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# Sex ratio effects on reproductive success of male and female *Daphnia*

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## Abstract

The intensity of mating competition and the opportunity for sexual selection are thought to depend on the operational sex ratio, the ratio of sexually active males to fertilizable females. Cyclic parthenogens, organisms that alternate between sexual reproduction and female-only parthenogenesis, show particularly high variation in sex ratios in natural populations but the effects of this variation on mating competition and reproductive success of each sex are poorly understood. In a series of experiments with *Daphnia magna*, we experimentally imposed five sex ratio categories, varying from one male per 81 females to an even sex ratio. We found that, in males, reproductive success strongly and monotonically decreased with decreasing number of females per male. In females, in contrast, mating success and reproductive success were reduced only at the most female-biased sex ratio (1:81), when many females remained unmated and unfertilized, and then again at equal sex ratios, probably due to negative effects of high density or stress induced by numerous males. Our results suggest that females experienced male limitation at heavily female-biased sex ratios below one male to about 50 females. As this is well within the sex ratio variation observed in natural *Daphnia* populations, we conclude that mating competition and the opportunity for sexual selection may exist not only in males but, at least periodically, also in females.

## KEYWORDS

cladocerans, cyclic parthenogenesis, *Daphnia magna*, mating competition, reproductive success, sex ratio, sexual selection

## 1 | INTRODUCTION

Sexual conflict theory predicts that, in species with sexual reproduction, separate sexes and conventional sex roles, reproductive success (RS) of females depends mainly on resources and is largely independent of the number of males available, whereas RS of males mainly depends on mating opportunities and should increase with the availability of fertilizable females (Bateman, 1948; Clutton-Brock, 2009; LeBas, 2006; Trivers, 1972). Female availability

depends on the operational sex ratio (OSR), that is, the ratio of sexually active males to receptive (fertilizable) females within the population (Emlen & Oring, 1977). However, increasing evidence is accumulating that mate limitation can increase competition for access to mates and negatively affect RS not only in males but also in females as well, in particular under female-biased sex ratios (Ah-King & Gowaty, 2016; Hare & Simmons, 2019; Kappeler, 2017; Rhainds, 2010; Rosvall, 2011; Stockley & Bro-Jørgensen, 2011; Vasilieva & Tchabovsky, 2015). For example, male limitation was

found to contribute to female reproductive failure in some insects (Fauvergue, 2013; Rhainds, 2010). In mammals, female reproductive failure due to a shortage of males at female-biased sex ratio was found in the Yellow ground squirrels (*Spermophilus fulvus*) (Vasilieva & Tchabovsky, 2015). To understand the mechanisms and effects of sexual selection from a broader perspective, the lag between empirical studies on male and female mating competition and opportunities for sexual selection should be further mitigated, in particular by reporting symmetrically on both sexes in the same study (Hare & Simmons, 2019).

In the cyclical parthenogenetic freshwater crustacean *Daphnia*, populations typically consist of parthenogenetic females for most of the year. Yet, before the onset of a season necessitating diapause (e.g. drought or freezing), females start producing sons and sexual eggs that require fertilization and will result in resting eggs (Ebert, 2005). However, the conditions that induce male production versus sexual production of resting eggs are, at least partially, uncoupled within populations (Cáceres & Tessier, 2004; DeMeester & Vanoverbeke, 1999; Galimov et al., 2011; Yampolsky, 1992). Likely as a result of this uncoupling, populations often show strong biases and temporal variation in operational sex ratios (Galimov et al., 2011; Gerber et al., 2018; Innes, 1997; Korpelainen, 1989; Spaak, 1995; Yampolsky, 1992).

The strong biases and variation in operational sex ratios suggest that Cladocerans, and the genus *Daphnia*, in particular, might be a highly interesting model taxon for research on the effects of OSR on sexual RS, mating competition and opportunities for sexual selection. Yet, compared to the large number of studies concentrating on the parthenogenetic part of the *Daphnia* life cycle, this research is still in its infancy. Only a few studies have started to interpret sex ratio variation in evolutionary terms (Booksmythe et al., 2018; Gerber et al., 2018; Innes & Dunbrack, 1993; Yampolsky, 1992). Even fewer studies have addressed sexual selection or the opportunity thereof in *Daphnia*.

