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A role for sexual conflict in the evolution of reproductive traits in *Nasonia* wasps?

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Abstract
Sexual conflict theory predicts that female and male reproductive traits coevolve resulting in disruption of reproductive behaviour upon mating of individuals from diverged populations. We used interfertile species of haplodiploid *Nasonia* wasps to compare re-mating frequency, longevity, oviposition rate and sperm use of conspecifically and heterospecifically mated females. Females that first mated with a heterospecific male re-mated more often a second time, indicating that conspecific males reduce female receptivity more. Mating did not affect female lifespan. Lifetime production of sons and daughters was significantly reduced in heterospecifically mated females. Dissection of females confirmed that heterospecific sperm survives equally well as conspecific sperm during storage in the spermatheca. Differences in daily fecundity and age at which females become sperm depleted could in part be explained by species differences in ovariole numbers. We conclude that sexual conflict may play a role in the evolution of female mating rate, fecundity and sex allocation in *Nasonia*.

Keywords
Haplodiploidy, interspecific mating, male-female competition, multiple mating, sex allocation, fecundity

Introduction

Sexual conflict occurs when the genetic interests of males and females do not coincide (Chapman et al., 2003). There is growing evidence that sexual conflict is an important driving force in evolution. The evolution of anisogamy, i.e. males producing many small gametes and females few large ones, has led to inequalities between the two sexes.

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The resulting differences in selection pressures on both sexes are manifested at multiple levels, such as at the individual and the genomic level. Examples of conflict that may arise at the individual level are mating frequency, fertilization rate and parental investment (Trivers, 1972; Parker and Macnair, 1979; Chapman et al., 2003). At the genetic level, conflict can occur between loci that experience different selection pressures between males and females. This has been termed inter-locus sexual conflict and may entail coevolution between competitive male traits that are harmful for females and female responses to reduce the harm (Rice, 1998). Sexual conflict can also be manifested at the intra-locus level. Opposite selection on the same locus in the two sexes can result in a tug-of-war between males and females with each sex favouring particular alleles and/or expression levels on that locus (Chippindale et al., 2001).

Although the notion of sexual conflict as an evolutionary force is now broadly acknowledged, the number of empirical studies that have unequivocally documented its role in causing evolutionary changes is still very limited. The best examples are studies by Rice and colleagues on experimental *Drosophila melanogaster* populations (Rice, 1992, 1996; Holland and Rice, 1999). The beauty of these studies is that they manipulate the inheritance pattern of the flies in such a way that genes are passed on through only one sex. By re-establishing the normal biparental inheritance one can test the fitness effects of loci that have undergone many generations of selection in one sex. These studies reveal strong negative genetic correlation between male and female reproductive success. Objections that can be raised against these studies are that they have been performed under artificial laboratory conditions and that they are very difficult to repeat for other organisms with less easily manipulative genetics.

The role of sexual conflict in promoting speciation has been considered by various authors (Parker and Partridge, 1998; Rice, 1998; Arnqvist et al., 2000; Martin and Hosken, 2003). Several models have shown that conflict between males and females can promote population divergence, both in allopatry and sympatry (Parker and Partridge, 1998; Gavrilets, 2000; Gavrilets and Waxman, 2002). Many studies have shown differences in, for example, morphological traits between the sexes that are beneficial to one sex at the expense of the other. Even though such rapid evolution of reproductive characters may come about through antagonistic coevolution between the sexes, linking this to speciation is often still difficult (Chapman et al., 2003). There is evidence that genes involved in reproduction undergo rapid evolution and that this may lead to speciation (Swanson and Vacquier, 2002). An example are reproductive tract proteins in many insects. During copulation *D. melanogaster* males transfer more than 130 different proteins to the female in their ejaculate (Findlay et al., 2008). These proteins directly affect female behaviour and physiology and their evolution appears to be mediated by sexual conflict. Rapid divergence of proteins involved in egg-sperm interaction may lead to reproductive incompatibilities between populations.

Much of the research on sexual conflict has been confined to a limited number of taxa, which currently precludes conclusion as to the range of phenotypic traits and organismal groups involved. An interesting group of organisms for studying the scope of sexual conflict are haplodiploids, such as the hymenopteran insects. Haplodiploidy confers a unique asymmetry in the relatedness between males and females (Normark,

2006). Females are diploid and of biparental origin, but males are haploid and develop from unfertilised eggs, hence they are only related to their mother. Since in many haplodiploids females have control over the fertilization process (by storing sperm in a spermatheca after mating) they have been used extensively in the study of adaptive sex allocation (Werren, 1983; Godfray and Hunter, 1994). It has been realised that haplodiploidy leads to sexual conflict, i.e. males are selected to increase the fertilization rate of their female partners as they only gain reproductive success through fertilized eggs (Hawkes, 1992). However, thus far only Shuker et al. (2006) have reported some evidence for a male effect on female sex allocation. Moreover, as far as we know, no studies are available on a possible role of reproductive proteins in haplodiploid sexual conflict.

