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# ***Daphnia* females adjust sex allocation in response to current sex ratio and density**

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## **Author contributions**

IB, NG and HK conceived the study; IB, NG and DE designed the experiments and sampling regime; IB and NG collected and analysed the data; IB, NG, DE and HK wrote the paper.

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## **Data accessibility**

All data from this study are archived in the DRYAD data repository, DOI:10.5061/dryad.sb269

1 **Abstract:** Cyclical parthenogenesis presents an interesting challenge for the study of sex allocation,  
2 as individuals' allocation decisions involve both the choice between sexual and asexual  
3 reproduction, and the choice between sons and daughters. Male production is therefore expected to  
4 depend on ecological and evolutionary drivers of overall investment in sex, and those influencing  
5 male reproductive value during sexual periods. We manipulated experimental populations, and  
6 made repeated observations of natural populations over their growing season, to disentangle effects  
7 of population density and the timing of sex from effects of adult sex ratio on sex allocation in  
8 cyclically parthenogenetic *Daphnia magna*. Male production increased with population density, the  
9 major ecological driver of sexual reproduction; however, this response was dampened when the  
10 population sex ratio was more male-biased. Thus, in line with sex ratio theory, we show that *D.*  
11 *magna* adjust offspring sex allocation in response to the current population sex ratio.

12

13 Sex allocation theory was developed to explain the observation, common across diverse taxa, of  
14 equal ratios of male to female progeny. Fundamentally, over-producing one sex reduces the  
15 expected fitness payoff from individuals of that sex, thereby selecting for increased production of  
16 the opposite sex (Fisher 1930; Düsing 1884 reported in Edwards 2000). This negative frequency-  
17 dependent principle has been successfully applied across an impressive range of life histories,  
18 breeding and genetic systems (West 2009). Sex allocation generalizes to cases where equilibrium  
19 offspring sex ratios (proportion of male offspring) are not 50%, in which case equal investment into  
20 production of each sex is predicted (if males and females are not equally costly to produce, more  
21 offspring of the cheaper sex are expected; Charnov 1982; but see Kahn *et al.* 2015). Trivers and  
22 Willard (1973; see also Charnov 1982) recognized that, given differential environmental effects on  
23 male and female fitness, reproducing individuals would benefit by adjusting their relative  
24 investment towards the sex with higher reproductive value. Fluctuations in population sex ratio over  
25 time, arising for example through seasonality, can make the reproductive values of sons and  
26 daughters differ. Thus, assuming that individuals can measure the current sex ratio or a correlate  
27 (e.g. the current season), the primary sex ratio (sex ratio among offspring at production) can evolve  
28 to respond to the changing reproductive values (Werren and Charnov 1978, West and Godfray  
29 1997, Kahn *et al.* 2013).

30 Werren and Charnov's (1978) models were built on specific scenarios of seasonal variation or  
31 unusual perturbations to sex-specific fitness expectations. Although the theory lacks extensive  
32 further development (West 2009), the idea of facultative sex ratio adjustment in response to  
33 population sex ratio remains popular in the empirical literature. Observational data provide mixed  
34 support (positive: lions, *Panthera leo*: Creel and Creel 1997; snow skinks, *Niveoscincus*  
35 *macrolepidotus*: Olsson and Shine 2001; northern goshawks, *Accipiter gentilis*: Byholm *et al.* 2002;  
36 negative: reed warblers, *Acrocephalus arundinaceus*: Bensch *et al.* 1999; gray-tailed voles,  
37 *Microtus canicaudus*: Bond *et al.* 2003). Robust experiments manipulating sex ratio while  
38 controlling for other potential cues are rare, although Southern green stink bugs, *Nezara viridula*

39 (McLain and Marsh 1990), parasitic mites, *Hemisarcoptes coccophagus* (Izraylevich and Gerson  
40 1996), a perennial herb, *Begonia gracilis* (Lopez and Dominguez 2003), and southern water-skinks,  
41 *Eulamprus tympanum* (Robert *et al.* 2003) have been shown to produce male-biased offspring sex  
42 ratios when kept in female-biased populations, and *vice versa*. However, equally many experiments  
43 have failed to find the expected pattern (guppies, *Poecilia reticulata*: Brown 1982; common lizards,  
44 *Lacerta vivipara*: Le Galliard *et al.* 2005; southern water-skinks, *E. tympanum*: Allsop *et al.* 2006;  
45 jacky dragons, *Amphibolurus muricatus*: Warner and Shine 2007).

46 Systems with unusual sex ratio dynamics may be useful in identifying general patterns and  
47 furthering understanding of when the sex ratio affects sex allocation. Cyclical parthenogenesis  
48 describes a lifecycle where females typically produce daughters asexually, but engage occasionally  
49 in (often environmentally induced) male production and subsequent sexual reproduction (Bell  
50 1982). This creates conditions for plastic adjustment of offspring sex, as producing males can in  
51 principle range from completely unprofitable when all females opt for asexuality, to highly  
52 profitable when many or all females are sexual. Cyclical parthenogenesis has several consequences  
53 for sex allocation theory. First, because daughters' reproductive value is not as tightly bound by  
54 frequency-dependence as in organisms where every individual has both a mother and father,  
55 cyclical parthenogens can show extremely female-biased sex ratios. Second, reproductive decisions  
56 in cyclical parthenogens involve not only the sex of offspring, but also whether and when to  
57 reproduce sexually. Fitness consequences of these decisions are intertwined: male production  
58 makes little sense unless there are sexually reproducing females in the population. In addition, sex  
59 can entail a range of genetic, demographic, and ecological costs and benefits compared to asexual  
60 reproduction (Halkett *et al.* 2006; Paland and Lynch 2006; Auld *et al.* 2016), and sexually-produced  
61 young sometimes face a different developmental fate: for example, in *Daphnia* only sexually-  
62 produced offspring undergo dormancy before hatching. Finally, additional complexity arises when  
63 only some individuals switch to sex, while others continue asexual reproduction. The co-occurrence

64 of asexually- and sexually-reproducing generations may make it difficult for females to measure the  
65 current sex ratio and base reproductive decisions on it when offspring fitness is realized later.