From behavioural studies, it is known that *Daphnia* males actively pursue and try to attach to other conspecific daphnids, both males and females (Brewer, 1998; La et al., 2014). Evidence for the presence of sexual pheromones and fighting between males in *Daphnia* and other Cladocera are rather controversial (Brewer, 1998; La et al., 2014; VanDamme & Dumont, 2006) but there is no direct data on preferential pursuit of sexual females. The duration of contact of males with sexual females is significantly higher than with males and parthenogenetic females (Brewer, 1998; Duneau et al., 2020a, 2020b; La et al., 2014). There is also no evidence for active search behaviour in female *Daphnia*. Rather, even sexual females sometimes exhibit escape reactions from male pursuits (Brewer, 1998). Limited evidence for direct male-male competition comes from reports of fighting, the fact that sometimes two or even three males can be found attached to the same female, and from sperm morphology, which varies in a manner consistent with the occurrence of sperm competition (Duneau et al., 2020a, 2020b; La et al., 2014). On the other hand, occasions of highly female-biased sex ratios may limit

mating success in female *Daphnia* (Duneau et al., 2020a, 2020b; La et al., 2014), and might open opportunities for female-female competition over mates.

Biased operational sex ratios are thought to provide a strong opportunity for sexual selection because access to mates is limited and mating competition is more intense in the sex in excess (Clutton-Brock & Parker, 1992; Emlen & Oring, 1977; Janicke & Morrow, 2018; Kvarnemo & Ahnesjö, 1996). However, it is now clear that the relationship between OSR and the extent of sexual selection is not as straightforward as it was originally thought (Clutton-Brock, 2007; Kokko et al., 2012). In particular, when mate monopolization patterns covary with OSR or when direct competition for mates involves investment into weaponry, the relationship between the OSR and the extent of sexual selection may be nonlinear, neutral or even negative (Klug et al., 2010; Kokko & Jennions, 2008). Nevertheless, the OSR does measure the ratio of the competitors to the contested resource and is, therefore, in many cases, expected to reflect the difficulty of achieving matings (Janicke & Morrow, 2018; Kokko et al., 2012). Moreover, in *Daphnia*, mating success is thought to mainly depend on the encounter rate between males and sexually receptive females, and there is little evidence for investment into weaponry, although some direct competition for mates and short-term mate monopolization may occur (Brewer, 1998; Duneau et al., 2020a, 2020b; La et al., 2014).

In any case, the fact that sex ratios in *Daphnia* are highly variable and, at least temporarily, strongly female-biased suggests that sexual selection may potentially act on both sexes, though likely at different times. In the current study, we, therefore, assess the opportunity for sexual selection in both sexes of *Daphnia magna* by assessing how male and female RS vary with OSR. In particular, we expected that male RS increases with an increasing relative abundance of females (i.e. decreasing OSR) while female RS might be lower at highly female-biased OSR (low male availability). In addition, we expected that population density might negatively influence the survival of both sexes and female investment in sexual reproduction due to lower food availability.

## 2 | MATERIALS AND METHODS

### 2.1 | Origin of *Daphnia* used in the experiments

The clones (i.e. parthenogenetic descendants of a single female) used in the experiment originated from the Moscow Zoo Cormorant pond (population 'MOS') that comprised both male-producing (MP) and non-male-producing (NMP) clones (Galimov et al., 2011; Molinier et al., 2018). Clones were obtained from sexually produced resting eggs that had been collected from the natural population and that were hatched in the laboratory. They were kept in separate clonal cultures for several months, in constant light, at 18°C, medium culture density (3–30 females per 150 ml of artificial *Daphnia* medium (ADaM (Klüttgen et al., 1994), and high food ( $5 \times 10^4$  *Scenedesmus acutus* cells ml<sup>-1</sup> day<sup>-1</sup>).

## 2.2 | Experiment design and setup

For the experiment, we set up replicated cultures with varying adult sex ratios and kept them for three weeks under conditions favouring sexual reproduction. We experimentally imposed five adult sex ratio categories (varying from heavily female-biased, one male to 81 females, to equal male-to-female ratios). We estimated the OSR (the ratio of ready-to-reproduce males to fertilizable females), taking into account that, in *Daphnia*, only a fraction of adult females are ready to reproduce sexually at any given moment. This fraction can be estimated from the number of 'ephippia' produced, that is, the number of diapause capsules containing up to two fertilized resting eggs (the latter are actually embryos arrested at the ~3500 cell stage (Chen et al., 2018)). Shortly, each fertilizable female initiates the production of a single ephippium prior to fertilization, and the ephippium is subsequently shed, independent of whether fertilization takes place (it is empty in case of no fertilization). Hence the production of an ephippium is a reliable indicator of the presence of a fertilizable female (Figure 1). We estimated male RS as the number of resting eggs divided by the number of males and female RS as the number of resting eggs divided by the number of ephippia produced (i.e. female RS for fertilizable females only).