In this study we explore the possible role of sexual conflict in the haplodiploid wasp *Nasonia*. *Nasonia* are pupal parasitoids of cycloraphous flies and have become a model organism in genetics and evolutionary biology (Beukeboom and Desplan, 2003; Pultz and Leaf, 2003; Shuker et al., 2003). The existence of three closely related species that can still interbreed under laboratory conditions allows one to investigate the extent to which divergence of reproductive characters have occurred between these species. There are however a number of issues that need to be addressed when testing for the scope of sexual conflict in heterospecific comparisons. It is important to distinguish whether species divergence is driven by sexual conflict or the result of other processes, such as random genetic drift in isolation. Even within species it has proven difficult to test the antagonistic coevolution hypothesis in interpopulation crosses, as populations may be at different stages of male and female reproductive optima resulting in different outcomes when breaking up their coevolution (Long et al., 2006). Indeed, experiments using crosses between natural populations have yielded inconsistent results (Chapman et al., 2003), but the use of laboratory adapted strains has been suggested as a possible means to avoid some of the confounding effects (discussed in Long et al., 2006). Another potential complication when testing for sexual conflict with different species is that fitness traits, such as offspring production, can be affected by hybrid incompatibilities.

Is the structure of the *Nasonia* mating system sensitive to sexual conflict and what are the predictions of the antagonistic coevolutionary theory for a number of life-history traits? In theory, because *Nasonia* females have facultative control over offspring sex ratio, males are expected to manipulate females by increasing oviposition and fertilization rate, and reducing remating rate. On the other hand, females are expected to have evolved counter adaptations to withstand male manipulation. Mating females with foreign males will then alter female fitness in terms of survival, offspring number and sex allocation, but the direction of fitness change is hard to predict (Rowe et al., 2003). Because *Nasonia* females typically mate only once, and the spermatheca is filled with one insemination (Holmes, 1974; Van den Assem and Feuth-De Bruijn, 1977; own observations), adaptations to sperm competition are less likely to be present. Typically, female receptivity is irreversibly turned off by a bout of post-copulatory behaviour of the male (Van den Assem and Visser, 1976), but multiple mating tends to increase in laboratory populations over time (Van den Assem and Jachmann,

1999; Burton-Chellew et al., 2007). This suggests that *Nasonia* mating behaviour is not unalterable. If males affect female remating rate through their seminal products, heterospecifically mated females are expected to remate more frequently. We use interfertile crosses between species to explore a possible role of sexual conflict in the evolution of mating behaviour and life history traits in *Nasonia*.

Material and methods

Nasonia *wasps*

Nasonia are 2-3 mm large parasitoid wasps that lay their eggs in pupae of various fly species, such as Protocalliphora, that occur at bird nests and carcasses (Whiting 1967). A clutch typically consists of 20-30 eggs, depending on host size, that are mostly fertilized (female) consistent with Local Mate Competition theory (Hamilton, 1967; Werren, 1983). Male offspring emerge first and stay at the natal patch where they mate with emerging females, some of which may be sisters depending on the number of founding parental females. Females typically mate only once and disperse in search of new host patches. There are three described species in the *Nasonia* genus, *N. vitripennis*, *N. longicornis* and *N. giraulti* (Darling and Werren 1990). They are reproductively isolated in nature due to infection with *Wolbachia* bacteria that cause paternal chromosome destruction in heterospecific crosses (Breeuwer and Werren 1990). Antibiotic curing of the bacteria allows for heterospecific crosses in the laboratory despite some pre- and postzygotic barriers between the species.

Strains and conditions

The experiments, apart from the re-mating test, made use of *Wolbachia*-cured lab strains. To test for female offspring production we used strains of *N. vitripennis* and *N. longicornis* because crosses between *N. vitripennis* and *N. giraulti* result in high levels of post-zygotic incompatibilities which obscure possible effects of sexual conflict. Re-mating tests were performed on *N. vitripennis* and *N. giraulti*, because strong prezygotic isolation barriers are in effect between *N. vitripennis* and *N. longicornis* which highly complicate multiple mating experiments. For *N. vitripennis* the lab strain AsymCHS was used. It originated from Leiden, The Netherlands, and has been maintained for more than 40 years (approximately 1000 generations). For *N. longicornis* the strain IV7R2 was used. It originated from Utah, USA, and has been maintained in the laboratory for over 20 years. The re-mating experiment made use of the cured and uncured field strains Nv-ITH-3F and Nv-ITH-4C (collected at Ithaca, New York, USA in 2006) for *N. vitripennis,* and NgVA-1 and NgVA-2 (collected in Virginia, USA in 2006) for *N. giraulti*. *Calliphora vicina* fly pupae were used as hosts.

All experiments were done at 25C under constant light conditions, unless noted otherwise. Every experiment used inexperienced males and virgin females. Virgin females were obtained by opening fly hosts and separating male and female wasp pupae prior to hatching. They were transferred to 15C and constant light after emergence at 25C, to keep them alive until they were actually used in experiments. Virgin males

were either used immediately in experiments, or stored in a refrigerator at 4C in darkness, from which they were taken at least three hours before the start of the experiment. Refrigeration of males is a standard procedure in our laboratory and does not affect their behaviour and fertility (L. Beukeboom, unpublished). Moreover, mobility of sperm from refrigerator-stored males was confirmed in dissection experiments. All experiments, with the exception of the ovariole counts and sperm survival experiment, used 2-3 day old females. Mate discrimination of females declines with age and reduces the amount of prezygotic isolation in heterospecific crosses (Velthuis et al., 2005).

