# Cryptic individual-level variation in propensity for selfing in a hermaphroditic snail

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## Abstract

For mating-system evolution, individual-level variation is as important as variation among populations. In self-compatible hermaphrodites, individuals may vary in their lifetime propensity for selfing, which consists of a fundamental, likely genetic and an environmental component. According to the reproductive assurance hypothesis explaining partial selfing, a key environmental factor is mate availability, which fluctuates with population density.

We quantified individual variation in selfing propensity in a hermaphroditic snail by manipulating mate availability in the laboratory, recording mating behaviour, estimating selfing rates from progeny arrays, and measuring female lifetime fitness. Our results revealed four classes of individuals with different selfing propensities: pure outcrossers, pure selfers, and two types of plastic individuals. These classes only became apparent in the laboratory; the field population is outcrossing. All classes were present both under low and increased mate availability; this large among-individual variation in selfing propensities meant that effects of the pairing treatment on the frequency and extent of selfing were non-significant despite large effect sizes and sufficient statistical power.

We believe that selfing propensities may have a genetic component and when selected on cause mean selfing rates to evolve. We propose that heritable variation in selfing propensities offers a reconciliation between the reproductive assurance hypothesis and its weak empirical support: distributions of selfing propensities vary temporally and spatially, thus obscuring the relationship between population density and realised selfing rates.

## Introduction

The mating system is the way a population or species is structured with respect to sexual behaviour. In hermaphroditic species capable of self-fertilisation (hereafter selfing), it primarily describes the relative frequency of selfing *versus* outcrossing. The mating system adopted by hermaphroditic species determines the quantity and genetic quality of offspring (Barrett 2014). Consequently, it has pervasive effects on individual fitness and on evolutionary and demographic properties of populations and species, influencing genetic diversity, effective population size, and the expression of inbreeding depression (Charlesworth and Charlesworth 1987; Barrett and Harder 2017), the strength of sexual selection, population growth rate, colonising ability, and adaptive potential (Baker 1955; Anthes et al. 2010; Noël et al. 2017; Felmy et al. 2020).

Estimates of variation in selfing rates have often been obtained at the level of species (Vogler and Kalisz 2001; Goodwillie et al. 2005; Jarne and Auld 2006; Moeller et al. 2017). The distribution of selfing rates among species has been found to be bimodal, with more outcrossers and selfers than mixed maters ($0.2 <$ selfing rate $s < 0.8$). Recent years have seen a flurry of studies showing variability between populations of a single species, particularly in plants (reviewed in Whitehead et al. 2018; e.g., Latron et al. 2020; Christopher et al. 2021). For example, in 51% of 105 surveyed plant species, populations did not fall into a single mating-system category (Whitehead et al. 2018). Our knowledge on animal populations is sparser, but where selfing rates or the delay in the onset of reproduction when selfing (termed “waiting time”, Tsitrone et al. 2003) have been estimated across populations, inter-population variation was the norm (Viard et al. 1997; Trouvé et al. 2003; Bousset et al. 2004; Charbonnel et al. 2005; Mackiewicz et al. 2006; Puurtinen et al. 2007; Escobar et al. 2009; Kupfernagel et al. 2010; Giannakara and Ramm 2020), with few exceptions (Tian-Bi et al. 2019). These studies

demonstrate the importance of characterising species by more than one population estimate, lest crucial differences between populations are overlooked.

We postulate that the same argument ought to be made for differences in selfing rates between individuals. Evolutionary change primarily happens at the level of the population, with intra-population variation in a trait constituting the raw material natural selection acts upon (Conner and Hartl 2004). If all individuals within a population of simultaneous hermaphrodites were uniform in their ability to and proclivity for selfing, and had equal opportunities to outcross, the selection gradient for the selfing rate would be zero: selection can only act on traits that vary (Lande and Arnold 1983).

Despite their importance, estimates of variation in individual selfing rates are rare. An obvious reason is that such estimates are data-hungry, since cost-effective but indirect estimation methods based on heterozygote deficiency (i.e., $F_{IS}$) or identity disequilibria (David et al. 2007) cannot be used to estimate variation in selfing rates among individuals (Bürkli et al. 2017). Instead, labour-intensive progeny arrays must be used, requiring large numbers of parents and offspring to be genotyped. Existing studies have shown that selfing rates varied within populations of trees (Feres et al. 2012; Gauzere et al. 2013) and snails (Coutellec-Vreto et al. 1997; Viard et al. 1997; Wiehn et al. 2002; Trouvé et al. 2003; Meunier et al. 2004; Charbonnel et al. 2005; Henry et al. 2005; Jokela et al. 2006). However, many of these estimates may be biased (Bernatchez and Duchesne 2000; Bürkli et al. 2017), as they are based on small numbers of weakly polymorphic loci, which hampers the detection of outcrossing events (but see Feres et al. 2012; Gauzere et al. 2013 for exceptions). Alternatively, researchers have used proxies of selfing rates: in animals, the waiting time prior to selfing (Escobar et al. 2007; Escobar et al. 2009; Ramm et al. 2012) and in plants, the presence of seeds in self-pollinated individuals (Himes and Wyatt 2005; Weber et al. 2012). Again, differences among individuals were found to be substantial.

Like other complex traits, an individual's selfing rate likely depends on genetic factors, environmental conditions, and genotype-by-environment interactions. Genetic self-incompatibility systems are well-known in plants (reviewed in Fujii et al. 2016; Muñoz-Sanz et al. 2020) and have recently been discovered in ascidians (Sawada et al. 2020) and oomycetes (Dussert et al. 2020). Differences in waiting times among families, suggestive of genetic variation in selfing rates, have also been found in snails (Tsitrone et al. 2003; Escobar et al. 2007; Escobar et al. 2009) and flatworms (Ramm et al. 2012).

It is plausible to expect that a key environmental determinant of individual selfing rates is mate availability. Selfing provides reproductive assurance when mates are scarce, but may not be common if outcrossing is possible, due to inbreeding depression and other costs of selfing (Jarne and Charlesworth 1993; Uyenoyama et al. 1993). In animals, the association between selfing rates and mate availability has mainly been studied at the population level, where it has consistently found to be weak (Trouvé et al. 2003; Henry et al. 2005; Puurtinen et al. 2007; Kupfernagel et al. 2010). The two studies conducted at the individual level, both lacking genetically estimated selfing rates, found no effects of mate availability and its proxies either (Vianey-Liaud et al. 1989; Henry et al. 2006). However, to the best of our knowledge, the impact of mate availability on variation in individual selfing rates has not yet been assessed directly in a hermaphroditic animal.

Individual variation in selfing and its dependence on ecological conditions can be assessed by estimating individuals' lifetime propensity for selfing. Here we define the "propensity for selfing" as a biological property of an individual (the ability and readiness to self) and its environment (the availability of mating partners), loosely following Robert Brandon's propensity definition of fitness (Brandon 1978). The propensity thus consists of a fundamental, individual-level component and a contextual component. This definition emphasises the environmental dependence of realised selfing rates, thereby allowing a separate exploration of

the individual (supposedly genetic) *versus* ecological determinants of variation in selfing rates, the need for which recent studies have abundantly shown (Escobar et al. 2011; Whitehead et al. 2018; Giannakara and Ramm 2020; Latron et al. 2020).

