Evidence for Costs of Mating and Self-Fertilization in a Simultaneous Hermaphrodite With Hypodermic Insemination, the Opisthobranch *Alderia willowi*

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Abstract. Simultaneous hermaphrodites offer the chance to study antagonistic coevolution between the sexes when individuals function in both roles. Traumatic mating by hypodermic insemination has repeatedly evolved in hermaphroditic taxa, but evidence for the fitness costs of such male-advantage traits is lacking. When reared in isolation, specimens of the sea slug *Alderia willowi* (Opisthobranchia: Sacoglossa) initially laid clutches of unfertilized eggs but 4 days later began self-fertilizing; this is only the third report of selfing in an opisthobranch. Hypodermic insemination may allow selfing in *Alderia* if penetration of the body wall bypasses internal mechanisms that promote outcrossing. Selfing specimens and slugs reared in pairs had reduced fecundity compared to isolated slugs laying unfertilized clutches, suggesting that hypodermic insemination imposes a cost of mating. Egg production increased for field-caught slugs separated after mating compared to slugs held in pairs, a further indication that accessibility to mates imposes a fitness cost to the female function. Such antagonism can confer a competitive advantage to slugs mating in the male role but diminish reproduction in the female role among hermaphrodites capable of long-term sperm storage. *Alderia willowi* is also a rare case of poecilogony, with adults producing either planktotrophic or lecithotrophic larvae. Our rearing studies revealed that most slugs switched between expressed development modes at some point; such reproductive flexibility within individuals is unprecedented, even among poecilogonous species.

Introduction

Sexually antagonistic traits that confer a male advantage are commonplace in animals (Rice, 1996; Partridge and Hurst, 1998; Arnqvist and Rowe, 2005). Physical features include genital armature and other piercing structures that effect fluid transfer to the mate (Stutt and Siva-Jothy, 2001; Arnqvist and Rowe, 2002; Hosken and Stockley, 2004; Koene and Schulenburg, 2005). Biochemical adaptations, such as proteins in seminal fluid or accessory secretions, can manipulate female reproductive behavior, often increasing female fecundity in the short term at a long-term cost to fitness (Chapman *et al.*, 1995, 2003; Johnstone and Keller, 2000; Gillott, 2002). Behaviors such as taxing courtship rituals can also harm females (Bateman *et al.*, 2006). Such features often result from intrasexual selection due to male-male competition, and inflict female harm as a pleiotropic by-product (Edvardsson and Tregenza, 2005; Ronn *et al.*, 2006). Male-advantage alleles can trigger counter-selection in females (Reinhardt *et al.*, 2003); eventually, both sexes may depart from their reproductive optima (Rice, 1996; Holland and Rice, 1999; Friberg *et al.*, 2005; Ronn *et al.*, 2007).

Simultaneous hermaphrodites present an intriguing opportunity to test predictions from sexual selection theory, because individuals function in both male and female roles (Charnov, 1979; Michiels, 1998; Leonard, 1999, 2004, 2006). Hermaphrodites may escape the genetic load associated with intra-locus conflicts that may occur when genders are separate (Morgan, 1994; Lew *et al.*, 2006; Prasad *et al.*, 2007). Reciprocal sperm exchange is thought to have evolved in many groups as an adaptation to avoid conflict between mating partners, at least over sex roles (Leonard and Lukowiak, 1984; Leonard, 1991; Michiels and Streng,

1998; Greef and Michiels, 1999; Anthes and Michiels, 2005; Anthes *et al.*, 2005, 2006a, b). However, theory predicts that if antagonistic traits conferring an advantage on the male function arise, they should quickly go to fixation in a population (Lessells, 2005). Examples include behaviors like hit-and-run mating in polyclad flatworms (Michiels and Newman, 1998); structures such as modified setae, love darts, and penial stylets (Gascoigne, 1976; Koene *et al.*, 2002, 2005; Koene and Schulenburg, 2005); and allohormone secretions that increase fertilization success (Koene and Chase, 1998; Koene and ter Maat, 2001; Rogers and Chase, 2001; Koene, 2005; Chase and Blanchard, 2006; Anthes and Michiels, 2007a, b). An individual may benefit from such traits when mating in the male role, yet suffer due to exploitation by other males when mating in the female role.

How such mating conflicts are reconciled remains unclear (Parker, 2006). Sperm storage is commonplace in many hermaphroditic invertebrates; females may thus maximize fitness by avoiding excessive mating and escaping the associated costs (Stutt and Siva-Jothy, 2001; Arnqvist *et al.*, 2005). However, hypodermic insemination, which diminishes female control over fertilization, has evolved in many hermaphroditic taxa (Michiels and Koene, 2006). If males can force unilateral insemination on partners, costs of mating may be unavoidable, and individuals may depart from their reproductive optima in both male and female roles (Michiels and Newman, 1998; Anthes and Michiels, 2007a, b).

Although overall fitness costs due to male-advantage traits are predicted by theory, evidence for such costs is lacking for hermaphrodites. Opisthobranch sea slugs—simultaneous hermaphrodites with internal fertilization and the potential for long-term sperm storage—are an ideal study system for examining the costs of traumatic insemination (Beeman, 1977; Hadfield and Switzer-Dunlap, 1984). Adults mate in both male and female roles, often with multiple partners successively or concurrently (Painter, 1992; Angeloni *et al.*, 2003). Features that promote conflict between sex roles, including penial stylets and hypodermic insemination, have evolved repeatedly within the group Sacoglossa (Grahame, 1969; Jensen, 2001). The opisthobranch orders Cephalaspidea and Anaspidea have figured prominently in classical and more recent studies on hermaphrodite mating strategies (Leonard and Lukowiak, 1984; Angeloni and Bradbury, 1999; Angeloni *et al.*, 2003; Anthes and Michiels, 2007a, b; Anthes *et al.*, 2008), but there has been little work on sacoglossans (Schmitt *et al.*, 2007). The Sacoglossa are therefore ripe for comparative studies of hermaphrodite mating strategies, and for testing assumptions of obligate outcrossing in opisthobranchs.