Re-mating

Females that were mated in a conspecific or heterospecific mating trial, were introduced to a second male 24 hours after the first copulation. The male was either a conspecific of the female's own strain or a heterospecific individual. Second males were either conspecific or heterospecific if the first male was conspecific, but when the first male was heterospecific only conspecific second males were used due to already low first male heterospecific mating frequencies. Observations lasted for 15 minutes and re-mating frequency was scored.

Copulation duration

Copulation duration was compared between conspecific and heterospecific crosses. Copulation duration can be an indication for the amount of sperm transferred during copulation (Vermeulen et al., 2008). Single inexperienced males were first introduced in a plastic tube (60 mm length, 10 mm diameter) and followed 15 minutes later by a single virgin female. The wasps were observed for 15 minutes. When copulation took place, the duration was recorded. Mated females were given two host pupae and kept on there until death, to assess female offspring production as a confirmation for copulation success.

Life-time offspring production

If male and female mating behaviour coevolved within a species, fecundity and sex allocation of heterospecifically mated females may be constrained compared to conspecifically mated females. We examined lifetime offspring production with a focus on the start and end point of daughter production. Because of haplodiploidy, the production of daughters over time reflects a female's sperm use. Directly after mating individual females were provided with two hosts for 1h and with two new hosts each subsequent hour for a total period of nine hours. It is known that *Nasonia* females have a refractory period directly after copulation during which they cannot use the sperm (Van den Assem and Feuth-De Bruijn, 1977). In this experiment we therefore compared the start of daughter production as a measure for sperm use in mated females. After the eight hourly transfers, females were put on two new pupae for the remaining hours of day one. Twenty-four hours after mating, females were transferred onto four new hosts for every 48 hours until death. Females that did not produce daughters during the first nine hours, but during the remaining hours of day 1 were assigned the first daughter

production on time point 16h (middle between 9 and 24 h), females that produced the first daughters between 24 and 72h after the start of the experiment were assigned 48h as midpoint. At the same time, virgin females of both species were provided with hosts under the same regime, as control for the possible effect of sperm on the oviposition rate in con- and heterospecific crosses. Progenies were scored in four categories: male and female, only male, only female, or no offspring. Time of the mother's death was also recorded to compare the longevity of unmated and con- and heterospecifically mated females. Females, other than the control virgin females, that did not produce daughters (N_{VxV}=1) or offspring (N_{VxV}=4, (N_{LxV}=1), (N_{LxL}=1), (N_{VxL}=0) at all were discarded. A subset of progenies was taken for counting all females and males.

Sperm survival

To determine whether heterospecific sperm and conspecific sperm survive equally well within spermathecae of the females, heterospecifically and conspecifically mated females were dissected at different time intervals after mating. Specimens were dissected alive in a drop of Beadle saline (128.3 mM NaCl, 4.7 mM KCl, 23 mM CaCl2) to determine the status of the sperm present in the spermatheca. This allows for a crude assessment of the number and mobility of spermatozoa as an indication of the viability of the sperm. Sperm was categorized as viable if the majority of visible spermatozoa appeared to be moving. To determine how fast the sperm enters the spermatheca, dissection was done immediately after copulation in conspecific crosses. Because the range of the end of daughter production in the life-time offspring production experiment was determined as day 9 for *longicornis* male x *vitripennis* female crosses and day 17 for *vitripennis* male x *longicornis* female crosses, we dissected females on day 1, 5, 9, 13, 17, 20 and 23 after start of oviposition. The dissection on day one was done after five hours of parasitizing – the time it took most females in the lifetime offspring production experiment to start producing daughters. Sperm survival was also measured by examining the viability of sperm inside the spermathecae in females that were provided with a 10% honey solution, but no hosts, at 15C after mating. Before dissection they were given two hosts for 24 hours to verify daughter production.

Ovariole counts

Because we found significant differences in daily production of offspring between the two species, we determined ovariole numbers of the two used strains. Wasps were cultured at 25C under constant light conditions and females were given two hosts for 48 hours prior to dissection. This was done to standardize development because ovariole number is phenotypically variable in *Drosophila*, with sensitivity to environmental conditions as temperature, rearing density and larval nutrition (Robertson et al., 1957; Delpuech et al., 1995; Hodin and Riddiford, 2000).

Data analysis

Re-mating frequencies were analysed with a binomial generalized linear mixed-effects model (GLMM) in version 2.6.2 of R (R Development Core Team, 2006) using the

lme 4 library (Bates et al., 2008). GLMM takes into account that the response variable is binary (e.g. re-mate or not re-mate) and errors are not normally distributed, and accounts for variation between strains within cross types. Strains were fitted in the model as random effect, while female species (*vitripennis* or *girault*i) and cross type (conspecific or heterospecific) were incorporated as fixed effects. Pairs in which second males made no attempt to court the female were excluded from analysis (<10% of males). Longevity, start of daughter production, start of offspring production, and duration of offspring production were analysed using a parametric survival analysis with a Weibull distribution. Copulation duration, and lifetime offspring production (after log-transformation) were tested using two-way ANOVAs. Survival analysis and ANOVA were done in R v2.8.1 (R Development Core Team, 2006) using the survival library. Differences in ovariole number were analysed using a Kruskal-Wallis test in Statistica.