The freshwater snail *Radix balthica* is an ideal species to study within-population variation in selfing propensity and the factors that maintain it. Laboratory studies have confirmed that self-compatibility is geographically widespread: many unmated snails reproduced via selfing in Swiss (Felmy et al. 2020) and Icelandic populations (Johansson et al. 2016). However, self-compatibility does not appear to be ubiquitous: the fraction of isolated snails that selfed stayed well below 100% in all populations (Johansson et al. 2016; Felmy et al. 2020). Additionally, there is tentative evidence that molecularly estimated selfing rates vary between populations in North-Western Europe (Pfenninger et al. 2011), and both between and within Swiss populations (Wiehn et al. 2002; Jokela et al. 2006). Although these estimates may come with a bias (Bürkli et al. 2017), some heterogeneity in selfing rates is probably real. The population studied here is almost fully outcrossing in the field (i.e., its realised selfing rate is near-zero: Bürkli et al. 2017), in accordance with its high density.

We derived individuals' lifetime propensity for selfing for two laboratory environments with differential mate availability. We isolated laboratory-reared, unmated snails to ascertain how successfully they selfed when forced to do so. We then assigned them to one of two mating treatments, involving either a single opportunity to mate with a single partner, or six opportunities, each with a different partner. The first treatment simulated a low population density and scarcity of mating opportunities, such as in newly colonised or ecologically disturbed habitats. The second treatment emulated conditions in the field population, where field-collected egg clutches had 2.1 fathers on average, with a range of one to nine (Bürkli and Jokela 2017). Note that only a fraction of mate encounters might result in siring success. For each snail (n = 274), we recorded the mating behaviour, production of eggs in isolation

(necessarily selfed) and after the start of mating trials (potentially outcrossed), and proportion of eggs that developed into embryos. In addition, for a subset of individuals (n = 57), we estimated individual selfing rates in eggs produced post-isolation using progeny arrays based on nine highly polymorphic microsatellites.

Assuming variation in post-isolation selfing rates, we had two *a priori* hypotheses: Selfing post-isolation is more common under reduced mate availability (hypothesis 1) and in snails that selfed in isolation (hypothesis 2). Hypothesis 1 reflects the notion that selfing has evolved to provide reproductive assurance at low population density but is not preferred when outcrossing opportunities are abundant (Jarne and Charlesworth 1993; Uyenoyama et al. 1993). If supported, hypothesis 1 suggests selfing propensities have a strong ecological component. Hypothesis 2, on the other hand, postulates that a period of isolation reveals classes of individuals with different propensity for selfing, and that these remain largely unaffected by a change in environmental conditions. Hypothesis 2 therefore assumes that selfing propensities are stable, fundamental properties based in genetic differences. The two hypotheses are not mutually exclusive; we expected ecological and genetic factors to work together in shaping individual selfing rates. To further explore how variation in selfing propensities is maintained, we investigated their relationship with individuals' sexual drive and female lifetime reproductive success.

# Methods

## Study system

*R. balthica* is a simultaneously hermaphroditic snail living in the shallow littoral zone of lakes throughout Europe (Cordellier and Pfenninger 2009; Pfenninger et al. 2011; Lawton et al. 2015). In Lake Zurich, Switzerland, the species is an annual with non-overlapping generations. Snails hatch from eggs in spring, reach sexual maturity at the end of the year, reproduce from March to May, and then die (Bürkli and Jokela 2017). During the single breeding season, individuals may copulate repeatedly in both sexual roles and lay hundreds of eggs in distinct egg clutches (Bürkli and Jokela 2017). Copulation is unilateral. Lymnaeid snails can store allosperm from copulations in a special organ and should not be allosperm-limited even if they do not mate continuously (Nakadera et al. 2014).

## Experimental snails

We estimated the selfing rate of 57 first-generation (F1) laboratory-born snails kept in the laboratory throughout their lives. They were part of a larger group of 274 F1 snails subjected to laboratory mating trials (details in Felmy et al. 2020) and were all derived from 38 snails (P0 mothers) collected at peak breeding season (24 April 2013) in Uerikon, Lake Zurich, Switzerland. The 57 snails with estimated selfing rates originated from 22 P0 mothers. F1 snails were reared in sibling groups and individually from age 19.4 ± 4.1 weeks onwards (mean ± SD) to preserve their virginity (age measured since oviposition here and henceforth). This is a very early start of isolation; paired control snails only began to mate at age 41.3-45.7 weeks.

## Mating trials

Mating trials began in early May 2014 when F1 snails were 52.1 ± 1.0 weeks old. In the ancestral field population, breeding started in early March (Bürkli and Jokela 2017), and laboratory control snails had started to copulate several weeks earlier (see previous paragraph). When mating trials began, 38.7% of 274 F1 snails had produced selfed eggs. The experiment then continued for seven weeks, by which time the mortality rate began to increase in accordance with the snails' natural, annual life cycle.

Two types of mating trials were conducted: snails either had one mating opportunity with one partner (n = 33/57 snails with selfing rate estimates), or six opportunities over the course of six weeks, each with a different partner (n = 24/57). Each mating opportunity lasted for 10.3 ± 0.8 hours, which is sufficiently long for copulations in both sexual roles. Mating trials were recorded on time-lapse movies (one frame/30 sec). From these we extracted the number of partners each snail had copulated with as a male and as a female.

We measured female lifetime reproductive success (hereafter "LRS"; number of eggs containing developed embryos 17 days after oviposition) for each snail, separated into an isolation and post-isolation phase (see Felmy et al. 2020for details).

## Genetic analysis

The condition for snails (n = 57) to be selected for selfing rate estimation was successful reproduction after mating trials had started. We genotyped F2 offspring that hatched from eggs that were laid after F1 snails had participated in mating trials. We genotyped juveniles from 33 snails that received a single mating partner and from 24 snails that received a sequence of mating partners. For logistic reasons, we did not genotype any juveniles from the last eggs laid before the experiment was terminated. Hence, the genotyped offspring of repeatedly paired

snails originate from eggs laid before mating opportunity 4 (n = 6 snails), 5 (n = 15 snails), and 6 (n = 3 snails), respectively. Offspring of these snails can thus be sired by three to five non-maternal fathers.

F2 offspring were reared in sibling groups until the age of 12.3 ± 0.5 weeks, when some were used in a field experiment and the rest frozen for genetic analysis. At that moment, 65.9 ± 16.0% of offspring born post-isolation had died. Because of the pre-sampling mortality, the selfing rates estimated in this study are "secondary" selfing rates, after the expression of potential in- or outbreeding depression in early development.

We aimed to genotype approximately 15 randomly chosen juveniles per F1 snail, or ≥ 80% of available juveniles when families were smaller, resulting in 13.7 ± 4.3 juveniles being genotyped per family. We also genotyped all P0 and F1 snails to estimate population-level inbreeding coefficients using Genetix version 4.05.2 (Belkhir et al. 1996-2004) and to ascertain whether F1 snails themselves were selfed (Table S1).

Snails were genotyped for ten highly polymorphic microsatellite loci (GenBank Accession No. KX830983-KX830992) developed specifically for this population by Ecogenics GmbH (Zurich, Switzerland). The genotyping and scoring protocol has been described in detail elsewhere (Bürkli et al. 2017). One locus showed a non-negligible frequency of null alleles (locus Rb_3, GenBank Accession No. KX830985) and was excluded from all analyses.