66 Cyclically parthenogenetic *Daphnia magna* Straus meet theoretical assumptions for facultative  
67 adjustment of offspring sex in response to the population sex ratio (Werren and Charnov 1978),  
68 exhibiting overlapping generations and temporal sex ratio variation. Female *Daphnia* can produce  
69 three kinds of offspring: asexually produced males and females, and sexually produced resting eggs,  
70 which require fertilization by males. Several generations fit into one summer growing season,  
71 during which individual females reproduce iteroparously, switching back and forth between sexual  
72 and asexual reproduction, and between producing male or female asexual clutches. *Daphnia*  
73 hatching from resting eggs (in subsequent growing seasons) are invariably female. The sex of  
74 asexually (ameiotically) produced offspring is environmentally determined: both males and females  
75 are genetically identical to their mothers. Male production starts before females begin to switch to  
76 the production of sexual eggs – an intuitively expected pattern when males need time to mature  
77 before they can fertilize eggs (N. Gerber, I. Booksmythe, H. Kokko, unpublished).

78 Once males are present in the population, predictions for subsequent sex allocation become less  
79 straightforward, as the option of asexual reproduction means that not all females ‘count’ in the  
80 manner assumed by Fisherian sex ratio theory. Previous work on *D. magna* ruled out the strict  
81 alternation of sexes of consecutive broods on detection of a ‘male-inducing’ cue, and hypothesised  
82 that an increase in population sex ratio over time was due to individual females adjusting, on a  
83 brood-by-brood basis, offspring sex in response to their current environment (Barker and Hebert  
84 1986). Although seasonal environmental cues play a role in male production (Stross and Hill 1965;  
85 Carvalho and Hughes 1983; Hobaek and Larsson 1990), population density is one of the best-  
86 known ecological predictors of male production (Hobaek and Larsson 1990; Kleiven *et al.* 1992,  
87 Berg *et al.* 2001) and sexual reproduction (Carvalho and Hughes 1983).

88 We test whether female *D. magna* adjust between the three possible offspring types according to the  
89 current population sex ratio. We consider both sex ratio adjustment, by which we mean the sex ratio  
90 among asexual offspring, and sex allocation, which we use when referring to allocation decisions  
91 between the production of males and fertilizable eggs. Our use of ‘sex allocation’ for the latter  
92 decision concurs with the standard use of this term in obligate sexuals with separate sexes. We  
93 examined sex ratio adjustment in both natural and experimental settings, documenting sex ratios in  
94 natural populations over the growing season, and manipulating density and sex ratio in  
95 experimental populations to disentangle the effects of these parameters on offspring sex. In the  
96 natural populations we additionally estimated sex allocation between male and female sexual  
97 function. Extrapolating from the literature on crowding effects, we expected increased male  
98 production with increasing population density. However, if *Daphnia* adjust offspring sex to  
99 optimise the reproductive value of offspring produced, theory predicts that male production should  
100 decrease with increasing population sex ratio. This creates an experimental opportunity to  
101 determine if the sex composition of conspecific density matters for individuals’ sex ratio adjustment  
102 and sex allocation, by manipulating population density and sex ratio separately.

## 103 **Methods**

### 104 *Natural populations*

105 Data on offspring sex ratios of individual females was collected during a study of the timing of sex  
106 in natural *Daphnia magna* populations (Gerber et al. 2018). We sampled 11 populations inhabiting  
107 separate rock pools distributed over 6 islands in the Finnish archipelago at Tvärminne Zoological  
108 Station (59.8420° N, 23.2018° E) over two months during the summer growing season of 2015.  
109 These rock pools are small, with surface area of less than 10 m<sup>2</sup>, but have *Daphnia* populations of  
110 several thousand individuals. Every three to four days we recorded the density and demographic  
111 structure of every population (14-18 sampling events/population). To estimate population density,

112 350-ml samples were collected at 15 haphazardly chosen locations spanning the pool area and  
113 depth. These were combined and stirred to distribute individuals evenly, and a 350-ml subsample  
114 was taken as the final density sample. The remaining animals were returned to the rock pool. After  
115 collecting the density sample a small hand net was swept through the pond to take a representative  
116 population sample. All *D. magna* individuals in the density sample were counted under a dissecting  
117 microscope and converted to an estimate of individuals/l. Population samples were categorised by  
118 age and reproductive status: juvenile males and females, adult males, and adult (reproductively  
119 mature) females, which were further classified as sexually reproducing (carrying an ephippium, the  
120 melanised capsule into which the fertilized resting eggs are deposited) or not (asexually reproducing  
121 and non-reproductive). Up to 10 females (where possible; median = 10, mean  $\pm$  SE =  $8.72 \pm 0.17$ )  
122 with an asexual clutch visible in the brood pouch were then isolated from the sample and  
123 maintained individually in 35 ml jars until they released their clutch. Clutch size and offspring sex  
124 were determined under a dissecting microscope, and we recorded whether the mother formed an  
125 ephippium for her next instar, visible by a darkening and change in shape of the female brood  
126 pouch.

