The Size Advantage Model of Sex Allocation in the Protandrous Sex-Changer Crepidula fornicata: Role of the Mating System, Sperm Storage, and Male Mobility

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Keywords: sequential hermaphrodite, protandry, reproductive success, gregarious behavior, mollusc

List of elements for expanded online edition: supplementary methods, supplementary figures S1 to S7, supplementary references, and color version of figure 1.

This manuscript is an article

Abstract

Sequential hermaphroditism is adaptive when the reproductive value of individuals varies with size or age and this relationship differs between males and females. In this case theory shows that the lifetime reproductive output of an individual is increased by changing sex (a hypothesis referred to as the size-advantage model). Sex-linked differences in size-fitness curves can stem from differential costs of reproduction, the mating system, and differences in growth and mortality between sexes. Detailed empirical data is required to disentangle the relative roles of each these factors within the theory. Quantitative data are also needed to explore the role of sperm storage, which has not yet been considered with sequential hermaphrodites. Using experimental rearing and paternity assignment, we report relationships between size and reproductive success of *Crepidula fornicata*, a protandrous (male-first) gastropod. Male reproductive success increased with size due to the polygamous system and stacking behavior of the species, but females nonetheless had greater reproductive success than males of the same size, in agreement with the size-advantage theory. Sperm storage appeared to be a critical determinant of success for both sexes, and modeling the effect of sperm storage showed that it could potentially accelerate sex-change in protandrous species.

Introduction

Sequential hermaphrodites mature first as one sex and then switch to the other sex at some point during their life. This strategy is adaptive when the lifetime reproductive output of an individual is maximized by changing sex during its life. Theory shows that this happens when the gender that provides the highest reproductive value (expected future reproduction) differs depending on the size or the age of the individual (the size advantage hypothesis, Ghiselin 1969; Warner 1988). This hypothesis has been formalized under the general framework of sex allocation theory (reviewed in Charnov 1982; West 2009), which correctly predicts both the direction and timing of sex-change in sequential hermaphrodites from several groups where this reproductive strategy is found.

The size advantage model has broad applicability and is seemingly simple since it can be outlined by the relationship between reproductive value and a unique predictor (body size or age). However, it is important to realize that this relationship integrates a range of complex biological processes that drive reproduction expectations of individuals of both sexes (discussed e.g. in Charnov 1982; Warner 1988; West 2009). The reproductive value of an individual has several components: it integrates both current and future reproductive output, and the probability of surviving to reproduce in the future. There are thus several mechanisms that can result in males and females displaying different reproductive value trajectories with respect to age/size.

First, females make large nutritious gametes and in many species the production and storage of these gametes requires space within the body (e.g. in fish and invertebrates). In addition the female function often entails higher energy costs than the male function. Female fecundity therefore increases sharply with body size in many species. If this size-effect results in large individuals having a higher reproductive value as females than as males, then protandry (male-first sex change) is selected for.

Second, sex-linked differences in growth and mortality rates can result in the effect of size on reproductive value being greater in one sex, thereby introducing selection for sex change. Theory shows that there is an advantage to starting life as the sex that has the fastest growth rate and/or the lowest mortality rate (e.g. Charnov 1982, p. 136; Iwasa 1991).

Finally, social systems can create conditions where a differential effect of size on male vs female reproductive success can be expressed (Munday et al. 2006). In this study we focused on this factor, which is perhaps more elusive than the first two factors because species that change sex have a variety of mating systems and sexual selection regimes. A general finding is that polygynous mating systems favor protogynous (female-first) sex change, because larger individuals have a significant advantage in male-male competition (Ghiselin 1969; Andersson 1994; Munday et al. 2006; West 2009). Specifically, protogyny is selected for when the advantage of large males is strong enough to result in an individual's reproductive success increasing more rapidly with size for males than for females. On the other hand, protandry (male-first sex change) is adaptive if the increase in male reproductive success as a function of size does not outweigh the fecundity advantage of large females. In line with this, mating systems where size has little or no effect on male reproductive success are associated with protandrous species (e.g. Charnov 1982, p.142, 182; West 2009, p.200, 223).

Empirical research has provided several illuminating examples that have validated the size-advantage theory and the expected effects of different mating systems within this theory (e.g. protandrous pandalid shrimps and protogynous labroid fish, reviewed and analyzed in details in Charnov 1982). However, other social systems remain to be examined and authors have repeatedly called for more quantitative data on sex-specific size-fitness functions (e.g. West 2009; Kazancioglu and Alonzo 2010).

The protandrous slipper limpet (*Crepidula fornicata*) is an interesting system for investigating the effect of social systems on sex change relative to other factors. Slipper limpets are bentho-

88 pelagic mollusks (i.e. sedentary adults producing free-living larvae). They form long-lived stacks of
89 typically 2 to 20 individuals with younger, smaller individuals (immature or males) piling up on older,
90 larger ones (females). These stacks are permanent mating associations of genetically unrelated
91 individuals of various ages and sexes that reproduce with each other (individuals may live up to ca.
92 10 years). All individuals mature first as males before changing sex at a time that depends strongly
93 on environmental (social) conditions (earliest work by Conklin 1897; Orton 1912; Coe 1936;
94 Hoagland 1978). Interestingly, parentage studies (starting with Gaffney and McGee 1992) have
95 repeatedly shown that the mating system is polygamous and male reproductive success may be
96 highly variable (suggested by results from Dupont et al. 2006; Proestou et al. 2008; Le Cam et al.
97 2009). This variance seems to be linked with the position of individuals within stacks: the stacking
98 process results in older larger males being closer to the females and having higher reproductive
99 success (Dupont et al. 2006; Proestou et al. 2008; Le Cam et al. 2009). The oldest / largest males are
100 thus possibly at a strong advantage, as suggested by Dupont et al. (2006) and Proestou et al. (2008)
101 but is not clear how this tendency in males compares with the strong size-fitness relationship
102 reported for females (Hoagland 1978; Richard et al. 2006; Li and Pechenik 2007; Proestou et al.
103 2008; Le Cam and Viard 2011). It is thus interesting to quantify the reproductive success of males
104 and females of different sizes and test whether the suspected increase of male reproductive success
105 with size is verified: a strong size-effect on male reproductive success could be compatible with
106 protandry if large females have even greater reproductive success than large males.

107 Moreover, reproductive success is only part of the story. The reproductive value of males and
108 females also depends on other factors that could be of importance (e.g. growth and mortality). In
109 particular, male slipper limpets grow faster than females of the same size (Collin 1995), a situation
110 that favors protandrous sex-change. Mollusks (and particularly Calyptraeidae, the family that
111 contains *Crepidula* species) have been the focus of much effort to disentangle the selective forces
112 driving sex-change (Collin 2013). Yet quantitative data on fitness have remained difficult to obtain
113 and the lack thereof is a persistent barrier to understanding the role of mating systems in sex

allocation. In *Crepidula fornicata* as in most other species, such investigations have been limited so far by the relatively low number of broods and adults used in parentage analyses and, consequently, the lack of precise quantitative estimates for male and female reproductive success.

One additional mating-system-linked factor that might be of relevance to both the direction and the timing of sex change in *Crepidula* and many other sex-changing species is sperm storage. Female *Crepidula fornicata* can mate repeatedly and produce several broods over a reproductive season using sperm from multiple males that has been stored, possibly over a long time period (at least one year according to Hoagland 1978, see also discussion in Conklin 1897). Although sperm storage does affect the variance in reproductive success of individuals of both sexes (e.g. Dupont et al. 2006), its effect on the evolution of sequential hermaphroditism has been largely ignored so far, not only in limpets (see, however, discussions in Dupont et al. 2006; Proestou et al. 2008) but to our knowledge also in any other organism. This is in strong contrast with the situation of simultaneous hermaphroditism, where sperm storage was shown to play an important role (Charnov 1996; Michiels 1998; Angeloni et al. 2002). Variations in sperm production, sperm-holding capacity, and sperm displacement have a marked effect on the optimal allocation of resources to male function in simultaneous hermaphrodites (Charnov 1996; Angeloni et al. 2002). In sequential hermaphrodites with internal fertilization, sperm storage could potentially reduce the cost of changing sex, and, more importantly, strongly affect the fitness of individuals of both sexes. This topic deserves investigation.

Finally, there is an interesting additional twist to the sex change system in *Crepidula fornicata*. There have been recurrent discussions in the literature regarding whether the mobility of a male affects its ability to fertilize females. As individuals grow the shell shape conforms to the substrate upon which they grow so that they quickly lose any possibility of movement (Conklin 1897; Orton 1912; Coe 1938). This led several authors to wonder whether small mobile could have a substantially higher success than larger, immobile males (e.g. Charnov 1982, p. 198). This hypothesis has been

139 argued against (Collin 1995) and is either not supported or only poorly supported by genetic
140 parentage studies (Dupont et al. 2006; Proestou et al. 2008; Le Cam et al. 2009). Yet these parentage
141 studies have repeatedly found that a small fraction of larvae were sired by males that were not
142 present in the maternal stack. The role of such small males, which may fertilize females by
143 wandering on the sides of other individuals and roving from stack to stack, remains controversial.

144 The primary goal of this study was to assess the role of the mating system in determining size-
145 reproductive success relationships in a protandrous sex changer in the context of the size advantage
146 theory. To attain this objective, we sampled *Crepidula fornicata* stacks in the wild and maintained
147 them in the lab in an experimental set-up that allowed us to: 1) sample larvae as they were released
148 (several successive broods per female), 2) estimate the reproductive success of males and females
149 through genetic parentage analyses, and 3) identify the determinants of reproductive success with
150 regard to the size advantage model of sex-change. Because sperm storage appeared to have a very
151 important influence on the reproductive success of the study species, we developed a model to test
152 whether sperm storage could theoretically have a significant effect on the timing of sex-change in
153 protandrous hermaphrodites. Finally, observations of an unexpected behavior for several small
154 mobile males prompted us to investigate the effect of male mobility on reproductive success and
155 sex-change.

156

Material and methods for the empirical study

Sampling of adults

Adult *Crepidula fornicata* were sampled in the wild within an area of a few hundred square meters (Baie de Morlaix, North-west Brittany, France) on January 12, 2011. This period corresponds to the very beginning of the reproductive season for *C. fornicata* in this area (Richard et al. 2006; Le Cam 2009, p. 132-133). Stacks were sampled one-by-one by scuba-divers at a depth of 14m within a period of one hour. Two sets of stacks were used in this study. First, 33 stacks of 4 to 15 individuals were selected for our rearing experiment (294 individuals overall). Second, 21 additional stacks of 4 to 15 ind. (total: 190 ind.) were fixed in ethanol and later used to estimate allelic frequencies in the source population. Data from the additional stacks provided an independent reference for downstream parentage analyses (see below and supplementary material).

Experimental set-up

We kept all adult *C. fornicata* (33 stacks) in a common tray with circulating seawater (conditions detailed in supplementary material). Each stack was placed in an individual plastic structure sealed near the bottom with a net (200 µm mesh size) through which water could freely circulate while all released larvae would be retained (Fig.1). Each event of larval release could thus easily be detected and associated with one particular parental stack.

This set-up was maintained from January 12 to September 20, 2011 (i.e. 251 days) for 25 out of 33 stacks. The eight remaining stacks were chosen for in-depth analyses of reproductive success (Table 1) and for logistic reasons they were removed earlier (after 85 days) or later (286 days). Many occurrences of mating were randomly observed over the course of the experiment. Mating partners were identified, and the distance between mating individuals was defined as the number of other limpets (male or female) separating them (i.e. two individuals in direct contact would be at a distance of 0). All larval releases were recorded (see below). Finally, the death of individuals was

recorded (especially during cleaning operations, when most dead individuals would detach from the stack).

Adult traits and genotyping

All adults were fixed in ethanol either when they were found dead during the course of the experiment (see Results), or at the end of the experiment. Each individual was then sexed according to Hoagland (1978), its position in a stack was recorded, the presence of brooded egg sacs was noted, and a tissue sample was taken for genetic analyses. All adults (along with the additional 190 individuals that had been simultaneously sampled in the wild) were genotyped at 9 microsatellite loci: DAYN22, BI13YE17, DA8YN14, CL322, DA5YM24, CL191, CL270, DA4Y003 (Riquet et al. 2011), and CfH7 (Dupont et al. 2006) following the protocols detailed by the authors.

The size of each individual, defined as the shell straight length *L* (largest antero-posterior shell length, following e.g. Richard et al. 2006) was measured with a caliper on the first day of the experiment and after the death of the individual. Shell growth was estimated as the difference between these two measurements. We used the individuals that were reared for 251 days (and alive at this date) to estimate shell growth as a function of the initial size of individuals and their sex. These data were further used to approximate Von Bertalanffy growth parameters without distinguishing between sexes in order to obtain an average growth model that was used in our modeling study (see details in the model section).