## Paternity analysis

Paternity analyses were performed using a custom-built R routine. We excluded 44 juveniles (5.6% of 783 juveniles genotyped) from the analysis because they did not meet the inclusion criteria (< 6 loci of both mother and offspring successfully genotyped, OR genotypes lacking a maternal allele at > 2 loci, OR genotypes with 6 successfully genotyped loci but 2 loci without a

maternal allele). For the remaining 739 juveniles, we counted the number of loci consistent with being outcrossed, i.e., those with one non-maternal allele. We then compared each juvenile's genotype to those of all its non-maternal candidate fathers, which we defined as all the potential mating partners of a juvenile's mother, irrespective of whether copulations had been observed. A candidate father was considered a perfect match if he possessed a juvenile's paternal allele at every locus successfully genotyped in the juvenile, the mother, and himself. Candidate fathers that lacked a juvenile's paternal allele at 1 ≥ loci were considered non-perfect matches and were deemed increasingly unlikely to be the true father as the number of loci containing father-offspring mismatches increased. Additionally, we repeated all paternity analyses using COLONY version 2.0.5.9 (Jones and Wang 2010, details in the Supporting Methods), a software known for its highly accurate parentage assignments (Jones et al. 2010; Harrison et al. 2013).

Using these procedures, paternity assignments were successful for 731 (98.9%) juveniles with high-quality genotypes, originating from 56 F1 snails (13.1 ± 3.8 assigned offspring per F1 snail). For the offspring of one F1 snail, paternity assignments were inconclusive. For 719 juveniles, our R routine and COLONY identified the same individual as the most likely father. Of the remaining twelve juveniles, five were left unassigned by the R routine, four by COLONY, and three were assigned by both methods but to different fathers, in which case we gave preference to the R routine (details in Table S2). Allelic diversity was high across loci (Table S1), yielding powerful paternity analyses. The mean probability of being selfed for offspring identified as selfed was 0.80 ± 0.22, while that of offspring identified as outcrossed was 0.00 ± 0.00 (Table S2). Outcrossed offspring were assigned to a father with a probability of 0.92 ± 0.16 (Table S2).

## Statistical analysis

We used two-tailed Pearson's Chi-square tests for count data, using either 2 x 2, 3 x 2, or 4 x 2 contingency tables, and generalised linear mixed models (GLMMs) for hypotheses where we could construct a response-predictor structure.

The likelihood of F1 snails producing at least some selfed juveniles post-isolation (binary response: no vs. yes) was analysed using a GLMM with binomial errors. As fixed effects we included the experimental treatment (once- vs. repeatedly paired), the number of developed embryos produced in isolation, and the number of male and female partners snails had copulated with. P0 mother identity was used as a random intercept.

We used GLMMs to test if snails with different propensities for selfing differed in their total number of eggs (female LRS), total number of developed embryos, or proportion of undeveloped embryos. We estimated these models for the subset of F1 snails whose offspring were successfully genotyped (n = 51, note that five outcrossers were excluded due to low sample size) and for the whole dataset of female fertile snails (n = 126). We fitted the GLMMs using negative binomial errors (nbinom 1 family) for the first two response variables and Gaussian errors for the proportion of undeveloped embryos as it was normally distributed. Adult body size was used as a continuous covariate and the propensity for selfing as a factor (three levels, see results). All models included P0 mother identity as a random intercept.

Finally, we tested if snails that mated in both sexual roles (binary response: no vs. yes) differed in their propensity for selfing using a GLMM with binomial errors. The propensity for selfing (four levels, see results), adult body size (continuous covariate) and P0 mother identity (random intercept) were used as independent predictors. This model was fitted for the subset of snails with successfully genotyped offspring (n = 51, see above).

Analyses were performed using R v. 4.0.5 (R Core Team 2021). GLMMs were fitted using R-package "glmmTMB" (Brooks et al. 2017). The model fit was assessed using a simulation-based approach implemented in R-package "DHARMa" (Hartig 2021). We tested the significance of the random effect with log-likelihood ratio tests (full model vs. model with no random effect). Values are given as mean ± SD. Scatter plots were prepared using R-package "beeswarm" (Eklund 2016) and depict data points superimposed on boxplots. Binomial standard errors were computed according to Zar (1996) as $SE = \sqrt{p(1-p))/(n-1)}$, where $p$ was the proportion tested and $n$ the total sample size. The entire code and model output is provided as an R Markdown document (Xie et al. 2018; Allaire et al. 2020).

# Results

## Frequency of selfing, mixed mating, and multiple paternity

Most genotyped F2 families (73.2%) contained 20% or fewer selfed offspring (Fig. 1a). At least one selfed offspring was found in 44.6% of F2 families, with 26.8% of families consisting of 83% or more selfed offspring (Fig. 1a). Overall, 23.0% of offspring were identified as selfed (168/731). There were no families with intermediate selfing rates ($0.20 < s < 0.80$, Fig. 1a).

Multiple paternity was significantly more common among the offspring of repeatedly paired (45.8%) than once-paired snails (15.6%, Pearson's Chi-squared test with Yates' continuity correction: observed counts: 5, 27, 11, 13, expected counts: 9.1, 22.9, 6.9, 17.1, $\chi^2_1 = 4.74$, $p = 0.0294$; Fig. 1a). On average, the progenies of repeatedly and once-paired snails had 1.7 ± 0.9 (max. = 4) and 1.2 ± 0.4 (max. = 2) fathers, respectively.

There was no evidence of selfing in either the F1 or P0 generation. All 57 F1 snails were outcrossed, as shown by their possession of a non-maternal allele at 1 ≥ loci (mean: 4.8 ± 1.5 loci; Table S1). P0 mothers were likely outcrossed too: their genotypes did not have reduced heterozygosity ($F_{IS}$ = -0.0009, 95% CI: -0.075, 0.046, Table S1). P0 mother identity did not explain any variance in F1 snails' probability of selfing post-isolation (Table 1).

## Effect of mate availability on selfing

Mean selfing rates did not differ significantly among once- (0.36 ± 0.47) and repeatedly paired snails (0.19 ± 0.36; Wilcoxon rank sum test with continuity correction: $W = 1452.5$, $p = 0.21$), nor did their variance (Levene's test for homogeneity of variance: $F_{1,24} = 2.48$, $p = 0.12$, Fig. 1b). Repeated mating opportunities did not significantly reduce the likelihood of a snail producing at least some selfed offspring (Table 1), nor the proportion of families with non-zero selfing rates

(once-paired: 50.0%, repeatedly paired: 37.5%, Pearson's Chi-squared test: observed counts: 16, 16, 9, 15, expected counts: 14.3, 17.7, 10.7, 13.3, $\chi_1^2 = 0.87$, $p = 0.35$; Fig. 1c). We thus did not find strong support for hypothesis 1, which predicted that selfing is more common in snails given limited mating opportunities.

However, as a group, once-paired snails produced significantly more selfed offspring than repeatedly paired snails: 33.6% vs. 8.9% (Pearson's Chi-squared test: observed counts: 140, 227, 28, 286, expected counts: 90.5, 276.5, 77.5, 236.5, $\chi_1^2 = 77.80$, $p < 0.0001$, Fig. 1d). Nevertheless, the distribution of selfing rates was similarly bimodal among once- and repeatedly paired snails (Fig. 2a).

