually shrinks as the animal changes sex. Females have a female genital papilla (fgp) and a capsule gland on the right side of the mantle cavity, which appears at about the same time. Transitional individuals show both or neither of these characters. Previous studies with *C. cf. marginalis* have demonstrated that experimental treatments can alter the duration of the sexual transition (M erot and Collin, 2012a, b). Therefore, we took a more conservative approach than in previous studies, and considered that an individual had achieved sex change when the fgp was present and the penis was absent. After 126 d, we removed from the experiment

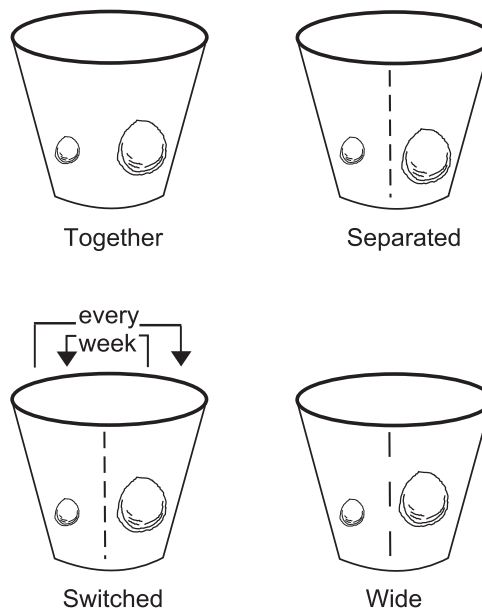


Figure 1. Experimental treatments: *Together*: a small and a large slipper snail pair in the same cup with no barrier; *Separated*: the pair is separated by a 350- μ m mesh barrier glued across the middle of the cup; *Switched*: the pair is separated with a 350- μ m mesh barrier switched from side to side every week; and *Wide*: the pair is separated by a 1-mm mesh barrier placed across the middle of the cup.

those cups in which both snails had already completed sex change.

Mortality in small snails often occurs within the first few days to weeks of being collected and placed in the treatments. Some injury can occur when the snails are removed from their rocks; and small snails, in particular, are prone to climbing out of the water and drying out. Cups in which one snail died during the first 2 wk were excluded from the experiment. Data from the cups in which one snail died after the first 2 wk were included in the analysis up to the date of the animal's death. At that point, its partner was also removed from the experiment. After the first 2 wk, mortality in Experiment 1 was 0.53% per wk over 32 wk (Separated group: 3/30 large and 9/29 small; Together group: 0/30 large and 8/29 small). After the first 2 wk, mortality in Experiment 2 was 0.34% per wk over 24 wk in the four treatments (Together: 0/38 large and 1/38 small; Separated: 1/28 large and 3/28 small; Switched: 2/29 large and 7/29 small; and Wide: 3/26 large and 3/26 small).

Statistical analysis

All statistical analyses were carried out using the R statistical package version 3.1.0 (R Development Core Team, 2012). The small and large animals of each pair were analyzed separately. For both sets of animals, a repeated measures ANOVA (RM-ANOVA), implemented as a mixed model (lmer) with likelihood ratio tests, was used to

test for significant effects of treatment, day, and the interaction between treatment and day on shell length over the course of the experiment. One-way ANOVA of shell length measurements, recorded each week, was used to identify the time at which the treatments began to show a significant effect on growth. A Kruskal-Wallis test was used when the data did not fit the assumptions of ANOVA.

To examine the time to sex change for each treatment, a Kaplan-Meier survival analysis was performed, using completed sex change as the event. Survival analysis is designed to account for individuals who drop out of the study, as well as those who do not experience the event of interest (Clark *et al.*, 2003). Those individuals that died, those whose partner had died, and those that had not changed sex by the end of the experiment were treated as right-censored. ANCOVA with Type III sum of squares for unbalanced data was used to determine the effect of treatment on size at the time of completed sex change, with time to sex change as a covariate.

Results

Experiment 1

Growth rate. Physical contact in the Separated treatment was effectively restrained. Occasionally, we observed both snails in a cup crawling onto the net and facing each other, but no animals crossed to the other side of the barrier. Moreover, none of the larger animals of each pair laid eggs after becoming female, indicating that no copulations occurred. In contrast, most females (21/30) in the Together treatment laid eggs at least once, as expected of females with unlimited access to a male. This experiment continued for 238 d, at which time all large snails had changed sex, while 4 of the remaining small snails in the Separated treatment and 11 of the remaining small snails in the Together treatment had not yet changed. It is not unusual for some small snails to suspend growth and remain male for years (R. Collin, pers. obs.).

Treatment had a significant effect on the length of the larger snail in each pair (RM-ANOVA, $\chi^2 = 6.93$, $P = 0.03$; Fig. 2a). The larger snails in the Together treatment grew significantly more than the larger snails in the Separated treatment, as reflected by the interaction between treatment and time (RM-ANOVA, $\chi^2 = 4.09$, $P = 0.04$). One-way ANOVA tests of data from each weekly measurement showed that the difference in shell lengths became significant around Day 60 (ANOVA, $F = 16.24$, $SS = 26.96$, $df = 1$, $P < 0.0002$; Fig. 2a).

There was no overall effect of the treatment on the length of the smaller snail in each pair (RM-ANOVA, $\chi^2 = 1.39$, $P = 0.49$; Fig. 2b). There was also no significant interaction between treatment and time on the length of the smaller snail ($\chi^2 = 1.55$, $P = 0.21$).

