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Parasitoids: metapopulation ecology and genetics, mating behaviour, and aphid host resistance

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Introduction

Introduction to problem statement

Insect parasitoids are ubiquitous members of natural insect communities and important agents of population regulation through biological control. Understanding, therefore, the parasitoid population structure and their ecological and evolutionary interactions with their hosts is of great practical and theoretical interest. Given that parasitoids need to parasitize hosts and that, by definition, they kill their host as part of their normal lifecycle, there is bound to be reciprocal coevolution whereby the hosts develop resistance with negative consequences for their parasitoids. The mating patterns of the solitary parasitoids of gregarious hosts may be influenced by adult parasitoid resource use behaviours on the natal patch, in addition to other interacting organisms such as trophobiotic ants on those natal patches. This section contains a review of literature on aphid hosts and their parasitoids' genetic metapopulation structure, evolving mating opportunities, aphid host resistance and its effect on parasitoid fitness, research questions, brief introduction to the methodologies, justification of the study and a brief overview of the manuscripts discussed in this thesis.

Parasitoid and host metapopulation genetic structure

The spatial distribution of interacting species affects their coexistence and co-evolution (Thompson, 1994). Althoff & Thompson (1999) found incongruent geographical genetic structures and subsequently established that the patchy distribution of the host food plant contributed to the genetic structure of the *Aganthis* parasitoids (Althoff & Thompson, 2001). Before, it had been realized that many species consist of assemblages of ephemeral subpopulations or demes that persisted over time in a dynamic balance between local extinction and recolonization termed 'metapopulations' (Levins, 1969). A metapopulation is defined as a population of populations with an age structure established by the birth of populations through colonization and their death through extinction, a description since broadened to cover any population system in which discrete subpopulations exchange genes with one another through dispersal (Pannell & Charlesworth, 2000).

In a field survey, Weisser (2000) established that an aphid host-parasitoid system comprising the specialist wasp parasitoid, *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae: Aphidiinae) attacking the specialist aphid, *Metopeurum fuscoviride* Stroyan (Hemiptera: Aphididae), which feeds exclusively on common tansy plants, *Tanacetum vulgare* L. (family Asteracae), has a classic metapopulation structure. The system was characterized by

s*ubpopulations that occurred in discrete resource patches* and *individuals dispersed between patches but such dispersal events were rare* (Harrison & Taylor, 1997; Hanski, 1999). Rauch & Weisser (2007) found parasitism in plants some 192m away from the release plant and concluded that, even though *L. hirticornis* could colonize plants at this distance, such events were rare. Given this seemingly limited dispersal, there was paucity of information on *L. hirticornis* population genetic structure.

Roslin (2001) showed, with the specialist dung beetle *Aphodius fossor* L. (Coleoptera: Scarabaeidae), that though the spatial population structure was highly aggregated, due to aggregated distribution of cattle pastures, the genetic structure was strikingly homogenous, suggesting extensive gene flow and homogenous populations. Johannesen & Seitz (2003) found higher population genetic sub-structuring of the univoltine oligophagous primary parasitoid, *Eurytoma robusta* Mayr (Hymenoptera: Eurytomidae) compared to its univoltine gall forming fly, *Urophora cardui* L. (Diptera: Tephritidae). Likewise, Anton *et al*. (2007) found higher overall genetic differentiation in the specialist univoltine parasitoid *Neotypus melanocephalus* Gmelin (Hymenoptera: Ichneumonidae) compared with its univoltine host, the Dusky large blue butterfly, *Maculinea nausithous* Bergsträsser (Lepidoptera: Lycaenidae). In spite of the fact that they share the same host, the Glanville fritillary butterfly, *Melitaea cinxia* L., *Cotesia melitearum* Wilkinson (Braconidae: Microgastrinae) showed substantial genetic structure compared with *Hyposoter horticola* Gravenhorst (Ichneumonidae: Campopleginae) (Kankare *et al.*, 2005). These four articles (Roslin, 2001; Johannesen & Seitz, 2003; Kankare *et al.*, 2005; Anton *et al.*, 2007) discuss the importance of dispersal and gene flow on population structuring in a metapopulation and show that, even in closely interacting species, the population genetic structure at one trophic level does not necessarily influence the genetic structure of another trophic level. Population genetic studies are therefore necessary to establish whether *L. hirticornis* is influenced by that of its host and possibly the host plant. High population differentiation in *M. fuscoviride* has been documented; Massonnet (2002), using six microsatellite markers on seven subpopulations (five from the Alsace region of France and two from Germany), reported high population differentiation and of the 21 pairwise F_{ST} comparisons, 18 were significant.

Massonnet *et al*. (2002b) found high genetic differentiation among a third of the local populations of the aphid *Macrosiphoniella tanacetaria* Kaltenbach (Homoptera: Aphididae) in Germany, as well as significant deviations from Hardy-Weinberg and linkage disequilibria in these populations. They concluded that frequent local extinction and colonization events had created a hierarchical metapopulation structure in the aphid. In host-parasitoid interactions,

studies show that parasitoids can cause host extinctions (Lei & Hanski, 1997; Weisser, 2000; van Nouhuys & Tay, 2001). For example, Weisser (2000) found that the specialist parasitoid, *L. hirticornis* caused up to 100% parasitism of 12% of the local *M. fuscoviride* populations on the common tansy. Obviously, if a monophagous parasitoid drives its host extinct locally, it too will disappear; the risk to both host and parasitoid is intensified if habitat patches become isolated and the metapopulation structure starts to break down.

The birth and death of population demes as a result of extinction and recolonization sets up a population age structure in the metapopulation (Wade & McCauley, 1988), and greater variation in the level of absolute within-deme diversity between demes in such a metapopulation than in a sub-divided population without recurrent extinction. Second, recurrent local extinction reduces levels of species-wide diversity (Slatkin, 1977; Maruyama & Kimura, 1980; Lande, 1992; Whitlock & Barton, 1997; Pannell & Charlesworth, 1999). Theoretical work by Wright (1940) suggested that a pattern of extinction and recolonization should enhance the genetic differentiation of local populations because the number of individuals colonizing a patch is likely to be much smaller than the local carrying capacity. Slatkin (1985) challenged this proposition by arguing that ongoing local extinction implies recolonization, recolonization constitutes gene flow, and gene flow will prevent local populations from becoming differentiated. However, Wade & McCauley (1988) showed that the outcome of extinction and recolonization was dependent on the mode of founding of new populations. If colonizing propagules tended to be large and contained individuals from many populations, then the turnover had the homogenizing effect as described by Slatkin (1985). But if propagules tended to be small and homogenous, emanating from only one or few source populations, then the turnover of local populations actually enhanced their differentiation, and redistributed total variation from 'within' to 'among' populations. The *L. hirticornis- M. fuscoviride* system is characterized by extinction and recolonization dynamics but it is not known what the consequences of these processes are for the population genetics in a single growing annual season. Population turnover is expected to cause dramatic reduction in the total diversity maintained in a metapopulation. In a metapopulation that has lost almost all its diversity, migration will clearly have little effect on within-deme diversity, just as recombination along the length of homozygous chromosomes has no genetic effect (Pannell & Charlesworth, 2000).

Solitary parasitoid mating system on the natal patch

In hymenopteran parasitoids, the natal patch forms an important factor and determinant of mating opportunities and fitness returns afforded to females versus males. If the hosts of solitary

parasitoid species are gregarious such as aphids or coccids, quasi-gregarious broods of the parasitoid offspring can be produced (van den Assem *et al.*, 1980), where partial local mate competition may occur (Mackauer & Völkl, 2002). Hamilton's (1967) theory of local mate competition (LMC) predicts that if offspring mate randomly on the natal patch before dispersing, mothers will produce only as many sons as are required to fertilize all available females.

In *Cotesia glomerata* L. (Hymenoptera: Braconidae), a gregarious parasitoid of first- to third-instar caterpillars of *Pieris* butterflies, Gu & Dorn (2003) detected that about 70% of the mating occurred on the natal patch between resident females and immigrant males. Loch & Walter (2002) studied *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae), a quasi-gregarious *Nezara viridula* L. (Heteroptera:Pentatomidae) egg parasitoid and found that more than 18% of emerging females were not mated by the dominant protandrous male upon emergence, 13% of females were not observed to be mated at all and left their natal site as virgins, and 25% of females were mated multiple times and sometimes by multiple males. Females remained near the natal site for up to several hours after emergence, before emigrating, and males dispersed away from the natal site during female emergence. It is not known if this is a general pattern for solitary parasitoids attacking gregarious hosts.

Schwörer & Völkl (2001) described *A. ervi* as having "low resource utilization" i.e. it parasitized only few aphids within a host colony: females oviposited on average less than two eggs although up to 10 hosts were available. Females of *L. hirticornis* oviposited on average more than 30 eggs in ant-attended colonies and is considered as having "high resource utilization" (Mackauer & Völkl, 2002). There was a seasonal pattern in resource utilization for *Pauesia pini* Haliday (Hymenoptera: Braconidae: Aphidiinae) attacking the aphid, *Cinara* spp on Norway spruce, i.e., the females' heavily parasitized colonies of the large-sized aphid generations on spruce early in the season, while the small summer generations were almost ignored (Mackauer & Völkl, 2002; Völkl *et al.*, 2007). *P. pini* is generally considered a "low resource user" (Völkl & Novak, 1997)**.** With such "resource use behaviours" solitary parasitoids may have subtle differences in mating systems on the natal patch.

Some Aphididae parasitoid hosts have developed mutualistic associations with trophobiotic ants: the ants obtain honey dew, an important source of nutrients and in return protect their hosts against predators and parasitoids (Addicott, 1978; Buckley, 1987). The association between ants and their partners may be highly specialized, with the survival of the ant partner impossible without the services provided by the ants (obligate myrmecophiles), or of more casual nature where the partners can survive without each other (facultative myrmecophiles) (Stadler *et al.*, 2001). However, studies have shown that this protection is incomplete, and that ants cannot

always provide an enemy free space for their mutualistic partners. A number of parasitoids have evolved morphological and or behavioural adaptations to gain access to ant attended resources (see Völkl, 1997 for review). *Lysiphlebus* species mimic the cuticular hydrocarbons of their aphid hosts and are able to deceive the ants (Liepert & Dettner, 1993, 1996). Ant behaviours towards foraging parasitoids might determine whether parasitoids stay or leave the natal patch and therefore affect the evolving mating system.

Resistance in aphid hosts and effects on parasitoids

Parasitoids, unlike most parasites, kill their host as part of their normal lifecycle and if the parasitoid kills the host before it becomes reproductively active, parasitoid attack equals genetic death for the parasitized host. This means that, unless attack rates by parasitoids are rare, there will be a strong selection pressure on the host to develop a resistance mechanism that either prevents parasitism taking place, or kills the parasitoid egg or larva before it can do further damage to the host. In turn, the evolution of a resistance mechanism in the host will exert a selection pressure on the parasitoid for a counter-resistance mechanism that somehow avoids or overcomes the host's resistance mechanism. Therefore, the parasitoids and their hosts are likely to exert strong and reciprocal selection pressure during their coevolutionary process (Godfray, 1994; Sasaki & Godfray, 1999).

In insect host–parasitoid systems, reciprocal selection between antagonists is particularly strong because interactions are always a matter of life or death. Adult female parasitoids seek hosts, and deposit their eggs in, on, or near these hosts. Hosts that support development of the parasitoid larvae are consumed and killed by the parasitoid larvae and give rise to adult parasitoids. Some hosts, however, are able to debilitate or destroy parasitoid eggs while surviving parasitoid attack. The physiological basis for this type of host resistance in many systems is a cellular immune response resulting in encapsulation of the parasitoid egg (Strand & Pech, 1995), and it has been demonstrated in the system consisting of the host *Drosophila melanogaster*, and two of its parasitoids, *Asobara tabida* (Nees) and *Leptopilina boulardi* (Poirie *et al.*, 2000; Dupas *et al.*, 2003).

In aphid host-parasitoid systems encapsulation has not been reported but variation for susceptibility and infectivity has been detected in a number of aphid species including the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), against its parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae: Aphidiinae) (Henter, 1995; Henter & Via, 1995). Interestingly, much of the observed variation for susceptibility to parasitoids in pea aphids is due to endosymbiotic bacteria among aphid clones (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et*

al., 2005). In addition to *Buchnera aphidicola*, which serves a nutritional function (Douglas, 1998), pea aphids may harbour several other endosymbiotic bacteria, collectively referred to as facultative or secondary symbionts, because they are not indispensable for survival (Sandström *et al.*, 2001; Tsuchida *et al.*, 2004; Moran *et al.*, 2005b). *Regiella insecticola*, in pea aphids affected host specialization and increased resistance to a fungal pathogen (Tsuchida *et al.*, 2004; Scarborough *et al.*, 2005), *Serratia symbiotica* improved thermal tolerance and slightly increased resistance to parasitoids (Montllor *et al.*, 2002; Oliver *et al.*, 2003), and *Hamiltonella defensa* provided protection against parasitoids (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Guay *et al.*, 2009). In addition to these three facultative secondary symbionts, pea aphid *Rickettsia* (PAR), *Spiroplasma* spp. (Chen *et al.*, 1996; Chen & Purcell, 1997; Fukatsu *et al.*, 2000; Tsuchida *et al.*, 2002) and *pea aphid X-type symbiont* (*PAXS*) (Guay *et al.*, 2009) have been reported. These facultative symbionts occur in multiple infections, and up to three co-infections have been recorded (Frantz *et al.*, 2009). All secondary symbionts are transmitted from mother to daughter, with some evidence of horizontal transmission (Sandström *et al.*, 2001; Russell *et al.*, 2003).

Although a study by Oliver *et al.* (2005) suggested that in pea aphids, all of the clonal variation for susceptibility to parasitoids is due to endosymbionts, recent studies on the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) (von Burg *et al.*, 2008) and the black bean aphid, *Aphis fabae fabae* (Hemiptera: Aphididae) (Vorburger *et al.*, 2009) suggest otherwise. In *M. persicae, v*on Burg *et al.* (2008) detected significant variation among clones without facultative endosymbionts. Vorburger *et al.* (2009) found reduced parasitism by *Lysiphlebus fabarum* (Marshall) (Hymenoptera: Braconidae: Aphidiinae) on clones of *Aphis f. fabae* infected with *H. defensa*, as well as significant variation between clones without *H. defensa.* This suggested that aphid parasitoids had to deal with two levels of defence, that of innate resistance and that of resistance conferred by endosymbionts. Studies to establish whether pea aphids possess innate resistance were necessary.

Host species attacked by a given parasitoid species can be classified as ''suitable'' if all or most individuals can complete development, or as ''partially resistant'', or ''marginal'' if a substantial fraction of host individuals are able to debilitate the immature parasitoids and survive. In resistant pea aphid clones, parasitoids have been shown to debilitate the resistance and emerge as adults.

General Objective

The general objective of this study was to investigate how the aphid host metapopulation affects the dynamics and genetics of its specialist parasitoid; how aphid hosts and parasitoid resource use behaviours affect parasitoid mating system, and aphid host resistance *vis-à-vis* parasitoid fitness.

Specific objectives

i.   Isolate microsatellites from *Lysiphlebus hirticornis,* and identify microsatellite markers of other *Lysiphlebus* species that cross amplify in *L. hirticornis* and test all those microsatellites for polymorphism.

ii. Determine the level of gene flow and population substructuring in *L. hirticornis* within a small spatial scale.

iii. Identify population heterogeneity among *L. hirticornis* parasitoid populations and determine whether parasitoid genetic variability was dependent on parasitoid population density.

iv. Establish how *L. hirticornis* genetic variability fluctuates during a single annual growing season.

v.   Establish how *L. hirticornis* genetic variability correlates with that of the aphid host and compare parasitoid gene flow to that of its aphid host.

vi. Determine whether solitary parasitoids have similar mating opportunities in their natal patches and how ant myrmecophily influences mating opportunities on the natal patch.

vii. Establish whether there is innate clonal variation in the pea aphid, *A. pisum* resistance to *A. ervi* in addition to that conferred by secondary symbionts, and establish any sublethal effects of the aphid resistance on emerging parasitoids.