## Infertility in isolation was linked to outcrossing post-isolation

Predominant selfing after the start of mating trials was restricted to snails that reproduced successfully in isolation, while snails without prior selfing exclusively outcrossed post-isolation (Fig. 2b). Yet, 70.6% of snails that successfully selfed in isolation switched to complete outcrossing as soon as it was possible or maintained only a low level of selfing post-isolation (Fig. 2b). Consequently, selfing in isolation was not a strong predictor of continued selfing, with its effect only being statistically significant when a single extremely prolific snail was included (Table 1) and non-significant otherwise ($b = 0.01$, $z = 1.93$, $p = 0.05$). We therefore rejected hypothesis 2, which posited that selfing post-isolation is more common in snails with a history of selfing, while acknowledging that the lack of that history reliably predicted outcrossing.

## Individual-level variation in propensity for selfing

Snails varied in their overall propensity for selfing. For the 56 F1 snails whose offspring were successfully genotyped, we ascertained the propensity for selfing from the combination of selfing in isolation (presence/absence) and the post-isolation selfing rate. This procedure

revealed four groups of snails: 8.9% “outcrossers” that never reproduced via selfing, 46.4% “plastic switchers” that selfed in isolation but switched to outcrossing as soon as they could mate, 17.9% “plastic mixers” that combined selfing in isolation with predominant yet incomplete outcrossing post-isolation, and 26.8% “selfers” that always reproduced fully or predominantly through selfing (Fig. 2c).

The subsample of snails with genotyped offspring underrepresented snails that reproduced only in isolation or only post-isolation (grey areas in Fig. 2d). To estimate the frequency of selfing propensities, we thus considered all snails (n = 274). We ascertained the apparent selfing propensity in snails without genotyped offspring based on when they laid eggs and how they mated. This revealed three groups: “apparent outcrossers” that only reproduced post-isolation and copulated as a female, “apparent plastic snails” that reproduced both before and after being paired and copulated as a female, and “apparent selfers” that only reproduced in isolation, or in isolation and post-isolation yet never copulated as a female. The propensity for selfing of snails without developed embryos (n = 148) is unknown. Among all snails with ascertained selfing propensity (n = 126), outcrossers and apparent outcrossers made up 31.0%, plastic and apparent plastic snails 42.9%, and selfers and apparent selfers 26.2%.

The pairing treatment did not influence the propensity for selfing of snails with genotyped offspring (Pearson’s Chi-squared test: observed counts: 11, 5, 15, 1, 4, 5, 11, 4, expected counts: 8.6, 5.7, 14.9, 2.9, 6.4, 4.3, 11.1, 2.1, $\chi^2_3 = 4.63$, $p = 0.20$). However, when using the full dataset, selfing propensities differed among experimental groups (observed counts: 29, 26, 11, 11, 20, 27, expected counts: 21.3, 24.5, 20.2, 18.7, 21.5, 17.8, $\chi^2_2 = 15.17$, $p = 0.0005$). Low mate availability led to more apparent selfers (43.9% vs. 19.0%) and fewer apparent outcrossers (16.7% vs. 46.6%) yet had no effect on apparent plastic snails (39.4% vs. 34.5%), lending a small amount of support to hypothesis 1.

## Reduced female lifetime reproductive success in selfers

Preferential selfing decreased female LRS by reducing both the quantity and quality of eggs. Selfers produced 46.0% fewer developed embryos than plastic mixers and 26.8% fewer than plastic switchers (Fig. 3a, Table 2). In part, this was because egg production was significantly reduced, by 37.4% and 19.1% compared to plastic mixers and switchers, respectively (Table 2). Additionally, selfers had a larger proportion of eggs that failed to develop (28.3%) than plastic mixers (17.9%); the difference to plastic switchers (21.4%) was non-significant (Fig. 3b, Table 2). Outcrossers were excluded because of low sample size (n = 5).

Analyses of all female fertile snails corroborated these findings and showed that selfers had a lower female LRS also compared to outcrossers. Selfers and apparent selfers produced 62.7% fewer developed embryos than plastic and apparent plastic snails, and 53.6% fewer than outcrossers and apparent outcrossers (Fig. 3c, Table 2). Again, the reduction was both due to a reduced fecundity (reductions by 56.8% and 46.3%, respectively) and higher proportion of undeveloped embryos in selfers (36.8% vs. 25.5% and 27.8%, respectively, Fig. 3d, Table 2).

These results were corrected for differences in body size and P0 mother identity (Table 2).

## Mating as a female does not prevent selfing

A strong propensity for selfing was not accompanied by a lack of sexual drive. Neither the number of male (i.e., potential sperm donors) nor female mating partners (i.e., potential sperm recipients) was significantly associated with the likelihood of selfing post-isolation (Table 1). Most selfers (53.3%) copulated in the female role and so might have received allosperm. Four out of five (80.8%) selfers mated in the male role, potentially siring outcrossed offspring. Of snails that selfed post-isolation, 72.0% mated as a female.

However, sexual activity and selfing propensity were not completely independent of one another. Significantly fewer selfers (40.0%) than plastic switchers (92.3%) copulated in both sexual roles ($b$ = 2.88, $z$ = 3.18, $p$ = 0.0015; Fig. 4); the difference to plastic mixers was non-significant (70.0%, $b$ = 1.27, $z$ = 1.45, $p$ = 0.15). Outcrossers (80.0%) were excluded from the model due to low sample size (n = 5, Fig. 4). Body size ($b$ = -0.03, $z$ = -0.15, $p$ = 0.88) and P0 mother identity ($\chi^2_1$ = 0.0, $p$ = 1.00) had no effect on the likelihood of mating in both sexual roles.

## Discussion

We found that propensities for selfing differed substantially within this snail population, ranging from exclusive outcrossing to exclusive selfing, despite identical exposure of individuals to mating opportunities. Individual-level variation in selfing propensity has not been fully acknowledged in the mating-system literature, which has traditionally been focused on the species (Vogler and Kalisz 2001; Goodwillie et al. 2005; Jarne and Auld 2006; Moeller et al. 2017) or population level (Whitehead et al. 2018; Christopher et al. 2021). The variation discovered here was cryptic in two ways. First, it only became apparent in the laboratory, owing to an initial period of isolation followed by a period of monitored access to mating partners. By contrast, in the field, both direct and indirect selfing rate estimates of the same population were near-zero, although obtained across three years and throughout the entire breeding season (Bürkli et al. 2017). Using the field results only, the population would have been deemed purely outcrossing and its considerable potential for selfing missed.

Second, at least half of the variation in individual propensity for selfing would have been overlooked had we only recorded selfing, or lack of selfing, during an isolation treatment, without estimating post-isolation selfing rates. This approach, used in pioneering earlier studies (e.g., Jarne and Delay 1990; Coutellec-Vreto et al. 1998), merely reveals an individual's ability to self. It does not uncover its propensity for selfing, which must be assessed in the face of outcrossing opportunities and using genetic markers. Here we show just how variable the propensity is, comprising two types of plastic individuals, selfers, and outcrossers. Selfing-outcrossing thus is a gradient, not a dichotomy – also and perhaps especially at the individual level.