Sampling of larvae

Swimming larvae were visible immediately after their release, but because they were contained within the aquariums with the adults they tended to be filtered by the adults (as observed by Pechenik et al. 2004), leading to their being lost over about a 24-hour period. As our goal was to estimate the reproductive success of individuals, we surveyed the presence of larvae as often as

possible: several times per day and at least once every night (around 23h) during the week, and at least once during week-ends.

The eight stacks chosen for in-depth analyses of reproductive success (Table 1) were surveyed intensively until the desired number of larval releases was observed. Note that a single release of larvae could have been a mixture of two broods if two females had released their larvae simultaneously. Similarly, two successive batches of larvae released over a short interval (a few hours) could have corresponded to a single brood. To avoid confusion, we will use the term "larval release" to designate an observation of a batch of larvae in the experimental setup, while the word "brood" will designate the actual brood of one given female (which could only be fully defined after parentage analyses). At this stage of the experiment we did not know the sex of the individuals within the stacks (live individuals cannot be sexed). Based on the number of individuals per stack and prior knowledge of sex-ratio distribution in the sampled population, we aimed for ca. 8 broods per stack in order to obtain more than one brood per female and thus estimate reproductive success of most males and females. To this end, we obtained data from the first 6 to 9 larval releases of each stack (62 in total, see Table 1 and Results).

Each observation of a larval release was treated as follows: 1) all larvae were immediately collected in a measuring cylinder filled with seawater (typical volume *V*=500 mL). 2) After gentle mixing, the larvae contained in several (at least five) 3 mL subsamples were exhaustively counted under a binocular microscope. An estimation of the number of larvae released is then $N = \bar{x} \times V$, with $\bar{x}$ the mean concentration of larvae across n samples. The standard error of *N* was estimated as $s_N = \frac{s_x}{\sqrt{n-1}} \times V$, where s_x is the standard error of larval concentrations estimated across the n 3mL samples where larvae were counted. 3) Finally, a sample of approximately 100-300 larvae was fixed in 100% ethanol for downstream genetic analyses.

Genotyping of larvae

We observed an average brood size of ca. 14000 larvae (see Results), of which only a minute fraction could be genotyped for parentage assignment and reproductive success analyses. The probability of detecting at least one offspring of a father that contributed a fraction *p* of a brood can be calculated as $1-(1-p)^n$ with sample size *n*. We therefore genotyped 48 larvae from each of the 62 larval releases mentioned above (in a few cases more larvae were genotyped). With this sample size the probability of detecting a father that sired e.g. 5% of a brood is 91.5% (99.3% for a father that contributed 10% of a brood, see supplementary material figure S1-A) and the number of larvae from this father expected to be present in one sample is 3 (5 for a father that contributed 10% of a brood, Fig. S1-B). This assumes that one larval release corresponds to one female's brood and each larva can be genetically assigned to its parents. The former assumption was not strictly fulfilled (see results), meaning that the actual power achieved varies slightly around this basic figure. Larvae were washed in phosphate buffer saline (PBS) prior to DNA extraction (Nucleospin96 Tissue Macherey Nagel kit) and genotyped using the same microsatellite loci as the adults.

Parentage analyses

Parentage analyses were facilitated by the fact that a small set of candidate parents (that is, all adults of a given stack) was clearly identified for each genotyped larva. However, previous parentage studies in *Crepidula fornicata* have repeatedly found that some larvae were fathered by males that were not present in the stack where the incubating mother was sampled (Gaffney and McGee 1992; Dupont et al. 2006; Proestou et al. 2008; Le Cam et al. 2009). Hence we used the method implemented in the software COLONY V2.0.4.5 (Jones and Wang 2010) where parent-offspring and sibship relations are simultaneously inferred (including siblings with no candidate parents in the sample pool). Each larval release (represented by 48 larvae) was analyzed independently. The parentage analysis methodology is detailed in the supplementary material.

Reproductive success

Hermaphrodites can produce offspring through the female function (production and brooding of larvae) and the male function (fertilization of eggs). Paradoxically, these two functions can occur simultaneously in sequential hermaphrodites. This happens when the sperm produced by a male has been stored and is being used by one or several females at a time when the male has itself switched to the female sex (note that selfing is not possible in *C. fornicata*: individuals do not store their own sperm after changing sex). The reproductive success of an individual slipper limpet at a given point in time has thus two components: the number of larvae produced through the female function $W^{\female}$ (this happens only in currently female individuals), and the number of larvae sired through the male function $W^{\male}$ (this happens in males and in currently female individuals that inseminated other females before changing sex). The sum of these two components gives the total reproductive success of an individual *i*:

$$W_i = W_i^{\female} + W_i^{\male} \quad (1)$$

In nature the breeding season of *C. fornicata* can extend over a long period (8 months in our sampling area, Le Cam 2009, p. 136), with ample time for females to produce several successive broods per year. In our experimental settings females produced on average one brood every 3 weeks, only a fraction of which was analyzed here (0-6 broods per female, see results). Hence we aimed to measure the "instantaneous" reproductive success of each individual rather than the total number of offspring parented, which would have been influenced by irrelevant experimental parameters (e.g. length of the experiment or date of death of an individual, and number of broods analyzed per female). Moreover, a few of the brood analyses did not produce a reliable estimation of the total number of larvae that they contained, because they were sampled too late after release (and thus some larvae were lost through filtration by the adults). For these reasons we defined estimators of average reproductive success that were independent of the length of the experiment and the number of broods sampled per female.

The reproductive success of a female *i* (through the female function) was therefore simply defined as the average brood size of that female:

$$W_i^{\female} = \frac{1}{B_i}\sum_b N_{ib} \quad (2)$$

where *B* is the number of broods produced by female *i* and analyzed in this study and N_{ib} is the size (total number of larvae) contained in brood *b* of that female. These numbers were directly obtained from the size estimates of larval releases and parentage analyses described above.

Reproductive success through the male function has several components. It depends on the number of successful mates (female partners that bear progeny), the fecundity of these mates, and the proportion of larvae fathered by each individual within each brood. Hence we define:

$$W_i^{\male} = \sum_{j \neq i} P_{ij} W_j^{\female} \quad (3)$$

where P_{ij} is the average proportion of larvae sired by an individual *i* when fertilizing broods produced by female *j*, and the product $P_{ij}W_j^{\female}$ is thus the average reproductive success of individual *i* with female mate *j* (that is, the expected number of larvae parented by individual *i* for an average brood of female *j*). Summed over all successful mates, this gives the total reproductive success of individual *i* through its male function. Average father contributions P_{ij} were estimated from the results of the parentage analyses.

These definitions of reproductive success for male and female functions give the average number of offspring parented per time unit, considering that one time unit = the time needed to produce one brood. These definitions are unbiased with regards to the length of the experiment and the number of broods sampled, but they do not take into account any potential variation in the frequency of brood production among females (see results and discussion). Moreover, fitness components such as the size of the larvae and their survival are also not taken into account here. With these data we analyzed the instantaneous reproductive success of an individual, how it was

299 partitioned into male and female functions, and how it related to the size of individuals and their
300 social environment.

301 Determinants of reproductive success

302 The relationship between reproductive success and adult size was tested in both sexes using
303 generalized linear models (GLM) with a log link function and quasi-Poisson error family. In *Crepidula*
304 *fornicata* size effects were confounded with the effect of the position of individuals within a stack
305 (Dupont et al. 2006; Proestou et al. 2008; Le Cam et al. 2009). We addressed position effects in two
306 different ways. First we drew the distribution of mating frequencies as a function of the distance
307 between mating partners (data observed in all stacks during the whole experiment) and compared
308 that to the distribution that would be expected if distance would not have any effect. The latter was
309 obtained by considering that a male could fertilize any female in its stack with equal probability.
310 Second, we looked at the proportion of offspring sired by a male within a brood as a function of the
311 distance between this male and the mother that produced the brood.

312 **Results of the empirical study**

313 Adult survival and growth

314 Survival of the 223 individuals that were reared for 251 days (25 stacks, see methods) was
315 78% at day 251. The growth of the surviving individuals, expressed as the difference between final
316 and initial shell length, is shown in Figure 2. Growth was markedly stronger in males than females,
317 and growth rate decreased with initial size. A good empirical fit to the data was provided by a
318 polynomial model that included initial shell size, sex, an interaction between initial size and sex, and
319 a quadratic term for the effect of initial size (shown in Fig. 2). This ad-hoc model predicts that a male
320 grows twice as fast as a female of similar size.

321 Parentage results

322 The eight stacks used for reproductive success analyses (Table 1) were composed of 37
323 females and 31 males (sex at the end of the experiment; excluding one small individual that was lost
324 during the experiment and two additional individuals for which the sex was uncertain due to a loss
325 of raw data for stack 7, see table 1). Parentage analyses were based on larval releases sampled
326 between February 4 (date of the first observation of larvae) and May 20 (i.e. days 23-128). In this
327 106 day period we sampled 62 larval releases, corresponding to between 6 and 9 of the first batches
328 of larvae released by each of our eight focal stacks (table 1). These batches contained between 2403
329 and 38860 larvae, with standard errors for these estimates in the range 160-2990.

330 From these 62 larval samples, we obtained a genotype at 5 or more loci for 3240 individual
331 larvae, 3229 of which (99.7%) could be unambiguously assigned to their two parents (including
332 several reconstructed father genotypes that were not found in the adult set, for example because
333 they were dead before the stacks were sampled in the field). These analyses indicated that nine
334 larval releases appeared to be a mixture of 2 broods from distinct females of the same stack.
335 Conversely, on three occasions two successive larval batches appeared to stem from the same brood
336 (larvae released within a short interval, i.e. in the order of hours). Over the entire experiment,
337 parentage data was obtained for between 5 and 11 broods for each stack (total 69 broods, table 1),
338 with an average of 46.7 larvae successfully genotyped per brood. With these data we calculated the
339 reproductive success of males (male function only) and females (female function + delayed male
340 function).

341 Reproductive success of males

342 Out of 31 males, 7 did not achieve any successful mating. The other males successfully mated
343 with up to 3 female partners (Fig. S2). These males sired between 0 and 100% of the larvae of a
344 given brood (Fig. S3; 0% corresponds to when a male did not sire any offspring of a particular brood
345 but successfully sired at least one other brood of the same mother). These numbers remained

similar when averaged over broods of a specific female: the average proportion of offspring P_{ij} sired by male *i* with a given female *j* ranged from 0.6 to 100% (median 27%, mean 35%).

The resulting distribution of total reproductive success for males is shown in figure 3: $W^{\male}$ varied between 0 and 25761 offspring (average 5441). Six males out of 31 were excluded from this computation because they sired offspring with at least one female whose average reproductive success $W^{\female}$ was not estimated (see below). We did not calculate the reproductive success $W^{\male}$ of these males (even though we knew their success with other females) because it would have been underestimated.

Reproductive success of females

Females may combine success through the female and male functions (via sperm stored and used by other females). Out of 37 females, 2 did not have any mates. Others successfully mated with up to 8 partners that may have been either males or females (Fig. S2). The reproductive success of these individuals is detailed below according to sexual functions.

Female function

Out of 37 females, 6 did not produce any broods during the 106 days sampling period (and were not found to incubate any larvae when the stacks were dismantled). We considered that these females had no reproductive success through the female function ($W^{\female}$=0).

The other 31 females produced between 1 and 6 broods. But we obtained an estimate of the number of larvae for only 44 out of the 69 broods that were genotyped (table 1, distribution of brood size shown in Fig. S4) because the remaining broods were sampled too late after release (see methods). These 44 broods were produced by 24 females, with 1 to 5 brood size estimates per female (average 1.83), yielding an estimate of average fecundity for each of these females $W^{\female}$ in the range 2458 – 26400 (average 14063, reduced to 11250 when the 6 females with no reproductive success were included). The seven remaining females produced only broods from which the total

number of larvae could not be properly estimated. We have no estimate of reproductive success through the female function for these individuals (although we know which males they reproduced with, and in what proportions, from the genotypes of their offspring).

Delayed male function

Nearly half of the females (17 out of 37) contributed offspring through the male function. Nine of them fathered offspring with a single other female and the remaining 8 fathered offspring with 2 females. Just like their male counterparts, they sired between 0% and 100% of the offspring of a given brood and average proportions P_{ij} ranged between 0.6 and 100% (median 32%, mean 44%). We were able to estimate the reproductive success through the male function for 12 of these 17 females: $W^{\male}$ ranged 350-21235 offspring (average 8195, average including the 20 females that had no success through delayed male function 3073).

The resulting total reproductive success for females (that is, combining both female and male functions) is shown in figure 3. Females produced from 0 to 32121 offspring (average 15438).