Three weeks prior to the start of the experiment, five females from each clone were used to establish two separate cultures containing mixtures of 71 MP and 46 NMP clones, respectively. The MP and NMP cultures were placed for a three-week period in conditions that are known to induce male and ephippia production in MOS clones (14°C, photoperiod gradually decreasing from 14:10 to 13:11 L:D, the cultures were fed  $10^9$  cells of *Scenedesmus acutus* per 1 L of culture per day). The population density was gradually increased from ca. 50 females  $L^{-1}$  at the start of treatment to 700 individuals  $L^{-1}$  by the end of the third week. After three weeks, 4860 adult and



**FIGURE 1** Mating in *D. magna*, female above, male below. Note the ephippium, the dense part of the female's carapace with two resting eggs already deposited inside. After mating, the ephippium will darken and will be shed during the next moult of the female [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

pre-adult females and 1452 adult and pre-adult males were used to establish 20 experimental populations using females from the NMP cultures and males from the MP cultures. By using only NMP females, we were able to exclude that experimentally imposed sex ratios were affected by parthenogenetic production of additional males during the experiment (NMP females are unable to produce males (Galimov et al., 2011)).

The twenty experimental populations represented five treatments with different sex ratios (four replicates per treatment). We chose an experimental design with a constant number of 243 females per aquarium. Sex ratios were adjusted by adding different numbers of males (3, 9, 27, 81, and 243 for 1:81, 1:27, 1:9, 1:3 and 1:1 male-to-female sex ratios, respectively). In this design, the 81-fold variation in sex ratio is correlated with a two-fold variation in initial density, and hence effects of sex ratio and density are confounded in the analyses. Yet, the advantage of this design is that female densities were constant, at least in the beginning of the experiment, ensuring that, with respect to the volume of the aquaria, females were not diluted at higher sex ratios. This is important because a dilution of females may affect encounter rates of individual males with females (or search times) and may, therefore, affect male RS independent of effects of sex ratio. Moreover, designs that keep total density constant have the caveat that density effects may not only depend on the number of individuals but also on their size, food consumption and other traits, which differ between male and female *Daphnia* (Lüring & Beekman, 2006). In our experiment, we first consider all results as a function of sex ratios and subsequently discuss potential effects of density (also taking into account mortality that occurred during the experiment, see below).

Throughout the 21 days of the experiment, all populations were cultured in 5L aquaria with 2L of ADaM. Aquaria were kept at 14°C and 12L:12D photoperiod changed to 11L:13D after seven days in order to prevent females from switching to parthenogenetic reproduction. Independent of the treatment, all populations were fed  $10^8$  cells of *Scenedesmus acutus* three times a week. Occasional parthenogenetic offspring were removed weekly. At the end of the experiment, we counted the numbers of males and females alive. From these numbers, we calculated realized adult sex ratios and the realized densities at the end of the experiment, as well as male and female mortality. We also counted all ephippia that had been produced during the 21 days of the experiment, which corresponds to about three moult cycles in adult female *D. magna* under experimental conditions (Figure S1). However, because almost no ephippia were produced during the first week of the experiment, and because their production may have been initiated prior to the transfer to the aquaria, we only used ephippia produced during the second and third week for the analysis. Each ephippium was opened with forceps and needles under a stereomicroscope, and the numbers of live and unviable resting eggs were counted. Unviable resting eggs are morphologically distinguishable by their colour and outer membrane position (Keller & Spaak, 2004).

## 2.3 | Data treatment and analysis

### 2.3.1 | Predictors

We used the five experimentally imposed adult sex ratio categories as a predictor in the first place. Second, to estimate the OSR (the ratio of ready-to-reproduce males to fertilizable females), we assumed that all adult males were ready to reproduce. However, this assumption is unlikely to be valid for female *Daphnia*: Under conditions favouring sexual reproduction, some females will nonetheless reproduce parthenogenetically or postpone reproduction (Galimov et al., 2011). The production of 'resting eggs' in *Daphnia* involves the deposition of one or two fertilized eggs into an 'ephippium', a maternally produced, protective structure, in which the developing embryos can survive harsh conditions (Figure 1). The deposition of sexual eggs into the brood pouch is stimulated by the contact with a male and/or mating. Because ephippium formation starts prior to fertilization, an empty ephippium is shed and ovaries are resorbed in the absence of fertilization (C.A.K., 1899; Hiruta & Tochinai, 2014; Svendsen et al., 2015; Zaffagnini & Sabelli, 1972). It is, therefore, evident that not all females were fertilizable at any given time in our experiment. However, because each fertilizable female initiates ephippium production (independent of whether fertilization will actually occur), we used the total number of ephippia produced (independent on whether they contained zero, one, or two embryos) as a proxy for the number of fertilizable females. We, therefore, measured the OSR as the number of males divided by the number of ephippia produced during the experiment. Note that this measure likely still underestimates the immediate OSR at any given time because the window during that a female is fertilizable may be relatively short, though not limited to the brief period just after moult, as is the case for parthenogenetic eggs (Hiruta & Tochinai, 2014). Nonetheless, the degree of this underestimation should be the same across all replicates so that the estimate should still represent a good measure of the relative OSR among experimentally imposed sex ratio classes and correlate with the immediate OSR. Third, in our design, effects of the sex ratio cannot be entirely disentangled from effects of density (see above). We, therefore, ran certain analyses with density (the number of *Daphnia*, both sexes combined, at the beginning of the experiment per volume culture medium) rather than sex ratio as a predictor. Since the compositions of experimental populations changed with time during the experiment due to mortality, we also ran analyses with realized density and sex ratio (measured as the average of initial and final estimates of density and sex ratio) as predictors. However, we do not report the details of these analyses because the results are very similar to results using initial density and imposed sex ratio categories. Indeed, both density measures and both sex ratio measures were highly correlated ( $r = 0.97$ ,  $p < 0.0001$  and  $r = 0.98$ ,  $p < 0.0001$ , respectively, Figure S2). The data (i.e. the number of males and females present at the end of the experiment) are reported in Table S1.

