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Male influence on sex allocation in the parasitoid wasp *Nasonia vitripennis*

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Abstract Sex allocation is an important reproductive decision for parents. However, it is often assumed that females have substantial control over sex allocation decisions, and this is particularly true in haplodiploid insects, in which females apparently determine sex by deciding whether to fertilise an egg (and produce a diploid daughter) or not (and produce a haploid son). Mechanisms by which males may influence sex allocation are not so straightforward, and their potential influence on sex ratios has been somewhat neglected. Here, we test whether males influence offspring sex ratios in the parasitoid wasp *Nasonia vitripennis*. We show that some of the variation in observed sex ratios can be attributed to males when comparing the affect of male strain on sex ratio. We did not find among-male variation in sex ratio with a less powerful experiment using males from only one strain or an effect of male mating environment. Our data suggest that males can influence female sex ratios and contribute to the variation around the sex ratios optimal for females. However, the influence is not large, suggesting that females have more influence on sex allocation than do males. We conclude by considering whether male influences on sex ratio represent differences in male reproductive competence or deliberate attempts by males to increase their fitness by influencing daughter production.

Keywords Adaptation · Constraints · Hymenoptera · Local mate competition · Sex ratio · Sexual conflict

Introduction

Sex allocation is an important reproductive decision for parents, and the study of sex ratios has proven to be one of the most productive areas in evolutionary biology (Charnov 1982; Frank 2002). Deciding the sex of the offspring, and how much to invest in those offspring, has received a great deal of theoretical and empirical attention (Charnov 1982; Hardy 2002). Our understanding of the evolution of sex ratios and sex allocation is helped enormously by the conceptually straightforward trade-off between the fitness obtained through sons and that obtained through daughters, a trade-off that is often very easy to measure empirically. Sex allocation theory is therefore one of the few areas in which evolutionary theory can be realistically tested in a quantitative fashion (West et al. 2000, 2002). This means that meaningful attempts to identify constraints on adaptive evolution can be made (Herre 1987; West and Sheldon 2002; Shuker and West 2004). Here, we consider one such constraint on adaptive female sex ratio behaviour, the role of males.

In many organisms, females have been assumed, explicitly or otherwise, to be in control of sex allocation. For example, the controversy over adaptive sex allocation in birds has been fuelled by the lack of an obvious mechanism by which females could shift sex ratios (Pike and Petrie 2003). In haplodiploid insects such as Hymenoptera on the other hand, females have been assumed to determine the sex ratio by the apparently simple mechanism of deciding whether or not to fertilise the eggs, producing either female or male offspring respectively. Males have been considered to have little influence on the sex ratio because it was not clear how males could influence sex ratio (e.g. Werren and Beukeboom 1998), although this view is perhaps the result of a lack of detailed studies. This has meant that attention on sex ratio evolution in the Hymenoptera has tended to concentrate on the female, although it has been argued that this assumption is unjustified (Orzack 1993, 2002).

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Males could influence female sex allocation in a number of ways in haplodiploids (Henter 2004). First, males may differ in fertilisation ability, with some producing sperm that were unable to successfully fertilise eggs. This would lead to an increased production of sons relative to daughters. Second, fertilisation may take place, but the paternal and maternal genomes could be incompatible, leading to the embryonic death of daughters. Third, males may actively attempt to influence sex allocation because males only pass genes into the next generation via daughters (Hawkes 1992). Here, we address these issues in the parasitoid wasp *Nasonia vitripennis* (Hymenoptera: Chalcidoidea). Females of this species have been repeatedly shown to facultatively alter their offspring sex ratios in the direction predicted by the local mate competition (LMC) theory of Hamilton (1967, 1979) (see also Suzuki and Iwasa 1980; Taylor and Bulmer 1980; Werren 1980). Females alter their sex ratios in response to (1) the presence of other females and (2) laying eggs on a host already parasitised by another female (e.g. Werren 1980, 1983; Orzack 1990; Orzack et al. 1991; King et al. 1995; Molbo and Parker 1996; Flanagan et al. 1998; Shuker and West 2004). In both cases females increase their offspring sex ratio with increasing numbers of offspring from other females on a patch because the level of local mate competition between their own sons is reduced as more unrelated individuals on a patch interact. However, for all the support of LMC theory in *N. vitripennis*, there remains unexplained variation around the optimal sex ratios. We consider whether males contribute in some way to this variation.