Study systems and methodologies

To investigate these specific objectives, aphid host-parasitoid systems were used. These were:

1. The metapopulation structure involving *Metopeurum fuscoviride* aphid host and the specialist parasitoid, *Lysiphlebus hirticornis*. A field survey was performed. *Lysiphlebus hirticornis* mummified aphids were collected and allowed to emerge for DNA extractions and genotyping. Microsatellite markers were isolated from *L. hirticornis*; in addition, microsatellites from *L. fabarum* and *L. testaceipes* were screened and identified for polymorphism in a cross amplification test on *L. hirticornis*. Afterwards, the polymorphic microsatellite markers were used in a population genetic study. Most

studies infer parasitoid local dynamics from the distribution of parasitized hosts (e.g., Eber & Brandl, 1994; Dempster *et al.*, 1995a; Dempster *et al.*, 1995b; Eber & Brandl, 1996, 1997; Weisser, 2000). The dynamics of *L. hirticornis* are based on the occurrence of aphid mummies, which provide a basis for reliable estimates of the presence of the developing offspring of parasitoids. Microsatellites have been successfully used in the study of genetic differentiation and gene flow of insects (Loxdale & Lushai, 1998; Simard *et al.*, 2000; Bailly *et al.*, 2004; Keyghobadi *et al.*, 2005; Endersby *et al.*, 2006; Franck *et al.*, 2007; Fuentes-Contreras *et al.*, 2008; Lozier *et al.*, 2009) including aphid wasp parasitoids (Hufbauer *et al.*, 2001; Baker *et al.*, 2003; MacDonald *et al.*, 2003; Fauvergue *et al.*, 2005; Sandrock *et al.*, 2007; Tentelier *et al.*, 2008) to test for aspects of the molecular ecology, including dispersal, host adaptation and range, relatedness and inbreeding and density dependence.

2. To determine whether solitary parasitoids had similar mating opportunities on the natal patch and whether there was variations between sexes of a species, *A. ervi*, *P. pini* and *L. hirticornis*, known for different "resource use behaviours" and ant myrmecophily were used. For each species a single mated female was allowed to parasitize aphids on their respective host plants, except *A. ervi* where aphids were exposed to the parasitoid in a Petri dish before transfer to bean plants, *Vicia faba*. Adult parasitoids eclosing from these parasitizations were observed on their natal patches for mating behaviour. In addition, for *L. hirticornis*, either ants or healthy living aphids were excluded and observations made under those conditions. The experimental procedure followed here has been reported for *A. ervi* and *P. pini* (Völkl & Novak, 1997; Schwörer & Völkl, 2001).

3. Using the pea aphid, *Acyrthosiphon pisum- Aphidius ervi* parasitoid system in a correlative study, 30 genetically distinct aphid clones harbouring secondary symbionts in up to 10 complements were screened for resistance to the parasitoid and how the resistance affects eclosing parasitoid fitness. Groups of aphids were exposed to parasitoids for a fixed period of time and the proportion parasitized through dissections and mummy formation measured. Fitness parameters for both healthy and parasitized aphids and for parasitoids eclosing from aphids were observed. The method of parasitization has been described by Henter & Via (1995) and recently used by Vorburger *et al.* (2009).

Justification of the study

Hymenopteran parasitoids are increasingly used as biocontrol agents due to seemingly inexhaustible source of wasp species known to parasitize a wide range of arthropod pests. However, the potential to reduce insect pest numbers in the field is not always realised in practice (Schmidt *et al.*, 2000). Genetic variation and genetic diversity are closely associated with important features of parasitoid host interactions, such as host adaptations and specificity. The problem is that some of the fundamental processes of how insect endoparasitoids interact with hosts are unknown. Kankare *et al.* (2005) noted that comparing the interacting species inhabiting the same landscape was a powerful approach to studying spatial population structures. Understanding the extent of spatial genetic structure of organisms might facilitate the identification of evolutionary or ecological factors that influence genetic structure (Dionne *et al.*, 2008). Understanding parasitoid behaviours that emanate from their interactions with hosts are necessary in order to curtail effects of inbreeding. Failure of some biological control introductions could be due to resistance (e.g., Henter & Via, 1995). Studies to unravel the mechanisms of resistance and sub-lethal effects of host-resistance on parasitoids are necessary.

Overview of manuscripts

Six articles are contained in this thesis. Below is a brief summary of each article, and each of the author's contribution.

Nyabuga FN, Loxdale HD, Sharbel TF, Todd M, and Weisser WW. 2009. Microsatellites from *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae), a specialist primary parasitoid attacking the specialist tansy aphid, *Metopeurum fuscoviride* Stroyan (Hemiptera: Aphididae). *Molecular Ecology Resources* **9:** 931-934.

This paper comprises data on the molecular marker isolation and characterization from *L. hirticornis*. Twenty three loci were obtained from an enriched partial genomic Library and only nine proved to be polymorphic and usable. In addition, the loci described for *L. fabarum* (Sandrock *et al.*, 2007) and *L. testaceipes* (Fauvergue *et al.*, 2005) were tested. Two loci, one each from the two publications, were found to be polymorphic in *L. hirticornis*. In total, eleven microsatellite markers tested on twenty *L. hirticornis* wasps collected from eight tansy genets on an area approximately 150m^2 in Jena, Central Germany are described. The number of alleles, observed and expected heterozygosity, and departures from Hardy-Weinberg equilibrium (HWE) are reported and discussed.

FNN, HDL and WWW conceived the study. FNN extracted the DNA with help of HDL. In consultation with WWW and HDL, the DNA was sent to Genetic Identification Services who performed the sequencing and developed primer sequences. FNN then tested the primers for polymorphism in consultation with TS and HDL on methodologies. FNN wrote the paper and MT was instrumental in terms of primer development methods.

Nyabuga FN, Loxdale HD, Heckel DG and Weisser WW. (2010). Spatial population dynamics of a specialist aphid parasitoid, *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae: Aphidiinae): evidence for philopatry and restricted dispersal. *Heredity.* **doi:10.1038/hdy.2009.190**

This paper describes the spatial differentiation and diversity of *L. hirticornis*, isolation by distance and dispersal at the local scale as found using the 11 microsatellite markers described in the article above. We found that populations were differentiated indicating limited dispersal; however, there was no evidence for isolation by distance. We argue that the patchy distribution of tansy plants and therefore the host aphid together with their ant myrmecophily discourages high parasitoid movement. We also discuss biological reasons for population differentiation and genetic diversity in this highly specialized system.

FNN, HDL and WWW conceived the study. FNN did the field survey, collected parasitoid samples that he sequenced in DGH's laboratory under supervision of HDL and DGH. FNN discussed the data with HDL and WWW and wrote the manuscript which was editted by HDL and WWW before submission to *Heredity*.

Nyabuga FN, Loxdale HD, Heckel DG and Weisser WW: The consequences of an unstable relationship: genetical metapopulation dynamics of *Lysiphlebus hirticornis* Mackauer, a specialist parasitoid attacking a specialist aphid host on common tansy. In preparation for publication in the *Biological Journal of the Linnean society*

Following detection of spatial structuring in the preceding article above, we investigated whether structuring occurred beyond the site level, i.e. whether there was population structure at the level of the tansy genet. That host extinctions and recolonization are a part of *L. hirticornis-M. fuscoviride* system was important to elucidate the impact of this phenomenon on the parasitoid genetics over one annual growing season. Reduced genetic diversity and increased differentiations over time at the site level was detected. Analysis of molecular variance indicated that the genet rather than site was the unit of population sub-structuring.

FNN, HDL and WWW conceived the study. FNN did the DNA extractions, genotyped the samples at DGH's lab, discussed the data with WWW and HDL. FNN wrote the manuscript.

Nyabuga FN, Loxdale HD, Heckel DG and Weisser WW: Population structure of the tansy aphid, *Metopeurum fuscoviride* and its specialist parasitoid *Lysiphlebus hirticornis.* In preparation for publication in *Proceedings of the Royal Society B*

In this manuscript, the interaction between *M. fuscoviride* and *L. hirticornis* continued persistence is discussed. The aphid host was genotyped at six microsatellite markers and the parasitoid at eleven such markers. The aphid host was greatly differentiated while the parasitoid was moderately differentiated. Positive mantel correlations between aphid and parasitoid pairwise F_{ST}, and between shared allele distances (*DAS*) were detected. We conclude that the parasitoid is tracking behind the aphid host and could be the mechanism behind the persistence of this aphid-parasitoid system.

FNN, HDL and WWW conceived the study. FNN did the parasitoid genotyping. HDL provided results of the aphid host, *M. fuscoviride*. FNN performed the data analysis, discussed the results with HDL and WWW. FNN wrote the manuscript.

Nyabuga FN, Völkl W, Schwörer U, Weisser WW and Mackauer M.: Mating strategies in solitary aphid parasitoids: effect of patch residence time and ant attendance. Submitted for publication in *Behavioural Ecology*

In this manuscript, males and females of three solitary parasitoids of gregarious hosts that differ in resource exploitation and ant mutualism are compared for mating opportunities on the natal patch. The results showed that *L. hirticornis* had a long residence time compared with *P. pini* and *A. ervi*. In *A. ervi*, females stayed longer in the natal patch compared with males. *L. hirticornis* had approximately 90% of the total parasitoids mating on the natal patch compared with less than 20% in *A. ervi.* Importantly, in all the three parasitoid species, a fraction of parasitoids did not mate in the natal patches. In *L. hirticornis*, removal of any of the interacting partners, either ants or aphids, reduced the natal patch residence time and mating success from approximately 90% to less than 43%. We conclude that: mating off the natal patch is a common phenomenon in solitary parasitoids; the degree of 'off-natal patch' mating differs among solitary parasitoid species and between sexes within a species, and that mating behaviour in solitary parasitoids is influenced by ant myrmecophily.

WV and MM conceived the study. WV and US conducted the experiments. FNN performed the data analysis and discussed the results with WV and WWW. FNN wrote the manuscript.

Nyabuga FN, Outreman Y, Simon JC, Heckel DG and Weisser WW: Effects of pea aphid secondary endosymbionts on aphid resistance and development of the aphid parasitoid *Aphidius ervi*: a correlative study. Submitted for publication in *Entomologia Experimentalis et Applicata*

It is known that resistance of the pea aphid, *Acyrthosiphon pisum* to the parasitoid *Aphidius ervi* is due to secondary endobacterial symbionts harboured by the aphid host. To date, six secondary symbionts have been reported and pea aphid clones may be infected by up to three symbionts in what is termed multiple or co-infections but only three symbionts and one co-infection are documented to confer resistance. In addition to the other symbionts in single or multiple infections, we hypothesized that there existed innate clonal variation. Since the resistance was rarely 100%, then parasitoids that emerged from the secondary symbionts were bound to be negatively affected. In this manuscript**,** data on aphid host resistance and sublethal effects on adult parasitoids is presented.

FNN, WWW and DGH conceived the study. FNN planned the experiment aided by WWW, YO and JCS. The aphid clones were provided by YO and JCS. FNN performed the experiment in YO's lab. FNN performed the data analysis in consultation with WWW, DGH and YO. FNN wrote the manuscript that has since been edited by the co-authors and submitted to *Entomologia Experimentalis et Applicata*.

Article I: Microsatellites from *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae), a specialist primary parasitoid attacking the specialist tansy aphid, *Metopeurum fuscoviride* Stroyan (Hemiptera: Aphididae)

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Microsatellites from *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae), a specialist primary parasitoid attacking the specialist tansy aphid, *Metopeurum fuscoviride* Stroyan (Hemiptera: Aphididae)

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Abstract

Nine polymorphic microsatellite loci were isolated from the specialist aphid parasitoid, *Lysiphlebus hirticornis*. In addition, two published loci from closely related *Lysiphlebus* species were also used. Allelic diversity and heterozygosity were quantified in samples collected from eight tansy plants growing in an area of approximately 150 m² in Jena, Germany.

Keywords: aphid parasitoid, Braconidae, DNA, *Lysiphlebus hirticornis*, microsatellite

The aphid, *Metopeurum fuscoviride* Stroyan, a specialist on tansy (*Tanacetum vulgare* L.), shows a metapopulation structure with colonization and extinction phases on its plant host (Weisser 2000; see also Massonnet *et al*. 2002). It is attacked by two specialist braconid parasitoids, *Aphidius tanacetarius* Mackauer and *Lysiphlebus hirticornis* Mackauer (Starý 1973). The population structure and dynamics of the aphid host may influence the population genetic structure and dynamics of the specialist parasitoids (Weisser 2000; Rauch & Weisser 2007). Here we investigate the population genetic structure of *L. hirticornis* in the light of the behaviour and ecology of *M. fuscoviride*, whose distribution reflects that of tansy, in effect a tri-trophic study.

Genomic DNA was isolated from a pool of 40 female wasps following Sunnucks & Hales (1996), resuspended in 100 μL TE buffer, pH 8.0, and stored at −20 °C. Cloning procedure followed Jones *et al*. (2002). Purified DNA was partially restricted using a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV). Fragments between 350 bp and 700 bp were isolated and ligated to adaptors containing a *Hin*dIII site. Magnetic bead capture (CPG Inc.) was used following the manufacturer's protocol to isolate microsatellite-containing fragments, using biotin-(CA)$_{15}$, biotin-(GA)$_{15}$, biotin-(ATG)$_{12}$ and biotin-(TAGA)$_8$ as capture molecules. These were amplified and digested with *Hin*dIII to remove the adaptors, and ligated

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into the *Hin*dIII site of pUC19 vectors. Recombinant molecules were electroporated into *Escherichia coli* DH5α (ElectroMaxJ, Invitrogen); a 2:1 ligation mix was used for each of the genomic libraries. After transformation and recovery incubation in SOC broth (Invitrogen), glycerol was added (20% final vol.) and the products plated out to produce 100–300 recombinant colonies. Colonies were isolated for sequencing on bluo-gal-IPTG/ampicillin LB (BIA-LB) plates, and incubated at 37 °C using 2× LB broth in a 96-well block. Plasmid DNA was purified from randomly selected recombinant clones using Millipore MultiScreen MAFB NOB plates (http://www.millipore.com/), and sequencing was performed using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) on an Applied Biosystems 377 Sequencer. Enrichment levels were expressed as the fraction of sequences containing a microsatellite, from which 23 polymerase chain reaction (PCR) primers were designed using DesignerPCR software version 1.03 (Research Genetics, Inc.).

An initial screening for polymorphism in the 23 primers with seven female parasitoid samples on 1.5% agarose gel yielded six polymorphic loci. Forward primers for the six were fluorescently labelled (LI-COR IRD 700 and 800) and routine survey begun. One primer was, however, discovered to have a duplicated primer-binding site and was thus discarded. In order to screen for more polymorphic loci, M13 adapter tails were made for the remaining 17 primers together with 11 loci developed from *Lysiphlebus fabarum* (Sandrock *et al*. 2007) and six from *Lysiphlebus testaceipes*

Table 1 Characteristics of 11 microsatellite markers in *Lysiphlebus hirticornis*, including locus name and GenBank Accession no., primer sequences, repeat motif in cloned allele, their size range in base pairs (bp) and annealing temperature (T_a). The number of amplified alleles (A), expected and observed heterozygosities (H_E/H_O) are reported for 20 diploid female parasitoids from one site in Jena, Germany (50°92′N–11°58′E). Na frequency, null allele frequency. HW, significance of deviation from Hardy–Weinberg equilibrium. NS, not significant; * = 0.05, ** = 0.01, *** = 0.001. These significance levels include a Bonferroni correction (Van Oosterhout *et al.* 2004)

Locus	GenBank Accession no.	Primer sequences and label (F, forward; R, reverse)	Repeat motif	Size range (bp)	T_a (°C)	A	H_O	H_E	Van Oosterhout Na frequency
*Lhirt*01	EU878532	F: 5′-TAGGCTGTAGTCATCGTAGTGA-3′ R: 5′-TTCGCTTTTCTCTGTCTGTT-3′	$(TG)_{30}$	182–270	54	4	0.350**	0.522	+0.124
*Lhirt*02	EU878533	F: 5′-TTGGTAAAAGATGTCGTTGAAC-3′ R: 5′-CACACGTCGATAGAAGATGC-3′	$(TG)_{22}$	126–140	46	2	0.250NS	0.224	−0.134
*Lhirt*03	EU878534	F: 5′-TATGTGCCCACGAATACTC-3′ R: 5′-AGGACTTTCAGACCATTTTAC-3′	$(GT)_{50}$	192–232	58	5	0.050***	0.635	+0.399
*Lhirt*04	EU878535	F: 5′-AAAGGACTAGACGTATTCCAG-3′ R: 5′-GTTGCATGATTTTATCACG-3′	$(CT)_{16}$	160–286	54	8	0.650***	0.849	+0.252
*Lhirt*06	EU878537	F: 5′-AGTATGCTTCCAATCAACACAC-3′ R: 5′-TGCAAGTACATTTTGTGGTTC-3′	$(CAA)_{24}$	226–270	46	3	0.400NS	0.465	−0.393
*Lhirt*08	EU910554	F: 5′-GCGTCTTTAGTTGTTTGAGTG-3′ R: 5′-ACGATGTTCTCTTTTCTCTGTC-3′	$(GT)_{13}$	218–222	51	2	0.550NS	0.467	+0.058
*Lhirt*10	EU910556	F: 5′-GGTGGTTGGGTGATTTTATG-3′ R: 5′-ACGGGAGTTGAAAGAGGAAG-3′	$(CT)_{15}$	295–300	46	3	0.450NS	0.540	−0.013
*Lhirt*15	EU910561	F: 5′-TCCAATCATCTCAACAAGC-3′ R: 5′-AACGAGTGAAGTACCACCAC-3′	$(CAA)_{13}$	320–330	46	3	0.400NS	0.376	−0.245
*Lhirt*23	EU910569	F: 5′-TTATTTACAACAGGACCAGTTG-3′ R: 5′-AGCAACAACAATTCTACCAAC-3′	$(GCT)_9$	246–274	51	4	0.250*	0.627	+0.158
*Lysi*08†	EF173292	F: 5′-TGACTGAACGTGGACTTTGAT-3′ R: 5′-TCGTTAAACGTCCAACCACAT-3′	$(GT)_{14}$	118–120	55	2	0.050NS	0.224	−0.134
*Lysi*6b12‡	AY670692	F: 5′- CATATGAAGAGTAAAGATCG-3′ R: 5′-CGATTATTCTAGGTAAAATG-3′	$(CA)_{10}$	158–166	46	3	0.138NS	0.142	+0.172

†from Sandrock *et al.* (2007); ‡from Fauvergue *et al.* (2005).