Total reproductive success and its relationship with shell length

The average total reproductive success of females was nearly 3 times higher than that of males (15438 *vs* 5441 offspring; the difference is 10-fold if one compares medians). Note that this is only possible because of sperm storage: many offspring had a female mother and a *female* father. The average reproductive success of females is greater because it combines female and male functions. The reproductive success of females with a male function was on average about 28% higher than that of females without a male function (17703 vs 13880).

Figure 4 shows the relationship between reproductive success and individual size in males and females (size at the onset of the experiment). Male shell length ranged from 7.6 to 51.3 mm while female shell length ranged from 35.6 to 57.3 mm. The reproductive success $W^{\male}$ of males smaller than 22 mm was nil, and it increased with male size (GLM, p=0.025). Female success through the

394 female function $W^{\female}$ or through both male and female functions $W^{\female} + W^{\male}$ did not significantly
395 increase with female size (GLM, p=0.68 and 0.786, respectively), although we note that the power of
396 this analysis was limited by the restricted range of female sizes, in addition to the intrinsic
397 uncertainty associated with fecundity estimates.

398 Other determinants of fitness

399 The vast majority of mating observed during the experiment (n=145, Fig. S5A) happened
400 between individuals that were directly in contact (distance=0, frequency=40%) or separated by only
401 one other individual (distance=1, frequency=42%), although one mating was observed while the
402 male was separated from the female by as many as 6 individuals. This distribution differed clearly
403 from expectations of random mating within each stack, where mating at distances of 0 to 3
404 intermediate individuals should represent ca. 15% each (Fig. S5A).

405 Within a given brood, the proportion of larvae sired by a male appeared strongly constrained
406 by the distance between this male and the mother of the brood (Fig. S5B). These proportions ranged
407 0 to 100% at distances of 0 and 1 and decreased strongly at larger distances. However, some outliers
408 corresponding to two males that fathered a large fraction of the offspring of a distant female were
409 identified (Fig. S5B).

410 Solitary mobile adults

411 As mentioned in the introduction, small individuals have the capacity to move and, in the field,
412 some males may use this ability to get closer to females and perhaps to fertilize females from
413 different stacks. During the experiment we observed 30 individuals (that is, 10% of the 294
414 individuals from the 33 stacks reared in the lab) that moved away from their initial position. Only
415 one of them changed position within its stack. That individual left its position at the top of a stack of
416 eight individuals and adopted a secondary position on the side of the fourth individual. All other
417 movements observed were rather surprising: 29 individuals left their original stack (as early as day

418 14) and attempted to leave the plastic structure in which each stack was contained. They could not
419 succeed because it would have meant going above water (Fig. 1), but none of them came back on
420 the stack. They remained on the plastic walls, sometimes halfway above water, for the rest of the
421 experiment. We will refer to these individuals as "solitary".

422 These individuals were found to be males when they were sexed except for two individuals
423 that were in the process of changing sex and one other that was immature. All of these solitary
424 individuals were either initially positioned on the side of another individual (secondary position) or
425 at the top of a stack (including some individuals that were initially 2nd or 3rd from the top before
426 males above them moved away). Their average size was 25.2 mm (range 9.3-38.8) at the onset of
427 the experiment. The movement of these individuals made it difficult to keep track of their identity
428 throughout the experiment, so that we know both the initial and final size with certainty for only
429 eight of them. They were reared for a variable period of time; hence we estimated their growth rate
430 per day, for comparison with the growth of others. The shell length of these eight solitary individuals
431 grew at an average rate of 0.008 mm per day. The average growth rate of all other males in the
432 same size range (9-40mm, n=53) was 0.031 mm per day.

433 We obtained reproductive success measurements for 6 such solitary individuals (included in
434 the overall fitness estimates produced above for males). Surprisingly, each of them successfully
435 fathered larvae during the experiment. They successfully sired offspring from one (n=4) or two
436 females (n=2), and the average proportion of offspring P_{ij} sired by solitary male *i* with a given
437 female *j* ranged from 1.4 to 100% (median 32%, mean 42%). As a result, they achieved a
438 reproductive success $W^{\male}$ of between 240 and 10335 offspring (mean 4534).

439 **Model**

440 Empirical results from this study suggested that sperm storage might favor individuals that
441 change sex at an earlier time than predicted from the size-advantage hypothesis because once a

male has inseminated several females, further gain in reproductive success may be limited (see discussion). However, this effect is predicted to depend on the dynamics of sperm replacement in the sperm storage organs of females. In order to investigate these ideas we developed a simple model of reproductive success for a sequential hermaphrodite species with or without sperm storage. Here we outline the model framework, and a more detailed description is given in the online supplementary material.

Model outline

To investigate the effect of sperm storage on the timing of sex-change we simulate the reproductive success of an individual (ind. number 3 in Fig. 5) that interacts with one male and two females. Time is divided into discrete units and each female in the system produces one brood per time unit. Our focal individual starts its benthic life at size *L*=0 and grows at each time step. We then calculate the reproductive success that this individual would have as a male or a female at each time step, with or without sperm storage.

In the absence of sperm storage, the reproductive success of our focal individual as a male ($W^{\male}$) is determined simply by the fecundity of its female partners (brood size *N*) and the fraction of sperm φ that our focal male provides to inseminate these females. With two females we have $W^{\male} = 2\varphi N$ at any time step and the male function stops as soon as the focal individual becomes female. In contrast with the male function, reproductive success through the female function depends on the size of the focal individual, following the size-fitness relationship reported by Li and Pechenik (2007).

The lifetime reproductive output (ω) of an individual is maximized by a protandrous strategy with the sex-change occurring when the individual reaches a size at which its reproductive success as a female becomes superior to its reproductive success as a male (in agreement with the size-advantage model, Ghiselin 1969). Sperm storage modifies reproductive success by allowing a female

466 to continue fathering offspring for some time after sex-change, thus combining fitness output
467 through male and female functions. However, success through the male function may decline
468 rapidly, depending upon the dynamics of sperm reserves in inseminated females. We consider that
469 females have sperm reserves that contain a proportion *p* of sperm from our focal male and the
470 reproductive output of our focal individual through the male function at any time step t is $W_t^{\male} =$
471 $2p_tN$. If the sex-change has not happened, the focal individual is a male and we assume that it
472 produces sperm continuously so that the fraction of its sperm in females' reserves is equal to the
473 fraction of sperm φ that our focal male provides to inseminate these females. After sex-change, we
474 consider two situations (referred to as models 1 and 2 in Fig. 5). First, our focal individual (now
475 female) stops producing sperm and thus the proportion of its sperm in the reserves decreases at
476 some rate *m* following $p_{t+1} = p_t(1 - m)$. In that case its reproductive success through the male
477 function $W_t^{\male} = 2p_tN$ will progressively decrease until all of it stored sperm is replaced by that of the
478 other male. In a second situation, the remaining male (number 4 in Fig. 5) shifts its mating effort
479 toward the newly available female as soon as our focal individual has changed sex. To illustrate this
480 point we consider the extreme situation where the top male no longer contributes sperm to the
481 most distant female (note that this extreme situation is quite realistic considering the effect of
482 distance between mating partners in our empirical results, see distance=2 in figure S5A). In that case
483 the proportion of sperm from our focal individual that is stored in female 1 remains constant after
484 the sex-change, because the female storage organ is not refilled by any male. Only in female 2 does
485 the proportion of sperm from the focal individual decrease at rate *m*. The reproductive success
486 (through the male function) of our focal individual after the sex-change thus becomes $W_t^{\male} = \varphi N +$
487 p_tN.

488 To test for the effect of sperm storage we considered a baseline scenario (Fig. 6) where the
489 parameters φ and N were held constant and we varied only sperm mortality (*m*) and the timing of
490 the sex-change. With this scenario the focal male sires 60% of the offspring produced by each female

($\varphi = 0.6$) and female fecundity is set to *N*=14000 so that $W_t^{\male} = 16800$ offspring (grey solid line in Fig. 6). If our focal individual is a female throughout its life, its reproductive success $W_t^{\female}$ varies from 0 at time *t*=0 to ca. 20700 offspring at time *t*=100 (black line in Fig. 6). Under these conditions the strategy that maximizes the reproductive value of the focal individual is to switch from the male to the female sex at time *t*=47, when $W_t^{\female}$ gets larger than $W_t^{\male}$. The optimal strategy is highlighted with a thick grey line in figure 6. Sperm storage changes this prediction by allowing a female to continue fathering offspring for some time after the sex-change, thus combining fitness output through male and female functions. An example is given in figure 6, where sex-change occurs at time *t*=20 (compare reproductive success with and without sperm storage, shown by the dotted line and the dashed line, respectively). We determined the optimal timing of the sex-change (that is, the timing that maximizes lifetime reproductive output) with different values of sperm mortality *m*=0, 0.01, 0.05, 0.1, 0.2, and 0.3.

To further understand the effect of sperm storage we calculated the lifetime reproductive output ω^t of an individual that changes sex at time *t* (with sperm storage occurring) and compared it to the reproductive output ω^* of an individual that follows the optimal strategy for a situation where there is no sperm storage (i.e. sex-change at time *t*=47). The difference $\Delta\omega = \omega^t - \omega^*$ is equal to the net difference in lifetime reproductive output between these two situations and can be visualized in figure 6 as the area between the dotted line and the thick grey line. We calculated $\Delta\omega$ for the different values of sperm mortality *m* listed above and with sex-change occurring at time *t*=10, *t*=70, and at every 5-generations interval between these two limits.

Model results

In the baseline conditions chosen to illustrate our point, an individual that follows the optimal strategy in absence of sperm storage has an expected lifetime reproductive output of $\omega^* \approx 1.84\times10^6$ offspring. Sperm storage increases this lifetime reproductive success in nearly all situations (Fig. S6), except when sex-change occurs very early and sperm mortality is high (*t*<25,

516 m>0.05). However, it does not necessarily mean that the optimal timing of sex-change is altered by
517 sperm storage. The moment when changing sex yields the highest lifetime reproductive output is
518 shown in Figure 7 (also visible as the mode of each curve in Fig. S6). The model indicates that earlier
519 sex-change is favored only when sperm mortality is quite low (m<0.1). This prediction changes if
520 sperm proportions remain constant in one of the females (model 2). In that case, the highest lifetime
521 reproductive success will be obtained for an earlier sex-change in all conditions (Figs. 7 and S7).

522

523

Discussion

Three notable patterns emerged from our reproductive success measurements on male and female slipper limpets: 1) male success increased strongly with size, 2) sperm storage was a remarkably strong driver of reproductive success, and 3) small mobile males were identified, and they had a significant level of reproductive success is. We will consider these results in light of the size advantage theory for protandrous sex-change.

Size-fitness curves in *Crepidula fornicata*

The reproductive success of females was on average 50% greater than that of males in the same size range (i.e. all individuals with shell length L > 35.5mm, n_{males}=9, $n_{females}$=27, average male success=10433 offspring, average female success=15438). This indicates that changing sex is beneficial in terms of immediate fitness. In agreement with the size advantage hypothesis, individuals change sex at a condition (linked with size or age) that allows them to produce more larvae through their own fecundity than they would sire as a male.

Remarkably, this result was obtained despite the fact that male success increased sharply with size, confirming results from Dupont et al. (2006) and Proestou et al. (2008). While we do not know whether male body size has a direct effect on reproductive output (e.g. through variations in sperm production), size is correlated with the position of males within a stack and this has a critical influence on reproductive success (Dupont et al. 2006; Proestou et al. 2008; Le Cam et al. 2009, this study). Due to the stacking process, males find themselves closer to females as they age/grow, and we found this to be a primary determinant of the male size-fitness curve. The two major components of male reproductive success, that is, mating success and the proportion of offspring sired within a given brood, are both strongly affected by the distance to females. More than 80% of all our mating observations occurred between individuals in direct contact or separated by only one other individual (Fig. S5A), and the male in such matings is expected to sire on average nearly half of

548 the offspring of a given brood (Fig. S5B with distance to female=0 or 1). However, this advantage of
549 large-males does not outweigh the gain in immediate fitness expected from the sex-change, since
550 females in this study had a greater average reproductive success. The situation found in *Crepidula*
551 *fornicata* therefore illustrates that changes in male reproductive success with size (here due to the
552 species' social system) are compatible with protandry, as expected from theory.

553 In contrast to males, a female's reproductive success depended almost exclusively on her own
554 fecundity. With the exception of solitary females (which occur when juvenile limpets are not in
555 contact with conspecifics and rapidly adopt the female sex, e.g. Coe 1938), all females in the wild
556 have at least one potential mating partner, and we found that above one the number of mating
557 partners has no effect on a female's total reproductive success (not shown). Female fecundity has
558 been shown to be linked with size (Hoagland 1978; Richard et al. 2006; Li and Pechenik 2007;
559 Proestou et al. 2008). However, here we did not observe this relationship because the females in our
560 stacks had a very limited size range (they were all rather large females) and perhaps also because of
561 the uncertainty in our fecundity estimates. Yet we found that female growth was much less rapid
562 than male growth (Fig. 2), confirming previous findings by Collin (1995). Hence very little additional
563 gain in reproductive success is to be expected once an individual has changed sex (at least in the size
564 range of females from our study), probably because most of the individual's energy is allocated to
565 the immediate production, encapsulation, and brooding of eggs rather than to additional somatic
566 growth.