The sacoglossan genus *Alderia* contains two species, the widespread *A. modesta* found throughout the north Atlantic and north Pacific, and *A. willowi* from California (Ellingson and Krug, 2006, 2007; Krug *et al.*, 2007). *Alderia willowi*

expresses a unique reproductive plasticity: it is the only known species in which an individual can switch from producing many small, feeding larvae (planktotrophy) to producing fewer, larger larvae that need not feed to complete metamorphosis (lecithotrophy) (Krug, 1998, 2007). Previous studies by Angeloni (2003) and Krug (1998, 2001) on what was identified as *A. modesta* from southern California actually used its then-cryptic sister species *A. willowi*. The penis in *Alderia* spp. is tipped with a sharp stylet used to inject sperm anywhere on the recipient's body; sperm can be stored for more than 3 weeks, and site of injection has no effect on fertilization success (Hand and Steinberg, 1955; Bleakney, 1988; Angeloni, 2003). Unilateral insemination is rare in *A. willowi*, but when it occurs, smaller individuals are twice as likely to act as sperm donors (Angeloni, 2003). In contrast, specimens of the larger *A. modesta* aggressively attempt unilateral inseminations with *A. willowi* (Krug, 2007).

For a study of reproductive isolation between species of *Alderia*, virgin specimens of *A. willowi* were reared in the laboratory: individual larvae were metamorphosed and juveniles were reared in isolation. We unexpectedly observed a high frequency of self-fertilization among the laboratory-reared "virgins." This in turn led us to investigate the potential costs of mating associated with hypodermic insemination, both for selfing and outcrossing individuals. The results provide empirical support for theory predicting that sexual selection can result in escalating male harm and fecundity costs in hermaphrodites.

Materials and Methods

Study system

The recently described *Alderia willowi* Krug *et al,* 2007, is an estuarine species restricted to back-bay habitats in southern and central California (Krug *et al.*, 2007). It exemplifies the stenophagy characteristic of the Sacoglossa, feeding exclusively on the heterokont alga *Vaucheria longicaulis* (Krug, 2001). Patches of *Vaucheria* filaments embedded in mud were collected and kept in an incubator at 16 °C on a 14:10 light/dark cycle; filaments were pulled free of the mud substrate, washed, and fed *ad libitum* to slugs. Seawater was obtained from the Cabrillo Marine Aquarium, adjacent to the mouth of the Los Angeles Harbor, and filtered to 0.45-μm filtered seawater (FSW) for all experiments. Adult slugs produce a string of eggs, each ovum in its own protective capsule, packaged into a gelatinous egg mass. Swimming larvae hatch from planktotrophic egg masses ≈3 days after deposition; lecithotrophic clutches hatch in 5–6 days (Krug, 1998). Lecithotrophic larvae are competent to metamorphose on blades of *V. longicaulis* immediately upon hatching, whereas planktotrophic larvae must feed on phytoplankton for one month before they can

metamorphose (Krug and Zimmer, 2000, 2004; Botello and Krug, 2006).

Self-fertilization and egg production in unmated sea slugs

Adult slugs were collected at low tide from exposed patches of *Vaucheria* in the Upper Newport Bay Ecological Reserve (Newport, CA) and placed individually in petri dishes of seawater to deposit egg masses. Development mode was typed 1–2 d later according to egg size (Krug, 1998), and lecithotrophic clutches were maintained until they hatched to yield competent veliger larvae. In a pilot study (July 2003), individual larvae ($n = 40$) were each transferred to a sterile plastic dish holding 4 ml of FSW; filaments of *Vaucheria longicaulis* were added to induce the larvae to settle and metamorpose, and as food for the juveniles (Krug and Zimmer, 2000). Slugs were transferred every day to clean dishes with new FSW and *ad libitum* algae, and 75% survived to maturity. About a week after metamorphosis, virgin slugs began laying atypically large clutches of unfertilized eggs. Three weeks later, several "virgins" began laying fertilized egg masses. All specimens had been individually isolated from the time of metamorphosis and thus had no opportunity for mating, suggesting that these slugs were self-fertilizing.

Two successive trials were then performed to compare the reproductive behavior of slugs reared in isolation or in pairs. Trial 1 was initiated in August 2003 and run for 5 weeks. Isolated virgin slugs ($n = 16$) were obtained as above. For paired treatments, two veliger larvae were put in each replicate dish ($n = 14$) with filaments of *Vaucheria*, and the juveniles were reared together. A second trial was initiated in January 2004 and run for 9 weeks, to increase sample size and examine long-term trends in egg production. Isolated individuals ($n = 31$) and pairs ($n = 29$) were obtained as before, and fed *Vaucheria* from Venice Beach, CA; new patches of algae were field-collected every 2 weeks to supply fresh food.

In both trials, egg masses were typed for development mode on the basis of egg and capsule size, and were checked for fertilization after one day at room temperature. Egg masses were partially flattened under a cover slip with clay "feet" on all vertices, and photographed with a Nikon CoolPix 950 camera; the number of eggs per clutch was scored from digital images, using Adobe Photoshop 7.0 software.

We hypothesized that egg production would be lower for paired slugs than for isolated individuals because tissue damage from repeated hypodermic insemination would impose a cost of mating. Mating rate is high in *A. willowi*; Angeloni (2003) observed inseminations in ≈50% of 124 pairs of laboratory-acclimatized slugs during a 30-min period, and most were reciprocal injections. Thus, paired slugs would have mated frequently during our trials. Because excess algae was supplied each day, there was no competition for food between paired individuals. However, paired slugs could instead have lost feeding time due to disruption by their partner, or may have invested more in their male function. Because isolated individuals also self-fertilized (see Results), we were able to test our hypothesis in two ways: first, by comparing the egg production of isolated *versus* paired slugs; and second, by comparing the egg production of isolated slugs producing unfertilized *versus* self-fertilized clutches. If rates of egg production were similar for self-fertilized and paired slugs but higher for slugs laying unfertilized eggs, this would support the hypothesis that reduced fecundity results from costs of mating rather than from other costs of co-habitation.