Results

Re-mating rate

N. vitripennis females have a higher conspecific re-mating rate than *N. girault*i females (*N. vitripennis*: 49.1%, N=59; *N. girault*i: 17.1%, N=41; Chi-squared=4.47, df=1, p=0.034). Both species females are very reluctant to re-mate a heterospecific male after having mated conspecifically (*N. vitripennis*: 7.5%, N=53; *N. girault*i: 2.2%, N=45), while re-mating frequencies increased when the first male was a heterospecific male and the second male a conspecific male (*N. vitripennis*: 56%, N=25; *N. girault*i: 56.5%, N=53). This is however only significant for *N. girault*i (*N. girault*i: Chi-squared=15.9, df=1, p<0.001; *N. vitripennis*: Chi-squared=0.33, df=1, p=0.566).

Longevity

We compared female longevity in respect to their mating status, i.e. conspecifically mated, heterospecifically mated or unmated. Survival analysis did not show an effect of mating status (Chi-squared=0.73, df=2, p=0.696), but did reveal a significant effect of female species (Chi-squared=10.26, df=1, p=0.001) (fig.1). Regardless of mating status, *N. vitripennis* females lived longer than *N. longicornis* females. Hence, there appears to be no strong effect of sperm on female survival in *Nasonia*.

Copulation duration

Copulation durations of conspecific and heterospecific crosses were compared to test for mating incompatibilities (e.g. morphological or behavioural) between the two species. Copulation durations of crosses between *N. vitripennis* males and *N. longicornis* females were shorter than crosses with *N. vitripennis* females, although the difference was less than two seconds on an overall duration of 12-14 sec (ANOVA: $F_{3,119}$=5.10, p=0.002) (fig. 2). In both species, heterospecific crosses did not differ from conspecific crosses in copulation duration ($F_{1,120}$=0.149, p=0.701). However, there was an effect of

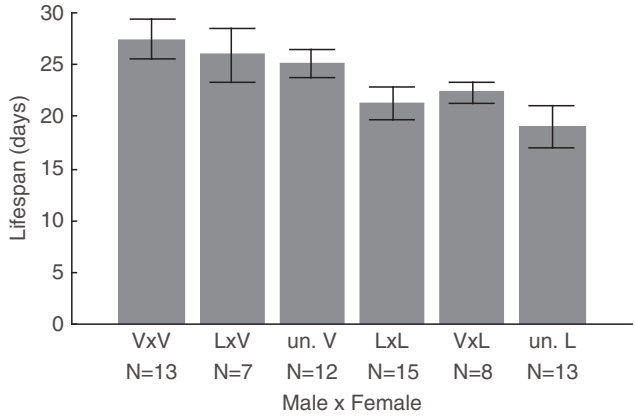

Figure 1. Mean lifespan of conspecifically and heterospecifically mated females, and unmated females, in the lifetime offspring production experiment. V = *N. vitripennis*, L = *N. longicornis*, un. = unmated.

female species ($F_{1,120}$=11.218, p=0.001), where copulations with *N. vitripennis* females lasted longer (mean±se=13.60±0.23 sec) than copulations with *N. longicornis* females (mean±se=12.47±0.25 sec). This shows that heterospecific crosses are only slightly impaired compared to conspecific crosses. Moreover, all copulations resulted in female offspring, which suggests that the speed at which sperm is transferred and the amount of transferred sperm does not differ much between conspecific and heterospecific crosses.

Start offspring production

The *N. vitripennis* females start producing offspring before the *N. longicornis* females (median *N. vitripennis*: 3.5 hours, 95%CI: 3-4; median *N. longicornis*: 16 hours,

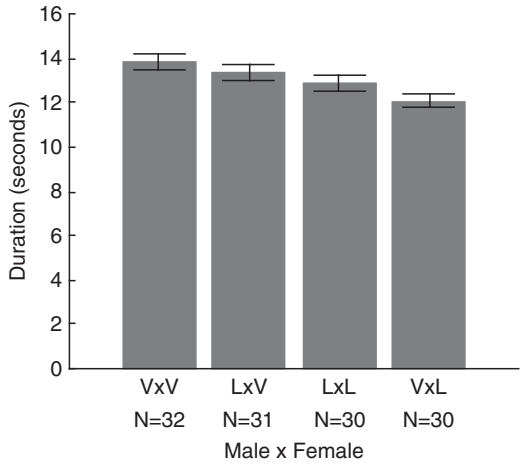

Figure 2. Mean copulation duration by species combination. V = *N. vitripennis*, L = *N. longicornis*.

95%CI: 6-16; Chi-squared=40.46, df=1, p<0.001). There is an effect of mating status (Chi-squared=11.90, df=2, p<0.001), with females mated to *N. vitripennis* males tending to start offspring production earlier (median: 4 hours, 95%CI: 3-5) than females mated to *N. longicornis* males (median: 5 hours, 95%CI: 4-16), or unmated females (median: 7 hours, 95%CI: 5-16) (fig. 3). Although the effect of mating status is significant, the differences between the mating classes are only slight. Our results therefore suggest a stronger effect of maternal species than of conspecific or heterospecific mating on first egg laying.

