

The costs and benefits of multiple mating in a mostly monandrous wasp

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The taxonomically widespread nature of polyandry remains a puzzle. Much of the empirical work regarding the costs and benefits of multiple mating to females has, for obvious reasons, relied on species that are already highly polyandrous. However, this makes it difficult to separate the processes that maintain the current level of polyandry from the processes that facilitate its expression and initiated its evolution. Here we consider the costs and benefits of polyandry in *Nasonia vitripennis*, a species of parasitoid wasp that is “mostly monandrous” in the wild, but which evolves polyandry under laboratory culture conditions. In a series of six experiments, we show that females gain a direct fecundity and longevity benefit from mating multiply with virgin males. Conversely, mating multiply with previously mated males actually results in a fecundity cost. Sexual harassment may also represent a significant cost of reproduction. Harassment was, however, only costly during oviposition, resulting in reduced fecundity, longevity, and disrupted sex allocation. Our results show that ecological changes, in our case associated with differences in the local mating structure in the laboratory can alter the costs and benefits of mating and harassment and potentially lead to shifts in mating patterns.

KEY WORDS: Local mate competition, polyandry, sexual selection, sex allocation.

Explaining the near ubiquity of female multiple mating across multiple partners (polyandry) remains a key problem in behavioral and evolutionary ecology (Parker and Birkhead 2013; Pizzari and Wedell 2013; Boulton and Shuker 2013; Snook 2014). Historically the focus was on females as a limiting resource over which males competed, with males maximizing their reproductive success by increasing their mating frequency (Bateman 1948). Until recently, Bateman’s principle was interpreted to suggest that females do not benefit from mating multiply (Hosken and Stockley 2003). Over the past two decades however it has become increasingly apparent that females can and do benefit from mating with multiple males and that polyandry is widespread (Arnqvist and Nilsson 2000; Taylor et al. 2014). The resulting “polyandry revolution” (Pizzari and Wedell 2013) has led to renewed interest in the evolution of polyandry, in particular why females mate multiply and what the ecological and evolutionary consequences of polyandry might be.

Many evolutionary explanations for polyandry have been proposed, from infanticide avoidance through to the direct

benefits females gain from ejaculate components (Boulton and Shuker, 2013). Direct benefits of polyandry emphasize what females can gain from re-mating immediately. These range from obtaining sufficient sperm for reproduction (Elzinga et al. 2011), through to using other ejaculate components to enhance reproductive success (e.g., water: in the seed beetle *Callosobruchus maculatus*: Paukku and Kotiaho 2005; proteins: in *Plodia interpunctella*: Greenfield 1982; see also Avila et al. 2011). Overall, in the insects at least, direct benefits alone appear to be sufficient to explain the maintenance of polyandry (Arnqvist and Nilsson 2000; South and Lewis 2011).

However, genetic benefits can also make additional matings profitable (Shuker 2014). First, mating with multiple males may be advantageous if females increase the chances that they mate with at least one male with whom they can produce viable offspring (i.e., with whom they are genetically compatible; Zeh and Zeh 1996, 1997; Tregenza and Wedell 1998, 2000). Second, if certain males are of a high genetic quality (i.e., they have “good genes”), females may benefit from mating multiply if by doing



so they produce offspring of similar high quality (reviewed by Andersson 1994; Kuijper et al. 2012; Hunt and Sakaluk 2014). The third possible indirect benefit arises from the sperm competition that females induce when they mate multiply (Parker 1970; Simmons 2001). The “sexy sperm” hypothesis suggests that males with the best or “sexiest” sperm will attain the highest insemination success. If sperm quality is heritable to some extent, a polyandrous female’s sons will inherit “sexy sperm” from their fathers (Fisher 1930; Parker 1970; Sivinski 1984; Curtsinger 1991; Yasui 1998; Simmons & Kotiaho, 2002). Of course, these indirect benefits are not mutually exclusive, and may be difficult in practice to separate, for instance if high-quality males produce high-quality ejaculates (Jennions and Petrie 2000).

Convenience polyandry differs from the benefit-driven explanations for multiple mating in that it emphasizes how females may mate at a rate higher than their intrinsic optima if by doing so they can mitigate the costs of rejecting superfluous matings (Thornhill and Alcock 1983; Arnqvist and Nilsson 2000; Rowe and Arnqvist 2002). For instance at high male densities, females of the water strider species *Aquarius remigis* experience a high level of harassment. Females that are less reluctant to mate when male density was high suffered reduced costs, by reducing energy expenditure in costly precopulatory struggles, increasing foraging time, and reducing predation risk (Rowe et al. 1994; Fairbairn 1991).

Much of the empirical work exploring the evolutionary causes and consequences of polyandry has relied on species that are already highly polyandrous (often for sensible logistical reasons). Furthermore, the ecological context under which we consider polyandry may have a significant influence on the economics of mating, but this has rarely been considered outside the convenience polyandry literature (but see Toft and Albo 2015). Much of what we know has come from testing potential processes of selection maintaining polyandry under controlled laboratory conditions, which may have limited bearing on female fitness in the conditions under which polyandry evolved (Cordero and Eberhard 2003). Moreover, the explanations underlying the maintenance of polyandry may be very different from why polyandry was initially beneficial; for instance, only when polyandry has been established will there be strong selection on “sexy sperm” through sperm competition.

