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Reproductive strategies under multiparasitism in natural populations of the parasitoid wasp Nasonia (Hymenoptera)

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diapause; local mate competition; microsatellites; Nasonia giraulti; Nasonia vitripennis; resource allocation; sex ratio; superparasitism.

Abstract
Parasitoid Nasonia wasps adjust their progeny sex ratio to the presence of conspecifics to optimize their fitness. Another trait under female control is the induction of offspring diapause. We analysed progeny sex ratios and the proportion of diapausing offspring of individual Nasonia females in host patches parasitized by two species, Nasonia vitripennis and Nasonia giraulti, in North American field populations using microsatellite fingerprinting. Both Nasonia species produced similar sex ratios on hosts that were co-parasitized by their own species as by the other species, indicating that females do not distinguish between con- and heterospecific clutches. The sex ratios of the diapause and adult fractions of mixed broods from single females were not correlated. We found further indications that N. vitripennis females take the emergence time of the offspring into account in their sex allocation. The reproductive strategies of Nasonia under multiparasitism are largely adaptive, but also partially constrained by information.

Introduction
The evolution of life-history traits leads to intricate adaptations to maximize fitness in a local environment (Stearns, 1992). Parasitoid wasps have been used extensively for studying adaptation, especially with respect to foraging behaviour and sex allocation (Shuker & West, 2004 and citations therein). Parasitoids have only a limited amount of resources to allocate for the development of their offspring. Therefore, the quality of the host plays a major role in the optimization of resource allocation (Harvey, 2005). If there are enough resources, a parasitoid should prefer to parasitize unparasitized hosts only. However, under natural conditions there is ample competition with con- and heterospecific foundresses. In addition, a founding parasitoid has to deal with variation in host quality and density as well as abiotic factors, such as temperature and seasonality. Facing all these challenges, every parasitoid uses certain reproductive strategies to maximize its fitness and, in the course of time, natural selection will lead to adaptation to the local environmental conditions.

The gregarious wasps (females lay more than one egg in a host) of the genus Nasonia are pupal parasitoids of cyclorrhaphous flies found on carcasses and in bird nests (Whiting, 1967). In this genus, three species are known: Nasonia longicornis that is only found in the west of North America, Nasonia giraulti that is restricted to eastern North America, and Nasonia vitripennis, which is cosmopolitan and occurs in close sympathy with its sister species in North America (Darling & Werren, 1990). The three species are closely related and phylogenetic studies revealed that the lineages split rather recently (between 0.2 and 1 Mya; Campbell et al., 1993). The species are reproductively isolated due to Wolbachia infection with incompatible strains (Breeuwer & Werren, 1990). However, cured strains produce viable and fertile hybrid offspring (Breeuwer & Werren, 1995). Behavioural studies have shown that there are clear differences in courtship between the three species (van den Assem & Werren, 1994; Beukeboom & van den Assem, 2001), which, together with Wolbachia infection, makes the occurrence of hybrids in nature very rare.
Nasonia vitripennis has been widely used as a model organism to study sex ratio adjustment in the framework of local mate competition (LMC) theory (Werren, 1984; Drapeau & Werren, 1999; Shuker et al., 2005, 2006). LMC theory assumes that a female has control over the sex ratio of its offspring and can maximize its fitness by reducing the competition between its sons. This is an evolutionarily stable strategy if males are not the dispersing sex and if mating only takes place at the natal patch (Hamilton, 1967). In such a mating system, all males are confined to mate with the females that are available at their natal patch. If the patch population consists of offspring of a single female, males are brothers and it is beneficial for the foundress female to shift the progeny sex ratio strongly towards daughters to reduce competition among its sons. With increasing foundress number, competition between unrelated males increases and therefore selection favours females that produce more males to increase the chance that their sons mate with daughters of other females as well. This leads to a less female-biased sex ratio. The resulting prediction is that the progeny sex ratio in a patch is a function of the number of females ovipositing on that patch (Hamilton, 1967). It has been shown in the laboratory (Werren, 1984; Drapeau & Werren, 1999; Shuker et al., 2005, 2006) and field (Werren, 1983; Molbo & Parker, 1996; Burton-Chellew et al., 2008) that females of all three Nasonia species follow the predictions of LMC theory quite closely, when they are the only Nasonia species in a patch.