Because of the low sample size in trial 1, statistical tests for egg production were run only on data from trial 2. We first tested the hypothesis that rate of egg production would be higher for isolated slugs producing unfertilized eggs than for isolated but self-fertilizing slugs. We could not compare total egg production over the whole trial because (a) many individuals switched development modes, and the two types of clutches differ 10-fold in egg number (Krug, 1998); and (b) individuals differed widely in how many self-fertilized *versus* unfertilized clutches they produced. We therefore calculated mean daily egg production per slug over a 1–2-week period when (a) specimens produced only unfertilized clutches, and (b) the same specimens laid self-fertilized eggs. The mean number of eggs per day was then compared within individuals ($n = 9$ lecithotrophic, 2 planktotrophic) using a nonparametric paired sign test. This allowed us to test for a change in egg production upon selfing, regardless of the development mode a slug expressed. Given the results of pilot studies, we tested a one-tailed hypothesis of higher fecundity when slugs laid unfertilized eggs than when selfing.

Next, we computed mean daily rates of egg production for paired slugs, to compare with isolated slugs. Data were restricted to specimens laying lecithotrophic eggs from trial 2, for which the most data were available. The daily rate of egg production per slug was calculated over a 1–2-week interval by taking (a) the average egg production for a pair if both members laid lecithotrophic eggs, or (b) the reproductive output from a single individual if its partner produced only planktotrophic eggs. This analysis was restricted to slugs laying lecithotrophic eggs and did not involve within-individual comparisons, thus permitting parametric tests. Data were log-transformed prior to analysis to improve normality and heteroscedasticity. To test the hypothesis that paired slugs would produce fewer eggs than unpaired slugs, we used a one-tailed Student's *t*-test to compare the mean daily egg production of isolated slugs laying unfertilized clutches ($n = 19$) to that of paired slugs ($n = 12$).

Egg number per clutch varies considerably between the two development modes, and preliminary results indicated

that eggs per clutch also varied among mating types (unfertilized, self-fertilized, or paired). However, there was no *a priori* expectation that rate of egg mass production would differ across development modes, or among mating types if slugs decreased the number of eggs per clutch rather than the number of clutches laid in response to any costs of mating. Clutch production per slug per week was scored for all individuals that laid multiple clutches from the second through the fourth week of trial 2. A two-way ANOVA was used to test the hypothesis that development mode and mating type had no effect on the rate of clutch production. A failure to reject the null hypothesis would be consistent with the hypothesis that clutch production did not differ among mating treatments, and that any differences between types were due to a change in egg number and not in how eggs were packaged into individual clutches.

Effects on fecundity of remating *versus* use of stored sperm

The disparity in number of eggs per clutch noted in previous trials (see Results) implied a cost of mating in *Alderia*. As noted, the rate of mating is high in *A. willowi*: slugs held in pairs mate frequently in the laboratory, whereas isolated slugs use stored allosperm for up to 2 weeks without a decline in fertilization (Angeloni, 2003; authors' unpubl. obs.). We therefore compared the fecundity of field-caught slugs held in pairs to that of slugs that were isolated after mating to eliminate costs of recurrent insemination. Three successive trials were performed, to assess any effects of field condition or size. The first trial (January 2007) used adult *A. willowi* collected from Long Beach, California; the second (December 2007), slugs from Mill Valley, San Francisco, California; and the third (January 2008), slugs from Newport, California. Slugs were typed for development mode within 1–2 days of collection (Krug, 1998), and only those laying planktotrophic clutches were used in trials.

Within 2 days of collection, slugs were haphazardly paired in glass dishes containing 12 ml of FSW and filaments of *Vaucheria*. All slugs were transferred to clean water with fresh algae every other day. Pairs were randomly assigned to one of two treatment groups: a "continuously paired" treatment or a "separated" treatment. In paired treatments, slugs were kept together for the 6-day duration of each trial. In "separated" treatments, slugs were initially held in pairs and allowed to mate for 2 days; the members of each pair were then isolated and kept in separate dishes for 4 days, during which each slug used stored allosperm to fertilize its eggs. Total egg production was scored for three successive 2-day intervals comprising each trial: (1) the initial 2 days, when all pairs were held together (no difference between treatment classes expected); (2) day 2 to day 4, when members of treatment pairs were separated while

paired controls were left together; and (3) day 4 to day 6, when members of treatment pairs were still separated and paired controls were still together. Total egg production was tracked for all initial pairs over each 2-day interval, whether individuals were continuously paired or separated from their partner. If one member of a pair died during the course of a trial, data for that pair were eliminated from the trial. Final samples sizes were Long Beach: $n = 6$ separated pairs, $n = 5$ continuous pairs; San Francisco: $n = 6$ separated pairs, $n = 9$ continuous pairs; and Newport: $n = 9$ separated pairs, $n = 11$ continuous pairs. There was no difference in mean weight of slugs allocated to the two groups in trial 2 (unpaired two-tailed *t*-test: df = 13, $t = 0.24$, $P = 0.81$) or trial 3 (df =18, $t = 0.69$, $P = 0.50$), for which weights were measured.

We expected no difference in egg production among pairs allocated to each treatment group for the initial 2 days of the trial—that is, prior to isolation of the members of "separated pairs." A two-way ANOVA, with source population as a random between-subjects factor and treatment group as a fixed factor, was used to test this hypothesis. There was no significant group $\times$ population interaction in the full ANOVA model and the *P* value was greater than 0.25 ($F = 1.00$, $P = 0.38$), hence the interaction term was removed from the final model as recommended (Quinn and Keough, 2002).

