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LETTER

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

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Abstract

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

Keywords

Cyclical parthenogen, *Daphnia magna*, population density, sex allocation, sex ratio adjustment.

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INTRODUCTION

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

Werren & Charnov's (1978) models were built on specific scenarios of seasonal variation or unusual perturbations to sex-specific fitness expectations. Although the theory lacks extensive further development (West 2009), the idea of facultative sex ratio adjustment in response to population sex ratio remains popular in the empirical literature. Observational data provide mixed support (positive: lions, *Panthera leo*: Creel & Creel 1997; snow skinks, *Niveoscincus microlepidotus*: Olsson & Shine 2001; northern goshawks, *Accipiter gentilis*: Byholm *et al*. 2002; negative: reed warblers, *Acrocephalus arundinaceus*: Bensch *et al*. 1999; gray-tailed voles, *Microtus canicaudus*: Bond *et al*. 2003). Robust experiments manipulating sex ratio while controlling for other potential cues are rare, although Southern green stink bugs, *Nezara viridula* (McLain & Marsh 1990), parasitic mites, *Hemisarcoptes coccophagus* (Izraylevitch & Gerson 1996), a perennial herb, *Begonia gracilis* (Lopez & Dominguez 2003) and southern water-skinks, *Eulamprus tympanum* (Robert *et al*. 2003) have been shown to produce male-biased offspring sex ratios when kept in female-biased populations, and *vice versa*. However, equally many experiments have failed to find the expected pattern (guppies, *Poecilia reticulata*: Brown 1982; common lizards, *Lacerta vivipara*: Le Galliard *et al*. 2005; southern water-skinks, *E. tympanum*: Allsop *et al*. 2006; jacky dragons, *Amphibolurus muricatus*: Warner & Shine 2007).

Systems with unusual sex ratio dynamics may be useful in identifying general patterns and furthering understanding of when the sex ratio affects sex allocation. Cyclical parthenogenesis describes a lifecycle where females typically produce

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daughters asexually, but engage occasionally in (often environmentally induced) male production and subsequent sexual reproduction (Bell 1982). This creates conditions for plastic adjustment of offspring sex, as producing males can in principle range from completely unprofitable when all females opt for asexuality, to highly profitable when many or all females are sexual. Cyclical parthenogenesis has several consequences for sex allocation theory. First, because daughters' reproductive value is not as tightly bound by frequency-dependence as in organisms where every individual has both a mother and father, cyclical parthenogens can show extremely female-biased sex ratios. Second, reproductive decisions in cyclical parthenogens concern not only the sex of offspring, but also whether and when to reproduce sexually. Fitness consequences of these decisions are intertwined: male production makes little sense unless there are sexually reproducing females in the population. In addition, sex can entail a range of genetic, demographic and ecological costs and benefits compared to asexual reproduction (Halkett *et al.* 2006; Paland & Lynch 2006; Auld *et al.* 2016), and sexually produced young sometimes face a different developmental fate: for example, in *Daphnia* only sexually produced offspring undergo dormancy before hatching. Finally, additional complexity arises when only some individuals switch to sex, while others continue asexual reproduction. The co-occurrence of asexually- and sexually reproducing generations may make it difficult for females to measure the current sex ratio and base reproductive decisions on it when offspring fitness is realised later.

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

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

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

METHODS

Natural populations

Data on offspring sex ratios of individual females was collected during a study of the timing of sex in natural *Daphnia magna* populations (Gerber *et al.* 2018). We sampled 11 populations inhabiting separate rock pools distributed over six islands in the Finnish archipelago at Tvärminne Zoological Station (59.8420 °N, 23.2018 °E) over 2 months during the summer growing season of 2015. These rock pools are small, with surface area of less than 10 m^2, but have *Daphnia* populations of several thousand individuals. Every 3 to 4 days we recorded the density and demographic structure of every population (14–18 sampling events/population). To estimate population density, 350-mL samples were collected at 15 haphazardly chosen locations spanning the pool area and depth. These were combined and stirred to distribute individuals evenly, and a 350-mL subsample was taken as the final density sample. The remaining animals were returned to the rock pool. After collecting the density sample, a small hand net was swept through the pond to take a representative population sample. All *D. magna* individuals in the density sample were counted under a dissecting microscope and converted to an estimate of individuals/*l*. Population samples were categorised by age and reproductive status: juvenile males and females, adult males and adult (reproductively mature) females, which were further classified as sexually reproducing (carrying an ephippium, the melanised capsule into which the fertilised resting eggs are deposited) or not (asexually