The expectation from LMC theory for a foundress that encounters heterospecific eggs in a host is that there is no sex ratio response, but only resource competition. The oviposition response of a parasitoid female to the presence of a female of a closely related species has been investigated by several authors in the laboratory: Vet et al. (1984) found that Asobara tabida and Asobara rufescens mutually avoided parasitism of hosts parasitized by the other species; Wylie (1973) found that N. vitripennis differentiate between hosts parasitized by a conspecific and Muscidifurax raptor; A.B.F. Ivens (unpublished data) found that N. vitripennis and N. longicornis differentiate between conspecific and heterospecific co-foundresses regarding host acceptance, but not with respect to progeny sex ratios. Here, the focus will be on the sex ratio response of N. vitripennis and N. giraulti in a sympatric field situation.

In temperate regions, most insects can only survive the winter in diapause. An important life-history decision is therefore when to enter diapause. The cues that lead to a switch from normal development to diapause appear highly variable among the insects. Photoperiod, light intensity, temperature, thermoperiod, food, moisture, density, mating status, chemical cues and even the presence of predators have been found to affect diapause (Danks, 2007 and citations therein). In most insects, the diapause inducing cues are experienced by the individual going into diapause. In Nasonia, however, the mother is in control of the developmental mode of its offspring. The main cue influencing the females’ decision is the photoperiod, whereas temperature, food shortage and age appear to have a modulating effect (Saunders, 1965a,b, 1966a,b).

In an evolutionary context, the question arises of how a Nasonia foundress uses the limited resources of a host for its sons and daughters, as well as for adult and diapausing offspring, in the presence of a closely related species that is competing for the same resources. We investigated this in natural populations of N. vitripennis and N. giraulti in North America by fingerprinting offspring of host patches (bird nests) that were parasitized by both species. We ask the following questions: (i) is the host usage within and between nests random, or do the species actively avoid each other? (ii) do the two species produce different sex ratios when encountering their own species vs. the other species on a host? (iii) does a foundress produce a different sex ratio when encountering their own species vs. the other species on a host? (iv) does a foundress produce the same sex ratio among its diapausing and adult offspring? (v) does a foundress produce a different sex ratio in the presence of diapaus- ing vs. adult offspring of another foundress? (vi) does a foundress produce the same brood size when producing diapausing vs. adult offspring?

**Material and methods**

**Sample collection**

In July 2005, we collected all fly host pupae out of 108 nest boxes from three regions in New York State in the USA (near Brewerton 32, Ithaca 53, Rochester 23). Most nest boxes were used by tree swallows (101), some by eastern bluebirds (6) and one by chickadees. The pupae were incubated individually at room temperature until either a fly or parasitoids emerged. If nothing emerged after 3 weeks, the hosts were opened to check for diapause larvae. Adult parasitoids and diapause larvae were stored in 96% alcohol and empty hosts were discarded. For all adult Nasonia, the species and sex were determined. As females of all Nasonia species are phenotypically similar, we determined the species on the basis of wing length of males; N. giraulti has long wings, N. vitripennis short wings (Darling & Werren, 1990). For a subset of the diapause larvae, the species and sex were determined after genetic analysis (see below).

**Genetic analysis**

For a more detailed analysis of the frequency and offspring sex ratios of the two species within a nest, genetic information was required. Three nest boxes from the study site close to Brewerton (BR12, BR23 and BR29) were chosen, which contained males from both species.
and had a number of progeny that could be logistically processed (up to a total of 2000 individuals). These three nest boxes contained a total of 84 parasitized hosts, which can be seen as independent data points regarding sex allocation as has been shown by Grillenberger et al. (2008). DNA was extracted from all individuals using a standard high salt protocol (Maniatis et al., 1982).

