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

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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

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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 cyclorraphous 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 sympatry 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 preparasitized by the other species; Wylie (1973) found that *N. vitripennis* differentiate between hosts preparasitized 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 higher proportion of diapausing brood when encountering other conspecific foundresses? (iv) does a foundress produce the same sex ratio among its diapausing and adult offspring? (5) does a foundress produce a different sex ratio in the presence of diapausing vs. adult offspring of another foundress? (6) 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 AA ACC ACG TTA CCC-3′, annealing temperature 52 °C, 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

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.

Locus	Chromosome*	Primer sequences	Allele no.	H_t	Ann. temp.	Species specific?	GenBank accession no.
Nv-104	IV	5′-GAT TTC CCG GAG CTT CCT AC-3′ 5′-GCC GTT AAT TGA ACC TGT CG-3′	8	0.859	55 °C	Yes	FJ156110
Nv-109	V	5′-GCT TAC TCT CGG GAA CTG GA-3 5′-CGA GCA TTA ACC ATC AGC AG-3′	10	0.880	55 °C	Yes	FJ156114
Nv-111	III	5′-AGG TCT CAG CCG CAC AAA-3′ 5′-GCA GCT GCT TTT GGC ACT-3′	16	0.962	55 °C	Yes	FJ156115
Nv-114	IV	5′-ATG GGC AAT AAA ACG AAA CG-3′ 5′-CAT CCT TGC GGA GAC ACT AA-3′	15	0.826	55 °C	No	FJ156231
Nv-300	II	5′-ACA TTC CGC AGA GCG ATT AT-3′ 5′-CGC GAC CGA TGA TTT ACT C 3′	3	0.676	55 °C	Yes	FJ156211
Nv-308	I	5′-ATT CGG AAT CCA CGA AAC G-3′ 5′-TAG GGC GCG TAT AGA TCG AG-3′	10	0.705	55 °C	Yes	
Nv-313	V	5′-GAA GCT GCG GGT TAA GTG TG-3′ 5′-CGC TAC TTT ATG CCA GTT ACG G-3′	19	0.902	55 °C	Yes	FJ156221

*Chromosome designation according to Rütten *et al.* (2004).

box level (Burton-Chellew *et al.*, 2008; Grillenberger *et al.*, 2008). Therefore our statistical analysis is mostly based on foundress numbers on a host level. To answer the questions posed in the Introduction, we performed the following statistical analysis on the genetic data obtained from the three fully analysed nest boxes:

1 To test whether the host usage of *N. vitripennis* and *N. giraulti* is random, we compared the observed foundress 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.

2 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 *H*-tests and Nemenyi *post hoc* tests for pairwise comparisons between the three brood classes (Zar, 1999).

3 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 χ^2-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.

4 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 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.

5 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.

6 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.

Nest	Total host no.	Host no. parasitized per nest	% Host multiparasitized	% Host superparasitized	Host no. parasitized	Species	UN ind.	NA ind.	Ind. no.	% Diap. of total offsp.	Foundress number	Avg. foundress no. per host	Nest sex ratio	Avg. brood size	Avg. host sex ratio	Avg. brood sex ratio
BR29	85	63	49	50	52	NV	5	23	926	46	9	1.94 ± 0.19	0.34	9.26 ± 0.71	0.36 ± 0.003	0.59 ± 0.003
				52	42	NG			483	0.4	10	1.81 ± 0.15	0.45	6.36 ± 0.66	0.53 ± 0.006	0.66 ± 0.005
BR23	64	14	7	0	4	NV	0	18	57	42	1	1.0 ± 0	0.05	14.25 ± 2.46	0.04 ± 0.004	0.04 ± 0.004
				0	11	NG			204	0.5	1	1.0 ± 0	0.12	18.54 ± 2.10	0.12 ± 0.0005	0.12 ± 0.0005
BR12	21	7	43	71	7	NV	2	10	152	0	5	2.0 ± 0.33	0.29	10.86 ± 2.57	0.43 ± 0.02	0.46 ± 0.02
				0	3	NG			26	0	1	1.0 ± 0	0.23	8.67 ± 3.84	0.39 ± 0.01	0.29 ± 0.01
Overall	170	84	42	45	63	NV	7	51	1135	39	15	1.81 ± 0.15	0.32	9.62 ± 0.68	0.34 ± 0.003	0.55 ± 0.003
				44	56	NG			713	0.4	12	1.68 ± 0.13	0.35	7.92 ± 0.75	0.43 ± 0.004	0.58 ± 0.004

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 vitripennis*; NG, *Nasonia giraulti*.

in approximately 60% more *N. vitripennis* than *N. giraulti* offspring (1135 vs. 713). This reflects a higher productivity of *N. vitripennis* in the study area. The superparasitism rate of *N. vitripennis* was comparable to that of *N. giraulti* (45% and 44% respectively; Table 2).