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reproducing and non-reproductive). Up to 10 females (where possible; median = 10, mean ± SE = 8.72 ± 0.17) with an asexual clutch visible in the brood pouch were then isolated from the sample and maintained individually in 35 mL jars until they released their clutch. Clutch size and offspring sex were determined under a dissecting microscope, and we recorded whether the mother formed an ephippium for her next instar, visible by a darkening and change in shape of the female brood pouch.

Experimental populations

Population sex ratio and density were manipulated in three separate experiments, in July 2015, June 2016 and July 2016. In July 2015, stocks of 20 *D. magna* clones that had been previously collected from the study population, treated with antibiotics to clear microsporidian infections, and maintained in the laboratory for a year (see Roulin *et al.* 2015) were established in 9-L buckets (one clone per bucket; 10–20 founding individuals per clone) outside, near natural rock pools containing *Daphnia*, so they were exposed to the natural climate and weather conditions. Buckets were filled with 8 L water from a rock pool not used in our observational study, in which no *Daphnia* were detected during the study period. The water was filtered through 48-μm mesh to avoid possible contamination with *Daphnia*, other large plankton or predators. Algae small enough to pass through the filter were allowed to grow and provided a food source for the populations. Each bucket was additionally inoculated with 20 mL of algae suspension (50 million *Scenedesmus* cells/mL) and left to stand for several days before adding *Daphnia*. Filtered water from the same source pool was added to all buckets on two occasions during stock growth to compensate for evaporation.

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

Populations were monitored for maturation of the founding females and the presence of neonates. After 2 weeks, roughly two juvenile cohorts were apparent in the populations (two size classes of neonates). Each entire population was collected and the number and sex of juveniles determined. The experimental period was kept deliberately short to ensure that the offspring sex ratios we recorded were produced under the manipulated density and sex ratio conditions, as newborn sons and daughters will quickly alter the population structure. The experiment was not designed to address allocation to ephippia production, as the short timeframe and use of newly matured females (ensuring similar reproductive history across populations) made ephippia production unlikely. As expected, no ephippia were produced during the experiment.

Statistical analysis

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

Analyses of the experimental populations were performed in MATLAB. We compared a set of candidate logistic regression models predicting offspring sex, based on model AIC scores. In addition to a 'null' model (intercept-only; neither density nor sex ratio was allowed to predict the proportion of males produced), we built models in which the total density of founders was included as a predictor, and models in which the densities of male and female founders were included as separate predictors that could independently affect the production of males. In each case, we also considered a model variant where estimates from the three experiments were allowed to vary in their effects.

RESULTS

Natural populations

The mean clutch size among asexually reproducing *D. magna* females sampled from natural rock pool populations was 11.97 ± 0.26 offspring ($N = 1614$; range: 1–116 offspring).

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Clutch sizes were smaller at higher population densities (LMM: -0.030 ± 0.013, $X^2 = 5.69$, $p = 0.017$). Asexual clutches are predominantly single-sex (Barker & Hebert 1986; 91.6% of clutches in our data) and mixed-sex clutches are usually strongly biased towards one sex. For the following analyses, we present results in which clutches were assigned their majority sex, ignoring sons produced in majority female clutches. However, results of all analyses were qualitatively extremely similar (no changes of sign or significance) if we used 'at least one male' as the criterion for male production.