### 2.3.2 | Response variables

Our experimental design did not allow us to directly measure individual mating and reproductive success neither in males nor in females but only the average reproductive success for all animals of a given sex in each replicate. We estimated average male *per capita* reproductive success (RS) for each replicate population as the number of resting eggs per male. For females, however, we were interested in the RS of only those females that were potentially fertilizable. Again (as for the OSR), we used the number of ephippia produced as a proxy of the number of these females. Consequently, we estimated the average *per capita* RS of fertilizable females as the number of live resting eggs per ephippium. RS estimates were square root-transformed to fit normal distribution (Shapiro-Wilk's test for transformed data:  $p > 0.05$ ).

During the third week of the experiment, we recorded data on ephippia with no eggs separately as an indication of female mating failure (C.A.K., 1899; Svendsen et al., 2015). To assess the effect of SR on female mating success, we analysed the variation in the proportion of empty ephippia (logit-transformed, Shapiro-Wilk's test:  $p > 0.05$ ) between SR categories.

As further response variables, we used egg viability, defined as the proportion of live eggs among all resting eggs ( $N$  live eggs was  $>1$ , and a constant of 1 was added to the total number of eggs to allow logit transformation for data normalization; Shapiro-Wilk's test for transformed data:  $p = 0.045$ ). We also used the number of ephippia per female (i.e. the number of ephippia produced during the experiment divided by the number of females at the beginning of the experiment; untransformed data) as a measure of the sexual activity of females, and mortality rate, estimated as the proportion of individuals that died during the three weeks of the experiment. Mortality rates for males and females were logit-transformed to fit normal distribution (Shapiro-Wilk's test for transformed data:  $p > 0.05$ ).

## 2.4 | Statistical analysis

Effects of the five experimentally imposed sex ratio categories were analysed using ANOVA, with Tukey's HSD-test for post hoc comparisons. To test for curvilinear relationships between OSR and response variables, we included both linear and quadratic terms for ln-transformed OSR in GLM models. To assess gender- and density-dependent variation in mortality, we categorized densities in three categories of low, medium and high (123 to 135, 165, and 243 individuals  $L^{-1}$ , respectively). In other words, we combined the first three experimentally imposed categories with the lowest sex ratios because they differed only a little in density. We used repeated-measures ANOVA with gender as within effect and density as between effect to analyse the mortality data. One-way ANOVA was applied to analyse the variation in the proportion of empty ephippia between SR categories. All statistical analyses were performed using STATISTICA, v. 8.0 (StatSoft, Tulsa, OK, USA).

### 3 | RESULTS

#### 3.1 | Male and female reproductive success and egg viability

The reproductive success (RS) of fertilizable females (i.e. the number of eggs per ephippium) significantly varied among sex ratio categories (Table 1, please see Table S2 for primary data). It was the lowest (1.1 egg per ephippium) in the lowest sex ratio category, then increased significantly and levelled off (1.6 to 1.7 eggs per ephippium in the following three categories), and then decreased again significantly (1.4 eggs per ephippium in the category with the highest sex ratio). Accordingly, the RS of fertilizable females varied quadratically with OSR, that is, with an increase in the relative abundance of males (GLM:  $R^2 = 0.80$ ,  $F_{2,17} = 35.1$ ,  $p = 0.000001$ , Figure 2a).

The proportion of empty ephippia varied significantly between SR categories (ANOVA:  $F_{4,15} = 26.8$ ,  $p < 0.00001$ , Figure 3). They were most abundant at the lowest (1:81) sex ratio (Tukey HSD-test:  $p < 0.01$  for comparisons with all other SR categories)—overall, there were 35% empty ephippia in contrast to 3–13% at other sex ratios. At the medium sex ratios of 1:3 and 1:9, there were only few empty ephippia (overall, 5% and 3%, respectively), significantly less than at 1:1 sex ratio (Tukey HSD-test:  $p < 0.01$ ).

