Host acceptance and sex allocation of *Nasonia* wasps in response to conspecifics and heterospecifics

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Species recognition is an important aspect of an organism’s biology. Here, we consider how parasitoid wasps vary their reproductive decisions when their offspring face intra- and interspecific competition for resources and mates. We use host acceptance and sex ratio behaviour to test whether female *Nasonia vitripennis* and *Nasonia longicornis* discriminate between conspecifics and heterospecifics when ovipositing. We tested pairs of conspecific or heterospecific females ovipositing either simultaneously or sequentially on a single host, using strains varying in their recent history of sympatry. Both *N. vitripennis* and *N. longicornis* rejected parasitized hosts more often than unparasitized hosts, although females were more likely to superparasitize their own species in the sequential treatment. However, sex ratio behaviour did not vary, suggesting similar responses towards conspecifics and heterospecifics. This contrasts with theory predicting that heterospecifics should not influence sex ratios as their offspring do not influence local mate competition, where conspecifics would. These non-adaptive sex ratios reinforce the lack of adaptive kin discrimination in *N. vitripennis* and suggest a behavioural constraint. Discrimination between closely related species is therefore context dependent in *Nasonia*. We suggest that isolating mechanisms associated with the speciation process have influenced behaviour to a greater extent than selection on sex ratios.

**Keywords:** species recognition; speciation; adaptation; sex ratios; superparasitism; multiparasitism

1. INTRODUCTION

Species recognition is an important aspect of an organism’s biology, not just in terms of mate recognition and any resulting reproductive isolation (e.g. Price 2008), but also in terms of a whole range of other behaviours. For instance, the level of competition for resources is likely to vary between intra- versus interspecific interactions, and discrimination of species identity among interaction individuals will affect their patterns of resource use. Similarly, reproductive allocation decisions may vary with the identity of the competitors. In organisms such as parasitoid wasps, populations are often highly structured with only one or a few females contributing offspring to a breeding group. Under such circumstances, the offspring of these founding females will compete among each other for, for example, resources and mates. The degree of competition is dependent on whether the founding females are heter- or conspecifics: competition for resources will be equal in both cases, but competition for mates is expected to be less in the heterospecific case. Therefore, the ability to discriminate between conspecifics and heterospecifics should be important for optimal behavioural decision-making. Using the wasp genus *Nasonia*, here we test whether or not females do discriminate based on species identity and whether this discrimination is context dependent.

Female parasitoid wasps face at least two important decisions upon encountering a host: first, whether to accept or reject the host, and second to determine the number and sex ratio of their progeny (Godfray 1994; Wäginberg et al. 2008). These decisions can be affected by many factors such as host quality (size and species), the number of competing females and whether the host has been previously parasitized (e.g. Salt 1935; Wylie 1965; King & Skinner 1991; Godfray 1994; van Baaren et al. 1994; Gauthier et al. 1999; Darrouzet et al. 2007; Lébreton et al. 2009). Both are also influenced by the species identity of competitors. For example, parasitoid wasps may encounter hosts that have been previously parasitized by other individuals of their own species (conspecifics, termed superparasitism) or by individuals of another species (heterospecifics, termed multiparasitism; reviewed in Godfray 1994). Host acceptance and sex ratios produced under superparasitism have been extensively studied in a variety of species, in particular, *Nasonia vitripennis* (Walker) (e.g. Wylie 1965, 1966; Werren 1980, 1983; King & Skinner 1991; Flanagan et al. 1998; Shuker & West 2004). Only a few studies, however, have investigated oviposition behaviour under multiparasitism, and these have dealt almost exclusively with host acceptance.

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Received 8 June 2009
Accepted 7 July 2009

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rather than sex ratios, mostly focusing on species of solitary parasitoid wasps (e.g. Godfray 1994; Gauthier et al. 1999). For example, *Anaphes* parasitoid wasps have been shown to avoid multiparasitism more often than superparasitism (van Baaren et al. 1994), in contrast to *Asobara* parasitoid wasps that have been shown to discriminate more strongly against hosts parasitized by conspecifics, or females of a closely related species, than of a less closely related species (Vet et al. 1984). Likewise, Wylie (1970) found that *N. vitripennis* females reject hosts that have been parasitized by other, distantly related species less often than hosts pre-parasitized by their own species. These wasps therefore behave differently in a super-versus a multiparasitism context, with the phylogenetic distance between interacting individuals mediating this difference.