### 127 *Experimental populations*

128 Population sex ratio and density were manipulated in three separate experiments, in July 2015, June  
129 2016 and July 2016. In July 2015, stocks of twenty *D. magna* clones that had been previously  
130 collected from the study population, treated with antibiotics to clear microsporidian infections, and  
131 maintained in the lab for a year (see Roulin *et al.* 2015) were established in 9-L buckets (one clone  
132 per bucket; 10-20 founding individuals per clone) outside, near natural rock pools containing  
133 *Daphnia*, so they were exposed to the natural climate and weather conditions. Buckets were filled  
134 with 8 L water from a rock pool not used in our observational study, in which no *Daphnia* were  
135 detected during the study period. The water was filtered through 48- $\mu$ m mesh to avoid possible  
136 contamination with *Daphnia*, other large plankton or predators. Algae small enough to pass through



137 the filter were allowed to grow and provided a food source for the populations. Each bucket was  
138 additionally inoculated with 20 mL of algae suspension (50 million *Scenedesmus* cells/mL) and left  
139 to stand for several days before adding *Daphnia*. Filtered water from the same source pool was  
140 added to all buckets on two occasions during stock growth to compensate for evaporation.

141 When stock populations were in the exponential growth phase, pre-reproductive females and males  
142 were collected separately and used to create a mixed-clone stock of each sex. While we attempted  
143 to include similar numbers of individuals from each clone, availability of individuals varied due to  
144 population size differences among the stocks, and the exact representation of each clonal genotype  
145 in the stock mixture is unknown. Using individuals haphazardly sampled from these stocks we set  
146 up two sets of experimental populations. The first set manipulated sex ratio across four treatment  
147 levels, from 0 to 74% male, while holding density constant at 50 individuals. The second set  
148 manipulated sex ratio and density simultaneously by adding 0, 10, 25 or 50 males to populations of  
149 50 females (Table S1, 'Exp 1', in Supporting Information). In June 2016, we repeated this  
150 experiment using pre-reproductive *D. magna* females and males collected directly from several  
151 natural populations at the study site; the genetic composition of 2016 stocks was therefore  
152 completely unknown. Numbers of females and males used in each treatment level differed slightly  
153 from the 2015 experiment, as we included a wider range of density treatments (from 25-100 total  
154 individuals, Table S1, 'Exp 2'). To ensure treatments had enough replication to comprehensively  
155 cover the range of population densities we had used, in July 2016 we set up additional replicated  
156 populations in a third experiment, again using animals collected from several natural populations at  
157 the study site, to manipulate the sex ratio (across four levels from 0 to 75% male) while holding  
158 density constant at a low (25 individuals) or high (100 individuals) level (Table S1, 'Exp 3').  
159 Experimental populations in both years were established in 9-L buckets containing 8 L filtered  
160 water from the same source used for the stock populations, each inoculated with 20 mL of  
161 *Scenedesmus* (50 million cells/mL) and left to stand for several days before adding *Daphnia*.

162 Populations were monitored for maturation of the founding females and the presence of neonates.  
163 After two weeks, roughly two juvenile cohorts were apparent in the populations (two size classes of  
164 neonates). Each entire population was collected and the number and sex of juveniles determined.  
165 The experimental period was kept deliberately short to ensure that the offspring sex ratios we  
166 recorded were produced under the manipulated density and sex ratio conditions, as newborn sons  
167 and daughters will quickly alter the population structure. The experiment was not designed to  
168 address allocation to ephippia production, as the short timeframe and use of newly-matured females  
169 (ensuring similar reproductive history across populations) made ephippia production unlikely. As  
170 expected, no ephippia were produced during the experiment.

### 171 *Statistical analysis*

172 Summary statistics are presented as mean  $\pm$  1 standard error (SE), unless otherwise specified. The  
173 relationship of clutch size with population density was tested in a linear mixed model (LMM)  
174 including population as a random factor. To analyse offspring sex ratio and sex allocation data from  
175 natural populations we used generalized linear mixed-effects models (GLMMs) with binomial error  
176 and logit link in the R package lme4 (Bates *et al.* 2015). Population density, adult sex ratio, and  
177 clutch size were included as fixed-effect covariates; the natural log of density and clutch size were  
178 used to normalize these variables. To account for repeated measurements population was included  
179 as a random factor. If binomial models were overdispersed an individual-level random factor was  
180 included (Harrison 2014). We initially included all two-way interactions between predictors, and  
181 sequentially excluded non-significant interactions to obtain final models.

182 Analyses of the experimental populations were performed in MATLAB. We compared a set of  
183 candidate logistic regression models predicting offspring sex, based on model AIC scores. In  
184 addition to a 'null' model (intercept-only; neither density nor sex ratio was allowed to predict the  
185 proportion of males produced) we built models in which the total density of founders was included  
186 as a predictor, and models in which the densities of male and female founders were included as

187 separate predictors that could independently affect the production of males. In each case, we also  
188 considered a model variant where estimates from the three experiments were allowed to vary in  
189 their effects.

## 190 **Results**

### 191 *Natural populations*

192 The mean clutch size among asexually reproducing *D. magna* females sampled from natural rock  
193 pool populations was  $11.97 \pm 0.26$  offspring ( $N = 1614$ ; range: 1 – 116 offspring). Clutch sizes were  
194 smaller at higher population densities (LMM:  $-0.030 \pm 0.013$ ,  $X^2 = 5.69$ ,  $p = 0.017$ ). Asexual  
195 clutches are predominantly single-sex (Barker and Hebert 1986; 91.6% of clutches in our data) and  
196 mixed-sex clutches are usually strongly biased towards one sex. For the following analyses, we  
197 present results in which clutches were assigned their majority sex, ignoring sons produced in  
198 majority female clutches. However, results of all analyses were qualitatively extremely similar (no  
199 changes of sign or significance) if we used ‘at least one male’ as the criterion for male production.