Sex change. Survival analysis indicated that the treatment had an effect on time to completion of sex change in the larger snail of each pair (Kaplan-Meier survival analysis, $\chi^2 = 12.37$, $P = 0.0004$; Fig. 3a). Large males in the Together treatment changed sex significantly more quickly than those in the Separated treatment. About 50% of the larger Together treatment males had changed sex (as assessed by the presence of the fgp) by Day 28; in contrast, it took 49 d for 50% of the larger Separated treatment males to change. Treatment also had a significant effect on time to completion of sex change for the smaller males (Kaplan-Meier survival analysis, $\chi^2 = 4.86$, $P = 0.027$). Smaller animals in the Separated treatment changed sex sooner and at smaller sizes than those in the Together treatment. More than 50% of the small snails in the Separated treatment had completed sex change by Day 140 compared to only 10% of the small snails in the Together treatment (Fig. 3b).

The relationship between size at sex change and time to sex change was complicated in the large snails by a significant interaction effect in the ANCOVA (Fig. 4a and Table 1; Engqvist, 2005). The significant interaction between treatment and time to sex change showed that, although size at sex change increased with time to sex change in both treatments, the increase was significantly greater in the Together treatment than in the Separated treatment. In the smaller snails, no significant interaction between size at sex change and time to sex change was detected, making interpretation of these results more straightforward. In the small snails, size at sex change also increased with time to sex change in both treatments, and size at sex change was significantly greater in the Together than in the Separated treatment (Fig. 4b and Table 1).

Experiment 2

Growth rate. In Experiment 2, some of the smaller snails (7 Separated, 4 Switched, and 4 Wide) were found unexpectedly on the wrong side of the partition, with the larger cup mate. In addition, one of the females of the Separated treatment produced broods, presumably indicating that copulation had occurred. Those animals and their cup mates were excluded from analysis. Experiment 2 continued for 182 d, at which time all large snails had changed sex. Of the small snails, 1 of the Separated, 0 of the Switched, 2 of the Wide, and 8 of the Together treatment had not yet changed sex.

Treatment had a significant effect on length of the larger snails (RM-ANOVA, $\chi^2 = 16.87$, $P < 0.01$), similar to what was seen in Experiment 1. The large snails of the Together treatment grew more quickly than the large snails in the other three treatments. A significant difference in growth rate was indicated by the significant interaction between treatment and time on shell length (RM-ANOVA, $\chi^2 = 9.87$, $P = 0.02$; Fig. 2c). By the 35th d, the larger

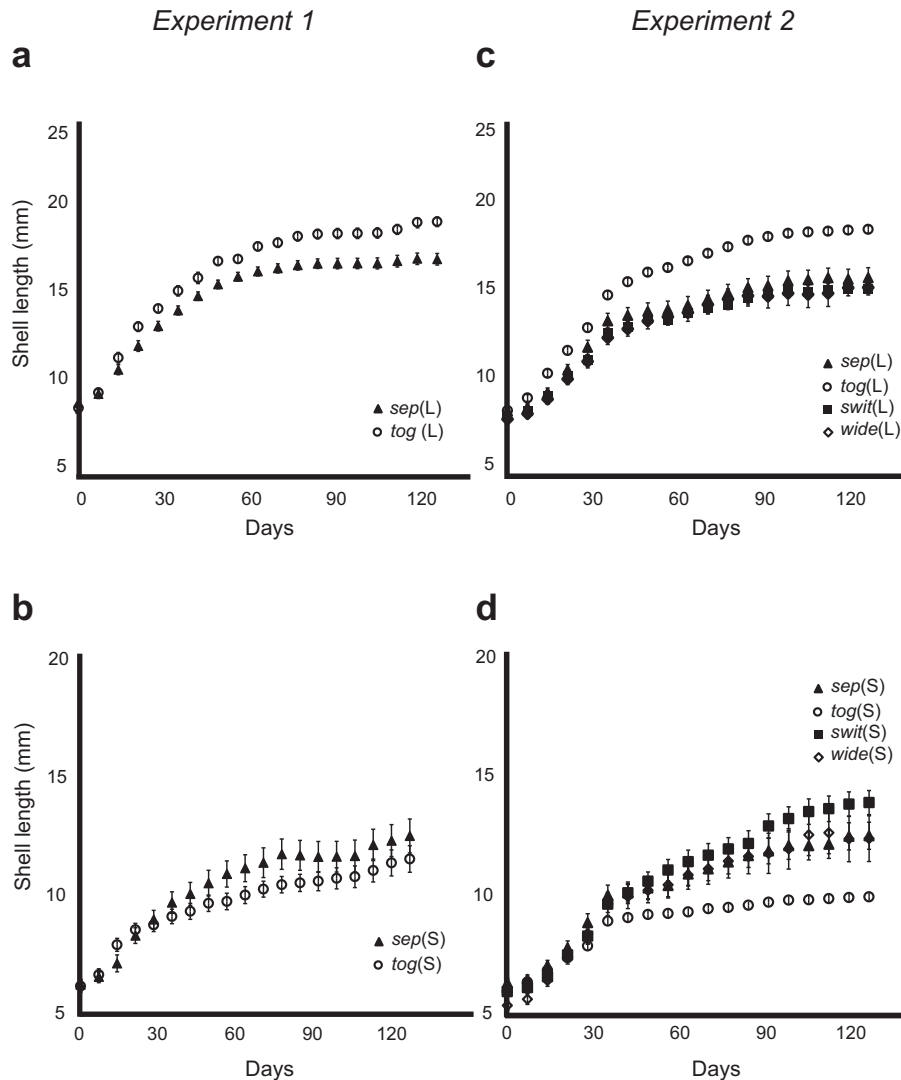


Figure 2. Growth rates in the two experiments. Growth of larger snails (a, c) and smaller snails (b, d) in *C. cf. marginalis* pairs in Experiment 1 (a, b) and in Experiment 2 (c, d). (L), larger male; (S) smaller male; sep, separated; tog, together; swit, switched; wid, wide. Bars represent standard errors.