We used two approaches to explore whether males can influence the sex ratio females produce. The first was to partition the variance in female sex ratio amongst males either from one outbred strain (“Experiment 1”) or from six strains established from collections from one natural population (“Experiment 2”). A significant effect of male mating partner would suggest that males influence the sex ratio, assuming that they vary in their ability to do so. We screened males for their effect on female sex ratio with females ovipositing either alone on fresh hosts (in which case females should produce large, highly female-biased clutches) or on parasitised hosts (leading to smaller, less female-biased clutches). In the third experiment, we used a different approach, experimentally manipulating the mating and oviposition environment of males and females, to see whether any effect of males depended on whether males had to inseminate one or several females in a short time period, as might be predicted if sperm limitation constrains female sex allocation. Females then either oviposited alone or together in groups.

Methods

Study organism

Nasonia vitripennis is a gregarious parasitoid of pupae of numerous species of large cyclorrhaphous Diptera (Whiting

1967). Males have small wings and are unable to fly, typically remaining close to the site of adult emergence to compete with each other for matings with emerging females. Females on the other hand mate before dispersing to parasitise fresh hosts. Females lay clutches of 20–40 eggs and limit oviposition in previously parasitised hosts (superparasitism) if possible (e.g. Werren 1980, 1984; Charnov and Skinner 1984; Shuker et al. 2005). Since *N. vitripennis* is haplodiploid, females are assumed to be able to facultatively alter the sex ratio by choosing whether or not to fertilise an egg (producing diploid females or haploid males, respectively). We used the outbred wild-type laboratory strain HV7 for experiments 1 and 3, derived from wasps from bird boxes collected from the field in 2001 and 2002 (Hoge Veluwe, Netherlands). For experiment 2 we used the strains C378, C289, C349, C194, C222A and C130, again collected from Hoge Veluwe (in 2003). We also used the red-eye mutant laboratory strain STDR to allow us to identify the broods of individual wild-type females when superparasitising (experiments 1 and 2) or in multi-foundress groups (experiment 3). All wasps were maintained prior to the experiment in mass culture, with *Calliphora vomitoria* pupae as hosts, at 25°C, 16:8 h light/dark conditions. Under these conditions, males emerge after 13 or 14 days, with females emerging soon after.

Experiment 1: sex ratio variation and male mating partner

We obtained mated HV7 females from mass culture and gave each one a separate host to parasitise. Before emergence of these broods (about 12 days post-oviposition), we opened the hosts and collected male and female pupae. Using these pupae, we set-up mating groups of one male and up to ten unrelated females in 25×75-mm glass tubes. Wasps emerged and mated for 2 days, and then we isolated individual females and pretreated them for 24 h with access to a fresh host followed by 24 h with honey-solution-soaked filter paper. This pretreatment allows the female to host-feed and mature eggs prior to the experiment. Females were then given either two fresh hosts to parasitise (treatment A), or two hosts that had been parasitised during the previous 24 h by similarly pretreated STDR females (treatment B). After 1 h, one-way escape tubes were fitted to the tubes to allow females to disperse from the experimental patch to allow females to limit the extent of superparasitism (Werren 1980; Godfray 1994). After 48 h all females were removed, and the hosts were incubated at 25°C. Following emergence of the brood, the number, sex and eye colour of all individuals were recorded. Broods where only males were produced, indicating female virginity, were discarded. In addition, the number of females emerging successfully from the collected pupae was unexpectedly low in some replicates, so sample sizes varied across males. In total, 134 females from 42 males provided data. Due to these emergence problems, treatment A was performed across two blocks.

There was no significant difference between blocks in sex ratio so the data were pooled for subsequent analysis (general linear mixed model $F_{1,102}=0.02$, $P=0.89$).