First sperm use

N. vitripennis females start producing fertilized eggs after mating earlier (median: 3.5 hours, 95%CI: 3-4) than *N. longicornis* females (median: 16 hours, 95%CI: 16-16, fig. 4). Survival analysis shows that not only female species significantly influences the time until first sperm use (Chi-squared=12.25, df=1, p<0.001), but that also male species has a significant effect (Chi-squared=41.45, df=1, p<0.001) on the time until first sperm use. In general, females mated to *N. longicornis* males delay the first sperm use (median: 16.0 hours, 95%CI: 16-16) compared to females that are mated to *N. vitripennis* males (median: 4.0 hours, 95%CI: 3-5). The interaction between male and female species was not significant (Chi-squared=2.94, df=1, p=0.08). However, our data indicate a trend that that *N. longicornis* females use sperm earlier when mated to *N. vitripennis* males (median: 10.5 hours 95%CI: 4-∞), than to *N. longicornis* males (median: 16.0 hours 95%CI: 16-∞). The difference in the onset of daughter production appears to be less influenced by the male partner in crosses with *N. vitripennis* females (*N. vitripennis* males: median: 3.0 hours 95%CI: 2-∞; *N. longicornis* males: median: 4.0 hours 95%CI: 3-∞). Both combinations with *N. longicornis* females also

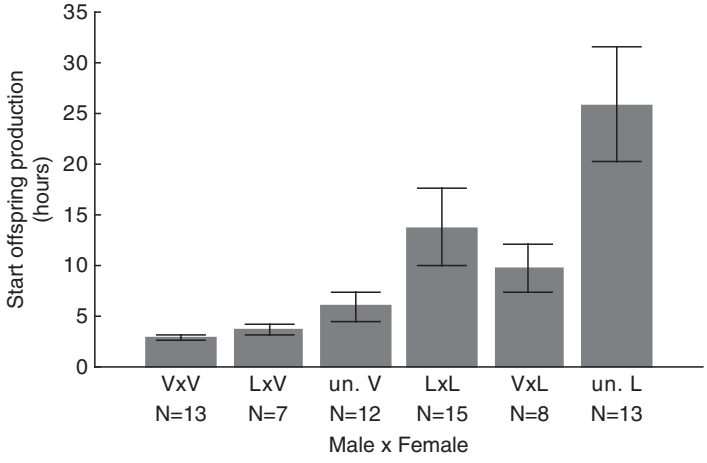

Figure 3. Mean start of offspring production in hours after copulation. V = *N. vitripennis*, L = *N. longicornis*, un. = unmated.

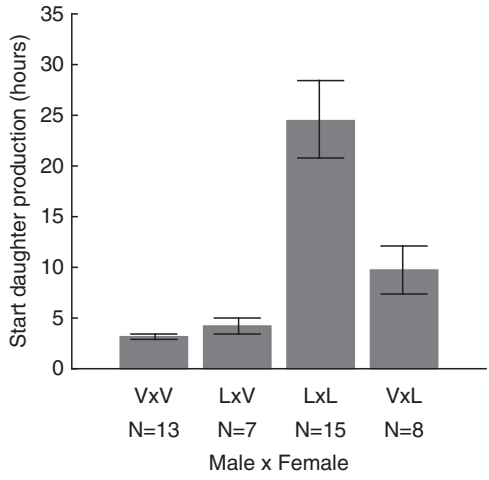

Figure 4. Mean start of daughter production in hours after copulation. V = *N. vitripennis*, L = *N. longicornis*.

show a greater variation (coefficient of variation CV=0.73) in the onset of daughter production compared to *N. vitripennis* females (CV=0.41) . The *N. longicornis* x *N. longicornis* cross is the only combination that consistently starts producing sons before daughters. The other three combinations have daughters present in the first tube with offspring, except for two *N. vitripennis* mated *N. longicornis* females.

To examine whether the delay in daughter production is caused by a slow transfer of sperm in the crosses with *N. longicornis* females, resulting in absence of sperm in the spermatheca during the first hours after copulation, wasps were dissected immediately and five hours after copulation. All dissected females had sperm in their spermathecae (N=6 and N=4 after 0h respectively N=3 and N=3 after 5h for *N. vitripennis* and *N. longicornis* respectively). Thus, the delay of daughter production of *N. longicornis* females is not caused by absence of sperm in the spermatheca. This leaves open the option that the *N. longicornis* female is not able to use the conspecific sperm shortly after copulation, or that she actively avoids to fertilize eggs during early stages of offspring production.

Lifetime sperm use

Lifetime daughter production was determined as a measure for sperm use and survival. Sexual conflict theory predicts that sperm use and survival may be altered in heterospecifically mated females due to a disruption of the interaction between eggs or the spermatheca and the sperm. We did not find a direct effect of male species on daughter production ($F_{1,19}$=2.76, p=0.113), but rather an effect of female species ($F_{1,19}$=58.58, p<0.001) and an interaction between female and male species ($F_{1,19}$=11.55, p=0.003). *N. vitripennis* females produce the highest number of female offspring irrespective of male species (fig. 5). However, for both species, mating to a heterospecific male reduces the overall daughter production.