Our hypothesis was that separated slugs would escape the costs of repeated mating, and hence their fecundity would increase compared to that of slugs held in pairs. Total planktotrophic egg production over 4 days was the response variable; we did not compare egg production at multiple time points by repeated measures ANOVA because inclusion of source population as a random factor complicates any interpretation of within-subjects effects. Data were analyzed by a two-way ANOVA with source population as a random factor and treatment group as a fixed factor. There was again no significant group $\times$ population interaction in the full ANOVA model ($F = 0.92$, $P = 0.41$), hence the interaction term was removed from the final model (Quinn and Keough, 2002).

RESULTS

Self-fertilization

In trial 1, age of first reproduction was about 10 days after metamorphosis for slugs raised in pairs (Table 1). Four days after paired slugs initiated egg-laying behavior, most isolated "virgins" began producing large clutches of unfertilized eggs (Table 1). Only 3 of 16 isolated slugs did not produce any clutches. Egg masses of both development modes were produced by 13 laboratory-reared individuals, with 7 laying mostly lecithotrophic clutches and 6 producing planktotrophic egg masses. Two isolated slugs produced partially fertilized clutches starting about 5 days after their

Table 1

Clutch production and self-fertilization for specimens of Alderia willowi

Trial	Treatment	Age of 1st clutch production (days)	Age of 1st selfing (days)	Proportion selfing
1	Individual	14.1 ± 2.5 (13)	19.5 ± 0.7 (2)	15.4%
	Pair	9.9 ± 1.4 (14)		
2	Individual	20.2 ± 6.4 (31)	27.3 ± 6.1 (24)	77.4%
	Pair	15.8 ± 5.4 (29)		

Slugs were reared in the laboratory from the time of metamorphosis, either as isolated individuals or in pairs. Data are means ± one standard deviation, with sample size (number of isolated individuals or pairs) in parentheses. Age in days is given from the time of metamorphosis into a juvenile slug. For isolated individuals, the first clutch produced was either unfertilized or self-fertilized.

Table 2

Clutch production and self–fertilization for specimens from Trial 2 that differed in development mode of their eggs

Reproductive characteristic	Lecithotrophic ($n = 22$)	Planktotrophic ($n = 9$)
% of self-fertilized clutches	$62.3\% \pm 8.3$	$39.0\% \pm 11.1$
Total clutches laid	18.1 ± 1.9	19.8 ± 5.2
Age at 1st laying (days)	19.8 ± 1.1	21.3 ± 2.9
Lifetime egg production	390.5 ± 52.6 (16)	2206.2 ± 375.5 (5)

Data are means ± one standard error. Age in days is given from the time of metamorphosis into a juvenile slug. Lifetime egg production combines unfertilized and self–fertilized eggs for individuals that survived 56 days; sample sizes are in parentheses.

first egg mass was laid and continuing for 5 and 10 days, respectively. Because these individuals had been isolated as non-reproductive larvae, they had no opportunity for juvenile mating, and fertilization of eggs suggested that selfing had occurred.

Specimens took longer to reach reproductive maturity in trial 2. Paired slugs initiated reproduction at age 15.8 d ± 5.4 SD, but as in trial 1, isolated specimens delayed reproduction by about 4 days (Table 1). Difference in age at first reproduction between isolated and paired slugs was significant (unpaired two-tailed *t*-test: df = 58, $t = 2.89$, $P = 0.005$). All 31 isolated specimens laid multiple clutches; the mean rate of clutch deposition was 4.1 ± 1.5 SD clutches per week. Twenty-two specimens produced mostly lecithotrophic eggs, while nine produced mainly or solely planktotrophic eggs.

In trial 2, selfing started about one week after initial clutch deposition, and 24 out of 31 isolated specimens self-fertilized one or more times (Table 1). Overall, a mean $32.2 \pm 28.4\%$ SD (range 0–100%) of clutches deposited by isolated individuals were at least partially self-fertilized. One specimen self-fertilized all 36 of its clutches. The mean number of consecutive self-fertilized clutches was 5.0 ± 6.9 SD ($n = 44$ intervals of one or more self-fertilized clutches, range 1–36 clutches). The mean interval between selfing events, during which at least one unfertilized clutch was laid, was 8.7 days ± 7.2 SD ($n = 21$ intervals, range 1–28). The total number of clutches an individual produced was positively corelated with the proportion of its clutches that were self-fertilized ($r = 0.431$, $z = 2.439$, $P = 0.015$).

Aside from expected differences in lifetime egg production, adults that expressed different development modes were remarkably similar in their reproductive characteristics (Table 2). Specimens producing planktotrophic *versus* lecithotrophic clutches did not differ in their propensity to self-fertilize (Table 2, and results of an unpaired, two-tailed *t*-test on arcsine square-root-transformed percentages of self-fertilized clutches: df = 29, $t = 1.56$, $P = 0.13$).

Effects of selfing and outcrossing on rates of egg production

When rates of egg production were considered for both planktotrophic and lecithotrophic egg producers, isolated slugs produced more unfertilized eggs than they did self-fertilized eggs (one-tailed Wilcoxon signed rank test: $n = 13$, $P < 0.01$). Rates of lecithotrophic egg production were significantly higher among isolated slugs laying unfertilized clutches than among paired slugs (Fig. 1 and results of a one-tailed unpaired *t*-test: df = 29, $t = 2.56$, $P = 0.008$). There was no difference in the rate of egg production for self-fertilizing slugs *versus* paired slugs (Fig. 1 and results of a one-tailed unpaired *t*-test: df = 23, $t = 0.91$, $P = 0.18$). The low rate of egg production in paired slugs therefore most likely reflects a cost of insemination also borne by self-fertilized slugs, and not some other cost of co-habitation with a partner.