All individuals were genotyped for seven polymorphic microsatellites using the Qiagen Multiplex kit (see Table 1 for primer details) and fragment lengths were determined on an ABI 3730XL sequencer (Applied Biosystems, Carlsbad, CA, USA). All individuals were assigned into fullsib families using three simple rules of haplodiploid inheritance (Grillenberger et al., 2008): (i) a female can maximally provide two alleles per locus; (ii) the father can only provide one allele per locus (being haploid) that is shared by all full sisters; (iii) sons can only have an allele from their mother, as they develop from unfertilized eggs. For a first assignment, the software COLONY 1.2 (Wang, 2004) was used. To correct for genotyping errors, the software output was then revised by hand and similar families were combined. Clear differences in allelic composition between the two species allowed species identification based on the microsatellite fingerprint. To ensure correct species identification, one individual per family was sequenced for a 400-bp fragment of the mitochondrial cytochrome oxidase I gene (COI). Primers were designed on the basis of the N. vitripennis COI sequence from GenBank (acc. no. LOC100113910) (fwd-primer: 5′-GTT ATA CCT KTW ATA ATW GGA GGA TT TGG-3′, rev-primer: 5′-CTT TGA CAG CCG CAC AAA-3′). The resulting sequence was aligned with sequences of known origin. B.K. Grillenberger, unpublished data). The resulting sequence was aligned with sequences of known origin. This used COI fragment differs in 15% of nucleotides between N. vitripennis and N. giraulti (B. K. Grillenberger, unpublished data). For diapause larvae, the sex was determined by the number of loci that were homozygous. Individuals homozygous for all seven loci were scored as males (hemizygous haploids), all other individuals as females (diploids). Using this information, clutch size and sex ratio of individual parental females were determined, as well as the number of co-founding females per host.

**Definition of terms**

To avoid confusion, we will define some terms that we use in the rest of the paper. A female that is laying eggs is called a foundress. The foundresses are parasitizing fly pupae that are called hosts and these hosts are found in bird nest boxes. In a nest box, we find both unparasitized and parasitized hosts. Hosts parasitized by a single foundress are called single foundress hosts. If two or more foundresses of the same species parasitized a host, this is called superparasitism, whereas if a host is parasitized by one or more foundresses of more than one species, the situation is called multiparasitism. A clutch is the eggs of an individual foundress laid in a single host. The foundress’ brood is the actual offspring of the foundress in a single host observed in this study. A foundress can parasitize several hosts and can therefore have several broods. A brood that consists of adult offspring only is called an adult brood while a brood that contains only diapausing offspring is called a diapausing brood. Mixed broods contain both diapausing and adult offspring.

**Statistical analysis**

Previous research has shown that a Nasonia foundress bases its sex ratio decision on a host level rather than nest

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**Table 1 Chromosomal location, primer sequences, number of alleles, Nei’s overall expected heterozygosity \( (H_t) \) (Nei, 1987) and annealing temperatures of seven microsatellites used.**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome*</th>
<th>Primer sequences</th>
<th>Allele no.</th>
<th>( H_t )</th>
<th>Ann. temp.</th>
<th>Species specific?</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nv-104</td>
<td>IV</td>
<td>5′-GAT TTC CGG GAG CCT CCT AC-3′</td>
<td>8</td>
<td>0.859</td>
<td>55 °C</td>
<td>Yes</td>
<td>FJ156110</td>
</tr>
<tr>
<td>Nv-109</td>
<td>V</td>
<td>5′-GCC GTT AAT TGA ACC TGT CG-3′</td>
<td>10</td>
<td>0.880</td>
<td>55 °C</td>
<td>Yes</td>
<td>FJ156114</td>
</tr>
<tr>
<td>Nv-111</td>
<td>III</td>
<td>5′-AGG TCT CGG CGG CAC AAA-3′</td>
<td>16</td>
<td>0.962</td>
<td>55 °C</td>
<td>Yes</td>
<td>FJ156115</td>
</tr>
<tr>
<td>Nv-114</td>
<td>IV</td>
<td>5′-GCA GCT GCT TTT GGC ACT-3′</td>
<td>15</td>
<td>0.826</td>
<td>55 °C</td>
<td>No</td>
<td>FJ156231</td>
</tr>
<tr>
<td>Nv-300</td>
<td>II</td>
<td>5′-ATG GGC AAT AAA ACG AAA CG-3′</td>
<td>3</td>
<td>0.676</td>
<td>55 °C</td>
<td>Yes</td>
<td>FJ156211</td>
</tr>
<tr>
<td>Nv-305</td>
<td>I</td>
<td>5′-GCA GAG CAG GAG ATT AT-3′</td>
<td>10</td>
<td>0.705</td>
<td>55 °C</td>
<td>Yes</td>
<td>FJ156221</td>
</tr>
<tr>
<td>Nv-313</td>
<td>V</td>
<td>5′-GCA GCT GCT GGT TAA GTG TG-3′</td>
<td>19</td>
<td>0.902</td>
<td>55 °C</td>
<td>Yes</td>
<td>FJ156221</td>
</tr>
</tbody>
</table>