(Fauvergue *et al.* 2005). M13 tags comprise two parts, an adaptor sequence (AGGGTTTTCCCAGTCACGACGTT) and a fluorescent-labelling with IRD700 or IRD800 dye (Oetting *et al.* 1995). Separation of PCR products on 6.5% polyacrylamide gels yielded a further four polymorphic loci from the original 17, plus *Lysi*08 from *L. fabarum* (Sandrock *et al.* 2007) and *Lysi*6b12 from *L. testaceipes* (Fauvergue *et al.* 2005). A total 11 polymorphic loci were therefore identified and used in this study (Table 1). The PCR protocol was as follows: 10 μL reaction mixture containing 1 μL (~8–20 ng) of template DNA, 0.2 μM of each locus-specific primer (see Table 1), 0.2 mM dNTPs (Promega), and 1 μL of 10× reaction buffer containing 1.5 mM MgCl$_2$ and 0.05 U *Taq* DNA polymerase (GeneCraft or Metabion). PCR amplifications were conducted with Eppendorf thermocyclers (Mastercycler) using a denaturation step at 94 °C for 3 min, followed by 27 cycles of denaturation at 94 °C for 40 s, a locus-specific annealing temperature (Table 1) for 40 s, elongation at 72 °C for 30 s; and a final extension step at 72 °C for 4 min. For M13-tagged, PCR was performed as above, except that two annealing temperature regimes were used, one primer-specific — 10 cycles for 60 s, (Table 1), the second for 15 cycles at 55 °C and 60 s. Final extension was at 72 °C for 10 min. PCR products were diluted with 15 μL Blue stop solution (LI-COR) and 15 μL double-distilled water, centrifuged for 10 s and further denatured at 94 °C for 10 min. Thereafter, all PCR products were separated on a LI-COR 4300 sequencer using 6.5% polyacrylamide gels with fluorescently labelled size markers (IRD 700 and 800; 50–350 bp).

Because braconid parasitoids are haplo–diploid, males and females were identified under a binocular microscope before DNA extraction and male sequences used as a check for null alleles (see also below). Here we present data for 20 female parasitoids collected from aphids infesting eight tansy plants at one site in Jena. Allelic diversity and Hardy–Weinberg equilibrium (HWE) probabilities were calculated using Arlequin 3.1 (Excoffier *et al.* 2005) and Micro-Checker (Van Oosterhout *et al.* 2004) was used to estimate the frequency of null alleles segregating at each locus and calculate deviation from HWE after Bonferroni correction. The number of alleles across 20 individuals ranged from 2 to 8, mean = 3.5 ± 0.520 (SE); observed heterozygosity from 0.050 to 0.650, mean = 0.345 ± 0.054

Table 2 *P* values for each locus pair across all populations (Fisher's exact method, Raymond & Rousset 1995)

	Lhirt01	Lhirt02	Lhirt03	Lhirt04	Lhirt06	Lhirt08	Lhirt10	Lhirt15	Lhirt23	Lysi08	Lysi6b12
Lhirt02	0.000										
Lhirt03	0.003	0.546									
Lhirt04	0.013	0.008	0.560								
Lhirt06	0.621	0.331	0.446	0.380							
Lhirt08	0.000	0.008	0.039	0.038	0.445						
Lhirt10	0.048	0.042	0.286	0.673	0.057	0.020					
Lhirt15	1.000	0.778	0.719	0.215	0.811	1.000	0.963				
Lhirt23	0.213	0.219	0.525	0.000	0.520	0.329	0.300	0.606			
Lysi08	0.461	0.074	0.511	0.015	0.700	0.651	0.888	1.000	0.045		
Lysi6b12	0.031	1.000	0.060	1.000	0.662	0.015	0.805	0.712	1.000	1.000	
No. of significant pairwise linkages ($P < 0.05$)	6	4	2	5	0	6	3	0	2	2	2

(SE); and expected heterozygosity from 0.142 to 0.849, mean = 0.461 ± 0.060 (SE) (Table 1). Positive significant ($P < 0.05$) F_{IS} values were recorded at four loci. Van Oosterhout's null allele frequencies are given in Table 1. All 55 possible pairwise tests of linkage disequilibrium were performed using GenePop 4.0 (Raymond & Rousset 1995), of which 16 were significant ($P < 0.05$, Fisher's exact test, Table 2). The deviations from HWE might have been affected by haplo–diploidy on breeding structure (Menken 1991) along with patch fidelity (Weisser 2000; Rauch & Weisser 2007) as well, in part at least, to the presence of null alleles. However, hemizygous male parasitoids (52 out of 59) showed no blanks on sequencing gels at all loci tested, suggesting a minimal role of null alleles. Hence, the homozygous excess appears to be due to inbreeding in these parasitoids as a result of oviposition and mating behaviour.

Interestingly, of the 11 *L. fabarum* primers, all showed amplification, but only *Lysi*08 was polymorphic with 2 alleles. Of the 6 *L. testaceipes* primers, only two showed amplification, and only *Lysi*6b12 was polymorphic with 3 alleles (Table 1). This is further evidence of conserved primer-binding sites, not necessarily polymorphic across closely related species (MacDonald *et al.* 2003). In relation to this, we tested the 11 *L. hirticornis* markers against *A. tanacetarius* and the hyper-parasitoid, *Dendrocerus* spp. Only four loci gave amplification with *A. tanacetarius*. The product lengths were as follows: 118 bp (*Lhirt*02); 255 bp (*Lhirt*04); 257 bp (*Lhirt*10) and 148–154 bp (*Lysi*6b12). None of the primers showed amplification with the four hyper-parasitoid samples.

Acknowledgements

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Article II: Spatial population dynamics of a specialist aphid parasitoid, *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae: Aphidiinae): evidence for philopatry and restricted dispersal

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ORIGINAL ARTICLE

Spatial population dynamics of a specialist aphid parasitoid, *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae: Aphidiinae): evidence for philopatry and restricted dispersal

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Within insect communities, the population ecology of organisms representing higher trophic levels, for example, hymenopterous parasitoids, may be influenced by the structure of their insect hosts. Using microsatellite markers and ecological data, we investigated the population structure of the specialist braconid wasp parasitoid, *Lysiphlebus hirticornis* Mackauer attacking *Metopeurum fuscoviride*, a specialist aphid feeding on tansy, *Tanacetum vulgare*. Previous studies revealed that *M. fuscoviride* has a classic metapopulation structure with high subpopulation turnover. In this study, up to 100% of ramets within a host plant genet colonized by aphids were colonized by the parasitoid, yet plants with aphids but no parasitoids were also observed. Genetic differentiation measured by F_{ST}, actual differentiation (D) and relative differentiation (G_{ST}) indicated highly structured parasitoid population demes, with restricted gene flow among and between parasitoid subpopulations at the various sites. Interestingly, both field data and population assignment analysis showed that the parasitoid is highly philopatric. Thus, despite the frequent local extinctions of the aphid host, the parasitoid continuously exploits its aphid host and contributes to the demise of local aphid subpopulations, rather than spreading its genes over many aphid populations. F_{ST} values for the haplodiploid parasitoid were similar to those found in an independent study of the diploid aphid host, *M. fuscoviride*, hence supporting the view that an insect herbivore's population structure directly influences the ecology and genetics of the higher trophic level, in this case the wasp parasitoid.
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Keywords: *L. hirticornis*; *M. fuscoviride*; microsatellites; philopatry; isolation by distance; gene flow

Introduction

In recent years, a number of empirical studies have shown that metapopulation structure influences the genetic makeup of subpopulations of various organisms studied (for example, Bay *et al.*, 2008; Orsini *et al.*, 2008; Purrenhage *et al.*, 2009), including aphids (Massonnet *et al.*, 2002). General predictions about the genetic structure of metapopulations are difficult to make because of factors such as the population dynamics of local populations, their extinction rate, and the rate and distance of dispersal of the species under consideration (Harrison and Hastings, 1996; Pannell and Charlesworth, 2000). Broadly speaking, a metapopulation with frequent extinctions is expected to show lower overall genetic variability compared with an unstructured population of a similar organism with similar natural history, and subpopulations (colonies or demes) are more or less genetically differentiated depending on the balance between dispersal, colonization and extinction dynamics

(Pannell and Charlesworth, 2000). For example, in insect species with metapopulation dynamics, genetic differentiation between populations range from: (1) significant differences between almost all subpopulation pairs investigated, for example, the tansy aphid, *Macrosiphoniella tanacetaria* Kaltenbach (Hemiptera: Aphididae) (Massonnet *et al.*, 2002); to (2) some level of genetic differentiation, such as that found in the wasp parasitoid, *Hyposoter horticola* Gravenhorst (Hymenoptera: Ichneumonidae), which attacks the Glanville fritillary butterfly, *Melitaea cinxia* L. (Lepidoptera: Nymphalidae) (Kankare *et al.*, 2005); to (3) no genetic differentiation despite the existence of discrete subpopulations, for example, in the dung beetle, *Aphodius fossor* L. (Coleoptera: Scarabaeidae) (Roslin, 2001).

In spatially structured multitrophic systems such as those formed on plants by insect herbivores and their natural enemies, the population ecology of the higher trophic levels, for example, wasp parasitoids, may be influenced by the metapopulation structure of the host insect. However, few studies have to date estimated dispersal and genetic population structure at one or more trophic levels or analysed the influence of species interactions on metapopulation structure of one or more of the organisms involved (Cronin and Reeve, 2005). The few studies performed (for example, Johannesen and

Seitz, 2003; Kankare et al., 2005; Anton et al., 2007) show that even though the herbivore host may have a distinct metapopulation structure, which also affects local survival of the parasitoid, the population genetic structure of the parasitoid can be very different.

The host–parasitoid system comprising the specialist wasp parasitoid, Lysiphlebus hirticornis Mackauer (Hymenoptera: Braconidae: Aphidiinae) attacking the specialist aphid, Metopeurum fuscoviride Stroyan (Hemiptera: Aphididae), which feeds exclusively on tansy, Tanacetum vulgare L. (family Asteracae), has a classic metapopulation structure (Weisser, 2000; Zheng et al., 2009). In a field study, Weisser (2000) reported frequent extinction events for the aphid, M. fuscoviride, and as a direct consequence, of the parasitoid L. hirticornis, at the level of tansy plants (= genets) and shoots (= ramets). At the local spatial scale (site), Weisser (2000) found that many tansy plants were not colonized by the aphid and many tansy genets with the aphid were not colonized by the parasitoid. He also reported a mean survival time of 3.1 and 4.8 weeks (mode = 1 week) for aphids at the level of ramet and genet, respectively.

In support of the view that M. fuscoviride indeed shows a metapopualtion structure, Massonnet (2002), using six microsatellite markers on seven subpopulations (five from the Alsace region of France and two from Germany), reported high population differentiation: F_{ST} values ranged from 0.029 to 0.416, and of the 21 pairwise F_{ST} comparisons performed, 18 were significant ($P < 0.05$).

L. hirticornis is a solitary parasitoid (that is, a single wasp develops from a single aphid host) that attacks aggregated aphids and will oviposit eggs in the same aphid colony until virtually all available hosts are parasitized or it becomes egg limited (Völkl, 1997). In addition, although winged, the adult parasitoids have a low dispersal ability (Rauch and Weisser, 2007), but this has not been tested directly using marked individuals—either fluoresecent dyes or molecular DNA markers.

In this study, the population dynamics of L. hirticornis on hand-labeled tansy plants colonized by M. fuscoviride were surveyed, and parasitoid samples genotyped at 11 polymorphic microsatellite loci to assess the extent of L. hirticornis relative isolation and dispersal, the relationship of flight behaviour (or lack of it) to philopatry, and the effect of aphid host population structure (that is, that of M. fuscoviride, the second trophic level) on the genetic diversity and differentiation of the parasitoid, L. hirticornis (the third trophic level). To investigate these variables, we estimated the level of L. hirticornis colonization and dispersal on tansy genets colonized by M. fuscoviride, the population genetic differentiation of the parasitoid subpopulations and isolation by distance (IBD), analysed L. hirticornis for philopatric behaviour, and compared the population structure of this haplodiploid parasitoid with that of its diploid aphid host. Given the limited knowledge on the population genetic structure of the parasitoid in relation to that of its host, we tested the following hypotheses: (1) there is significant population genetic differentiation between local colonies of L. hirticornis; (2) as in the case of the host, there is no IBD in the parasitoid; and (3) the level of genetic differentiation in the parasitoid is as high as in the host aphids.

Materials and methods

Study system
Tansy is a perennial composite with bright yellow flowers native to Europe and Asia (Mitich, 1992). It grows mainly along river valleys, but also occurs in well-drained poor soils and on wastelands. It propagates by both sexual and asexual means and a single genet consists of numerous 'genetically identical' ramets produced by vegetative propagation.

The tansy aphid, M. fuscoviride, is a monophagous, holocyclic species (that is, with an autumnal/winter sexual phase involving the laying of cold-hardy eggs) that feeds exclusively on tansy (Massonnet, 2002). M. fuscoviride colonies are normally attended by ants, especially Lasius niger L. (Hymenoptera: Formicidae) (Mackauer and Völkl, 1993). From the aphid, ants obtain an important source of nutrients in the form of honeydew; in return, they act as guards, warding off predators and parasitoids (Völkl, 1997). The parasitoid L. hirticornis, is monophagous on M. fuscoviride (Starý, 1973) and is thought to mimic the cuticular hydrocarbons of its aphid host to evade ant attacks (Dettner and Liepert, 1994; Liepert, 1996).

Field sites
Parasitoid samples in the form of aphid mummies were collected between early June and late October, 2007 from 11 sites in and around Jena, Germany (50° 54′ N, 11° 35′ E; Figure 1). The sites were composed of ruderal habitats (see Supplementary Appendix 1 for site location and description). The geographical positioning system was used to estimate distances between sampling sites, the furthest points being Göschwitz-Bhf and Dornburg-Bhf, ~15 km apart.

Field surveys and population sampling
Genets at all sites were visited and inspected for M. fuscoviride every 10 days, starting June 2007 and ending October 2007. When colonized by aphids, the genet was marked with a label; the numbers of ramets on a genet were then counted along with the number of ramets colonized by aphids. Each aphid colony was estimated numerically (data not shown) and further inspected for aphid mummies. The aphid mummies formed by L. hirticornis are dark brownish in colour and readily distinguished from other parasitoid species attacking M. fuscoviride, in particular Aphidius tanacetarius Mackauer and Ephedrus spp. Haliday (Hymenoptera: Braconidae: Aphidiinae).