Although rate of egg production varied among treatments, there was no difference in the number of clutches per week produced by slugs, regardless of development mode or
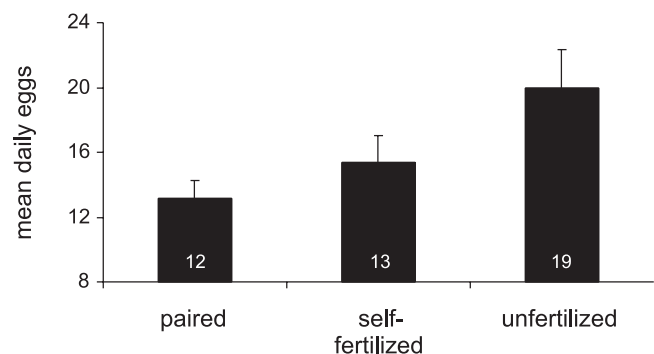
Figure 1. Effect of mating status on rate of daily egg production in laboratory–reared specimens of *Alderia willowi*. Data are the mean number of lecithotrophic eggs produced per slug per day (± one standard error) in trial 2. Specimens reared in isolation produced either unfertilized or self–fertilized eggs; "paired" denotes egg production by one member of a pair reared together from the time of metamorphosis. Number of individuals used in analyses is given on the corresponding bar.
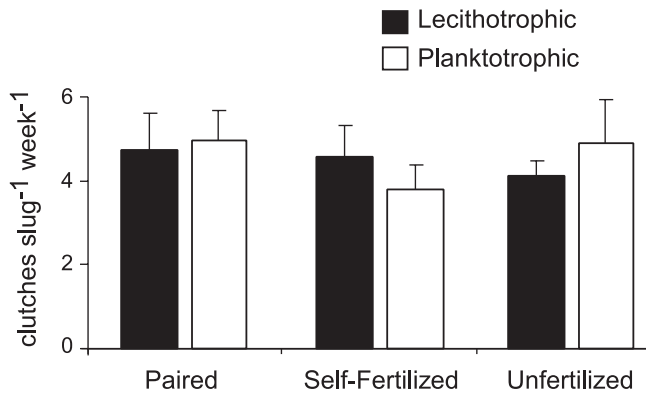
Figure 2. Rate of clutch production per week for paired slugs *versus* isolated slugs either selfing or producing unfertilized egg masses. Data are mean weekly rates of clutch deposition $\pm$ one standard error, for slugs producing either planktotrophic or lecithotrophic egg masses. Means were calculated from the 2nd to 4th week of trial 2.

mating class (Fig. 2 and results of a two-way ANOVA: effect of development mode, $F_{1,77} = 0.002$, $P = 0.96$; effect of mating class, $F_{2,77} = 0.54$, $P = 0.59$).

Given the initial delay in production of unfertilized eggs among isolated slugs, specimens reared in isolation could have been storing eggs for 4–5 days and including those "extra" eggs in their initial clutches; since early clutches were unfertilized in more than 80% of individuals, stored eggs could inflate rates of egg production for isolated slugs. However, most isolated slugs (27 out of 31 slugs in trial 2) did not lay their largest clutch during the initial few days of egg production.

Costs of mating versus use of stored sperm

To test the hypothesis that repeated insemination carries a fitness cost, we compared total egg production among field-caught slugs that were either held in pairs or separated after an initial mating period. The experiment was repeated

three times with slugs from different source populations; only slugs that produced planktotrophic larvae were used, and trials were limited to 6 days. As expected, there was no initial difference in egg production between pairs randomly assigned to treatment groups during the initial 2-day mating period, when all slugs were paired (Table 3, Fig. 3). After slugs from some pairs were separated, there was a significant effect of treatment on fecundity ($P = 0.018$); egg production was higher among slugs using stored allosperm than among paired slugs with constant mating opportunity (Table 3, Fig. 3). There was a significant effect of source population on initial fecundity ($P = 0.004$), likely due to the large size of slugs from San Francisco (2.16 mg $\pm$ 0.07 SE) *versus* Newport (0.53 mg $\pm$ 0.05 SE); however, there was no effect of source during the treatment period (Table 3).

For comparative purposes, we broke up the treatment period into 2-day intervals and plotted mean egg production per treatment group for each trial (Fig. 3). In trial 1 (Long Beach) and trial 2 (San Francisco), total egg production was much higher for pairs of separated slugs during the first treatment interval (days 2–4), but declined to the level of paired slugs from days 4 to 6 (Fig. 3A, B). In trial 3 (Newport), paired slugs experienced a peak in egg production during the first 2 days of the treatment, but separated slugs had higher fecundity from days 4 to 6 (Fig. 3C). Thus, while the time course of response varied by source population, the overall trend was higher egg production for slugs using stored allosperm than for paired slugs, consistent with a cost of regular mating.

Transitions in development mode

A unique feature of *Alderia willowi* is that individual specimens can produce both planktotrophic and lecithotrophic egg masses. Long-term monitoring of laboratory-reared individuals provided the opportunity to test whether specimens changed the development mode of their offspring

Table 3

Effects of repeated mating on egg production in Alderia willowi

Treatment period	Source	SS	df	MS	F	P
Pre-treatment	Treatment group	54403.52	1	54403.52	1.93	0.17
	Population	356615.31	2	178307.65	6.33	0.004
	Residual	1183102.70	42	28169.11		
Post-treatment	Treatment group	236044.71	1	236044.71	6.04	0.018
	Population	24316.17	2	12158.09	0.31	0.73
	Residual	1641242.60	42	28169.11		