200 As population densities increased, the adult sex ratio (ASR, adult males:adult females) became  
201 more male-biased (GLMM:  $0.646 \pm 0.079$ ,  $z = 8.19$ ,  $p < 0.001$ ). The overall mean probability that  
202 an asexual female produced a male-biased clutch was  $0.22 \pm 0.01$ . Clutches were more likely to be  
203 male-biased when they were larger, when sampled from higher-density populations, and when the  
204 ASR was more male-biased (Table 1, Fig. 1). The likelihood that a female that released an asexual  
205 clutch subsequently produced an ephippium (mean  $\pm$  SE probability:  $0.14 \pm 0.01$ ) increased when  
206 the ASR was more male-biased (Fig. 1), when the female’s clutch was male-biased (Table 2), and  
207 when the female’s clutch was small in absolute terms (Table 2). However, the size of a female’s  
208 asexual clutch relative to others in the same population sample had a positive effect on ephippia  
209 production: females that produced relatively large clutches were more likely to subsequently  
210 produce an ephippium. When accounting for these effects, the effect of density on ephippia

211 production was not significant (Table 2), but excluding them for comparison with previous studies,  
212 the effect of density alone was significantly positive (GLMM:  $0.740 \pm 0.091$ ,  $z = 8.09$ ,  $p < 0.001$ ).

213 We also considered sex allocation, the ratio of investment into male function (producing sons  
214 asexually) vs. sexual female function (switching to sexual reproduction, i.e. producing ephippia).  
215 Adult males and ephippial females are the ‘sexual individuals’ that make up the mating pool. Note  
216 that allocation towards sexual female function is not the production of daughters through ephippia,  
217 but the likelihood of ephippia production – that is, the likelihood that the female herself switches to  
218 sexual reproduction. To obtain this ratio, we treat a clutch (asexual or ephippial) as the ‘unit’ of  
219 offspring. Our population samples give the proportion of currently-reproducing females carrying  
220 ephippia ( $0.17 \pm 0.02$ ) and the proportion carrying asexual clutches ( $0.83 \pm 0.02$ ). Multiplying the  
221 proportion of asexually-reproducing females by the probability that an asexual clutch was  
222 predominantly male or female ( $0.22 \pm 0.01$  or  $0.78 \pm 0.01$ , respectively), we obtain estimates of  
223 relative allocation towards clutches of ephippia, sons, and daughters (e.g. using mean values,  
224 ephippia:sons:daughters =  $0.17 : 0.18 : 0.65$ ). We can use these proportions to approximate relative  
225 allocation among sexual functions, i.e. sons:ephippia. Defined this way, sex allocation decreased at  
226 higher population densities (Table 3) and when the current ASR was male-biased (Table 3, Fig. 1).  
227 Across all populations over the sampling period, the mean sex allocation ratio was approximately  
228 even ( $0.18 : 0.17 =$  sex allocation ratio of 0.51).

### 229 *Experimental populations*

230 To test the effects of population density and ASR on sex ratio adjustment among asexually  
231 produced offspring, we quantified the proportion of sons among all offspring produced after two  
232 weeks (~2 clutches). Model selection based on AIC scores identified an unambiguous best model:  
233 that in which male and female density independently affected the proportion of sons produced, and  
234 in which their effects were allowed to vary between the three experiments (Table 4). Despite the  
235 best model estimating the effects of male and female density separately for each experiment, these

236 effects showed a remarkably consistent pattern across the three experiments: increasing female  
237 density always predicted a greater increase in the likelihood of producing sons than did increasing  
238 male density (Fig. 2: isoclines are steeper when female than when male density changes). In  
239 experiment 1, adding one male was equivalent to adding 0.51 females in terms of the sex allocation  
240 response; in experiments 2 and 3, the corresponding numbers are 0.37 and 0.45.

## 241 **Discussion**

242 While much of sex allocation theory enjoys good empirical support, evidence of primary sex ratios  
243 responding to the current sex ratio in natural populations remains relatively scant (see West 2009,  
244 chapter 8). Our study is conducted in a system where sex itself is facultative, creating strong  
245 opportunities for phenotypically plastic sex allocation responses: male production yields little  
246 fitness benefit during periods when most females are not reproducing sexually. Sex ratio adjustment  
247 conceivably extends to allow females to respond to the current ASR, which also influences the  
248 success of any sons produced. However, if females use conspecific density as a cue for sex (and not  
249 only seasonal cues such as day length, e.g. Roulin *et al.* 2013, 2015), it is not straightforward to  
250 predict how females should respond to an increased density of females or males. Female abundance  
251 does not reliably indicate future mating opportunities for males, as these females might continue  
252 reproducing asexually. Male presence can indicate that the population has already partially  
253 transitioned to sexual reproduction, and females can gain fitness by producing males who then  
254 fertilize sexual eggs. On the other hand, a high ASR also means that any males produced will  
255 experience high competition, lowering their expected reproductive success. The former effect  
256 predicts that male presence could trigger females to produce more males, the latter argues for  
257 inhibition.

258 In natural populations, a link between high ASR and production of sons is supported at first sight,  
259 as females from more male-biased populations were more likely to produce sons than were females

260 from female-biased populations (Table 1, Fig. 1). Our experiments showed that females respond to  
261 male presence in a remarkably consistent manner: increasing density by adding males led to  
262 increased production of sons, but only by 37% – 51% of the increase observed if the additional  
263 density consisted of females. This suggests that both factors play a role: females produced more  
264 sons when densities were high, with a dampened response if the ASR was already high.