snails in the Together treatment were significantly larger than those in the other three treatments (Together = 15.1 ± 0.23 mm, Separated = 13.6 ± 0.43 mm, Switched = 12.9 ± 0.30 mm, and Wide = 12.7 ± 0.38 mm; Kruskal-Wallis, $\chi^2 = 33.16$, $P < 0.0001$; Fig. 2c). No significant difference in size was detected among snails in the three barrier treatments.

A significant effect of treatment on overall shell length was also observed for the smaller snail of a pair (RM-ANOVA, $\chi^2 = 24.13$, $P < 0.001$). Once again, differences in growth rates were reflected by the interaction of treatment and time ($\chi^2 = 20.32$, $P < 0.001$; Fig. 2d). Animals in the Together treatment differed from the other three groups, as seen by their significantly smaller shell lengths after the 105th d (Together = 9.80 ± 0.20 mm;

Separated = 12.0 ± 0.60 mm; Switched = 13.5 ± 0.50 mm; and Wide = 12.5 ± 0.90 mm; Kruskal-Wallis, $\chi^2 = 20.4$, $P = 0.0001$; Fig. 2d).

Sex change. Treatment had a significant effect on time to sex change. More than half of the larger snails in both the Together and Switched treatments had changed sex after 35 d. Half of the Separated treatment snails changed sex after 56 d, and half of the Wide treatment snails changed sex after 63 d (Kaplan-Meier survival analysis, $\chi^2 = 19.99$; $P < 0.0002$; Fig. 3c). The effect of the barrier was also evident in the timing of sex change in the smaller snails of the three treatments; they changed sex sooner than their counterparts in the Together treatment (Kaplan-Meier survival analysis, $\chi^2 = 57.27$; $P < 0.0001$; Fig. 3d). More than 50% of the

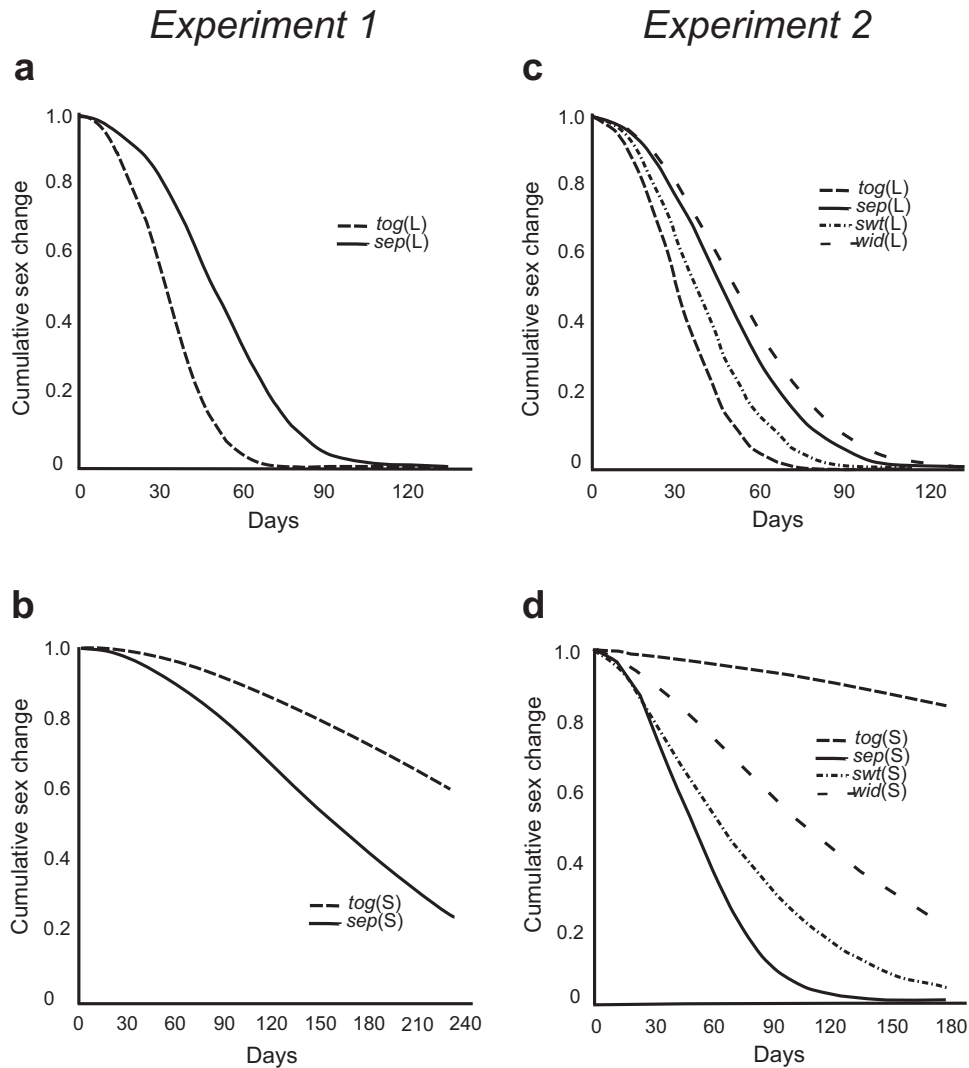


Figure 3. Time to sex change in the two experiments. Survival analysis of the percentage of large (a, c) and small (b, d) snails that changed sex in Experiment 1 (a, b) and in Experiment 2 (c, d). (L), larger male; (S), smaller male; sep, separated; swt, switched; tog, together; wid, wide. Lines represent the fit of the survival model.

small snails in the Switched, Separated, and Wide treatments had changed sex by Days 63, 49, and 98, respectively. However, fewer than 20% of the small males in the Together treatment had changed sex by Day 182, when the experiment was terminated (Fig. 3d).