Results are from two–way ANOVAs, with source population as a random factor, treatment group as a fixed factor, and total egg production as the response variable. "Pre–treatment" results are for egg production over 2 days when all specimens were paired in both treatment groups (no difference expected between groups). "Post–treatment" results are for total egg production over 4 days when slugs in the separated treatment were isolated from their mate, while continuously paired slugs were held together. In both ANOVAs the group $\times$ population interaction was not significant in the full model and was therefore omitted from final ANOVA models.
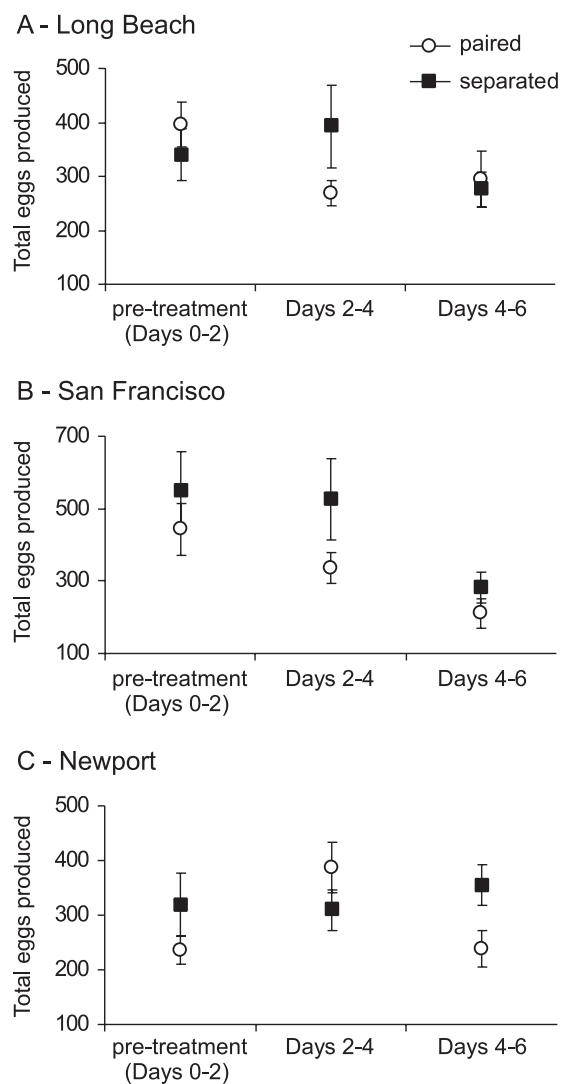
A - Long Beach

B - San Francisco

C - Newport

Figure 3.   Fecundity cost of prolonged pairing in *Alderia willowi*. Data are the total number of planktotrophic eggs laid by each pair of slugs, over three successive 2–day intervals, plotted as means ± one standard error. In three trials using different source populations, field-collected adults were randomly paired for 2 days (days 0–2; no treatment applied). Members of some pairs were then separated and held in isolation while remaining pairs were kept together; egg production was scored after 2 and 4 days. (A) Egg production in trial 1, using slugs from Long Beach (*n* = 6 separated pairs; *n* = 5 continuous pairs). (B) Egg production in trial 2, using slugs from San Francisco Bay (*n* = 6 separated pairs; *n* = 9 continuous pairs). (C) Egg production in trial 3, using slugs from Newport Bay (*n* = 9 separated pairs; *n* = 11 continuous pairs).

under constant conditions. In trial 1, few isolated and no paired individuals switched development mode of their eggs over a 5-week period. Five of 16 isolated slugs initially laid two to four planktotrophic clutches before switching to lecithotrophic clutch production. Two laid "mixed" clutches, containing a range of egg sizes, while transitioning between pure planktotrophy and lecithotrophy. One slug laid planktotrophic eggs for 22 days, then produced no

clutches for 11 days, and finally deposited a single lecithotrophic egg mass.

In trial 2, two slugs each produced one initial planktotrophic egg mass before thereafter producing only lecithotrophic eggs. Twelve individuals initially laid lecithotrophic eggs but transiently switched to planktotrophy for 1–5 clutches mid-trial before reverting back to lecithotrophy; this may have been caused by a decline in the quality of available food algae. Four slugs switched from lecithotrophic to planktotrophic egg production a few days before dying; only four specimens produced exclusively lecithotrophic clutches throughout the trial. In contrast, seven individuals produced solely planktotrophic clutches, while two produced a single lecithotrophic clutch out of 9 and 31 planktotrophic clutches, respectively. Thus, while most individuals expressed predominantly one mode of development under laboratory rearing conditions, two-thirds of isolated specimens switched between development modes at least once.

DISCUSSION

Costs of mating in hermaphrodites

Costs of reproduction underlie many life-history trade-offs and may be mediated by similar mechanisms across species (Sgro and Partridge, 1999; Harshman and Zera, 2006). In contrast, costs of mating (*i.e.,* sperm transfer or receipt) often vary widely among related taxa, due to the vagaries of mating systems (Arnqvist and Rowe, 2002; Ronn *et al.*, 2006). Simultaneous hermaphrodites may evolve harmful male behavior more readily than gonochorists if the paternity advantage conferred upon the male function offsets the fecundity loss in the female function (Michiels and Koene, 2006). However, such antagonistic adaptations can lead to a pronounced conflict of interest between sex roles. When a male trait both harms a female and decreases her investment in the male's offspring, it is likely a pleiotropic consequence of intrasexual selection for an advantage in sperm competition, rather than an intrinsically adaptive trait. This is supported by evidence from insects, but comparative data for hermaphrodites is lacking (Morrow *et al.*, 2003; Edvardsson and Tregenza, 2005).

Our study was initiated by the observation that virgins produced large clutches of unfertilized eggs, yet fecundity dropped when individuals selfed and was consistently lower for paired slugs. We interpret the depressed rate of egg production among paired and selfing slugs as evidence for a cost of mating in *Alderia willowi*. Hypodermic insemination necessarily inflicts tissue damage, and slugs mate at a high rate from the time they are juveniles (Angeloni, 2003). We hypothesize that in *A. willowi*, hypodermic insemination imposes an intrinsic cost: energy that would otherwise go toward egg production is reallocated to somatic tissue repair, due to damage inflicted by the sperm donor. The rate

of unfertilized egg-laying would represent the maximum egg production possible for *A. willowi*. Analogous results were obtained for field-caught specimens: isolation from conspecifics promptly increased egg production. To our knowledge, these are the first data indicating an immediate cost of mating in a hermaphrodite with hypodermic insemination.