*Chromosome designation according to Rutten et al. (2004).
To test whether a foundress produces a higher sex ratio in single- vs. multi-parasitism, we compared the observed sex ratio frequencies with the expectation under a binomial distribution using Fisher’s exact test. To do this, the total number of hosts available for parasitism at one moment in the patch has to be known. In this study, it was only observed whether a host was parasitized, or not, but it is unknown for what reason a host is unparasitized. Hence, it was not possible to distinguish between hosts that might have been available for parasitism but were not chosen (the number that is required for estimating the random distribution), and the hosts that were not suitable for parasitism. As host number appears to be the limiting factor for Nasonia, we excluded in our analysis hosts that had not been parasitized by at least one species, assuming that they were not available for parasitism.

To compare sex ratios produced under single-, multi- and superparasitism, we distinguished three classes of broods: (i) The single foundress case, where a host contains offspring of one foundress only; (ii) the multiparasitism case, where a single brood of the focal species foundress is accompanied by one or more broods of the other species; (iii) the superparasitism case, where two or more broods of the same species are present in a host without the presence of the other species.

For both species, we used Kruskal–Wallis post hoc tests for pairwise comparisons between the three brood classes (Zar, 1999).

To test whether a foundress produces a higher proportion of diapausing offspring when encountering other foundresses, we first compared the observed frequencies of diapausing broods with the expectation under a binomial distribution with a χ²-test. Additionally, we investigated whether the proportion of diapausing offspring per host is correlated with the number of foundresses, using a Spearman’s rank correlation.

To test for differences between the sex ratio of diapausing and adult broods, we investigated whether the sex ratio of adult offspring resembles that of the sex ratio of diapausing offspring produced by the same female using a Spearman’s rank correlation. Furthermore, we compared the sex ratio of adult and diapausing broods in single- and superparasitism hosts, using Mann–Whitney U-tests.

To test whether a foundress produces a different sex ratio in the presence of diapausing vs. adult offspring of another foundress, we compared the brood sex ratio of the following classes using Kruskal–Wallis H-tests: (i) single foundress diapausing broods; (ii) single foundress adult broods; (iii) hosts with diapausing broods of a single foundress accompanied by one or more adult broods; (iv) hosts that contained multiple diapausing and at least one adult brood. Categories (iii) and (iv) were also compared using a Mann–Whitney U-test.

To test whether a foundress produces the same brood size when producing diapausing vs. adult offspring, we compared the brood size of diapausing, mixed and adult broods, using Kruskal–Wallis H-tests and Nemenyi post hoc tests.

All statistical tests were performed with R (R Development Core Team 2006). All sex ratios are given as the proportion of males, and averages are calculated after arcsin square root transformation and back transformation following Wilson & Hardy (2002). As N. giraulti hardly produced any diapause larvae, the diapause analysis was performed on N. vitripennis data only.

Results

Species abundance and distribution across hosts

Out of the 108 nests collected, 64 contained fly pupae (a total of 2043 pupae), of which 58 (91%) were parasitized by Nasonia. Seventeen nests (29%) contained both N. vitripennis and N. giraulti, 39 (67%) contained only N. vitripennis and no nests contained exclusively N. giraulti (for two nests the species could not be determined). The number of hosts per infested nest ranged from 1 to 138 (mean 31.9). The proportion of hosts parasitized per nest ranged from 1% to 100% (mean 48%), excluding infested nests that did not yield Nasonia.