The foundress distributions across hosts differed between the species [Fisher's exact test with low-represented classes combined (≥ 4 foundresses), $P = 0.03$; Fig. 1a]. More hosts parasitized by *N. vitripennis* were unused by *N. giraulti* than vice versa. There was no evidence for female preference for or against patches or hosts used by other females. We found no significant deviation from a random distribution of the foundresses across used hosts within a patch [Fisher's exact test against binomial distribution using data of the largest patch (BR29) only and combining the low-represented classes (≥ 4 foundresses), for both species: $P = 0.20$ (excluding unused hosts); for *N. vitripennis* only: $P = 0.67$; for *N. giraulti* only: $P = 0.79$; Fig. 1b–d].

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*: $H_2 = 7.29$, $P = 0.03$; *giraulti*: $H_2 = 16.04$, $P < 0.001$; 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

Fig. 1 Frequency distribution of foundress number per used host in nest BR29. (a) The relative frequencies of hosts used by either both species (black bars), *Nasonia vitripennis* (grey bars, NV) or *Nasonia giraulti* (white bars, NG). (b–d) Comparison of the observed frequency of foundress number per host (black bars) with the expected frequencies assuming a binomial distribution (grey bars, see text for details): (b) both species combined (there are no observed empty hosts in the graph, as the analysis only included used hosts; see text), (c) only *N. vitripennis* and (d) only *N. giraulti*. Fisher's exact test showed a significant difference between the host usage of the two species (see text for details).
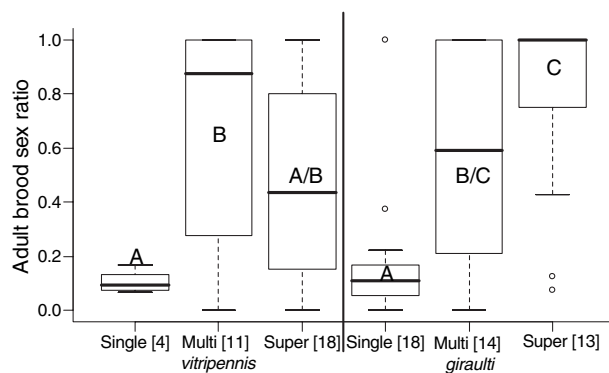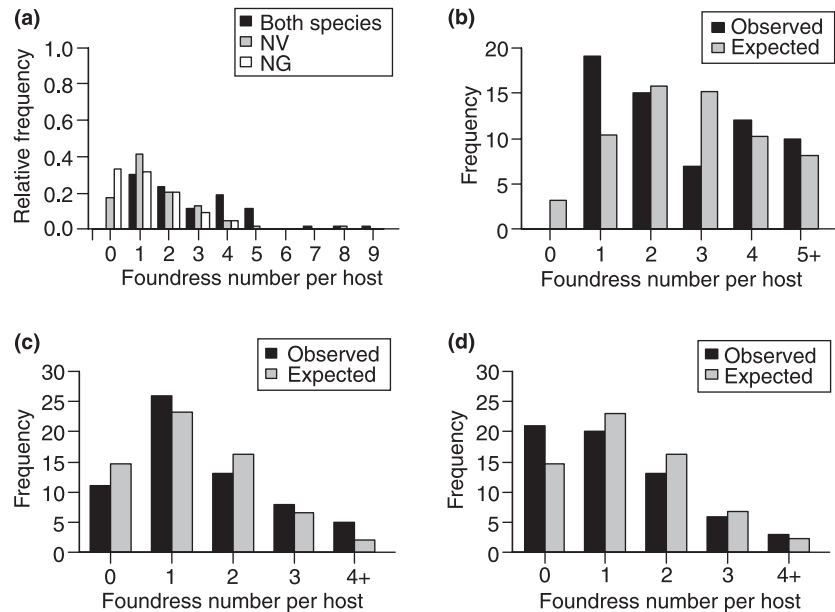

Fig. 2 Box and Whisker plots showing brood sex ratio of adult offspring for the two species in three different situations: (i) single foundress broods of the focal species, without the presence of any other broods in that host (single); (ii) the sex ratio of the focal brood, when there is one brood of the focal species together with at least one brood of the other species (multi = multiparasitism); (iii) multiple broods of one species (super = superparasitism). Numbers in brackets refer to sample size, and capital letters indicate significant differences between groups within one species, or within groups between species, using Nemenyi's test.

