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ABSTRACT

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

Keywords: cyclical parthenogenesis, monogonont rotifers, reproductive mode, sexual 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-

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

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

they produce diploid diapausing eggs formed by regular gamete fusion. These diapausing "eggs" actually consist in multicellular embryos that can resist dessication and adverse environmental conditions, and may remain viable for several years (Lubzens et al., 2001). After a dormant phase, they can hatch when the environmental conditions become favorable again.

In rotifers from the *Brachionus* genus, the switch from asexual to sexual reproduction is mainly controlled by population density, through a form of quorum sensing mechanism involving a protein (the "mixis-inducing protein" or MIP) produced by the females themselves (Carmona et al., 1993; Stelzer and Snell, 2003, 2006; Snell et al., 2006). The threshold population density at which sexual females start being produced (called the "mixis threshold") was shown to vary among species, and also among strains from the same species (Gilbert, 2017 and references therein). Variations in the mixis threshold among isolates from the same natural population was demonstrated in the brackish-water species *Brachionus plicatilis* (Carmona et al., 2009; Gabaldon and Carmona, 2015), and in the freshwater species *Brachionus calyciflorus* (Becks and Agrawal, 2010). A second component of investment in sex is the "mixis ratio", corresponding to the proportion of sexual females among offspring (once the mixis threshold has been reached — see Figure 1); variation for the mixis ratio among strains has also been demonstrated, but to what extent the mixis ratio correlates with the mixis threshold remains unclear (Gilbert, 2017).

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

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

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

MATERIAL AND METHODS

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

parental clones).

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

Effect of the number of clonal generations after diapausing egg hatching. A subset of 4 P-clones displaying constrasted mixis thresholds was chosen based on

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

juvenile female was sampled from each sub-clonal line to measure its mixis ratio. For this, the tested female was placed in a well of a 48-well plate (Greiner Bio-One™) with 0.3mL of sex-inducing medium, consisting in standard culture medium with an algal concentration of 4×10^5 cells/mL, mixed in equal proportions with filtrate obtained from a previous rotifer culture that had reached a density of approximately 20 females/mL (filtrated on a 0.2μm mesh), and that was stored at at 5°C (a unique batch of this medium was used throughout the experiment). The concentration of mixis-inducing protein in the resulting medium should thus be approximately equivalent to its concentration in a population at 10 females/mL density, which is well above the density required to induce sex in most populations (Gilbert, 2017). Every day until its death (which generally occurred after 10 to 15 days), the tested female was transferred into a new well containing 0.3mL of fresh sex-inducing medium, and its offspring were collected and individually transferred to a single well of a 96-well plate (Greiner Bio-One™) containing 0.2mL of standard culture medium. When offspring started to reproduce, they were typed as asexual (if they produced females) or sexual (if they produced males).