During subsequent visits, the fate of marked genets with aphid colonies was followed and any new genet colonization was marked and recorded. The dynamics of L. hirticornis are based on the occurrence of aphid mummies, which provide a basis for reliable estimates of the presence of the developing offspring of parasitoids. During visits, a maximum of 10 aphid mummies were sampled per genet. These were held in 1.5-ml Eppendorf tubes for transport to the laboratory. Here, they were left to eclose and the sex of emerging adult wasps was determined under a stereo binocular microscope. Males and females from a single genet were stored separately in Eppendorf tubes in 100% ethanol at 4 °C before DNA extraction. The study continued for the
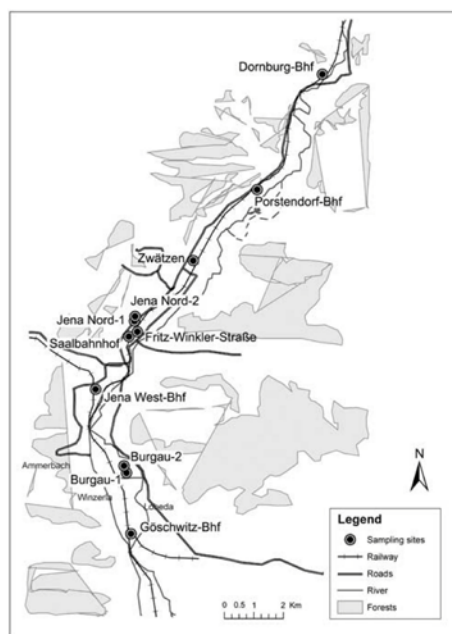
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Figure 1 Schematic representation of the sampling sites of *L. hirticornis*. The closest distance was between Jena Nord-1 and Jena Nord-2, 217 m apart, and the furthest, 15 106 m, between Göschwitz-Bhf and Dornburg-Bhf.

entire growing season, until the shoots senesced and the aphid colonies collapsed.

Microsatellite analysis

For genetic analyses, only female parasitoids were used. In total, DNA from 321 female individuals (that is, between 25 and 30 wasps per site, Table 2) was extracted using the 'salting-out' method of Sunnucks and Hales (1996). Eleven polymorphic microsatellite loci were used as follows: nine (*Lhirt 01*, *Lhirt02*, *Lhirt03*, *Lhirt04*, *Lhirt06*, *Lhirt08*, *Lhirt10*, *Lhirt15*, *Lhirt23*) developed from *L. hirticornis*, and two others derived from published results for other *Lysiphlebus* species, that is, *Lysi08* from *L. fabarum* (Marshall) and *Lysi6b12* from *L. testaceipes* (Cresson) (see Nyabuga *et al.*, 2009 for details and references therein).

Genetic diversity in *L. hirticornis* populations

Genetic diversity was quantified from observed heterozygosity (H_O) and the unbiased estimates of expected heterozygosity (H_E) calculated using ARLEQUIN (v.3.1; 2006, Excoffier *et al.*, 2005). The number of alleles per locus (A) and allelic richness was calculated using FSTAT version 2.9.3.2 (Goudet, 2002). Values for allelic richness were subjected to analysis of variance in SPSS v. 16 (SPSS, 2007) to determine differences in population

genetic diversity. Within-sample deviation from Hardy–Weinberg equilibrium (HWE) was tested in GENEPOP (V.4.0; Raymond and Rousset, 1995) under the dual null hypotheses of both heterozygote excess and deficiency for individual loci as well as over all loci. An estimate of private (unique) allele frequency was also performed using GENEPOP as described by Barton and Slatkin (1986).

The hierarchical partitioning of the genetic variance was calculated with ARLEQUIN using an analysis of molecular variance framework with 11 populations (Weir and Cockerham, 1984; Excoffier *et al.*, 1992). Pairwise and global F_{ST} according to Weir and Cockerham (1984) were calculated in ARLEQUIN. Absolute differentiation (D_{ST}, the proportion of gene diversity present among populations), relative differentiation (G_{ST}, a measure of genetic differentiation estimated as the gene diversity between populations relative to the combined population) (Nei, 1973) and actual differentiation (D) (Jost, 2008) were calculated using SMOGD, version 1.2.5 (Crawford, 2009). In brief, D_{ST} is the difference between total heterozygosity (H_T, heterozygosity of the pooled subpopulations) and the mean within-subpopulation heterozygosity (H_S, the mean heterozygosity of the individual subpopulations): $D_{ST} = H_T - H_S$. Nei (1973) called D_{ST} a measure of absolute differentiation, and he divided this by total diversity (H_T) to obtain what he termed the relative differentiation, G_{ST}: $G_{ST} = D_{ST}/H_T$. Nei's G_{ST} is equivalent to Wright's F_{ST} (Nei, 1973) and has a theoretical minimum of zero indicating no genetic divergence and a theoretical maximum of one indicating fixation for alternative alleles in different subpopulations. The observed maximum, however, is usually much less than one (see Wright, 1978 for further details). Actual differentiation, D by Jost (2008) is calculated as: $D = (J_T/J_S - 1)/((1/n) - 1)$ or $((H_T - H_S)/(1 - H_S)/(1 - H_S))(n/(n-1))$, where J_T is the gene identity of the pooled subpopulations, J_S is the gene identity within subpopulation and *n* the number of subpopulations. Values of D range between zero (no genetic differentiation) to one (subpopulation fixation). Jost (2008) argued that G_{ST} and its relatives did not rank species correctly in terms of differentiation because they used expected heterozygosity in their calculations and discusses a number of shortcomings. Thus he proposed that actual differentiation (D) was a better estimate (see Jost, 2008 for details). In spite of the shortcomings of F_{ST} and its relatives, nevertheless, the index continues to prove useful in many population genetic studies, hence its calculation here.

For each parasitoid population, the proportion of unique genotypes D^* was calculated as the number of genotypes present only in that population divided by the total number of parasitoid wasps analysed (Hunter, 1993).

Bottleneck analysis

A graphical technique was used to test for severe reductions in population size, that is, bottlenecks (Luikart *et al.*, 1998). The method 'allele distortion by graphical technique' involves comparing the distribution of allele frequencies observed in a population suspected of having gone through a bottleneck with the distribution expected in a population not having done so. For

selectively neutral loci in a natural population, allele number and frequency distribution result from a dynamic equilibrium between mutation and genetic drift. The graphical method groups alleles from a sample of at least five polymorphic loci into ten allele frequency classes (0.001–0.100, 0.101–0.200, 0.201–0.300, and so on) and then plots a frequency histogram. Populations that have not gone through a bottleneck and are near mutation-drift equilibrium for selectively neutral loci are expected to have a large proportion of alleles at low frequency allele classes (Luikart *et al.*, 1998). Low-frequency allele classes are defined as those between 0–0.1; high-frequency allele classes between 0.901–1.00; and the eight intermediate classes between 0.101 and 0.900.

The Garza–Williamson index (G–W index; Garza and Williamson, 2001) was also used to estimate bottlenecks. The index is calculated as: $G-W = k/R+1$, where k is the number of alleles at a given locus in a population sample and R is the allelic range of that particular locus. The statistic has a very low value in a population that has passed through a bottleneck and is close to unity in a stationary population.

IBD

The measure of population differentiation $F_{ST}/(1-F_{ST})$ developed by Rousset (1997) was used to test for IBD. F_{ST} values were used to compute Rousset's (1997) genetic distance, and thereafter all IBD calculations were computed using an application program, Isolation by Distance Web Service (IBDWS Version 3.15; Jensen *et al.*, 2005). IBDWS assesses whether there is a statistically significant relationship between the genetic distance (or similarity) matrix and the comparable matrix of geographical distances using Mantel tests (Bohonak, 2002).

Relatedness and assignment of individual genotypes to populations for philopatry analysis

Population assignment was performed using logarithm of likelihood measures calculated in ARLEQUIN. To assign a multilocus genotype (MLG) to a population, an individual's allele frequencies are estimated at each locus. The genotypes' expected frequency at each locus in its sampled population is determined and the value is multiplied across all loci and the product logarithm transformed. The same calculations to estimate the genotypes' frequency in other putative source populations are repeated and the MLG assigned to the population in which it has the highest logarithm likelihood (that is, greatest probability) of occurrence (Paetkau *et al.*, 1995; Waser and Strobeck, 1998).

Estimation of relatedness was performed using the program ML-RELATE (Kalinowski *et al.*, 2006). Each individual in each population was related to all other individuals and the maximum likelihood values obtained (= 102720 pairwise relatedness comparisons) were categorized as follows: 0 = unrelated; 0.0001–0.25 = weakly related; 0.2501–0.5000 = moderately related; 0.5001–0.9999 = highly related; and 1.0 = fully related. Within- and between-population comparisons were then performed using the category frequencies.
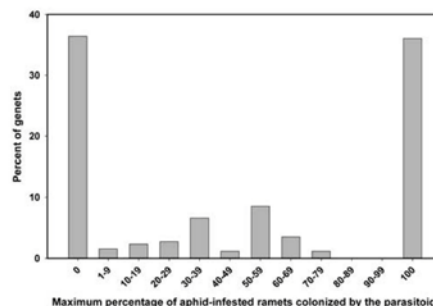
Figure 2 Maximum *L. hirticornis* occupation of aphid colonies within genets. The graph shows the maximum percentage of aphid-infested ramets per genet that also had parasitoids ($N = 258$ genets with aphids). For each genet, the maximum derives from the 12 inspections during the season.

Field data and correlations

Field data means and all Pearson's correlations were performed by SPSS version 16 for windows (SPSS, 2007). The significance level was set at $\alpha = 0.05$.

Results

L. hirticornis population ecology

Parasitoid dynamics: Mummified aphids were found at all sites, indicating the presence of *L. hirticornis*. After pooling data of genets with aphids from all sites over the entire season; 36% of the genets were not colonized by parasitoids, 36% of the genets had had 100% of all aphid colonies colonized by the parasitoid, and on the remaining 28% of genets, between 1 and 79% of their aphid colonized ramets were colonized by the parasitoid (Figure 2). The percentage of genets with aphids colonized by the parasitoid increased from 6% in early June (4–13) to 41.1% in mid-July (4–13), and thereafter began to steadily decline to about 3% by the end of the season (30 October). Within genets colonized by both aphid and parasitoid, the mean percentage of ramets with aphids and colonized by parasitoids increased from 2% in June (4–13) to 40% by the end of the season (30 October), thus showing the infection to have spread locally. Within sites, the percentage of aphid-infested genets with mummified aphids on all aphid-infested ramets ranged from 12% at Porstendorf-Bhf to 69% at Fritz–Winkler–Straße (Supplementary Appendix 1). A total of 1796 *L. hirticornis* parasitoids emerged from the mummies collected; of these, 553 were males, an average sex ratio of 0.31 (sex ratio is calculated as the number of males to total parasitoids). Generally, female biased sex ratios were calculated on all sites and ranged from 0.17 in Porstendorf-Bhf to 0.49 in Saalbahnhof (Table 2).

Microsatellite overall diversity

The observed number of alleles ranged from 2 at locus *Lysi08* and *Lhirt08* to 21 at *Lhirt01* and *Lhirt04*, mean = 8.0 (s.d. = 6.67). Overall expected heterozygosity ranged from 0.186 at *Lysi6b12* to 0.858 at *Lhirt01* (Table 1). Private alleles were recorded at 5 of the 11 loci tested

(Supplementary Appendix 2). Allelic size distributions varied among loci resulting in differences in their overall variances, for example, the variance of *Lhirt04* was 63 times greater than that of *Lysi08* (Table 1). A few loci showed disjunct allelic size distributions in which size classes were separated by several base pairs. This was particularly so at locus *Lhirt04*, which had an allelic size range of 160–286 bp, but no alleles were found between 160–244 bp; locus *Lhirt15* (allele size range 300–330 bp) had no alleles between 300 and 320; and locus *Lhirt01* (allele size range 182–270 bp) had gaps between 182–192, 196–216 and 238–252 bp.

Within-population genetic diversity

The mean number of alleles per locus for each population ranged from 2.9 (Zwätzen) to 4.4 (Burgau-2) (Table 2). Allelic frequencies ranged from 2% at locus *Lysi08* in Burgau-1 to 100% (fixation) at locus *Lysi6b12* in Fritz–Winkler–Straße (Supplementary Appendix 2). The observed differences in allelic richness among populations sampled were not significant ($F_{10,100} = 51.03$, $P = 0.085$).

Of the MLGs observed, 310 were found for the 321 parasitoids analysed at the 11 loci. Ten MLGs were repeated (multiple repeats): nine were duplicated, and one was found three times. The repeated MLGs were sampled from ten tansy genets from various sites (four

from Zwätzen, two from Burgau-1, and one each from Burgau-2, Jena West-Bhf and Fritz–Winkler–Straße). The proportion of unique genotypes (D^*) found per population ranged from 0.87 (Zwätzen) to 1.0 (populations Jena Nord-1, Göschwitz-Bhf, Saalbahnhof, Jena Nord-2 and Porstendorf-Bhf; Table 2). The number of genets from which parasitoids were collected and the proportion of unique genotypes were not correlated ($r = 0.43$, $F_{1,10} = 2.01$, $P = 0.190$). The number of alleles per population and the number of MLGs were marginally correlated ($r = 0.61$, $F_{1,10} = 5.35$, $P = 0.046$); and the number of genets from which the parasitoids were collected was correlated with the number of alleles per population ($r = 0.61$, $F_{1,10} = 5.44$, $P = 0.045$). The correlation between the number of alleles per population and number of parasitoids genotyped was not significant ($r = 0.03$, $F_{1,10} = 0.01$, $P = 0.938$).

Of the 121 Fisher's exact tests performed (11 populations at 11 loci) to measure deviations from HWE, 74 (61.2%) were significant ($P < 0.05$, Table 2); moreover, significant heterozygote deficits were found in 65 of the 74 (87.8%) significant deviations. Only one test showed significant heterozygote excess (Saalbahnhof at locus *Lhirt06*; $P = 0.029$, s.e. = 0.002) Over all loci, all the 11 populations had significantly positive F_{IS} values (Table 2). H_O ranged from 0.367 in subpopulation Jena Nord-2 to 0.170 in Burgau-2, mean = 0.28 ± 0.18 (s.d.); H_E from 0.533 in Jena Nord-2 to 0.423 in Burgau-2, mean = 0.54 ± 0.23 (s.d.).