567 Bringing together these observations for male and female reproductive success, we found that
568 large individuals had an advantage when they were female but that there was no clear advantage for
569 small individuals in being male. Our data indicate that small males have very little or no reproductive
570 success. Based upon two previous studies that reported quantitative relationships between shell
571 length and fecundity, expressed as the number of offspring (Li and Pechenik 2007; Proestou et al.
572 2008), small males from our study would have been predicted to have had the same or greater

success if they had been females. In terms of immediate fitness, there is thus no indication that being a male is a fitter option than being a female at any point during an individual's life. What is important for the evolution of sequential hermaphroditism, however, is the trajectory of sex-specific reproductive values, rather than immediate success. The faster growth of males is one factor that will create a difference in reproductive values between sexes. Because there is no clear immediate advantage in being male at any point in life, we conclude that the faster growth rate of males is an important factor that will favor protandry, by allowing individuals to rapidly reach a size that confers high female-fitness. That conclusion however needs to be pondered in light of the effect of sperm storage and accrued mobility of small males.

Does sperm storage influence the timing of sex-change in sequential hermaphrodites?

A striking result from this study is the influence of stored sperm on reproductive success. This is particularly interesting from the females' point of view: nearly half of the females contributed offspring through the male function, and these females had an average reproductive success about 28% higher than the others. This seems to have important consequences for the timing of the sex-change, because without this fitness component the advantage of being a female is reduced (compare the dotted and dashed lines in Figure 4). Females that did not father any offspring had an average reproductive success of 13880 offspring, compared with 10433 for males in the same size range. This difference is rather small given the number of individuals for which we could estimate reproductive success and the number of broods on which each of these estimates was obtained.

Moreover, sperm storage might also affect the success of the males closest to the females. Because these males monopolize most mating opportunities one can imagine that the sperm storage organs of the females below them are filled mostly with their sperm. If further sperm transfer has diminishing returns in terms of the proportion of offspring sired in upcoming broods, then it would

be beneficial to switch to the female sex earlier than predicted from body size. This benefit, however, is likely to be temporary, depending upon the duration of sperm storage, direction of sperm precedence, and the modalities of sperm replacement.

Modeling the simplest possible system, we found that sperm storage alone does not automatically promote an earlier sex-change. In many situations the fitness benefit obtained through sperm storage is highest at the same moment as the optimal timing of sex-change in the absence of sperm storage (Fig. 7). That is, sperm storage increases an individual's fitness but does not necessarily alter the timing of the sex-change that would maximal lifetime reproductive success.

This result holds as long as sperm replacement is rapid (i.e. m>0.05 in Fig. 7). If sperm is lost or replaced at a slower pace, then sperm storage can result in earlier sex-change because under these conditions the delayed male function persists throughout a female's lifetime. Sperm viability and replacement rate are thus key factors, as was shown to be the case in the context of sex allocation in simultaneous hermaphrodites (e.g. Charnov 1996). Empirical estimates, however, are difficult to obtain. Using our parentage data, we found one situation where a ballpark figure for our parameter m could be estimated (Fig. 8): the female at the base of stack 1 (individual 1 in figure 8) repeatedly produced broods partly sired by the second female of that stack (individual 2). That is, individual 2 was already a female when the experiment began, but its sperm stored in female 1 was repeatedly used to fertilize that female's eggs. Four consecutive broods produced by female 1 were analyzed for this study (16 to 19 days between successive broods). We genotyped and analyzed one additional brood produced 180 days later by the same female (n=87 larvae). A decay model fitted to these data (see supplementary methods and figure 8) gave an estimate of m=0.14. This rough estimate falls into the region where sperm storage might not have any effect on the timing of the sex-change, under the conditions of our model. While this estimate illustrates only one situation (namely, a four-individual stack where the female father that we looked at was in competition with two other potential fathers), it suggests that sperm storage could have little effect on the timing of the sex-

622 change if stacks generally contain a small number of individuals (because in such stacks females
623 would never be far from males and they would receive new sperm continuously).

624 A situation where sperm storage would favor an earlier sex-change, even for high rates of
625 sperm mortality, would occur when the sex-change prompts the remaining males in the stack to
626 shift their copulation effort towards that new female (model 2 in Fig. 5). In that case sperm
627 competition would be relaxed in the older females because they would receive less sperm (possibly
628 none). Previous sperm donors would thus benefit from sperm storage for longer periods of time.

629 Parentage analyses from our empirical study revealed at least one situation where sperm
630 storage must have benefited one father over a long time period. The female at the base of our
631 largest stack (stack 8, *n*=15 ind., including 6 females) produced two broods that we analyzed. With
632 the exception of one larva (out of 47), the first brood appeared to be fertilized by sperm from a
633 single female (third individual in the stack, starting from the base). The second brood, produced
634 three weeks later, gave the same result (46/47 larvae fathered by female 3). Because the first six
635 individuals of this stack were females at the end of the experiment, it is likely that the basal female
636 had not been receiving any sperm for a long time, giving female 3 a long term advantage (*m* was
637 effectively equal to 0 in the three week interval between the two broods that we analyzed). Such a
638 pattern may be common in the wild: a nine-year long monthly survey of our source population
639 showed that 25% of the stacks contained 4 females or more (*n*=3693 stacks analyzed, S. Le Cam, F.
640 Riquet, F. Viard, unpublished data).

641 Finally, we note two additional effects of sperm storage that have a bearing on sex-change.
642 First, sperm storage reduces the immediate cost of changing sex to nearly zero. Second, females
643 with little or no access to males (e.g. in stacks containing more than 4 or 5 females) can continue to
644 produce offspring as long as their sperm stores are not emptied. We conclude that sperm storage
645 might influence the timing of the sex-change in protandrous species with internal fertilization (e.g.
646 all calyptraeids), a topic that deserves a more detailed theoretical treatment. Empirical research

focusing on processes of sperm viability and competition (including sperm precedence and postcopulatory sexual selection) would also be particularly informative.

Small mobile males: an alternative strategy, or is it?

It was quite surprising to watch small males (range 9.3-38.8 mm) rapidly moving away from their stack and remain isolated for months without any attempt to return to their original stack. It was all the more surprising to realize that all six such individuals that were included in our fitness measurements actually had successfully sired a rather large quantity of offspring (average reproductive success estimated to 4534 offspring, which is just below the average for all the other males, 5727). Had these males successfully integrated a new stack, it is very likely that they would have achieved better reproductive success. Note that sperm storage is fundamental to this strategy.

These results therefore settle the debate as to whether small mobile males exist, and whether these males father a significant number of offspring. This behavior does exist, and it explains why a fraction of offspring were assigned to unsampled males in all the paternity analyses that have been performed so far with *Crepidula fornicata* stacks (Dupont et al. 2006; Proestou et al. 2008; Le Cam et al. 2009, this study). The fitness of small mobile males was no better than that of large males that were in direct contact with females (as wondered by Charnov 1982), but neither was it markedly worse, and fitness could be further increased if small mobile males were able to rove freely from stack to stack, an hypothesis that remains to be tested. Based upon our data, the advantage conferred by mobility to small individuals did not alter the general pattern of increase in male reproductive success with size (solitary males were included in all calculations). But these data ignore the success that mobile males could have had in neighboring stacks.

An interesting question is left unanswered: does the behavior of the small mobile males represent an alternative strategy (see reviews e.g. in Taborsky et al. 2008; Neff and Svensson 2013),

671 in which individuals would engage in exploratory movement, grow more slowly, and perhaps change
672 sex later or not at all, or do most or all individuals adopt this behavior when they are young before
673 associating with a stack and waiting for their turn to become close to the females and eventually
674 change sex? The fact that many of the solitary males observed in this study were actually first
675 observed on top of a stack (in some cases after the male above them had left) suggests that males
676 may pile up and then temporarily leave their position at some point to rove down the stack towards
677 the females or try to reach other stacks. Also in favor of the second hypothesis, it seems unlikely that
678 the reproductive value of an individual that remains a small male for all of its life would exceed that
679 of a male that grows up quickly and at some point monopolizes fertilization with one or two females
680 and then switches to the female sex. The mobility strategy could pay off, however, in very dense
681 populations with large stack sizes, a situation that has been observed in some areas in the
682 introduced range of *Crepidula fornicata*. The density of the population where our adults were
683 collected was estimated twice per year in 2002, 2003, and 2004 by L. Dupont and colleagues
684 (Dupont 2004 p. 69), yielding an average density of 21 ind.m^{-2}, although with a very patchy
685 distribution (maximum local density = 201 ind.m^{-2}). It seems possible that mobile males could
686 fertilize females from distinct stacks under these conditions.

687

688 Limits of this study

689 One original contribution of this study in terms of the data produced is that reproductive
690 success was measured from sequences of broods (up to 5 broods from a single female), and that a
691 large number of larvae from each brood was analyzed (46.7 larvae on average). However, we could
692 not estimate brood size in all cases, a frustrating limitation that led us to work only with the average
693 reproductive success for males and females (calculated from a small number of broods per female)
694 and prevented more detailed analyses (e.g. covariance between brood size and father identities).
695 Notwithstanding our efforts to analyze multiple broods, the data presented here give only a

696 snapshot of an individual's total reproductive output (*Crepidula fornicata* reproduces continuously
697 during an extended breeding season in the wild). Thus one main limit of this study is that it ignores
698 several components of fitness. We do not know whether the frequency of brood production is
699 variable among females, and, most importantly, if that variance is linked with size. There is no
700 obvious reason why the development of larvae should be quicker for larger mothers, but such a
701 relationship could alter some of our conclusions (e.g. regarding the female size-fitness curve).
702 Furthermore, while our data are informative on the role of sex and size on the "instantaneous"
703 reproductive success per brood produced, downstream components of fitness such as larval growth
704 and survival remain unexplored (see Le Cam et al. 2009 for an effect of multiple paternity on the
705 variance in larval growth).

706 Finally, as mentioned above, the success of males outside the stack where they were sampled
707 is unknown. If male mobility between stacks is more extensive than currently thought, then it would
708 be important to take extra-stack paternity into account to refine the male size-fitness curve. Male
709 mobility between stacks could be relevant to female fitness as well, because mobile males could
710 have some (currently unknown) reproductive success through delayed male function in neighboring
711 stacks. In theory this point could be tested by genotyping all adults from within a certain radius in a
712 moderately high density population, and seeking paternity for a sample of broods from incubating
713 mothers.

714

715 **Conclusion**

716 Size-fitness curves established for male and female *Crepidula fornicata* were essentially in
717 agreement with predictions from the size advantage hypothesis of sex change. Most importantly,
718 females were large individuals and had a higher average reproductive success than males of equal
719 size. On the other hand, the immediate advantage of being male for small individuals is less

apparent, mostly because the mating system of *Crepidula fornicata* is advantageous to large males, as their size and/or position grant them both a higher mating success and fertilization success. We suggest that the higher growth rate of males, and thus the increased reproductive value of individuals which start their life as males, confers a decisive advantage to protandry in *Crepidula fornicata*. Male mobility might also contribute to the immediate reproductive success of small males if they succeed in copulating with females from distinct stacks, a hypothesis that is yet to be tested. We found that sperm storage affects strongly the reproductive success of males and females, and that it could theoretically promote earlier sex change in protandrous species. Missing information on the production of sperm, holding capacity in female organs, and sperm precedence and displacement processes are determinant in this regard. Overall, *Crepidula fornicata* provides an example of a protandrous species where male fitness increases quite sharply with age/size but protandry is still adaptive because large females have an even higher fitness, in part due to sperm storage effects.

Acknowledgements

We thank the following people for their input in this study through invaluable discussions, suggestions, or technical help: Mark Cock, Thierry Comtet, Claire Daguin-Thiébaut, Regis Lasbleiz, Sabrina Le Cam, Nicolas Perrin, Florentine Riquet, Denis Roze, Eric Thiébaut, and Jon Yearsley. Editors Susan Kalisz, Peter Nonacs, and two anonymous referees helped improve the manuscript. We are grateful to the divers of the Marine Operation Department of Roscoff biological station for sampling adult limpets in the wild and we thank the Biogenouest Genomics and Genomer plate-forme facilities for technical support. This study was funded by Region Bretagne through a grant from the SAD funding program to TB.

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811

Tables

Table 1: Description of the eight stacks chosen for reproductive success analyses. We report the total number of individuals in each stack, the number of females, the number of larval releases analyzed per stack, and the number of actual broods identified from these batches after parentage analyses (see text). The number of broods from which female fecundity (i.e. brood size) could be estimated is given in the last column.