The reproductive success of males strongly and monotonically decreased among sex ratio categories, from 85 eggs per male in the lowest sex ratio category to just 0.8 eggs per male in the category with the highest sex ratio and with all decreases between subsequent categories being significant (Table 1). As the decrease in RS slowed towards higher sex ratio categories, there was also a significant quadratic fit with OSR (GLM:  $R^2 = 0.99$ ,  $F_{2,17} = 1177.2$ ,  $p < 0.000001$ , Figure 2b).

#### 3.2 | Egg viability

Unviable eggs were almost only found in the lowest sex ratio category. Accordingly, the proportion of live eggs (among all resting eggs) was lower in that category (about 0.9) compared to all other

categories (where the proportion was close to 1, Table 1). When egg viability was fitted to ln-transformed OSR, the quadratic fit was significant (GLM:  $R^2 = 0.58$ ,  $F_{2,17} = 11.8$ ,  $p = 0.0006$ , Figure 4, Table 1).

#### 3.3 | Mortality and sexual activity of females

Mortality and the degree of investment into sexual reproduction by females were considered to be more likely affected by density than directly by the sex ratio (please see Materials and Methods and Discussion). Both density and gender strongly and significantly influenced the mortality rate (repeated measures ANOVA:  $F_{2,17} = 77.0$ , partial eta-squared = 0.82,  $p < 0.000001$  and  $F_{2,17} = 15.1$ , partial eta-squared = 0.64,  $p = 0.0002$ , respectively; interaction:  $F_{2,17} = 6.3$ , Partial eta-squared = 0.42,  $p < 0.01$ , Table 2). In males, the mortality rate was, on average, three times higher than in females ( $0.60 \pm 0.18$  vs.  $0.17 \pm 0.17$ , CI95% = 0.51–0.68 and 0.09–0.25, respectively) but only slightly varied with density (Table 2, Figure S3). In females, the mortality rate significantly varied with density. It was minimal at low and intermediate densities and reached almost 50% at the highest density (Table 2). The number of ephippia produced per female during the experiment averaged 0.87 across all densities and was the lowest (0.56 ephippia per female) at high density, while close to one ephippium per female at low and intermediate densities (Table 2).

### 4 | DISCUSSION

#### 4.1 | Reproductive success of males and females

The experimentally imposed sex ratio categories and the estimated OSR affected both male and female RS but in different ways. In agreement with classic sexual conflict theory, male RS rapidly and monotonically decreased with the decreasing number of fertilizable females per male. This indicates intense male–male competition for mates when the OSR is not strongly female-biased and provides

TABLE 1 Mean  $\pm$  SD reproductive success of fertilizable females (N eggs/ephippium), reproductive success of males (N eggs/male), and egg viability (proportion of live eggs) in the different sex ratio categories

Response variable	Sex ratio category					ANOVA test statistics
	Very low (1:81 M:F)	Low (1:27 M:F)	Medium (1:9 M:F)	High (1:3 M:F)	Very high (1:1 M:F)	
N eggs / ephippium	1.09 $\pm$ 0.11 <sup>a</sup>	1.64 $\pm$ 0.09 <sup>b</sup>	1.71 $\pm$ 0.03 <sup>b</sup>	1.67 $\pm$ 0.03 <sup>b</sup>	1.42 $\pm$ 0.05 <sup>c</sup>	$F_{4,15} = 50.7$ , $p < 0.000001$ <sup>†</sup>
N eggs / male	84.9 $\pm$ 12.0 <sup>a</sup>	43.5 $\pm$ 5.0 <sup>b</sup>	14.6 $\pm$ 0.7 <sup>c</sup>	4.6 $\pm$ 0.3 <sup>d</sup>	0.8 $\pm$ 0.2 <sup>e</sup>	$F_{4,15} = 384.3$ , $p < 0.000001$ <sup>†</sup>
Proportion of live eggs	0.895 $\pm$ 0.036 <sup>a</sup>	0.989 $\pm$ 0.009 <sup>b</sup>	0.985 $\pm$ 0.007 <sup>b</sup>	0.991 $\pm$ 0.004 <sup>b</sup>	0.988 $\pm$ 0.002 <sup>b</sup>	$F_{4,15} = 14.7$ , $p = 0.00005$ <sup>‡</sup>

Note: <sup>a,b,c,d,e</sup>Different superscripts indicate significant pairwise contrasts according to Tukey HSD-tests for estimates in the same row.