Experiment 2: sex ratio variation and male strain

We used six strains of recently collected *N. vitripennis*: C378, C289, C349, C194, C222A and C130. Randomly chosen virgin females from C378 were individually mated to males from one of the six lines and then either given a single fresh host to parasitise (treatment A) or one STDR pre-parasitised host (treatment B) following pretreatment as before. Again, we fitted one-way escape tubes and removed the females after 48 h. Females mated to males from each strain were split between racks and between shelves to reduce confounding positional effects in the incubator. The only difference between females was the strain identity of the male with which they mated.

Females who produced all-male broods in treatment A were considered virgins and were excluded from the sex ratio analysis. We checked whether females in treatment B who produced male-only broods were virgins by subsequently giving a fresh host to oviposit upon to check virginity status (since optimal sex ratios when ovipositing may include very small, male-only clutches; Werren 1980). From 39 putative virgins, 29 produced females when ovipositing on a fresh host. In total, eight females from treatment B could not be assigned virgin/non-virgin status as they died before ovipositing in the virgin-check treatment. Inclusion or not of these females did not influence the results (so results with them are presented below). Three females who produced more than ten diapause larvae were also excluded (again, results did not change if they were included). In total, 412 females provided oviposition data ($N=26-40$ for each treatment and strain combination), with 292 of these providing sex ratio data ($N=9-34$ for each treatment and strain combination).

Experiment 3: sex ratio variation and the mating environment

In the third experiment we checked whether male effects are influenced by the mating environment. All males and females were collected as pupae from a large sample of hosts parasitised by HV7 females and were therefore virgins prior to the mating treatments. We kept them individually in 10×75-mm glass tubes and fed them with honey-soaked filter paper for 2 days. We then randomly allocated them to mating treatments, putting males and females together in 25×75-mm tubes. We used a mating treatment with two levels: (1) one male and one female, with three fresh hosts present; and (2) one male and five females, again with three fresh hosts present. The presence of hosts substituted for the pretreatment of experiment 1. We allowed the wasps to mate for 48 h, after which we placed females in one of two oviposition treatments: (1) oviposition alone with three fresh hosts; or (2) oviposition with four red-eye STDR

females of the same age and mating status, again with three fresh hosts. Only one of the five females from each replicate from the multi-female mating treatment, chosen at random, was used for oviposition to avoid pseudoreplication. After 1 h in the oviposition tubes, we again fitted one-way escape tubes to allow females to disperse away from the patch. All females were removed after 48 h, and the hosts were then incubated at 25°C. As before, we sexed and counted all wild-type and red-eye offspring that emerged. Broods with no females, indicating virginity, were discarded, as were a limited number of broods with ten or more diapausing larvae or undeveloped pupae. In total, we used broods from 175 females, with sample sizes per treatment ranging from 38 to 50.

Statistical analysis

We defined sex ratio as the proportion of offspring that were male. Sex ratio data are characterised by binomially distributed data, and are most appropriately analysed with generalized linear models (GLMs), with binomial error structures and logit link functions (Crawley 2002; Wilson and Hardy 2002). However, problems in significance testing can arise if the data are overdispersed; dispersion is measured by calculating the so-called dispersion parameter by dividing the residual deviance by the residual degrees of freedom following model fitting (Crawley 2002). The data from the first and second experiments were somewhat overdispersed (the dispersion parameter from the full model was >4), so we used general linear models with arcsine-square root transformed sex ratios weighted by clutch size. For experiment 1 we used a mixed model with male as a random factor, and we tested the significance of the male effect by comparing the change in deviance explained by models with or without the random factor using a likelihood ratio test (Pinheiro and Bates 2000). For the fixed factors (treatment and clutch size), model simplification was carried out following Crawley (2002) to test the significance of main effects and interactions. In the third experiment, the dispersion parameter was close to 4. We therefore analysed experiment 3 using both generalized linear models with binomial errors and general linear models with transformed data. The results are similar, so only the former are reported here, except when considering multi-foundress groups alone, as the data were more overdispersed in this subset of the data. Linear modelling was performed with S-Plus 6 (Insightful Corporation, Seattle, WA, USA) and Genstat 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK).