Stack	No. Ind.	No. females	No. larval releases	No. broods	No. brood size estimates
1	4	2	8	11	9
2	5	3	6	5	4
3	6	5	8	9	8
4	8	4	8	8	5
5	10	4	8	9	3
6	11	6	9	9	4
7	12	7[a]	7	9	3
8	15[b]	6	8	9	8
Total	*71*	*37*	*62*	*69*	*44*

a) The sex of two individuals from stack 7 was unknown due to a loss of raw data. The minimum number of females in this stack was 7.

b) Including one small individual (10.6 mm) that was lost during the experiment, probably during cleaning operations. This individual is not included in any of the analyses.

Figure legends

Figure 1: Experimental set-up. Thirty-three stacks of 4 to 15 *Crepidula fornicata* sampled in the wild were kept in the lab. Each stack was reared in an individual structure allowing us to collect larvae as they were produced, without any mixing of larvae from other stacks. Photo credit: Wilfried Thomas, Marine Operation Department, Station Biologique de Roscoff.

Figure 2: Shell growth observed during the experiment for the individuals from 25 stacks that were kept alive in the lab for 251 days. Shell length at the start of the experiment was measured from living individuals in stacks, hence there is some uncertainty associated with such measures. Growth predictions from a polynomial model (lines) highlight the difference in growth rates between sexes.

Figure 3: Distribution of the reproductive success of adult *Crepidula fornicata* (24 males and 27 females from 8 stacks, that is, males for which an estimate of the male function was obtained and females for which estimates of the male and female functions were both obtained). Reproductive success is defined as the average number of offspring parented per brood produced (see text). Some individuals (black bars) that recently changed sex from male to female combine reproductive success through the female function (production of eggs) and the male function (male gametes produced prior to sex-change and now used by older females through sperm storage). Here we see that such females are frequent in the group of individuals showing the highest success (e.g. > 20000).

Figure 4: Reproductive success of adult *Crepidula fornicata* as a function of their sex and size. A female's reproductive success has two components: the number of larvae produced ("female function": empty black dots) and the number of larvae fertilized by male gametes produced prior to sex-change and stored by older females ("delayed male function"). The sum of these two components is shown by the solid black dots. Arrows show how this delayed male function (sperm storage) increases a female's fitness. The fitness of females does not increase significantly with their size, whether one considers only the female function (dotted line) or their total fitness (combining

male and female functions, dashed line). Male fitness (solid grey dots) increases significantly with shell size (solid grey line).

Figure 5: Cartoon of the system considered for modelling the effect of sperm storage on reproductive success. We start with a situation where 2 males contribute sperm to 2 females at each time step in proportions φ and $1-\varphi$. We follow the reproductive success of individual number 3, which may change sex at some point. We consider two cases. In model 1, sex-change of our focal individual results in male 4 contributing all sperm to females 1-3. In model 2, reproductive effort from male 4 will switch towards the newly available female, so that the most basal female does not get any sperm and relies only on stored sperm to produce larvae.

Figure 6: Example dynamics of reproductive success from the model 1 depicted in Figure 5, with or without sperm storage. The solid lines represent the reproductive output of the focal individual considered in our model if it remains a male (grey line) or a female (black) throughout its life. The strategy highlighted with a thick grey line shows the optimal strategy in absence of sperm storage (sex-change at time t=47). The dashed and dotted lines represent the reproductive success in case of sex-change at an earlier time t=20 without or with sperm storage, respectively. In this example m=0.05, φ=0.6.

Figure 7: Optimal time of sex-change as a function of sperm mortality rate m. In absence of sperm storage lifetime reproductive success is maximized with sex-change happening at time t=47 (dashed grey line). Earlier sex-change is predicted in a situation where sperm is stored and does not disappear too rapidly (model 1, solid black curve) or is not fully replaced by sperm from other males (model 2, solid grey curve).

Figure 8: Decay of the proportion of larvae from stack 1 mother 1 sired by individual 2 (which was already female when the first brood was produced, here represented at time t=0). Each dot represent data from one brood from mother 1, and grey bars show standard error of the proportion

of larvae sired by individual 2. The first four data points come from four consecutive broods, starting February 10, 2011, with an average interval of 18 days (= one time unit). The final brood was produced August 9, 2011 (exactly 180 days after the first one). The black line shows a model of the form $p_t = p_0(1-m)^t$, with best fit obtained for p_0=0.21 and m=0.14 (adjusted R^2= 0.93). This observation suggests that the fertilization success of individual 2 with female 1 decreases at rate m=0.14. Under the simplest rules of sperm replacement (fair raffle, no sperm precedence, as in our model) it can be interpreted as a sperm mortality rate of 0.14. This decay rate can also result from more complex, unknown, sperm replacement rules.

Figure 1

Figure 2

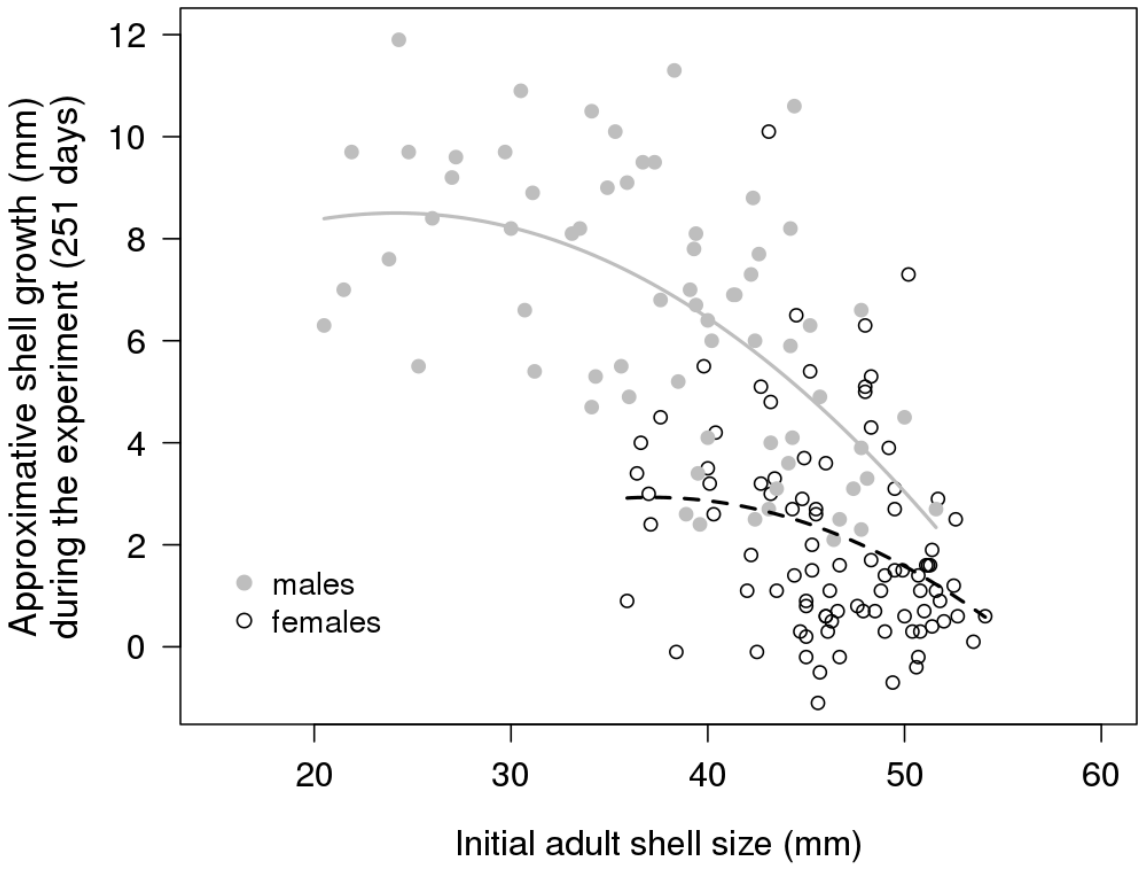

Approximative shell growth (mm)
during the experiment (251 days)
12
10
8
6
4
2
0
males
females
20
30
40
50
60
Initial adult shell size (mm)

Figure 3

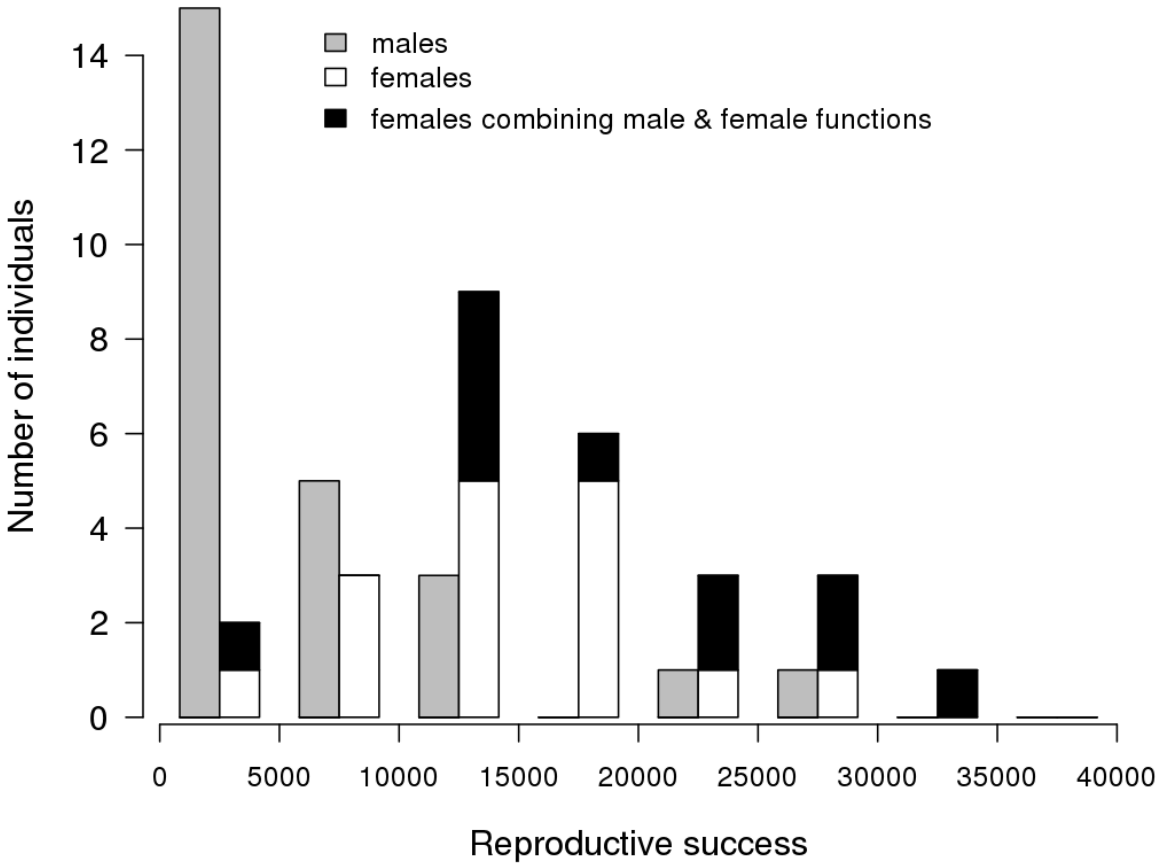

males
females
females combining male & female functions
Number of individuals
0
2
4
6
8
10
12
14
0
5000
10000
15000
20000
25000
30000
35000
40000
Reproductive success