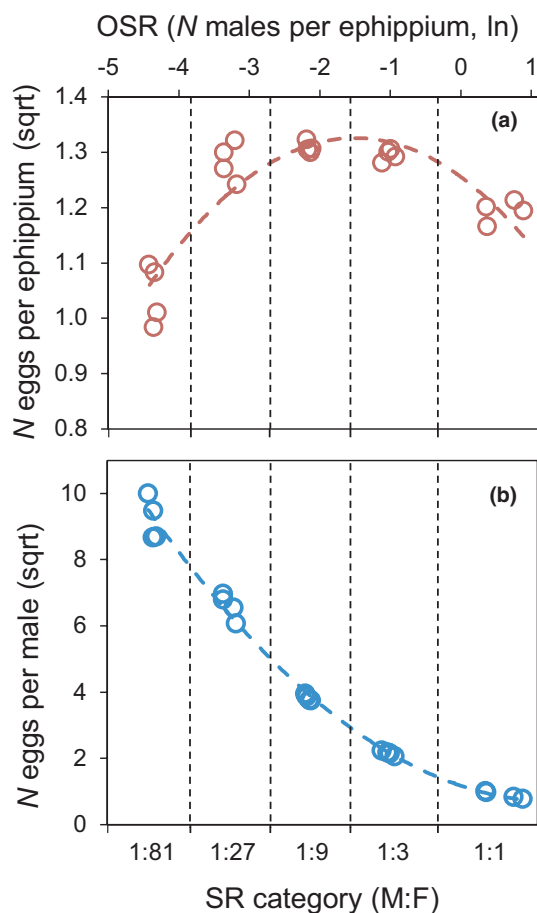
\*M:F - male:female sex ratio. The corresponding average estimates for OSR (male:ephippium) are 1:78, 1:26, 1:9, 1:3, and 2:1 for very low, low, medium, high, and very high sex ratio categories, respectively.

<sup>†</sup>Statistics for sqrt-transformed response variable.

<sup>‡</sup>Statistics for logit-transformed response variable.

conditions for sexual selection in male *D. magna* as has been suggested recently (Duneau et al., 2020a, 2020b).

Our results also show clear evidence for male limitation at the lowest sex ratio, leading to reduced female mating success and



**FIGURE 2** Reproductive success for (a) fertilizable females (ratio of the number of eggs to the number of ephippia produced, square-root-transformed) and (b) males (number of eggs per male, square-root-transformed) in relation to the experimentally imposed sex ratio categories (lower, primary x-axis, vertical dashed lines) and the estimated OSR (ln-transformed, upper, secondary x-axis) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

fertilization rates (number of resting eggs per ephippium) as well as reduced viability of produced resting eggs. Female mating failure as indicated by high proportion of empty ephippia apparently contributed to the overall low female RS at the lowest sex ratio (Figure 2a, Figure 3). This implies that at heavily female-biased sex ratio, there were too few males to mate with all fertilizable females and stimulate egg deposition (egg deposition into the ephippium is stimulated by mating, REF). Moreover, at the lowest sex ratio, a substantial fraction of deposited eggs was unviable, that is unfertilized (Figure 4). This supports possible sperm depletion (Winsor & Innes, 2002), despite high sperm production in males (Dufresne et al., 2019). As a result, female RS rapidly increased with increasing male availability at highly female-biased sex ratios as predicted by models that assume that females may experience mate limitation (Kokko et al., 2012).

Male limitation occurred only in the lowest sex ratio category (1 male per 81 females), but not in the second-lowest sex ratio category (1 male per 27 females). In these two classes, each female produced, on average, 0.96 and 0.98 ephippia, respectively, during the two weeks of the experiment. This suggests that a single male was able to successfully fertilize at least 1.9 (i.e.  $0.98 \times 27/14$ ) ephippia per day, but not 5.6 (i.e.  $0.96 \times 81/14$ ) ephippia per day. Moreover, ephippia in the lowest sex ratio category contained, on average, 67% fewer live resting eggs compared to ephippia produced in the second-lowest sex ratio category (1.09 vs. 1.64, respectively, see Table 1). Thus, our best estimate is that, under the conditions in our experiment, a single male was able to successfully fertilize about 3.7 (i.e.  $0.67 \times 5.6$ ) ephippia per day. This is consistent with data provided by Winsor and Innes (2002) for *Daphnia pulex* (0–13 females fertilized in 20h by one male). Male mating efficiency is hardly limited by duration of copulation (Brewer, 1998; La et al., 2014) and thus suggests that search times for fertilizable females (Winsor & Innes, 2002) or sperm limitation also play a role.

