



HAL
open science

Not just mutations: Inbreeding depression persists without genetic variation

Nicolás Bonel, Christoph Grunau, Patrice David

► To cite this version:

Nicolás Bonel, Christoph Grunau, Patrice David. Not just mutations: Inbreeding depression persists without genetic variation. 2025. <hal-04977368>

HAL Id: hal-04977368

<https://hal.science/hal-04977368v1>

Preprint submitted on 5 Mar 2025

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

1 **Not just mutations: Inbreeding depression persists without genetic**
2 **variation**

3

4 Nicolás Bonel ^{1,2*}, Christoph Grunau ³, Patrice David ²

5

6

7 ¹ Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS—CCT—CONICET Bahía
8 Blanca), Camino de la Carrindanga km 7, Bahía Blanca 8000, Argentina.

9

10

11 ² Centre d'Écologie Fonctionnelle et Évolutive, UMR 5175, CNRS—Université de Montpellier,
12 Université Paul-Valéry Montpellier—École Pratique des Hautes Études—IRD, 34293 Montpellier Cedex
13 05, France.

14

15 ³ IHPE, Univ Perpignan Via Domitia, CNRS, IFREMER, Univ Montpellier, Perpignan, France

16

17 * To whom correspondence should be addressed: nicobonel@gmail.com (N. Bonel)

18

19

20

21

22 **Running head:** Persistence of inbreeding depression in genetically uniform strains

23

24

25

26

27

28

29 **Key words:** Inbreeding depression, *Physa acuta*, epigenetics, inbred lines, self-
30 fertilization, fitness traits, evolutionary dynamics

30

31 **ABSTRACT**

32 Inbreeding depression (ID), the decline in fitness upon inbreeding, is thought to result
33 from a decrease in genetic heterozygosity enhancing phenotypic effects of recessive
34 deleterious mutations. However, emerging evidence suggests that mutations may not
35 explain ID completely. In this study, we test whether ID can emerge even in contexts
36 where genetic heterozygosity does not vary. To that end, highly inbred lines
37 ($F=0.99999997$) of the freshwater snail *Physa acuta* were used to produce individuals
38 with varying levels of parental relatedness (self-fertilization, sibling crosses, and cousin
39 crosses), though with identical genomic heterozygosity. Several fitness traits declined
40 significantly with increasing parental relatedness, a pattern characteristic of ID, and
41 quantitatively representing a non-negligible fraction of the ID usually observed in
42 natural, genetically diverse populations of *Physa acuta*. Individual-based simulations
43 showed that mutation rates compatible with values of ID found in natural populations
44 are way too low to generate as much ID as observed in our experiment. These findings
45 are consistent with the hypothesis that epigenetic changes, in addition to mutations,
46 could contribute to a rapid regeneration of ID and explain the persistence of detectable
47 ID in sets of genetically identical individuals.

48

49

50 INTRODUCTION

51 Inbreeding depression (ID) is the loss in fitness that occurs in offspring of
52 consanguineous matings and is one of the most general observations in evolutionary
53 biology^{1,2}. Understanding the mechanisms of ID is important because it strongly
54 constrains the evolution of mating systems (self-fertilization rates, self-incompatibility)
55 and is a major concern in plant breeding and animal conservation³. ID is classically
56 explained by the presence of recessive deleterious mutations in the genome, whose
57 effects are exposed by homozygosity². Most of the mutations that occur spontaneously
58 at every generation are indeed deleterious and partly recessive^{2,4-6}. In large diploid
59 organisms, however, genomic mutation rates are typically small, implying that the
60 standing inbreeding load is accumulated through many generations until a steady state
61 of mutation, selection, and drift is reached. This view inspired the idea that a substantial
62 fraction of standing ID could be removed in conditions that drastically reduce
63 heterozygosity by increasing the elimination or fixation rates of alleles. The so-called
64 genetic purging is expected to occur by selection when unusual amounts of systematic
65 inbreeding (such as high selfing rates) are applied to large populations, exposing
66 deleterious alleles to selection^{7,8}. ID can also be lost by drift when population size is
67 severely reduced^{8,9}, as alleles (deleterious or not) get fixed and heterozygosity is lost.

68 Inbreeding depression is, however, surprisingly difficult to eliminate
69 completely¹⁰. First, while some evidence of genetic purging has come from comparative
70 studies in natural populations as well as a few experiments¹¹⁻¹⁴, some residual ID
71 frequently persists even in populations subject to recurrent inbreeding^{12,13,15,16}. Second,
72 ID is also not systematically eliminated by drift, even within lines with extremely
73 reduced effective size and highly homozygous, such as those propagated by continuous
74 sib-mating or continuous self-fertilization (e.g.¹⁷). A classical argument explaining the
75 difficulty of purging refers to the genetic architecture of the mutation load. If ID relies
76 on the joint effect of many loci with very small effects, allele frequency changes under
77 selection are expected to be very slow^{18,19}. This argument, however, does not apply to
78 fixation by drift. Purging and fixation may also be impeded by linkage and selective
79 interference between loci¹⁹. Indeed, linkage disequilibrium among deleterious mutations
80 may preserve heterozygosity (and ID) in some regions of the genome, when two
81 deleterious alleles with strong effects (such as lethals) are in repulsion, or when
82 stretches of weak-effect deleterious mutations collectively form pseudo-overdominant

83 blocks¹⁰. Thus, linkage can delay the loss of heterozygosity in small populations and
84 inbred lines. However, this loss eventually takes place given sufficient time, as
85 recombination ends up breaking the associations that prevent homozygosity. Once
86 homozygosity is attained in a genomic region, the sole mechanism that may reintroduce
87 heterozygosity, and regenerate ID, is the occurrence of spontaneous mutations.
88 Mutations, however, are rare and, within a selfing or sib-mating line, they are quickly
89 lost or fixed, so they are not expected to yield substantial levels of ID. Overall, an
90 extended period of very low effective population sizes, such as in an anciently
91 established inbred line, makes it unlikely to observe significant levels of ID. In other
92 words, aside from a few recent mutations, two individuals from the same inbred line are
93 essentially genetic clones. As a result, crossing them is genetically equivalent to self-
94 fertilization, and the fitness of offspring from crossbreeding and selfing should not
95 differ.

96 Recent studies, however, suggest another process that could regenerate
97 inbreeding depression in genetically uniform lines faster than spontaneous mutation:
98 epigenetic change (reviewed by²⁰). Support for a role of epigenetics in ID comes from
99 several lines of evidence^{21–24}. First, ID can be greatly reduced by applying a
100 demethylating enzyme²⁴. Second, in maize, the continued decline in performance within
101 selfing lines from generations 7 to 11 is accompanied by a global increase in DNA
102 methylation while residual heterozygosity is extremely low¹⁷. This suggests that
103 epigenetic change can co-occur with sustained phenotypic deterioration even when
104 genetic homozygosity itself does not increase anymore. Similarly, in *Arabidopsis*,
105 heterosis (the reciprocal of ID) is observed when crossing isogenic epi-RILs (i.e. selfing
106 lines differing only in methylation patterns;²⁵).

107 Epigenetic modifications can be independent of genetic variation (“pure” or
108 autonomous epigenetic variants, *sensu* Richards²⁶) thus being a possible source of
109 heritable phenotypic variation in the absence of DNA sequence change²⁷. A growing
110 body of evidence suggests that incomplete reprogramming and erasure of epigenetic
111 marks during gametogenesis and zygote development could promote stable
112 transgenerational transmission of some epigenetic variants²⁸. Epimutations are
113 generated at higher rates than genetic mutations and tend to revert to the wild-type in a
114 few generations²⁹. A fraction of the standing ID could therefore reflect fast-generated
115 epigenetic changes transmitted over one or a few generations. Phenotypic consequences
116 of autonomous epigenetic inheritance have been classically studied using systems with a

117 known lack of genetic variation (e.g.²¹). In a similar way, if autonomous epigenetic
118 changes play a role in ID, differences in offspring fitness should persist among different
119 types of crosses (self-fertilized, sib-mating, cousin-mating) even when all parents used
120 are genetically identical (i.e. clonemates). Yet, this approach has not been undertaken
121 yet.

