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Effects of constrained females on offspring sex ratios of *Nasonia vitripennis* in relation to local mate competition theory

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Abstract: Empirical studies of how constrained females affect sex ratio are few. Constrained females are those that can produce only sons (e.g., in haplodiploid species, females that have not mated or older females that have used up their sperm). In the parasitoid wasp *Nasonia vitripennis* (Walker, 1836), failure to mate soon after emergence increased the probability of a female being constrained and thus affected sex ratio directly. Local mate competition theory shows that whether a female is constrained can also affect sex ratio indirectly by affecting what sex ratio other females produce. However, this was not the case in *N. vitripennis*. A female's sex ratio was not significantly different when she was with another young mated female versus a virgin female or an old mated female depleted of sperm. These results suggest that *N. vitripennis* females may be unable to recognize whether another female is constrained. The increased proportion of sons in response to other females relative to when alone did not persist the day after exposure.

Résumé : Il y a peu d'études empiriques qui cherchent à savoir comment les femelles « restreintes » affectent le rapport des sexes. Les femelles restreintes sont des femelles qui ne peuvent produire que des rejetons mâles (e.g., chez les espèces haplodiploïdes, des femelles qui ne se sont pas accouplées ou des femelles plus âgées qui ont épuisé leur réserve de sperme). Chez la guêpe parasitoïde *Nasonia vitripennis* (Walker, 1836), l'absence d'accouplement tôt après l'émergence augmente la probabilité qu'une femelle devienne restreinte et affecte donc directement le rapport des sexes. La théorie de la compétition sexuelle localisée montre que le fait qu'une femelle soit restreinte affecte aussi le rapport des sexes indirectement, en déterminant quel rapport des sexes est produit par les autres femelles. Cependant, ce n'est pas le cas chez *N. vitripennis*. Le rapport des sexes produit par une femelle n'est pas significativement différent lorsqu'elle est en présence d'une autre jeune femelle accouplée plutôt qu'une femelle vierge ou qu'une vieille femelle accouplée n'ayant plus de sperme. Ces résultats laissent croire que les femelles de *N. vitripennis* sont incapables de reconnaître si une femelle voisine est restreinte. La production d'une proportion plus élevée de rejetons mâles en présence d'autres femelles qu'en leur absence a déjà disparu le jour qui suit la rencontre.

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Introduction

Evolutionary theory has made clear how adjusting offspring sex ratio in response to certain environmental factors can be advantageous in terms of the mother's fitness (reviewed in Charnov 1982). One of the best known and most frequently cited species in relation to sex-ratio theory, specifically in relation to local mate competition theory (LMC), is the parasitoid wasp *Nasonia vitripennis* (Walker, 1836) (Thornhill and Alcock 1983; Krebs and Davies 1993). LMC was designed to explain female-biased sex ratios and moth-

ers that increase their sex ratios in the presence of other mothers (Hamilton 1967). LMC has been most studied in parasitoid wasps, but it is relevant to a diverse array of taxa (e.g., Trouve et al. 1999; West et al. 2000; Peer and Taborsky 2004). LMC assumes that some or all mating takes place at the natal site, resulting in competition for mates among males, including among brothers (Hamilton 1967; Nunney and Luck 1988). This assumption is widely accepted as being met in *N. vitripennis* (e.g., Werren 1980; Flanagan et al. 1998), and there have been literally dozens of studies of the sex-ratio response to other mothers or their hosts in *N. vitripennis* (e.g., references in Godfray 1994). It has even been called the *Drosophila* of sex-ratio research (Crozier and Pamilo 1993).

As predicted by LMC, overall sex ratios tend to be female-biased in *N. vitripennis*, and females generally increase the proportion of sons that they produce in response to the number of other females present (e.g., Wylie 1967) and in response to a host having been previously parasitized by another female (e.g., Holmes 1972; Werren 1980). Also

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consistent with LMC, a female oviposits a greater proportion of sons when she parasitizes a host previously parasitized by a conspecific female than when she parasitizes a host previously parasitized by herself (King 1992). To make these sex-ratio adjustments, females must somehow recognize when another female is ovipositing, or has oviposited, in a host. Females also appear to assess the other female's clutch size relative to their own (Suzuki and Iwasa 1980; Werren 1980; but see Orzack 1993), apparently by assessing the other female's size (Flanagan et al. 1998).