There was a significant effect of the interaction between treatment and time to sex change on size at sex change in the ANCOVA of large snails, similar to that in Experiment 1. The large snails in the Together treatment had a steeper increase in size at sex change with time than in the other treatments (Fig. 4c and Table 1). For the smaller snails, the complete model showed no significant effects. However, when the non-significant interaction term was removed, a significant effect of time to sex change was detected: size at sex change decreased with the time to sex change. No

significant difference between treatments was seen in mean size at sex change. A similar decrease in size at sex change with time was also apparent for the large animals in the three barrier treatments (Fig. 4c, d).

Overall, these results were consistent. The larger snails of the Together treatment grew faster and generally changed sex sooner than did the larger snails of the barrier treatments. In the larger snails, size at sex change depended on an interaction between time to sex change and treatment. The smaller snails in the Together treatment had significantly slower growth than smaller snails of the other treatments in Experiment 2, but this finding was not seen in Experiment 1. Nevertheless, in both experiments, the small snails of the Together treatment delayed sex change compared to those snails in the barrier treatments. Those that

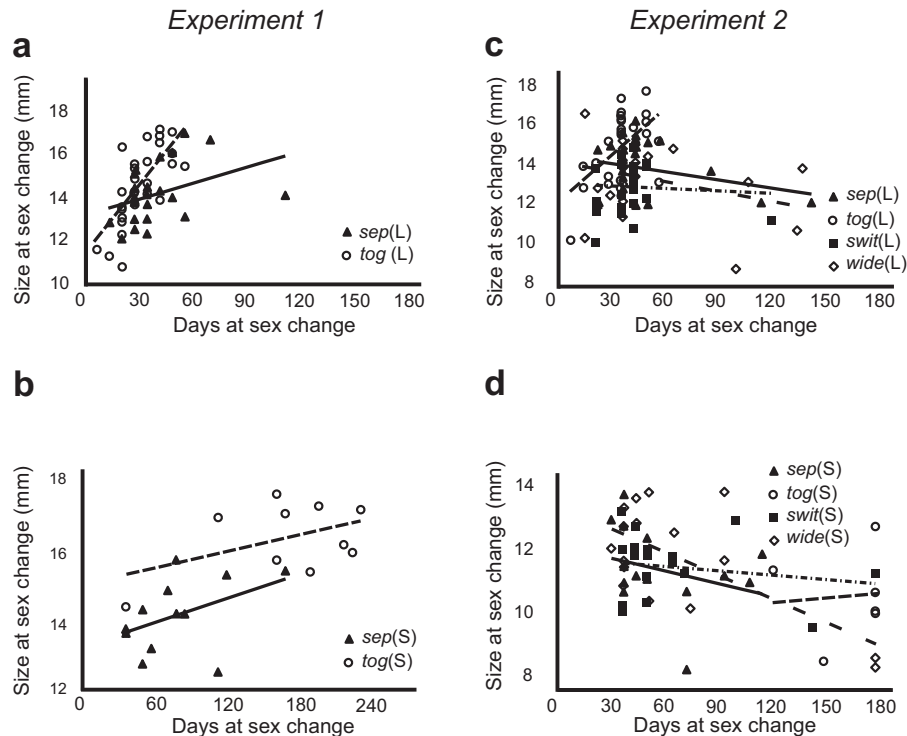


Figure 4. Relationship between size at sex change and time to sex change. A significant effect of the interaction between treatment and time to sex change was noted for large snails (L) in both Experiments 1 and 2 (a, c), but not for the small snails (S) (b, d). Solid lines, separate; dashed lines, together; dot-dash line, switched; wide-spaced dash lines, wide.

were separated by the wide mesh showed an intermediate response to snails in the Separated and Together treatments. Those snails that were switched and allowed contact with the pedal mucus of their cup mate showed little difference from those that were separated with the fine mesh barrier.

Discussion

How do large snails inhibit sex change in smaller snails?

The results of our experiments show that the associations and interactions between slipper snails have complex effects on their growth and sex allocation. Based on previous experiments with various calyptraeids, we expected that the presence of a larger animal would delay the sex change of the small animal. Our experiments were designed to determine if this inhibitory influence of the larger animal was mediated *via* waterborne cues, contact with pedal mucus, or close physical contact. In one, but not both of our experiments, when animals were allowed contact, the small animal grew more slowly. In both experiments, the smaller animals that were allowed contact delayed sex change compared to those that had no contact. Our results indicate that this delay depends on some kind of direct physical interaction between the pairs: small animals exposed to waterborne cues alone or waterborne cues and contact with pedal mucus

changed sex sooner and, in Experiment 1, at smaller sizes than those snails allowed physical contact.