122 Here we tested whether significant ID can be detected even when differences in
123 genomic heterozygosity are extremely low. To that end, we created “outbred clones” by
124 intercrossing inbred lines. We produced two highly inbred lines of the hermaphrodite
125 freshwater snail *Physa acuta* (a naturally outcrossing species) that have undergone more
126 than 28 generations of enforced self-fertilization, ensuring minimal residual
127 heterozygosity. We chose this species because it is easy to raise in the laboratory, ID
128 has been repeatedly observed in its natural (outbred) populations, and it can be forced to
129 self-fertilize (the most efficient way to eliminate heterozygosity). Individuals within
130 each line are genetically homozygous and identical, except for neo-mutations in the last
131 few generations. We crossed individuals from two different inbred lines and obtained a
132 set of offspring that were heterozygous and identical to one another (“outbred clone”).
133 We then performed different types of matings with individuals from this outbred clone:
134 self-fertilization, sibling pair-crossing, and cousin pair-crossings. These treatments
135 yielded second-generation offspring with the same expected genomic heterozygosity
136 (ignoring recent mutations), but that differed in pedigree inbreeding, i.e. the number of
137 generations tracing back to the common ancestor of the two uniting gametes. We chose
138 to start with outbred parents (though genetically belonging to the same “clone”) rather
139 than directly from inbred ones, because highly inbred individuals are rather weak, as
140 expected, a condition we deemed “unnatural”. In contrast, the results obtained with
141 normally heterozygous, outbred individuals, are representative of processes that could
142 contribute to ID in natural contexts.

143 To evaluate whether parental relatedness (mother and father being either the
144 same individual, or siblings, or cousins) affected progeny fitness, we measured juvenile
145 survival, adult body size, probability of laying eggs (in isolation) at the age of 50 days,
146 and female fecundity after insemination. Classical genetic theory predicts the same
147 average performances in all three types of parental relatedness as offspring all have the
148 same genomic heterozygosity.

149

150 RESULTS

151 Figure 1 recapitulates the pedigree of the parents (F_2) we used to produce F_3 snails by
152 either self-fertilization, sib-mating or cousin-mating. Table 1 and Figure 2 summarize
153 our main results, while linear models are reported in detail in Tables S1-S3.

154 Juvenile survival significantly differed across treatments. The highest survival
155 was in the offspring of cousin-cross (average 0.76) whereas survival in the selfing and
156 sib-cross treatments was reduced by 18% and 9%, respectively, compared to cousin-
157 cross (Table 1, Fig. 2A). Body weight showed a similar pattern, the cousin-cross
158 treatment had the highest mean, with a reduction of 20% and 14% in the self-
159 fertilization and sib-cross treatment, respectively (Table 1, Fig. 2B). Despite this
160 difference in body weight, there was no significant effect of parental relatedness on
161 female fecundity at 53 days, after insemination (Table 1, Fig. 2C). In contrast, we found
162 a strong treatment effect on the probability of having produced eggs by self-fertilization
163 at the age of 50 days. This probability was the highest in cousin-cross treatment and it
164 decreased by 77% and 35% in the selfing and sib-cross treatments, respectively (Table
165 1, Fig. 2D).

166 We genotyped F_2 individuals, which represent inter-line crosses in our design
167 (their offspring are F_3), using microsatellites fixed for alternative alleles in the two
168 inbred lines. As expected, almost all F_2 were fully heterozygous, with rare exceptions
169 where one or two loci appeared homozygous in a few individuals, likely due to
170 genotyping errors (Fig. 3). In the F_3 generation, we found no significant differences in
171 heterozygosity among mating treatments ($X^2_2 = 0.475$, $P = 0.789$, binomial model on the
172 proportion of heterozygous loci). The multilocus heterozygosity distribution within each
173 treatment was centered around 0.5, consistent with expectations for $F_2 \times F_2$ progeny:
174 selfing ($H_O = 0.51 \pm 0.05$), sib-cross ($H_O = 0.49 \pm 0.16$), and cousin-cross ($H_O =$
175 0.52 ± 0.16 ; Fig. 3).

176 We conducted simulations replicating our experimental design to determine
177 whether the observed differences between treatments could result from incomplete
178 fixation, linkage, or spontaneous mutation. The mutational parameters were selected to
179 align with previous estimates of ID in natural, outbred populations of *Physa acuta*³⁰.
180 The simulations showed that offspring fitness from full-sib and cousin crosses was only
181 slightly higher than that from the selfing treatment, with differences of 0.8% and 2.0%,
182 respectively—well below the observed differences in juvenile survival, body weight,

183 and the probability to lay eggs in isolation (Fig. 4). We tested various parameter sets
184 compatible with naturally observed levels of ID, adjusting linkage, contributions of
185 lethal mutations, and selection or dominance coefficients, but these had minimal effects
186 on the results. The largest predicted difference in fitness between cousin-cross, sib-
187 cross, and self-fertilization occurred when the source of ID was driven quasi-exclusively
188 by mutations to semi-lethals ($d_1=0.49$, $d_2=0.01$) and when these mutations were nearly
189 fully lethal ($s_1=0.99$). However, even under these extreme conditions, average fitness
190 differences never exceeded 5% (see Fig. S7). While individual replicate runs varied
191 considerably within each parameter set, the mean predictions consistently fell well
192 below the observed ratios in all situations. Simulations could match the experimental
193 results only when the mutation rate was artificially increased by approximately 10- to
194 12 fold beyond the levels of ID estimated for natural populations of *P. acuta* (Fig. S8).

195

196 **DISCUSSION**

197 Our study reveals substantial inbreeding depression (ID) in the progeny of
198 experimental lines, characterized by distinct levels of parental relatedness. Three fitness
199 traits —juvenile survival, body mass, and self-fertility—declined as parental relatedness
200 increased (self<sibs<cousins), while female fecundity showed no significant
201 differences. The amount of ID in juvenile survival (0.18) and body mass (0.20) in the
202 selfing treatment, relative to the cousin cross, is lower than typical values reported for
203 outbred and self-fertilized offspring of wild-caught *Physa acuta*, where ID estimates are
204 approximately 0.55 for juvenile survival^{30,31} and 0.38 for body mass³¹. Comparable data
205 for self-fertility are not available. Despite these lower ID values, the levels observed in
206 our experiment—amounting to one-third to one-half of those in natural outbred
207 populations for key traits—are striking and somewhat paradoxical given the unique
208 design of our study: all three types of offspring have identical genomic homozygosity.
209 During the establishment of the inbred lines, deleterious mutations have been either
210 eliminated or fixed, resulting in identical levels of heterozygosity across all F₃
211 categories (selfed, sib-cross, and cousin-cross)—all expected to retain half the
212 heterozygosity of the genetically uniform F₂. Under mutation-based ID theory, we
213 would predict similar average fitness across treatments, yet our results clearly indicate
214 otherwise.

215 One potential explanation for this result is that some F₂ or F₃ individuals may
216 have failed to successfully cross-fertilize, possibly due to a loss of cross-fertilization

217 ability after 28 generations of enforced selfing. However, two key arguments dispel this
218 possibility. First, additional crosses confirmed that these individuals could both fertilize
219 and be fertilized by their partners (see Supporting Information: *Outcrossing ability of*
220 *inbred lines*). Second, microsatellite genotyping supported the expected patterns, with
221 F₂ individuals showing full heterozygosity for the diagnostic markers. Occasional
222 apparent homozygosity at one or two loci is likely due to reading errors and does not
223 indicate selfed offspring, which would be fully homozygous like their parents.
224 Moreover, observed microsatellite heterozygosity did not differ among the three classes
225 of F₃ offspring, and was consistently at 0.5, as expected. Note that in classical ID
226 experiments, self-fertilized broods typically exhibit half the heterozygosity of
227 outcrossed ones.

228 In the absence of differences in genomic heterozygosity, what explains the
229 observed ID? A first hypothesis is that the reduced fitness in the selfing treatment may
230 stem from maternal effects³². However, such maternal effects in this case would not be
231 linked to variation in maternal quality, as mothers were randomly assigned to all
232 treatments. Instead, they might reflect a parental component of ID, where less energy is
233 allocated to self-fertilized eggs compared to outcrossed ones (e.g.²⁰). While plausible,
234 this argument fails to account for the fitness differences between the sib- and cousin-
235 cross treatments. Moreover, fitness traits in *Physa acuta*, including juvenile survival, are
236 not known to be strongly influenced by maternal effects. For instance, outcrossed
237 offspring of inbred mothers do not exhibit lower fitness than offspring of outbred
238 mothers (*cf.*^{14,31}), even though the inbred mothers themselves are less fit than outbred
239 ones.