Here we consider the costs and benefits of polyandry in *Nasonia vitripennis*, a species of parasitoid wasp that is “mostly monandrous” in the wild, but that evolves polyandry under laboratory conditions (Burton-Chellew et al. 2007; Grillenberger et al. 2008; van den Assem and Jachmann 1999). Ridley (1993) found that monandry occurred far more often in the parasitoids than in other groups, with 70% of species having females that (apparently) only mate once. *Nasonia vitripennis* is a gregarious parasitoid, and females often oviposit alone on their dipteran

hosts, such that sibling mating is common (Godfray 1994). Although inbreeding has no deleterious effects, it does lead to local mate competition (LMC), which selects for female-biased sex ratios that maximize grand-offspring production through reduced fraternal competition over mates (which are sisters when LMC is high). Under laboratory culture conditions local mating patches are broken down, outbreeding dominates, and reduced LMC results in sex ratios approaching 50:50. Sex allocation under LMC has the potential to shape the mating system considerably. In the typically female-biased conditions of local mating patches in the wild, the encounter rate between males and females will be low and monandry might be reflective of limited mating opportunities (Kokko and Mappes 2013).

We performed six experiments to assess some of the direct and indirect costs and benefits of mating and male harassment in female *N. vitripennis*. The first experiment examined whether there is a direct fecundity benefit from mating multiply. If polyandry is favored by direct benefits, then we predict that polyandrous females will have increased fecundity compared to monandrous females. In the first experiment, all males were virgins. In our second experiment, we compared the costs and benefits of mating when females had access to virgins versus nonvirgin males. Together these two experiments test whether any benefits of polyandry are context-dependent, that is, whether they depend on male mating status. *Nasonia* males are prospermatogenic and males emerge with their full complement of sperm, and so one prediction is that previously mated males transfer less sperm. If this is the case then mating with a nonvirgin versus a virgin male will result in reduced daughter production. In our third experiment, we explore the potential for indirect benefits via genetic compatibility. Following the methodology of Tregenza and Wedell (1998), we allowed females to mate twice to the same male or once each to two different males (see Slatyer et al. 2012). Under the genetic compatibility hypothesis, we predict that truly polyandrous females (two different males) will produce more viable offspring, than multiply mated females (two matings to the same male). In our fourth experiment, we examine the costs of male harassment under the mass mating conditions observed in our stock populations, focusing in particular on the potential for mating failure. Mating failure may arise through repeatedly disrupted courtship (van den Assem and Visser 1976); as *Nasonia* is protandrous (males emerge before females; Moynihan and Shuker 2011) we may expect that early-emerging females will be subject to the greatest harassment and interrupted matings, resulting in a higher proportion of mating failures. In our fifth and sixth experiments, we investigate the costs of male mating and harassment under mass conditions in more detail, manipulating access to males and testing for longevity costs (experiment V) and fecundity costs (experiment VI), when males either were and were not present during subsequent oviposition. In the wild, females will rarely

experience harassment during oviposition because males, unlike females, are unable to disperse away from the natal patch. We predict that harassment will be less costly when females are only exposed to males before oviposition.

Methods

STUDY SPECIES

Nasonia vitripennis (Chalcidoidea: Pteromalidae) is a gregarious idiobiont parasitoid that attacks dipteran pupae (Whiting, 1967). As with all Hymenoptera, *N. vitripennis* is haplodiploid and so inseminated females can produce both haploid sons (from unfertilized eggs) and diploid daughters (from fertilized eggs), while uninseminated females can only produce haploid sons. Females allocate sex according to the predictions of LMC theory (Hamilton 1967; Werren 1980, 1983; Shuker and West 2004; Shuker et al. 2005; Burton-Chellew et al. 2008). Sex allocation studies have generated a wealth of data regarding reproductive behaviour in *N. vitripennis*. For instance, Grillenberger et al. (2008) found, using microsatellite data, that around 4% of females mated multiply in the wild. However, polyandry is heritable in *Nasonia* (Shuker et al. 2007), and evidence from two studies suggests that polyandry evolves under laboratory culture conditions (Van den Assem and Jachmann 1999; Burton-Chellew et al. 2007).

In the wild, females typically lay mostly daughters due to LMC and infrequent superparasitism (when multiple females parasitize a single host sex ratios are less biased). In the wild, mating (typically between siblings) occurs on the natal patch and males exhibit pre and postcopulatory courtship, the latter of which serves to reduce female receptivity to further matings (van den Assem and Visser 1976). After mating, the females disperse from the natal patch to find hosts, but the brachypterous males are philopatric; as such, females will rarely experience any harassment by males during oviposition (King 1993), unless there are over-lapping host generations in close spatial proximity (around large vertebrate carcasses, animal-rearing and -processing plants, or refuse dumps: Werren 1983). Conditions in the laboratory differ greatly from those typical in the wild. In our laboratory (and representative of other *Nasonia* research groups), typically around 40 females are provided with hosts together, and males and females are maintained together for somewhere between two and four days after emergence. Synchronous female emergence results in the breakdown of local mating patches. As such, sex ratios are less female biased and the lack of dispersal means that males may be present during oviposition.