An inhibitory influence of conspecifics on sex change has been shown in a variety of taxa (Koeller *et al.*, 2000; Bauer, 2002; Morrey *et al.*, 2002; Baldwin and Bauer, 2003; Baeza, 2007). In coral reef fishes, visual, behavioral, and chemical cues can all influence sex change (Ross *et al.*, 1983; Cole and Shapiro, 1995). However, the importance of visual cues and behavioral interactions has been demonstrated far more often than has the role of waterborne chemical communication. The cues by which these interactions are mediated in sex-changing marine invertebrates have not been identified. We expected that waterborne cues would play a greater role in these sedentary, non-visual snails, since waterborne cues from conspecifics mediate settlement behavior in *Crepidula* (McGee and Targett, 1989; Zhao and Qian, 2002). However, our results were surprising; they suggest that slipper limpets, like fishes, show a greater response to behavioral interactions or contact-based chemical cues than to waterborne chemical cues. In slipper limpets, it is likely that behavioral cues are tactile rather than visual, as their eyes are small and, most of the time, are covered by the shell. The biology of slipper limpets suggests two main related and non-exclusive possibilities for the kind of physical contact that could mediate the influence of conspecifics on

Table 1

Results of the analysis of covariance showing the effects of experimental treatment and time to sex change on size at sex change in males of *Crepidula cf. marginalis*

	<i>F</i>	SS	df	<i>P</i> -value ¹
Experiment 1: Large snails				
Treatment	5.15	8.03	1	0.027
Time to change	20.61	32.14	1	<0.0001
Treatment × time to change	12.35	19.26	1	<0.0001
Residuals		77.95	50	
Experiment 1: Small snails				
Treatment	6.03	4.43	1	0.024
Time to change	4.48	3.29	1	0.047
Treatment × time to change	0.16	0.12	1	0.69*
Residuals		13.95	19	
Experiment 2: Large snails				
Treatment	2.39	14.87	3	0.07
Time to change	1.69	3.51	1	0.19
Treatment × time to change	5.11	31.82	3	0.002
Residuals		224.05	108	
Experiment 2: Small snails				
Treatment	1.84	8.13	3	0.15
Time to change	1.48	2.17	1	0.23#
Treatment × time to change	1.62	7.16	3	0.19
Residuals		75.07	51	

¹ Values in bold are statistically significant ($P < 0.05$).

* Factor and covariate remain significant when the nonsignificant interaction term is removed.

Becomes significant at $P < 0.002$ when the interaction is removed from the model.

sex change: (1) proximity or position relative to conspecifics mediates the influence of same sex or opposite sex conspecifics; and (2) copulation mediates the influence of opposite-sex conspecifics. Either of these possibilities could involve tactile and/or chemical cues.

Calyptreids, particularly species of *Crepidula*, are gregarious, and are often found in clusters or stacks attached to patches of discontinuous hard substrate (e.g., shells, cobbles, bottles). In some species, such as *Crepidula fornicata*, these stacks can include as many as 20 to 30 individuals; but the majority of species occur in pairs or triplets, with one to two small males attached to the shell of a larger female (Collin, 2006). *Crepidula cf. marginalis* lives in clusters under rocks, with pairs, or, occasionally, stacks of three animals in close proximity to other pairs or singletons. The following evidence supports the idea that sex change may occur sooner in unstacked animals than in males attached to females. Logistic regression on size and sex of 15 calyptreid species shows a larger L_{50} for the loss of the penis in stacked compared to unstacked animals in 5 species (Collin, 2006). Field-collected *Crepidula cf. marginalis* show a similar nonsignificant trend, with the L_{50} for the loss of the penis occurring at a smaller size: 10.81 mm (95% CI: 8.58–11.74; $n = 70$) in singletons than in stacked snails:

12.25 mm (95% CI: 10.95–13.96; $n = 77$). It thus seems possible that the male's position (on top vs. adjacent to or near a female) influences sex change.

Proximity to a female may also influence a male's likelihood of mating successfully. Slipper limpets have internal fertilization, during which the penis reaches under the margin of the female's shell, and the thin terminal papilla is inserted into the opening at the distal end of the female genital papilla (Starr & Collin, 2013). Although copulation often occurs when a male is attached to the female's shell, this positioning is not necessary. A small male is capable of moving and stretching its penis to copulate with a female (Hoagland, 1978). *Crepidula navicella* males are capable of reaching the female from a considerable distance, without being on top of the female's shell (see fig. 1 in Henry *et al.*, 2010), as are males of *Crucibulum personatum* (R. Collin, pers. obs.). In our observations of various *Crepidula* species, copulation appears to last for several hours; video footage of copulation in *Crepidula incurva* suggests that complex copulatory courtship may occur in this species (e.g., Eberhard, 1996; Starr & Collin, unpubl. data). The details of copulation have not been observed in *Crepidula cf. marginalis* or in any calyptreid species other than *C. incurva*. If there were a reliable way to observe copulation without disturbing the animals, it would be interesting to test if chemical cues passed by physical contact with a female and/or direct physical stimulation during mating inhibited sex change in a small male, or to test if sperm transfer was necessary.

During the first experiment, we observed that animals in the (350- μ m mesh) Separated treatment sometimes faced each other through the mesh. We designed the second experiment with the idea that the larger, 1-mm mesh barrier (Wide treatment) might allow copulation without allowing stacking. However, this did not appear to be the case. No Wide treatment females produced eggs, the most definitive evidence of copulation. The Wide treatment did, however, have an effect on sex change that differed from that of the other two barrier treatments. Survival analysis showed that the large animals in the Wide treatment changed sex at a similar time to their counterparts in the fine-mesh Separated treatment; but for the small males, the Wide treatment had an effect that was intermediate to the Together and Separated treatments. This finding suggests that the greater degree of physical contact allowed by the wide mesh influences sex change, and supports the conclusion that the influence of conspecifics on sex change is mediated by direct physical contact.