The variation in selfing propensity was only partially caused by variation in mate availability. As expected, selfing was most prevalent when snails were isolated from one another, yet it

often persisted once snails could mate. Moreover, pairing snails repeatedly rather than once had a surprisingly subtle effect, significantly affecting neither the mean selfing rate, the proportion of selfing individuals, nor the likelihood of selfing. Effects of the pairing treatment were non-significant despite large effect sizes and sufficient statistical power: doubling our sample size would not have rendered any of these effects significant (see R Markdown document). Instead, differences were non-significant because variation among snails was large even within treatment groups. The only significant effects of the pairing treatment were a reduced overall number of selfed offspring among repeatedly paired snails, showing that increased mate availability reduced selfing in high-fitness individuals, and an overabundance of apparent outcrossers, and scarcity of apparent selfers, among repeatedly paired snails when considering the full dataset.

Superficially, the subtlety of these effects contradicts ecological models of selfing, which assume an increased advantage and thus higher prevalence of selfing at low population density (Jarne and Charlesworth 1993; Uyenoyama et al. 1993). Earlier studies have struggled to find support for the reproductive assurance hypothesis of partial selfing in snails. Selfing rates were not increased in low-density, disturbed, or temporary habitats (Trouvé et al. 2003; Henry et al. 2005; Puurtinen et al. 2007; Kupfernagel et al. 2010), neither the frequency nor duration of outcrossing depended on the length of a mating opportunity (Vianey-Liaud et al. 1989), and snails did not adjust their waiting time to the perceived density of conspecifics (Henry et al. 2006). And yet, it is hard to imagine a scenario in which the ability to reproduce independently does not feature among the primary evolutionary drivers of selfing.

We think our study offers a potential reconciliation between the reproductive assurance hypothesis and its weak empirical support. First, reproductive assurance matters, but only in extreme situations. Consequently, there is an ascertainment bias: if a population is dense enough to be sampled, it might be too dense for selfing to be common. This is illustrated by the

near-complete lack of selfing in our study population under field conditions (Bürkli et al. 2017) despite its evident self-fertility. Clearly, the field density of this population is sufficient to prevent selfing in virtually everybody – especially since prolonged outcrossing is often possible after a single mating opportunity, as shown here and elsewhere (Vianey-Liaud et al. 1989; Nakadera et al. 2014). At this stage, we do not know what caused our snails to self after mating trials had begun: either a shortage of compatible mates even if paired repeatedly, or the initial phase of isolation, which may have primed snails for selfing. A study where snails are paired as soon as they reach maturity will need to distinguish between these options.

The second part of the reconciliation is understanding that a population's selfing rate depends on the distribution of individual selfing propensities among its members, which we advocate is a key trait in hermaphroditic species. As the selfing propensity consists of a fundamental, likely genetically based component and an environmental component (e.g., population density), the relationship between density and population-level selfing rates is complicated. How much a population's mean selfing rate reflects current density depends on the relative frequency of individuals that can respond to density variation with increased or decreased selfing, i.e., on the frequency of plastic individuals and selfers compared to outcrossers. Consequently, the selfing rate can be low because a population mainly consists of outcrossers that either outcross or fail to reproduce (even if density is low), or because density is high enough for outcrossing to occur even in individuals with high affinity for selfing. Interpreting high population-level selfing rates is perhaps easier, as they necessitate a sizeable proportion of plastic individuals or selfers. However, also in highly selfing populations density is difficult to predict, as the threshold density beneath which selfing is initiated may be population- or species-specific. Finally, a high selfing rate does not mean that all members of the population are self-compatible: there may still be surviving outcrossers from a previous phase of higher density, or outcrossers may keep being produced if self-incompatibility were to be encoded on a recessive allele.

Our results hint at a genetic background to variation in selfing propensity, in accordance with findings in other hermaphroditic animals (Tsitrone et al. 2003; Escobar et al. 2007; Escobar et al. 2009; Ramm et al. 2012; Sawada et al. 2020). A non-negligible proportion of snails did not reproduce in isolation: 37% of female fertile snails and 9% of the subset with genotyped offspring. These individuals all had post-isolation selfing rates of zero. An aversion to selfing so strong as to risk reproductive failure when deprived of mating partners is so clearly maladaptive that a genetic self-incompatibility mechanism appears plausible. Simultaneously, some snails were at the other end of the spectrum, extensively selfing after being paired and even after mating as a female. Invariably, these snails had already successfully selfed in isolation. Lastly, we found intermediate, plastic snails that selfed in isolation but not, or only a little, when outcrossing was possible. Our results thus imply that a detailed investigation of the genetic basis of variation in self-compatibility would be interesting in this population.

So, what maintains self-compatibility alleles in our study population, and why have they not gone to fixation? The cost of not having them is obvious – an occasional crash of population size will quickly wipe out any individuals that lack self-compatibility alleles. The benefit of not having them, as demonstrated here, is a higher female LRS, partly resulting from increased fecundity (likely due to increased clutch size, Felmy et al. 2020), and partly from decreased embryo mortality in the egg (potentially arising from early-acting inbreeding depression, Charlesworth and Charlesworth 1987). Male LRS might be higher too, particularly if selfers have sperm discounting (Porcher and Lande 2005) or a reduced energy allocation to the male function (Winkler and Ramm 2018). However, the strong sexual drive of selfers documented here (80% mated in the male role) suggests that the difference in siring success between selfers and outcrossers might be more modest. Additionally, selfed individuals may suffer from inbreeding depression in juvenile and adult life; inbreeding depression has not yet been

estimated in our study population, but has been found in other populations (Evanno et al. 2006) and in a close relative (Jarne and Delay 1990; Coutellec-Vreto et al. 1998).

Finally, it is worth pointing out the dangers of assuming that providing individuals with mating opportunities results in all their offspring being outcrossed, as done in some early work (e.g., Jarne and Delay 1990; Coutellec-Vreto et al. 1998). In the present study, 45% of paired snails selfed at least partially, and female copulations were observed in 72% of snails that selfed post-isolation. Hence, outcrossing is neither guaranteed by pairing individuals once or repeatedly, nor by observing female copulations; to know that paired individuals are outcrossing, genetic paternity analysis is unavoidable. The most obvious risk of failing to verify outcrossing events is underestimating the frequency and extent of selfing. Another risk pertains to estimates of inbreeding depression, often obtained by comparing obligately selfed individuals produced by isolated parents with purportedly outcrossed individuals produced by paired parents (e.g., Henry et al. 2003; Puurtinen et al. 2004; Weeks and Bernhardt 2004; Escobar et al. 2011). If some “outcrossed” individuals were, in truth, selfed, the strength of inbreeding depression would be underestimated. We here add to a growing number of studies that tested the assumption of (near-)exclusive outcrossing after pairing empirically, and found it sometimes confirmed (Physa acuta: Pélissié et al. 2012), but more commonly refuted (Bulinus globosus: Njiokou et al. 1992; Bulinus truncatus: Doums et al. 1996; Galba truncatula: Meunier et al. 2004; Lymnaea stagnalis: Nakadera et al. 2014).

## Ethics

We performed our study in accordance with the national laws.

## Data accessibility

Microsatellite markers: GenBank Accession nos. KX830983-KX830992. Microsatellite genotype and phenotypic data: Dryad X.

## Authors' contributions

A.F. designed the study, performed experiments, carried out molecular work, and analysed the data. A.F. and A.S. drafted the manuscript, with input from J.J. All authors contributed to manuscript revision and gave final approval for publication.

## Conflict of interest

The authors declare no conflict of interest.