As population densities increased, the adult sex ratio (ASR, adult males:adult females) became more male-biased (GLMM: 0.646 ± 0.079, $z = 8.19$, $P < 0.001$). The overall mean probability that an asexual female produced a male-biased clutch was 0.22 ± 0.01. Clutches were more likely to be male-biased when they were larger, when sampled from higher density populations, and when the ASR was more male-biased (Table 1, Fig. 1). The likelihood that a female that released an asexual clutch subsequently produced an ephippium (mean ± SE probability: 0.14 ± 0.01) increased when the ASR was more male-biased (Fig. 1), when the female's clutch was male-biased (Table 2) and when the female's clutch was small in absolute terms (Table 2). However, the size of a female's asexual clutch relative to others in the same population sample had a positive effect on ephippia production: females that produced relatively large clutches were more likely to subsequently produce an ephippium. When accounting for these effects, the effect of density on ephippia production was not significant (Table 2), but excluding them for comparison with previous studies, the effect of density alone was significantly positive (GLMM: 0.740 ± 0.091, $z = 8.09$, $P < 0.001$).

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

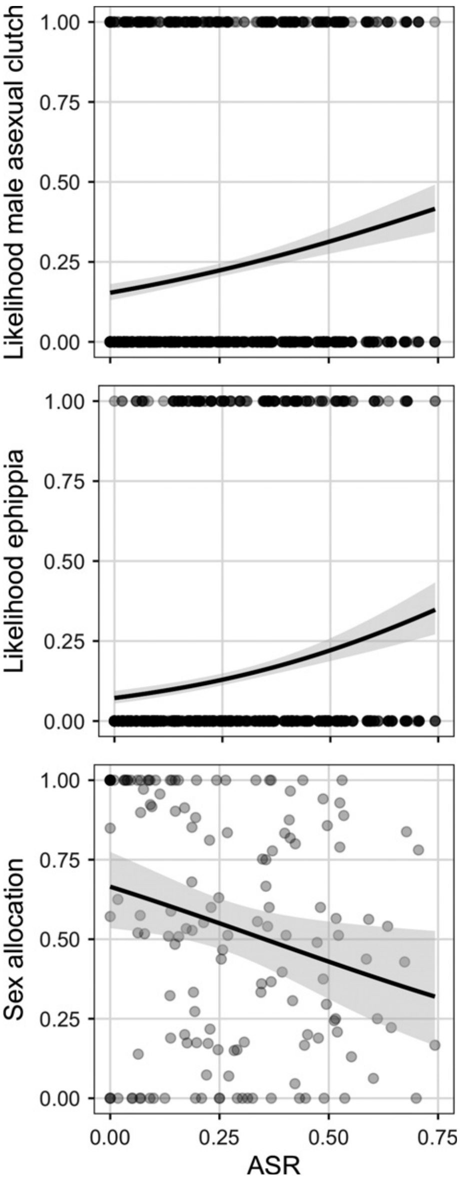

Figure 1 Relationships between the adult sex ratio (ASR) of natural populations and the likelihood a sampled female's asexual clutch was predominantly male (top), the likelihood a female produced an ephippium (middle) and the sex allocation ratio in the population (production of male-biased clutches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

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

Fixed effects	ß	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

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

Likelihood of individual female ephippia production				
Fixed effects	ß	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 × log previous clutch size	0.289	0.114	2.54	0.011
Log previous clutch size × relative clutch size	−0.278	0.131	−2.12	0.034
Random effects				SD
Population ID				0.694

*Relative clutch size is the clutch size standardised within a sample, that is, relative to the mean clutch size for females collected from the same population at the same sampling point.

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

Fixed effects	ß	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

allocation ratio was approximately even (0.18: 0.17 = sex allocation ratio of 0.51).

Experimental populations

To test the effects of population density and ASR on sex ratio adjustment among asexually produced offspring, we quantified the proportion of sons among all offspring produced after 2 weeks (*c*. 2 clutches). Model selection based on AIC scores identified an unambiguous best model: that in which male and female density independently affected the proportion of sons produced, and in which their effects were allowed to vary between the three experiments (Table 4). Despite the best model estimating the effects of male and female density separately for each experiment, these effects showed a remarkably consistent pattern across the three experiments: increasing female density always predicted a greater increase in the likelihood of producing sons than did increasing male density (Fig. 2: isoclines are steeper when female than when male density changes). In experiment 1, adding one male was equivalent to adding 0.51 females in terms of the sex allocation response; in experiments 2 and 3, the corresponding numbers are 0.37 and 0.45.