Small snails induce life-history changes in larger snails

Our experimental treatments also demonstrated that interactions with a smaller companion can affect the larger snail. In both experiments, we obtained the surprising result

that the larger snails grew faster when allowed to interact with smaller males. The larger snails also changed sex sooner than those that were separated from the small animals. In sex-changing fishes, the presence of large, dominant animals inhibits sex change by smaller animals. However, the presence of smaller animals sometimes induces change in the larger animals (Ross, 1990; Lutnesky, 1994).

The effect of smaller snails on both the growth rate and earlier sex change of the larger animals is a marked shift in life-history strategy, and it makes sense in the context of sex allocation theory. Sex allocation theory predicts that individuals should change sex to optimize lifetime reproductive success (Ghiselin, 1969; Charnov, 1982). Changing sex is optimal compared to two separate sexes, because reproductive success depends on size in different ways in the two sexes, with reproductive success increasing more steeply with size for the second sex (Charnov, 1982). Thus, in protandrous animals, it is predicted that reproductive success should increase more with size in females than it does in males. This, in turn, suggests that once the process of sex change is initiated, it is advantageous for a snail to grow as large as possible before beginning to reproduce as a female. In fact, *Crepidula cf. marginalis* often show a sharp increase in growth during sex change (Collin, 2013), supporting this theory. The cause of this increased growth during sex change is not known. Increased growth during sex change could result in the observed pattern of higher early growth rates in large snails that are in contact with small snails, because these snails undergo sex change sooner than do those with lower growth rates (*i.e.*, large snails not in contact with small snails). Still, there is no explanation for the earlier sex change of these large males.

As sex change is irreversible, changing sex too early risks reducing reproductive success in several ways. In the absence of any females, it may be optimal for the largest male to change sex **only** if another, smaller male is present to fertilize it (*i.e.*, the Together treatment). Since male slipper limpets are more mobile than females (Chaparro *et al.*, 1998, 2001; Le Cam *et al.*, 2014), an isolated male may have higher reproductive success if he can move and encounter a receptive female than if he changes sex in the absence of a smaller male. Second, calyptraeids store sperm from multiple males and for long periods of time (Dupont *et al.*, 2006; Le Cam *et al.*, 2014). The presence of smaller males may indicate that potential reproductive success through male function is likely to be reduced *via* sperm competition, further increasing the benefit of early sex change when smaller males are present. Therefore, our surprising result—that contact with a small male accelerates sex change and increases growth in larger males—may be explained by assured female mating opportunities in the presence of a small male. This, in turn, results in the earlier

increased growth rate observed during the transitional phase.

These and other arguments about optimal sex allocation imply that each animal is free to make its own optimal “decisions,” and that other individuals do not control or somehow manipulate their conspecific neighbors for their own benefit (Charnov, 1982). However, a number of animals do manipulate their sexual partners (Eberhard, 1996; Koene, 2005, 2006; Arnqvist, 2006). Land snails and other invertebrates use allohormones that are transferred in the ejaculate, and sometimes in pre-copulation injections of fluids *via* love darts, to alter their mate’s reproductive investment (reviewed in Koene, 2005). Although our results can be explained in terms of optimal sex allocation theory, the possibility that sex allocation is shaped by biochemical sensory exploitation by manipulative partners cannot be ruled out.

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Literature Cited

- Arnqvist, G. 2006. Sensory exploitation and sexual conflict. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **361**: 375–386.
- Atema, J. 1995. Chemical signals in the marine environment: dispersal, detection, and temporal signal analysis. *Proc. Natl. Acad. Sci. USA* **92**: 62–66.
- Atema, J., and D. Stenzler. 1977. Alarm substance of the marine mud snail, *Nassarius obsoletus*: biological characterization and possible evolution. *J. Chem. Ecol.* **3**: 173–187.
- Avise, J. C., and J. E. Mank. 2009. Evolutionary perspectives on hermaphroditism in fishes. *Sex. Dev.* **3**: 152–163.
- Baeza, J. A. 2007. Male mating opportunities affect sex allocation in a protandric-simultaneous hermaphroditic shrimp. *Behav. Ecol. Sociobiol.* **61**: 365–370.
- Baldwin, A. P., and R. T. Bauer. 2003. Growth, survivorship, life-span, and sex change in the hermaphroditic shrimp *Lyssmata wurdemanni* (Decapoda: Caridea: Hippolytidae). *Mar. Biol.* **143**: 157–166.
- Bauer, R. T. 2002. Tests of hypotheses on the adaptive value of an extended male phase in the hermaphroditic shrimp *Lyssmata wurdemanni* (Caridea: Hippolytidae). *Biol. Bull.* **203**: 347–357.
- Boal, J. G., and S. E. Marsh. 1998. Social recognition using chemical cues in cuttlefish (*Sepia officinalis* Linnaeus, 1758). *J. Exp. Mar. Biol. Ecol.* **230**: 183–192.
- Burke, R. D. 1986. Pheromones and the gregarious settlement of marine invertebrate larvae. *Bull. Mar. Sci.* **39**: 323–331.
- Chaparro, O. R., I. Bahamondes-Rojas, A. M. Vergara, and A. A. Rivera. 1998. Histological characteristics of the foot and locomotory activity of *Crepidula dilatata* Lamarck (Gastropoda: Calyptraeidae) in relation to sex changes. *J. Exp. Mar. Biol. Ecol.* **223**: 77–91.
- Chaparro, O., S. Pereda, and I. Bahamondes-Rojas. 2001. Effects of protandric sex change on radula, pedal morphology, and mobility in