Figure 4

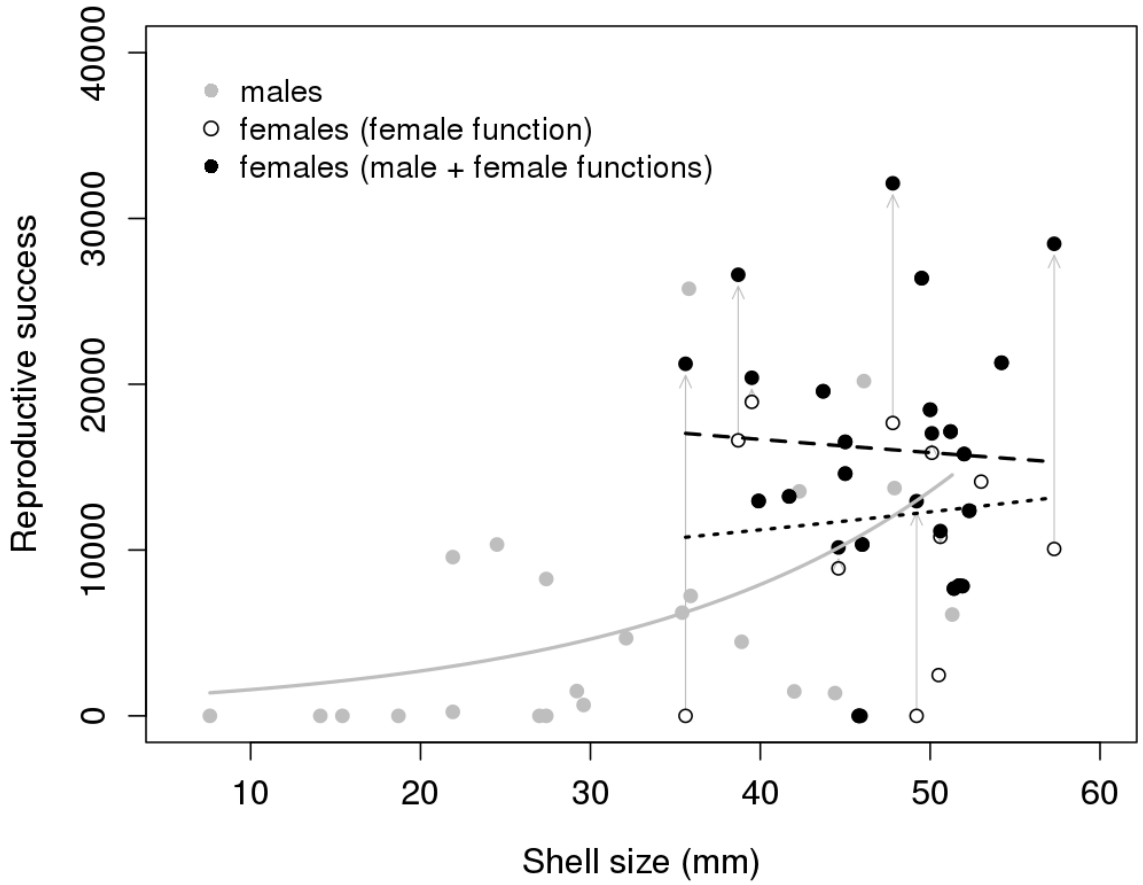

males
females (female function)
females (male + female functions)
Reproductive success
0
10000
20000
30000
40000
10
20
30
40
50
60
Shell size (mm)

Figure 5

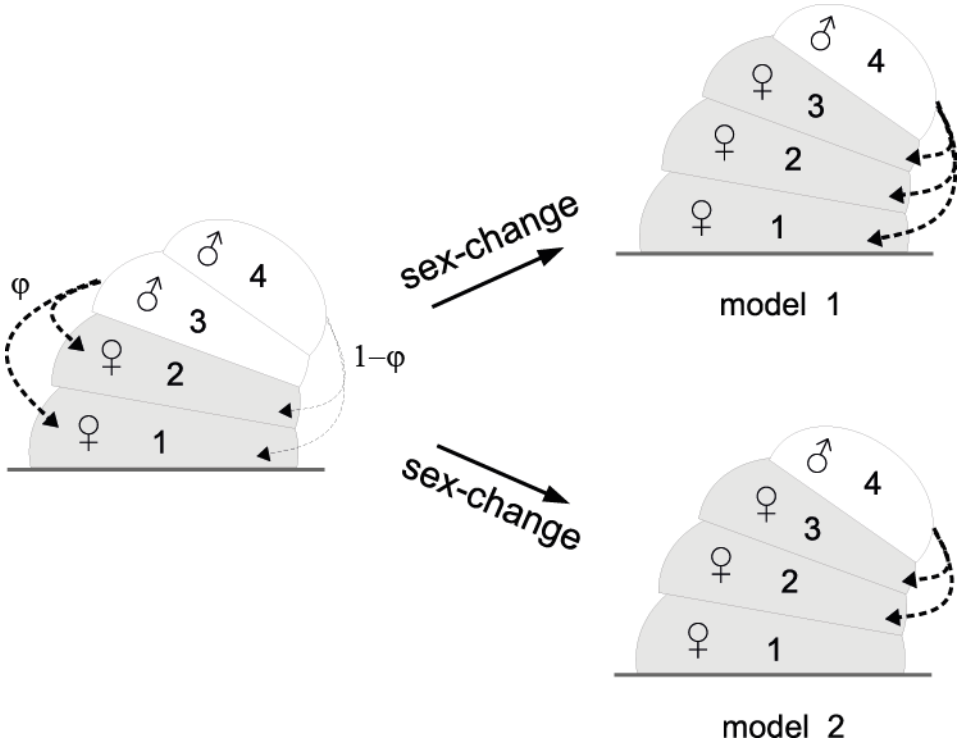


Figure 6

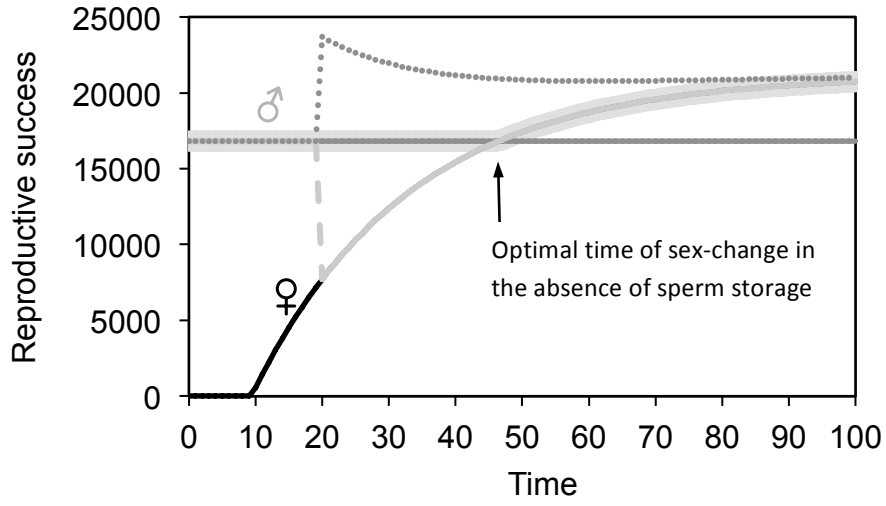

25000
20000
15000
10000
5000
0
Reproductive success
♂
♀
Optimal time of sex-change in
the absence of sperm storage
0
10
20
30
40
50
60
70
80
90
100
Time

Figure 7

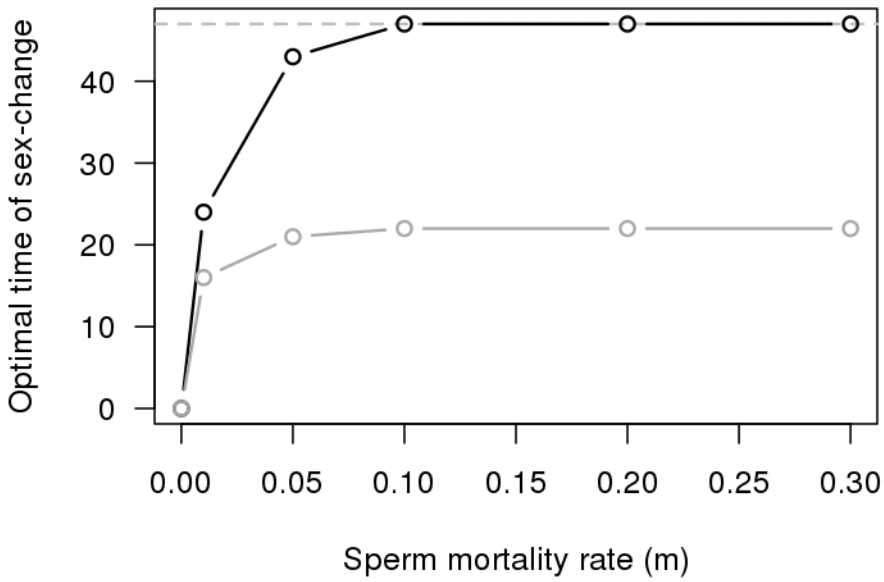
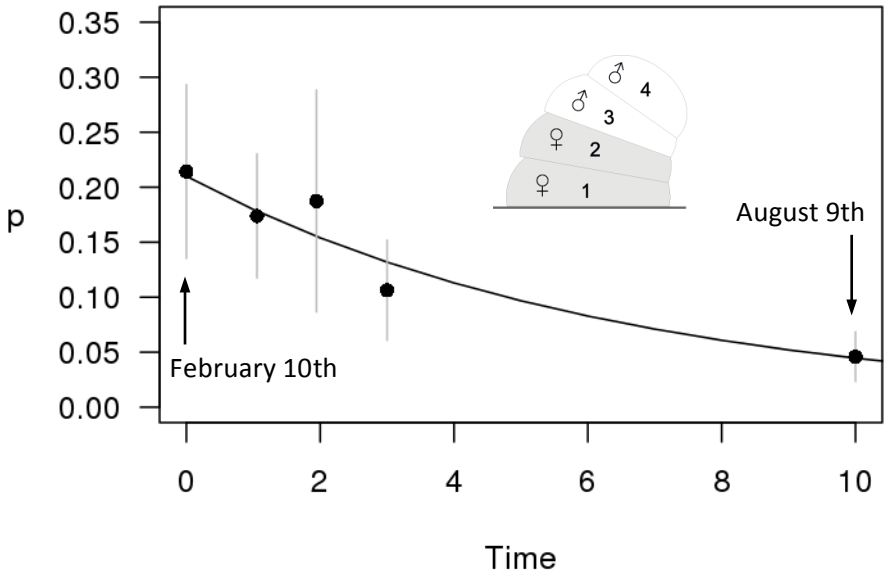

Optimal time of sex-change
0
10
20
30
40
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Sperm mortality rate (m)

Figure 8


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
p
0
2
4
6
8
10
Time
February 10th
August 9th
♂ 4
♂ 3
♀ 2
♀ 1

Supplementary methods

Experimental set-up

We kept all adult *C. fornicata* (33 stacks) in a common tray with circulating 5µm-filtered seawater, constantly renewed at a rate of 10 $L.h^{-1}$. The stacks were placed randomly within the tray just after their collection in the wild. Seawater temperature was 9°C at the collection site. Water temperature was gently increased from 11 to 16°C during the first 8 days of the experiment and then kept constant to 16°C afterwards. The limpets were fed daily with an average of 5.4 L of *Isochrysis galbana* [$24.6 \cdot 10^9$ cells per liter] mixed with 5.2 L of *Chaetoceros gracilis* [$15.6 \cdot 10^9$ cells per liter]. Half of this mixture was given at once during the day, after which water renewal was stopped for a few hours. The other half was distributed continuously during the night (slow automatic distribution). During week-ends ca. 10L of the same microalgae mixture was distributed continuously. All plastic structures containing the stacks (main text Fig.1) were cleaned every 1-3 days.

Parentage analyses with software COLONY (Jones and Wang 2010)

Each larval release (represented by 48 larvae) was analyzed independently with the full-likelihood approach with one run of "medium length" and "high likelihood precision". Since the experimental set-up (Fig. 1) allowed us to associate each larval release with a unique stack, all females of that particular stack were designed as candidate mothers in COLONY. The set of candidate fathers included all individuals of the stack but the most basal one (because any female but the most basal one in the stack could have produced male gametes prior to sex-change in the recent past and thus, through sperm storage, be the father of larvae produced during the experiment). We allowed both for polygyny and polyandry, setting the parentage prior (guess probability that the parent of an offspring is included in the candidate pool) to 1 for mothers. For fathers we assumed that ca.1-2 males outside a given stack could have contributed to reproduction

(based upon results from Dupont et al. 2006; Le Cam et al. 2009), hence we set parentage prior to $(I-1)/(I+2)$ where I is the number of individuals in the focal stack. This choice of prior is straightforward, but we note that this level of refinement for father parentage prior is inconsequential here because all mothers and a very large fraction of the fathers were genotyped.

We used reference allelic frequencies estimated over 480 adults, combining 290 individuals from the 33 stacks used in the breeding experiment (three individuals were disposed by error or lost before genotyping) and the 190 individuals that had been simultaneously sampled in the wild (that is, the second set of adults detailed in the main text). Random genotyping repetitions (20 to 98 per locus, data not shown) allowed us to detect a very high error rate for locus DAYN22, which was therefore removed from all analyses. With the remaining loci we set COLONY error settings to 1% for allelic dropouts and 0.1% for other genotyping errors (e.g. mutations or PCR-generated false alleles).

Null alleles are another source of genotyping errors that are very frequent in highly polymorphic species such as many marine invertebrates (e.g.Hare et al. 1996; Lemer et al. 2011). Allowing for a small proportion of genotyping errors does not fully control for the effect of null alleles in COLONY analyses, because a single non-amplified allele in a parent is expected to be present in half of its progeny. However, such patterns are easily detected with parent/offspring data, especially with large family sizes. In our case the fact that the mother was necessarily in the pool of candidate parents greatly facilitated the detection of null alleles in the parents of the two sexes (see also Proestou et al. 2008).