Fig. 3 Bubble plot of the proportion of diapause offspring per host as a function of the foundress number per host. Spearman's rank correlation was not significant. Bubble sizes indicate sample size.

(BR29) did not differ from a random distribution (χ^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
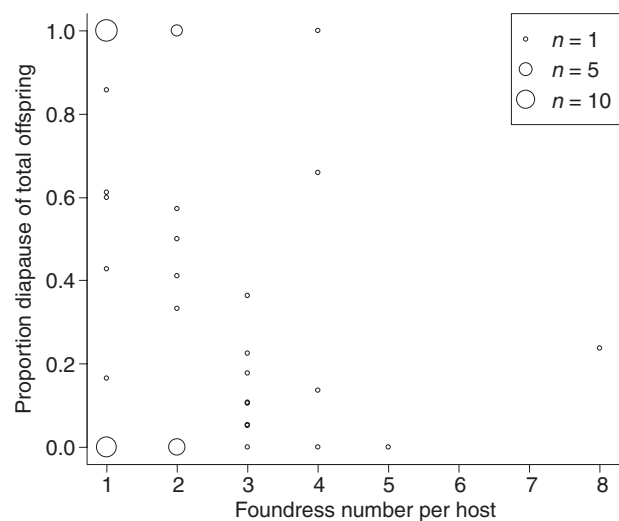
diapausing and adult offspring (Fig. 4; Spearman's rank correlation: $S = 1082.4$, $P = 0.43$, $n = 20$ broods of 9 foundresses). This indicates that the sex ratio of the diapausing and adult fractions of a brood is independent. The brood sex ratios of single foundresses and foundresses under superparasitism 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.
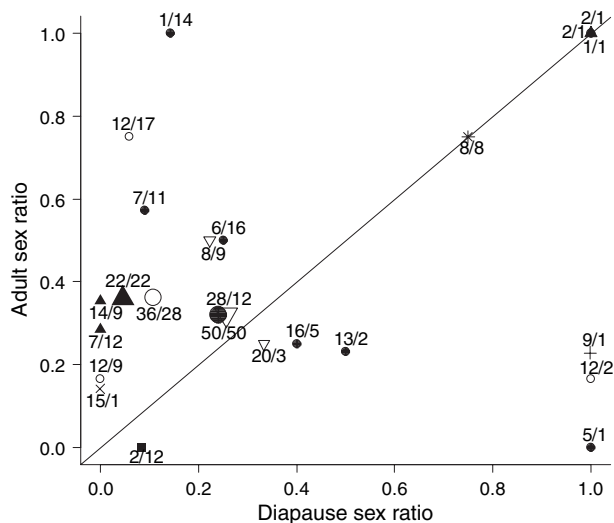
Fig. 4 Sex ratio of adult offspring vs. diapausing offspring within clutches of one foundress (*n* = 20). The solid line represents the theoretical expectation when sex ratio is equal for diapausing and adult offspring. Different symbols represent different foundresses (*n* = 9), the first number near the label is the number of adults in that clutch, the second is the number of diapause larvae. There is no correlation between the sex ratio of diapausing and adult clutches (Spearman's rank correlation: $S = 1082.399$, $\rho = 0.186$, $P = 0.4319$). The larger symbols and numbers represent the pooled values for the four foundresses that contributed more than one mixed clutch. These values are omitted from the correlation analysis.
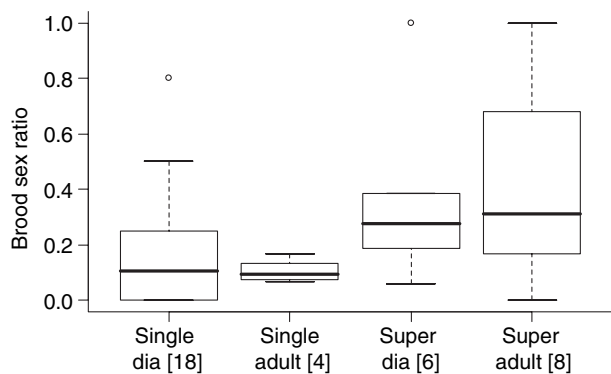

Fig. 5 Box and Whisker plots showing *Nasonia vitripennis* brood sex ratios of single foundresses producing pure diapausing (single dia) or pure adult (single adult) broods, as well as for foundresses under superparasitism (super dia and super adult). Numbers in brackets refer to sample size. Pairwise comparisons between diapausing and adult clutches within single and superparasitism classes were not significant (Mann–Whitney *U*-test).