Werren's (1980) LMC model predicts that a female's sex-ratio response to another female should be affected not only by their relative clutch sizes, but also by the other female's sex ratio. Werren's (1980) equation 2 is as follows:

$$X_2^* = \frac{(2X_1(T+1))^{0.5} - 2X_1}{2T}$$

where X_2^* is the predicted sex ratio (proportion of sons) of female 2, X_1 is the sex ratio of female 1, and T is the number of eggs oviposited by female 2 relative to female 1. (Werren (1980) described his model in terms of sequential oviposition, but it does not assume sequential oviposition.)

Werren (1984) showed that *N. vitripennis* females do not assess the sex ratio of another female's already oviposited eggs directly. However, females might assess another female's sex ratio indirectly by assessing whether the other female is constrained. By definition, constrained females can produce only sons. As in other hymenopterans, *N. vitripennis* females require sperm to produce daughters but produce sons parthenogenetically. Thus, constrained females include virgin females as well as old sperm-depleted females.

Support for the idea that females might be able to distinguish between virgin and mated females comes from a confamilial (King 2002) and from the observation in *N. vitripennis* that mated females appear to be more active than virgin females (King 1993b; King et al. 2000). In addition, at least in some other insects, mated and virgin females differ pheromonally (Ayasse et al. 2001). A female might determine that another female is constrained even before the constrained female oviposits and then might respond to the expected sex-ratio value for that class of females.

Here we expand on the growing body of data on sex ratios in *N. vitripennis* by exploring the effects of constrained females. First, we examine whether delayed mating affects sex ratio by increasing the risk of becoming a constrained female (e.g., through loss of female receptivity). Then we examine whether the presence of constrained females affects the sex ratios produced by unconstrained females. We look at two types of constrained females, virgin females and older females that are becoming sperm-depleted. Finally, we examine whether the sex-ratio response to another female persists even after the females are isolated.

Methods

We conducted experiments with *N. vitripennis* from laboratory stocks of a scarlet-eyed strain, using a wildtype strain as well when we needed to assign offspring from the same hosts to two different mothers. Support for LMC has come from both strains (e.g., Werren 1980, 1983, 1984). We main-

Table 1. Description of treatment of females in the effect of delayed mating on sex-ratio experiment of *Nasonia vitripennis*.

| | Female age (d) | | |
|----------------------------|----------------|-------|-------|
| | 0 | 1 | 2 |
| Delayed-mating treatment | Alone | Mate | Hosts |
| Immediate-mating treatment | | | |
| Hosts-1-d-later | Mate | Hosts | |
| Hosts-2-d-later | Mate | Alone | Hosts |

Note: Two immediate-mating subtreatments were necessary to match the delayed-mating treatment in terms of (i) the number of days between mating and receiving hosts (hosts-1-d-later subtreatment) and (ii) the female's age when she received hosts (hosts-2-d-later subtreatment).

tained the wasps on pupae of the blue bottle fly, *Calliphora vomitoria* (L., 1758), which we obtained commercially from Grubco, Inc. (Hamilton, Ohio) and stored in the refrigerator as pupae. Our care of our animals was in accordance with the principles and guidelines of the Canadian Council on Animal Care.

For experiments (described below), we obtained virgin wasps by separating females and males as pupae. Females had emerged within the last 24 h at the start of each experiment unless otherwise specified. When we used mated females, matings were observed and were between virgins. When females were presented with hosts, they were placed with the hosts in 4 dram (70 mm high × 21 mm diameter) glass shell vials with cotton plugs. Number of adult females and males from those hosts were counted and used to calculate offspring sex ratios (proportion of sons).

We used analyses of variance (ANOVA) and Student's *t* tests to compare means among treatments. However, when assumptions of normality were not met, we compared means of ranks by Mann-Whitney *U* or Kruskal-Wallis tests. Under certain theoretical conditions, proportions are not normally distributed (Zar 1984), but real sex-ratio data often fit assumptions of normality reasonably well (B.H. King, personal observation). We formally tested for normality with an α of 0.001 because Student's *t* tests and ANOVA are robust to deviations from normality (Scheffé 1959). Conclusions are unaffected by using nonparametric tests throughout. For statistical tests of means, we used the traditional α of 0.05. Two-tailed *P* values are given throughout.