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

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

RESULTS

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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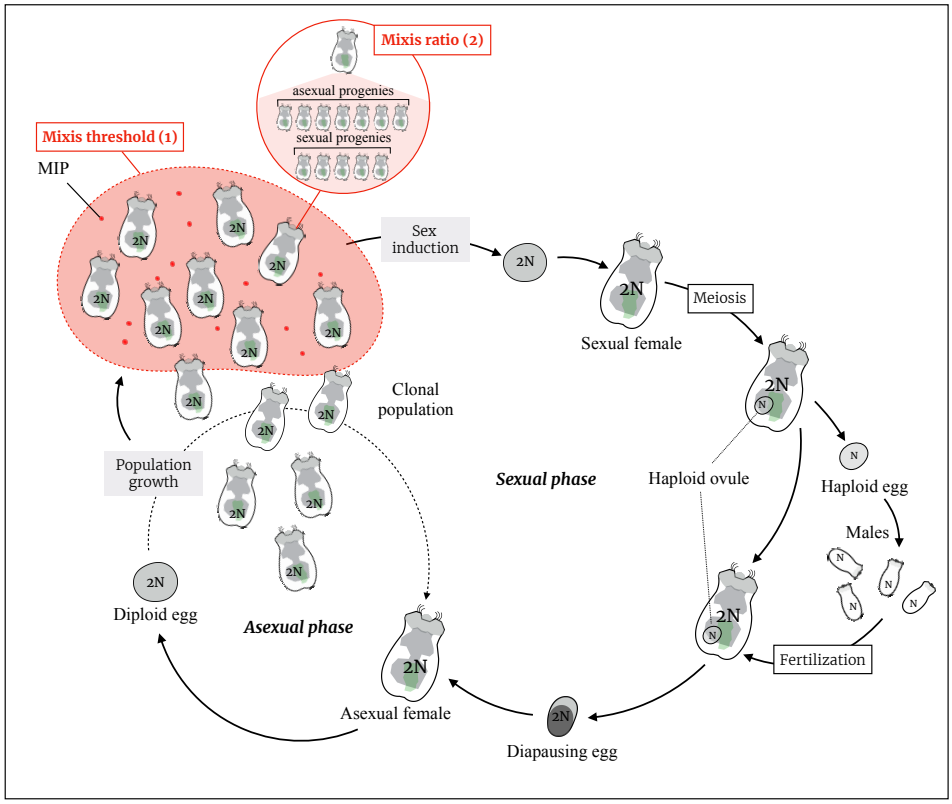
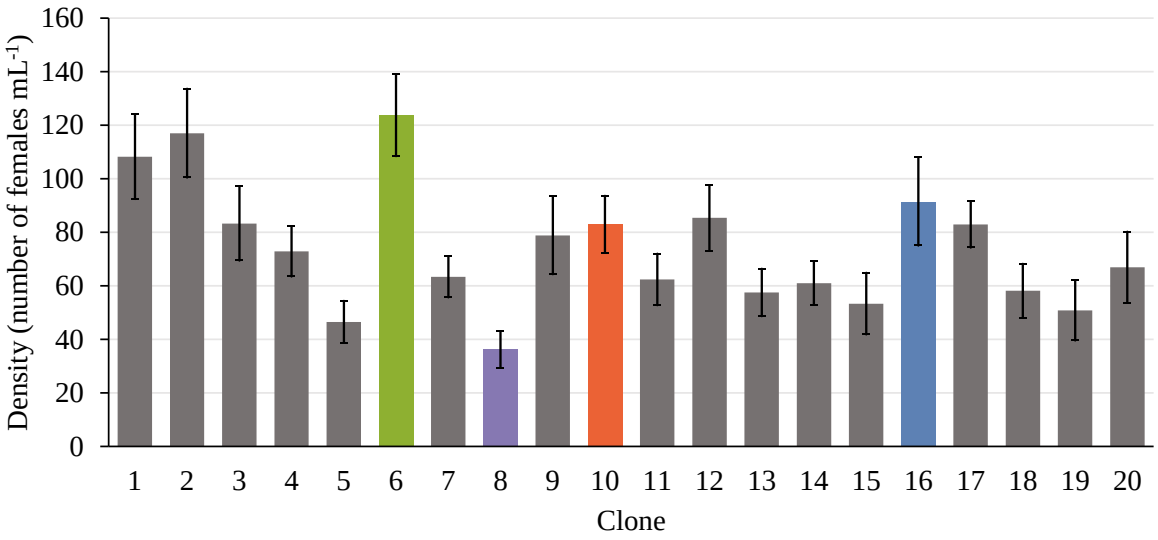


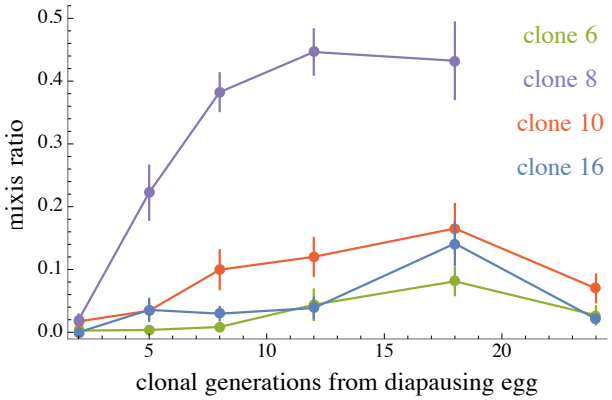
519

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).