EXPERIMENTAL STRAIN

The strain of *N. vitripennis* used for all experiments was HVRx. This line was created from wild caught wasps collected from Hoge

Veluwe national park in the Netherlands and is maintained as a large outbred population (by mixing stock tubes containing pupae each generation). HVRx was derived from five lines collected in 2001 (van de Zande et al. 2014).

STANDARD LABORATORY CULTURE

Six replicate stock tubes of each line were maintained by transferring 40 females (per replicate) and several males from the tube that the wasps emerged from into a fresh stock tube (23 mL, 75 × 23.5 mm) 48 h after emergence. Forty *Calliphora vicina* pupae were put into each tube as hosts for females to oviposit on. After seven days the hosts were removed from the tubes, mixed with the hosts from all other replicates of the same strain, and redistributed into six fresh tubes (to maintain an outbred population). The parasitized hosts were kept in an incubator at 25°C, and the next generation emerged after 2 weeks and were re-hosted again 48 h later (see van de Zande et al. 2014 for further details regarding the initiation of HVRx).

EXPERIMENT I. DIRECT BENEFITS OF RE-MATING

To investigate whether *N. vitripennis* females that re-mate gain a reproductive advantage, we measured longevity and fecundity for females that were: (1) virgin, (2) once mated, (3) twice mated, (4) three times mated. After an initial mating, females in treatments 3 and 4 were exposed to another male after 24 h (and once more 24 h later for females in treatment 4). To standardize the experimental individuals, males and females used were reared from a grandparental generation of virgins (removed from stock tubes prior to emergence; these females provided the males) and mated females (removed 48 h after emergence) that were provided with hosts. The hosts parasitized by these grandmothers were opened two days prior to emergence, and virgin males and females were isolated and maintained alone in small vials for experiments. Initially 60 females per treatment were set up, but due to refusals (see next), accidental escapes, and deaths sample sizes range from 16 to 46.

To increase the likelihood that females would re-mate, postcopulatory courtship was prevented by moving the male away using a paintbrush after he had finished inseminating the female. Despite this, females did not always accept all mates that they were offered. Females that refused a mating were recorded and provided with hosts to rule out the possibility that naturally more fecund females were more likely to re-mate (Torres-Vila et al. 2004). Six hosts were provided to all experimental females on day 2, 24 h after their final mating and then again on days 5, 8, and 10. The females were checked three times daily (at 0900 h, noon, and 1700 h) for mortality. Females that survived until the end of the experiment received a total of 24 hosts. Parasitized hosts were maintained in an incubator at 25°C and 12:12 light:dark cycle. Progeny emerged after 14 days and were counted after death.

The total number of sons and daughters was counted for each batch of hosts. Fecundity was measured as the total number of offspring and the sex ratio the proportion of males laid by each female.

EXPERIMENT II. MALE MATED STATUS

In experiment II, we tested for differences in the costs and benefits of mating with a virgin (as in experiment I) as compared to a previously mated male (as in experiment III, see next). The experimental procedure was identical to that given above for treatment (ii), except that females either mated with a virgin male or with a male who had mated to another female 24 h previously. All matings were observed and males were removed after insemination. Six hosts per female were provided on days 2, 5, and 8 and maintained as mentioned above, and progeny counted after death. Unsuccessful matings were determined by the presence of all male progeny and removed from the data set. Sample sizes were $N = 23$ (virgin males) and $N = 25$ (mated males).

EXPERIMENT III. INDIRECT BENEFITS OF POLYANDRY

To increase the chances of a genetically compatible mating (and gaining additional genetic benefits for her offspring) a female must mate not just multiple times, but with multiple males. To investigate indirect genetic benefits, we provided females with multiple mating opportunities either with the same or different males. Focal females either: (1) remained virgin, (2) mated once, (3) mated twice to the same male, or (4) mated twice to different males. As before, experimental males and females used were taken from a grandparental generation to standardize rearing effects. All males used for matings had been previously mated once to standardize male mating status across treatments. As such, males used for the second mating in treatments (3) and (4) had mated twice before. As in the previous experiment examining direct benefits, postcopulatory courtship was prevented and females that refused to mate were retained and provided with hosts. Six hosts per female were provided on days 2, 5, 8, and 10 and maintained as mentioned previously and progeny counted after death. Initially 60 females per treatment were set up, with final sample sizes ranging from 43 to 50 per treatment.

EXPERIMENT IV. COSTS OF HARASSMENT: MATING FAILURES IN MASS CULTURE CONDITIONS

To determine whether the frequency of mating failures (a failure to be successfully inseminated) in laboratory stock conditions is high enough that it may influence female re-mating, we removed experimental females from stock tubes (six replicates per strain; see standard laboratory culture above) every hour after emergence. The stock populations were monitored starting one day prior to emergence (14 days after host provision at 25°C). When females began to emerge, 12 were removed every hour (from

9:00 to 17:00) from each of the six stock tubes (i.e., two wasps from each tube). The date, time, and tube identity were recorded. This was continued for three days after first emergence (females would normally be provided with hosts two days after the emergence date). Collected females were isolated and provided with three hosts to parasitize. Females were removed from these hosts after three days. Son and daughter production was recorded. A mating failure was classed as the production of an all-male brood. A reproductive failure was noted as when the female failed to parasitize any host at all.