Population differentiation and IBD

In all, 18 out of the total 88 alleles were private (Supplementary Appendix 2). There were six such alleles at locus *Lhirt01* distributed among six populations and eight at locus *Lhirt04*, distributed as follows: three in Burgau-1, two in Saalbahnhof, two in Jena Nord-2 and one in Jena West-Bhf. Two private alleles at locus *Lhirt06* were found in populations Jena Nord-1 and Saalbahnhof. A single private allele each at loci *Lhirt15* and *Lysi6b12* was recorded in populations at Fritz–Winkler–Straße and Saalbahnhof, respectively. A mean frequency of 0.097 private alleles was calculated. Analysis of molecular variance in ARLEQUIN revealed that 15% of the

Table 1 Allelic richness and allelic size variance for the 11 microsatellite loci used in population analysis

Microsatellite loci	Number of alleles	Allelic size variance (bp)	Expected heterozygosity
Lhirt01	21	88	0.858
Lhirt02	8	14	0.447
Lhirt03	10	40	0.815
Lhirt04	21	126	0.854
Lhirt06	9	24	0.612
Lhirt08	2	3	0.336
Lhirt10	3	5	0.591
Lhirt15	4	30	0.417
Lhirt23	5	28	0.640
Lysi08	2	2	0.202
Lysi6b12	3	8	0.186

Table 2 Details of population genetic diversity in *L. hirticornis* samples at the 11 microsatellite loci

	N_{genets}	Sex ratio	$N_{parasitoids}$	$N_{genotypes}$	D^*	MNA (s.d.)	H_E (s.d.)	H_O (s.d.)	F_{IS}	HW	HD	HE	G_{ST}	D_{ST}	D
Burgau-1	3	0.39	30	28	0.93	3.64 (2.46)	0.42 (0.26)	0.17 (0.14)	0.603***	8	8	0	0.5	0.416	0.784
Burgau-2	17	0.35	30	29	0.97	4.36 (2.46)	0.46 (0.20)	0.25 (0.13)	0.473***	7	7	0	0.459	0.386	0.779
Dornburg-Bhf	2	0.39	30	29	0.97	3.91 (2.07)	0.44 (0.24)	0.25 (0.12)	0.442***	7	5	0	0.494	0.419	0.807
Fritz–Winkler–Straße	5	0.42	25	28	0.93	3.91 (2.43)	0.51 (0.27)	0.27 (0.26)	0.473***	9	8	0	0.417	0.361	0.802
Göschwitz-Bhf	7	0.23	26	26	1	3.64 (1.37)	0.52 (0.20)	0.35 (0.22)	0.325***	8	6	0	0.393	0.33	0.740
Jena Nord-1	11	0.26	30	30	1	4.00 (2.22)	0.48 (0.22)	0.29 (0.18)	0.414***	6	5	0	0.447	0.383	0.802
Jena Nord-2	19	0.29	30	30	1	4.27 (2.86)	0.53 (0.20)	0.37 (0.16)	0.316***	5	4	0	0.381	0.323	0.748
Jena West-Bhf	11	0.27	30	29	0.97	4.09 (2.31)	0.44 (0.26)	0.25 (0.14)	0.426***	6	5	0	0.494	0.418	0.803
Porstendorf-Bhf	7	0.17	30	30	1	3.55 (1.67)	0.44 (0.23)	0.27 (0.20)	0.385***	6	5	0	0.487	0.406	0.782
Saalbahnhof	10	0.49	30	25	1	3.64 (1.92)	0.44 (0.20)	0.31 (0.16)	0.285***	4	5	1	0.495	0.418	0.801
Zwätzen	6	0.28	30	26	0.87	2.91 (1.08)	0.43 (0.19)	0.27 (0.12)	0.390***	8	6	0	0.493	0.416	0.799

Abbreviations: D^*, proportion of unique genotypes found per parasitoid population; D, actual differentiation (Jost, 2008); D_{ST}, absolute differentiation (Nei, 1973); F_{IS}, individual inbreeding coefficient in relation to subpopulation; G_{ST}, relative differentiation (Nei, 1973); HD, number of heterozygote deficits; H_E, expected heterozygosity; HE, number of heterozygote excesses; H_O, observed heterozygosity; HW, number of loci showing significant deviations from HWE; MNA, mean number of alleles per locus.
For each population, number of genets (N_{genets}) from which the sample ($N_{parasitoids}$) were collected; sex ratio (males: total parasitoids); number of different multilocus genotypes deduced ($N_{genotypes}$).
***$P < 0.001$.

variance was due to 'among-populations' differences, 35% due to 'among individuals within populations' and 50% due to 'within individuals' differences.

Absolute differentiation, D_{ST}, ranged from 0.419 in Dornburg-Bhf to 0.323 in Jena Nord-2 (Table 2). Pairwise F_{ST} values ranged from 0.065 (between Jena Nord-2 and Dornburg-Bhf) to 0.233 (between Zwätzen and Saalbahnhof; Table 3) and overall global F_{ST} was 0.148. Relative differentiation, G_{ST}, showed Burgau-1 to be the most genetically differentiated parasitoid subpopulation (0.5) and Jena Nord-2 the least (0.381) with mean G_{ST} value of 0.46. Actual differentiation, D, ranged from 0.74 in Göschwitz-Bhf to 0.807 in Dornburg-Bhf with a mean of 0.786, indicating high levels of subpopulation differentiation (Table 2).

A Mantel test performed for matrix correlation between genetic distance ($F_{ST}/(1-F_{ST})$) and logarithm of geographical distance showed no IBD relationship ($Z = 34.88$, $r = -0.21$, intercept $= 0.84 \pm 0.09$ s.e.; slope $= -0.18 \pm 0.03$ s.e.; $N = 55$: one-sided $P = 0.86$ from 1000 randomizations).

Bottlenecks

Graphical distortions of allele frequencies showed that the majority of alleles in all populations studied to be in the low-frequency class (0.001–0.1) and therefore had an ordinary distribution of alleles, an indication that bottlenecks were not important in determining the population genetic makeup observed. In contrast, at the level of individual loci, the G–W index revealed some low values (<0.1) at three loci to a mean 0.636 at *Lysi08* (Table 4). Over all loci, means ranged from 0.203 in population Burgau-1 to 0.264 in Burgau-2. At loci *Lhirt08* and *Lhirt10*, all populations had a mean of 0.5, and at locus *Lysi08*, ten populations had a mean of 0.667. The low values of the G–W index sometimes encountered (3 of 11 values; Table 4) suggests that these populations may have gone through bottlenecks at the various loci tested.

Philopatry

Population assignment using ARLEQUIN revealed that in 4 of the 11 populations studied (that is, Burgau-2, Jena Nord-1, Saalbahnhof and Zwätzen), all analysed individuals originated from their natal populations (Supplementary Appendix 3, Figures a–d). In the remaining subpopulations, most individuals were most likely to have originated from their natal patches, and only a few individuals (1–6) are predicted to have come from other subpopulations, for example, in Burgau-1, one individual may have originated from either Göschwitz-Bhf or Dornburg-Bhf, and in Fritz–Winkler–Straße, six individuals showed a high likelihood of having originated from other subpopulations (Supplementary Appendix 3, Figures e–k).

Analysis of within- and between-population relatedness using ML-RELATE showed that all populations had high proportions of full (1.0000), high (0.5001–0.9999) or moderate (0.2501–0.5000) degrees of relatedness within the population (Figure 3). In contrast, relatedness among populations was very low, as seen from the high proportions of unrelatedness (that is, zero) and low relatedness (0.0001–0.2500) values among pairs of individuals of different populations.

Table 3 Pairwise F_{ST} values (under the diagonal) of each population comparison according to Weir and Cockerham (1984) and their level of significance (above the diagonal)

	Burgau-1	Burgau-2	Dornburg-Bhf	Fritz–Winkler–Straße	Göschwitz-Bhf	Jena Nord-1	Jena Nord-2	Jena West-Bhf	Porstendorf-Bhf	Saalbahnhof	Zwätzen
Burgau-1		***	***	***	***	***	***	***	***	***	***
Burgau-2	0.111		***	***	***	***	***	***	***	***	***
Dornburg-Bhf	0.105	0.121		***	***	***	***	***	***	***	***
Fritz–Winkler–Straße	0.161	0.141	0.154		***	***	***	***	***	***	***
Göschwitz-Bhf	0.145	0.159	0.226	0.119		***	***	***	***	***	***
Jena Nord-1	0.196	0.161	0.065	0.187	0.226		***	***	***	***	***
Jena Nord-2	0.101	0.070	0.165	0.124	0.094	0.169		***	***	***	***
Jena West-Bhf	0.174	0.089	0.078	0.137	0.176	0.198	0.103		***	***	***
Porstendorf-Bhf	0.091	0.096	0.221	0.130	0.140	0.204	0.069	0.093		***	***
Saalbahnhof	0.219	0.202	0.221	0.207	0.236	0.220	0.198	0.214	0.223		***
Zwätzen	0.206	0.126	0.122	0.159	0.170	0.233	0.099	0.158	0.160	0.244	

*** = $P<0.001$.

Table 4 Garza–Williamson indices per locus for each parasitoid population sample and means per locus for each population

	Lhirt01	Lhirt02	Lhirt03	Lhirt04	Lhirt06	Lhirt08	Lhirt10	Lhirt15	Lhirt23	Lysi08	Lysi6b12	Mean G–W index/population
Burgau-1	0.056	0.133	0.171	0.071	0.120	0.500	0.500	0.065	0.172	0.333	0.111	0.203
Burgau-2	0.079	0.200	0.195	0.063	0.280	0.500	0.500	0.065	0.138	0.667	0.222	0.264
Dornburg-Bhf	0.090	0.267	0.146	0.055	0.120	0.500	0.500	0.065	0.138	0.667	0.222	0.252
Fritz–Winkler–Straße	0.112	0.267	0.146	0.047	0.120	0.500	0.500	0.097	0.103	0.667	0.111	0.243
Göschwitz-Bhf	0.067	0.267	0.122	0.039	0.120	0.500	0.500	0.097	0.172	0.667	0.222	0.252
Jena Nord-1	0.090	0.200	0.146	0.063	0.160	0.500	0.500	0.065	0.138	0.667	0.222	0.250
Jena Nord-2	0.112	0.267	0.171	0.071	0.080	0.500	0.500	0.065	0.138	0.667	0.222	0.254
Jena West-Bhf	0.101	0.267	0.122	0.063	0.160	0.500	0.500	0.065	0.138	0.667	0.222	0.255
Porstendorf-Bhf	0.079	0.200	0.146	0.039	0.080	0.500	0.500	0.097	0.138	0.667	0.222	0.243
Saalbahnhof	0.045	0.200	0.122	0.071	0.120	0.500	0.500	0.097	0.138	0.667	0.222	0.244
Zwätzen	0.045	0.133	0.122	0.031	0.080	0.500	0.500	0.065	0.138	0.667	0.222	0.228
Mean G-W index/locus	0.080	0.218	0.146	0.056	0.131	0.500	0.500	0.076	0.141	0.636	0.202	0.244

Abbreviation: G–W index, Garza–Williamson index.
Values close to zero = population or locus in bottleneck, whereas values close to one = non-bottlenecked locus or population.

Discussion

This study highlights variations in levels of genetic diversity and differentiation among different *L. hirticornis* subpopulations (demes) comprising the metapopulation. Another main finding is the restricted level of aerial dispersal of the winged adult parasitoids and the lack of significant IBD as calculated from the molecular genetic data over the geographical scale studied. Population assignment measures indicate small amounts of genetic 'leakage' (gene flow) between populations, but with highly related individuals within sites compared with between sites, a characteristic of philopatric behaviour. Given the similar F_{ST} and heterozygosity results for *L. hirticornis* in this study to those of the earlier independent study of *M. fuscoviride* by Massonnet (2002), we suggest that *L. hirticornis* population genetic/ecological structuring and dynamics are directly influenced by the metapopulation genetic structure of its aphid host.

Parasitoid metapopulation ecology in the field
In the field study, winged adult *L. hirticornis* were observed to fly as well as walk between ramets within individual tansy genets harbouring aphid colonies. It is therefore highly probable that *L. hirticornis* flies, at least occasionally, to nearby genets and ramets. Our field results show that the number of ramets occupied by the parasitoid increased over time. Although cases of up to 100% aphid mummification on a ramet or genet were recorded, aphid colonies on some ramets and genets had no mummies throughout the season (Figure 2). These results thus confirm those of Weisser (2000) who also found that many genets colonized by aphids within a tansy stand were not attacked by the parasitoid, even though instances in which 100% of aphids in a ramet and/or nearby genet were parasitized. Rauch and Weisser (2007) similarly reported up to 100% rates of parasitism and noted that *L. hirticornis* was rarely found on plants on which it had not been released.

Genetic variability and differentiation between populations
Genetic diversity for *L. hirticornis* in our study measured as observed heterozygosity was between 0.170–0.367, and hence is in the range of other parasitoid populations studied using microsatellites, for example,

$H_O = 0.171–0.629$ in *Neotypus melanocephalus* Gmelin (Hymenoptera: Ichneumonidae) (Anton *et al.*, 2007); 0.486–0.594 in *Aphidius ervi* Haliday (Hymenoptera: Braconidae: Aphidiinae) (Hufbauer *et al.*, 2004); and 0.250–0.538 in *Diaeretiella rapae* M'Intosh (Hymenoptera: Braconidae: Aphidiinae) (Baker *et al.*, 2003). Global population differentiation in *L. hirticornis* ($F_{ST} = 0.148$) is slightly higher than in *N. melanocephalus* ($F_{ST} = 0.116$) (Anton *et al.*, 2007). *Cotesia melitaearum* Wilkinson (Hymenoptera: Braconidae) and *H. horticola* had F_{ST} values of 0.378 and 0.063, respectively (Kankare *et al.*, 2005). It seems, however, that *L. hirticornis* has dispersal behaviour similar to *N. melanocephalus* and *C. melitaearum*. Population assignment measures indicate small amounts of genetic leakage between the subpopulations and relatedness analysis shows that individuals within a site were highly related compared with between sites, findings that further support the view that *L. hirticornis* displays philopatric behaviour.

Compared with G_{ST} and F_{ST} values, actual differentiation (D) values were higher and closer to unity, consistent with Jost (2008). The three measures indicated high levels of differentiation within populations and unlike Jost (2008), our results show that despite the presence of private alleles at a number of loci, *L. hirticornis* metapopulations shared many alleles.

With regard to population bottlenecks in our study, distorting allele frequencies and using graphical method (Luikart *et al.*, 1998) to test for severe reduction in population size failed to show such restrictions. In contrast, the G–W index gave low values (means <0.3), and as values close to unity are expected in populations that have not been through bottlenecks (Garza and Williamson, 2001), it is probable that bottlenecks are at least partially responsible for the population genetic structuring seen in *L. hirticornis*.

Departures from HWE, as found in all populations, may be due to either null alleles or violations of other assumptions inherent in fulfilling HW expectations. These include non-random mating, migration, selection, genetic drift, mutation and severe bottlenecks (discussed in preceding paragraph), and, in the case of the essentially neutral molecular markers used here, could be due to close linkage to areas under selection (hitchhiking). Failed amplifications were experienced in some individual parasitoids at some loci, even when the
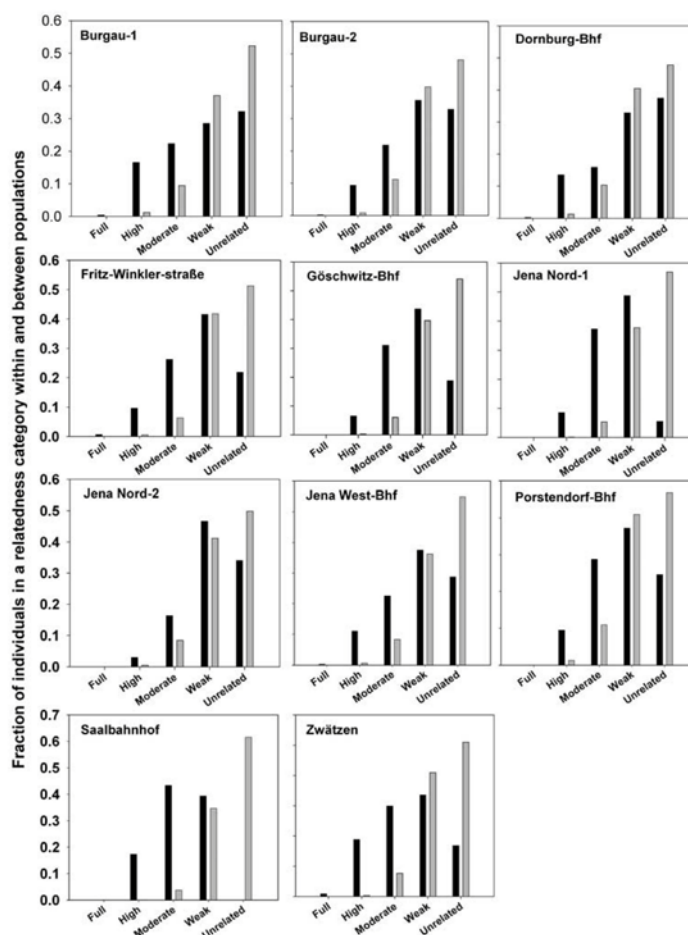
Lysiphlebus hirticornis, philopatry and dispersal
FN Nyabuga *et al*

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Figure 3 Within- and among-population relatedness of *L. hirticornis* individuals. For each of the 11 populations, pairwise levels of relatedness were calculated between individuals of the same population (within-population, full black bars) and between individuals of a population to individuals of other populations (among-population, light grey bars). Relatedness values were grouped as follows: 0 = unrelated; 0.0001–0.25 = weakly related; 0.2501–0.5000 = moderately related; 0.5001–0.9999 = highly related and 1.0 = fully related.

same individuals amplified satisfactorily at other loci. Nyabuga *et al.* (2009) report the possibility of null alleles at 6 of the 11 loci used in this study. It is unlikely, however, that null alleles were a main source of HW deviation because 88% of the male haploid parasitoids genotyped consistently gave the expected hemizygous genotype pattern at each of the 11 loci tested.

The F_{IS} values were all positive (homozygote excess) in all subpopulations, indicating that non-random mating occurred. Private or unique alleles were also recorded for the different subpopulations at different loci and these results support the view that the parasitoid tends to

undergo inbreeding at a very local level. The 10 repeated MLGs derived from parasitoid populations on aphids infesting a total of 10 tansy plants at 5 of the 11 sites examined. One site (Zwätzen) had four repeated MLGs deriving from four genets. This implies, importantly, that the unit of random mating is within a genet or a group of closely located genets, rather than at the level of the whole site.