265 In the sampled natural populations, the likelihood of ehippia production — the other aspect of  
266 sexual reproduction — increased with the ASR. This is not a response to mating, as *Daphnia*  
267 females commit to ehippia production several days before mating. The results match our  
268 expectations that investment in sexual female function increases when males are more common.  
269 This effect is not solely due to ehippia and male production increasing independently in response  
270 to the same conditions promoting sexual reproduction. The negative effect of male presence on  
271 production of sons, and its positive effect on ehippia production, was clearly evident when  
272 considering female investment in sexual function only (i.e. sex allocation). Females from more  
273 male-biased populations were less likely to produce males relative to ehippia than were females  
274 from female-biased populations (Table 3, Fig. 1).

275 The argument from sex ratio theory that increasing male-male competition reduces the expected  
276 reproductive value per son (Frank 1990) provides an evolutionary argument for why male  
277 abundance inhibits further male production, relative to the effect of female abundance. Male *D.*  
278 *magna* take around 10-12 days to mature, and this delay can help in understanding why responding  
279 to the population sex ratio can be adaptive, even without information on the current reproductive  
280 status of females in the population. All else being equal, more females mean more future  
281 fertilization opportunities, given that each female is able to switch multiple times back and forth  
282 between reproductive modes.

283 The only other cyclical parthenogens in which sex allocation has been investigated are the  
284 monogonont rotifer genus *Brachionus*, haplodiploids in which sexually- and asexually-reproducing

285 individuals are produced in distinct generations (Aparici *et al.* 2002). In *Brachionus*, the trait  
286 underlying sex allocation is the threshold age at which females lose the capacity to be fertilized.  
287 Females that reach this age unfertilized produce only males; increasing male frequency increases  
288 fertilization rates, thereby reducing male production in a negative frequency-dependent process.  
289 Furthermore, earlier fertilization thresholds increase the likelihood a female's lifetime sex  
290 allocation will be male-biased (Aparici *et al.* 1998). While there is no such direct effect of male  
291 frequency on *Daphnia* sex ratio adjustment, there are similarities: increasing densities induce sexual  
292 generations in *Brachionus* (Serra and Snell 2009) and production of males and ephippia in *Daphnia*  
293 (Carvalho and Hughes 1983). Models of the rotifer system find the threshold fertilization age is  
294 evolutionarily stable at the point where, for a given density, it results in equal production of males  
295 and sexual eggs – i.e., even sex allocation (Aparici *et al.* 1998, Serra *et al.* 2008). Data from field  
296 and laboratory populations match this prediction (Aparici *et al.* 2002). For *Daphnia*, where there is  
297 no unique temporal threshold, it is difficult to place as much significance on the roughly equal sex  
298 allocation we observed, as theory does not single out this value as the sole prediction under plastic  
299 sex allocation in general (Frank 1990) or for *Daphnia* specifically (N. Gerber, I. Booksmythe, H.  
300 Kokko, unpublished).

301 Sex ratios also covary with density in species with strong local mate competition (LMC). In fig  
302 wasps, females on low-density patches with few founders produce only enough sons to fertilise  
303 their daughters, while females at high density produce higher offspring sex ratios (e.g. Herre 1985,  
304 1987). Female fig wasps adjust the sex ratio in a local patch plastically, as is the case in *Daphnia*.  
305 However, LMC seems unlikely to explain the sex–density link in *Daphnia*. Population density does  
306 not reflect local *Daphnia* genetic diversity, making it an unlikely proxy for LMC. An earlier  
307 experiment in the same *D. magna* metapopulation (Altermatt and Ebert 2008) found no relationship  
308 between the initial size of experimental populations (i.e. number of founders) and the total  
309 production of ephippia over the growing season. Similarly, sex ratios in experimental outdoor

310 populations with low (single clone) and high clonal diversity did not differ, further suggesting an  
311 absence of LMC mechanisms in *Daphnia* (D. Ebert, unpublished data).

312 Our results agree with the expectation that ephippia production should not begin before male  
313 production. This helps explain the remaining conflict between our observational and experimental  
314 results: adding females led to a stronger male-producing response than adding males, yet the net  
315 effect in natural populations is more male production by more male-biased populations. Our short-  
316 term experimental populations might have simulated ‘early season’ conditions, with an absence of  
317 ephippial females, while our natural population sampling extended over the growing season and  
318 included periods when ephippial females were relatively common. Female age plays a role in  
319 ephippia production: a female’s early clutches are much less likely to be ephippial, although  
320 females are certainly able to produce an ephippium for their second clutch (Roulin *et al.* 2015).  
321 There may be energetic constraints on females’ ability to produce an ephippium (Lynch 1983), an  
322 idea further supported by our finding that ephippia production was more likely in females that had  
323 produced relatively large asexual clutches, an indicator of good condition (Tessier and Goulden  
324 1982; Ebert and Yampolsky 1992). Costs of ephippia production could thus contribute to the sex  
325 ratio adjustments we observed. If other individuals are reproducing sexually, a female who cannot  
326 afford to produce an ephippium can participate by asexually producing males.

327 Alternatively, it is possible that the results in natural populations were driven by density, while our  
328 experimental results aimed to disentangle male and female effects and thus included strongly male-  
329 biased sex ratio treatments. As sex ratio was strongly correlated with density in natural populations,  
330 increases in relative male density coincided with increases in overall density, explaining the  
331 observed increase in male production (analogous to moving from the lower right towards the upper  
332 left edge of the surfaces in Figure 2). Additionally, our most male-biased experimental populations  
333 had ASRs of 0.75, and 22% of experimental populations had ASRs greater than 0.5 (Table S1).  
334 While such ASR values occur in natural populations, only 188 of 1614 measured clutches (12%)  
335 came from natural population samples with  $ASR > 0.5$ , and only 17 clutches came from populations



336 with  $ASR > 0.7$ . Our experimental populations might therefore have better captured a hypothetical  
337 sex ratio ‘switch-point’ above which producing males loses value.