EXPERIMENT V. COSTS OF HARASSMENT: LONGEVITY

Harassment by males may represent a significant cost in mass culture conditions and females that re-mate may be able to offset some of these costs. To determine whether harassment by males reduces female survival we measured longevity for females that were maintained: (1) Alone, (2) with one male, (3) with five males, (4) with 10 males in large stock tubes (23 mL, 75 × 23.5 mm) at 25°C. Each replicate was provided with honey solution (on filter paper) as a food source for the first seven days of the experiment. Sample sizes ranged from 38 to 41 per treatment. Replicates were checked three times daily (0900 h, noon, and 1700 h) and both female and male mortality was recorded (any dead individuals were removed from the vial).

EXPERIMENT VI. COSTS OF HARASSMENT: FECUNDITY

Harassment by males may also directly influence female reproduction (not just through reduced survival). To assess the reproductive costs of harassment on females we placed single females in large stock tubes (23 mL, 75 × 23.5 mm) with either (1) 1 or (2) 10 virgin males. These tubes were checked twice daily and male and female mortality was recorded. If male mortality had occurred (and the focal female remained alive), the appropriate number of virgin males (maintained with brothers at 18°C) were added to the tube containing the female. After 48 h hosts were added and males were removed from half of the tubes containing only one male and half containing 10 males. There were therefore four treatments in total: (1a) females with one male for 48 h, alone during oviposition; (1b) females with one male before and during oviposition; (2a) females with 10 males for 48 h, alone during oviposition; (2b) females with 10 males before and during oviposition. Sample sizes ranged from 52 to 55 per treatment. We provided batches of six hosts on days 2, 5, and 8. Tubes were checked three times daily (0900 h, noon, and 1700 h) for female and male mortality. In the treatments where males were present during oviposition, we replaced them as necessary. As before hosts were incubated at 25°C and progeny were counted after death.

STATISTICAL ANALYSES

Cox proportional hazards regression was carried out to determine whether treatment (number of matings/males) had any effect on survival. In some cases, we lacked complete information on the longevity of individuals surviving past the end of the experiments. To account for this, these data were treated as surviving for as long as the duration of the experiment with an unknown time of death (termed “censored” data in the survival analysis literature). When we considered the survival cost of harassment (without reproduction) there was no specific experimental end point, and so in this case data were analyzed with censoring after seven days (when food provisioning stopped) and without censoring. Son and/or daughter production was analyzed using mixed models in R (package nlme, version 2.15.2). The outcome variable was always total offspring production and treatment was the between-subjects fixed factor. We also included host batch in the model as a repeated measure (nested within female identity) to control for differences in longevity (and thus the number of host batches each female had oviposited on). We used generalized linear mixed models with a binomial error structure and logit link function (using the package lme4 in R version 2.15.2) to determine whether treatment had any effect on the sex ratio (proportion of sons) produced. Female ID (nested within host batch) was again entered as a random factor to control for any differences in longevity. Including host batch in the model also allowed us to test whether females became sperm depleted over time (i.e., if they began to produce more males in later host batches). To analyze the change in frequency of mating failures (determined by production of an all-male brood) and reproductive failure (failure to parasitize any host), we coded these as dichotomous variables (success = 1, failure = 0). Generalized linear mixed models with a binomial error structure and logit link function were used to determine whether the time a female had spent in mass culture had a significant effect on the probability of reproductive failure. We also included tube identity as a fixed factor in the analysis; females were removed from one of six tubes and local conditions within the tube (for instance a sex ratio biased extremely toward males or females) could affect the frequency of failure.

Results

EXPERIMENT I. DIRECT BENEFITS OF RE-MATING

There was a significant effect of polyandry on survival (Mantel-Cox $\chi^2 = 12.61$, $df = 3$, $P = 0.006$). Females that mated twice lived significantly longer than females that remained virgin (Bonferroni corrected $P = 0.048$) or mated only once ($P = 0.002$). Polyandry led to improved fecundity independent of any survival benefit ($LR_3 = 8.53$, $P = 0.03$). Females that mated twice or three times produced approximately 10% more offspring than virgin females and those mated once. Pairwise tests (corrected for multiple

comparisons) indicated that this was only statistically significant for females that mated twice ($P = 0.02$; Fig. 1A), this may be an artifact of sample size as very few females were willing to accept a third mating ($N = 16$). The main effect of host batch ($LR_3 = 8.96$, $P = 0.03$) was also statistically significant (clutches were larger in the fourth and final batch of hosts compared to all preceding batches). Females that refused a second or third mating did not differ significantly in fecundity or longevity to females that were only offered one or two mates, respectively (both $P > 0.5$); this means that females that accepted additional matings did not have higher fecundity or longevity, regardless of treatment. These results remained the same if only daughter production was considered (i.e., eggs that were fertilized). There was no significant effect of treatment or host batch on the sex ratio (proportion of male offspring: mean = 0.15 ± 0.02 SE) such that females did not appear to run out of sperm; likewise multiple mating did not affect sperm availability (i.e., daughters: $LR_2 = 1.57$, $P = 0.73$; Fig. 1E) and daughter production did not change with successive host batches ($LR_3 = 1.26$, $P = 0.45$).