240 Alternatively, the observed variation in ID among mating treatments could result
241 from the long-term persistence of localized heterozygosity in specific genomic regions
242 within our inbred lines. This may occur due to linkage disequilibrium among deleterious
243 mutations, constituting pseudo-overdominant blocks¹⁰. Such blocks would not be
244 detected by the seven microsatellites used in our study, which represent only a small
245 fraction of the genome. However, our simulations do not support the hypothesis that
246 pseudo-overdominance, driven by tightly linked deleterious alleles, could persist or
247 accumulate over the 25+ generations of extreme inbreeding experienced by our lines.
248 Specifically, our simulations show that mutations generate negligible fitness differences
249 among offspring from selfing, sib-, and cousin-crosses. Even when we artificially
250 increased linkage by reducing the genome size to the unrealistic value of 1 Morgan

251 (despite *Physa acuta* having 19 pairs of chromosomes), the resulting fitness ratios
252 remained virtually unchanged. This suggests that linkage disequilibria among mutations
253 are insufficient to maintain heterozygosity in highly inbred lines such as those used in
254 our study. In contrast, simulations suggest that fitness contrasts are maximized when
255 inbreeding depression arises from semi-lethal mutations. While these mutations may not
256 persist for long, they can occasionally be present in a heterozygous state in the parents
257 at the time of experimentation. It is important to note that lethal mutations are lost at a
258 rate of one-third per generation in selfing lines. Indeed, homozygous lethal individuals
259 are unviable, so that among viable offspring produced by a heterozygote, one-third are
260 homozygous wild-type, and two-thirds heterozygous. As a result, the half-life of lethal
261 mutations in selfing lines is approximately 2-3 generations. Heterozygosity resulting
262 from mutations with milder effects has an even shorter half-life, as homozygous
263 mutants are not completely eliminated from the population. Thus, in long-established
264 selfing lines, the heterozygous load is likely to reflect only a few generations (2-3) of
265 accumulated spontaneous mutations.

266 Even in the most favorable scenario, which assumes an unusually high
267 proportion of lethal mutations, our findings remain highly unlikely. In our reference
268 scenario, we modeled an average per-haploid genome rate of spontaneous semi-lethal
269 mutations of $U_1 = 0.028$. Although direct estimates of U_1 are unavailable for *Physa*
270 *acuta*, this value is close to those reported for *Drosophila melanogaster*: 0.003 to 0.017
271 for the large chromosomes 2 and 3, and 0.006 to 0.034 for the full genome³³⁻³⁵. Across
272 all simulations, cousin-crossed or sib-crossed offspring consistently showed, on
273 average, a small but positive viability advantage (<5%) over self-fertilized offspring.
274 This aligns with the persistence of spontaneous mutations over two or three generations,
275 as discussed earlier. However, this predicted advantage is far smaller than the observed
276 differences in juvenile survival. Notably, fitness ratios across simulations exhibit
277 significant stochastic variation (Fig. 4), particularly when lethal mutations are frequent.
278 This variability is expected, given the stochastic nature of recent mutations in the lines
279 just a few generations before the experiment. While the observed survival estimates
280 remain improbable, a small fraction of simulations occasionally yield survival ratios
281 that are similar to those observed: 3.8 % for Sibling/Selfing, 0.8% for Cousin/Selfing,
282 and 6% for Cousin/Sibling (Fig. 4). To definitively rule out a purely mutational origin
283 for these survival differences, replication with independent line pairs would be
284 necessary.

285

286 Body size and the proportion of self-fertile individuals at the age of 50 days also
287 showed clear signs of ID, with ratios even higher than those observed for juvenile
288 survival. These traits expressed later in life, typically contribute less to overall ID than
289 juvenile survival in wild-caught *Physo acuta* and are less susceptible to purging^{14,30,31}.
290 This suggests that the genetic load affecting these traits is more strongly influenced by
291 small-effect alleles than by semi-lethal mutations. Consequently, simulating these traits
292 may require parameter adjustments, such as lower per-generation genomic mutation
293 rates and a higher proportion of small-effect mutations. However, such adjustments
294 would inevitably reduce residual heterozygosity within the lines, further decreasing all
295 ratios and pushing simulated values even farther from the observed results.

296 Taken together, the unexpectedly high ID observed across three independent
297 traits in our experiment is highly improbable under a mutational genetic model. To
298 reconcile our findings with such a model, one would need to assume a strong (> 10-
299 fold) increase in mutation rates in the inbred lines compared to the original populations
300 (see Fig. S8). While there is evidence that mutation rates can increase in individuals in
301 poor condition, as may be the case in our inbred lines, the magnitude required remains
302 implausible. For instance, studies in *Drosophila* have shown that individuals carrying a
303 load of fixed deleterious mutations—those in poor condition—tend to accumulate
304 spontaneous mutations at higher rates than healthier individuals^{35,36}. However the
305 observed increases in mutation rates were modest (up to 2-fold) and did not extend to
306 lethal mutations³⁵.

307 Our results therefore suggest either an increase in mutation rates much higher
308 than previous measures, or a source of fast-renewed ID unrelated to mutation.
309 Differences in fitness-related traits could instead arise from the accumulation of
310 deleterious epimutations, such as DNA methylations, small RNAs, and histone
311 modifications. For instance, in maize, inbred lines exhibit a persistent decrease in fitness
312 alongside a consistent rise in genomic methylation over generations of inbreeding¹⁷,
313 likely due to disruptions in methylation resetting mechanisms. However, this is unlikely
314 in our study since the founders (F₂) of the experimental lines were all highly outbred
315 individuals, a condition that would typically restore the overall methylation level back
316 to normal¹⁷. Instead, our findings support the hypothesis that the observed ID may be
317 driven by epigenetic similarity between uniting gametes.

318 Epigenetic marks can be transmitted across generations with incomplete fidelity,
319 suggesting that this information is stored within gametes through mechanisms that
320 remain poorly understood (but see³⁷, and references therein for the role of small RNAs
321 in epigenetic inheritance). Two gametes produced by the same individual are expected
322 to be more similar in their epigenetic information than two gametes produced by full-
323 sibs, while the latter are more similar than those of cousins. This gradient of similarity
324 arises from the increasing number of meiotic cycles, along with subsequent zygote
325 development and epigenetic resetting events, separating paternal and maternal gametes
326 from selfing to sib-cross to cousin-cross (Fig. 1).

327 Although no direct evidence currently supports this hypothesis, some studies
328 suggest that increased fitness (heterosis) can result from epigenetic dissimilarity
329 between parents, as observed in the crossing of isogenic epi-RILs in *Arabidopsis*²⁵. In
330 *Physa acuta*, as well as in other mollusks, parental environmental history can influence
331 offspring's traits through trans-generational plasticity (e.g.,³⁸⁻⁴⁰). In *Biomphalaria*
332 *glabrata*, a freshwater pulmonate gastropod closely related to *Physa*, DNA
333 methyltransferase inhibitors have been shown to disrupt methylation patterns, leading to
334 changes in growth and gene expression that persist in the untreated progeny of exposed
335 individuals⁴¹. Such environmental effects may contribute to epigenetic differences in
336 gametes produced by different individuals. More broadly, considering that epimutations
337 are generated at higher rates than genetic mutations²⁹, it is plausible that a fraction of the
338 standing ID originates from rapidly generated epigenetic changes. These changes,
339 transmitted over one or a few generations, could lead to a decline in performance as
340 parental relatedness increases.