Alternative costs could also potentially lower fecundity in paired slugs, including lost feeding time due to disturbance by the partner slug, or reallocation of energy to the male function (DeVisser *et al.,* 1994). However, it seems unlikely that there are significant costs of cohabitation with a partner slug other than those associated with mating, since self-fertilized but isolated slugs produced essentially the same number of eggs per day as paired slugs. Reallocation of resources to the male role also seems unlikely to limit egg production in this system. Separation of field-caught individuals after mating resulted in a significant increase in egg production in 2 days (Long Beach and San Francisco slugs) or 4 days (Newport slugs). It seems improbable that individuals would immediately stop allocating energy to the production of sperm or accessory secretions within a day of isolation; the boost in fecundity following separation was likely due to the cessation of injections and hence reduced costs of tissue repair.

Data from two laboratory rearing trials returned similar estimates of mating costs in *Alderia willowi*. We assume that the output of unfertilized eggs by virgins represents the optimum rate of egg production. Paired slugs had a 31% or 38% reduction in the number of lecithotrophic eggs per clutch, and a 55% or 58% reduction in planktotrophic eggs per clutch, compared to virgin slugs in the two trials. Based on fecundity data from trial 1 (when egg production approximated that of field-caught slugs), this difference translates as a cost of about 20 lecithotrophic eggs or 200 planktotrophic eggs per clutch. A regularly mated specimen would have to fertilize one clutch for every clutch it produced, to compensate for the cost of mating imposed on its female function. This could represent the selective pressure behind the evolution of reciprocal mating in *Alderia willowi*, the predominant mode of copulation in this species (Angeloni, 2003).

Disruptive selection on female mating rate can result if fitness is maximized by either mating rarely, to avoid costs, or mating often, to receive the benefits of polyandry (Arnqvist *et al.*, 2005). Reproduction in the female role would be optimized at a very low mating rate in *Alderia willowi* as specimens need only mate once every 1–2 weeks for 100% fertilization of their eggs (Angeloni, 2003). Slugs may express behaviors to avoid mating unnecessarily or to escape unilateral insemination, which is rare in this species (Angeloni, 2003). When individuals of its larger sibling species *A. modesta* are placed together with *A. willowi*, the larger slugs often "bully" small *A. willowi* and aggressively inseminate

them; smaller slugs attempt to crawl away rather than reciprocating (Krug, 2007). Thus, *A. willowi* may avoid situations where it will be forced into the female role or receive disproportional tissue damage from a larger mate. Analogously, the Caribbean sacoglossan *Elysia zuleicae* engages in group mating behavior that approximates penis fencing, and individuals flap their parapodia to swim away when another penis makes contact with their body, to avoid receiving an injection of sperm (Krug, per. obs.). Hypodermic insemination may commonly result in cycles of chase-away selection that amplify conflicts between gender roles and diminish overall fitness.

Hypodermic insemination in sacoglossans is often, but not always, associated with a sharp penial stylet for piercing the partner's body tissue (Reid, 1964; Gascoigne, 1976; Jensen, 2001). The presence of stylets in most sacoglossan families, but not in most species, suggests multiple independent origins. A molecular phylogenetic hypothesis of the Sacoglossa will enable statistical tests of correlated trait evolution and ancestral character state reconstruction, revealing how traits that favor male reproductive success evolve and drive counter-evolution of female traits (Morrow and Arnqvist, 2003; Anthes *et al.*, 2008).

Role of selfing in Alderia

Two unexpected findings from our rearing studies were the initial production of large unfertilized clutches by isolated slugs, followed by the production of fertilized egg masses. Self-fertilization is rare among opisthobranchs, yet common in their sister taxon, the pulmonates (Jarne and Charleswoth, 1993). Selfing has only been reported from two congeneric sacoglossans, *Berthelinia limax* from Japan (Kawaguti and Yamasu, 1961) and *B. caribbea* from Jamaica (Grahame, 1969). *Berthelinia* belongs to the Oxynooidea, the superfamily of shelled sacoglossans, and is only distantly related to *Alderia*, a derived genus in the superfamily Limapontioidea. Grahame (1969) reared individual *B. caribbea* in isolation beginning a few hours after metamorphosis, and reported selfing during a 60–70-day spawning period, but no frequency of selfing was reported; he also documented a penial stylet used in hypodermic insemination. Kawaguti and Yamasu (1961) isolated 5-day-old juveniles of *B. limax*, which therefore had opportunities for juvenile mating because the ability to donate sperm develops before female maturation in diverse opisthobranchs (Reid, 1964; Todd *et al.*, 1997; Angeloni, 2003). However, isolated specimens laid fertilized clutches for 70 days without remating, which supports the inference of selfing, unless allosperm survive for exceptional periods in *Berthilinia*.

The complex reproductive systems of opisthobranchs appear to preclude selfing in the majority of species, and it has been hypothesized that opisthobranch sperm are activated

only upon receipt by the seminal receptacle of a mating partner (Thompson, 1976; Beeman, 1977; Jarne and Charlesworth, 1993). Hypodermic insemination is relatively common among sacoglossans, and the penis of many sacoglossans is extremely long and flexible, often half the body length or more. These factors may facilitate selfing, allowing an individual to inject sperm into its own tissues and by-pass any barriers to internal self-fertilization. The proposed correlation between hypodermic insemination and the ability to self could be tested through rearing studies with other sacoglossans. Alternatively, the documentation of selfing in three sacoglossans may reflect the high frequency of lecithotrophy in this group, which facilitates metamorphosis and rearing of isolated individuals, making it easier to detect selfing by ruling out juvenile mating and sperm storage.