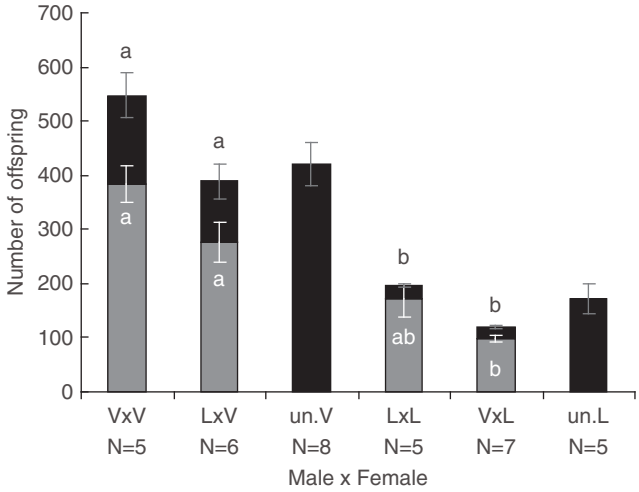

Figure 5. Total number of male (black bars) and female (light grey bars) offspring that a female produces during her lifetime. The number of sons produced by an unmated female is compared to the overall production of a mated female. Only offspring production of mated females is statistically compared. V = *N. vitripennis*, L = *N. longicornis*, un. = unmated.

For the lifetime production of sons, we found a strong effect of mating status ($F_{2,30}$=65.92, p<0.001), largely because unmated females will exclusively produce sons. *N.* vitripennis females produce more sons than the *N. longicornis* females ($F_{1,30}$=82.99, p<0.001), but this is largely due to male production at the end of their life, when no fertilized eggs are laid anymore. The interaction between female species and male partner species was significant as well ($F_{2,30}$=3.93, p=0.03), suggesting that mating to a heterospecific male reduces son production like it does for daughter production.

End of sperm use

As a measurement of the duration of sperm use, we compared the time of daughter production in respect to their male partner species. Survival analysis did not show an effect of male species on the number of days during which daughters were produced (Chi-squared=1.21, df=2, p=0.545), but did reveal a significant effect of female species (Chi-squared=11.95, df=1, p<0.001) (fig. 6). Regardless of male species, *N. longicornis* females produce daughters over a longer time (median: 16 days, 95%CI: 14-20) than *N. vitripennis* females (median: 12 days 95%CI: 10-14). For the time of son production, no effect of mating status (including unmated females) was found either (Chi-squared=0.06, df=2, p=0.973), only of female species (Chi-squared=16.78, df=1, p<0.001). However, the observed pattern was opposite for sons than for daughters, with *N. vitripennis* females producing sons over a longer period (median: 22 days, 95%CI: 20-24) than *N. longicornis* females (median: 14 days, 95%CI: 12-18).

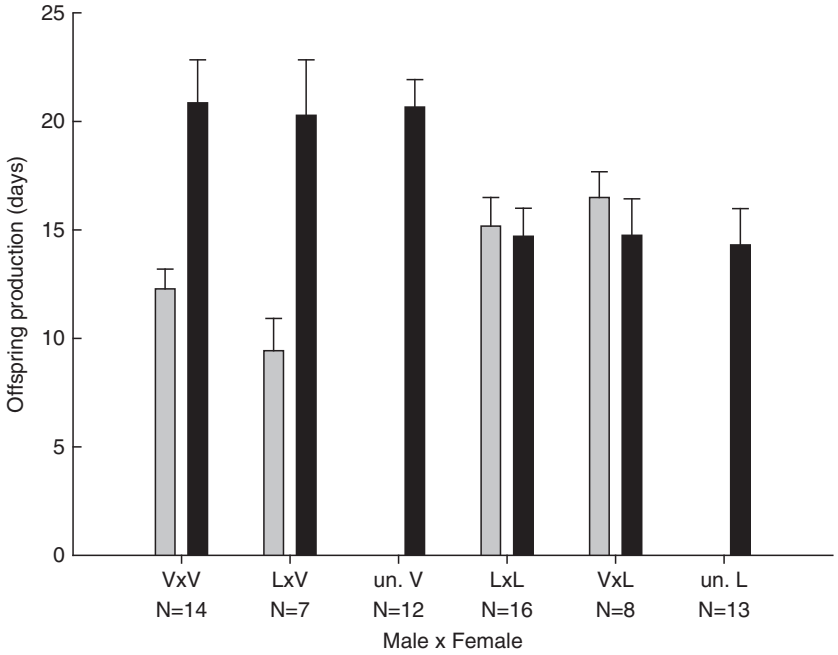

Figure 6. Total number of days that a female produces male (black bars) and female offspring (light grey bars). V = *N. vitripennis*, L = *N. longicornis*, un. = unmated.