338 Mechanistically, it remains to be determined how females detect and differently respond to  
339 densities of females and males. In *Brachionus*, the male-female encounter rate directly determines  
340 whether daughters (fertilized eggs) or sons (unfertilized) are produced (Aparici *et al.* 1998). Apart  
341 from mating, *Daphnia* individuals show little direct interaction; however, they are sensitive to flow  
342 disturbances in the water around them. *Daphnia pulicaria* exhibit escape (females) or pursuit  
343 (males) behaviour at detection distances of around 4 mm (~3 male body-lengths; Brewer 1998).  
344 Female *Daphnia* are larger than males, and the size of individuals affects the wake they produce  
345 (their ‘footprint’, Gries *et al.* 1999). It is not known whether this allows individual perception of the  
346 sex ratio. It is also possible that females ‘count for more’ in density assessments if they use more  
347 space or take up resources faster than males. The effect of density on reproduction is possibly  
348 chemically mediated: exposure to water that has previously contained dense *Daphnia* populations  
349 increases male production (Kleiven *et al.* 1992). *Daphnia* females could conceivably produce more  
350 of the chemical cue of conspecific presence, such that a population of 10 females and 10 males  
351 would be perceived as more crowded than a population of 5 females and 15 males. Alternatively,  
352 chemicals could be sex-specific, as in copepods (Heuschele and Selander 2014), enabling more  
353 precise estimation of the presence and abundance of different types of conspecifics. Chemical sex  
354 identification appears important in the few systems in which sex ratio assessment mechanisms have  
355 been investigated (water striders, *Gerris gracilicornis*: Han *et al.* 2012; eastern red-spotted newts,  
356 *Notophthalmus viridescens*: Rohr *et al.* 2005). *Daphnia* males may be able to chemically detect the  
357 presence of sexual females among a population of asexuals (La *et al.* 2014). Although we know of  
358 no relevant chemical study, it appears plausible that females could use sex-specific olfactory cues to  
359 estimate the sex ratio. This does not preclude a role for direct physical encounters with males, who  
360 frequently attempt to cling to females.

361 Comparing the reproductive value of a son, daughter, or ephippial clutch is not straightforward. The  
362 returns on these reproductive investments are measured in different currencies and over different  
363 timescales (as sexually produced eggs hatch in later seasons). Facultative sexual *Daphnia* differ  
364 from obligate sexuals, for which sex allocation theory has been developed, in that allocation  
365 between male and female sexual function is not a ‘zero-sum’ trade-off. Increasing investment in  
366 males does not automatically reduce ephippia production, as both can increase at the expense of  
367 asexual females. Our data from natural populations show that this occurs, and additionally reveal  
368 changes in the relative frequencies of males and ephippia produced that are consistent with  
369 predictions from sex allocation theory. Our experiments support this interpretation of plastic  
370 adjustment of offspring sex by demonstrating a causal relationship between ASR and male  
371 production, which makes up one part of the three-way allocation trade-off. Offspring sex allocation  
372 in the cyclical parthenogen *D. magna* is influenced not only by factors such as population density  
373 that drive the timing of investment in sex as a whole, but also by the current population sex ratio,  
374 which adds *Daphnia* to the list of organisms (see Introduction) that can respond to temporal  
375 variations in sex ratio by adjusting the sex of offspring they produce.

#### 376 **Data accessibility**

377 All data from this study are archived in the DRYAD data repository, DOI:10.5061/dryad.sb269

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498 **Figure legends**

499

500 **Figure 1.** Relationships between the adult sex ratio (ASR) of natural populations and the likelihood  
501 a sampled female's asexual clutch was predominantly male (top), the likelihood a female produced  
502 an ephippium (middle), and the sex allocation ratio in the population (production of male-biased  
503 clutches relative to ephippia, bottom). Darker shading indicates higher density of overlapping raw  
504 data points.

505

506 **Figure 2.** Effect of increasing male and female density on the proportion of male offspring  
507 produced in the experimental populations. Although our experiments did not include populations  
508 with more than 75 males, predictions for these cases (above the dashed lines) are included to ease  
509 visual comparison of male and female slopes.

510



511 **Table captions**

512

513 **Table 1.** Effects of log population density, adult sex ratio (ASR) and log clutch size on the  
514 likelihood an asexual clutch was male-biased ( $N = 1614$ ) in females collected from natural  
515 populations; estimated by binomial GLMM with logit link.

516

517 **Table 2.** Effects of log population density, adult sex ratio (ASR), previous clutch size and sex, and  
518 relative previous clutch size on the likelihood a female produced an ephippium ( $N = 1356$ ) in  
519 females collected from natural populations; estimated by binomial GLMM with logit link.

520

521 **Table 3.** Effects of log population density and adult sex ratio (ASR) on the production of male  
522 clutches relative to ephippia ( $N = 185$ ) in natural populations; estimated by binomial GLMM with  
523 logit link.

524

525 **Table 4.** Comparison of candidate models predicting the likelihood of producing males in  
526 populations manipulating the density and sex ratio of founding adults.

527

528 **Table 1.** Effects of log population density, adult sex ratio (ASR) and log clutch size on the  
 529 likelihood an asexual clutch was male-biased ( $N = 1614$ ) in females collected from natural  
 530 populations; estimated by binomial GLMM with logit link.

Fixed effects:	$\beta$	SE	z	p
(Intercept)	-3.821	0.423	-9.04	< 0.001
Log density	0.247	0.061	4.03	< 0.001
ASR	0.879	0.413	2.13	0.033
Log clutch size	0.467	0.099	4.70	< 0.001
Random effects:	SD			
Population ID	0.474			
Individual ID	0.00002			

531

532

533 **Table 2.** Effects of log population density, adult sex ratio (ASR), previous clutch size and sex, and  
 534 relative previous clutch size on the likelihood a female produced an ephippium ( $N = 1356$ ) in  
 535 females collected from natural populations; estimated by binomial GLMM with logit link.