The three nests that were chosen for the detailed analysis (BR29, BR23, BR12) contained 170 hosts, 84 (49%) of which yielded Nasonia offspring (Table 2). We genotyped a total of 1906 individuals for seven microsatellites. Fifty-one samples did not amplify and seven could not be assigned to a foundress. Six of the seven markers showed clear allelic differences between the two species (detailed information upon request from the author), and species assignment based on the microsatellite data was always in concordance with the mitochondrial sequence data (Table 1). For the remainder of the analysis, we focused on the three genetically analysed nest boxes.

There were large differences in the composition of the three nests. The largest nest contained 63 parasitized hosts, yielding 19 foundresses. The smallest nest contained seven parasitized hosts and six foundresses. Thirty-five of 84 parasitized hosts yielded both N. vitripennis and N. giraulti (41.7% multiparasitation rate, all three nests pooled). The number of broods and the average brood size were slightly higher for N. vitripennis than for N. giraulti (118 vs. 90 broods, 9.62 ± 0.68 vs. 7.92 ± 0.75 average brood size, Mann–Whitney U-test: U = 5988.5, P = 0.11). Additionally, there were more N. vitripennis than N. giraulti foundresses (N. vitripennis: 15, N. giraulti: 12). These differences result...
Table 2: Overview of the three fully analysed nests.

<table>
<thead>
<tr>
<th>Host no.</th>
<th>% Host parasitized</th>
<th>% Host multiparasitized</th>
<th>% Host superparasitized</th>
<th>No. of hosts used</th>
<th>No. of hosts parasitized</th>
<th>No. of hosts multiparasitized</th>
<th>No. of hosts superparasitized</th>
<th>Average sex ratio per host</th>
<th>Average brood sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR29</td>
<td>85</td>
<td>63</td>
<td>49</td>
<td>5</td>
<td>32</td>
<td>19</td>
<td>5</td>
<td>0.45 ± 0.05</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>BR22</td>
<td>64</td>
<td>44</td>
<td>32</td>
<td>0</td>
<td>18</td>
<td>10</td>
<td>2</td>
<td>0.50 ± 0.05</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>BR12</td>
<td>21</td>
<td>14</td>
<td>14</td>
<td>0</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>0.50 ± 0.05</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>Overall</td>
<td>170</td>
<td>84</td>
<td>71</td>
<td>15</td>
<td>63</td>
<td>45</td>
<td>15</td>
<td>0.45 ± 0.03</td>
<td>0.36 ± 0.03</td>
</tr>
</tbody>
</table>

Shown on the left are the total number of hosts found in a nest, the total number of hosts parasitized per nest and the fraction of hosts multiparasitized. The right shows per species and nest: the number of hosts parasitized, the fraction of hosts superparasitized, the total number of offspring, the fraction of offspring in diapause, the total and average foundress number per host, the nest-wide sex ratio and brood size, and the average host and brood sex ratio. UN ind., the number of individuals unassigned; NA ind., the number of individuals that gave no PCR amplification; NV, Nasonia giraulti; NG, Nasonia vitripennis.

Sex ratios

Overall sex ratios (given as proportion males) of *N. vitripennis* were lower than that of *N. giraulti*. Mean sex ratios per host for *N. vitripennis* were 0.34 ± 0.003 (n = 63, median = 0.3), and were 0.43 ± 0.004 (n = 57, median = 0.33) for *N. giraulti*; however, the Mann–Whitney U-test was not significant (U = 1666, P = 0.5) (Table 2). The sex ratios from single foundress hosts were significantly lower than broods in multi- and superparasitized hosts in *N. giraulti*, as expected from the stronger degree of LMC in the former case. In *N. vitripennis*, only the difference between single parasitism and multiparasitism was significant (Kruskal–Wallis H-test within species: *vitripennis*: H2 = 7.29, P = 0.03; *giraulti*: H2 = 16.04, P < 0.001; pairwise comparisons with Nemenyi test: *vitripennis*: single-multi: P = 0.03, single-super: P = 0.42, super-multi: P = 0.72; *giraulti*: single-multi: P = 0.008, single-super: P < 0.001, super-multi: P = 0.61; Fig. 2). This indicates that under multiparasitism both species are producing a sex ratio more similar to the superparasitism case than to the single foundress case, which is in contrast to expectation, as only conspecific competitors play a role in LMC. The variance in the multi- and superparasitism classes is much larger than in the single foundress class. The comparison between the two species within each class showed a significantly higher sex ratio in the case of superparasitism involving *N. giraulti* (Mann–Whitney U-test: *giraulti* super-*vitripennis* super, U = 166.5, P = 0.043). This means that *N. giraulti* shows a stronger within-species LMC response than *N. vitripennis*.