Results

Experiment 1: sex ratio variation and male mating partner

There was no significant effect of male mating partner on female sex ratio (likelihood ratio test $LR=0.50$, $df=1$,

$P=0.48$). This was also true if only superparasitising females were considered ($LR < 0.01$, $df=1$, $P > 0.9$). There was a highly significant effect of oviposition treatment on sex ratio, with females ovipositing on fresh hosts producing a more female-biased sex ratio (sex ratio=0.18) than superparasitising females (sex ratio=0.41; $F_{1,132}=12.56$, $P=0.0005$). There was no significant effect of clutch size on sex ratio ($F_{1,131}=2.20$, $P=0.14$), and amongst superparasitising females there was no effect of the red-eye clutch size ($F_{1,26} < 0.01$, $P=0.98$). There was one outlier in the data set, where the female produced a clutch of 82 offspring, but it only included one female (giving a sex ratio of 0.987; this female was clearly not a virgin, although she may have been sperm-limited). Removal of this data point did not change the results.

Experiment 2: sex ratio variation and male strain

There was a significant effect of male strain on the sex ratio produced by a female ($F_{5,273}=2.34$, $P=0.04$; Fig. 1), but the proportion of variance explained by male strain was small ($R^2=0.023$). There was also a significant effect of treatment on sex ratio ($F_{1,273}=171.26$, $P < 0.0001$; Fig. 1) as expected from theory. This GLM included female clutch size to control for both wild-type and STDR clutch sizes in this analysis (the latter is not useful for an analysis of both treatments because it is 0 in treatment A, but wild-type and STDR clutch sizes in treatment B are significantly negatively correlated: $P < 0.0001$, $R^2=0.31$). Clutch size was not significant as a main effect on sex ratio ($F_{1,273}=1.84$, $P=0.18$), but there was a significant interaction between treatment and clutch size ($F_{1,273}=19.86$, $P < 0.0001$). Other interactions were not significant (all $P > 0.1$). The final model explained 45.6% of the variance in sex ratio.

In terms of specific contrasts, within treatment A (females ovipositing alone), males from strain C289 produced a significantly more female-biased sex ratio

than males from the other five strains (controlling for clutch size $F_{1,186}=7.38$, $P=0.007$). Within treatment B (females superparasitising), males from strains C289, C194 and C349 produced sex ratios significantly more female-biased than males from the other three strains (controlling for STDR and wild-type clutch sizes $F_{1,98}=6.53$, $P=0.01$). It is worth noting that females mated to males from their own strain (C378) produced sex ratios among the least female-biased in the experiment (Fig. 1).

There was no effect of male strain on clutch size ($F_{5,280}=1.35$, $P=0.24$; Fig. 2) so that differences in sex ratio are not due to differential female mortality attributable to parental strain. As expected, there was a significant effect of treatment on clutch size ($F_{1,280}=345.48$, $P < 0.0001$), with superparasitising females producing smaller clutches (Fig. 2). There was also a non-significant interaction between male strain and treatment ($F_{5,280}=2.17$, $P=0.06$). However, within each treatment separately there was no effect of male strain [treatment A $F_{5,185}=1.34$, $P=0.25$; treatment B (controlling for STDR clutch size) $F_{5,95}=1.55$, $P=0.18$].

Male strain did influence whether or not a female oviposited, at least when superparasitising. Using logistic regression, there was a significant effect of treatment (i.e. fresh vs already-parasitised hosts) on the probability that a female would oviposit in the first place ($\chi^2_{1,410}=54.67$, $P < 0.001$), with females being more likely to oviposit on fresh hosts (88.7 vs 57.1% of females, respectively). There was no significant effect of male strain ($\chi^2_{5,405}=9.05$, $P=0.11$) and no interaction between treatment and strain ($\chi^2_{5,400}=8.79$, $P=0.11$). However, if we take STDR clutch size into account in treatment B, there was a significant effect of male strain on the probability of ovipositing ($\chi^2_{5,176}=16.92$, $P=0.005$). The influence of STDR clutch size differed between male strains ($\chi^2_{5,170}=11.31$, $P=0.05$).