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# Tables

## Table 1

**Generalised linear mixed model on the probability of selfing post-isolation.** Results are shown for the subset of F1 snails with genotyped offspring (n = 56). The response variable was the likelihood of F1 snails producing at least one selfed juvenile after the isolation treatment had ended (no vs. yes), modelled using binomial errors. Fixed effects were the experimental treatment (once- vs. repeatedly paired; test of hypothesis 1, predicting that selfing is less common under increased mate availability), the number of developed embryos produced in isolation (test of hypothesis 2, predicting that selfing is more common in snails that selfed in isolation), and the number of male partners (potential sperm donors) and female partners (potential sperm recipients) as snail had copulated with. P0 mother identity was included as a random effect; its effect was non-significant ($\chi^2_1$ = 0.0, $p$ = 1.0). Results are provided on a logit scale. S.E.: standard error.

| Predictor | Estimate (S.E.) | *z*-value | *p*-value |
|---|---|---|---|
| Intercept | -0.13 (0.80) | -0.17 | 0.87 |
| Treatment (repeated mating opportunities) | 1.47 (1.73) | 0.85 | 0.40 |
| Number of developed embryos produced in isolation | 0.01 (0.01) | 2.16 | 0.0310 |
| Number of male mating partners | -0.77 (0.55) | -1.40 | 0.16 |
| Number of female mating partners | -0.16 (0.40) | -0.40 | 0.69 |

## Table 2

**Generalised linear mixed models on female LRS and its components.** Results are shown for six models, three using the subset of F1 snails with genotyped offspring (n = 51; five outcrossers were excluded due to low sample size) and three using all F1 snails that had produced at least one developed embryo (n = 126). Response variables were female LRS, the lifetime number of developed embryos, and the lifetime proportion of undeveloped embryos. For the first two response variables, negative binomial errors were fitted, and for the third, Gaussian errors, as the proportion of undeveloped embryos was approximately normally distributed, as judged from QQ-plots and two-sided Kolmogorov-Smirnov tests (subset: $D$ = 0.12, $p$ = 0.45; whole dataset: $D$ = 0.08, $p$ = 0.36). Fixed effects were propensity for selfing (reference level: selfers) and body size (measured as shell length). P0 mother identity was included as a random intercept; its effect was non-significant in the subset ($\chi^2_1 \leq 1.53$, $p \geq 0.22$) but significant in the full dataset ($\chi^2_1 \geq 5.50$, $p \leq 0.0190$). Results for the first two response variables are provided on the log scale. Note that outcrossers could not be included in models of the subset because of low sample size (n = 5). S.E.: standard error, PFS.: propensity for selfing.

| Data | Response | Predictor | Estimate (S.E.) | *z*-value | *p*-value |
|---|---|---|---|---|---|
| Subset | Number of eggs (LRS) | Intercept | 4.41 (0.51) | 8.58 | < 0.0001 |
| | | PFS (plastic mixer) | 0.44 (0.15) | 3.00 | 0.0027 |
| | | PFS (plastic switcher) | 0.27 (0.13) | 2.12 | 0.0342 |
| | | Body size | 0.10 (0.03) | 2.84 | 0.0045 |
| Subset | Number of developed embryos | Intercept | 4.30 (0.58) | 7.43 | < 0.0001 |
| | | PFS (plastic mixer) | 0.58 (0.17) | 3.51 | 0.0004 |
| | | PFS (plastic switcher) | 0.36 (0.15) | 2.41 | 0.0158 |
| | | Body size | 0.08 (0.04) | 2.13 | 0.0329 |
| Subset | Proportion undeveloped embryos | Intercept | 14.51 (16.69) | 0.87 | 0.38 |
| | | PFS (plastic mixer) | -11.34 (5.25) | -2.16 | 0.0309 |
| | | PFS (plastic switcher) | -4.98 (4.22) | -1.18 | 0.24 |

| Data | Response | Predictor | Estimate (S.E.) | *z*-value | *p*-value |
|---|---|---|---|---|---|
| | | Body size | 0.91 (1.13) | 0.81 | 0.42 |
| All | Number of eggs (LRS) | Intercept | 2.71 (0.59) | 4.60 | < 0.0001 |
| | | PFS (likely plastic snail) | 1.01 (0.17) | 6.12 | < 0.0001 |
| | | PFS (likely outcrosser) | 0.85 (0.17) | 4.95 | < 0.0001 |
| | | Body size | 0.16 (0.04) | 3.90 | < 0.0001 |
| All | Number of developed embryos | Intercept | 2.55 (0.63) | 4.06 | < 0.0001 |
| | | PFS (likely plastic snail) | 1.08 (0.18) | 6.13 | < 0.0001 |
| | | PFS (likely outcrosser) | 0.92 (0.18) | 4.96 | < 0.0001 |
| | | Body size | 0.14 (0.04) | 3.35 | 0.0008 |
| All | Proportion undeveloped embryos | Intercept | 31.07 (14.39) | 2.16 | 0.0308 |
| | | PFS (likely plastic snail) | -12.67 (3.80) | -3.34 | 0.0009 |
| | | PFS (likely outcrosser) | -11.57 (3.90) | -2.97 | 0.0030 |
| | | Body size | 0.46 (1.03) | 0.44 | 0.66 |

# Figure legends

## Figure 1

**Experimental variation in mate availability did not have an effect on individual-level selfing rates.** Snails either had one mating opportunity with a single partner, or three to five subsequent mating opportunities, each with a different partner. (a) Paternity distributions among the progeny of 57 F1 snails. Each vertical bar depicts one F2 family, with sections in different colours corresponding to juveniles sired by different fathers. Father 1 refers to F1 snails' first and father 5 to their last mating partner, but identical colours in different F2 families do not indicate the same father identity. Within mating treatments, F2 families are arranged according to selfing rate and number of genotyped juveniles. (b) Differences in mean selfing rates between once- and repeatedly paired snails were not statistically significant. White triangles on boxplots show group means. (c) The proportion of F2 families containing at least some selfed juveniles was similar among once- and repeatedly paired snails. (d) However, once-paired snails produced significantly more selfed juveniles overall. In (c) and (d), error bars are binomial standard errors (± 1 SE).

## Figure 2

**Variation in individual propensity for selfing.** We ascertained the propensity for selfing of snails with genotyped offspring based on the presence/absence of selfing in isolation and the post-isolation selfing rate. (a) The distribution of post-isolation selfing rates was bimodal among both once- and repeatedly paired snails. (b) Successful selfing in isolation was not a reliable predictor of continued selfing: most previously selfing snails switched to near-complete outcrossing once they could mate. (c) Snails with genotyped offspring belonged to one of four groups with differential selfing propensity: "outcrossers" that never reproduced via selfing,

"plastic switchers" that selfed in isolation but switched to outcrossing once mating trials began, "plastic mixers" that mixed selfing in isolation with a low level of selfing post-isolation, and "selfers" that selfed fully or predominantly throughout their lives. (d) The subset of snails whose offspring were genotyped underrepresents snails that only reproduced before or after the start of mating trials (areas shaded in grey).

## Figure 3

**Reduced female LRS in preferential selfers.** Individuals with a strong propensity for selfing tended to produce the lowest total number of developed embryos (a, c) and the highest proportion of embryos that failed to develop (b, d). These patterns were evident both within the subset of snails whose offspring were genotyped (a, b) and within the larger dataset of female fertile snails (c, d). In (c, d), snails whose offspring were genotyped and whose post-isolation selfing rates are thus known with certainty are shown as black dots. The apparent propensity for selfing of snails without genotyped offspring was ascertained based on the timing of egg production and the mating behaviour. White triangles on boxplots show group means.

