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Genetic variability and transgenerational regulation of investment in  
sex in the monogonont rotifer *Brachionus plicatilis*

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*Running title:* Sex regulation in monogonont rotifers

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## ABSTRACT

1  
2 In cyclical parthenogens such as aphids, cladocerans and rotifers, the coupling  
3 between sexual reproduction and the production of resting stages (diapausing eggs)  
4 imposes strong constraints on the timing of sex. While induction of sex is generally  
5 triggered by environmental cues, the response to such cues may vary across individuals  
6 according to genetic and non-genetic factors. In this study, we explored genetic and  
7 epigenetic causes of variation for the propensity for sex using a collection of strains from  
8 a Spanish population of monogonont rotifers (*Brachionus plicatilis*) in which variation  
9 for the threshold population density at which sex is induced (mixis threshold) had been  
10 documented previously. Our results show significant variation for the mixis threshold  
11 among 20 clones maintained under controlled conditions for 15 asexual generations.  
12 The effect of the number of clonal generations since hatching of the diapausing egg  
13 on the mixis ratio (proportion of sexual offspring produced) was tested on 4 clones  
14 with contrasted mixis thresholds. The results show a negative correlation between the  
15 mixis threshold and mixis ratio, as well as a significant effect of the number of clonal  
16 generations since fertilization, sex being repressed during the first few generations after  
17 hatching of the diapausing egg.

18  
19 *Keywords:* cyclical parthenogenesis, monogonont rotifers, reproductive mode, sexual  
20 reproduction, zooplankton

## INTRODUCTION

Cyclical parthenogenesis (the alternation of phases of asexual propagation with sexual reproduction events) occurs in seven taxonomic groups of animals (Hebert, 1987), but has been best described in cladocerans, aphids and monogonont rotifers (e.g., Simon et al., 2002; Wallace et al., 2006; Decaestacker et al., 2009). In all three taxa, individuals reproduce asexually while environmental conditions are favorable, sex being often triggered by deteriorating conditions and leading to the production of diapausing eggs that can survive adverse conditions. This coupling between sexual reproduction and resting stage formation imposes a strong constraint on the timing of sex (e.g., Gerber et al., 2018): sex should occur early enough to allow the production of diapausing eggs before the environment becomes too hostile, but not too early — otherwise it will have an important cost in terms of reduced clonal propagation. The timing of sex is thus tightly regulated in cyclical parthenogens, using different types of environmental cues such as temperature (Simon et al., 2002), photoperiod (Zhang and Baer, 2000), food quality (Lubzens et al., 1993; Koch et al., 2009) or crowding (Stelzer and Snell, 2003; Gerber et al., 2018). Sensitivity to those cues may vary among populations and reflect local adaptation to different environmental conditions (e.g., Roulin et al., 2013; Franch-Gras et al., 2017); whether genetic variation also exist within populations remains less well known (but see Carmona et al., 2009; Becks and Agrawal, 2010).

Facultatively sexual organisms are of particular interest for designing experimental tests of the evolutionary significance of sex and recombination. While many of these tests compared the rates of adaptation of sexual vs. asexual populations of uni-

44 cellular organisms (e.g., Kaltz and Bell, 2002; Colegrave, 2002; Goddard et al., 2005;  
45 Lachapelle and Bell, 2012; McDonald et al., 2016), several experiments on brachionid  
46 rotifers took advantage of the existence of variability in investment in sex among indi-  
47 viduals, in order to explore under which conditions increased (or decreased) investment  
48 in sex may be favored (e.g., Smith and Snell, 2006; Becks and Agrawal, 2010, 2012;  
49 Luijckx et al., 2017). While these studies have lead to important insights, different  
50 components of investment in sex may vary between individuals (threshold level of the  
51 cue triggering sex, proportion of sexual offspring produced once the threshold has  
52 been reached); furthermore, this variation may be genetic or epigenetic, with some  
53 non-genetic effects possibly lasting over several generations (Gilbert, 2017). The aim  
54 of the present paper is to provide a better understanding of these different sources of  
55 variation (and how they may covary), which appears of particular importance for the  
56 design and interpretation of evolution experiments.

57         Monogonont rotifers are small invertebrates (50 to 2000  $\mu\text{m}$ ) living in a variety  
58 of aquatic or moist habitats (Wallace et al., 2006), often reaching very large popula-  
59 tion sizes due to high rates of clonal reproduction. Rotifer populations are typically  
60 temporary at temperate latitudes, the growing season starting by the hatching of sex-  
61 ually produced diapausing eggs present in the sediment. The hatchlings are diploid  
62 asexual (amictic) females, producing other females by ameiotic parthenogenesis. After  
63 an initial phase of population growth, sexual and asexual reproduction co-occur within  
64 populations: sex is induced by an environmental factor, causing parthenogenetic fe-  
65 males to produce some sexual (mictic) females among their offspring (see Figure 1).  
66 These sexual females produce haploid eggs by meiosis, which, if not fertilized, develop  
67 into dwarf haploid males. If sexual females are inseminated while they are still young,

68 they produce diploid diapausing eggs formed by regular gamete fusion. These dia-  
69 pausing “eggs” actually consist in multicellular embryos that can resist desiccation and  
70 adverse environmental conditions, and may remain viable for several years (Lubzens et  
71 al., 2001). After a dormant phase, they can hatch when the environmental conditions  
72 become favorable again.