We can also ask whether females were more likely to mate with males from some strains more than others. Virginity, or possible virginity, was generally low (2–12% of females). Taking females across both treatments, there

Fig. 1 Sex ratio with respect to paternal strain for females ovipositing as single foundresses or as superparasites. Error bars are binomial standard errors

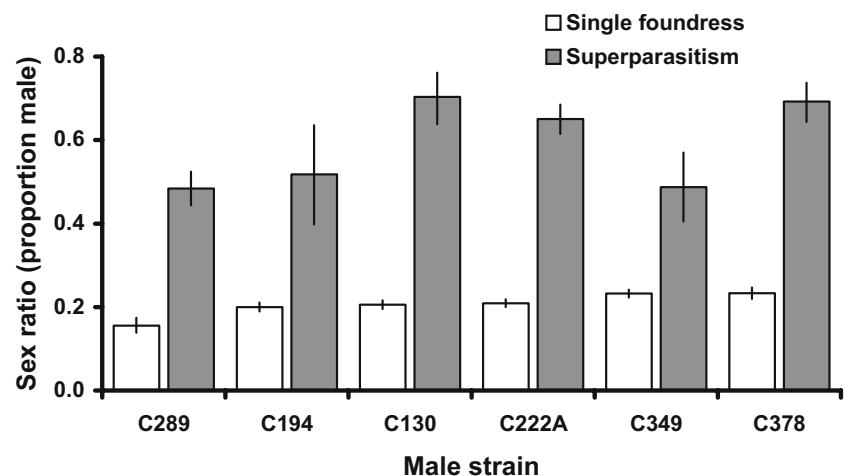
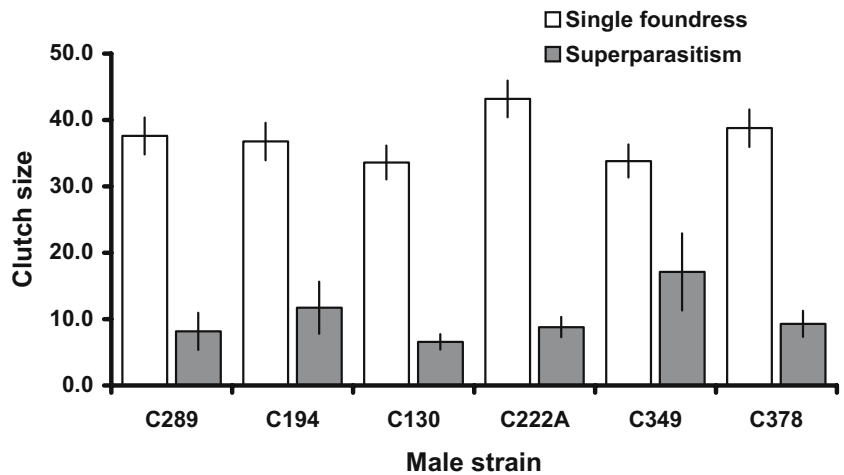


Fig. 2 Clutch size with respect to paternal strain for females ovipositing as single foundresses or as superparasites. Error bars are standard errors



was no significant effect of male strain on likelihood of a female remaining virgin (G test including the eight “possible virgins” $G_5=3.95$, $P=0.56$), so females did not seem to prefer one strain to another.