Detailed description of the model

To understand the effect of sperm storage we picture a *C. fornicata* stack composed by 2 females and 2 males (Fig. 5) and we will focus only on the reproductive success of the male that is

closest to the females (individual number 3 in Fig. 5). We are interested in following the dynamics of reproductive success of this focal individual as a function of sex and time.

Time is divided into discrete units corresponding to the frequency of brood production by the females. Each female produces one brood per time unit, and we take for example one time unit = one month. For simplicity the fecundity of the two females is equal and remains constant (depicting e.g. a situation where these two females are already quite large (and old) and do not grow anymore). Each female produces *N* larvae at each time step. In all simulations we set *N*=14000 offspring (average fecundity from the empirical study).

Our focal individual will start its benthic life at time *t*=0 with shell length *L*=0 and obey a Von Bertalanffy growth increment of the form:

$$L_{t+1} = L_t + (L_\infty - L_t)(1 - \exp(-K)) \quad \text{(S1)}$$

where parameters L_∞ and K were approximated from our growth data using Ford-Waldford method, that is, the linear regression of shell length taken at the onset of the experiment against shell length of all surviving individuals at day 251 (i.e. approximately 8 month). For simplicity and because there are no small females we used a unique growth model fitted with empirical data from both sexes, giving L_∞≈56.48 and *K*≈0.043. Our focal individual will thus grow at each time step. Because we want to look at the effect of sperm storage alone (and not the complexities associated with other aspects of the mating system or stacking behavior), we considered that our focal individual is directly in contact with a female (Fig. 5). Hence size/age will have no influence on reproductive success until the individual turns female. After that, the fecundity of this individual will depend directly on its size (details below).

At each time step we calculate the reproductive success of our focal individual through its male function, and, if it has already changed sex, through both male and female functions (similarly to the measure of reproductive success defined with our empirical data). The timeframe of our

model is arbitrarily set to 100 time steps (a period that is long enough to include the optimal timing of sex change in all simulations and is in the range of *C. fornicata* life expectancy).

Reproductive success through the male function

Before sex-change of our focal individual, the two males compete for fertilizing female broods. Each female has a sperm storage reserve that contains a proportion *p* of sperm from our focal male. In a first step (referred to as model 1, Fig. 5) we assume that the two female sperm reserves follow the same dynamics. At each time step *t*, a fraction *m* of sperm is lost through sperm mortality (that includes consumption for fertilization of the eggs) and replaced by sperm produced by each male in proportions φ from our focal male and $1-\varphi$ from its competitor (Fig. 5). Within each female's sperm storage organ, the proportion *p* of sperm from our focal male thus follows:

$$p_{t+1} = p_t(1-m) + m\varphi \quad \text{(S2)}$$

This model depicts a situation where the sperm storage organ is elastic and new sperm accumulate as a random mixture, or storage is limited but sperm is replaced proportionally to male contributions (akin to a "fair raffle" type of sperm competition, Parker 1998, without sperm precedence).

At each time step *t* the reproductive success of our focal male is:

$$W_t^{\male} = 2p_tN \quad \text{(S3)}$$

that is, the combined fecundity of the two females weighted by the proportion that the sperm of the focal male represents in the sperm stores. With this definition, the reproductive success through male function depends only on male-male competition. It is independent of the size or age of the focal male (in particular there is no immature phase, we take the simplification that the focal male is fully mature at time *t*=0). The equilibrium state for the proportion of sperm from our focal male stored in each female is $p_{eq} = \varphi$ and the reproductive success of this male at equilibrium is $W_{eq}^{\male} = 2\varphi N$.

By contrast, if our focal male changes sex it stops refilling the females' storage organs (i.e. φ=0 in equation S2) and the dynamics of sperm reserves becomes:

$$p_{t+1} = p_t(1 - m) \quad \text{(S4)}$$

The fitness (eq. S3) of our focal individual (now female) through male function will thus decline at rate *m* until it becomes null when all its sperm has been replaced by sperm from the other male.

Reproductive success through the female function

Contrary to male fitness, female's fecundity is constrained by its size (Li and Pechenik 2007; Proestou et al. 2008; Richard et al. 2006). Here we used the linear relationship reported by Li and Pechenik (2007) to define the reproductive success of our focal individual as a function of shell length when it has turned female: $W_t^{\female} = 558.4 \times L_t - 10380$. Negative values, predicted for $L_t < 18.6$ mm, were set to 0.

Estimation of sperm mortality *m* from empirical data

In our models a proportion *m* of sperm is lost from a female's sperm store each time that a new brood is produced. That sperm may (model 1 in Fig. 5) or may not (model 2) be replaced with new sperm. This parameter *m* appears to be very influential for the effect of sperm storage on the timing of the sex-change. There is one situation where *m* could be estimated from our parentage data. The female at the base of stack 1 repeatedly produced broods partly sired by the second individual of that stack, which was a female (see a cartoon of this stack in Fig. 8; we know that individual 2 was a female because it produced broods itself early in the experiment). Hence the father of these broods was already a female when the experiment began in January, but its sperm stored in the basal female (female 1) was repeatedly used to fertilize that female's eggs. For the reproductive success analyses presented in this study we already had parentage data for 4 consecutive broods produced

by female 1 (with 16 to 19 days between successive broods, Fig. 8). We genotyped and analyzed one additional brood produced 180 days later by the same female. We estimated the proportion *p* of larvae fathered by female 2 in each brood (*n* = 16 to 87 larvae genotyped per brood). The standard error of each estimate is given by $s_p = \sqrt{\frac{p(1-p)}{n-1}}$.

We used these data to fit a decay model of the form $p_t = p_0(1-m)^t$ where p_0 is the fitted proportion of sperm from female 2 used by female 1 for the first brood analyzed (we arbitrarily set this first brood at time *t*=0, Fig. S8). This model gives a remarkable fit (p-value=0.005, adjusted R^2= 0.93) for p_0=0.21 and *m*=0.14. Note that the timing of the first brood (and hence p_0) doesn't have any importance. For instance we obtain *m*=0.14 also without considering the first brood (p-value=0.03, adjusted R^2=0.90). We don't know the rules that drive the decay in the contribution of individual 2 (e.g. sperm mortality with random replacement or more complex mechanisms involving sperm precedence or postcopulatory competition) but it gives us a remarkable quantitative example of how sperm storage allows a male to continue fathering offspring after sex-change.

Supplementary figures

Figure S1: We observed an average brood size of ca. 14000 larvae, of which only a minute fraction could be genotyped for parentage assignment and reproductive success analyses. Assuming that one observation of a larval release corresponds to one brood produced by a single female and that each sampled larva can be genotyped and correctly assigned to its parents, how many larvae should be sampled to detect all the fathers that contributed to a given brood? The probability of detecting at least one offspring of a father that contributed a fraction *p* of a brood can be calculated as $1 - (1 - p)^n$ with sample size *n*. Panel A shows this probability for sample sizes 32, 48, and 96 larvae for theoretical sire contributions [0-0.25]. Panel B shows the expected number of offspring of a given father as a function of that father's contribution to a brood, within sample of size 32, 48, or 96 larvae.

Figure S2: Distribution of mating success (number of successful mating partners) of adult *Crepidula fornicata* (31 males and 37 females from 8 stacks). Females that reproduced only through the female function are pictured with white bars. Some females (black bars) have both male and female mating partners (or even female partners only), because before changing sex they produced male gametes that are still used by other females (sperm storage effect).

Figure S3: Distribution of the proportion of larvae sired by a given male in a given brood.

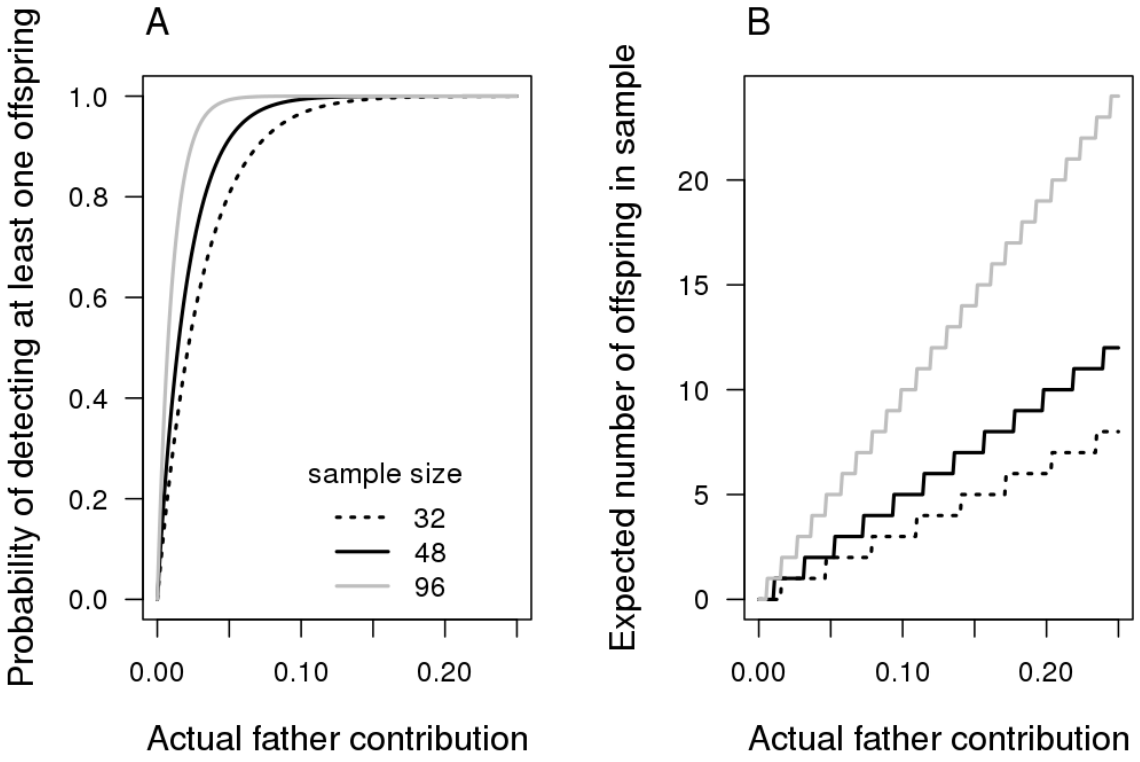
Figure S4: Distribution of brood size (n=44 broods produced by 24 females from 8 stacks).

Figure S5: Effect of stack structure on different components of reproductive success. A) Distribution of the distance between mating partners (that is, the number of other individuals that separate two mating partners in a stack). During the experiment we randomly observed 145 matings involving 101 individuals from 22 stacks. The distribution of distances for these observations (dark grey bars) can be compared to the expected distribution (light grey) under the null hypothesis of random mating between males and females within the same 22 stacks, taking into account the composition of each stack. B) Effect of the distance between parents of a given brood on the proportion of larvae sired by the father (n=125 proportions involving 32 broods and 16 fathers). The two kinds of triangle symbols correspond to apparent outliers that were all due to only two males within a single stack showing a particularly strong curvature that may have facilitated internal fertilization between distant individuals.

Figure S6: Effect of sperm mortality (m) on the gain in lifetime reproductive output ($\Delta\omega$) for an individual that would change sex at some time t (with sperm storage occurring), by comparison with an individual that follows the optimal strategy in the absence of sperm storage. These results were obtained under the conditions of model 1 (see main text and Figure 5), that is, where the sperm of the focal male that is stored in females is progressively replaced by sperm from another donor.

Figure S7: Effect of sperm mortality (m) on the gain in lifetime reproductive output ($\Delta\omega$) for an individual that would change sex at some time t (with sperm storage occurring), by comparison with an individual that follows the optimal strategy in the absence of sperm storage. These results were obtained under the conditions of model 2 (see main text and Figure 5), that is, where only one female progressively replaces the sperm from the focal male by sperm from another donor, while the other female does not receive any new sperm.