Sperm survival

To examine if the end of daughter production was caused by a limitation in the amount of sperm, and thus an empty spermatheca, *N. vitripennis* and *N. longicornis* females from heterospecific and conspecific crosses were dissected at specific time points. In 21 out of 24 cases where an empty spermatheca was found, only males were present in the final hosts that were parasitized. The other three tubes, that still contained females, already had male-biased sex ratios, indicating that the production of females likely would have stopped soon. Each female that still had a filled spermatheca also still produced female offspring in their final hosts (N=32). This confirms that the production of female offspring was determined, and limited, by the presence of sperm in the spermatheca. In addition, it was checked whether sperm would remain alive in the spermatheca when newly emerged females were kept without hosts for 10 days, after which they were given the chance to reproduce once for 24 hours, followed by dissection. All these females had spermatheca filled with moving sperm (N=6 and N=3 in conspecifically and heterospecifically mated *N. vitripennis* females respectively), indicating that the spermatheca is not a hostile environment for heterospecific sperm at least for the first 10 days after mating.

Ovariole counts

The results of the life time offspring production experiment suggested that *N. longicornis* females have a lower oviposition rate than *N. vitripennis* females. This prompted

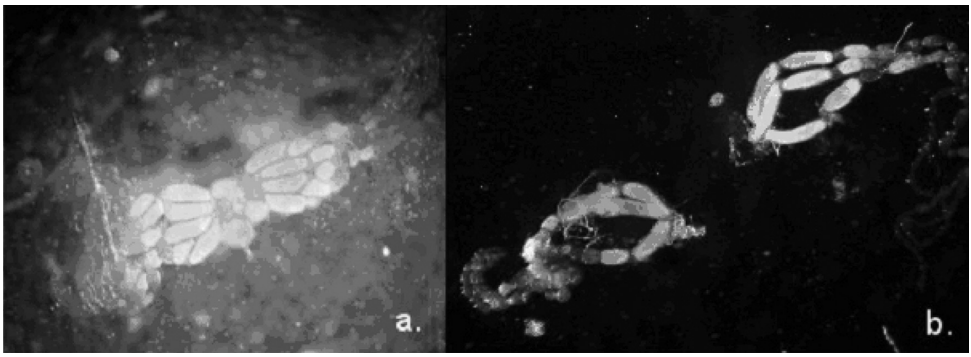

Figure 7. Ovaries of *N. vitripennis* with four ovarioles each (a) and *N. longicornis* with three ovarioles each (b). Each of the ovarioles has one mature egg at the base, followed by several eggs that are in different stages of development.

us to look for differences in ovariole numbers between the two species. Females of the *N. longicornis* strain invariably had two ovaries with three ovarioles each (fig. 7), in contrast to two times four ovarioles in *N. vitripennis* (KW-test: $H_{1,60}$=57.335, p<0.001). Two out of 29 *N. vitripennis* females had seven ovarioles (one ovary with four and one with three ovarioles).

Discussion

We considered whether *Nasonia* wasps can be used for studying the role of sexual conflict in the evolution of mating systems. From a theoretical point of view its haplodiploid reproduction calls for conflict between males and females over sex allocation, since males only gain reproductive success through daughters that develop from fertilized eggs. The existence of three interfertile species allows one to cross males and females of different species and hence disrupt mating interactions that potentially have co-evolved within species. We tested whether female receptivity and lifetime egg production and sex allocation (sperm use) differed between conspecifically and heterospecifically mated females.

We used crosses between *N. vitripennis* and *N. girault*i to determine whether female receptivity is influenced by the male partner. Although single mating appears to be the rule in nature and the spermatheca is typically filled with one copulation (Van den Assem and Feuth-De Bruijn, 1977), multiple mating is often observed in laboratory strains (Van den Assem and Jachmann, 1999; Burton-Chellew et al., 2007). We found a clear effect of male species on female re-mating rate, both *N. vitripennis* and *N. girault*i females mated more frequently after having first mated a heterospecific male. However, this increase was only significant for *N. girault*i females, which may in part be due to the higher conspecific re-mating rate of *N. vitripennis* females under lab conditions. This shows that heterospecific males are less efficient in decreasing female receptivity. At present it is not possible to differentiate between the mechanisms that

could reduce receptivity, such as differences in male postcopulatory behaviour or physiological effects of sperm in the reproductive tract of the female. Interestingly, *N. giraulti* males have longer post-copulatory courtship than *N. vitripennis* males, which may reduce re-mating rate of *N. giraulti* mated females. More information about remating rates in nature, in particular between the different species, is required to fully evaluate a possible role of conflict in the evolution of *Nasonia* mating systems.

Female survival has been found to be influenced by mating in many Diptera (Chapman et al., 1998) and by male ejaculates in *Drosophila* (Chapman et al., 1995). We found no difference in survival of either unmated and mated females, or conspecifically and heterospecifically mated females. This lack of mating costs corresponds to findings in the parasitoid *Leptopilina clavipes*, where no effect on female longevity was found in matings with arrhenotokous or thelytokous males (Reumer et al., 2007). Even though longevity data are difficult to analyse and our samples sizes are limited, our data suggest that mating in *Nasonia* does not affect female lifespan, and that ejaculates are neither harmful nor beneficial to female survival.