The proportion of offspring in diapause differed greatly between the two species. Thirty-nine per cent of all *N. vitripennis* offspring were in diapause compared to only 0.4% in *N. giraulti*. The distribution of *N. vitripennis* diapausing broods across hosts within the largest nest box...
(BR29) did not differ from a random distribution ($\chi^2$-test against binomial distribution, combining the low-represented classes, $\chi^2_2 = 1.20$, $P = 0.55$). There was no correlation between the proportion of diapausing offspring per host and the total foundress number (all *N. vitripennis* foundresses) per host (Spearman: $S = 49 \, 947.8$, $\rho = -0.199$, $P = 0.118$; Fig. 3).

The sex ratio of adult and diapausing offspring of an individual foundress was not correlated, and there is no consistent direction in the difference in sex ratio between diapausing and adult offspring (Fig. 4; Spearman’s rank correlation: $S = 1082.4$, $P = 0.43$, $n = 20$ broods of 9 foundresses). This indicates that the sex ratio of the diapausing and adult fractions of a brood is independent. The brood sex ratios of single foundresses and foundresses under superparasitism producing diapausing or adult offspring did not differ from each other (Mann–Whitney U-test: single dia–single adult: $U = 25.5$, $P = 0.919$; super dia–super adult: $U = 21.5$, $P = 0.796$; Fig. 5). This indicates that superparasitism does not differentially affect the sex ratio of diapausing and adult offspring.
The brood sex ratios of single diapausing broods, single adult broods and single diapausing broods accompanied by adult broods did not differ significantly (Kruskal–Wallis $H$-test: $H_2 = 0.0127, P = 0.99$; Fig. 6). The sex ratios of diapausing broods accompanied by other diapausing and adult broods, however, were significantly higher than the sex ratio of single diapausing broods accompanied by only adult broods (Mann–Whitney $U$-test: $U = 7$, $P = 0.031$; Fig. 6). This suggests that females producing diapausing broods increase their sex ratio as a function of the presence of other diapausing broods in a host.

Mixed diapause–adult broods were significantly larger than pure adult broods, whereas the difference between pure diapause and mixed broods was not significant (Kruskal–Wallis $H$-test: $H_2 = 11.1228$, $P = 0.004$; pairwise comparison with Nemenyi’s test: adult–mixed: $P = 0.007$, diapause–mixed: $P = 0.19$, diapause–adult: $P = 0.44$; Fig. 7). This could indicate that mixed broods are the product of foundresses that sequentially produced an adult and a diapause brood in the same host (self-superparasitism).

Discussion

Species distribution in the field

Our data clearly show that, in the three nests that were chosen for detailed analysis, the two species, *N. vitripennis* and *N. giraulti*, encounter each other very frequently, as the multiparasitism rate is very high (42%). The level of superparasitism in *N. vitripennis* (45%) is comparable to that found in a European *N. vitripennis* population (39%, Grillenberger et al., 2008), as well as that found in *N. giraulti* (42%). These results are in agreement with the data on foundress numbers, which appear to be comparable for the two species. Hence, the species densities...
appear to be similar in our research area. For *N. vitripennis*, the density is also more or less equal to the allopatric population in Europe (assuming an equal abundance of hosts), where similar foundress numbers of one to seven per nest box were found (Grillenberger et al., 2008).

The distribution of foundresses across hosts between species was significantly different. The main difference is in the number of single-species parasitized hosts (*N. vitripennis* occurs more often alone in a host than *N. giraulti*), which could indicate that *N. giraulti* is more selective regarding the host quality. The across host distribution within species was not different from a random distribution. This gives no indication of clumping of individuals of the same species, or avoidance of the other species in host acceptance. It is conceivable that the random distribution is a product of the lack of choice between available hosts, where similar foundress numbers of one to seven per nest box were found (Grillenberger et al., 2008).