In terms of sex ratios, under these reproductive circumstances, female-biased sex ratios are favoured in order to reduce the competition between related males over mates (termed local mate competition (LMC) by Hamilton (1967)). The role of LMC in sex ratio behaviour in many taxa is now well established (West et al. 2002, 2005; Ode & Hardy 2008). Females should vary their sex ratios with the number of co-founding females on their patch, or, more fundamentally, with the relative clutch size of those females (Hamilton 1967, 1979; Suzuki & Iwasa 1980; Werren 1980; Shuker et al. 2005). Indeed, factors such as foundress number and relative clutch size are well known to influence sex ratio in *Nasonia* (e.g. Werren 1980, 1983; Orzack & Parker 1990; Shuker & West 2004; Shuker et al. 2005, 2006) and in other organisms experiencing forms of LMC (e.g. fig wasps: Raja et al. 2008; ants: Cremer & Heinze 2002; snakes: Madsen & Shine 1992; fishes: Petersen & Fisher 1996 and even malaria parasites: Reece et al. 2008). Individuals from a different species should, however, not influence the level of LMC experienced by male offspring, generating a clear-cut difference in sex ratio between superparasitism and multiparasitism, but only if a foundress can discriminate between these conditions. To date, sex ratios in a multiparasitism situation have only been investigated in *N. vitripennis* by Wylie (1973). This study has shown that *N. vitripennis* sex ratios vary with co-foundress species. Sex ratios were more female-biased when females oviposited on hosts previously parasitized by *Spalangia cameroni* compared with *Muscidifurax zaraptor*, suggesting that *Nasonia* distinguishes between hosts that have been parasitized by different, un-related species (unfortunately, direct comparisons with the sex ratios produced by *N. vitripennis* under superparasitism were not made). This raises the question whether *N. vitripennis* females are also able to distinguish between hosts parasitized by a female of one of their closely related sibling species, *Nasonia longicornis* (Campbell et al. 1993). These two species are sympatric in North America, with *N. vitripennis* being more broadly distributed across America and northern Europe (Darling & Werren 1990). The species are known to share hosts in the wild (micro-sympathy, Darling & Werren 1990; B. K. Grillenberger & A. B. F. Ivens 2005, unpublished data), with reproductive isolation arising from *Wolbachia*-induced nucleo-cytoplasmatic incompatibilities (e.g. Breeuwer & Werren 1990; Bordenstein 2001) and from divergence in courtship behaviour (Van den Assem & Werren 1994; Beukeboom & Van den Assem 2001). How the two species interact in terms of influencing their reproductive decision-making is not yet known.

In this study, we therefore consider host acceptance and sex allocation of both *N. vitripennis* and *N. longicornis* under conditions of superparasitism and multiparasitism. We ask whether these parasitoids are able to distinguish between hosts parasitized by their own species and hosts parasitized by a closely related species, and if so, to what extent they adjust their host acceptance and sex ratio behaviour. To begin to explore how females might discriminate foundress identity, we use females from strains that vary in the history of coexistence (e.g. the number of generations since sympatry). We also present the host to females either simultaneously, so that females can behaviourally interact with the host and each other, or sequentially, such that cues from the host are the only ones available. Finally, our experiments include the first test of LMC theory in *N. longicornis*.

2. MATERIAL AND METHODS

(a) Study organism

*Nasonia* is a genus of gregarious wasps that parasitize pupae of blowflies that occur in bird nests and carcasses. *Nasonia vitripennis* and *N. longicornis* are sympatric across the western USA, occurring micro-sympathetically in the same parasitized fly pupae (Darling & Werren 1990; B. K. Grillenberger & A. B. F. Ivens 2005, unpublished data). Both species are characterized by a highly localized mating population structure, with males emerging from hosts first to then mate with emerging females, including their sisters (although in *N. longicornis* there is some degree of within-host mating, prior to emergence from the puparium: Drapeau & Werren 1999).

Females of both species are the dispersive sex, leaving the natal patch to find new hosts after mating. Male *N. vitripennis* are brachypterous with limited dispersal abilities, resulting in highly structured mating populations counteracted by random dispersal of females over patches (Grillenberger et al. 2008). *Nasonia longicornis* males have wings more nearly approaching those of females, but their flight abilities are believed to be very low (Lehmann & Heymann 2006).