73 In rotifers from the *Brachionus* genus, the switch from asexual to sexual repro-  
74 duction is mainly controlled by population density, through a form of quorum sensing  
75 mechanism involving a protein (the “misis-inducing protein” or MIP) produced by  
76 the females themselves (Carmona et al., 1993; Stelzer and Snell, 2003, 2006; Snell et  
77 al., 2006). The threshold population density at which sexual females start being pro-  
78 duced (called the “misis threshold”) was shown to vary among species, and also among  
79 strains from the same species (Gilbert, 2017 and references therein). Variations in the  
80 misis threshold among isolates from the same natural population was demonstrated  
81 in the brackish-water species *Brachionus plicatilis* (Carmona et al., 2009; Gabaldon  
82 and Carmona, 2015), and in the freshwater species *Brachionus calyciflorus* (Becks and  
83 Agrawal, 2010). A second component of investment in sex is the “misis ratio”, corre-  
84 sponding to the proportion of sexual females among offspring (once the misis thresh-  
85 old has been reached — see Figure 1); variation for the misis ratio among strains has  
86 also been demonstrated, but to what extent the misis ratio correlates with the misis  
87 threshold remains unclear (Gilbert, 2017).

88 Other environmental factors such as salinity or food quality may also affect  
89 investment in sex in monogonont rotifers (e.g., Lubzens et al., 1993). Interestingly,  
90 Gilbert (2002, 2003) showed that in *B. calyciflorus*, the misis ratio is affected by  
91 endogenous factors that may persist over several generations: in particular, sexual re-

92 production is inhibited during the first clonal generations following fertilization, with a  
93 gradual increase in the mixis ratio over the first 10-12 clonal generations after hatching  
94 of a diapausing egg. The same pattern was observed in different monogonont species,  
95 but was absent in others (e.g., Schröder and Gilbert, 2004; Gilbert, 2017). Although  
96 Gilbert (2003) and Schröder and Gilbert (2004) observed variation between genotypes  
97 from the same natural population in the rate of increase of the mixis ratio over clonal  
98 generations, the heritable component of this variation cannot be assessed from these  
99 experiments, since only a single replicate per genotype was performed. Conversely,  
100 this type of transgenerational effect may possibly have affected previous estimates of  
101 genetic variation for the mixis threshold, as the number of clonal generations since  
102 hatching of the diapausing egg is generally not strictly controlled in the experiments  
103 in which this variation is measured.

104 In this study, we first quantified genetic variation for the mixis threshold among  
105 a collection of strains originating from the same natural population of *Brachionus*  
106 *plicatilis*, measured after 15 clonal generations under controlled conditions. In a second  
107 experiment, we used a subset of 4 strains with contrasted mixis thresholds, to assess  
108 the effect of the number of clonal generations after fertilization on the mixis ratio. This  
109 allowed us to asses how these two measures of investment in sex (mixis threshold and  
110 mixis ratio) may covary, as well as to test the existence of possible transgenerational  
111 effects on induction of sex that have been described in other species. The results from  
112 our second experiment also allowed us to test for the effect of another non-genetic  
113 factor, the age of the mother, on the proportion of sexual offspring produced.

115 **Isolation and culture conditions of rotifer strains.** Clones of *B. plicatilis* were  
116 obtained from the hatching of diapausing eggs present in sediment sampled from Salo-  
117 bralejo Lake (Eastern Spain) in September 2013, and kindly provided by the Labora-  
118 tory of Evolutionary Ecology of the University of Valencia. This particular population  
119 was chosen because genetic variation for the mixis threshold had been documented in  
120 previous studies (Gabaldon and Carmona, 2015; Franch-Gras et al., 2017). Diapausing  
121 eggs were extracted from the sediment using the sugar flotation technique (Gómez and  
122 Carvalho, 2000), and hatched by placing them in artificial seawater (Instant Ocean<sup>®</sup>,  
123 Aquarium Systems) at 6g/L salinity, under constant illumination and at 22°C. Upon  
124 hatching, individuals were transferred to culture medium (referred hereafter as stan-  
125 dard culture medium) consisting in f/2-enriched artificial seawater (Guillard, 1975)  
126 at 12g/L salinity, containing  $2 \times 10^5$  cells/mL of the microalga *Tetraselmis suecica*  
127 used for food (our algal culture was maintained in exponential growth in a chemo-  
128 stat throughout the experiment). Because two cryptic species of rotifers (*B. plicatilis*  
129 and *B. manjavacas*) coexist in Salobralejo Lake (Montero-Pau et al., 2011), the first  
130 offspring of each hatched individual was collected for species identification using the  
131 RFLP-PCR method described in Gabaldon et al. (2013) — in parallel, we devised a  
132 quicker identification method using COI DNA primers (described in the Supplemen-  
133 tary Material) that yielded identical results. Twenty hatched *B. plicatilis* individuals  
134 were identified, and individually transferred into 30mL glass tubes containing standard  
135 culture medium to maintain clonal growth. Clones were then maintained by weekly  
136 transfers to fresh medium. Hereafter, these 20 clones will be denoted “P-clones” (for

137 parental clones).