Highly female-biased sex ratios, comparable to our lowest sex ratio category, are not untypical for natural *Daphnia* populations, including at times when ephippia production occurs (Galimov et al., 2011; Korpelainen, 1989; Sppak, 1995) Our results thus suggest that, at times when sex ratios are strongly female-biased, male

**TABLE 2** Mortality rate and ephippia production (Mean  $\pm$  SD) in relation to population density

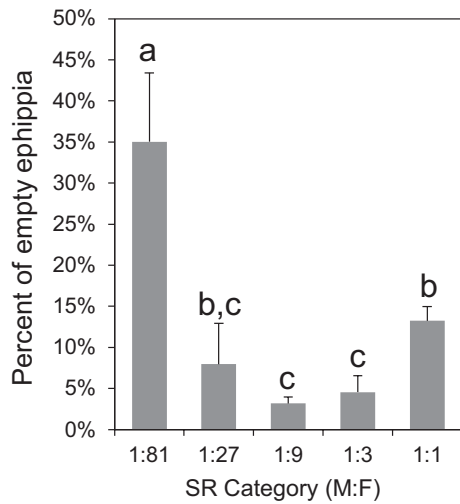
Response variable	Density category			One-way ANOVA test statistics <sup>†</sup>
	Low	Intermediate	High	
Mortality rate				
Males	0.52 $\pm$ 0.17 <sup>a</sup>	0.74 $\pm$ 0.15 <sup>a</sup>	0.67 $\pm$ 0.12 <sup>a</sup>	$F_{2,17} = 3.7, p = 0.05^{\dagger}$
Females	0.08 $\pm$ 0.05 <sup>a</sup>	0.13 $\pm$ 0.05 <sup>a</sup>	0.48 $\pm$ 0.17 <sup>b</sup>	$F_{2,17} = 20.2, p = 0.00003^{\dagger}$
Sex difference <sup>‡</sup>	$p = 0.0002$	$p = 0.0002$	$p = 0.5$	
N eph/female	0.96 $\pm$ 0.06 <sup>a</sup>	0.92 $\pm$ 0.07 <sup>a</sup>	0.56 $\pm$ 0.15 <sup>b</sup>	$F_{2,17} = 35.3, p = 0.000001$

Note: <sup>a, b</sup>Different superscripts indicate significant pairwise contrasts between density categories according to Tukey HSD-tests (one-way ANOVA) for estimates in the same row.

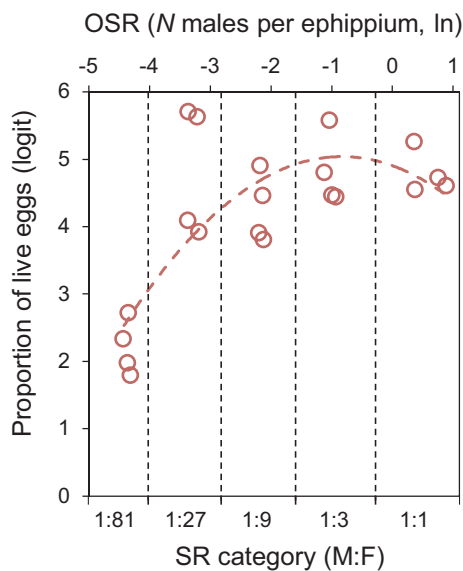
\*One-way ANOVA effects of density, tested for sex separately.

<sup>†</sup>Statistics for logit-transformed response variable.

<sup>‡</sup>Tukey HSD-test (repeated measures ANOVA) for sex differences within density categories.



**FIGURE 3** Average proportions of empty ephippia across SR categories. Error bars indicate standard deviations. Different letters indicate significant pairwise contrasts according to the post hoc Tukey HSD-tests (one-way ANOVA for logit-transformed proportions)



**FIGURE 4** Egg viability (logit-transformed) in relation to the experimentally imposed sex ratio categories (lower, primary x-axis, vertical dashed lines) and the estimated OSR (ln-transformed, upper, secondary x-axis) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

limitation can cause reproductive failure in female *Daphnia* and additional mates increases female RS. This finding contributes to growing evidence that male availability plays an important role in female RS when sex ratios are heavily female-biased and that females may not only compete for resources but for mating opportunities as well. Moreover, models predict that at heavily female-biased OSR, when females face shortage of mates, the sexual selection to increase the mating rate should be stronger on females, than males (Kokko et al., 2012), which is consistent with our results. Given that males can be strongly limited in wild populations, this suggests that

female–female competition for mates and the opportunity for female sexual selection may exist in natural populations of *Daphnia*. In this respect, it is important to understand patterns of sex allocation, timing and synchrony of sexual egg production—aspects of sexual reproduction poorly known in *Daphnia* and other cyclic parthenogens. For example, at highly female-biased OSRs, females may benefit from asynchronous production of ephippia because asynchrony likely would reduce male limitation and hence increase mating and egg fertilization success.