Superparasitism occurs frequently with up to 40 per cent of the hosts being parasitized by more than one female in allopatric *N. vitripennis* as well as in sympatric *N. vitripennis* and *Nasonia giraulti* populations (no data for sympatric *N. vitripennis* and *N. longicornis*) (Grillenberger et al. 2008, 2009). Sex allocation in *Nasonia* has been largely studied in terms of responses to LMC (see §1). However, sex ratios may be under selection as a result of other factors such as host quality or asymmetric larval competition (King 1992; Rivero & West 2005; Sykes et al. 2007). Evidence is limited for the former though, and theory suggests that the importance of the latter is smaller compared with LMC (Sykes et al. 2007). Moreover, *Nasonia* lay smaller clutches in hosts that already contain eggs of other females (this study: *N. vitripennis* 26.03 ± 0.70 s.e. in unparasitized hosts versus 5.97 ± 0.89 s.e. in parasitized hosts and *N. longicornis* 24.22 ± 0.91 s.e. in unparasitized hosts versus 8.80 ± 0.81 s.e. in parasitized hosts), thereby reducing the level of resource competition by adjusting clutch sizes to the amount of resources available. Being haplodiploid, in *Nasonia* sex is determined by whether or not eggs are fertilized prior to

*Proc. R. Soc. B* (2009)
oviposition, with unfertilized eggs developing into haploid males and fertilized eggs into diploid females.

(b) Lines and culturing
We used three lines of *N. vitripennis* and *N. longicornis*: a wild-type laboratory line, a red-eyed mutant laboratory line and a field line. For *N. vitripennis*, these were, respectively, AsymCHS, STDRTET and NVBTMONE05-3 and for *N. longicornis* IV7R2, NLSTCA-JB and NLBTMONC08-4. All laboratory lines are standard lines that have been kept in culture for at least 15 years. AsymCHS is originally from Leiden, The Netherlands and IV7R2 from Utah, USA. They have been cured of *Wlobachia* and are known to be free of sex ratio distorters (Skinner 1982, 1985; Gherna et al. 1991; Werren 1991). Both field lines have been collected in July 2005 in the same area near Huntsville, USA (coordinates: 41°14′37″ N; −111°42′80″ W) and thus were sympatric. Wasps were baited using *Sarcophaga* and after emergence in the laboratory, they were cultured in mass cultures (more than 100 wasps) at constant light at 25°C. *Sarcophaga* pupae, and after emergence in the laboratory, they were cultured on *Calliphora vicina* hosts for two generations prior to the experiments. These field lines were found to be free of sex ratio distorters, but have not been cured from *Wlobachia*. When not used in an experiment, all lines were maintained in mass cultures (more than 100 wasps) at constant light at 25°C. *Calliphora vicina* pupae were used as host. All females used in the experiments were offspring from individually hosted females presented with two hosts in small tubes (height 6.5 cm and diameter 0.9 cm), so we expected similar degrees of within-line relatedness for the randomly chosen experimental females.

(c) Experimental set-up
All females were 1 or 2 days old at the start of the experiment. We used a design that allowed us to manipulate: (i) recent experience of sympathy (field versus laboratory line, for each of the two species); (ii) presence or absence of a co-founding cue (the latter as a control); (iii) co-founding identity (conspecific versus heterospecific); and (iv) discrimination cues (both females presented with the host simultaneously, or else sequentially, with the focal female presented with the host second). Focal females belonged to either the field line or the wild-type laboratory line. In order to distinguish between the offspring of two females, co-founding females were always red-eye mutants. Each experimental group underwent four steps similar to that of Shuker & West (2004): (i) pre-treatment, in which all females were given a host for 24 h followed by 24 h honey solution, to allow host feeding and egg maturation; (ii) focal females were randomly assigned to one of the treatments for 24 h and presented with one host (either fresh in the simultaneous oviposition treatments or parasitized in the sequential treatments); (iii) all females were then given another host to test for virginity (with haplodiploid sex determination, unmounted females are constrained to produce only males). Replicates in which the focal female was a virgin were discarded from the analysis of sex ratio data (12 females in total). Finally, two weeks later; (iv) offspring emerged and were sexed and genotyped (wild-type or not) by eye colour. For each focal female, we recorded whether the host had been accepted (the presence of offspring) and the number and sex ratio of the emerging offspring. Host quality was standardized by selecting fly pupae of the same size and colour. To limit forced self-parasitism, all females were given a one-way dispersal tube when presented with the test host (Werren 1983; Shuker & West 2004). As *N. vitripennis* females are faster in ovipositing than *N. longicornis* (A. B. F. Ivens 2005, unpublished data), *N. vitripennis* females were given an escape tube after 1 h and *N. longicornis* females after 6 h. For similar reasons, *N. longicornis* females were offered the host pupa 3 h ahead of *N. vitripennis* females in the simultaneous treatment. In total, 881 females were tested, with sample sizes for host acceptance data for the species and treatments ranging from 20 to 69 for *N. vitripennis* and from 24 to 69 for *N. longicornis* and sample sizes for sex ratio data ranging from 7 to 66 for *N. vitripennis* and from 10 to 31 for *N. longicornis*.