341 In summary, our study demonstrates that inbreeding depression (ID), typically
342 characterized by reduced offspring fitness with increasing parental relatedness, can
343 appear even in genetically uniform populations. This finding challenges traditional
344 genetic models, as in such contexts individuals cannot differ in genomic heterozygosity.
345 The levels of ID we observed, impacting both juvenile and adult traits, are equivalent to
346 a substantial fraction of the ID typically reported in natural polymorphic populations for
347 the same traits. Although spontaneous mutations continuously arise and create minor
348 genetic differences among individuals, these mutations alone seem insufficient to
349 account for the magnitude of effects we observed across three distinct traits. By
350 definition effects that are not due to genetic differences, should be called epigenetic,
351 irrespective of their material basis. While genetic mutations undoubtedly contribute to

352 ID in populations, it would be premature to assume they account for the entirety of its
353 effects, particularly given the notorious difficulty of fully eliminating ID through purge
354 or fixation. Our findings suggest that epigenetic mechanisms may play a more
355 prominent role in ID than previously recognized – in line with emerging evidence that
356 epimutations and epigenetic inheritance contribute to heritable phenotypic variation in
357 general. Future studies, encompassing a broader range of lines and species, are essential
358 to fully unravel the role and impact of epigenetics to inbreeding depression.
359

360 **METHODS**

361 **Study organism and experimental conditions**

362 *Physa acuta* is a widespread simultaneously hermaphroditic freshwater snail that
363 reproduces mostly through cross-fertilization. Snails that have no access to mates can
364 resort to self-fertilization^{42,43}. When a potential mate is present, however, individuals
365 nearly always cross-fertilize^{44,45}. These snails mate frequently, with unilateral
366 copulations (*i. e.*, they adopt either the male or the female sex role during a given
367 copulation⁴⁶), and can store sperm for long periods. Sexual maturity is reached in
368 around 6 weeks under laboratory conditions at 25 °C.

369 Throughout the experiments—and also during the establishment of experimental
370 lines—snails were maintained under standard laboratory conditions (25 °C, a 12:12
371 photoperiod, water renewed twice a week, and *ad-libitum* food in the form of boiled
372 ground lettuce). Each individual was kept in a 75 mL plastic box. In addition, albino
373 lines of *P. acuta* are maintained in our laboratory. Albinism (a single-locus recessive
374 trait), can be used to assess paternity success of wild-type focal individuals because
375 wild-type offspring produced by albino mothers must have a wild-type father.

376

377 **Field sampling and breeding design**

378 The first step was to collect snails from the field and generate two inbred lines through
379 continuous self-fertilization over many generations. Self-fertilization was enforced by
380 keeping mature individuals isolated until they lay eggs, usually two or three weeks after
381 the age at which they normally start laying eggs when they have mates available⁴⁷. The
382 two ancestral wild-caught snails were part of a larger sample taken from a wild
383 population at the “Pont Romain” in the Lirou river (43°43'47.0"N, 3°49'50.4"E), located
384 close to the village of Les Matelles, 15 km north of Montpellier (France) on October 23,

385 2013. The initiation of 44 inbred lines is described in Janicke et al.⁴⁸, who used them for
386 an experiment after the third generation of self-fertilization. A subset of the lines were
387 propagated by continuous self-fertilization until May 2019, a time at which they had all
388 reached at least 25 generations of selfing. The inbreeding coefficient at these stage is
389 therefore expected to be, at least, $1-1/2^{25} = 0.99999997$. To evaluate whether inbred
390 individuals were still capable of cross fertilization, we performed a preliminary analysis
391 described in Supporting Information. Once confirmed that individuals from inbred lines
392 were fully capable of performing cross-fertilization, we conducted the main assay.

393

394 **Estimating inbreeding depression in outbred clones**

395 We selected two inbred lines named 120 and 110. At this stage, these lines had
396 undergone 28 and 29 generations of self-fertilization, respectively. The residual
397 heterozygosity is expected to be $< 4 \cdot 10^{-9}$ times that of the wild-caught individuals,
398 ignoring recent mutations. As a first approximation, we therefore consider inbred lines
399 as fully-homozygous clones. The pedigree and the mating protocol described below are
400 depicted in figure 1 and in figures S1 to S6 from the appendix.

401

402 *First step: obtaining of F₂ snails*

403 One individual from each inbred line, 120 and 110, was isolated and forced to self-
404 fertilize to produce the F₁. F₁ offspring were isolated at day 28 after egg-laying (still
405 immature) and then raised in isolation until maturity for three more weeks. We assigned
406 a code letter to the F₁ siblings for identification (120_a, 120_b, 120_c, and so on; and, 110_a,
407 110_b, 110_c, and so on), constituted inter-line pairs of F₁ individuals (e.g. 120_a x 110_a,
408 120_b x 110_b), and let them mate for one week. To differentiate the 120 snail from its 110
409 partner in each pair, we added small dots of harmless car-body paint of different colors
410 on their shell⁴⁹. After mating, F₁ individuals were re-isolated to lay F₂ eggs. We
411 successfully collected and raised the resulting F₂ hatchlings for 10 families *a* to *j* (Fig.
412 1).

413 F₂ snails belonged to two different types, hereafter type S and type T. The S-type
414 F₂ offspring were laid by a mother from the 120 line, and sired by fathers from the 110
415 line, while T-type offspring came from the reciprocal combination (mother 110 – father
416 120). As these two types are present within all 10 families *a* to *j*, we end up with 20
417 categories (S_{*a-j*} and T_{*a-j*}), each represented by several offspring. Note that irrespective of
418 the category they belonged to, all F₂ were expected to be heterozygous and genetically

419 identical to one another (i.e. they form a heterozygous clone; Fig. 1 and S1). We
420 proceeded until we obtained at least 40 F₂ descendants of type S and 10 of type T for
421 each family. This procedure sometimes required taking several successive clutches or
422 even re-mating the same pair to stimulate egg-laying. These descendants were raised
423 and isolated before maturity, as previously mentioned, and kept until fully mature and
424 ready for mating. Then, we used them to generate different types of F₃ snails.

425

426 *Second step: self-fertilization, sib-, and cousin pair-crosses*

427 We submitted each F₂ snail to one of three mating treatments: (i) selfing, (ii) sib-cross,
428 and (iii) cousin-cross. Sib and cousin crosses were themselves divided in two subtypes
429 depending on direction of exchange of genetic material (one- and two-ways), giving
430 five treatments in total. We explain this in more detail in the following.

431 The first treatment included S-type individuals that were forced to self-fertilize
432 (Selfing, Figs. 1 and S2). Thus, ten F₂ S-type individuals per family (i.e., S_{a,1-10}, S_{b,1-10},
433 ..., S_{j,1-10}) were isolated and eggs were collected three days after we detected the first
434 clutch. The second treatment consisted in pair-crossings between two S-type snails from
435 the same family (called “one-way sib cross”). So, for instance, in family *a*, we crossed
436 individuals S_{a,11} ... S_{a,20}, according to the following pairs: S_{a,11}/S_{a,16}, S_{a,12}/S_{a,17}, and so on
437 (and similarly for S_b to S_j) (Figs. 2 and S3). Note that in these crosses the two parents
438 are full sibs and they have the same mother and the same father. The third treatment
439 consisted in pair-crossings within the same family (e.g. family *a*) but between S and T
440 individuals, which we defined as “two-way sib cross”. For instance, we paired snails
441 from S_a (numbered from 21 to 25) with snails from T_a (numbered from 1 to 5), which
442 gave us the following combinations: S_{a,21} / T_{a,1}, S_{a,22} / T_{a,2}, and S_{a,23} / T_{a,3}, and so on.
443 The two parents in these crosses are full-sibs but not obtained in the same way (the
444 father of one is the mother of the other and reciprocally; Figs. 2 and S4). The fourth
445 treatment involved pair-crossings between two F₂ S-type snails (numbered from 26 to
446 35) from different families, which we called the one-way cousin cross (the two parents
447 are cousins, belonging to the same type). Because of insufficient individuals in families
448 *h* and *j* we were able to do this for four pairs of families. The resulting combinations
449 were: S_{a,26-35} / S_{b,26-35}, S_{c,26-35} / S_{d,26-35}, S_{e,26-35} / S_{f,26-35} and S_{g,26-35} / S_{i,26-35} (Figs. 2 and
450 S5). Finally, the fifth treatment consisted in pair-crossings between snails of the S-type
451 (36 to 40) and of the T-type (6 to 10) from different families, which we defined as the
452 two-way cousin cross. The combinations were: S_{a,36-40} / T_{b,6-10}, S_{c,36-40} / T_{e,6-10} or S_{d,36-40} /

453 $T_{f,6-10}$, and so on (Figs. 2 and S6). When necessary, we added different color dots of car-
454 body paint on each individual's shell to differentiate snails from each family and origin.