Likelihood of individual female ephippia production				
Fixed effects:	$\beta$	SE	z	p
(Intercept)	-1.685	1.397	-1.21	0.228
Log density	0.106	0.236	0.45	0.656
ASR	1.436	0.554	2.59	0.010
Log asexual clutch size	-2.235	0.685	-3.26	0.001
Relative clutch size *	0.625	0.290	2.16	0.031
Asexual clutch sex (0=female, 1=male)	0.809	0.188	4.31	< 0.001
Log density $\times$ log previous clutch size	0.289	0.114	2.54	0.011
Log previous clutch size $\times$ relative clutch size	-0.278	0.131	-2.12	0.034
Random effects:				
	SD			
Population ID	0.694			

536 \* **Relative clutch size** is the clutch size standardized within a sample, i.e. relative to the mean  
 537 clutch size for females collected from the same population at the same sampling point.

538

539 **Table 3.** Effects of log population density and adult sex ratio (ASR) on the production of male  
540 clutches relative to ephippia ( $N = 185$ ) in natural populations; estimated by binomial GLMM with  
541 logit link.

Fixed effects:	$\beta$	SE	z	p
(Intercept)	5.952	1.343	4.43	< 0.001
Log density	-0.831	0.272	-3.06	0.002
ASR	-3.432	1.747	-1.97	0.049
Random effects:	SD			
Population ID	0.593			
Individual ID	3.102			

542

543

544 **Table 4.** Comparison of candidate models predicting the likelihood of producing males in  
 545 populations manipulating the density and sex ratio of founding adults.

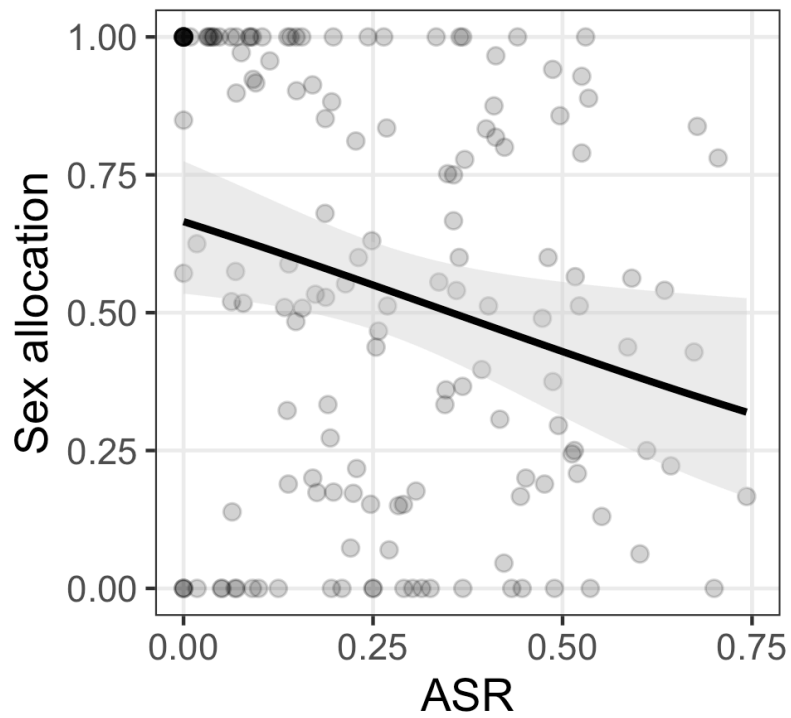
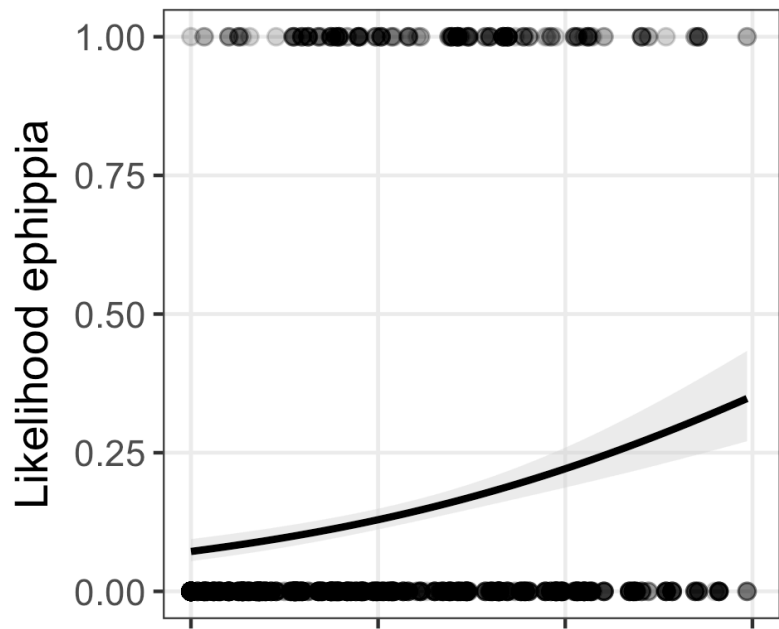
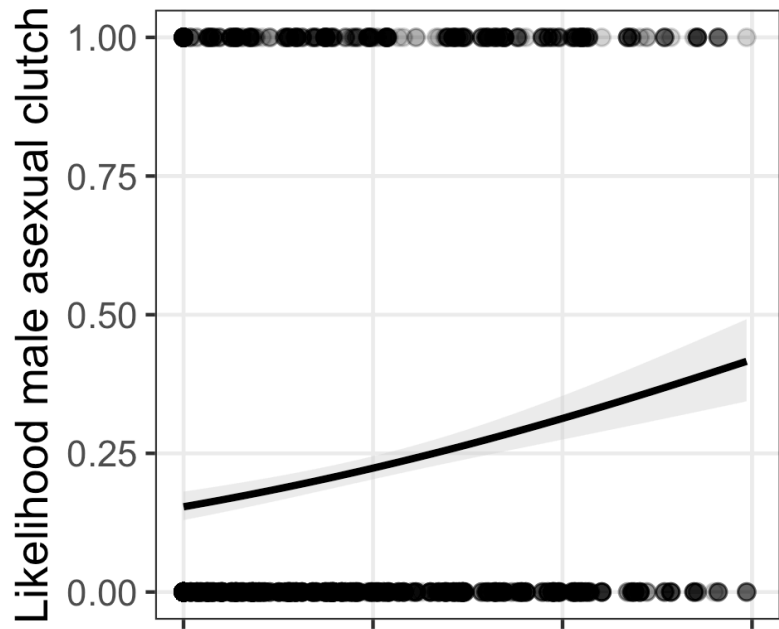
Model predictors	AIC *	$\Delta_i$ †	Estimated parameters ‡
Female density, male density experiments separate	45521	0	$-2.023 + 0.013*D_{f1} + 0.007*D_{m1} + 0.013*D_{f2} +$ $0.005*D_{m2} + 0.010*D_{f3} + 0.005*D_{m3}$
Female density, male density experiments combined	45542	21	$-1.968 + 0.012*D_f + 0.006*D_m$
Total density experiments separate	45562	41	$-1.858 + 0.009*D_{(f+m)1} + 0.006*D_{(f+m)2} + 0.006*D_{(f+m)3}$
Total density experiments combined	45591	70	$-1.833 + 0.008*D_{(f+m)}$
Null (intercept only) experiments separate	45724	203	$-1.197 + D_{(f+m)1} + D_{(f+m)2} + D_{(f+m)3}$
Null (intercept only) experiments combined	45774	253	$-1.197 + D_{(f+m)}$