- Crepidula fecunda* (Gastropoda: Calyptraeidae). *N. Z. J. Mar. Freshw. Res.* **35**: 881–890.
- Charnov, E. L. 1982.** *The Theory of Sex Allocation*. Princeton University Press, Princeton, NJ.
- Charnov, E. L., and P. J. Anderson. 1989.** Sex change and population fluctuations in pandalid shrimp. *Am. Nat.* **134**: 824–827.
- Clark, T. G., M. J. Bradburn, S. B. Love, and D. G. Altman. 2003.** Survival analysis Part I: Basic concepts and first analyses. *Br. J. Cancer* **89**: 232–238.
- Coe, W. R. 1938.** Influence of association on the sexual phases of gastropods having protandric consecutive sexuality. *Biol. Bull.* **75**: 274–285.
- Coe, W. R. 1953.** Influences of association, isolation, and nutrition on the sexuality of snails of the genus *Crepidula*. *J. Exp. Zool.* **122**: 5–19.
- Cole, K. S., and D. Y. Shapiro. 1995.** Social facilitation and sensory mediation of adult sex change in a cryptic, benthic marine goby. *J. Exp. Mar. Biol. Ecol.* **186**: 65–75.
- Collin, R. 1995.** Sex, size, and position: a test of models predicting size at sex change in the protandrous gastropod *Crepidula fornicata*. *Am. Nat.* **146**: 815–831.
- Collin, R. 2003.** Worldwide patterns in mode of development in calyptraeid gastropods. *Mar. Ecol. Prog. Ser.* **247**: 103–122.
- Collin, R. 2006.** Sex ratio, life-history invariants, and patterns of sex change in a family of protandrous gastropods. *Evolution* **60**: 735–745.
- Collin, R. 2013.** Phylogenetic patterns and phenotypic plasticity of molluscan sexual systems. *Integr. Comp. Biol.* **53**: 723–735.
- Collin, R., M. McLellan, K. Gruber, and C. Bailey-Jourdain. 2005.** Effects of conspecific associations on size at sex change in three species of calyptraeid gastropods. *Mar. Ecol. Prog. Ser.* **293**: 89–97.
- Croll, R. P. 1983.** Gastropod chemoreception. *Biol. Rev.* **58**: 293–319.
- Dupont, L., J. Richard, Y.-M. Paulet, G. Thouzeau, and F. Viard. 2006.** Gregariousness and protandry promote reproductive insurance in the invasive gastropod *Crepidula fornicata*: evidence from assignment of larval paternity. *Mol. Ecol.* **15**: 3009–3021.
- Eberhard, W. G. 1996.** *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press, Princeton, NJ.
- Engqvist, L. 2005.** The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim. Behav.* **70**: 967–971.
- Ghiselin, M. T. 1969.** The evolution of hermaphroditism among animals. *Q. Rev. Biol.* **44**: 189–208.
- Gould, H. N. 1952.** Studies on sex in the hermaphrodite mollusk *Crepidula plana*. IV. Internal and external factors influencing growth and sex development. *J. Exp. Zool.* **119**: 93–163.
- Hadfield, M. G., and V. J. Paul. 2001.** Natural chemical cues for settlement and metamorphosis of marine-invertebrate larvae. P. 431–461 in *Marine Chemical Ecology*, J. B. McClintock, B. J. Baker, eds. CRC Press, Boca Raton, FL.
- Hay, M. E. 2009.** Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Ann. Rev. Mar. Sci.* **1**: 193–212.
- Henry, J. J., R. Collin, and K. J. Perry. 2010.** The slipper snail, *Crepidula*: an emerging lophotrochozoan model system. *Biol. Bull.* **218**: 211–229.
- Hoagland, K. E. 1978.** Protandry and the evolution of environmentally-mediated sex change: a study of the Mollusca. *Malacologia* **17**: 365–391.
- Hoch, J. M., and A. E. Cahill. 2012.** Variation in size at sex-change among natural populations of the protandrous hermaphrodite, *Crepidula fornicata* (Gastropoda, Calyptraeidae). *Mar. Biol.* **159**: 897–905.
- Jacobsen, H. P., and O. B. Stabell. 2004.** Antipredator behaviour mediated by chemical cues: the role of conspecific alarm signalling and predator labelling in the avoidance response of a marine gastropod. *Oikos* **104**: 43–50.
- Jensen, R. A., and D. E. Morse. 1984.** Intraspecific facilitation of larval recruitment: gregarious settlement of the polychaete *Phragmatopoma californica* (Fewkes). *J. Exp. Mar. Biol. Ecol.* **83**: 107–126.
- Koeller, P., R. Mohn, and M. Etter. 2000.** Density dependent sex change in northern shrimp, *Pandalus borealis*, on the Scotian Shelf. *J. Northwest Atl. Fish. Sci.* **27**: 107–118.
- Koene, J. M. 2005.** Allohormones and sensory traps: a fundamental difference between hermaphrodites and gonochorists? *Invertebr. Reprod. Dev.* **48**: 101–107.
- Koene, J. M. 2006.** Tales of two snails: sexual selection and sexual conflict in *Lymnaea stagnalis* and *Helix aspersa*. *Integr. Comp. Biol.* **46**: 419–429.
- Krug, P. J., and A. E. Manzi. 1999.** Waterborne and surface-associated carbohydrates as settlement cues for larvae of the specialist marine herbivore *Alderia modesta*. *Biol. Bull.* **197**: 94–103.
- Lambert, W. J., and C. D. Todd. 1994.** Evidence for a water-borne cue inducing metamorphosis in the dorid nudibranch mollusc *Adalaria proxima* (Gastropoda: Nudibranchia). *Mar. Biol.* **120**: 265–271.
- Le Cam, S., F. Riquet, J. A. Pechenik, and F. Viard. 2014.** Paternity and gregariousness in the sex-changing sessile marine gastropod *Crepidula convexa*: comparison with other protandrous *Crepidula* species. *J. Hered.* **105**: 397–406.
- Lorenzi, V., R. L. Earley, and M. S. Grober. 2006.** Preventing behavioural interactions with a male facilitates sex change in female bluebanded gobies, *Lythrypnus dalli*. *Behav. Ecol. Sociobiol.* **59**: 715–722.
- Lutnesky, M. M. F. 1994.** Density-dependent protogynous sex change in territorial-harem fishes: models and evidence. *Behav. Ecol.* **5**: 375–383.
- McGee, B. L., and N. M. Targett. 1989.** Larval habitat selection in *Crepidula* (L.) and its effect on adult distribution patterns. *J. Exp. Mar. Biol. Ecol.* **131**: 195–214.
- Mérot, C., and R. Collin. 2012a.** Effects of food availability on sex change in two species of *Crepidula* (Gastropoda: Calyptraeidae). *Mar. Ecol. Prog. Ser.* **449**: 173–181.
- Mérot, C., and R. Collin. 2012b.** Effects of stress on sex change in *Crepidula* cf. *marginalis* (Gastropoda: Calyptraeidae). *J. Exp. Mar. Biol. Ecol.* **416-417**: 68–71.
- Míner, B. G., D. A. Donovan, and K. E. Andrews. 2010.** Should I stay or should I go: predator- and conspecific-induced hatching in a marine snail. *Oecologia* **163**: 69–78.
- Morrey, C. E., Y. Nagahama, and E. G. Grau. 2002.** Terminal phase males stimulate ovarian function and inhibit sex change in the protogynous wrasse *Thalassoma duperrey*. *Zool. Sci.* **19**: 103–109.
- Nolen, T. G., P. M. Johnson, C. E. Kicklighter, and T. Capo. 1995.** Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. *J. Comp. Physiol. A* **176**: 239–254.
- R Development Core Team. 2012.** *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Rittschof, D., P. Sawardecker, and C. Petry. 2002.** Chemical mediation of egg capsule deposition by mud snails. *J. Chem. Ecol.* **28**: 2257–2269.
- Ross, R. M. 1990.** The evolution of sex-change mechanisms in fishes. *Environ. Biol. Fishes* **29**: 81–93.
- Ross, R. M., G. S. Losey, and M. Diamond. 1983.** Sex change in a coral-reef fish: dependence of stimulation and inhibition on relative size. *Science* **221**: 574–575.
- Schleicherová, D., M. C. Lorenzi, and G. Sella. 2006.** How outcrossing hermaphrodites sense the presence of conspecifics and suppress female allocation. *Behav. Ecol.* **17**: 1–5.
- Schleicherová, D., M. C. Lorenzi, G. Sella, and N. Michiels. 2010.**