Experiment 3: sex ratio variation and mating environment

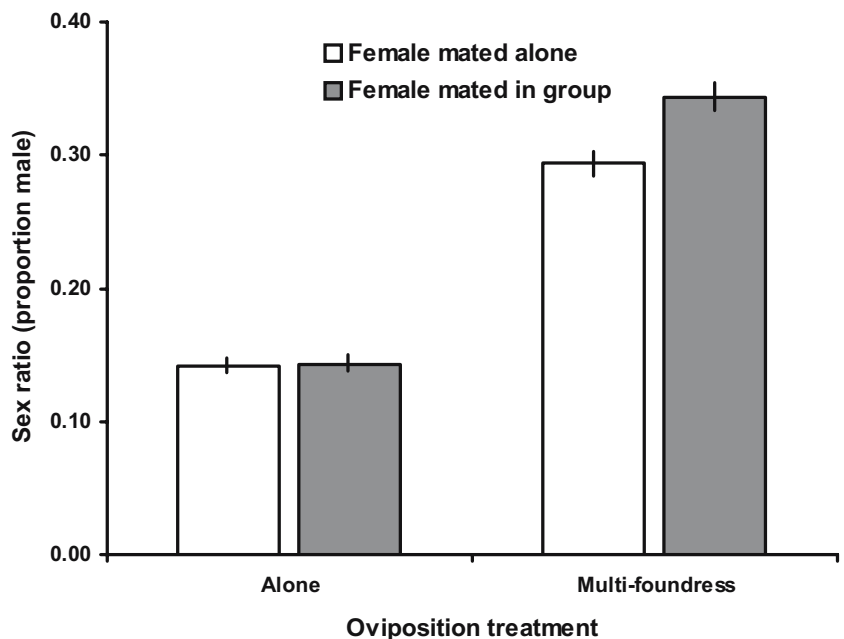
There was a highly significant effect of oviposition treatment, with females ovipositing alone producing a much more female-biased sex ratio than females in multi-foundress groups as expected (GLM $F_{1,173}=137.52$, $P<0.0001$; Fig. 3). There was no significant effect of mating group ($F_{1,172}=2.21$, $P=0.14$) and no significant effect of wild-type clutch size ($F_{1,171}=0.03$, $P=0.87$). Importantly, there were no significant interactions between the main effects (all removed from the model with $P>0.39$). For the females in multi-foundress oviposition groups,

there was no effect of number of red-eye brood on sex ratio (GLM with arcsine-square root transformed data $F_{1,84}=0.58$, $P=0.45$). Within this subset of the data, again, there was no significant effect of wild-type clutch size ($F_{1,83}=0.03$, $P=0.87$) or mating group treatment, although this was close to significance at the 5% level ($F_{1,85}=3.60$, $P=0.06$).

Discussion

The role of males in influencing sex allocation has been neglected, particularly in haplodiploid organisms in which females seem to have a clear mechanism with which to allocate sex (Henter 2004). Here, we have considered what role males may play in explaining variation in patterns of sex allocation in *N. vitripennis*. In the first two experiments we used variation amongst males from either the same outbred strain or from several different strains to assay

Fig. 3 Male influence on sex ratio with respect to mating environment. Females mated males either alone (open bars) or in groups of five (shaded bars), and then females either oviposited alone or in groups. Error bars are binomial standard errors



male influence on sex ratio. In the first experiment we failed to resolve any variation associated with males, although the sample sizes were small. In the second experiment we looked to see whether male strain influenced sex ratio, providing a more powerful test. Variation in sex ratio was associated with male strain as predicted, although the effect was small. In our third experiment we found no evidence that the number of females a male mated with influenced sex allocation, whether females oviposited alone or in groups.

What are the possible causes of the male strain effect we saw in experiment 2? The most straightforward is that male strains vary in their fertilisation success, for instance with males in some strains passing sperm of either insufficient quantity or quality to fertilise as many eggs as the females attempt to (Eberhard 1996). In terms of quantity, since we excluded females that produced only sons (and so could have been virgin in the sense of not having copulated), all the males in the experiments passed at least some sperm. However, if there was variation in the quantity of sperm between males, we would only expect to have seen a male effect in the situation where females produced large clutches, in which sperm was needed to produce 20 or more daughters and so might have been limiting, such as when females oviposited alone. We would perhaps not have expected to see such an effect when females produced the smaller, less female-biased clutches they did when superparasitising. On average, females in treatment A produced 29.53 daughters, and in treatment B they produced 3.98 daughters. Since we saw no treatment \times strain interaction, this suggests the strain effect was similar when females oviposited alone or when superparasitising, and thus argues against males not passing sufficient numbers of sperm. More generally, there was no male strain \times clutch size interaction that would again have been expected if sperm quantity was the cause. In addition, in experiment 3, females mated to males that could mate with four other females did not produce more males, indicative of sperm limitation, when compared with females who mated with just one male alone.