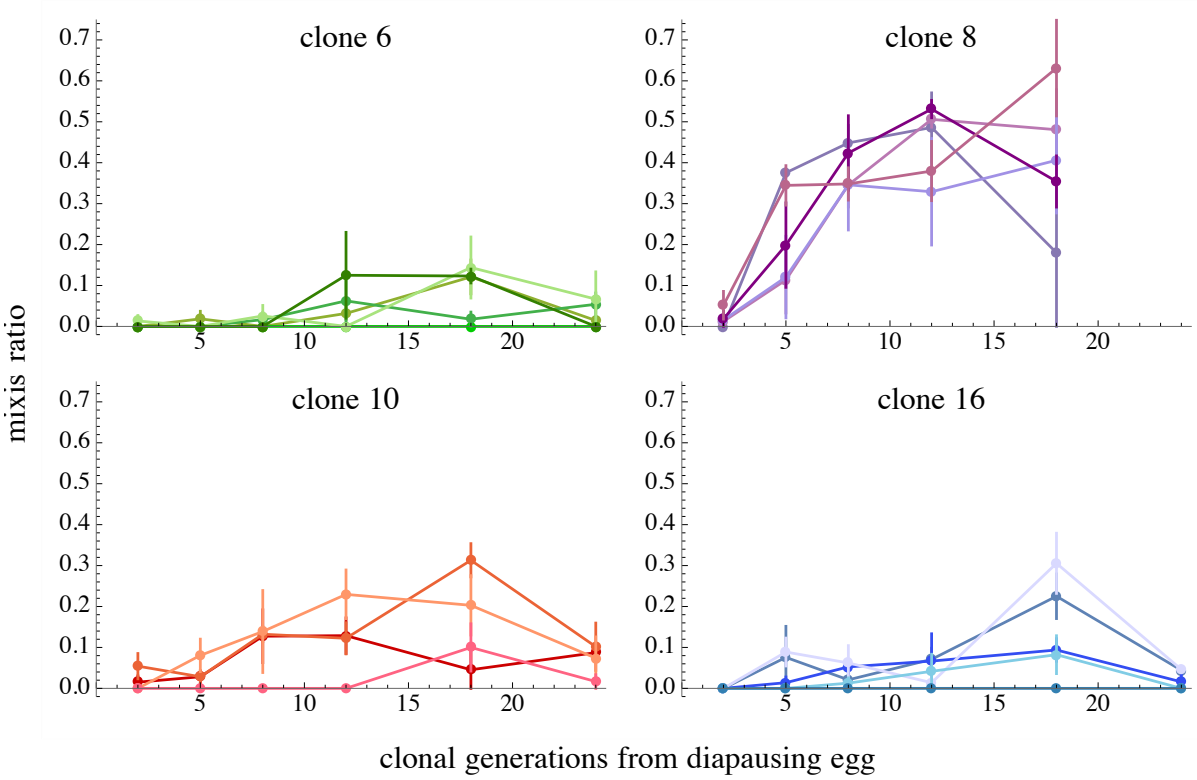
528

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 ±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 ±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 ±1 S.E.

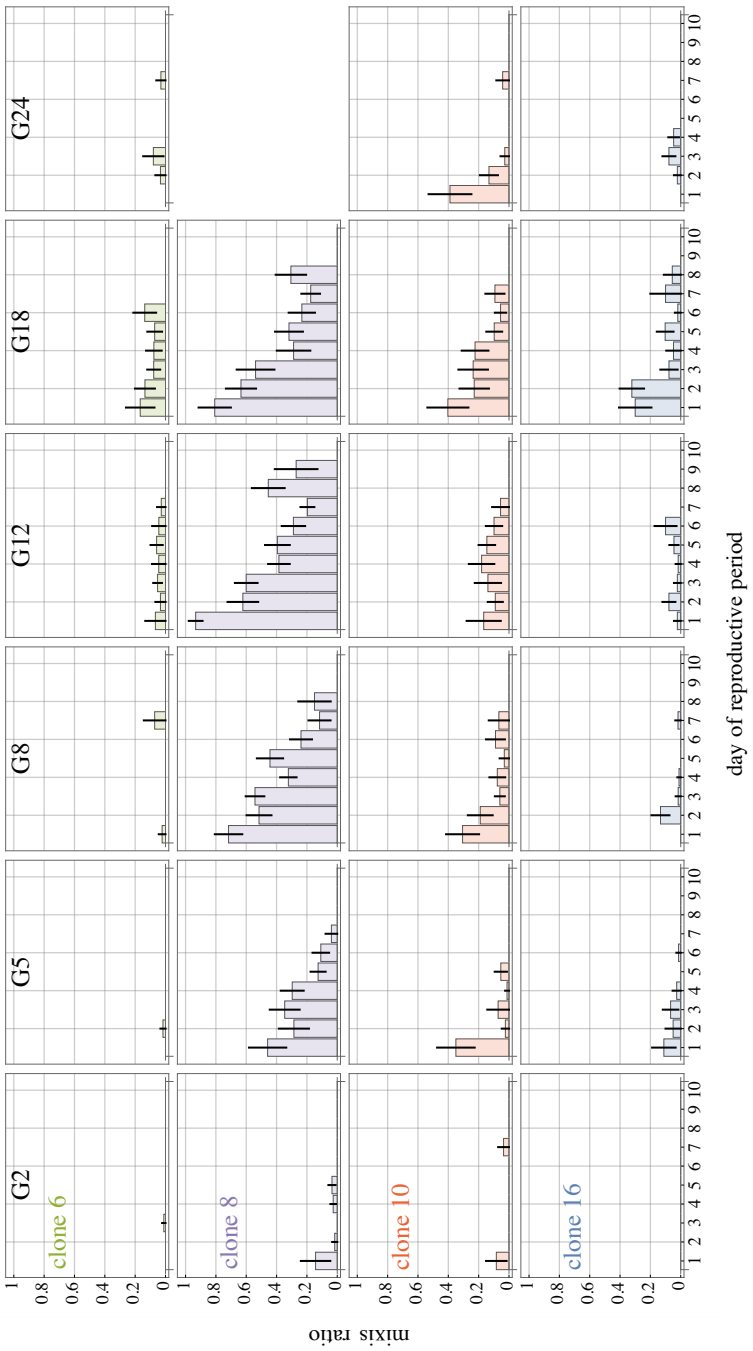


Figure 5. Mixis ratio (measured as the proportion of sexual females among offspring produced per day) as a function of the day of the reproductive period (day 1 corresponding to the day of first reproduction), for the different P-clones and numbers of clonal generations after hatching of the diapausing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