Figure S1



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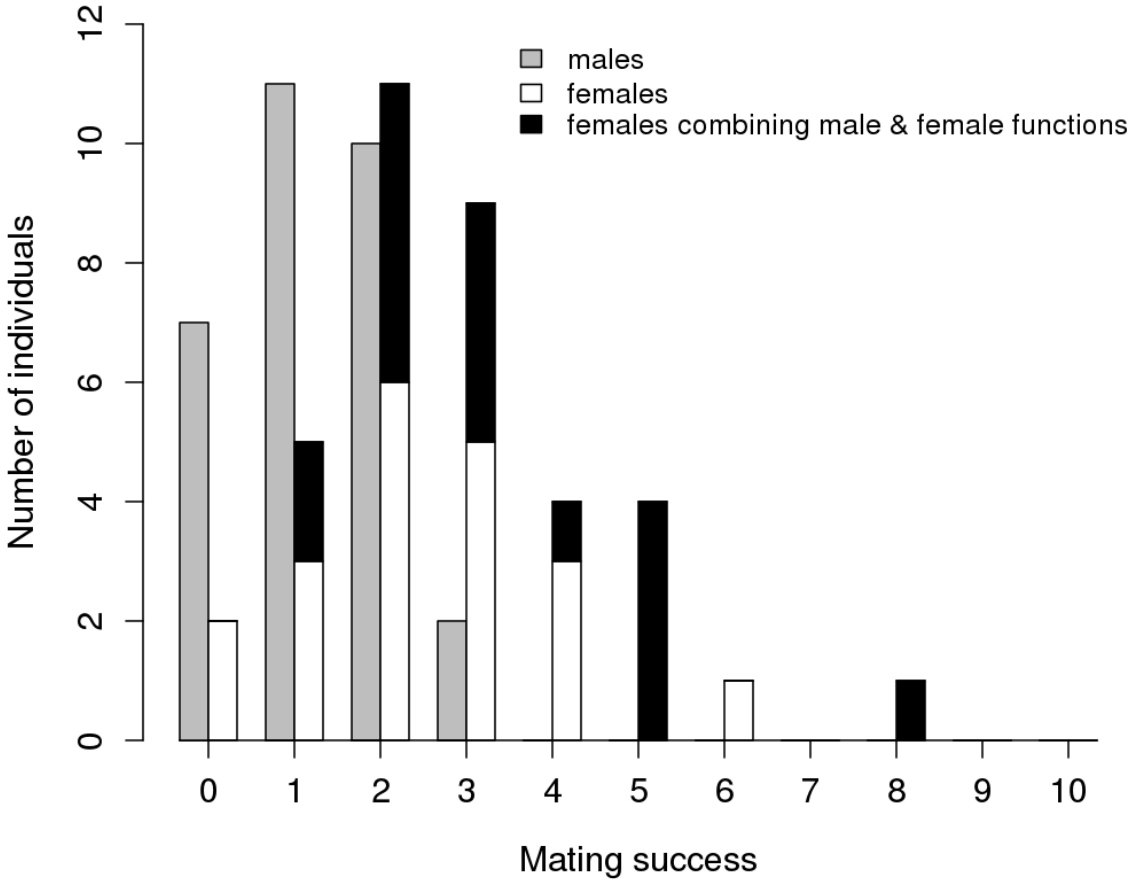

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Figure S3

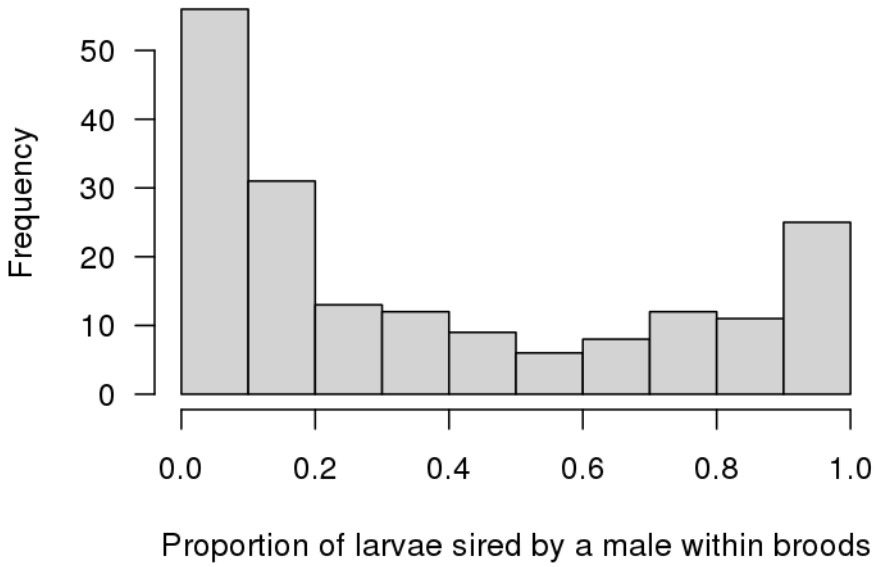

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Figure S4

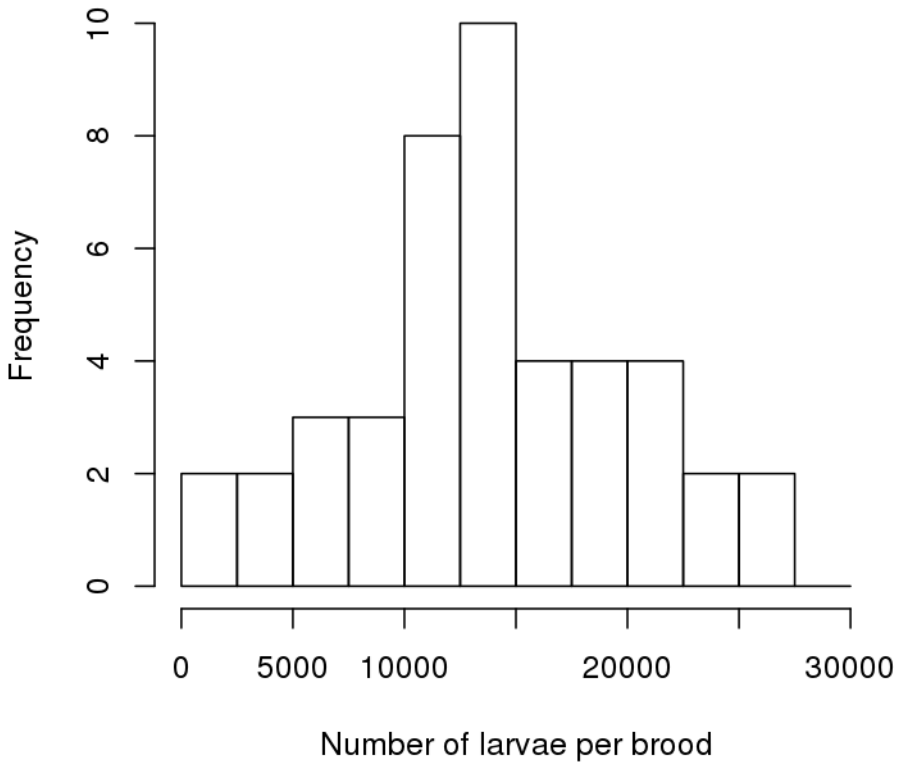

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Figure S5

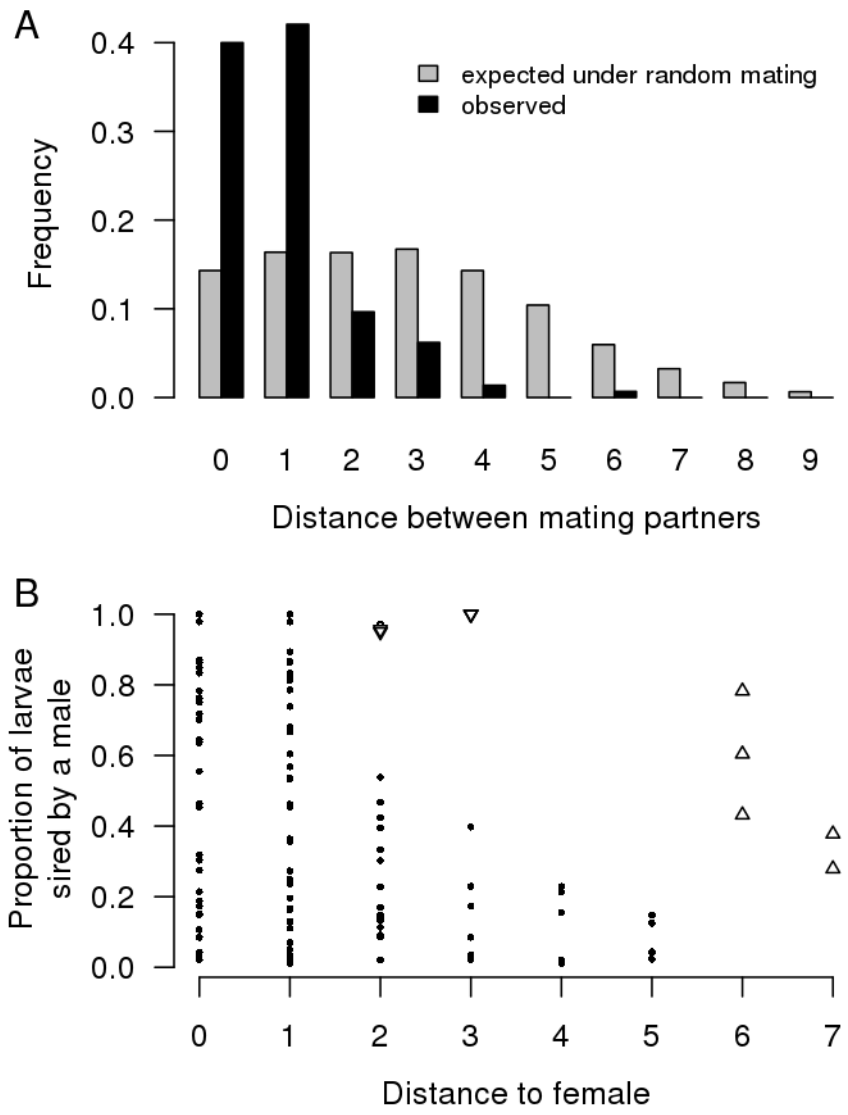

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Figure S6

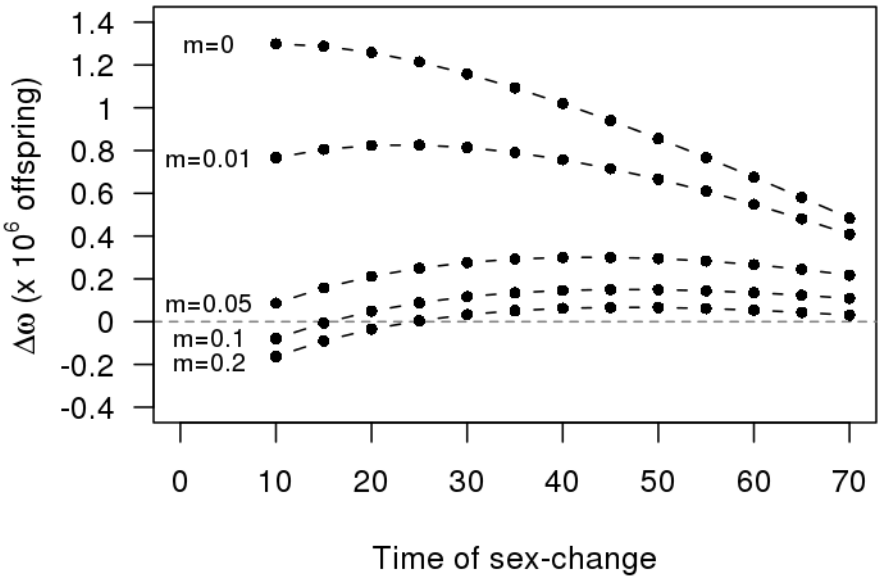

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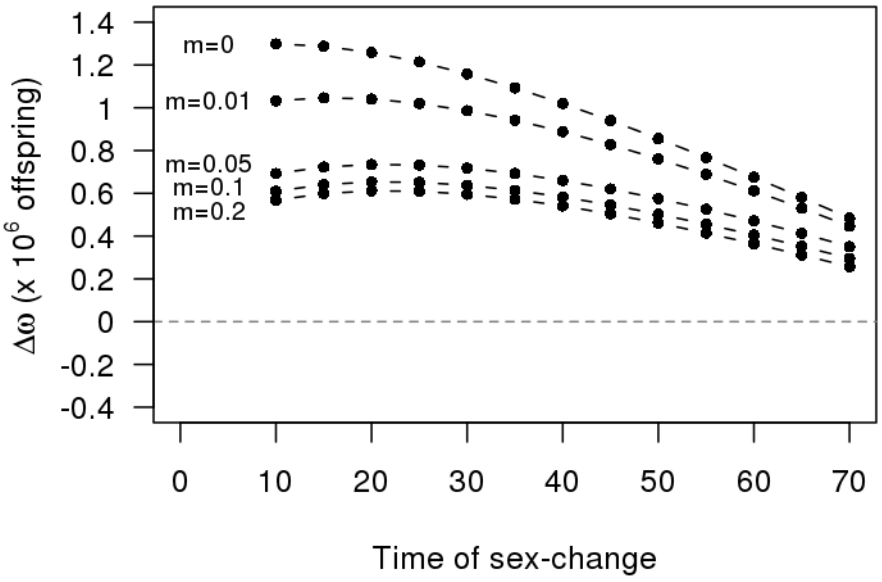
Figure S7



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