DISCUSSION

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

In natural populations, a link between high ASR and production of sons is supported at first sight, as females from more male-biased populations were more likely to produce sons than were females from female-biased populations (Table 1, Fig. 1). Our experiments showed that females

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

Model predictors	AIC*	Δ_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	$-1.197 + D_{(f+m)}$

*AIC, Akaike Information Criterion.

†Δ_i, difference in AIC score from the lowest score.

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

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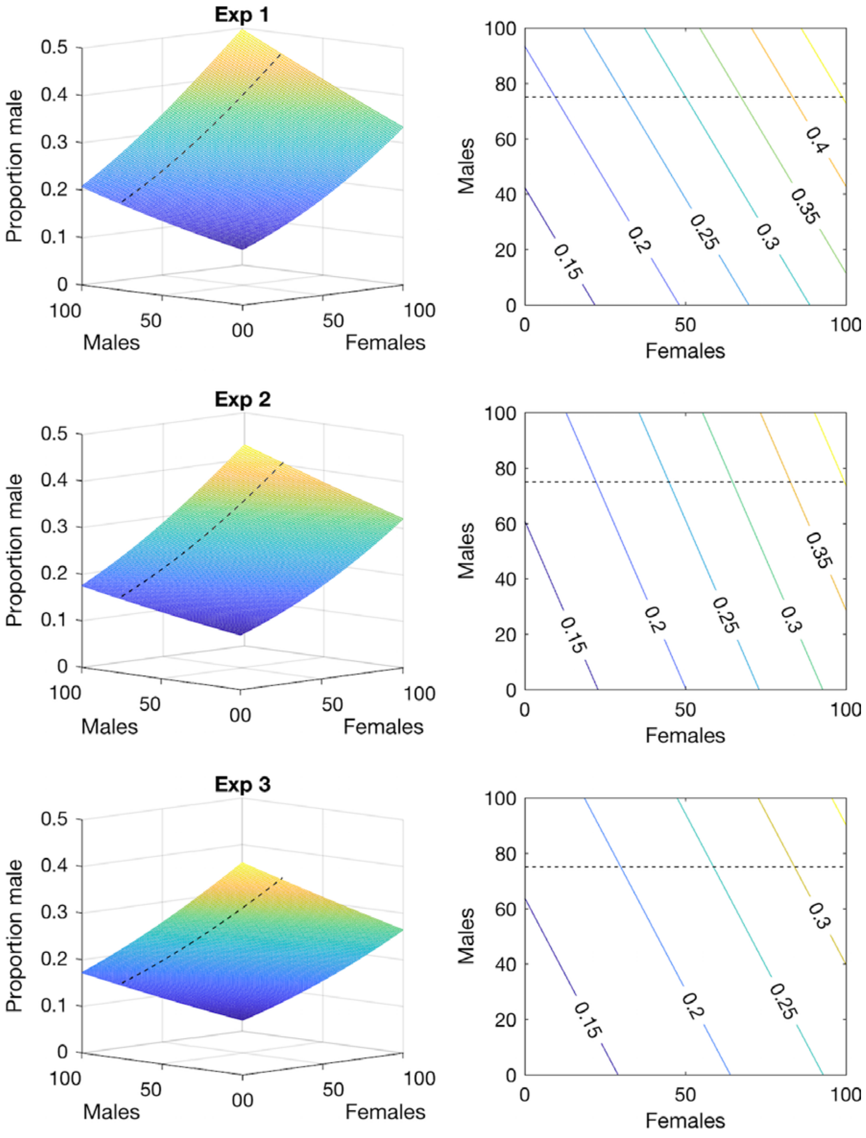

Figure 2 Effect of increasing male and female density on the proportion of male offspring produced in the experimental populations. Although our experiments did not include populations with more than 75 males, predictions for these cases (above the dashed lines) are included to ease visual comparison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

respond to male presence in a remarkably consistent manner: increasing density by adding males led to increased production of sons, but only by 37–51% of the increase observed if the additional density consisted of females. This suggests that both factors play a role: females produced more sons when densities were high, with a dampened response if the ASR was already high.