## Figure 4

**Poor correspondence between propensity for selfing and sexual drive.** Although selfers (n = 15) were less likely to mate in both sexual roles than plastic switchers (n = 26), they showed considerable sexual activity, with 53.3% mating as a female and 80.0% as a male. Error bars are binomial standard errors (± 1 SE).

# Figures

## Figure 1

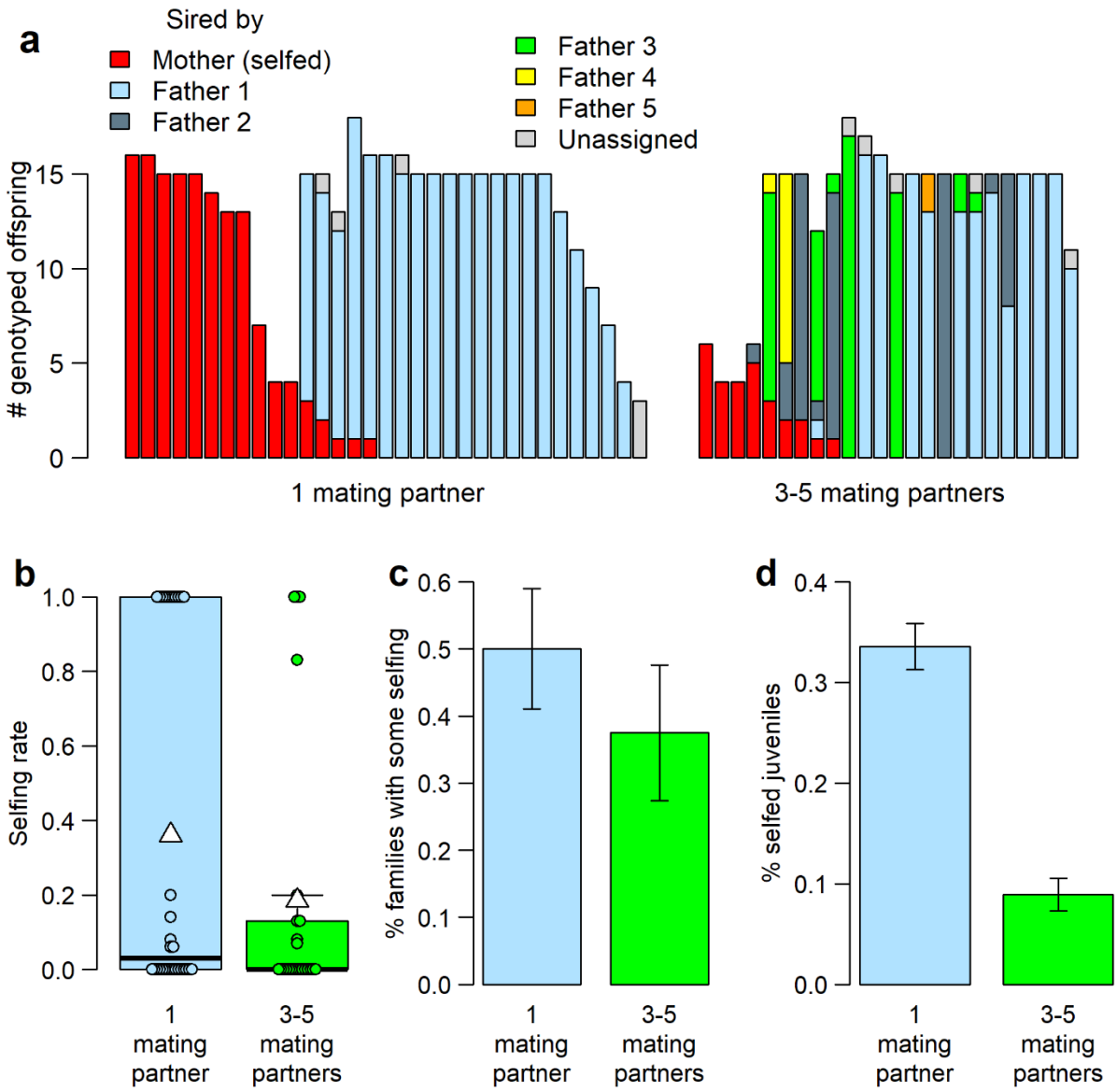


**Figure 2**

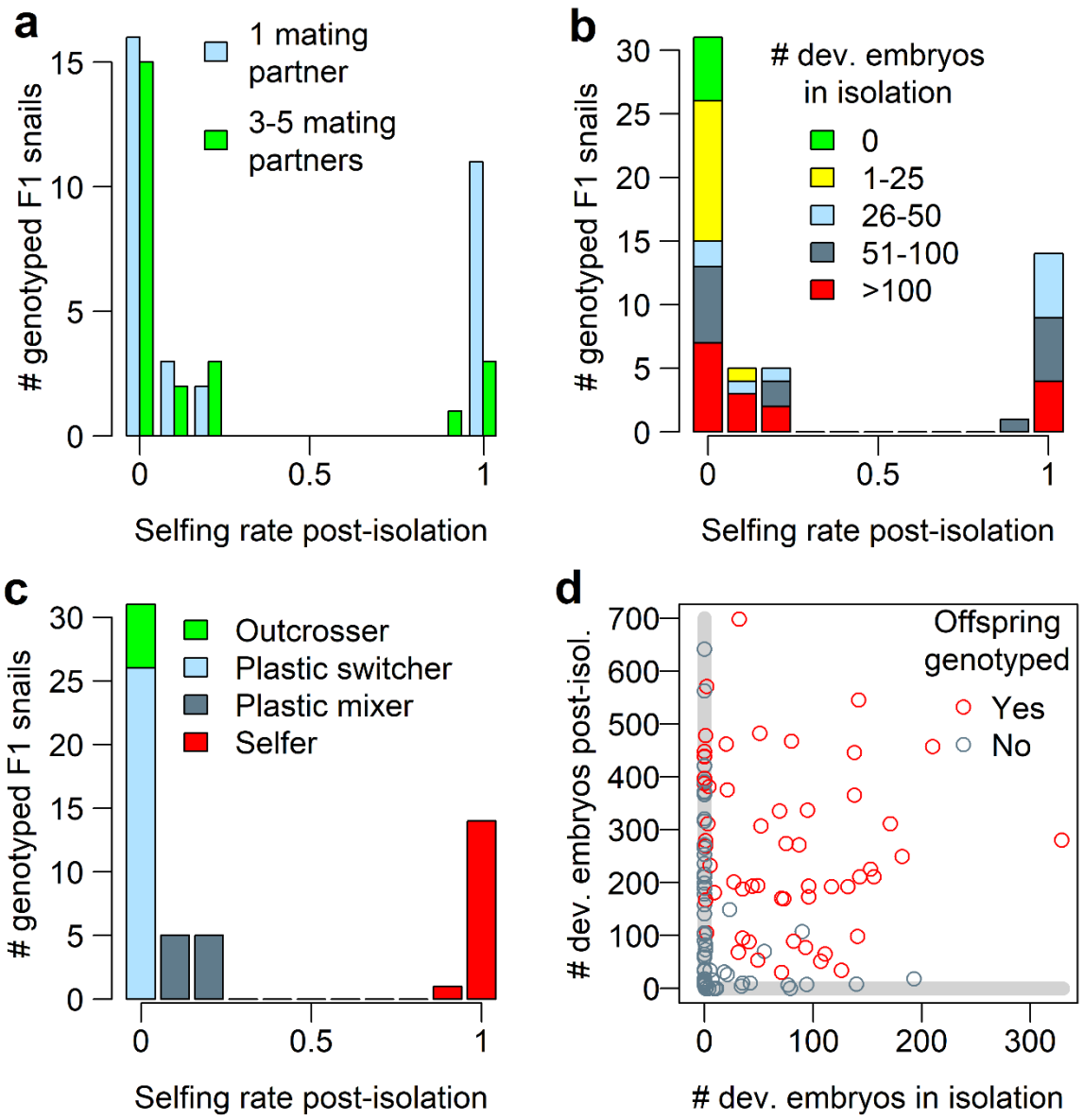

a
1 mating partner
3-5 mating partners
# genotyped F1 snails
Selfing rate post-isolation
b
# dev. embryos in isolation
0
1-25
26-50
51-100
>100
# genotyped F1 snails
Selfing rate post-isolation
c
Outcrosser
Plastic switcher
Plastic mixer
Selfer
# genotyped F1 snails
Selfing rate post-isolation
d
Offspring genotyped
Yes
No