**Fig. 7** Box and Whisker plots showing *Nasonia vitripennis* brood sizes of pure diapausing (100% dia), pure adult (100% adult) and mixed broods. Numbers in brackets refer to sample sizes (Kruskal–Wallis H-test: $H_2 = 13.69, P < 0.05$; pairwise comparison with Nemenyi’s test, significance indicated by different capital letters in the plot).

Surprisingly, the sex ratios produced by *N. giraulti* are higher than those produced by *N. vitripennis*. This is in contrast to the findings of King & Skinner (1991) and Drapeau & Werren (1999), who found lower sex ratios in *N. giraulti* in a laboratory experiment under both single- and superparasitism conditions, and interpreted this as the consequence of frequent within host mating in *N. giraulti*. The difference in sex ratios seems to be largely due to the significantly higher sex ratios of *N. giraulti* under superparasitism (Fig. 2). This indicates that *N. giraulti* shows a stronger LMC towards conspecifics than heterospecifics, and that its superparasitism response is stronger than in *N. vitripennis*.

Sex ratios in a two species situation

Our results show no indication that either *N. vitripennis* or *N. giraulti* is able to distinguish between conspecific or heterospecific broods in their sex allocation (Fig. 2). If they distinguished, the multiparasitism sex ratios would be expected to be indifferent from the single foundress sex ratios. An issue with studies using field data is the lack of information on parasitization sequence, as the first foundress on a patch is expected to produce a strongly female-biased sex ratio and later foundresses higher proportions of males (e.g. Hamilton, 1967; Werren, 1980; Shuker et al., 2005). To avoid this problem, we compared single foundress broods as one extreme case with superparasitized broods as the counterpart. As the multi-foundress classes contain both first and subsequent foundresses, the variance of sex ratios produced is large.

The multiparasitism sex ratios resemble those of superparasitism more than the single foundress sex ratios. Wylie (1965) found that *N. vitripennis* foundresses cannot detect whether a host has been parasitized previously, until its ovipositor has been inserted into the puparium. In a later study, Wylie (1973) showed that *N. vitripennis* females are able to distinguish between eggs of their own species and eggs of the heterospecific competitor *M. raptor*, as they showed a stronger sex ratio response when encountering conspecific eggs. A.B.F. Ivens (unpublished data) found in a laboratory experiment with *N. vitripennis* and *N. longicornis* that both species show a lower acceptance of hosts that have been parasitized by a heterospecific female compared with a conspecific. However, in agreement with our data, once multiparasitism occurs, there is no influence of multiparasitism on the sex ratio adjustment. This means that *N. vitripennis* is able to distinguish the eggs of *M. raptor* from its own species eggs (Wylie, 1973), but not the eggs of its sibling species *N. longicornis*, or *N. giraulti* as shown by our data. Martel & Boivin (2004) found in a lab...
experiment that Trichogramma minutum produced a different sex ratio when encountering heterospecific competitors, compared to conspecific competitors, but a closely related species (Trichogramma pintoi) did not show a differential response. They explained this by differences in the distribution range, as T. minutum lives sympatrially with other Trichogramma species in its natural habitat, whereas T. pintoi does not. As N. vitripennis and N. giraulti live microsympatrially in our research area, the most likely explanation for our results is that both species have not diverged far enough to discriminate between con- and heterospecific eggs.

Offspring in diapause

We found a difference in the occurrence of diapause between the two species. Nasonia vitripennis produced 39% diapause larvae, while N. giraulti produced only 0.4% (Table 2). This is surprising as both species have been collected at the same time of the year at the same location. Hence, the climatic factors potentially inducing the production of diapausing offspring by adult females were identical. Considering that the sampling took place early July, and the active season for Nasonia in the area runs until September, the difference is even more puzzling. In a European N. vitripennis population (the Netherlands) at approximately the same time of year in 2004, few diapause larvae were found (T. Koevoets, unpublished data). This indicates that there is large intra- and interspecific variation for the response of Nasonia to diapause inducing cues, and/or in the actual cues used.