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

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

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The only other cyclical parthenogens in which sex allocation has been investigated are the monogonont rotifer genus *Brachionus*, haplodiploids in which sexually- and asexually reproducing individuals are produced in distinct generations (Aparici *et al*. 2002). In *Brachionus*, the trait underlying sex allocation is the threshold age at which females lose the capacity to be fertilised. Females that reach this age unfertilised produce only males; increasing male frequency increases fertilisation rates, thereby reducing male production in a negative frequency-dependent process. Furthermore, earlier fertilisation thresholds increase the likelihood a female's lifetime sex allocation will be male-biased (Aparici *et al*. 1998). While there is no such direct effect of male frequency on *Daphnia* sex ratio adjustment, there are similarities: increasing densities induce sexual generations in *Brachionus* (Serra & Snell 2009) and production of males and ephippia in *Daphnia* (Carvalho & Hughes 1983). Models of the rotifer system find the threshold fertilisation age is evolutionarily stable at the point where, for a given density, it results in equal production of males and sexual eggs – that is, even sex allocation (Aparici *et al*. 1998; Serra *et al*. 2008). Data from field and laboratory populations match this prediction (Aparici *et al*. 2002). For *Daphnia*, where there is no unique temporal threshold, it is difficult to place as much significance on the roughly equal sex allocation we observed, as theory does not single out this value as the sole prediction under plastic sex allocation in general (Frank 1990) or for *Daphnia* specifically (N. Gerber, I. Booksmythe, H. Kokko, unpublished).

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

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

Alternatively, it is possible that the results in natural populations were driven by density, while our experimental results aimed to disentangle male and female effects and thus included strongly male-biased sex ratio treatments. As sex ratio was strongly correlated with density in natural populations, increases in relative male density coincided with increases in overall density, explaining the observed increase in male production (analogous to moving from the lower right towards the upper left edge of the surfaces in Fig. 2). Additionally, our most male-biased experimental populations had ASRs of 0.75, and 22% of experimental populations had ASRs greater than 0.5 (Table S1). While such ASR values occur in natural populations, only 188 of 1614 measured clutches (12%) came from natural population samples with ASR > 0.5, and only 17 clutches came from populations with ASR > 0.7. Our experimental populations might therefore have better captured a hypothetical sex ratio 'switch-point' above which producing males loses value.

Mechanistically, it remains to be determined how females detect and differently respond to densities of females and males. In *Brachionus*, the male-female encounter rate directly determines whether daughters (fertilised eggs) or sons (unfertilised) are produced (Aparici *et al*. 1998). Apart from mating, *Daphnia* individuals show little direct interaction; however, they are sensitive to flow disturbances in the water around them. *Daphnia pulicaria* exhibit escape (females) or pursuit (males) behaviour at detection distances of around 4 mm (*c*. 3 male body-lengths; Brewer 1998). Female *Daphnia* are larger than males, and the size of individuals affects the wake they produce (their 'footprint', Gries *et al*. 1999). It is not known whether this allows individual perception of the sex ratio. It is also possible that females 'count for more' in density assessments if they use more space or take up resources faster than males. The effect of density on reproduction is possibly chemically mediated: exposure to water that has previously contained dense *Daphnia* populations increases male production (Kleiven *et al*. 1992). *Daphnia* females could conceivably produce more of the chemical cue of conspecific presence, such that a population of 10 females and 10 males would be perceived as more crowded than a population of 5 females and 15 males. Alternatively, chemicals could be sex-specific, as in copepods (Heuschele & Selander 2014), enabling more precise estimation of the presence and abundance of different types of conspecifics. Chemical sex identification appears important in the few systems in which sex ratio assessment mechanisms have been investigated (water striders, *Gerris gracilicornis*: Han *et al*. 2012; eastern red-spotted newts, *Notophthalmus viridescens*: Rohr *et al*. 2005). *Daphnia* males may be able to

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chemically detect the presence of sexual females among a population of asexuals (La *et al.* 2014). Although we know of no relevant chemical study, it appears plausible that females could use sex-specific olfactory cues to estimate the sex ratio. This does not preclude a role for direct physical encounters with males, who frequently attempt to cling to females.

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

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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.

DATA ACCESSIBILITY STATEMENT

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

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