We first measured whether copulation duration in *N. vitripennis* and *N. longicornis* was affected by heterospecific mating. No differences were detected which suggests that sperm transfer is not mitigated in heterospecific crosses. This was further substantiated by occurrence of sperm in the spermatheca of heterospecifically mated females immediately after copulation. Thus, the transfer of sperm through the vagina and the speed with at which the sperm enters the spermatheca seem to be no obstacles in heterospecific crosses. Moreover, dissection of females that were interrupted in their copulation showed sperm present in their spermatheca which indicates that sperm is gradually transferred during copulation (data not shown). There appears to be no mechanical removal of sperm by the female, consistent with earlier findings (Holmes, 1974). The survival of sperm over time is also not affected, as females that had their reproductive period delayed by denying them access to hosts for the first ten days of their life, still appeared to have sperm in good condition in their spermatheca, regardless of whether they were heterospecifically or conspecifically mated. We therefore conclude that the spermatheca is not a hostile environment for heterospecific sperm in *Nasonia*.

The next level at which sexual conflict could be manifested is sperm use by the female. Males are predicted to increase the rate at which females fertilize their eggs. We measured lifetime egg production and fertilization rate in *N. vitripennis* and *N. longicornis*. *Nasonia* females are known to have a refractory period after copulation during which they do not use the received sperm yet (Van den Assem and Feuth-De Bruijn, 1977). We found that *N. longicornis* females take longer to start fertilizing eggs after copulation than *N. vitripennis* females, suggesting that *N. vitripennis* sperm speeds up daughter production in *N. longicornis* females. With dissection experiments we showed that the conspecific delay is not due to lack of received sperm. This result could therefore reflect decreased control of the female over the manipulative, fertilization promoting, effect of male sperm.

Heterospecific mating was found to reduce lifetime production of number of sons and daughters in both species, but this was only significant in terms of the interaction

between female and male species. Male species did not affect the duration, in terms of number of days, during which daughters and sons were produced. In addition, there were clear differences between the species in life time egg production and fertilization pattern. *N. vitripennis* produced more female and male offspring and stopped daughter production earlier. With dissection experiments we could attribute this latter effect to differences in the rate at which females become sperm depleted. These results are most easily explained with sperm depletion at the end of life of *N. vitripennis* females whereas *N. longicornis* females appear to die before they become depleted of sperm due to a lower per-day oviposition rate. However, these species differences in fecundity cannot explain the male species effect on offspring numbers of both sexes. Hence, heterospecific sperm appears to affect oviposition rate and sex allocation.

The difference in egg production between the species was supported by an ovariole number of six in *N. longicornis* and eight, with two exceptions, in *N. vitripennis* Some variation in ovariole number of *N. vitripennis* was also reported by King and Ratcliffe (1969), who found few individuals with fewer or more ovarioles than eight. The lower number of ovarioles of *N. longicornis* corroborates the lower fecundity of this species.

There are a number of potential complications with using interpopulation crosses and two different species when studying the scope of sexual conflict (for more details see Long et al., 2006). Except for the re-mating rate experiments, we used one highly inbred strain of each species that had been maintained in the laboratory for several hundreds of generations. We can therefore not exclude that our results have been influenced by laboratory culturing, nor that variation in the observed traits exist in nature. Reduced fitness of hybrids between two species may also influence our results, for example if hybrid females deriving from heterospecific crosses have reduced viability. This would result in an underestimation of the number of daughters, and overestimate a potential effect of sexual conflict. To rule out the possibility that the reduction of daughters in heterospecific crosses was caused by inviability of the hybrid daughters, the numbers of eggs laid were compared to the number of adult offspring emerging from the host pupa of conspecifically and heterospecially mated females. Females were given two host pupae for 24h after which one pupa was fixated (in Carnoy's fixative, 3 parts ethanol and 1 part acetic acid for 24 hours at -20C) to count the wasps' eggs and the second pupa was left to develop at 25C to count the emerging offspring. None of the crosses showed a significant difference between the number of eggs and the number of adult offspring (data not shown). We could thus rule out that our results were affected by hybridization artifacts,

A more fundamental issue is how population genetic differentiation influences the outcome of sexual conflict studies (Chapman et al., 2003). Different species are expected to accumulate reproductive incompatibilities over time as a result of genetic drift or adaptation, which would in part yield similar predictions as sexual conflict (Rice, 1998; Gavrilets and Waxman, 2002). As in many other studies involving interpopulation crosses (Long et al., 2006) it is hard to distinguish between these alternative explanations for our results without a better understanding of the genetic basis of female receptivity and egg fertilization behaviour. The availability of whole genome sequences of all three *Nasonia* species may be very a useful in this respect.

Thus far the number of empirical studies of sexual conflict are still scarce and mostly limited to *Drosophila* (Chapman et al., 2003). We have found that a number of female mating behaviour and life history traits are altered by mating with foreign males. This shows that the *Nasonia* sibling species complex is a potential candidate for further experimental testing of predictions of sexual conflict theory. Haplodiploidy confers an extra dimension to the study of sexual conflict because of the asymmetries in relatedness between parents and offspring. More studies on different haplodiploid species are needed to determine how important sexual conflict is in driving the evolution of haplodiploid mating systems.

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