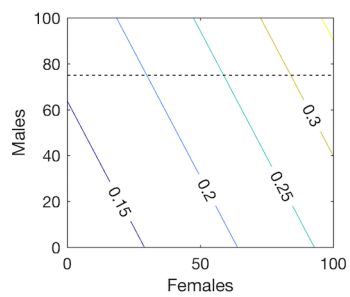
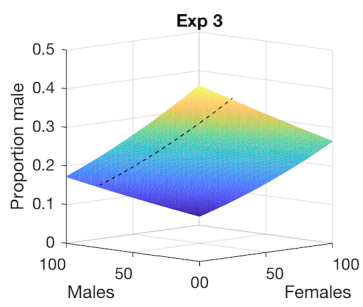
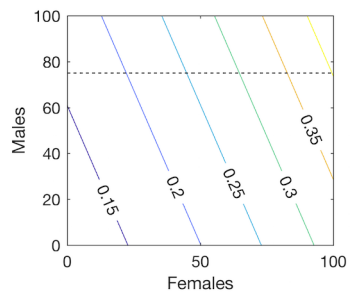
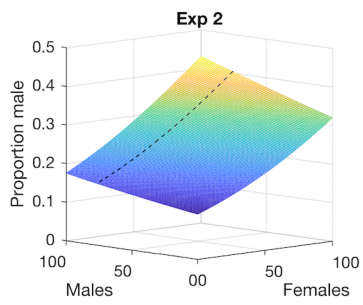
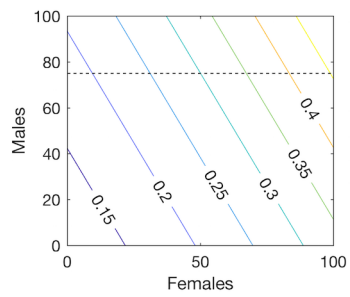
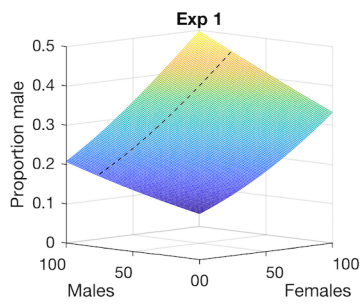
546 \* AIC, Akaike Information Criterion.

547 †  $\Delta_i$ , difference in AIC score from the lowest score.

548 ‡ D, density; subscripts *f* and *m* indicate female and male densities, respectively; subscript numerals  
 549 indicate the experimental block.

550





**Table S1.** Details of the population density and sex ratio manipulations used in three experiments.

Experiment	Manipulation	Treatment level details:				N *
		Female density	Male density	Total density	Sex ratio (% male)	
Exp1 (July 2015)	Constant density, varying sex ratio	50	0	50	0	10
		37	13	50	0.26	10
		25	25	50	0.5	10
		13	37	50	0.74	10
	Covarying density and sex ratio	50	0	50	0	8
		50	10	60	0.17	8
		50	25	75	0.33	8
		50	50	100	0.5	8
Exp2 (June 2016)	Constant density, varying sex ratio	50	0	50	0	8
		37	13	50	0.26	8
		25	25	50	0.5	8
		13	37	50	0.74	8
	Covarying density and sex ratio	25	0	25	0	8
		25	13	38	0.34	8
		25	75	100	0.75	8
Exp3 (July 2016)	Constant low density, varying sex ratio	25	0	25	0	8
		18	7	25	0.28	8
		12	12	24	0.5	8
		7	18	25	0.72	8
	Constant high density, varying sex ratio	100	0	100	0	8
		75	25	100	0.25	8
		50	50	100	0.5	8
		25	75	100	0.75	8

\* N: number of replicate populations.