138

139 **Variability in the mixis threshold among clones.** Three asexual females from  
140 each of the 20 P-clones were sampled and individually transferred into wells of 48-well  
141 culture plates (Greiner Bio-One™) containing 0.3mL of standard culture medium and  
142 maintained in a culture chamber at 22°C, in order to generate sub-clonal lines (3 per  
143 P-clone). Their first offspring were transferred into new wells with fresh medium, until  
144 15 clonal generations were reached (when a sampled offspring was a sexual female, it  
145 was replaced by another offspring from the same mother until obtaining an asexual  
146 female). This high number of clonal generations was chosen to ensure that none of  
147 the tested females could be just a few generations away from hatching of a diapausing  
148 egg, given that diapausing eggs may hatch spontaneously under culture conditions  
149 (e.g., Martínez-Ruiz and García-Roger, 2015). For each sub-clonal line, 6 neonates of  
150 the fifteenth generation were isolated and individually transferred into wells of 24-well  
151 culture plates (Greiner Bio-One™) containing 0.5mL of culture medium with an algal  
152 concentration of  $5 \times 10^5$  cells/mL, where they were let to reproduce. If the sampled  
153 female was sexual, it was replaced whenever possible by another fifteenth generation  
154 female from the same mother. Wells were inspected visually every 24h until the first  
155 males were observed, in which case the population density was measured by counting  
156 the number of females present in the well. This density corresponds to the estimated  
157 mixis threshold (Carmona et al., 2009).

158

159 **Effect of the number of clonal generations after diapausing egg hatching.**

160 A subset of 4 P-clones displaying contrasted mixis thresholds was chosen based on

161 the results of the previous experiment (clones 6, 8, 10 and 16, see Results section).  
162 Diapausing eggs were collected from the bottom of the tubes in which those clones  
163 were maintained, transferred into Petri dishes containing artificial seawater at 12g/L  
164 salinity, and maintained in the dark and at 4°C during 3 months. These diapaus-  
165 ing eggs were produced by intracloal mating, which is genetically equivalent to self-  
166 fertilization in hermaphroditic organisms. Diapausing eggs were then isolated into  
167 single wells of 48-well plates (Greiner Bio-One™) with 0.3mL of standard medium,  
168 and placed at 22°C and under constant illumination to induce hatching. For each  
169 P-clone, five hatched females were sampled at random to form our first generation  
170 (G1, see Supplementary Figure). Note that these five females have been produced by  
171 different intracloal fertilization events, and thus carry different genotypes (however,  
172 two females from the same P-clone are more related than females from two different  
173 P-clones). The first three juveniles (G2) produced by each G1 female were collected  
174 to initiate sub-clonal lines. Hereafter, we will denote “F1-clone” the set of 3 sub-clonal  
175 lines originating from the same G1 female (5 per P-clone); note that all individuals  
176 from the same F1-clone are genetically identical, since they are produced asexually.  
177 Sub-clonal lines were maintained in 48-well plates (Greiner Bio-One™) that were in-  
178 spected daily. When a female of a given generation had produced its first juvenile,  
179 the juvenile was transferred into a new well with 0.3mL of fresh standard culture  
180 medium. If the juvenile developed into a sexual female or died before reproducing,  
181 it was replaced by another juvenile produced by the same mother. Sub-clonal lines  
182 were maintained until the 24th generation (G24), at the exception of sub-clonal lines  
183 from clone 8, which took more time as more sexual females were produced, and which  
184 were maintained for 18 generations only. At generations 2, 5, 8, 12, 18 and 24, one

185 juvenile female was sampled from each sub-clonal line to measure its mixis ratio. For  
186 this, the tested female was placed in a well of a 48-well plate (Greiner Bio-One™)  
187 with 0.3mL of sex-inducing medium, consisting in standard culture medium with an  
188 algal concentration of  $4 \times 10^5$  cells/mL, mixed in equal proportions with filtrate ob-  
189 tained from a previous rotifer culture that had reached a density of approximately  
190 20 females/mL (filtrated on a  $0.2\mu\text{m}$  mesh), and that was stored at  $5^\circ\text{C}$  (a unique  
191 batch of this medium was used throughout the experiment). The concentration of  
192 mixis-inducing protein in the resulting medium should thus be approximately equiva-  
193 lent to its concentration in a population at 10 females/mL density, which is well above  
194 the density required to induce sex in most populations (Gilbert, 2017). Every day  
195 until its death (which generally occurred after 10 to 15 days), the tested female was  
196 transferred into a new well containing 0.3mL of fresh sex-inducing medium, and its  
197 offspring were collected and individually transferred to a single well of a 96-well plate  
198 (Greiner Bio-One™) containing 0.2mL of standard culture medium. When offspring  
199 started to reproduce, they were typed as asexual (if they produced females) or sexual  
200 (if they produced males).

201

202 **Data analyses.** The mixis threshold (measured in number of females per mL at  
203 the time of first male appearance) in the first experiment was log-transformed and  
204 analyzed by fitting a mixed effects linear model, with ‘P-clone’ as a fixed effect (with  
205 20 levels corresponding to the different P-clones) and ‘sub-clonal line’ (three for each  
206 P-clone) as a random effect. Mixis ratios in the second experiment were analyzed  
207 using a generalized linear mixed effects model (GLMM), in which the numbers of  
208 sexual/asexual females produced per day by each tested female was modelled as a bi-