# dev. embryos post-isol.
# dev. embryos in isolation

**Figure 3**

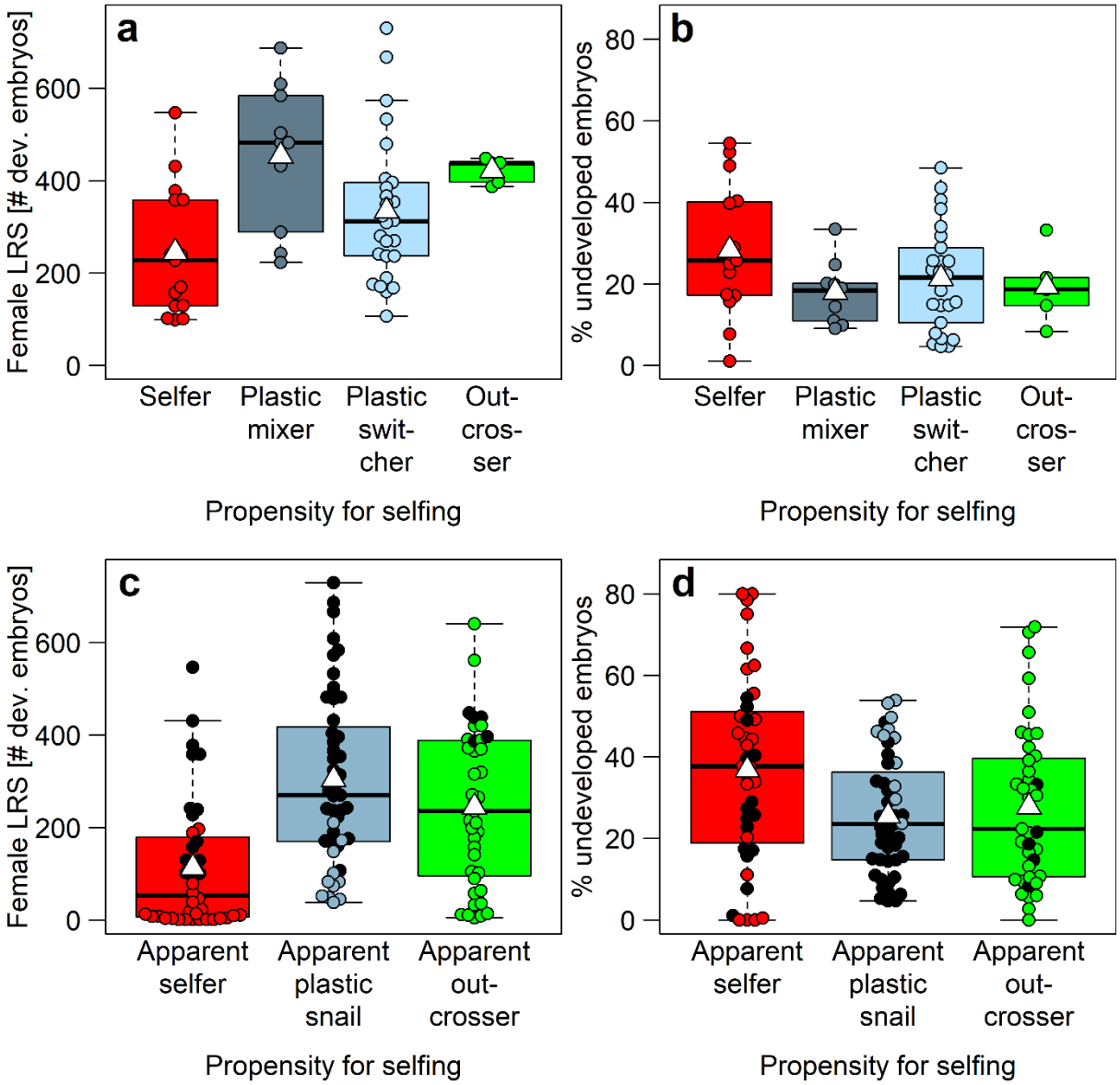
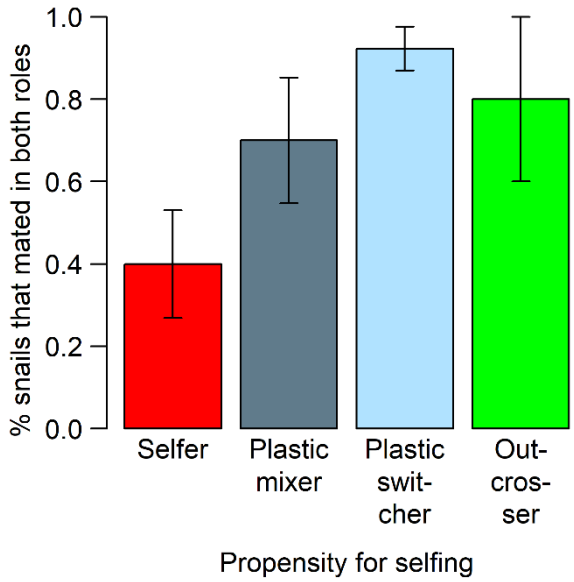

a
Female LRS [# dev. embryos]
0
200
400
600
Selfer
Plastic mixer
Plastic swit-cher
Out-cros-ser
Propensity for selfing
b
% undeveloped embryos
0
20
40
60
80
Selfer
Plastic mixer
Plastic swit-cher
Out-cros-ser
Propensity for selfing
c
Female LRS [# dev. embryos]
0
200
400
600
Apparent selfer
Apparent plastic snail
Apparent out-crosser
Propensity for selfing
d
% undeveloped embryos
0
20
40
60
80
Apparent selfer
Apparent plastic snail
Apparent out-crosser
Propensity for selfing

**Figure 4**


% snails that mated in both roles
1.0
0.8
0.6
0.4
0.2
0.0
Selfer
Plastic mixer
Plastic swit-cher
Out-cros-ser
Propensity for selfing