The production of uniparental clutches may reflect apomixis (reproduction without fertilization) triggered by the injection of autosperm, rather than true self-fertilization (D'Souza *et al.,* 2008). However, 20% of clutches deposited by isolated slugs contained some eggs that never underwent cleavage, while the rest developed normally. If self-injection triggered apomixis as a reproductive strategy, one would expect all eggs to cleave and develop. Instead, the high proportion of clutches containing some undeveloped eggs suggests that selfing was indeed occurring, but was less frequent than insemination by a partner, less successful at effecting fertilization, or both. Although hypodermic insemination can occur anywhere on the body, the injection site can have a considerable effect on fertilization success (Angeloni, 2003); both the frequency and site of self-insemination may therefore determine how many eggs are ultimately fertilized in selfing individuals.

In two trials, *Alderia willowi* individuals reared without access to a mate laid large clutches of unfertilized eggs starting 4 days after paired slugs first reproduced; slugs thereafter produced 1–2 clutches per day whether outcrossing, selfing, or laying unfertilized eggs. Populations of *Alderia* spp. can exceed 5000 slugs per square meter (M. Garchow, B. Shimer, P. Krug, unpubl. data). Such high densities may have selected for constitutive egg production since slugs will usually have ample allosperm reserves (Trowbridge, 1993; Angeloni, 2003). Density-dependent effects such as likelihood of insemination *versus* costs of mating may strongly influence the evolution of mating strategies and reproduction in taxa like *Alderia* spp. (Michiels, 1999; Kokko and Rankin, 2006); future studies will examine how density of conspecific and heterospecific slugs affects fitness in *A. willowi* and *A. modesta.*

Most isolated slugs selfed frequently, starting 5–7 days after initial deposition of unfertilized clutches. Delayed selfing in the absence of a mate has been reported in other systems, and is predicted by theory which holds that hermaphrodites may adaptively adjust the timing of selfing according to mate density. An optimal delay period before selfing is expected when (a) inbreeding depression carries a strong fitness cost, and (b) the odds of eventually encountering a mate are favorable (Tsitrone *et al.,* 2003a). In accordance with model predictions, the freshwater snail *Physa acuta* delays selfing for 2 weeks, investing in future growth and reproduction when reared in isolation (Tsitrone *et al.,* 2003b). Similarly, a parasitic cestode worm delays selfing relative to paired worms, minimizing the costs of low hatching success in self-fertilized eggs (Schjørring, 2004). The 5–7 day delay prior to selfing in laboratory-reared *A. willowi* is in accordance with the hypothesis that reproductive assurance strategies balance costs of inbreeding against costs of delayed reproduction. A virgin specimen of *A. willowi* has a high probability of future access to mates, due to high adult densities in natural populations; this should favor the observed delay of selfing.

It remains unclear whether selfing is an adaptive alternative to outcrossing in *Alderia willowi*. Although most individuals selfed, they did so only about a third of the time. The positive correlation between number of clutches laid and percentage of self-fertilized clutches suggests either that (a) some individuals were reproductively "primed" for both egg production and male functions, or (b) a positive feedback loop exists in which receipt of sperm triggers egg-laying behavior. Specimens of *A. willowi* isolated for several days frequently extend the penis (normally held inside the body) while crawling about. Prolonged penial deployment in the absence of a mate may lead to chance self-injection should the stylet-tipped penis contact an individual's own epithelium; self-fertilization may thus result from accidental penetration of the body wall by the penis of a "frustrated" virgin. However, selfing could be adaptive for initial founders of new habitat patches. The muddy intertidal zone where *A. willowi* lives is prone to dramatic fluctuations in temperature and salinity, and patches of the host alga *Vaucheria* spp. appear and disappear on a scale of weeks throughout California. This ephemeral, stressful habitat leads to regular extinction of local slug populations (authors' unpubl. data). As recolonization proceeds by recruitment of planktonic larvae from other estuaries, selfing may allow early colonists to avoid negative Allee effects stemming from the lack of potential mates. The observed delay of about a week prior to selfing could allow time for the settlement of incoming larvae before selfing behavior is initiated (Baker, 1955).

Shifts in development

Few marine species are capable of expressing both larval development modes, a phenomenon termed poecilogony; well-supported cases are limited to a few polychaete worms and sacoglossans (Hoagland and Robertson, 1988; Chia *et al.,* 1996; Ellingson and Krug, 2006; Krug, 2007; Krug,

unpubl. data). Even among poecilogonous species, production of both planktotrophic and lecithotrophic larvae by an individual is known only in *Alderia willowi* (Krug, 1998). However, specimens of *A. willowi* had previously been observed to vary the development mode of their offspring only under starvation conditions, shifting from lecithotrophy to planktotrophy (Krug, 1998). Our rearing data show that over 2 months, most individuals predominantly produced a single type of larvae. However, occasional egg masses of the alternative type were produced by about two-thirds of all isolated specimens, which may function as a bet-hedging strategy to vary offspring dispersal (Krug, 2001). When food quality transiently declined, many specimens switched from lecithotrophic to planktotrophic larvae, as noted previously. This change may adaptively increase dispersal of offspring when the adult environment deteriorates. Ocean currents can transport long-lived larvae along the coast, allowing them to colonize distant estuaries; the month-long maturation period of planktotrophic larvae therefore confers greater dispersal potential, compared to lecithotrophic larvae that can settle immediately upon hatching (Krug, 2001). Poecilogony thus acts as a dispersal dimorphism akin to the production of winged and wingless morphs in many insects (Harrison, 1980; Zera and Denno, 1997; Crnokrak and Roff, 1998).

Krug (1998) reported that a few field-caught individuals produced "mixed" clutches containing a normally distributed range of egg sizes, intermediate between planktotrophy and lecithotrophy. In the present study, laboratory-reared individuals occasionally produced mixed clutches while switching between development modes, suggesting they are an aberrant product of a female reproductive system in transition. In support of this hypothesis, field-collected adults produced mixed clutches only in spring months, a time when the field population in San Diego, California, is switching between larval types (Krug, unpubl. data). The extraordinary reproductive flexibility of *A. willowi* should continue to provide insight into the evolution of adaptive plasticity in life-history traits.

Acknowledgments

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