455 After pairing for three days, mated snails were re-isolated to lay eggs for three
456 days. We collected two successive F_3 clutches for each F_2 parent, by transferring the
457 parent to a new box after three days (Fig. S9). These F_3 clutches constitute the
458 generation on which we evaluated fitness traits.

459

460 *Third step: assessing and comparing fitness components in F_3 snails*

461 Eggs were counted in all F_3 clutches right after collection and live juveniles were
462 counted in the boxes 14 days later. We estimated juvenile survival as the ratio of
463 juveniles over eggs. Next, we randomly selected one of the surviving F_3 individuals per
464 box and raised it in isolation until age 50 days. At this age we weighed each snail and
465 determined whether it had initiated egg laying or not). Given that each individual was
466 kept in isolation, this egg-laying represents self-fertilization. Then, we paired it with a
467 same-age albino partner for 3 days. Once inseminated, we isolated the F_3 individual
468 once more, weighed it a second time, and allowed it to lay eggs for 3 days. Finally, we
469 counted these eggs that represent female fecundity of F_3 under cross-fertilization
470 conditions. We therefore have four traits on the F_3 : juvenile survival, body mass at 50-
471 53 days (the mean of the two measures), self-fertilization ability at 50 days (yes/no),
472 female fecundity after insemination at 54-57 days (number of eggs).

473

474 **Heterozygosity**

475 We genotyped a subset of the F_2 and F_3 individuals of different categories using 7
476 microsatellite loci chosen because preliminary data indicated that the inbred lines 110
477 and 120 were fixed for different alleles. The loci were AF108762, AF108764⁵⁰, Pac1,
478 Pac2,⁵¹ Pasu1-2, Pasu1-9, Pasu1-11 (*cf.*³⁰) grouped into two multiplexes. We expected
479 that the F_2 would be fully heterozygous (except for a portion of misreadings), whereas
480 in the F_3 generation, half of the loci would, on average, exhibit heterozygosity. We
481 assessed the number of heterozygous and homozygous loci for each individual.
482 However, it was inevitable that a small fraction of the genotypes remained unamplified,
483 leading to missing data. Moreover, in microsatellite readings heterozygous genotypes
484 are frequently misconstrued as homozygous, i.e. one allele behaves as partly
485 "dominant"⁵². To avoid any bias, we kept the readings blind to the sample's origin.

486 Also, we refrained from making corrections, even in instances where the reading error
487 was evident (e.g. one homozygous locus and all other loci heterozygous in a F_2).

488

489 **Statistical analyses**

490 All analyses were performed with *R* 4.0.2 packages *lme4*⁵³ and *nlme*⁵⁴ by fitting
491 generalized linear mixed models (GLMMs) for binomial variables (juvenile survival,
492 ability to self-fertilize, proportion of heterozygous loci) or linear mixed models (LMMs)
493 for Gaussian continuous variables (body weight, female fecundity). Our linear models
494 included as fixed effects: (i) mating treatment (selfing, sib-cross, and cousin-cross), (ii)
495 the cross type (one vs. two ways) when appropriate (i.e. for sib- and cousin-cross), and
496 (iii) the maternal type (S vs. T) within two-way crosses (recall that in the selfing and
497 one-way crosses all mothers are S). We proceeded by first running separate analyses
498 within the sib-mating and cousin-mating treatments in order to test the mating treatment
499 and maternal type effects. As these effects were not significant, we pooled all
500 subcategories within the sib-cross and cousin-cross treatments in order to test the
501 mating treatment effect using the whole dataset. In each model we added an appropriate
502 structure of random effects to account for various levels of replication within each level
503 of fixed factors (e.g. identity of the maternal family: *a*, *b*, *c*; pair of parental families:
504 *a/b*, *c/d*; identity of the mother: 1 to 35; and/or of the parental pair: 21/1, 22/2 described
505 for each model in the results). Observation number was also added as a random factor in
506 the binomial models on juvenile survival in order to account for overdispersion^{55,56}.
507 Tests of fixed effects were made using LRT (Likelihood-ratio tests, which compare the
508 likelihood of models with and without the factor of interest) keeping all random effects
509 present in the model. Random effects were tested using LRT with the corrections
510 indicated by Zuur et al.⁵⁷, chap. 5, pp. 123–125). Post hoc tests were performed when
511 the treatment effect was significant using Holm-Bonferroni correction for multiple-
512 testing to test effect sizes among different treatments.

513

514 **Simulations**

515 We made a set of individual-based simulations to evaluate whether the observed
516 changes in fitness between the three type of mating treatments (selfing, sib-cross,
517 cousin-cross) could be compatible with a purely genetic model of inbreeding depression
518 (*i.e.* deleterious-mutation based). Each individual genome was modelled as a single
519 chromosome on which deleterious mutations were located. The deleterious mutations

520 were of two types: small-effect and large-effect (quasi-lethal), each type with its own
521 rate, degree of recessivity, and effect. We modelled both the process of extraction from
522 a large population and the propagation of two inbred lines over 28 generations with the
523 following events at each generation: mutation, meiosis, recombination, selection, and
524 drift within each line. Then, we simulated the creation of F_1 , F_2 , and the three types of
525 crosses giving the F_3 as in our experiment. The output of each simulation was a set of
526 three average fitness values for each of the three types of F_3 . For each set of parameter,
527 1000 independent replicates were made to produce expected distributions of the relative
528 fitnesses of the three categories. The details on the simulations are explained in Support
529 Information in *Simulation Details*.

530

531

532 **ACKNOWLEDGMENTS**

533 We thank Tim Janicke for starting the inbred lines. This work was funded by CeMEB
534 ("Projets de Recherche Exploratoires du CeMEB" grant to project "Epigenetics of
535 inbreeding depression (EPID)" by P. David and C. Grunau) and ANR (FRIDA project,
536 grant ANR-23-CE02-0033-01 to P. David). N. Bonel was partially supported by the
537 "Programa de Financiamiento Parcial de Estadías en el Exterior para Investigadores
538 Asistentes", National Scientific and Technical Research Council CONICET (Res. N°
539 558 1236/08; 4118/16).

540

541 **AUTHORS' CONTRIBUTIONS**

542 Conceptualization, CG and PD; methodology, NB and PD; formal analysis, NB and PD;
543 data curation, NB and PD; writing—original draft preparation, NB and PD; writing—
544 review and editing, NB and PD; supervision, PD; project administration, PD; funding
545 acquisition, PD. All authors have read and agreed to the published version of the
546 manuscript.

547

548 **REFERENCES**

- 549 1. Charlesworth, B. & Charlesworth, D. The genetic basis of inbreeding depression. *Genet.*
550 *Res.* **74**, 329–340 (1999).
- 551 2. Charlesworth, D. & Willis, J. H. The genetics of inbreeding depression. *Nat Rev Genet*
552 **10**, 783–796 (2009).
- 553 3. Charlesworth, B. & Charlesworth, D. Some evolutionary consequences of deleterious
554 mutations. *Genetica* **102/103**, 3–19 (1998).
- 555 4. Simmons, M. J. & Crow, J. F. Mutations affecting fitness in drosophila populations.
556 *Annu. Rev. Genet.* **11**, 49–78 (1977).
- 557 5. Agrawal, A. F. & Whitlock, M. C. Inferences about the distribution of dominance
558 drawn from yeast gene knockout data. *Genetics* **187**, 553–566 (2011).
- 559 6. Huber, C. D., Durvasula, A., Hancock, A. M. & Lohmueller, K. E. Gene expression
560 drives the evolution of dominance. *Nat Commun* **9**, 2750 (2018).
- 561 7. Lande, R. & Schemske, D. W. The evolution of self-fertilization and inbreeding
562 depression in plants. I. Genetic models. *Evolution* **39**, 24–40 (1985).
- 563 8. Glémin, S. How are deleterious mutations purged? Drift versus nonrandom mating.
564 *Evolution* **57**, 2678–2687 (2003).
- 565 9. Kirkpatrick, M. & Jarne, P. The effects of a bottleneck on inbreeding depression and the
566 genetic load. *The American Naturalist* **155**, 154–167 (2000).