Previous behavioural studies (see review by Völkl, 1997) established that a single *L. hirticornis* female oviposited many eggs (up to 30) within aggregated aphid colonies. Recent laboratory studies showed that

L. hirticornis stayed on the natal patch for up to 4 h after emergence, and 52 out of 58 individuals (~90%) observed had matings on the natal patch (FN Nyabuga *et al.*, unpublished), emphasizing the philopatric nature of *L. hirticornis* involving inbreeding.

Comparison of *L. hirticornis* genetic differentiation with that of its aphid host

Previous studies indicate that the haplodiploid nature of parasitoids (haploid males are produced from unfertilized eggs, diploid females from fertilized eggs) results in higher population genetic differentiation than in diploid organisms (Johannesen and Seitz, 2003; Anton *et al.*, 2007). For example, Anton *et al.* (2007) reported higher F_{ST} values in the parasitoid *N. melanocephalus* (0.115) compared with its host butterfly, *M. nausithous* (0.087). Using a simulation study, they found that only part of the higher genetic differentiation of the parasitoid can be attributed to haplodiploidy. Massonnet (2002), working on *M. fuscoviride*, reported an overall F_{ST} of 0.222, with values ranging from 0.066 to 0.230 in Germany (Bayreuth and Münster) and an even lower F_{ST} (0.148) for populations from Alsace in France. Populations of the aphid host *M. fuscoviride* (Massonnet, 2002) and *L. hirticornis* (this study; $F_{ST} = 0.148$) are thus both highly genetically differentiated, with no difference in F_{ST} values between the two species, the parasitoid at small spatial scale and the aphid on a larger geographical scale. Perhaps the failure to find higher F_{ST} values for the parasitoid compared with the host is due to the different sampling period or to the different sampling parameters, that is, localities and spatial levels. Massonnet (2002) reported H_E values ranging from 0.27 to 0.52 for *M. fuscoviride*, whereas our results ranged from 0.423 to 0.533 for *L. hirticornis*, essentially rather similar.

Philopatry and survival in a metapopulation context

The ecological and genetic data presented here, in addition to unpublished behavioural data (Nyabuga *et al.*, in preparation), reveal a high level of *L. hirticornis* philopatry within *M. fuscoviride* colonies with little aerial dispersal between them. Thus, even though local extinctions of the aphid host are frequent, the parasitoid continues to exploit its host and contributes greatly to the demise of local aphid colonies, rather than spreading its genes over many aphid subpopulations, thereby (potentially at least) increasing genetic heterogeneity. In fact, this study system represents one of the few field examples involving metapopulations in which host colony extinction is directly due to natural enemy attack (Weisser, 2000). The low probability of the host population's survival raises the question as to why this particular behaviour involving philopatry has evolved, rather than a behaviour that would spread the risk, that is, an oviposition strategy that distributes eggs over several aphid colonies, as observed in many other parasitoids (Mackauer and Völkl, 1993). The parasitoid is apparently able to evade ant attacks by chemical mimicry, as found in *Lysiphlebus cardui* (Marshall) attacking the black bean aphid, *Aphis fabae cirsiiacanthoidis* Schrank on creeping thistle (Liepert and Dettner, 1996). We argue that a key trait of the parasitoid—the ability to use chemical mimicry to evade attack by aphid-tending ants—explains this counter-intuitive behaviour.

As in many *Lysiphlebus* species, chemical signals on the epicuticular surface of *L. hirticornis* mimic those of its aphid host, hence making the parasitoid chemically undetectable to ants (Dettner and Liepert, 1994; Liepert, 1996). As a consequence, the parasitoid is able to exploit ant-tended aphid colonies and the offspring obtain protection by ants from attack by even higher trophic levels, that is, hymenopterous hyperparasitoids (Völkl, 1992). Ant-tended colonies also persist longer than untended ones. By concentrating ovipositions into already encountered ant-tended colonies, parasitoids apparently realize higher reproductive success than would otherwise be possible by costly dispersal and oviposition into several aphid colonies, many of which do not survive for very long (a mean survival time of 4.8 weeks has been reported for aphids at the genet level; Weisser, 2000).

In conclusion, this study, the first on an aphid–parasitoid metapopulation system, shows that *L. hirticornis* has limited dispersal between resource patches or interpopulation movement coupled with philopatry and inbreeding. Genetic differentiation measurements clearly identified similarities between genetic structuring in *L. hirticornis* and its aphid host, *M. fuscoviride*.

Conflict of interest

The authors declare no conflict of interest.

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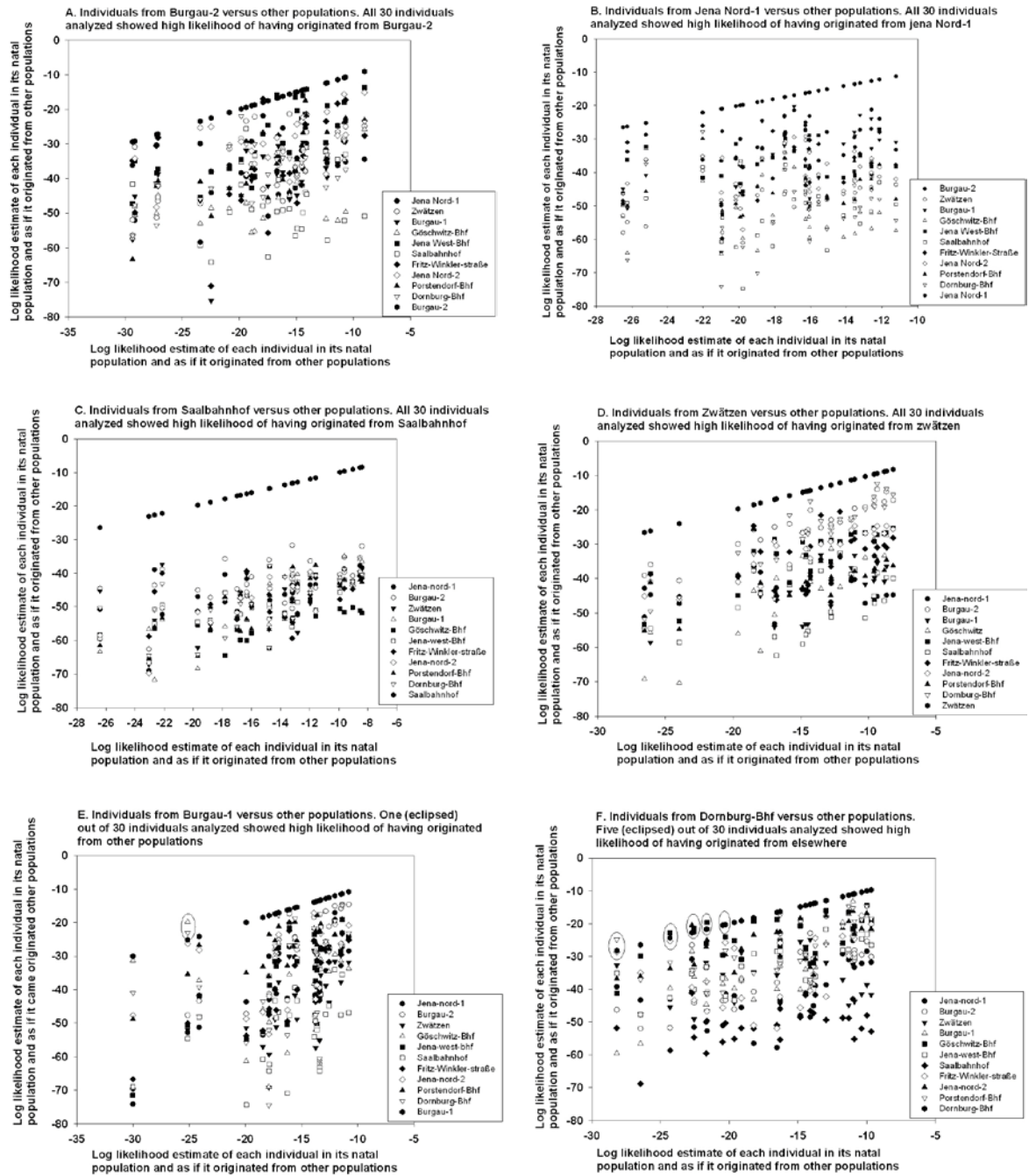
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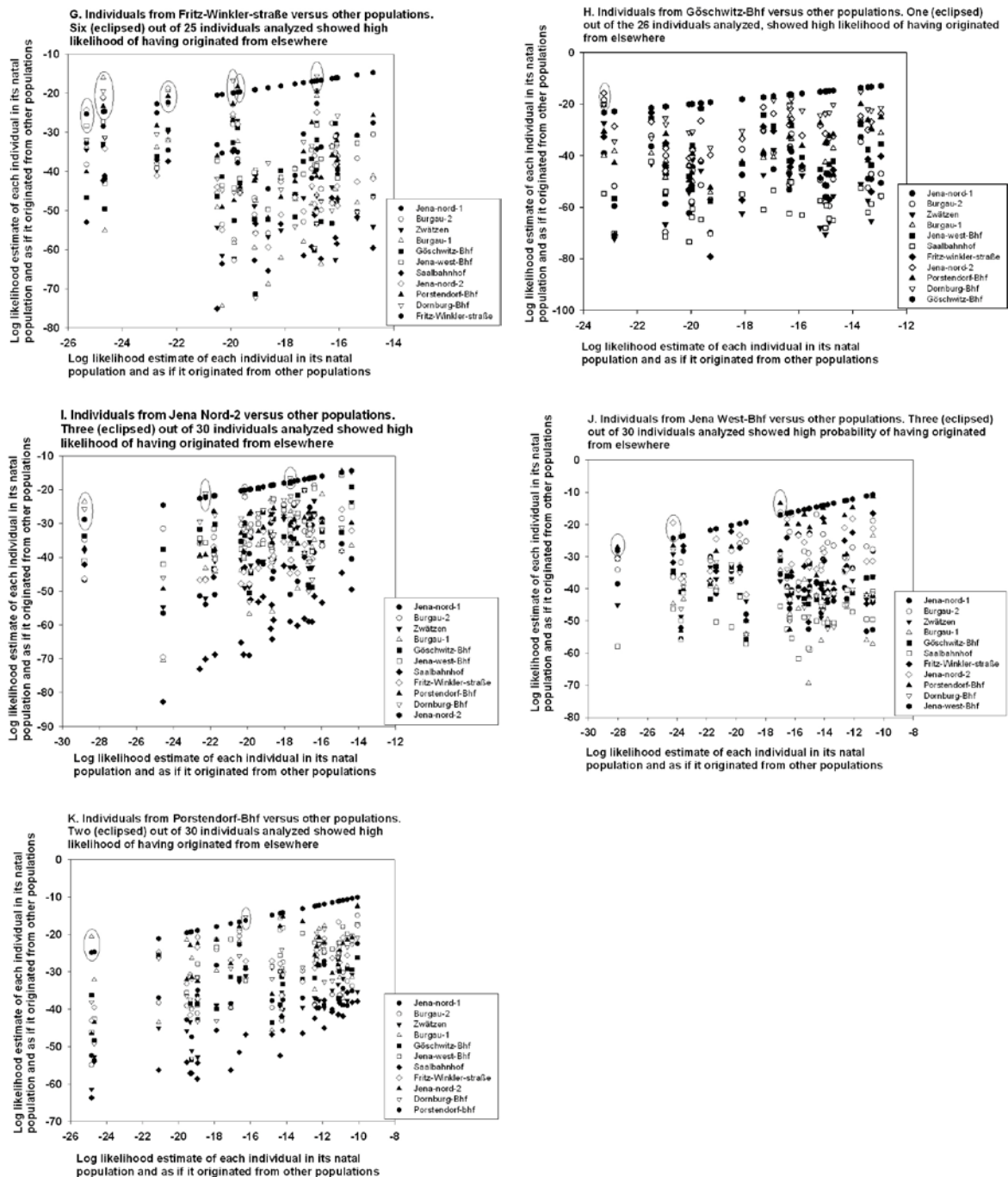
Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)

Supplementary material for article II next

Supplementary 1 Details of sampling sites for *L. hirticornis* in and around Jena between June and October, 2007. A short description of the site; approximate size of the area; GPS coordinates; number of genets counted per site (GOS); number of genets with aphids counted at least once by end of the season (GWAP); number of genets with aphids and without parasitoids by end of the season (GWAPNOP); and number of genets with aphids and parasitoid on each ramet with aphids by the end of the season (GWAPWP)

Site	Location and description	Area	GPS points		GOS	GWAP	GWAPNOP	GWAPWP
			X-coordinates	Y-coordinates				
Burgau-1	Located besides Burgau Wasserwerk on Burgau Park in Jena. Site mowed in late October.	1166m^2	4471378	5640463	19	9	4	4
Burgau-2	Wasteland between a bicycle track and railway lines to Burgau Park. A 2m mowing along the edges destroyed marked plants.	1700m^2	4470951	5640856	132	32	8	13
Dornburg-Bhf	Located by the rail lines at Dornburg Bahnhof to the north of Jena.	1852m^2	4477397	5652717	250	10	5	3
Fritz-Winkler-straße	Located between the Obi complex and buildings on one side near Fritz-Winkler-straße.	488m^2	4471877	5645018	35	16	3	11
Göschwitz-Bhf	Located on the southern part of Jena, Göschwitz Saale Bahnhof.	370m^2	4471466	5638875	87	20	6	10
Jena Nord-1	Open field on Zeitzer straße and Camburger straße. Site mowed in mid-October.	8160m^2	4472202	5645742	105	20	2	9
Jena Nord-2	Open field between Zeitzer straße and Altenburger straße, off Dornburger straße. Site mowed in mid-September.	3257m^2	4472222	5645883	107	55	17	17
Jena West-	Besides platform 2 on the Jena West Bahnhof.	1355m^2	4470401	5643197	168	32	15	14

Site	Location and description	Area	GPS points		GOS	GWAP	GWAPNOP	GWAPWP
Bhf								
Porstendorf-Bhf	Located at the Porstendorf Bahnhof along the main Jena-Dorburg Road.	500m^2	4475292	5649233	34	25	16	3
Saalbahnhof	Along old railway lines at the Saalbahnhof. Sections of the site were regularly mowed destroying marked aphid infested plants.	150m^2	4471669	5644884	45	22	9	5
Zwätzen	An open field on Am Flutgraben. Site mowed in early October.	12075m^2	4473819	5647012	45	17	8	4

A. Individuals from Burgau-2 versus other populations. All 30 individuals analyzed showed high likelihood of having originated from Burgau-2

B. Individuals from Jena Nord-1 versus other populations. All 30 individuals analyzed showed high likelihood of having originated from jena Nord-1

C. Individuals from Saalbahnhof versus other populations. All 30 individuals analyzed showed high likelihood of having originated from Saalbahnhof

D. Individuals from Zwätzen versus other populations. All 30 individuals analyzed showed high likelihood of having originated from zwätzen

E. Individuals from Burgau-1 versus other populations. One (eclipsed) out of 30 individuals analyzed showed high likelihood of having originated from other populations

F. Individuals from Dornburg-Bhf versus other populations. Five (eclipsed) out of 30 individuals analyzed showed high likelihood of having originated from elsewhere

G. Individuals from Fritz-Winkler-straße versus other populations. Six (eclipsed) out of 25 individuals analyzed showed high likelihood of having originated from elsewhere

H. Individuals from Göschwitz-Bhf versus other populations. One (eclipsed) out of the 26 individuals analyzed, showed high likelihood of having originated from elsewhere

I. Individuals from Jena Nord-2 versus other populations. Three (eclipsed) out of 30 individuals analyzed showed high likelihood of having originated from elsewhere

J. Individuals from Jena West-Bhf versus other populations. Three (eclipsed) out of 30 individuals analyzed showed high probability of having originated from elsewhere

K. Individuals from Porstendorf-Bhf versus other populations. Two (eclipsed) out of 30 individuals analyzed showed high likelihood of having originated from elsewhere

Supplementary 2 The graphs display results of population assignment using ARLEQUIN. Figures A-D show populations in which all the individuals analyzed showed high probabilities of having originated from those natal populations, whilst in Figures E-K some individuals (eclipsed) had a high likelihood of having originated from other populations

Supplementary 3 Allele frequencies at each locus for each population. Highlighted values indicate private (unique) alleles