Saunders (1965b) showed that a female’s age plays a major role in the proportion of diapause larvae produced. From our data, it is not clear whether all eggs of one female were laid at once, or whether sequential parasitization events by the same foundress occurred. It could be that the first clutch contained mostly adult offspring and a later clutch contained mostly diapausing offspring, as the female aged one or more days in the meantime. Self-supersparasitism would presumably result in a larger mixed brood. This explanation is consistent with the larger brood size of females in mixed (diapause–adult) broods compared with pure broods of either type (Fig. 7).

Saunders (1962, 1965b, 1966a, 1973) also showed that stressed females (high and low temperatures, host shortage) tend to produce more diapausing offspring. A correlation between the total number of foundresses per host, which could be considered a stress situation in the form of crowding, and the proportion of diapausing offspring was not found in our study. Diapause is usually seen as a state to survive the winter (Danks, 2007 and citations therein). However, in the case of diapause larvae in July, it is questionable why N. vitripennis females do not produce another adult generation. This suggests that there are other cues triggering the production of diapausing offspring. One possibility could be the presence of its sibling competitor N. giraulti in a nest box. Whether there is a difference in the proportion of diapausing individuals among N. vitripennis offspring in allopatric and sympatric populations remains to be tested.

Sex ratio and diapause

We did not find a correlation between the sex ratio of adult and diapausing offspring within one brood. Although this could be due to low statistical power, it could be the outcome of different sex allocation among adult and diapausing offspring. If foundresses adjust the sex ratio of their offspring no matter whether the eggs are going into diapause or not, and mixed broods are the result of a single clutch, the sex ratio of the diapause and the adult fraction of one clutch should be identical. Therefore, our results suggest a physiological link between diapause induction and sex allocation, or in case of multiple clutches by ageing females, a change in sex allocation in diapausing clutches produced later in life.

From an evolutionary point of view, sex allocation according to LMC theory applies only to the fraction of offspring that encounters each other. This means that the fraction of a brood that is in diapause is expected to compete only with other diapausing individuals, assuming simultaneous emergence out of diapause after the winter. The prediction would then be that a foundress separates the sex ratio adjustment for adult and diapausing offspring. Indeed, we found that the sex ratio of single diapausing broods in hosts that also contained adult broods did not differ from the sex ratios produced in single foundress diapausing or adult broods (Fig. 6). In addition, the sex ratios of superparasitism diapausing clutches were significantly higher than those of single foundresses. This observation fits the expectation of a foundress adjusting its progeny sex ratio only to broods that are expected to emerge at the same time (e.g. after the winter), and suggests that a foundress is able to distinguish between eggs of co-foundresses that will develop into adult or diapausing offspring.

Conclusion

We did not find evidence that Nasonia foundresses differentiate between hetero- and conspecific co-foundresses in their sex allocation response. As interspecific matings do not yield any hybrid female offspring, Nasonia foundresses should be selected to differentiate between con- and heterospecific co-foundresses to avoid costly interspecific mating among their progeny. There are clear differences in courtship behaviour between the three sister species (van den Assem & Werren, 1994) resulting in different degrees of prezygotic isolation. Why species differentiation in sex allocation has not yet evolved can
only be speculated upon. One possible explanation is that *N. vitripennis* is a non-native species in the sampled area (B.K. Grillenberger, unpublished data), originating from an allopatric population, and adaptation has not yet progressed this far.

We found a large difference in the production of diapausing offspring between *N. vitripennis* and *N. giraulti* in North America as well as between North American and European *N. vitripennis* populations. The data point to variation in the response to environmental cues between as well as within species. We also found evidence for an interaction between diapause induction and sex allocation. We are not aware of any studies evaluating the adaptive potential of various diapause strategies in a life-history evolution context. The *Nasonia* system appears promising for further research into the adaptive significance of diapause strategies in nature. Laboratory experiments on sex allocation and different diapause conditions are also needed, as field studies also have their drawbacks (e.g. no information about the sequence of parasitism).

In the history of LMC research, a transition took place from simplistic models towards more complex models that capture some of the observed variation. The current view is that sex allocation is largely adaptive, but that there are certain limits due to information constraints (Shuker & West, 2004). Our study also shows that *Nasonia* females are not omniscient, but constrained by the information they can use in their sex allocation decision, such as the inability to discriminate against their closely related species. The further transition from evolutionary models that assume perfect adaptation towards models that include information constraints will be a challenge for the future.

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