Effect of delayed mating on sex ratio

This experiment examined whether failure to mate soon after emergence affects a female's sex ratio by affecting the proportion of sons that she produces after mating and (or) by preventing her from mating at all. This experiment included an immediate-mating treatment, in which the female was paired with a male within 1 d of emergence, and a delayed-mating treatment, in which the female was paired with a male 1 d later. The number of pairs that mated within 30 min was determined by observation and compared between treatments by Fisher's exact test. Then each female that mated received 10 hosts for 1 d. In the delayed-mating treatment, the female received these hosts the day after mating (Table 1). The immediate-mating treatment was broken into two subtreatments, differing in when the hosts were provided (Table 1): one subtreatment controlled for female age at the time of oviposition and the other controlled for the

Table 2. In the response to constrained- versus unconstrained-female experiment, a focal female *N. vitripennis* was always alone (i.e., "other female" absent) or was with another female on day 1 and then alone on day 2.

| | Day 1 | | | Day 2: alone | | | | |
|--------------|----------|----------------------------|----------|--------------|----------------------------|----------|----------------------------|----------|
| | <i>T</i> | X_1^a (mean \pm SE) | <i>N</i> | X_2^* | X_2^b (mean \pm SE) | <i>N</i> | X_2^c (mean \pm SE) | <i>N</i> |
| Other female | | | | | | | | |
| Absent | | | | 0.00 | 0.19 \pm 0.058 | 15 | 0.25 \pm 0.070 | 17 |
| Young virgin | 1.08 | 1.00 \pm 0.000 | 13 | 0.02 | 0.44 \pm 0.100 | 12 | 0.24 \pm 0.059 | 16 |
| Old mated | 6.24 | 0.68 \pm 0.076 | 12 | 0.14 | 0.39 \pm 0.068 | 15 | 0.25 \pm 0.068 | 17 |
| Young mated | 0.98 | 0.44 \pm 0.069 | 16 | 0.22 | 0.35 \pm 0.070 | 16 | 0.34 \pm 0.091 | 17 |

Note: Sample-size discrepancies within rows result from sex ratios not being available when females produced no offspring and from the exclusion of focal females that produced only sons. *T* is the observed ratio of mean clutch size of focal females to mean clutch size of "other females"; X_1 is the observed sex ratio (proportion of sons) of the "other female"; X_2^* is the predicted sex ratio of the focal female; and X_2 is the observed sex ratio of the focal female.

^aOld mated versus young mated female treatment: independent Student's *t* test, $t = 2.33$, $df = 26$, $P = 0.03$.

^bTwo-female treatments (young virgin, old mated, young mated): ANOVA, $F_{[2,40]} = 0.34$, $P = 0.72$; two-female treatments combined versus absent treatment: Mann-Whitney *U* test, $U = 93.5$, $N_1 = 43$, $N_2 = 15$, $P < 0.001$.

^cTwo-female treatments: Kruskal-Wallis test, $\chi^2 = 0.94$, $df = 2$, $P = 0.62$; two-female treatments combined versus absent treatment: independent Student's *t* test: $t = 0.33$, $df = 65$, $P = 0.74$.

number of days between mating and exposure to hosts. Both factors affect sex ratio in some parasitoid wasps (King 1987). Males in all treatments had emerged within the last 24 h when mated to the female, to control for any effects of male age on eagerness to mate. The offspring sex ratios of the females that mated were compared between the delayed-mating treatment and each of the immediate-mating sub-treatments.

Response to constrained versus unconstrained females

This experiment examined the sex-ratio response of a female when she was with a constrained female versus an unconstrained female and when she was alone. This experiment also examined the persistence of a female's sex-ratio response after the other female was gone. The focal female was a scarlet-eyed mated female (following Werren 1983). She was given 10 hosts for 1 d in one of four treatments. Each focal female was either (1) alone, or in the presence of (2) a young wildtype virgin female, (3) a young wildtype mated female, or (4) an old wildtype mated female. We refer to the wildtype female as the "other female". When the latter three treatments are referred to as a group, they are called the "two-female treatments". The old mated "other female" had been given 10 hosts daily for her first 16 d to deplete her supply of sperm or at least reduce her production of daughters (Velthuis et al. 1965). To determine the persistence of the effect of exposure to another female, we subsequently isolated the focal female with 8 hosts for an additional day.