Locus	Allele length (bp)	Burgau-1	Burgau-2	Dornburg-Bhf	Fritz-Winkler-straße	Göschwitz-Bhf	Jena Nord-1	Jena Nord-2	Jena West-Bhf	Porstendorf-Bhf	Saalbahnhof	Zwätzen
Lhirt01	182	0	0	0	0	0.038	0	0	0	0	0	0
	192	0.05	0.017	0.1	0.1	0.269	0.033	0.067	0.017	0.117	0	0
	194	0.017	0.033	0	0	0	0	0.017	0.017	0	0	0.217
	196	0	0	0	0.033	0	0	0	0	0	0.1	0
	216	0	0	0	0	0	0	0	0	0	0.74	0
	222	0	0	0.033	0.05	0.115	0	0.133	0.6	0.383	0	0
	224	0.567	0.483	0.45	0.283	0.462	0.267	0.367	0.1	0.35	0	0
	226	0	0.317	0.133	0.15	0	0	0	0	0	0.08	0.633
	228	0	0.1	0.033	0	0	0.133	0.05	0	0	0.08	0
	230	0	0	0.067	0	0	0	0	0	0	0	0
	232	0.033	0	0.083	0.067	0.077	0	0.15	0	0.033	0	0
	234	0.333	0.033	0	0.15	0	0.117	0	0.117	0.05	0	0
	236	0	0.017	0	0.033	0	0	0	0	0.033	0	0
	238	0	0	0	0	0	0	0	0	0	0	0.033
	252	0	0	0	0	0	0	0.033	0	0	0	0
	254	0	0	0.1	0	0.038	0	0.05	0.05	0.033	0	0
	256	0	0	0	0.033	0	0.033	0.1	0.017	0	0	0
	258	0	0	0	0	0	0.067	0.033	0	0	0	0
	260	0	0	0	0.1	0	0.233	0	0.033	0	0	0
	262	0	0	0	0	0	0.117	0	0	0	0	0.117
	270	0	0	0	0	0	0	0	0.05	0	0	0
Lhirt02	126	0	0	0	0	0.135	0	0.05	0	0	0	0

Locus	Allele length (bp)	Burgau-1	Burgau-2	Dornburg-Bhf	Fritz-Winkler-straße	Göschwitz-Bhf	Jena Nord-1	Jena Nord-2	Jena West-Bhf	Porstendorf-Bhf	Saalbahnhof	Zwätzen
	128	0	0	0.067	0	0.096	0	0.133	0	0	0	0
	130	0	0.033	0	0	0	0	0	0.033	0	0	0
	132	0.367	0	0	0	0	0	0	0	0.133	0	0
	134	0.633	0.917	0.8	0.233	0.404	0.867	0.783	0.933	0.833	0.84	0.8
	136	0	0	0.083	0.5	0.365	0.1	0	0.017	0.033	0	0
	138	0	0.05	0.05	0.033	0	0.033	0.033	0.017	0	0.1	0.2
	140	0	0	0	0.233	0	0	0	0	0	0.06	0
Lhirt03	192	0.117	0.05	0	0	0.019	0	0	0	0	0	0
	208	0.067	0.033	0.067	0.133	0.077	0.117	0.167	0.15	0.167	0.08	0.317
	212	0.017	0.55	0	0.367	0	0.267	0.1	0.517	0.35	0.06	0.167
	214	0.333	0.133	0.6	0.283	0.192	0.167	0.333	0.15	0.333	0.62	0.317
	218	0.217	0.067	0	0	0	0.383	0.233	0	0.033	0	0.033
	222	0.133	0	0.15	0	0.635	0	0.033	0	0.033	0.12	0
	224	0.117	0.083	0.017	0.033	0	0.033	0.1	0.033	0.083	0	0.167
	228	0	0.067	0	0.1	0.077	0.033	0.033	0.15	0	0.12	0
	230	0	0.017	0.083	0	0	0	0	0	0	0	0
	232	0	0	0.083	0.083	0	0	0	0	0	0	0
Lhirt04	160	0	0	0	0	0	0	0.017	0	0	0	0
	244	0.117	0	0.067	0	0.058	0	0.1	0	0	0	0
	246	0	0.017	0	0	0	0	0	0.05	0	0	0
	248	0	0	0	0	0	0	0	0.067	0	0	0
	250	0	0.033	0	0	0	0	0	0.033	0	0	0
	252	0	0.4	0.267	0	0.327	0	0.3	0.3	0	0	0.667
	254	0.233	0.267	0	0.283	0	0.183	0.283	0.3	0.467	0.1	0

Locus	Allele length (bp)	Burgau-1	Burgau-2	Dornburg-Bhf	Fritz-Winkler-straße	Göschwitz-Bhf	Jena Nord-1	Jena Nord-2	Jena West-Bhf	Porstendorf-Bhf	Saalbahnhof	Zwätzen
	256	0.383	0.15	0.05	0.133	0	0.583	0.133	0.1	0.017	0.32	0.2
	260	0.05	0.033	0.083	0.217	0.154	0.067	0.067	0.117	0.033	0.2	0.033
	262	0	0.083	0	0	0	0.017	0	0	0	0.06	0
Lhirt04	264	0	0	0.1	0.167	0	0.05	0	0	0.367	0.12	0.1
	266	0	0	0	0	0	0	0	0	0	0.1	0
	268	0.017	0	0.4	0.183	0.423	0.033	0.05	0.033	0.117	0	0
	270	0	0.017	0	0.017	0	0.05	0.017	0	0	0.04	0
	272	0	0	0	0	0	0	0.033	0	0	0	0
	274	0.117	0	0	0	0	0	0	0	0	0	0
	276	0.017	0	0	0	0	0	0	0	0	0	0
	278	0	0	0.033	0	0.038	0.017	0	0	0	0	0
	280	0.017	0	0	0	0	0	0	0	0	0	0
	282	0	0	0	0	0	0	0	0	0	0	0.02	0
	286	0.05	0	0	0	0	0	0	0	0	0.04	0
Lhirt06	246	0	0.017	0	0	0	0	0	0	0	0.02	0
	250	0.3	0.033	0.083	0	0.269	0	0.1	0.033	0.2	0	0
	252	0	0.083	0.017	0.367	0	0.033	0	0	0	0	0.1
	254	0	0	0	0	0	0.017	0	0	0	0	0
	256	0	0	0	0	0	0	0	0	0	0.3	0
	260	0	0.083	0	0	0	0	0	0.4	0	0	0
	262	0	0.05	0	0	0	0	0	0	0	0.68	0
	266	0.633	0.533	0.9	0.567	0.712	0.017	0.9	0.517	0.8	0	0.9
	270	0.067	0.2	0	0.067	0.019	0.933	0	0.05	0	0	0
Lhirt08	218	0.067	0.117	0.117	0.317	0.385	0.267	0.283	0.15	0.083	0.28	0.317

Locus	Allele length (bp)	Burgau-1	Burgau-2	Dornburg-Bhf	Fritz-Winkler-straße	Göschwitz-Bhf	Jena Nord-1	Jena Nord-2	Jena West-Bhf	Porstendorf-Bhf	Saalbahnhof	Zwätzen
	221	0.933	0.883	0.883	0.683	0.615	0.733	0.717	0.85	0.917	0.72	0.683
Lhirt10	295	0.2	0.167	0.167	0.217	0.154	0.15	0.033	0.15	0.217	0.08	0.183
	298	0.683	0.7	0.45	0.45	0.519	0.467	0.633	0.483	0.55	0.62	0.433
	300	0.117	0.133	0.383	0.333	0.327	0.383	0.333	0.367	0.233	0.3	0.383
Lhirt15	300	0	0	0	0.017	0	0	0	0	0	0	0
	320	0	0	0	0	0.038	0	0	0	0.017	0.04	0
	326	0.333	0.217	0.5	0.033	0.308	0.417	0.317	0.067	0.417	0.16	0.3
	330	0.667	0.783	0.5	0.95	0.654	0.583	0.683	0.933	0.567	0.8	0.7
Lhirt23	246	0.033	0	0	0	0.038	0	0	0	0	0.02	0
	250	0.05	0.15	0.1	0	0.269	0.067	0.133	0.1	0.05	0.12	0.033
	259	0.733	0.667	0.583	0.35	0.346	0.283	0.433	0.433	0.55	0.54	0.35
	266	0.033	0.017	0.067	0.1	0.077	0.117	0.15	0.167	0.033	0	0.067
	274	0.15	0.167	0.25	0.55	0.269	0.533	0.283	0.3	0.367	0.32	0.55
Lysi08	117	0	0.2	0.05	0.15	0.038	0.15	0.3	0.1	0.05	0.1	0.1
	119	1	0.8	0.95	0.85	0.962	0.85	0.7	0.9	0.95	0.9	0.9
Lysi6b12	157	0	0	0	0	0	0	0	0	0	0.06	0
	163	0	0.183	0.05	0	0.135	0.2	0.283	0.05	0.067	0	0.1
	165	1	0.817	0.95	1	0.865	0.8	0.717	0.95	0.933	0.94	0.9

Article III: The consequences of an unstable relationship: genetical metapopulation dynamics of *Lysiphlebus hirticornis* Mackauer, a specialist parasitoid attacking a specialist aphid host on common tansy

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Abstract

In insect species characterized by inbreeding and metapopulation structure, high genetic differentiation and reduced genetic diversity within a local population are expected. Using the model system *Lysiphlebus hirticornis,* a specialist parasitoid of the tansy aphid, *Metopeurum fuscoviride,* we examined within-site temporal population dynamics and genetics, including differentiation at the tansy whole plant (=genet) level. Aphid-parasitoid metapopulation dynamics were surveyed and parasitoids sampled from 72 genets at 11 sites in and around Jena over a single annual growing season. Thereafter, the parasitoid samples were genotyped at 11 microsatellite loci. Results showed that colonization and extinction events occurred during the season. Allele numbers were highly variable, and while some disappeared, new alleles appeared over time. Globally (i.e. samples from all sites pooled together), allele number over all loci showed a decreasing trend with time. Analysis of molecular variance showed that samples at the genet level explained the highest variance compared with site level; hence, the genet is the unit of parasitoid population sub-structuring. We conclude that the genetic structuring of this insect is very fine and the temporal genetic diversity is explained by a combination of extinction and re-colonization events, as well as inbreeding.

Introduction

Genetic diversity is the material on which selection acts and its amount in a population results from a balance between the gain and loss of allelic variants (Amos & Harwood, 1998). Increase in genetic diversity can come from; the creation of new alleles by mutation, through migration into the population bearing different alleles, and through sexual reproduction by mixing and recombining via crossing over the alleles present in a population. Loss of genetic diversity arises due to the action of natural selection, through the effects of random genetic drift and emigration (Amos & Harwood, 1998).

In structured populations characterized by low dispersal among patches, genetic variability in space and time is influenced by colonization and extinction cycles (Whitlock & McCauley, 1990; Husband & Barrett, 1996) and variations in population density (Lynch *et al.*, 1995). Wright (1940) suggested that patterns of extinction and recolonization would enhance genetic differentiation of local populations because the number of individuals colonizing a patch is likely to be much smaller than the local carrying capacity. Slatkin (1985) in contrast, argued that such inter-demic selection is inherently unlikely because ongoing local extinction also implies ongoing recolonization and that recolonization constitutes gene flow which prevents local population differentiation. Wade & McCauley (1988), however, reported that the outcome of extinction and recolonization depended on the mode of founding of new populations: If colonizing propagules are large and contain individuals from many populations, turnover has homogenizing effects, whereas if propagules are small and homogenous, coming from one or a few source populations, turnover of local populations enhanced their differentiation. Even so, neither Slatkin (1985) nor Wade & McCauley (1988) based their arguments on empirical data.

The seasonal decline of population size or density and founder events may reduce variability and create spatio-temporal genetic differentiation (Lynch *et al.*, 1995). Such decline may also reduce variability and create differentiation between populations due to genetic drift resulting from bottlenecks and founder effects, although this reduction is strongly influenced by gene flow between populations. Natural selection and drift are major forces responsible for temporal genetic changes in populations, particularly those with low effective size (Hartl & Clark, 1989). Drift promotes loss of alleles among isolated populations, potentially affecting the evolutionary response of selected traits influenced by such alleles. In addition, directional selection may reduce effective population size, enhancing the impact of drift on variability within populations and differentiation among them (Falconer & Mackay, 1996).

In social animals generally, Chesser (1991a) noted that the mating system affects the distribution of genetic variation on a local scale and that female philopatry plays an

important role in the disparity of variance within lineages relative to random individuals within the population. He continues that polygyny together with female philopatry produces high gene correlations among progeny and adults within social groups. Polygynous groups or local subpopulations consisting of mating pairs prevent the complete admixture of genes amongst groups (Sugg *et al.*, 1996). In primary Hymenopteran wasp parasitoids, especially the gregarious species as well as the solitary species attacking gregarious hosts, local mating competition (LMC) due to mating between siblings and brothers competing for mates at natal/ emergence patches has been observed (Godfray, 1994). In solitary parasitoids attacking colony forming hosts, quasi-gregarious broods of parasitoids are produced which favour sib-mating or inbreeding on the natal patch (Mackauer & Völkl, 2002). Inbreeding enhances reduced levels of genetic diversity and increases the probability of extinction of local populations (Saccheri *et al.*, 1998). Inbreeding may also lead to fine-scale genetic structure or the non-random spatial distribution of individuals with respect to relatedness or allele frequencies (Chesser, 1991b).

To date, few molecular ecological studies of insects have examined changes over the course of several seasons (e.g., Loxdale & Brookes, 1990 on aphids; Abbott *et al.*, 2008 on damselfly), even more rarely, within a single season (e.g., Rhomberg *et al.*, 1985 on rose aphids; Goodisman *et al.*, 2007 on fire ants; Brevault *et al.*, 2008 on cotton aphid) and none on primary hymenopterous parasitoids that we are aware of. In the present work, we explore the effect of local mating behaviour and inbreeding on population genetic diversity and structure over a single annual season using a host-parasitoid system comprising the specialist parasitoid *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae: Aphidiinae*)* attacking the specialist aphid, *Metopeurum fuscoviride* Stroyan (Hemiptera: Aphididae) on tansy, *Tanacetum vulgare* L. (Family Asteraceae). These model system is characterized by a metapopulation structure, i.e., limited dispersal and, colonization and extinction events (Hanski, 1999; Weisser, 2000; Zheng *et al.*, 2009). The life cycle of the parasitoid involves multiple overlapping generations in one year, i.e. it is multivoltine with about 14-17 generations *per annum*. Females have been observed to oviposit large number of eggs (40-70), and often to forage until all available hosts are parasitized, independent of colony size on a tansy ramet= shoot (Mackauer & Völkl, 2002). Extinctions of the aphid and in consequence the parasitoid, due to predation at the level of tansy ramets and genets, are common in a single season (Weisser, 2000; Weisser & Härri, 2005).

Ecological and DNA genetic variability (microsatellite) studies of the *spatial* structure of *L. hirticornis* populations have revealed low dispersal rates, inbreeding and partial sib-mating, population sub-structuring and philopatry within demes (colonies), and confirmed the existence of a metapopulation in the *L. hirticornis-M. fuscoviride* model system

(Mackauer & Völkl, 2002; Rauch & Weisser, 2007; Nyabuga *et al.*, 2010 [Article II]). In light of these earlier results, we here further describe the spatio-temporal dynamics within parasitoid population demes as well as determine the extent of fine scale spatial structuring. Specifically, we asked the following two main questions: (1) how does *L. hirticornis* molecular variance at the tansy genet level compare with that at the site level i.e., a spatial unit with many tansy genets? (2) How does the population genetic diversity of the parasitoid fluctuate over time?

Materials and methods

Field sites: Between June and October 2007, *M. fuscoviride* and its parasitoid, *L. hirticornis* population dynamics were surveyed and parasitoids sampled from 11 sites in and around Jena, Germany (50° 54' N, 11°35' E) The tansy plant was mainly found along the course of the River Saale, and on the sidelines of railway tracks sites on ruderal habitats separated by human development, agricultural land and forests, the closest sites being between Jena Nord-1 and Jena Nord-2, 217m apart. For details on site description, see Appendix 1 and Figure 1 in Nyabuga *et al*. (2010 [*Article II*]).