In terms of quality, males might have passed sperm that proved less capable of fertilising eggs, for instance through a lack of sperm viability or motility, leading to reduced fertilisation. This would lead to more males being produced than a female attempted to allocate. Again, if males had passed sperm completely incapable of fertilising any eggs, then they would have been excluded because we could not distinguish them from cases in which the male and female just did not copulate (since 100% males would be produced in both cases), so all males in the experiments passed some sperm capable of fertilising eggs. However, variation between strains in male sperm characteristics might explain the data, with male ejaculate quality thus being a potentially important constraint on facultative sex allocation.

Alternative explanations involve one or more side effects of the interaction between the genotypes of individuals from different strains. However, male strain did not influence the number of offspring produced, as we

might expect if the results were due to differences in genetic compatibility between male and female strain influencing the survival of fertilised eggs or larvae. Females could also vary in their preference for males of different strains (Andersson 1994). In terms of pre-copulatory choice, there should have been variation in the number of females who remained unmated (i.e. virgin) with respect to male strain, but there was no such effect. In terms of post-copulatory choice, females could have chosen to either limit or not use sperm from males from certain strains, but it is worth noting that the most male-biased sex ratios were produced by females mated to males from their own strain, with which they are presumably compatible. Instead, more eggs were fertilised when females mated with males from strains different to their own. It is also worth remembering that whilst each strain was initiated from wasps from a different bird nest, they were all collected in the same locality. Therefore it would actually be quite surprising to find incompatibility between males and females from different strains.

One other possibility is that males are attempting to deliberately influence sex ratio to maximise their genetic contribution to the next generation by increasing daughter production (Hawkes 1992). In haplodiploids, daughters are the only way for males to pass on genes, and so they could be selected to increase fertilisation rate. In species with LMC, when females oviposit alone they produce highly female-biased sex ratios, but these sex ratios become increasingly less biased as more females oviposit together or as the relative brood size of a superparasitising female decreases (Hamilton 1967; Werren 1980). In these latter situations, optimal male and female sex ratios may differ, leading to a sexual conflict over sex allocation (Hawkes 1992). We are currently exploring to what extent male effects are the result of reproductive incompetence, or male attempts are to deliberately shift female sex ratios. One of our most intriguing results was that males may influence the likelihood of females ovipositing, at least when faced with an already parasitised host (females with fresh hosts oviposit very readily). Oviposition rate is one of the female reproductive traits males are known to influence via seminal products in some insects (Eberhard 1996; Chapman 2001).

The importance of clarifying the effect of males on the sex ratio is threefold. First, possible male effects represent a further class of constraint on the production of adaptive sex ratios. We have been examining constraints on adaptive sex allocation in *N. vitripennis* as part of a long-term research program, revealing in particular how information processing constrains female sex ratio behaviour (Reece et al. 2004; Shuker et al. 2004a,b; Shuker and West 2004). That males can passively or actively influence female sex ratio behaviour adds significantly to our understanding of sex allocation in *N. vitripennis*. Second, models of LMC assume, often implicitly, that females are the sole arbiters of sex ratio decisions (Orzack 1993, 2002). Whilst this may seem a reasonable assumption, evidence from *Muscidifurax raptorellus* suggests that male genotype can influence female oviposition behaviour (Legner 1988, 1989, al-

though recent data question this result, Legner and Stouthamer, personal communication), and surprisingly this has yet to be tested more widely (Hawkes 1992; Orzack 1993, 2002; but see Henter 2004). Given the often cryptic influence males can have on a variety of female reproductive behaviours (Eberhard 1996; Chapman 2001), we need to be sure whether males influence females when testing LMC models. The third reason for investigating hidden male effects on sex ratio is that they could provide an unexpected source of error in quantitative genetic studies of sex allocation. Male influence via sperm quality or seminal products therefore represents another possible environmental effect that may confound attempts to dissect genetically sex ratio behaviour (Antolin 1992).

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