(d) Data analysis
Host acceptance was based on the emergence of focal female offspring. In total, 881 replicates were used to test host acceptance. Of the 599 focal females who accepted their host, the sex ratios of their emerging offspring were analysed. Throughout, we consider sex ratio as the proportion of offspring that are male. Both host acceptance and sex ratios were modelled with logistic regression, using the generalized linear model (glm) procedure of R v. 2.7.1 (R Development Core Team 2008). To correct for over-dispersion, we used the option ‘family=quasi-binomial’ and tested significance of variables with F-tests (Crawley 2007). For the four combinations of focal species and treatment, we used backward elimination to arrive at final statistical models, starting with a model including all two-way and three-way interactions between three explanatory variables: co-founder species (conspecific versus heterospecific), line (field versus laboratory) and absolute (host acceptance data) or relative (sex ratio data) number of offspring of the co-founding species. Specifically, in the sex ratio analyses, we used the relative number of offspring of the first laying female as a predictor of the second female’s sex ratio.

3. RESULTS

(a) Host acceptance for oviposition
Females invariably showed high host acceptance levels (80–100%) when hosts were unparasitized or when ovipositing simultaneously (figure 1a). In the sequential treatment, females were less likely to oviposit (*N. vitripennis* $F_{1,266} = 103.8$, $p < 0.00001$; *N. longicornis* $F_{1,264} = 53.4$, $p < 0.00001$). *Nasonia vitripennis* accepted hosts in approximately 50 per cent of the cases when the co-founding female was also *N. vitripennis*, but with just over 20 per cent, significantly less often when the co-founding was *N. longicornis* ($F_{1,165} = 8.62$, $p < 0.005$). There was no significant difference in host acceptance between the *N. vitripennis* laboratory and field line in the sequential treatment ($F_{1,165} = 0.16$, $p = 0.69$). On average, *N. longicornis* was equally prone to accept pre-parasitized hosts as *N. vitripennis* ($F_{1,532} = 1.19$, $p = 0.28$), but the *N. longicornis* field line accepted at a higher rate than the laboratory line ($F_{1,165} = 39.02$, $p < 0.00001$). However, the *N. longicornis* field line did not discriminate between conspecifics and heterospecifics ($F = 0.04$, $p = 0.84$), unlike the laboratory line ($F_{1,104} = 24.81$, $p < 0.00001$); significant interaction in table S1, electronic supplementary material: $F_{1,105} = 6.49$, $p < 0.02$). These data show that previously parasitized hosts are accepted at a lower rate than unparasitized hosts if oviposition is sequential. Moreover, host
acceptance rate is lower if hosts have been pre-parasitized by the sibling species.

(b) Sex ratios
Among control N. vitripennis females, the laboratory line produced a more female-biased sex ratio than the field line (0.140 ± 0.009 s.e. laboratory versus 0.244 ± 0.023 s.e. field; F_{1,164} = 62.8, p < 0.00001; figure 1b), whereas in N. longicornis, the field line produced a slightly more female-biased sex ratio (0.116 ± 0.013 s.e. laboratory versus 0.084 ± 0.008 s.e. field; F_{1,107} = 4.43, p < 0.05).