- 567 10. Waller, D. M. Addressing Darwin's dilemma: Can pseudo-overdominance explain
568 persistent inbreeding depression and load? *Evolution* **75**, 779–793 (2021).
- 569 11. Barrett, S. C. H. & Charlesworth, D. Effects of a change in the level of inbreeding on
570 the genetic load. *Nature* **352**, 522–524 (1991).
- 571 12. Carr, D. E. & Dudash, M. R. The effects of five generations of enforced selfing on
572 potential male and female function in *Mimulus guttatus*. *Evolution* **51**, 1797–1807 (1997).
- 573 13. Chelo, I. M., Carvalho, S., Roque, M., Proulx, S. R. & Teotónio, H. The genetic basis
574 and experimental evolution of inbreeding depression in *Caenorhabditis elegans*. *Heredity* **112**,
575 248–254 (2014).
- 576 14. Noël, E. *et al.* Reduced mate availability leads to evolution of self-fertilization and
577 purging of inbreeding depression in a hermaphrodite. *Evolution* **70**, 625–640 (2016).
- 578 15. Byers, D. L. & Waller, D. M. Do plant populations purge their genetic load? Effects of
579 population size and mating history on inbreeding depression. *Annu. Rev. Ecol. Syst.* **30**, 479–
580 513 (1999).
- 581 16. Crnokrak, P. & Barrett, S. C. H. Perspective: purging the genetic load: a review of the
582 experimental evidence. *Evolution* **56**, 2347–2358 (2002).
- 583 17. Han, T. *et al.* An epigenetic basis of inbreeding depression in maize. *Sci. Adv.* **7**,
584 eabg5442 (2021).
- 585 18. Charlesworth, D. & Charlesworth, B. Inbreeding depression with heterozygote
586 advantage and its effect on selection for modifiers changing the outcrossing rate. *Evolution* **44**,
587 870–888 (1990).
- 588 19. Lande, R., Schemske, D. W. & Schultz, S. T. High inbreeding depression, selective
589 interference among loci, and the threshold selfing rate for purging recessive lethal mutations.
590 *Evolution* **48**, 965–978 (1994).
- 591 20. Cheptou, P.-O. The evolutionary ecology of inbreeding depression in wild plant
592 populations and its impact on plant mating systems. *Front. Plant Sci.* **15**, 1359037 (2024).
- 593 21. Bossdorf, O., Richards, C. L. & Pigliucci, M. Epigenetics for ecologists. *Ecology*
594 *Letters* **11**, 106–115 (2008).
- 595 22. Cheptou, P.-O. & Donohue, K. Epigenetics as a new avenue for the role of inbreeding
596 depression in evolutionary ecology. *Heredity* **110**, 205–206 (2013).
- 597 23. Biéumont, C. Inbreeding effects in the epigenetic era. *Nat Rev Genet* **11**, 234–234 (2010).
- 598 24. Vergeer, P., Wagemaker, N. (C. A. M.) & Ouborg, N. J. Evidence for an epigenetic
599 role in inbreeding depression. *Biol. Lett.* **8**, 798–801 (2012).
- 600 25. Dapp, M. *et al.* Heterosis and inbreeding depression of epigenetic Arabidopsis hybrids.
601 *Nature Plants* **1**, 15092 (2015).
- 602 26. Richards, E. J. Inherited epigenetic variation — revisiting soft inheritance. *Nat Rev*
603 *Genet* **7**, 395–401 (2006).
- 604 27. Johannes, F. *et al.* Assessing the Impact of Transgenerational Epigenetic Variation on
605 Complex Traits. *PLoS Genet* **5**, e1000530 (2009).
- 606 28. Preite, V. *et al.* Increased transgenerational epigenetic variation, but not predictable

- 607 epigenetic variants, after environmental exposure in two apomictic dandelion lineages. *Ecology*
608 *and Evolution* **8**, 3047–3059 (2018).
- 609 29. Van Der Graaf, A. *et al.* Rate, spectrum, and evolutionary dynamics of spontaneous
610 epimutations. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 6676–6681 (2015).
- 611 30. Escobar, J. S., Nicot, A. & David, P. The different sources of variation in inbreeding
612 depression, heterosis and outbreeding depression in a metapopulation of *Physa acuta*. *Genetics*
613 **180**, 1593–1608 (2008).
- 614 31. Janicke, T., Vellnow, N., Sarda, V. & David, P. Sex-specific inbreeding depression
615 depends on the strength of male-male competition: inbreeding depression in a hermaphrodite.
616 *Evolution* n/a-n/a (2013) doi:10.1111/evo.12167.
- 617 32. McCarthy, T. M. & Sih, A. Relatedness of mates influences mating behaviour and
618 reproductive success of the hermaphroditic freshwater snail *Physa gyrina*. *Evolutionary*
619 *Ecology Research* **10**, 77–94 (2008).
- 620 33. Fry, J. D., Keightley, P. D., Heinsohn, S. L. & Nuzhdin, S. V. New estimates of the
621 rates and effects of mildly deleterious mutation in *Drosophila melanogaster*. *Proc. Natl. Acad.*
622 *Sci. U.S.A.* **96**, 574–579 (1999).
- 623 34. Charlesworth, B., Borthwick, H., Bartolomé, C. & Pignatelli, P. Estimates of the
624 genomic mutation rate for detrimental alleles in *Drosophila melanogaster*. *Genetics* **167**, 815–
625 826 (2004).
- 626 35. Sharp, N. P. & Agrawal, A. F. Evidence for elevated mutation rates in low-quality
627 genotypes. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 6142–6146 (2012).
- 628 36. Agrawal, A. F. & Wang, A. D. Increased transmission of mutations by low-condition
629 females: evidence for condition-dependent DNA repair. *PLoS Biol* **6**, e30 (2008).
- 630 37. Cecere, G. Small RNAs in epigenetic inheritance: from mechanisms to trait
631 transmission. *FEBS Letters* **595**, 2953–2977 (2021).
- 632 38. Luquet, E. & Tariel, J. Offspring reaction norms shaped by parental environment:
633 interaction between within- and trans-generational plasticity of inducible defences. *BMC Evol*
634 *Biol* **16**, 209 (2016).
- 635 39. Fallet, M., Luquet, E., David, P. & Cosseau, C. Epigenetic inheritance and
636 intergenerational effects in mollusks. *Gene* **729**, 144166 (2020).
- 637 40. Tariel, J., Plénet, S. & Luquet, É. Transgenerational plasticity of inducible defences:
638 Combined effects of grand-parental, parental and current environments. *Ecology and Evolution*
639 **10**, 2367–2376 (2020).
- 640 41. Luviano, N. *et al.* The methylome of *Biomphalaria glabrata* and other mollusks:
641 enduring modification of epigenetic landscape and phenotypic traits by a new DNA methylation
642 inhibitor. *Epigenetics & Chromatin* **14**, 48 (2021).
- 643 42. Henry, P.-Y., Bousset, L., Sourrouille, P. & Jarne, P. Partial selfing, ecological
644 disturbance and reproductive assurance in an invasive freshwater snail. *Heredity* **95**, 428–436
645 (2005).
- 646 43. Jarne, P., Pointier, J.-P., David, P. & Koene, J. M. Basommatophoran gastropods. in
647 *The evolution of primary sexual characters in animals* 173–196 (Oxford University Press,
648 2010).