This brings us back to the question of what the immediate OSRs were achieved in the different imposed adult sex ratio categories of our experiment. As explained above, the time window during which a female is fertilizable may be relatively short, certainly shorter than one moult cycle (seven days under the experimental conditions), and likely longer than one day as it is not limited to a brief post-moult period for parthenogenetic eggs (Hiruta & Tochinai, 2014). We can thus estimate limits of the immediate OSR (i.e. the ratio of the number of males to the number of fertilizable females at any given moment), considering that each ephippium indicates the presence of a fertilizable female for a duration of somewhere between one day and seven days. We thus obtain an immediate OSR of somewhere between 1:6 and 1:39 for the most female-biased sex ratio class and of somewhere between 4:1 and 14:1 for the class with equal adult sex ratios. This suggests that the immediate OSR in our experiment varied from female-biased to male-biased and, because adult sex ratios in natural populations vary roughly by the same amount as in the experiment, we can expect female-biased as well as male-biased OSRs also to occur in natural populations.

## 4.2 | Density effects

While male RS decreased monotonically with OSR, female RS was highest at intermediate sex ratios. In particular, in the highest sex ratio category (1:1 male-to-female sex ratio), females produced a lower number of resting eggs per ephippium and a lower number of ephippia, compared to intermediate sex ratio categories. Both these effects may have been caused by the negative influence of high density on available food resources rather than by high sex ratios (population density in the highest sex ratio category was 50% higher than in the second-highest sex ratio category - 243 and 165 individuals  $L^{-1}$ , respectively). Indeed, negative effects of low food supply on parthenogenetic reproduction have been demonstrated in numerous studies (Orcutt & Porter, 1984). So far, no experimental study has addressed density effects or food limitation effects on the production of resting eggs in *Daphnia*, though an increasing percentage of empty ephippia with decreasing food levels was observed in a natural population of *D. pulicaria* (Conde-Porcuna et al., 2014). A possible negative effect of high density on female performance also manifested itself via reduced ephippia production. However, because female mortality also increased at high density, it remains unknown whether the lower number of ephippia per female indeed indicates a lower sexual activity of females at



high population densities or whether it is explained by a lower number of females being present during sexual reproduction. Finally, the higher numbers of males at high densities may also have had a direct negative effect on female reproduction via induced stress (Ismail et al., 2010).

Besides female reproduction, also female survival was negatively correlated with density, while male mortality was more or less constant, though high, across all experimental categories. Negative effects of low food levels on female survival are known in *Daphnia* (McCauley et al., 1990), suggesting that this effect may again have been mediated by decreased *per capita* food levels at high densities. Density-independent and generally relatively high mortality of males compared to females may be attributed to the higher metabolic activity of males (MacArthur & Bailie, 1926), which may limit their survival even at relatively high food levels, especially when mating effort is high. In turn, high mortality rate of males relative to females should bias adult sex ratio and, therefore, OSR, thus influencing mating competition.

High densities of 200–300 individuals L<sup>-1</sup>, similar to those in our experiments, are frequently observed in *D. magna* rockpool and pond populations, as well as in aquacultures (DePauw et al., 1981; Galimov et al., 2011; Ghilarov, 1967). This suggests that density effects on female survival and sexual reproduction observed here are relevant for natural *Daphnia* populations and should be taken into account in industrial *Daphnia* cultures.

## 5 | CONCLUSIONS

The results of our study suggest that female performance during sexual reproduction in *D. magna* is optimized at intermediate female-biased sex ratios, that is, with a sufficient number of males to fertilize the available resting eggs, but not too high numbers to avoid negative effects (likely mediated via density and food availability) on survival and reproduction. Males, on the other hand, have the highest individual performance at strongly female-biased sex ratios, presumably because negative effects of male–male competition can be avoided when males are relatively rare. More generally, our results suggest that, given the sex ratio variation in natural *Daphnia* populations, not only males but also females may periodically experience mating competition, creating opportunities for sexual selection. We need further studies on sexual reproductive biology and ecology of *Daphnia* and other cyclic parthenogenes to understand whether the opportunity for sexual selection in females is realized, and if it yes, what female traits are under selection. Our results represent a first step towards an evolutionary interpretation of sex ratios and their effects on mating and reproductive success in male and female *Daphnia*.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## AUTHOR CONTRIBUTIONS

YRG and AVT conceived and planned the study. YRG and ART carried out the experiment. AVT, CRH and YRG analysed the data, AVT, CRH, ART and YRG wrote the paper, and all authors approved the final version.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/jeb.13942>.

## DATA AVAILABILITY STATEMENT

We confirm that the data supporting the findings of this study are available within the article, supplementary materials and/or Dryad repository at <https://doi.org/10.5061/dryad.gf1vhhmqr>.

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