209 nominal variable with a logit link function. The model included ‘P-clone’ (with 4 levels),  
210 ‘tested generation’ (number of clonal generations from the diapausing egg, treated as  
211 a continuous variable), ‘day of the reproductive period’ (day 1 corresponding to the  
212 first day the tested female produced a juvenile, treated as a continuous variable) as  
213 fixed effects, as well as an interaction between ‘P-clone and ‘tested generation’, and an  
214 interaction between ‘P-clone’ and ‘day of reproductive period’. Effects of the F1-clone  
215 (5 for each P-clone) and of the sub-clonal line (3 for each F1-clone) were included in  
216 the model as random effects. The correlation between the mixis threshold (measured  
217 in the first experiment) and mixis ratio (measured in the second experiment) over the  
218 4 P-clones used in the second experiment was assessed using a modified version of the  
219 model for the second experiment, in which ‘tested generation’ and ‘mixis threshold’  
220 (one estimate for each P-clone) were included as fixed effects, while ‘F1-clone’ and ‘sub-  
221 clonal line’ were included as random effects. For this last analysis, we only included  
222 mixis ratios measured at generations 8, 12 and 18. The significance of fixed effects  
223 and their interactions was assessed by comparing models with or without the tested  
224 effect or interaction using likelihood ratio tests. Analyses were carried out using R v.  
225 3.3.3 (R Core Team, 2017), and the *lmer*, *glmer* and *anova* functions from the “lme4”  
226 package (Bates et al., 2015). Marginal coefficients of determination (proportions of the  
227 variance explained by fixed effects) were obtained using the *r.squaredGLMM* function  
228 of the “MuMIn” package (Bartoń, 2018).

230 **Variability in mixis threshold.** Figure 2 shows the average density at first male  
231 appearance for the different P-clones, measured after 15 asexual generations. As ex-  
232 plained in the Methods, three sub-clonal lines were maintained for each of the P-clones,  
233 and 6 measures were performed for each sub-clonal line (yielding 18 measures per P-  
234 clone). However, some sub-clonal lines were lost (due to the death of individuals that  
235 could not be replaced), and as a consequence, we obtained results from only two sub-  
236 clonal lines (instead of three) from P-clones 1, 2 and 15. The number of measures  
237 from some of the other sub-clonal lines was less than 6 due to the early death of  
238 individuals, yielding a total of 326 mixis threshold estimates (instead of 360). The  
239 statistical analysis showed significant variation among P-clones for their mixis thresh-  
240 olds ( $\chi^2(19) = 36.17, p = 0.01$ ). The proportion of total variance explained by the  
241 ‘P-clone’ effect was 0.16.

242

243 **Transgenerational effect on the mixis ratio.** From the previous results, we se-  
244 lected two P-clones with high and low estimated mixis thresholds (clones 6 and 8,  
245 respectively — see Figure 2), and two P-clones with near-average mixis thresholds  
246 (clones 10 and 16) to perform the experiment on the effect of the number of clonal  
247 generations after fertilization on investment in sex. A total of 15 sub-clonal lines were  
248 maintained for each of these P-clones (see Material and Methods, Supplementary Fig-  
249 ure), starting from 5 diapausing eggs produced by intraclonal mating (3 sub-clonal lines  
250 per diapausing egg). However, the lines originating from one of the diapausing eggs  
251 from clone 10 had low fitness (high death rates of individuals and low fecundity, which

252 may be caused by inbreeding depression) and these lines could not be maintained:  
253 therefore, data from clone 10 consist in measurements over 4 distinct genotypes (F1-  
254 clones) produced by intracloal mating (instead of 5). Furthermore, one data point  
255 was missing for clone 8 at generation 5, and two at generation 18 (leading to 14 and  
256 13 measures instead of 15), due to the premature death of tested females.

257 Figure 3 shows the effect of the number of generations since hatching of the  
258 diapausing egg on the mixis ratio (measured as the proportion of sexual females pro-  
259 duced among all offspring produced by a female), averaged over each P-clone (results  
260 for all F1-clones are shown in Figure 4). The results show a significant increase of the  
261 mixis ratio with the number of clonal generations ( $\chi^2(4) = 241.60, p < 0.001$ ), G2  
262 individuals (that is, the offspring of individuals that hatched from diapausing eggs)  
263 producing very few sexual females, while the mixis ratio increases to reach a plateau  
264 after about 8 to 10 clonal generations. The results also show significant differences  
265 among P-clones ( $\chi^2(9) = 910.29, p < 0.001$ ), with a much higher mixis ratio of indi-  
266 viduals from clone 8 (observed over all 5 F1-clones, see Figure 4), while the mixis ratio  
267 is lowest in individuals from clone 6. The model also detected a significant interaction  
268 between the tested generation and P-clone ( $\chi^2(3) = 41.64, p < 0.001$ ), reflecting the  
269 fact that the mixis ratio increases more rapidly with the number of clonal generations  
270 in some P-clones than others. The model in which different coefficients were attributed  
271 to the four P-clones was significantly better than a model in which clones 6 and 16 were  
272 treated as identical ( $\chi^2(3) = 15.53, p = 0.0014$ ), and was also better than a model in  
273 which clones 10 and 16 were treated as identical ( $\chi^2(3) = 28.89, p < 0.001$ ), reflecting  
274 the fact that the four P-clones displayed different behaviors. Finally, our modified sta-  
275 tistical model including the estimated mixis threshold of P-clones as a fixed factor (see

276 Material and Methods) detected a significant, negative effect of the mixis threshold on  
277 the mixis ratio ( $\chi^2(1) = 504.61, p < 0.001$ ), indicating that P-clones with lower mixis  
278 thresholds tend to have higher mixis ratios.