Replicates in which the focal female produced only sons were excluded from analyses because females cannot adjust their sex ratios if they can produce only sons. There was no significant difference among two-female treatments in the number of such females ($\chi^2 = 0.16$, $df = 2$, $P = 0.92$) or between two-female treatments (16 of 66 females) versus the absent treatment (2 of 22 females) ($\chi^2 = 2.33$, $df = 1$, $P = 0.13$). Including such females would not affect our conclusions.

Our major goal in this experiment was to determine whether females vary their sex ratios depending on the type of other female present. Thus, in our statistical analyses, we first compared sex ratios on day 1 among the two-female treatments. The two-female treatments did not differ, so we

then combined them prior to confirming the well-established observation that sex ratios differ in the presence versus absence of another female. We then repeated these analyses for day 2 to test for persistence in the patterns of sex-ratio response. To test whether older other females were sperm-depleted, we compared the proportion of sons produced by young versus old other females.

In addition to these analyses, we generated predicted sex ratios based on Werren's (1980, eq. 2) LMC model. For X_1 , we used the mean observed sex ratios of other females (Table 2). For *T* we used the ratio of averages (i.e., we first computed mean clutch size for each type of female and then calculated the ratio of these means); however, the relative ranking of the predicted sex ratios among the four treatments was unaffected when a ratio was computed for each pair of females and then the ratios were averaged. Relative ranking of the predicted sex ratios among the four treatments was also the same when we calculated a predicted sex ratio separately for each replicate (i.e., using each replicate's observed clutch sizes and other female sex ratio). In comparing observed sex ratios to predicted sex ratios, we focussed on the general pattern of differences predicted among treatments because such patterns are more robust to deviations from the exact assumptions of the specific LMC model (Nunney and Luck 1988).

Results

Effect of delayed mating on sex ratio

The 8 of 14 females that mated in the delayed-mating treatment was less than the 25 of 26 females (13 of 13 in the hosts-1-d-later subtreatment, 12 of 13 in the hosts-2-d-later subtreatment) that mated in the immediate-mating treatment (Fisher's exact test, $P = 0.004$). Fewer females mated in the delayed-mating treatment because they were less receptive (i.e., they failed to open their genital orifices despite male mounting and courtship). Of those females that mated, offspring sex ratio did not differ between the delayed-mating treatment and either immediate-mating subtreatment (Table 3).

Table 3. Observed sex ratios (proportion of sons) among treatments in the effect of delayed mating on sex-ratio experiment of *N. vitripennis*.

| | <i>N</i> | Mean | Median | Range |
|----------------------------|----------|------|--------|-----------|
| Delayed-mating treatment | 8 | 0.30 | 0.20 | 0.11–1.00 |
| Immediate-mating treatment | | | | |
| Hosts-1-d-later | 13 | 0.19 | 0.18 | 0.12–0.37 |
| Hosts-2-d-later | 12 | 0.30 | 0.18 | 0.13–0.92 |

Note: Hosts-1-d-later versus delayed-mating treatments: $U = 46.0$, $N_1 = 8$, $N_2 = 13$, $P = 0.66$. Hosts-2-d-later versus delayed-mating treatments: $U = 43.0$, $N_1 = 8$, $N_2 = 12$, $P = 0.70$.

Response to constrained versus unconstrained females

A female's sex-ratio response to another female was not significantly affected by whether the other female was mated or a virgin, and if the other female was mated, by whether she was young or old (X_2 in Table 2). The old mated other females were becoming sperm-depleted as seen by their producing a greater proportion of sons than the young mated other females (X_1 in Table 2).

Given the observed values of T and X_1 (Table 2), Werren's (1980) LMC model predicted that the sex-ratio response to constrained females, particularly to virgin females, should be considerably more female-biased than the response to unconstrained females (young mated females) (X_2^* in Table 2). However, the observed sex ratios did not differ significantly and were not even in the predicted direction (compare the relative ranking of sex ratios in X_2 versus in X_2^* in Table 2).

Although females produced a greater proportion of sons during direct exposure to another female relative to when alone, after the females were all alone, there was no sex-ratio difference among treatments (last column in Table 2). Females that had always been alone did not change their sex ratios significantly (paired t test: $t = 0.81$, $df = 14$, $P = 0.43$). Females that had been with another female decreased their sex ratios (sign test: 40 of 43 females, $P < 0.001$).