EXPERIMENT II. MALE MATED STATUS

Females that mated once to a previously mated male had significantly lower fecundity than females mated once to virgin males ($LR_1 = 5.92$, $P = 0.02$; Fig. 1B). Offspring production decreased over successive host batches ($LR_2 = 9.09$, $P = 0.01$) but this decrease occurred regardless of male mated status (Interaction effect: $LR_2 = 0.15$, $P = 0.93$). The overall sex ratio laid was constant over host batches ($LR_2 = 0.05$, $P = 0.98$) but females mated to virgin males did produce more male-biased broods over time (Interaction effect: $LR_2 = 7.78$, $P = 0.02$; Fig. 1F). When we consider lifetime sex ratio though (pooled across host batches) treatment had no effect ($LR_1 = 0.97$, $P = 0.32$) and female survival did not differ with respect to male mated status ($\chi^2 = 0.0001$, $df = 1$, $P = 0.99$).

EXPERIMENT III. INDIRECT BENEFITS OF POLYANDRY

There was no effect of re-mating (with the same or a different male) on female longevity (Mantel-Cox $\chi^2 = 5.95$, $df = 3$, $P = 0.11$). However, unexpectedly females mated twice to different males had reduced fecundity compared to females mated only once ($LR_2 = 7.81$, $P = 0.02$; Fig. 1C). These results remained the same if only daughter production was considered. Fewer offspring were laid in host batches two and four compared to one and three ($LR_3 = 301.52$, $P < 0.0001$). In terms of sex ratio, females that mated twice, either to one or two males, produced a more male-biased sex ratio than singly mated females ($LR_2 = 13.91$, $P \leq 0.001$; Fig. 1G). Females appeared to become sperm-depleted in final host batch as the sex ratio became more male biased ($LR_3 = 18.15$, $P < 0.001$) however females across all treatments

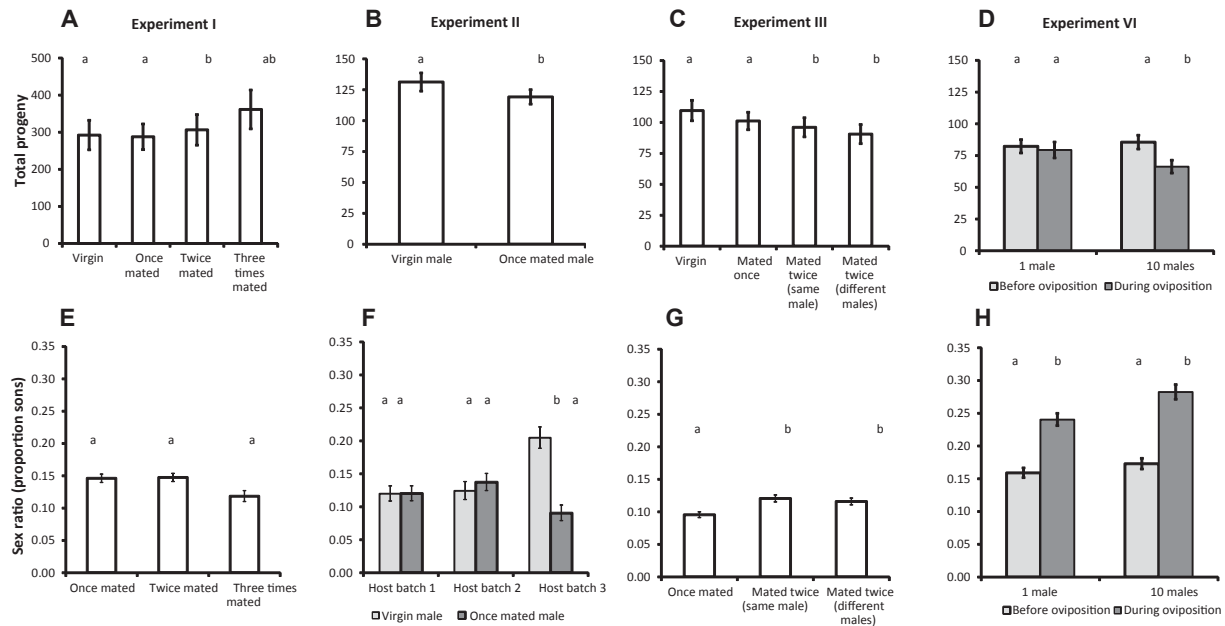


Figure 1. Total progeny production and sex ratio in experiments I, II, III, and VI. (A) Female *Nasonia vitripennis* that mated multiply with virgins males gained a fecundity benefit. (B) Females mated once to a previously mated male produced fewer offspring than those mated once to a virgin male. (C) Females of *N. vitripennis* that mated twice to different males suffered a fecundity cost. (D) Females that experienced high levels of harassment during oviposition had significantly reduced fecundity. (E) Mating multiply with virgin males had no significant effect on the sex ratio. (F) Females mated once to a virgin male laid a less female-biased sex ratio in the last host batch than those mated once to a previously mated male. (G) Females that mated twice (to the same or different males) produced a less female-biased sex ratio than once-mated females. (H) Females that were harassed by 1 or 10 males during oviposition laid a less female-biased sex ratio than females that were not harassed during oviposition. Error bars represent 95% CIs (A–D) or binomial CIs (E–H). Statistically significant differences are indicated by different lower case letters.

suffered this apparent sperm-depletion (no significant interaction effect; $LR_6 = 11.37$, $P = 0.08$).