- 649 44. Péliissié, B., Jarne, P. & David, P. Sexual selection without sexual dimorphism: bateman
650 gradients in a simultaneous hermaphrodite: sexual selection in a hermaphrodite. *Evolution* **66**,
651 66–81 (2012).
- 652 45. Noël, E. *et al.* Reduced mate availability leads to evolution of self-fertilization and
653 purging of inbreeding depression in a hermaphrodite: selfing evolution in a hermaphroditic
654 snail. *Evolution* **70**, 625–640 (2016).
- 655 46. Wethington, A. R. & Dillon, Jr, R. T. Gender choice and gender conflict in a non-
656 reciprocally mating simultaneous hermaphrodite, the freshwater snail, *Physa*. *Animal Behaviour*
657 **51**, 1107–1118 (1996).
- 658 47. Tsitrone, A., Duperron, S. & David, P. Delayed Selfing as an Optimal Mating Strategy
659 in Preferentially Outcrossing Species: Theoretical Analysis of the Optimal Age at First
660 Reproduction in Relation to Mate Availability. *The American Naturalist* **162**, 318–331 (2003).
- 661 48. Janicke, T. *et al.* Environmental effects on the genetic architecture of fitness
662 components in a simultaneous hermaphrodite. *Journal of Animal Ecology* **91**, 124–137 (2022).
- 663 49. Henry, P. & Jarne, P. Marking hard-shelled gastropods: tag loss, impact on life-history
664 traits, and perspectives in biology. *Invertebrate Biology* **126**, 138–153 (2007).
- 665 50. Monsutti, A. & Perrin, N. Dinucleotide microsatellite loci reveal a high selfing rate in
666 the freshwater snail *Physa acuta*. *Molecular Ecology* **8**, 1076–1078 (1999).
- 667 51. Sourrouille, P., Debain, C. & Jarne, P. Microsatellite variation in the freshwater snail
668 *Physa acuta*. *Molecular Ecology Notes* **3**, 21–23 (2003).
- 669 52. David, P., Pujol, B., Viard, F., Castella, V. & Goudet, J. Reliable selfing rate estimates
670 from imperfect population genetic data. *Molecular Ecology* **16**, 2474–2487 (2007).
- 671 53. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models
672 using lme4. *arXiv:1406.5823 [stat]* (2014).
- 673 54. Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team. nlme: linear and
674 nonlinear mixed effects models. R package version 3.1-131. (2017).
- 675 55. Elston, D. A., Moss, R., Boulinier, T., Arrowsmith, C. & Lambin, X. Analysis of
676 aggregation, a worked example: numbers of ticks on red grouse chicks. *Parasitology* **122**, 563–
677 569 (2001).
- 678 56. Browne, W. J., Subramanian, S. V., Jones, K. & Goldstein, H. Variance partitioning in
679 multilevel logistics models with over-dispersion. *Journal of the Royal Statistical Society* **168**,
680 599–613 (2005).
- 681 57. Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A. & Smith, G. M. *Mixed Effects*
682 *Models and Extensions in Ecology with R*. (Springer New York, New York, NY, 2009).
683 doi:10.1007/978-0-387-87458-6.

684

TABLES

Table 1. Summary of means (\pm SE) and statistical significance of the mating treatment type effects on fitness traits measured on F₃ individuals of the hermaphrodite snail *Physa acuta*. Number of observations are indicated in parentheses. In this table, as in fig.2, we did not distinguish, within each mating treatment, the different possible types of crosses (one-way versus two-way, see fig.1) because they were not statistically different. Detailed results are reported in supplementary tables S1-S3.

Variable	Mating treatment			Treatment effect	Treatment comparison	Effect size
	Selfing (SF)	Sib-cross (SC)	Cousin-cross (CC)			
Juvenile survival	0.62 \pm 0.03 (93)	0.69 \pm 0.03 (193)	0.76 \pm 0.02 (153)	$X^2_2 = 15.40$; $P < 0.001$	SF vs. CC	-4.04***
					SF vs. SC	-2.74*
					SC vs. CC	-2.33*
Body weight	41.6 \pm 5.3 (82)	44.9 \pm 2.6 (152)	52.3 \pm 1.8 (134)	$X^2_2 = 10.06$; $P = 0.007$	SF vs. CC	-2.98**
					SF vs. SC	-1.94 .
					SC vs. CC	-2.23 .
Fecundity rate	12.0 \pm 0.4 (79)	11.5 \pm 0.5 (146)	11.3 \pm 0.4 (127)	$X^2_2 = 1.53$; $P = 0.466$	—	—
					—	—
					—	—
Probability of producing selfed eggs at the age of 50 days	0.10 \pm 0.06 (82)	0.28 \pm 0.05 (152)	0.43 \pm 0.07 (134)	$X^2_2 = 19.69$; $P < 0.001$	SF vs. CC	-4.25***
					SF vs. SC	-3.09**
					SC vs. CC	-2.0 *

Treatment means (\pm SE, with) are reported in natural scale for the four traits (proportion of eggs surviving, milligrams, eggs laid per day, and proportion of laying individuals respectively); however statistical models are generalized linear mixed models on the appropriate scale (Binomial for juvenile survival and probability of producing selfed eggs, with logit link; Gaussian for body weight and fecundity, identity link), and include appropriate random effects to represent the structure of our design (not shown here, see methods and detailed results in Supplementary materials and tables S1-S3). Mating treatment effect sizes (estimated mean difference on transformed scale divided by its SD) were tested using post hoc tests with the Holm-Bonferroni correction for multiple testing. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ after correction.

FIGURES

Figure 1. Pedigree of the F₂ snails used in this study, and how they were crossed to produce the F₃ on which fitness traits were measured. Parents were two individual snails extracted from independent inbred lines (named 120 and 110) derived from wild-caught ancestors through 28 or 29 successive generations of self-fertilization in the laboratory, respectively. F₁ individuals (inbred sublimes) resulted from self-fertilization of parents, and were also homozygous. The F₁ pair crossing yielded a set of individuals that constituted the experimental strains (F₂ snails), which are all heterozygous and clonal to one another but with different type of origins, S and T. F₂ snails from the S type had their mothers from the 120 line and their fathers from the 110 line, and *vice versa* for those from type T. The experimental strains were constituted by ten families (*a* to *j*) per type of origin.