Discussion

Failure to mate soon after emergence increased the proportion of constrained females and thus affected sex ratio directly in *N. vitripennis*. The increased sex ratio in response to delayed mating resulted from fewer females mating and not from an increased proportion of sons among females that did mate. However, with a 2-d delay and using the wildtype strain, delayed mating also caused an increased proportion of sons among females that mated (B.H. King, unpublished data).

The reason that fewer females mated when mating was delayed is that by then females were less receptive. There may be no selection on virgin females to remain receptive as they age if there is selection to instead fly to a new site (e.g., a new carcass) so that they can begin ovipositing. Because males cannot fly, they will not be present at new carcasses. How quickly virgin females disperse from their natal site is unknown.

This increased proportion of sons in *N. vitripennis* in response to delayed mating is not consistent with other species. There is no effect on sex ratio in the confamilials *Spalangia endius* Walker, 1839 and *Spalangia cameroni*

Perkins, 1910 (King 2002; B.H. King, unpublished data), and sex ratio decreases in other species (Hoelscher and Vinson 1971; Rotary and Gerling 1973; Fauvergue et al. 1998).

Females increased their offspring sex ratios equally in response to the presence of young mated females as to virgin or sperm-depleted females. An increase in response to young mated females has been demonstrated repeatedly before in *N. vitripennis* (Velthuis et al. 1965; Wylie 1966; Walker 1967; Werren 1983; King and Skinner 1991; Orzack et al. 1991), as well as in many other parasitoid wasps (reviewed in King 1993a; Godfray 1994), and this increase is predicted by LMC. However, that the sex-ratio response of *N. vitripennis* did not persist upon subsequent isolation is in contrast to the other species that have been examined (Viktorov 1968; Strand 1988; King and Seidl 1993; King 1996, 2002). The reason for this difference is unclear.

That females increased their proportion of sons equally in response to constrained females versus unconstrained females was contrary to predictions of Werren's (1980) LMC model. The response to sperm-depleted females has not previously been examined and the response to virgin females has been examined in only two other species. Like *N. vitripennis*, *Bracon hebetor* (Say, 1836) increase their proportion of sons equally in the presence of mated and virgin females (Ode et al. 1997). In contrast, *S. endius* females produce a greater proportion of sons in response to mated females than virgin females (King 2002). How *S. endius* females recognize whether a female is constrained has not been demonstrated.

One explanation for the lack of a differential sex-ratio response to constrained versus unconstrained females in *N. vitripennis* is that females cannot recognize whether a female is constrained. If this is the case, selection should favour a fixed sex ratio that is optimal given the mean proportion of females that are constrained in the population (Nunney and Luck 1988; Abe et al. 2003). The difference in activity between virgin and mated *N. vitripennis* females that has been observed in the laboratory (King 1993b; King et al. 2000) seemed like a plausible recognition mechanism. However, perhaps selection on sex-ratio behavior occurred under conditions in which this difference in activity was absent because it was overwhelmed by other sources of variation in activity.

The lack of a recognition mechanism also appears to be responsible for failure to support another prediction of the sex-ratio theory in *N. vitripennis*. Females do not produce a different sex ratio when they mate with a sibling versus a nonrelative, presumably because they lack kin recognition (Reece et al. 2004; Shuker et al. 2004).

It seems unlikely that the failure of females to differentiate between constrained and unconstrained females is a result of too few constrained females in nature, and hence no selection to differentiate. In collections of *N. vitripennis* from natural oviposition sites in Utah (Skinner 1983), about 18% of females were constrained, which is not a trivial number: 6.5% of females appeared to lack sperm and an estimated 11% of females had mated with males with the sex-ratio factor psr (such females receive sperm yet produce only sons; Skinner 1983). Both psr and sperm-lacking females oviposit. The sex-ratio response to psr females appears not to have been examined.

Just as there was no difference in the sex-ratio response to the presence of mated versus virgin females in our study, there also is no difference in response to hosts parasitized previously by virgin versus mated females (Werren 1984). Examining the response both to hosts parasitized previously by virgins as well as to the presence of virgins is important because each can affect a female's sex-ratio response independently of the other (King et al. 1995; Shuker and West 2004). Which will have a greater effect will likely depend on the amount of previous parasitization versus the extent to which females physically interact. The results of this study and Werren's (1984) study indicate that among the different LMC models the best match to the behavior of *N. vitripennis* is provided by those models that do not assume females have knowledge of other females' sex ratios.

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