Nasonia longicornis sex ratios were more female-biased than those of N. vitripennis (F_{1,273} = 35.0, p < 0.00001). In N. vitripennis, larger broods were significantly less female-biased (F_{1,164} = 10.80, p < 0.002), but in N. longicornis, no effect of brood size was evident (F_{1,107} = 0.12, p = 0.73).

In line with LMC theory, when ovipositing simultaneously with another female, N. vitripennis produced less female-biased sex ratios than when alone (F_{1,160} = 49.2, p < 0.00001). The same was true on average for N. longicornis, but only significant in the field line (interaction with line F_{1,135} = 5.06, p < 0.002). The N. vitripennis field line produced less female-biased sex ratios than the laboratory line. No other significant effects on sex ratios of simultaneously ovipositing females were found (table S2, electronic supplementary material). In particular, whether the co-foundress was a conspecific or heterospecific had no effect on the focal females’ sex ratio.

In sequentially ovipositing females, both species clearly produced less female-biased sex ratios compared with the control groups (figure 1b). In N. vitripennis, the laboratory line responded more strongly in the sequential treatment compared with the simultaneous treatment, in contrast to the field line (three-way interaction control × line × treatment F_{1,317} = 10.2, p < 0.002). No such effect was apparent in N. longicornis (F_{1,207} = 2.89, p = 0.091). As in the simultaneous treatment, there was no significant effect of co-foundress species (table S2, electronic supplementary material). In both species, the relative number of offspring of the co-foundress had a strong positive effect on the proportion of sons produced by the focal female (figure S1, electronic supplementary material).

4. DISCUSSION
Host acceptance and sex allocation, two major decisions female parasitoid wasps face when ovipositing, were investigated under superparasitism (two females of the same species) and multiparasitism (two females of different species) in N. vitripennis and N. longicornis. Our results show that information about the species identity of a co-foundress is used in a context-dependent manner. Females varied their host acceptance behaviour in part due to co-foundress identity, but they did not change their sex ratio behaviour, contradicting LMC theory. Instead, the presence of eggs and/or ovipositing adults were treated equally in terms of shifting the sex ratio, regardless of them being either conspecific or heterospecific.

Specifically, females of both species typically accept fresh hosts, either alone or simultaneously with a co-foundress. However, when confronted with a host that was parasitized 24 h before, females of both species reject the host more often. This response is stronger in N. vitripennis than in N. longicornis. Interestingly, apart from the N. longicornis field line, the rate at which a host is rejected is higher if this host is pre-parasitized by a heterospecific than by a conspecific (i.e. they are more likely to superparasitize their own species than a heterospecific). This suggests that females are, at least after 24 h, able to distinguish between cons- and heterospecifics.
Oviposition decisions in Nasonia wasps

A. B. F. Ivens et al.

in this context. This confirms earlier observations of Wylie (1970), Vet et al. (1984) and van Baaren et al. (1994), who showed that N. vitripennis, Asobara and Anaphes parasitoid wasps can distinguish between hosts parasitized by con- or heterospecifics. However, the pattern we observed of multiparasitism avoidance is only exhibited in Anaphes wasps (van Baaren et al. 1994). In contrast to the studies by Wylie (1970) and Vet et al. (1984), the wasps showed a lower level of acceptance of hosts parasitized by conspecifics than by hetero-
specifics (i.e. superparasitism avoidance). From an adaptive point of view, heterospecific competition may be more disadvantageous than intra-specific competition because multiparasitism is purely competitive whereas superparasitism also involves additional mating opportu-
nities. There are a number of important biological differences between the study of Wylie (1970) and the cur-
cent study, including the phylogenetic distance between the co-foundresses (sister species in our case and different genera in his) and the outcome of larval competition (usually competitive exclusion with Spalangia or Muscidifurax). The host species used by Wylie (Musca domestica) is also a less favourable host for Nasonia. How these factors might influence the nature of intra- versus interspecific larval competition clearly merits further study.