EXPERIMENT IV. COSTS OF HARASSMENT: MATING FAILURES IN MASS CULTURE CONDITIONS

The frequency of mating failures was very low (1.6%; four out of 278 females were unseminated) and did not change over time ($LR_1 = 0.64$, $P = 0.42$). Despite the infrequency of mating failures the effect of tube was close to significance ($LR_5 = 10.42$, $P = 0.06$); out of the four females that produced all male broods, three were from a single tube (tube 4). Twenty-five females failed to reproduce at all (8.71%) and the probability of reproductive failure appeared to increase over time (Fig. 2A) but this was not statistically significant ($LR_1 = 3.22$, $P = 0.07$). The mean offspring production was 44.90 ± 1.26 SE ($N = 254$). Females removed from different stock tubes did not differ in their number of offspring ($LR_5 = 7.91$, $P = 0.16$) but over time offspring production decreased even after reproductive failures were removed from the dataset ($LR_1 = 5.03$, $P = 0.02$, Fig. 2B). The sex ratio laid (mean = 0.14 ± 0.01 , excluding mating failures and reproductive failures) also remained constant over time ($LR_1 = 2.60$, $P = 0.11$), although females removed from tube 4 did lay a more male-biased

sex ratio even after all male broods were excluded ($LR_5 = 18.28$, $P = 0.003$).

EXPERIMENT V. COSTS OF HARASSMENT: LONGEVITY

Harassment by males had no effect on female survival. The mean female longevity across all treatments was 192.94 h (± 4.34 SE). There was no effect of the number of males present on survival when individuals were censored at seven days (Mantel-Cox $\chi^2 = 4.07$, $df = 3$, $P = 0.25$) or uncensored ($\chi^2 = 1.12$, $df = 3$, $P = 0.77$; Fig. 3A). Male survival (mean = 130.75 h ± 3.48 SE) on the other hand varied with respect to the number of other males present ($\chi^2 = 22.70$, $df = 2$, $P = <0.0001$). Males that were maintained alone with a female survived for longer than males maintained with competitors and a female ($P < 0.0005$; Fig. 3B). This suggests a sizeable cost to male–male competition for mates.

EXPERIMENT VI. COSTS OF HARASSMENT: FECUNDITY

In contrast to the previous experiment, females that were maintained with 10 males during oviposition had significantly reduced longevity ($\chi^2 = 10.52$, $df = 3$, $P = 0.01$) and fecundity

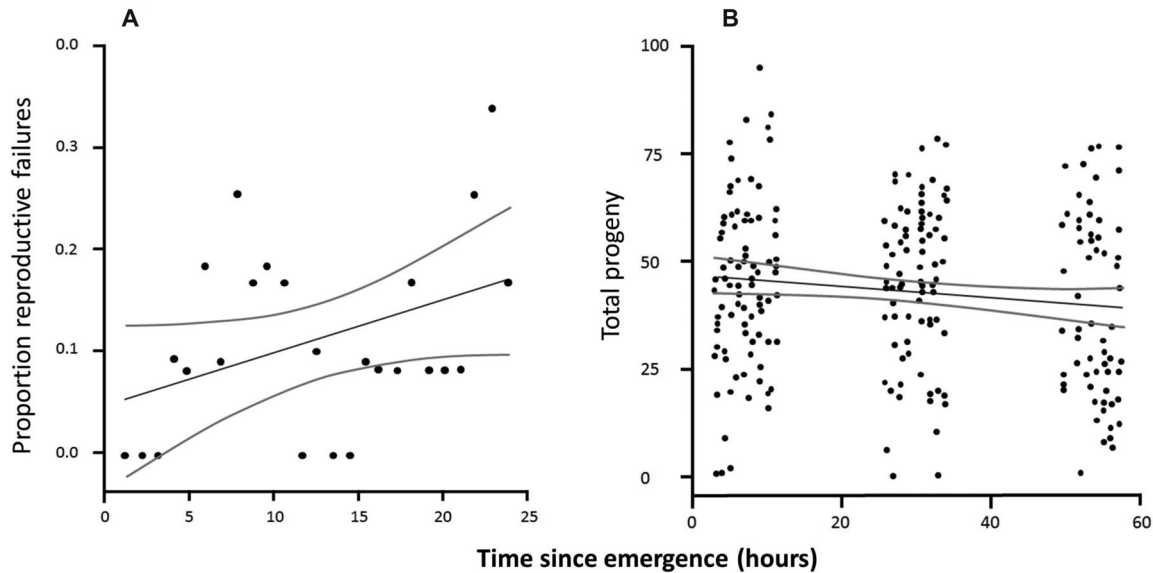


Figure 2. (A) The proportion of reproductive failures (females that failed to parasitize any hosts) over time in experiment IV. (B) Offspring production decreased over time for females collected from stock tubes in experiment IV. Gray lines represent 95% CIs.