- Gender expression and group size: a test in a hermaphroditic and a gonochoric congeneric species of *Ophryotrocha* (Polychaeta). *J. Exp. Biol.* **213**: 1586–1590.
- Shapiro, D. Y. 1983.** Distinguishing behavioral interactions from visual cues as causes of adult sex change in a coral reef fish. *Horm. Behav.* **17**: 424–432.
- Sleeper, H. L., V. J. Paul, and W. Fenical. 1980.** Alarm pheromones from the marine opisthobranch *Navanax inermis*. *J. Chem. Ecol.* **6**: 57–70.
- Starr, M. J., and R. Collin. 2013.** Snail Porno: *Crepidula* mating behavior. YouTube video available at: <http://youtu.be/Cg1OAPU08EI?list=UUw0tqBUIRp5zADhPYj-ESTw> [2015, November 5].
- Toonen, R. J., and J. R. Pawlik. 1996.** Settlement of the tube worm *Hydroides dianthus* (Polychaeta: Serpulidae): cues for gregarious settlement. *Mar. Biol.* **126**: 725–733.
- Velando, A., J. Eiroa, and J. Domínguez. 2008.** Brainless but not clueless: earthworms boost their ejaculates when they detect fecund non-virgin partners. *Proc. Biol. Sci.* **275**: 1067–1072.
- Warner, R. R., D. L. Fitch, and J. D. Standish. 1996.** Social control of sex change in the shelf limpet, *Crepidula norrisiarum*: size-specific responses to local group composition. *J. Exp. Mar. Biol. Ecol.* **204**: 155–167.
- Webster, D. R., and M. J. Weissburg. 2009.** The hydrodynamics of chemical cues among aquatic organisms. *Annu. Rev. Fluid Mech.* **41**: 73–90.
- Zhao, B., and P.-Y. Qian. 2002.** Larval settlement and metamorphosis in the slipper limpet *Crepidula onyx* (Sowerby) in response to conspecific cues and the cues from biofilm. *J. Exp. Mar. Biol. Ecol.* **269**: 39–51.
- Zimmer, R. K., and C. A. Butman. 2000.** Chemical signaling processes in the marine environment. *Biol. Bull.* **198**: 168–187.