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
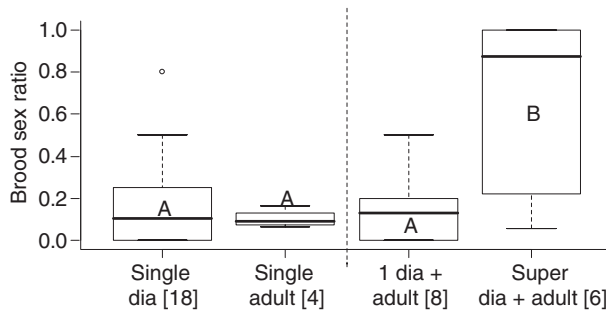

Fig. 6 Box and Whisker plots showing *Nasonia vitripennis* brood sex ratios of single foundresses producing pure diapausing (single dia) and pure adult broods (single adult), as well as of single foundresses producing diapausing brood in the presence of one or more adult broods in the same host (1 dia + adult; the reverse 1 adult + dia, was not found), and of foundresses producing a diapausing brood in the presence of at least one other diapausing brood and one other adult brood (super dia + adult). Numbers in brackets refer to sample size (Kruskal–Wallis *H*-test across the first three groups was not significant; Mann–Whitney *U*-test between 1 dia + adult and super dia + adult was significantly different; significance indicated by different capital letters in the plot).

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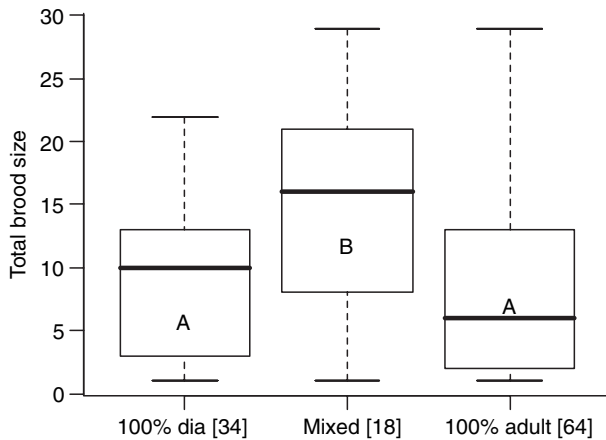
Fig. 7 Box and Whisker plots showing *Nasonia vitripennis* brood sizes of pure diapausing (100% dia), pure adult (100% adult) and mixed broods. Numbers in brackets refer to sample sizes (Kruskal–Wallis *H*-test: $H_2 = 13.69$, $P < 0.05$; pairwise comparison with Nemenyi's test, significance indicated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

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, when a female arrives in a nest box. Note that the composition of a patch (here a nest box) is variable over time. The hosts are only available for parasitism for a few days (depending on temperature), and therefore there are potentially three classes of hosts in a given patch: hosts that are too young to be parasitized, hosts that are too old to be parasitized and hosts that are in the correct stage. Hence a foundress might have a limited number of hosts to choose from and therefore has limited opportunity to actively avoid or prefer hosts that have already been parasitized by either a con- or heterospecific. Superparasitism is then often its only option. The final outcome of such competition for hosts could be the random distribution that we observed. A.B.F. Ivens (unpublished data) found in a laboratory study on *N. vitripennis* and *N. longicornis* that foundresses of both species do not reject hosts that are parasitized by the other species, when parasitizing synchronously, but

do reject preparasitized hosts of the other species after 24 h. This indicates that either most multiparasitism in our study happened synchronously or the species interaction between *N. vitripennis* and *N. giraulti* differs from *N. vitripennis* and *N. longicornis*.

Sex ratios in a two species situation

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*.

Our results show no indication that either *N. vitripennis* or *N. giraulti* is able to distinguish between con- 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 preparasitized 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 sympatrically with other *Trichogramma* species in its natural habitat, whereas *T. pintoi* does not. As *N. vitripennis* and *N. giraulti* live microsympatrically 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-superparasitism 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 between adult and diapause 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.

Acknowledgments

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