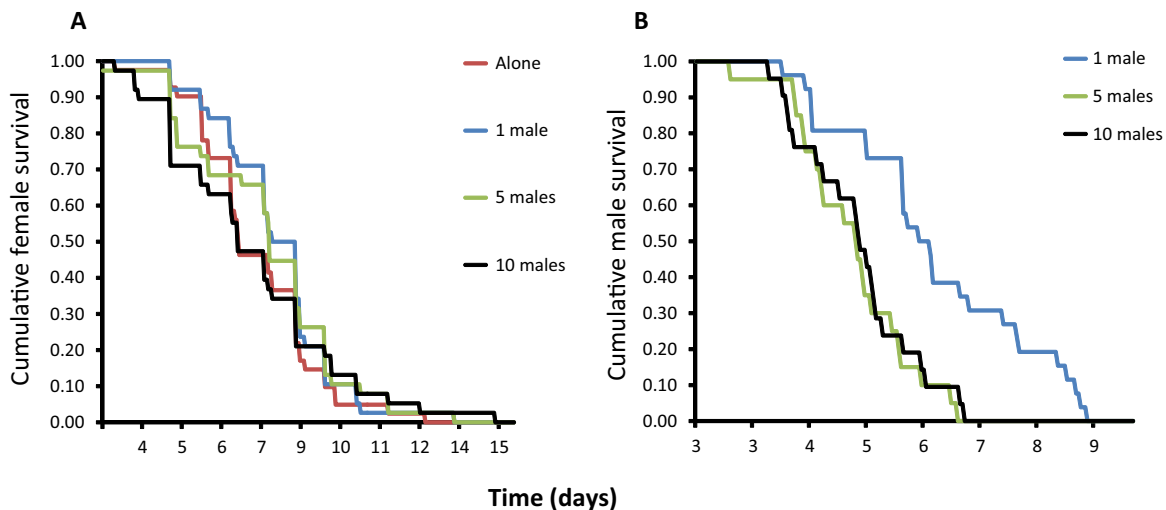


Figure 3. (A) There was no effect of harassment by males on survival of *Nasonia vitripennis* females in experiment V, when males were present before oviposition. (B) There was a significant reduction in male survival when *N. vitripennis* males were maintained in groups of 5 or 10 males during interactions with a female (experiment V).

(independent of longevity; mean = 78.45 ± 1.42 ; $LR_3 = 17.10$, $P < 0.0001$; Fig. 1D) than females in other treatment groups. Moreover, the presence of 1 or 10 males during oviposition resulted in increased production of sons compared to treatments where males were absent during oviposition ($LR_3 = 20.04$, $P < 0.001$; Fig. 1H).

Discussion

We have shown that multiple mating and the opportunity for multiple interactions with males has a range of consequences for female *N. vitripennis*, some positive in terms of fitness, and some

negative. First, females gained a fecundity benefit by mating multiply with virgin males, a benefit that was not due to a female effect (i.e., where more fecund females are naturally more receptive to additional matings: Torres-Vila et al. 2004). The availability of virgin males will be greater in laboratory culture, as sex ratios are less female biased (due to the breakdown of local mating structure and relaxation of LMC), and this direct benefit may contribute to higher rate of polyandry in laboratory adapted *N. vitripennis*. Second, there was no indirect compatibility benefit of polyandry: females mated to multiple males did not produce more offspring than those mated multiply to the same male. However, any such benefit would likely be masked by the reduction

in fecundity that we saw which comes with mating to previously mated males rather than virgin males. Furthermore, females suffered a fecundity cost of polyandry when both of their mating partners had mated previously. Third, male harassment alone had no effect on female longevity, but the presence of males during (but not before) oviposition resulted in reduced female survival and fecundity, as well as disrupted sex allocation (male-biased broods).

Our observations suggest that although insemination failure is rare for *N. vitripennis* females maintained in mass culture, the local conditions (encapsulated here as stock tube “identity”) may influence the likelihood of sperm depletion or “pseudovirginity.” There are two potential routes to mating failure in mass mating conditions. First, *N. vitripennis* males are prospermatogenic (i.e., they eclose as adults with their full complement of sperm) and will continue mating once they are sperm depleted (van den Assem 1986). This means that if the sex ratio within the stock tube is female biased, females may not gain sufficient sperm to maximize daughter production. This might be more likely for later-emerging females, as *N. vitripennis* is protandrous (Moynihan and Shuker 2011; although with so little mating failure, we saw no such pattern in our data). However, some degree of sperm limitation may explain why females in certain stock tubes produced less female-biased sex ratios than others. Second, under mass mating conditions there may be an elevated risk of females being courted but not inseminated, for instance if several males attempt to mate with a single female at once (such “mating scrums” are commonly seen in the laboratory). The abundance of courting males in mass culture conditions may also mean that females are exposed to the male oral pheromone without receiving courtship and insemination, with female receptivity to future mating drastically reduced independently of sperm transfer or courtship (Ruther et al. 2010; Ruther and Hammerl 2014). However, in the more controlled experiments (experiments I, II, and III), mating failure (lack of insemination) and sperm depletion were not common outcomes, as singly mated females did not experience reduced daughter production compared to multiply mated females.