As predicted by LMC theory, sex ratios of both N. vitripennis and N. longicornis were less female-biased in two-foundress situations compared with the control single foundress situation. In addition, second females in pairs of sequentially ovipositing females adjusted the sex ratio with respect to the relative clutch size of the first female in the expected direction. A similar effect of relative clutch size on sex ratios was recently found in field populations of N. vitripennis (Burton-Chellew et al. 2008). However, in contrast to the effect on host acceptance, whether a co-foundress was a conspecific or heterospecific had no detectable effect on sex ratio. Nasonia females in a multiparasitism situation are thus able to respond to the circumstances regarding host acceptance: they clearly distinguish between hosts parasitized by con- or heterospecifics. However, with regard to sex ratios, the females respond suboptimally; they adjust their sex ratio to heterospecifics as if they were conspecifics, thereby overinvesting in male offspring. This is not the first reported case in which sex allocation related to the recognition of co-foundresses in Nasonia appears to be maladaptive: earlier Shuker et al. (2004a,b) and Reece et al. (2004) showed that N. vitripennis females did not adjust their sex ratios with respect to either the relatedness of their mating partner or the relatedness of their oviposition partner. Taken together, these findings suggest that Nasonia females may indeed be constrained in their information use of cues about their social environment when it comes to their sex ratio decisions.

The next important question is why is information available in one context not used in another. How N. vitripennis females gain information has been extensively considered by Wylie (1965, 1966, 1970, 1973) and King & Skinner (1991). Females appear to rely on internal cues of the fly puparia that are associated with the oviposition of a previous female. These cues are most probably produced by the venom that is injected upon oviposition and detected by subsequent females, but may also involve the eggs themselves. Interestingly, King & Skinner (1991) found evidence for a difference in the cues used for clutch size and sex ratio decisions. The extent to which cues are used or become available over time is also of interest. Previous work by Werren (1984), King & Skinner (1991), King (1992) and Shuker et al. (2006) on Nasonia has shown that time since prior oviposition (and the type of cue) is associated with changes in sex ratio behaviour. The strengths of these cues increase over time, but the cue for sex ratio seems to diffuse more slowly or less far through the host. Our observed differences in host acceptance and sex ratio between the simultaneous and sequential treat-
ment are consistent with this observation. A similar effect might explain why heterospecific cues influence host acceptance but not sex ratio, especially if compo-
nents of the venom have diverged between the two species. However, host cues cannot explain all the simul-
taneous oviposition treatment results, as the presence of another (conspecific) female in the environment, without any oviposition from them, is sufficient for N. vitripennis females to decrease the female bias of their brood (Shuker & West 2004). More generally, it is becoming clear that hosts change both in quality and in terms of the cues available for ovipositing females to gauge quality across a range of parasitoids (e.g. Lебретон et al. 2009).

The lack of sex ratio adjustment to interspecific interactions was apparently not influenced by the history of coexistence of the lines. At first sight, this might be the result of the species rarely having experienced multiparasi-
tism in the field. However, the field lines used were collected in an area where N. vitripennis and N. longicornis occur micro-sympathetically (B. K. Grillenberger & A. B. F. Ivens 2005, unpublished data), so experience with multiparasi-
tism is likely. Thus, although N. longicornis appears to be more specialized on Protocalliphora hosts in bird nests than N. vitripennis (Darling & Werren 1990), competition for oviposi-
tion resources probably occurs frequently in the field. Interestingly, some N. longicornis lines tend to mate within the host, which may be considered as a strategy to avoid interspecific matings (Drapeau & Werren 1999). Such behaviour may render the need for flexible sex allocation behaviour superfluous because offspring will only mate within the brood and will therefore always experience high levels of LMC. The N. vitripennis laboratory line is of Dutch origin and has never experienced competition with N. longicornis. It, nevertheless, does discriminate between con- and hetero-
specifically parasitized hosts in terms of acceptance. As such, host acceptance behaviour and the use of cues under-
lying that behaviour may have played a role in the speciation process, with selection on host acceptance having been more important than selection on sex ratios. More generally, relating host discrimination behaviour to other aspects of the Nasonia mating system, such as species differences in mating behaviour, interspecific mate discrimination and costs of hybridization, will provide a unique opportunity to test how reproductive isolation affects reproductive strategies in the round.

The authors like to thank J. Prij for practical advice and assistance and S. West for extensive discussion on the experimental design. This work benefited from a short visit grant to A.B.F.I. from the European Science Foundation (ESF)—Behavioural Ecology of Insect Parasitoids (BEIPAR) scientific programme. The collection of field lines in Utah, USA, was funded by a grant of the Schure-Beijerinck-Popping

Proc. R. Soc. B (2009)
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