279

280 **Effect of maternal age on the mixis ratio.** The model detected a significant effect  
281 of the age of the tested mother ('day of the reproductive period' effect) on the propor-  
282 tion of sexual females produced per day ( $\chi^2(4) = 177.54, p < 0.001$ ), and a significant  
283 interaction between 'P-clone' and 'day of the reproductive period' ( $\chi^2(3) = 14.70,$   
284  $p = 0.002$ ). Indeed, Figure 5 shows that the tested females tended to produce a higher  
285 proportion of sexual offspring during their first days of reproduction, the decline in  
286 mixis ratio with the age of the mother being most apparent for clone 8.

287

## DISCUSSION

288 Although one may expect that the timing of sex should be under strong selec-  
289 tion in cyclical parthenogens, substantial genetic variation in the rate of response to  
290 the sex-inducing stimulus may exist within natural populations (e.g., Carmona et al.,  
291 2009; Becks and Agrawal, 2010). Our results confirm the existence of genetic vari-  
292 ability for the threshold population density to induce sex within a single population  
293 of the monogonont rotifer *Brachionus plicatilis*, after controlling for the number of  
294 clonal generations since fertilization (at least 15 in our first experiment). Our esti-  
295 mate for the proportion of variance in the mixis threshold explained by the genotype  
296 of individuals (0.16) is lower than the heritability estimates obtained by Gabaldon  
297 and Carmona (2015) and Franch-Gras et al. (2017) from the same natural population

298 (0.51 and 0.36, respectively). This difference may partly be due to the fact that the  
299 number of clonal generations since hatching of the last diapausing egg was not strictly  
300 controlled in these previous experiments (although three clonal generations were per-  
301 formed before the estimation of density thresholds). Perhaps more likely, it may be  
302 caused by a higher environmental variance in our experiment. In particular, males  
303 were observed at densities much higher than in Gabaldon and Carmona's experiment,  
304 which may stem from the fact that density thresholds were assessed in smaller volumes  
305 in our experiment (0.5mL, vs. 15mL in Gabaldon and Carmona, 2015, Franch-Gras  
306 et al., 2017). Indeed, the mixis threshold estimate is known to be negatively corre-  
307 lated to the culture volume, which may be due to the fact that in smaller volumes,  
308 population density may reach higher values before the mixis-inducing protein reaches  
309 the concentration needed to induce sex (Carmona et al., 2011). A higher number of  
310 reproductive events during the time needed to reach the mixis threshold may possi-  
311 bly have enhanced the effect of environmental factors on the estimated density at the  
312 threshold. Furthermore, our test populations were observed once per 24h (vs. twice  
313 in Gabaldon and Carmona's study), which may also have inflated the variance caused  
314 by measurement error.

315 Our second experiment showed important genetic variation in the mixis ratio  
316 (proportion of sexual offspring produced under a strong sex-inducing stimulus), and a  
317 correlation between the two components of propensity for sex: the clone in which the  
318 density at first male appearance was the lowest (respectively, highest) in the first exper-  
319 iment displayed the highest (respectively, lowest) mixis ratio in the second experiment  
320 (clones 6 and 8, Figures 1 and 2). This result shows that genotypes do indeed differ  
321 in their overall investment in sexual reproduction; a different conclusion would have

322 been reached if the genotypes engaging early in sexual reproduction also had tended to  
323 show a lower investment in sex under a strong sex stimulus (that is, if the correlation  
324 between mixis threshold and mixis ratio had been positive). One may notice that our  
325 sex-inducing medium was equivalent to a density of 10 individuals/mL, which is much  
326 lower than the mixis thresholds shown in Figure 1. However, this discrepancy again  
327 stems from the fact that when estimating mixis thresholds by the density at first male  
328 appearance in growing populations, the measured density is likely to be much higher  
329 than the density that would be required to produce the threshold concentration of  
330 mixis-inducing protein in a steady-state population, particularly when measurements  
331 are performed in small volumes (Carmona et al., 2011). In order to test whether a  
332 higher density would increase the mixis ratio, we ran additional tests on 10 females  
333 from clone 6, after 18 generations from the diapausing egg, exposing them to a sex-  
334 inducing medium corresponding to 25 individuals/mL (instead of 10), but we did not  
335 observed any significant increase of the mixis ratio (results not shown). Our results  
336 also show that young asexual females tend to produce a higher proportion of sexual  
337 offspring than older females, in agreement with previous observations on *B. plicatilis*  
338 (Carmona et al., 1994), *B. calyciflorus* (Rougier and Pourriot, 1977) and *Synchaeta*  
339 *tremula* (Timmermeyer and Stelzer, 2006) — however, a maximal investment in sex  
340 in the middle of the reproductive period of individuals was observed in one study on  
341 *B. calyciflorus* (Fussmann et al., 2007).

342         The selective forces allowing the maintenance of genetic polymorphism for in-  
343 vestment in sex within natural populations remain unknown. Carmona et al. (2009)  
344 showed that clones investing less in sex tend to increase in frequency during the grow-  
345 ing season (since they invest more in parthenogenetic reproduction), and hypothesized

346 that temporal fluctuations in the length of growing seasons may allow the mainte-  
347 nance of polymorphism, as genotypes investing more in the production of diapausing  
348 eggs may be favored when growing seasons are short, while genotypes investing more  
349 in parthenogenetic growth may be favored under longer growing seasons (see also  
350 Franch-Gras et al., 2017). Theoretical models have shown that temporal environ-  
351 mental fluctuations coupled with a dormant stage can indeed allow the maintenance  
352 of polymorphism (the “storage effect”, e.g., Warner and Chesson, 1985; Ellner and  
353 Hairston, 1994; Turelli et al., 2001), and a model by Spencer et al. (2001) showed  
354 that reproductive strategies differing in the timing of diapausing egg production may  
355 coexist within the same population when the time length of the growing season is  
356 uncertain.