Females mated to virgin males do appear to become sperm limited sooner than if the male had mated previously, although the reason for this is unclear. We would expect previously mated males to have less sperm to transfer than virgin males, not more. These apparent differences could relate to the costs and benefits of polyandry for *N. vitripennis* in terms of fecundity and longevity, which also depend on the mating history of the male: polyandrous females only gain direct benefits when mating with virgin males. When females were mated to previously mated males, polyandry actually resulted in a fecundity cost (Experiment III). Females mated to virgin males may run out of sperm sooner if these males increase fecundity or oviposition. On a proximate level, this pattern could be explained by differences in the ejaculate

composition of virgin and mated males. Ejaculates are comprised of sperm and seminal fluid, with the latter being an often complex mix of components that have been shown to influence female insect reproductive physiology in a number of ways (Poiani 2006). Although sometimes harmful to female fitness (Gillott 2003), seminal fluid proteins can provide females with substantial benefits (as a kind of nuptial gift: see Introduction). Polyandry may become costly when males that have mated previously transfer ejaculates that are not sufficiently beneficial to outweigh the chemical or mechanical costs incurred during mating (Daly 1978). We know very little about ejaculate composition in *N. vitripennis*; analysis of the *Nasonia* genome has not revealed any evidence of genes encoding accessory gland proteins (ACPs) such as sex peptide (SP) or its receptors (Hauser et al. 2010; Watt and Shuker 2010). In *N. vitripennis*, there is some evidence that males can manipulate sex allocation, but whether this is mediated by ejaculate components is not yet known (Shuker et al. 2006; see also Shuker and Cook 2014). In any case, the consequences of this mating status dependent cost of mating warrants further investigation. For instance, we might expect there to be selection on earlier female emergence time to maximize the probability of encountering a virgin male (Burton-Chellew et al. 2007).

We did not find any evidence for genetic compatibility benefits of re-mating (experiment III). Females that mated twice to different males did not have greater offspring production than females mated twice to the same male. The lack of any compatibility benefit in *N. vitripennis* is not entirely unexpected. Sib-mating is the norm in *N. vitripennis* and there is no evidence for kin discrimination or inbreeding avoidance (e.g., Shuker et al. 2004). The results of the current study are, however, limited to discrete compatibility benefits and do not address the continuous nature of genetic compatibility. Some matings may be less severely incompatible than others and still result in surviving offspring, although of low quality. What is more, the possibility that *N. vitripennis* females can gain “good genes” or “sexy sperm” benefits remains untested (Zeh and Zeh 1996, 1997; Newcomer et al. 1999; Slatyer et al. 2012). Although it is likely that indirect benefits of this nature only accumulate once polyandry has been established, investigating the occurrence of these processes in a previously monandrous species may yet prove fruitful.

As mentioned above, a further possibility is that polyandry is adaptive, not due to any benefit but rather to reduce the costs incurred by persistent males attempting to court and copulate. The presence of males alone does not appear to be costly for females of *N. vitripennis* in terms of survival or fecundity. However, when females were allowed access to hosts under conditions of high levels of harassment, they did suffer a longevity cost and an independent fecundity cost. Additionally, these females appeared unable to allocate sex adaptively, laying a more male-biased sex ratio than would be predicted under single foundress conditions

(Hamilton 1967; Werren 1983). This means that male harassment is costly for females, but that the costs only accrue during oviposition. Similarly, in female seaweed flies (*Coelopa frigida*) the presence of the oviposition substrate (brown algae) in combination with the presence of courting males results in a substantial reduction in female survival (Meader and Gilburn 2008). The ecological context in which mating costs are measured is extremely important. Both in the current study and in the work of Meader and Gilburn (2008) the costs of mating and harassment are contingent on whether females have the opportunity to oviposit. Other environmental factors may also modify mating costs, for instance in the water striders the key factors appear to be predation risk and foraging efficiency (e.g., Rubenstein 1984; Wilcox 1984; Rowe 1992, 1994).

Adaptations to LMC are probably the most notable feature of the *Nasonia* system (Werren 1980, 1983; Shuker and West 2004; Shuker et al. 2005; Burton-Chellew et al. 2008). Here we have shown that reducing LMC in the laboratory environment can substantially modify the costs and benefits of mating, namely by increasing the availability of virgin males. Furthermore male presence during oviposition renders females unable to allocate sex according to the predictions of LMC. However, only if females are laying under conditions of high LMC will this cost be realized; under laboratory culture conditions, this sex allocation cost may not actually be that costly.

Females will vary in their ability to withstand the costs and sequester the benefits of mating, which are not fixed and vary with environmental context. As such females are expected to modulate their mating rate based on what is optimal for them under the current conditions (Arnqvist and Nilsson 2000; see also Toft and Albo 2015). Whether the tendency of *N. vitripennis* females to re-mate is subject to plasticity based on the demands of the environment remains to be tested. Ecological changes in the laboratory that alter economics of multiple mating and the frequency of mating opportunities may elevate the frequency of re-mating that may allow other benefits to accrue, ultimately leading to shifts in female mating patterns. The ecological changes that we inflict on *Nasonia* when we culture them in the laboratory are clearly related to the mating system changes that we see, with a move from monandry to greater polyandry. Our findings offer valuable insight into the circumstances under which polyandry can benefit a mostly monandrous species, and will guide more explicit research into how females respond to context-dependent costs and benefits of mating, and how these circumstances ultimately influence the evolution of polyandry in *N. vitripennis*.

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