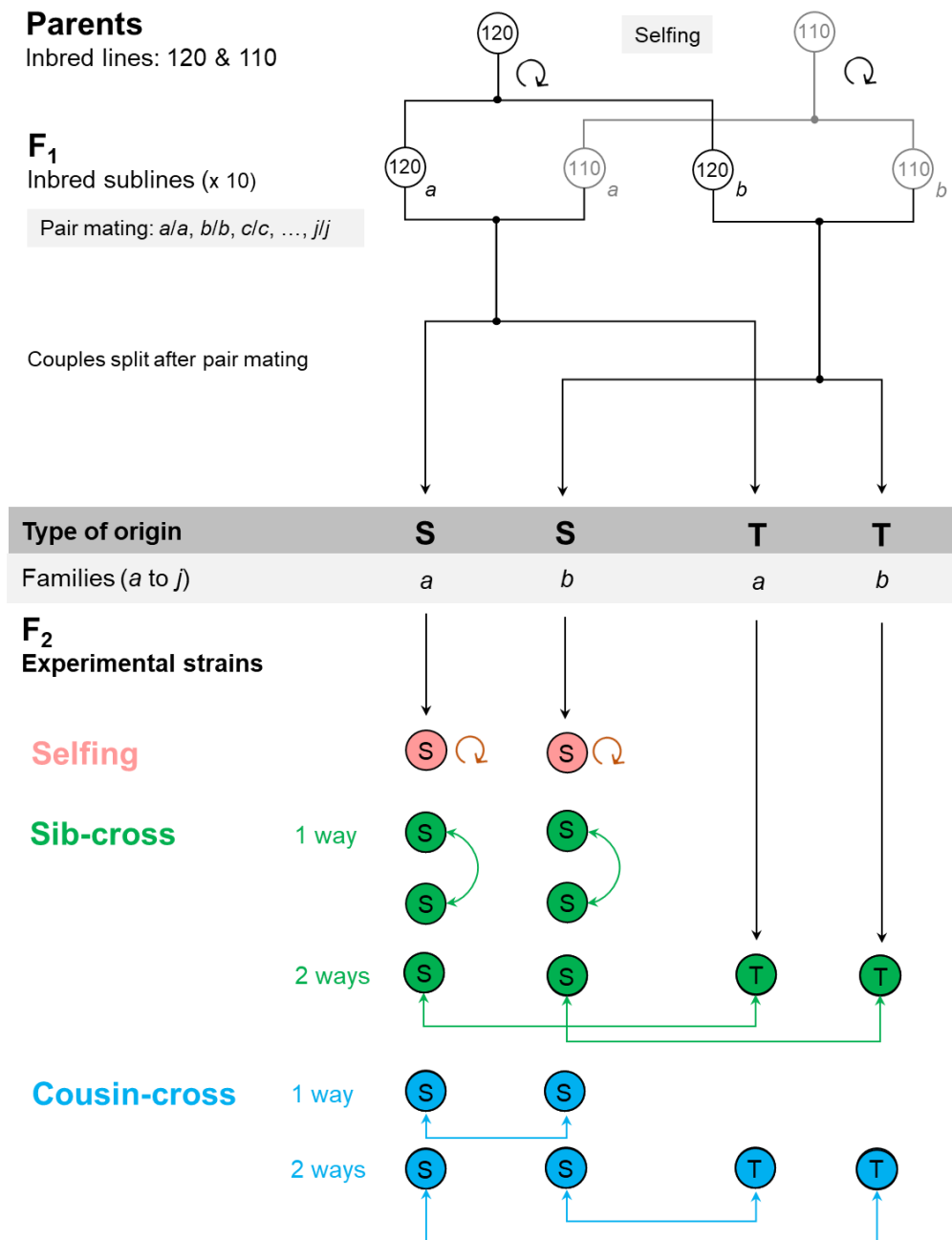


Figure 2. Fitness traits measured on F₃ individuals of the hermaphrodite snail *Physa acuta*. **A.** Juvenile survival (proportion of eggs that become juveniles alive after 14 days). **B.** Body weight at 50-53 days (the mean of the two measures, before and after pairing the focal snail with a virgin albino partner). **C.** Female fecundity rate after insemination (number of eggs per day produced by the focal snail). **D.** Probability of having produced self-fertilized eggs at 50 days just before pair mating. Mating treatment effect sizes (difference between two types divided by its SD) were tested using post hoc tests with the Holm-Bonferroni correction for multiple-testing (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). SF, selfing; SC, sib-cross; CC, cousin-cross. Black dots are mean values \pm 1SE. We did not separate one-way and two-way crosses in the SC and CC category as differences between them were small and not significant (see supplementary tables S1-S3).

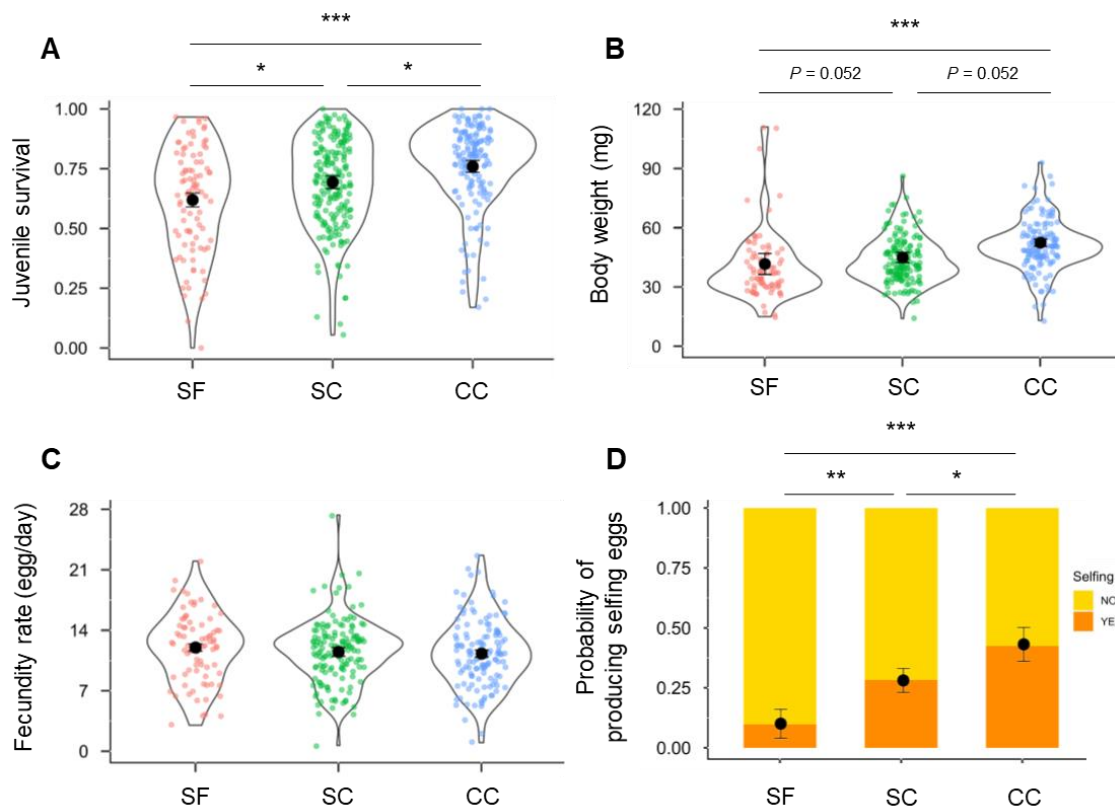


Figure 3. Distributions of multilocus heterozygosity for a subset of F₂ and F₃ individuals of different categories using 7 microsatellite loci (AF108762, AF108764, Pac1, Pac2, Pasu1-2, Pasu1-9, Pasu1-11) chosen because preliminary data indicated that the inbred lines 110 and 120 were fixed for different alleles. The microsatellites were grouped into two multiplexes. Overall, F₂ individuals were fully heterozygous (with a few exceptions) whereas in the F₃ heterozygosity was spread around 0.5 within each mating treatment. Red dots are mean values \pm 1SE.

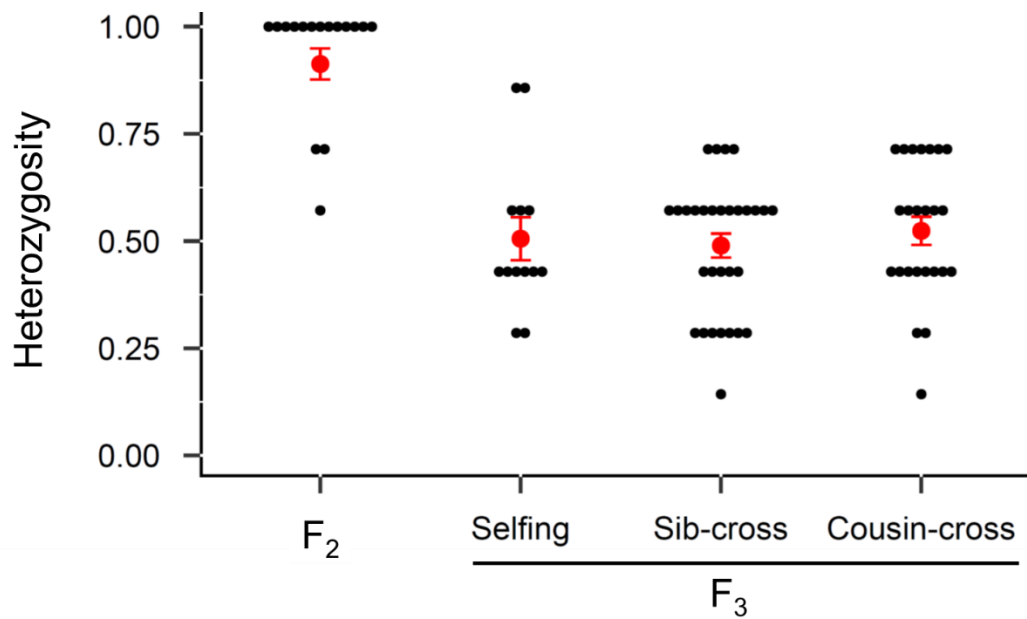


Figure 4. Violin plots of the distribution of fitness ratios from 1,000 simulations of our experiment, using the reference parameter set, compatible with available data on ID in *Phylla acuta* (see Methods and Simulations in Supporting Information). Parameters include the length of the genome ($L = 10$ Morgans), the dominance coefficient and effect size of semi-lethals ($h_1 = 0.02$, $s_1 = 0.9$), and those of small-effect mutations ($h_2 = 0.2$, $s_2 = 0.05$), with both mutation types contributing to a total inbreeding depression of $d=0.5$ in natural populations ($d_1 = 0.3$ for semi-lethals and $d_2 = 0.2$ for small-effect mutations). Black dots represent the mean fitness ratio, with 95% confidence intervals (CI) shown. The observed ratios for juvenile survival (red line), body mass (blue line), and the proportion of fertile individuals at 50 days (green line) were for Sib/Self: 1.11, 1.08, and 2.80 (not shown, out of range); for Cous/Self: 1.23, 1.26, and 4.30 (not shown); and for Cous/Sib: 1.10, 1.16, and 1.54, respectively. Simulations with different parameter sets are in Figure S7 from Supporting Information.