357 Finally, our results demonstrate a gradual increase in the propensity for sex over  
358 clonal generations following the hatching of diapausing eggs. This confirms previous  
359 indications that a transgenerational maternal effect repressing sexual reproduction,  
360 and similar to the one observed in several other monogonont species (Gilbert, 2002;  
361 Schröder and Gilbert, 2004) occurs in *B. plicatilis* (Hino and Hirano, 1977; Hagiwara et  
362 al., 2005). This delayed-mixis mechanism may have evolved to increase the chances of  
363 establishment of newly hatched lineages, by promoting parthenogenetic growth (Serra  
364 et al., 2005) — while an increasing proportion of sexual daughters produced over  
365 the lifetime of the mother could also have evolved in order to postpone sexual re-  
366 production, our results demonstrate that this is not the case, since young mothers  
367 tend to produce higher frequencies of sexual offspring (Figure 5). Several hypotheses  
368 have been proposed concerning the mechanism underlying this transgenerational effect  
369 (DNA methylation, cytoplasmic compound present in decreasing concentration over

370 generations, e.g., Gilbert, 2017), but it currently remains unknown. While similar  
371 sex-repressing mechanisms may be favoured in other cyclical parthenogens (such as  
372 aphids or cladocerans) at the start of the growing season, their existence has (to our  
373 knowledge) not been tested. Interestingly, the existence of such a mechanism in mono-  
374 gonont rotifers raises the possibility that the effect of other factors known to affect  
375 investment in sex, such as population density or food stress, may persist over a given  
376 number of clonal generations (evidence that food stress may affect the mictic response  
377 of females over several generations can be found in Hagiwara et al., 2005; Kamizono et  
378 al., 2017). These effects should be explored in order to better understand the selective  
379 forces that may act on the evolution of the propensity for sex in monogonont rotifers,  
380 in both natural and experimental environments.

381

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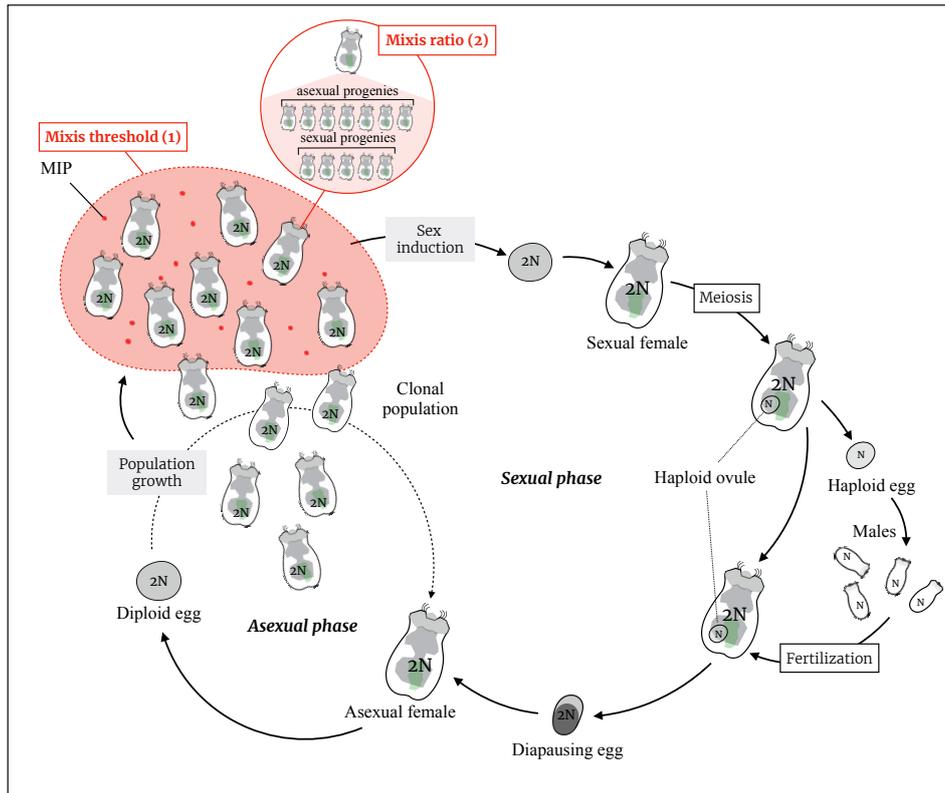
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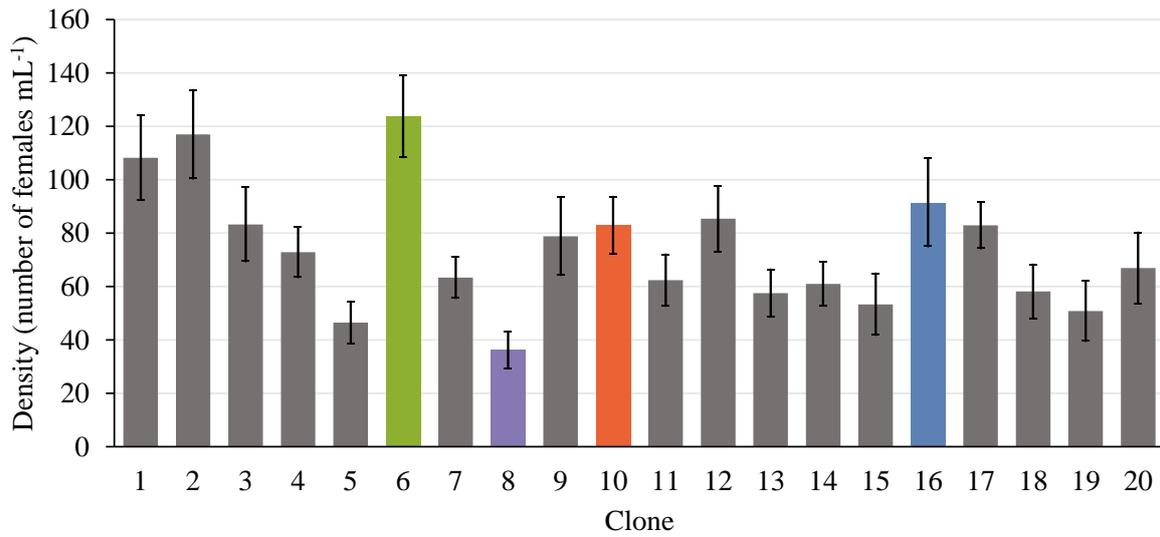
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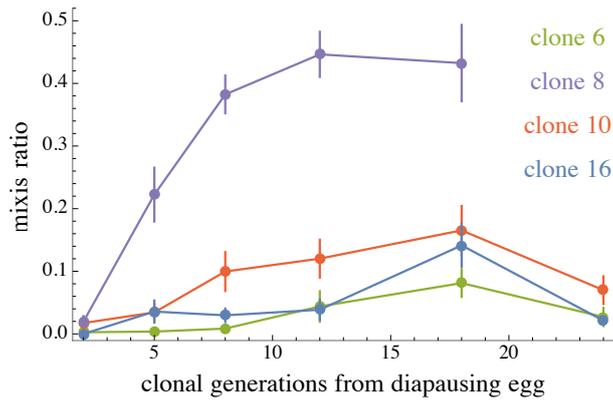
519

520 **Figure 1.** *Brachionus plicatilis* life cycle. Asexual reproduction leads to clonal pop-  
 521 ulation growth until the accumulation of the mixis inducing protein (MIP) in the  
 522 environment triggers the production of sexual females. A sexual female produces hap-  
 523 loid males if unfertilized, and one or several diploid diapausing eggs if fertilized by a  
 524 male. The threshold population density required for the production of sexual females  
 525 is termed the mixis threshold (1). When sex is induced, asexual females can give birth  
 526 to both sexual and asexual females; the proportion of sexual progenies produced by a  
 527 given female is referred to as its mixis ratio (2).



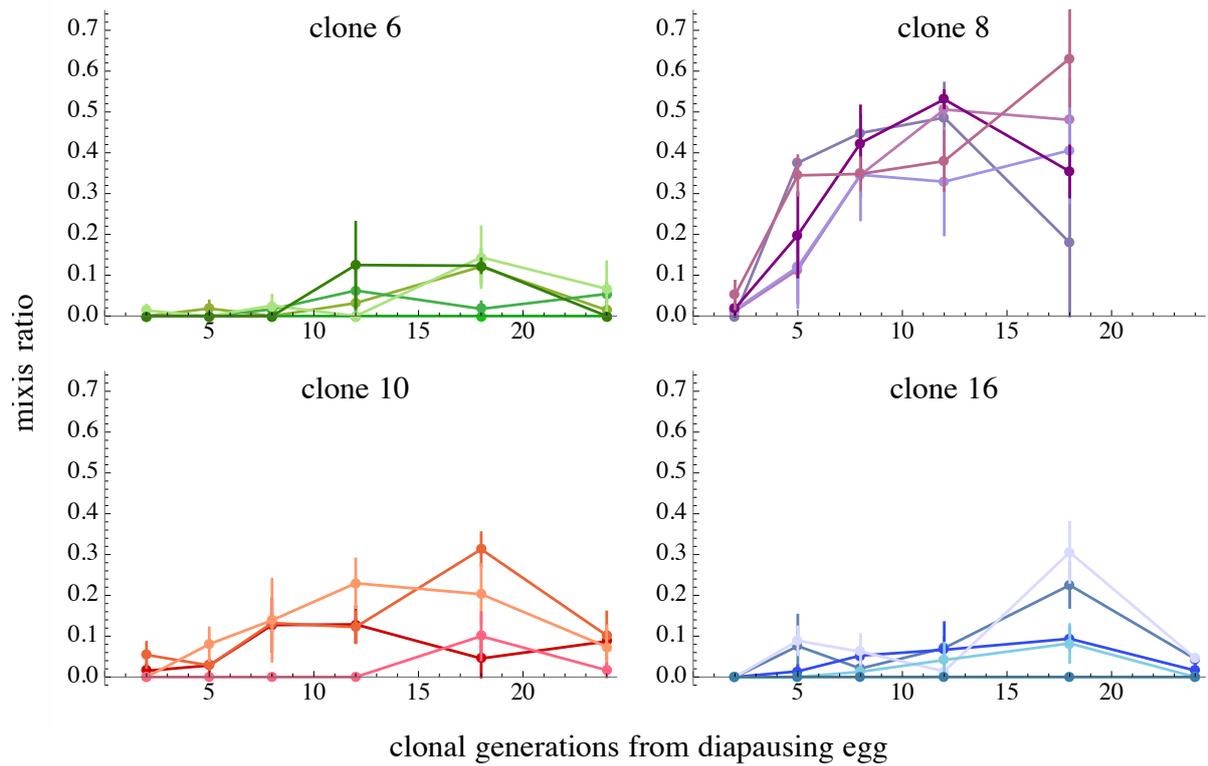
528

529 **Figure 2.** Average mixis threshold of the different P-clones (density at which the  
 530 first males were observed), measured after 15 asexual generations. Error bars show  
 531  $\pm 1$  S.E. The colored bars show the clones selected for the second experiment (effect of  
 532 the number of generations after fertilization on the mixis ratio).



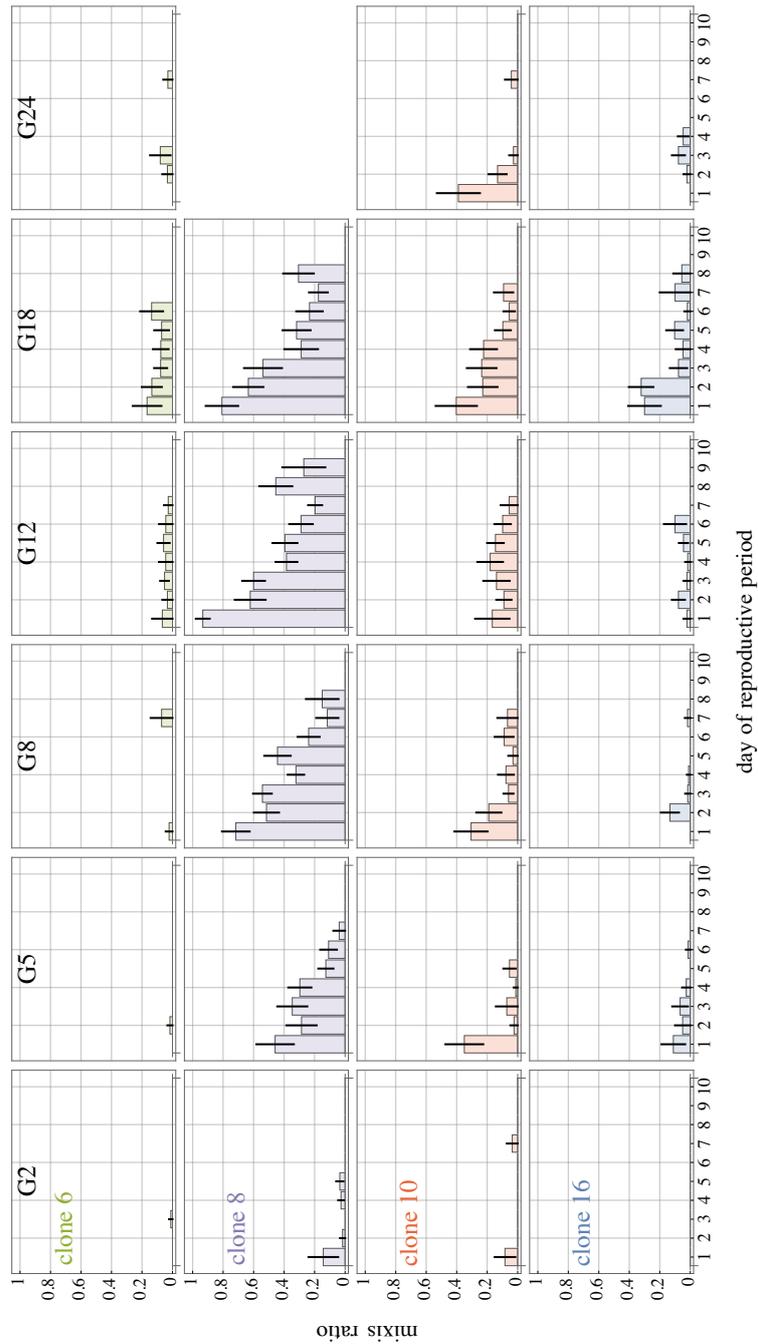
533

534 **Figure 3.** Mixis ratio (proportion of sexual females among the whole progeny of  
 535 an individual) averaged over each P-clone, and as a function of the number of clonal  
 536 generations since hatching of the the diapausing egg. Error bars show  $\pm 1$  S.E.



537

538 **Figure 4.** Mixis ratio (proportion of sexual females among the whole progeny of  
 539 an individual) as a function of the number of clonal generations since hatching of the  
 540 diapausing egg, averaged over each F1-clone (initiated from a different diapausing egg)  
 541 produced by intraclonal mating within each P-clone (5 F1-clones from clones 6, 8 and  
 542 16, and 4 F1-clones for clone 10). Error bars show  $\pm 1$  S.E.



544 **Figure 5.** Mixis ratio (measured as the proportion of sexual females among offspring  
543 produced per day) as a function of the day of the reproductive period (day 1 cor-  
545 responding to the day of first reproduction), for the different P-clones and numbers  
546 of clonal generations after hatching of the diapausing egg. Error bars show  $\pm 1$  S.E.  
547 Averages were computed only when more than 10 juveniles were produced for a given  
548 mother's age class, over the whole P-clone and for a given generation.  
549