Field surveys and population sampling

At all sites, tansy plants were visited and inspected for *M. fuscoviride* every ten days during the growing season. If colonized by aphids, a genet was labeled and its phenology noted; the numbers of ramets per genet were counted, along with the number of ramets colonized by the aphid (Nyabuga *et al.*, 2010 [Article II]). The size of each aphid colony was estimated and further inspected for *L. hirticornis* aphid mummies. The mummies provide reliable estimates of the presence or absence of the developing *L. hirticornis* larvae and were therefore used to determine parasitoid population dynamics (e.g., Eber & Brandl, 1994; Dempster *et al.*, 1995a; Dempster *et al.*, 1995b; Eber & Brandl, 1996, 1997; Weisser, 2000). The dark brownish coloration of *L. hirticornis* aphid mummies easily distinguished them from those of other parasitoid species attacking *M. fuscoviride*, in particular *Aphidius tanacetarius* Mackauer and *Ephedrus* Haliday spp. (Hymenoptera: Braconidae: Aphidiinae) (Weisser, 2000). During sampling visits which were destructive in nature, a maximum of ten aphid mummies were sampled per genet to avoid a impacting on parasitoid population densities and genetics. Mummies were collected in 1.5ml eppendorf tubes for transport back to the laboratory where they were left until the adult winged wasps emerged; these were then sexed under a stereo binocular microscope. Males and females from a single genet were stored separately in eppendorf tubes in 100% ethanol at 4°C until DNA extraction. During subsequent visits, the fate of marked genets with aphid colonies was followed and any new

genet colonization was marked, measurements and sampling proceeded as described above. The study was continued for the entire growing season, until the shoots senesced and the aphid colonies collapsed.

Microsatellite analysis

Results of only the diploid female parasitoids are reported here even though both males and females were genotyped. This is because males are haploid, and only diploid females provided genotypes for Hardy-Weinberg Equilibrium (HWE) analysis. DNA was extracted using the 'salting-out' method of Sunnucks & Hales (1996) from 610 female individuals collected from the 11 sites (see Appendix 1 for number of samples at each site). Eleven polymorphic microsatellite loci were used in genotyping; of these, nine (*Lhirt 01, Lhirt02, Lhirt03, Lhirt04, Lhirt06, Lhirt08, Lhirt10, Lhirt15, Lhirt23*) were developed from *L. hirticornis*, and a further two derived from published primer sequences for other *Lysiphlebus* species, namely *Lysi08* from *L. fabarum* (Marshall), and *Lysi6b12* from *L. testaceipes* (Cresson) (see Nyabuga *et al.*, 2009 [Article I] and references therein for details).

Statistical analysis

First, the 610 parasitoids genotyped from the 11 sites were pooled together as if a single population and microsatellite variability determined. Secondly, the 610 parasitoids as if a single population were grouped into three according to time of sampling as follows: early summer (June-15 July); mid-summer (16 July-end of August) and late summer (September-October). These three 'time groups' were analyzed for global diversity measures. Third, the 610 parasitoids according to the sites of collection were grouped into genets (=whole plants) from which they were sampled, and with this 72 groups (genets) were attained. Fourth, the 72 genets were split further over time, and here 103 groups with range four to ten samples was attained (Appendix 1). Lastly, the 610 genotyped parasitoids grouped according to the 11 sites and time of collection i.e., early summer, midsummer or late summer were checked for the minimum sample size at each of these three time points. Only five of the 11 sites could realize minimum 10 or more samples for each of the three time points. These were: Jena Nord-1, Burgau-2, Göschwitz-Bhf, Jena West-Bhf and Jena Nord-2 (Appendix 1). Using the minimum number of samples per time point per site, i.e., Jena Nord-1=24, Burgau-2=23, Göschwitz-Bhf=12, Jena West-Bhf=11 and Jena Nord-2=45, random sampling was performed using the statistical package R (R Development Core Team, 2008) in order to randomly select a uniform sample number.

Hierarchical partitioning of the genetic variance of parasitoid samples was performed in ARLEQUIN (v. 3.1; 2006, Excoffier *et al.*, 2005) using an analysis of molecular variance

(AMOVA) framework (Weir & Cockerham, 1984; Excoffier *et al.*, 1992). Pairwise and global F_{ST} was calculated according to Weir & Cockerham (1984) in ARLEQUIN. Population genetic diversity was quantified as observed heterozygosity (H_O) and with the unbiased estimates of expected heterozygosity (H_E), calculated using ARLEQUIN. The number of alleles per locus (A) and allelic richness (R) was calculated in FSTAT. Within-sample deviation from HWE was tested in GENEPOP (v. 4.0; Raymond & Rousset, 1995) using the dual null hypotheses of both heterozygote excess and deficit at individual loci as well as over all loci.

Results

Metopeurum fuscoviride and *Lysiphlebus hirticornis* population dynamics

The number of aphids and parasitized (mummified) aphids showed different patterns and trends at each of the 11 sites sampled (Table 1). At the start of sampling (4-13 June), between 285 and 6,755 aphids were counted at Burgau-1 and at Jena Nord-2, respectively. The rates of increase and fluctuations varied from one site to another (Table 1). At all sites examined, a low number of aphid mummies were counted throughout the season. From 24 June-3 July, aphid mummies were recorded at all sites sampled and found to be present until late August when three of the 11 sites had no aphid mummies, thereafter the sites with no mummified aphids increased over time (Table 1). When all the sites were aggregated and analyzed as a function of time, up to *ca*. 11,300 aphids were counted at the start of season, dropping to around 6,900 by mid June and building up in late June-early July before undergoing a continuous decline in early September (Figure 1). The peak of aphid mummification was recorded between 4-13 July and thereafter showed a general declining pattern (Figure 1). There was no relationship between peak aphid and peak parasitoid abundance (R=0.069, $F_{1, 257}$=1.223, p=0.270).

The highest percentage of genet colonization by the aphid was observed over the period 24 June -3 July and new colonization occurred until mid September (Figure 2). Aphid extinction events on genets were noted from 14-23 June and continued throughout the season, peaking from 11-20 September. A number of genets in which aphid extinction had occurred were again recolonized (Figure 2). At the level of genets over all sites, aphid survival ranged from three to 20 weeks, mean 8.9±0.33 (SE) weeks, with a modal survival time of three weeks. Tansy plants of different phenologies were observed at different times of the season. Flowering was observed starting late June, and while some plants were wilting and senescing, others were seen to be sprouting (Figure 3).

Table 1. The total number of aphids/aphid mummies due to *L. hirticornis* counted on each of the 11 sites at various time periods

Sampling period	Burgau-1	Burgau-2	Dornburg-Bhf	Fritz-Winkler-straße	Göschwitz-Bhf	Jena Nord-1	Jena Nord-2	Jena West-Bhf	Porstendorf-Bhf	Saalbahnhof	Zwätzen
Jun 4-13	285/0	---	---	---	---	---	6755/15	490/0	---	3400/5	375/0
Jun 14-23	265/10	755/95	---	---	4850/70	---	---	---	---	---	1025/15
Jun 24- Jul 3	---	---	3860/5	1305/110	---	4335/115	6140/145	585/100	11255/10	1515/110	---
Jul 4-13	195/25	272/210	6330/45	750/210	3270/245	4040/450	11650/290	3490/160	28710/30	815/110	550/5
Jul 14-23	80/0	2800/250	7580/50	270/185	2835/175	975/295	6525/305	820/50	35020/60	550/65	540/70
Jul 24- Aug 2	115/10	4420/235	4050/25	185/50	1345/90	365/95	6870/255	280/35	41365/45	865/20	1435/25
Aug 22- 31	370/30	3455/220	2420/100	300/0	710/10	4830/70	11100/195	270/65	39655/0	300/0	2010/45
Sept 1 -10	360/60	4025/90	2510/35	30/0	1595/5	4650/70	8820/160	330/40	19990/25	300/0	2380/0
Sept 11-20	30/30	1360/30	---	30/0	965/70	4555/230	7585/100	215/35	---	70/30	---
Sept 21-30	---	---	150/35	---	---	---	---	---	6320/0	---	1200/10
Oct 1- 10	0/0	135/0	5/0	0/0	695/150	---	---	30/5	1225/0	0/0	35/5
Oct 21-30	---	---0	0/0	---	135/85	---	---	0/0	155/0	0/0	---

---=no data were collected
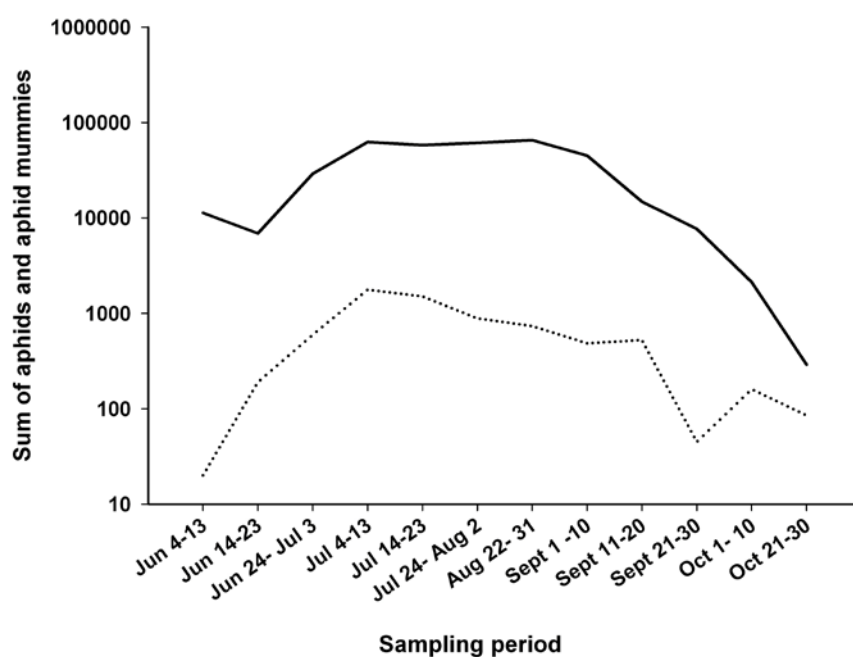
Figure 1. Over all the 11 sites sampled over time, the number of aphids, *M. fuscoviride* (solid line) was always higher that the mummified aphids (dotted line) due to the parasitoid *L. hirticornis*
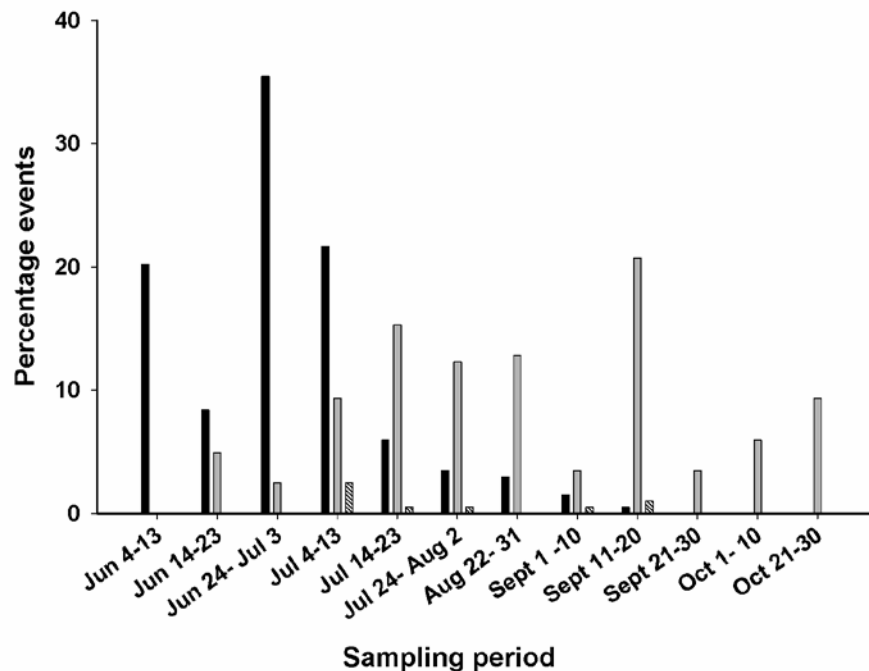
Figure 2. Over all the 11 sites sampled, aphid colonization events (black bars) continued until 11-20 September; aphid extinction events (light gray bars) began in June 14-23 and continued throughout the season, and aphid recolonization following extinction events (patterned) were observed starting July 14-23 until September 11-20
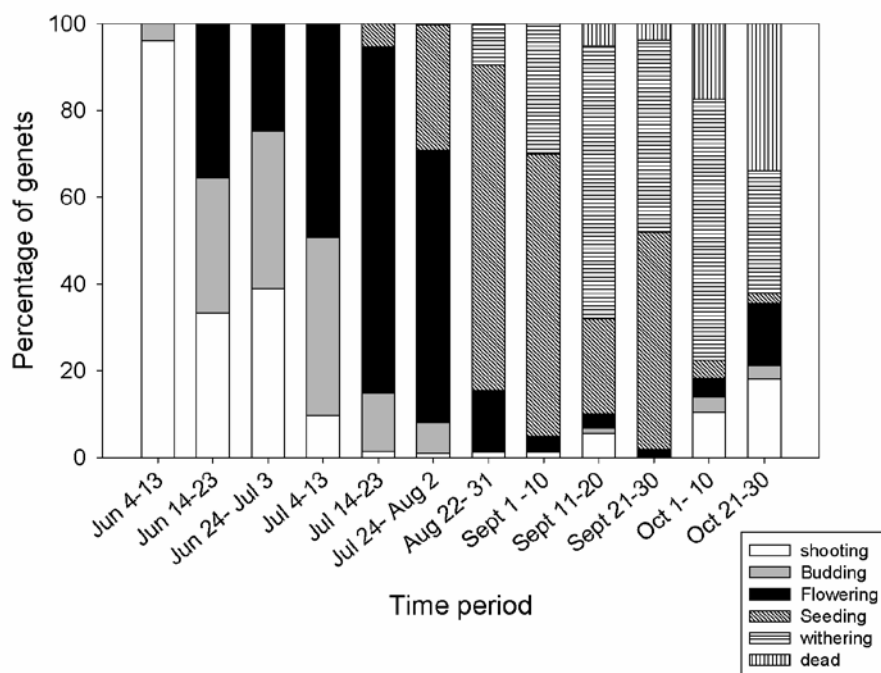
Figure 3. Over all 11 sites sampled over time, tansy (*Tanacetum vulgare*) plants of varying phelonogies were sampled. While some were sprouting, others were senescing and dying

Overall microsatellite diversity

Over all the 610 parasitoids grouped as one population, the number of alleles varied from two at *Lysi08* and *Lhirt08* to 23 at *Lhirt01*, mean 8.3±7.07 (SD). Overall expected heterozygosity ranged from 0.215 at *Lysi08* to 0.828 at *Lhirt04* (Table 2). Loci *Lysi08* and *Lysi6b12* showed significant HW deviations (p<0.05) in one population each (Table 2), but over all populations, did not deviate significantly from expectations (*Lysi08;* χ2=20.7, DF=20, p=0.416 and *Lysi6b12;* χ2=20.1, DF=16, p=0.216). All other loci (nine) had between two and 11 populations deviating from HW expectations (Table 2), and significantly so over all populations. Between one and 11 populations showed significant heterozygote deficit at the various microsatellite loci tested (Table 2). Exact tests for genotypic linkage disequilibrium using Fisher's method showed 43 significant (p<0.05) values out of the total of possible 55 pair-wise combinations.

Analysis of molecular variance

Analysis of molecular variance for the 610 parasitoids with time, i.e., 'early summer', N=179, 'mid-summer', N=279 and 'late summer', N=152 with 10,000 permutations revealed that percent variance explained by: 'among populations=three time points' was 0.77% (df=2, p<0.001); 'among individuals within populations' was 40.28% (df=607, p<0.001); and

'within individuals' was 58.95% (df=610, p<0.001). AMOVA of the 11 sites split into 72 genets revealed that 9% (df=10, p<0.001) of the variance was accounted for by 'among groups (=sites)' and 11.8% (df=61, p<0.001) 'among populations (genets) within groups', 20.9% (df=538, p<0.001) 'among individuals within populations' and 58.3% (df=610, p<0.001) 'within individuals'. At the level of genets at all sites (72 populations) over time, 13.3% (df=71, p<0.001) of the variance was accounted for by 'among groups (genets)', 10.1% (df=31, p<0.001) 'among populations within groups', 17.6% (df=507, p<0.001) 'among individuals within populations' and 58.9% (df=610, p<0.001) 'within individuals'.

For each of the five sites (Jena Nord-1, Burgau-2, Göschwitz-Bhf, Jena West-Bhf, Jena Nord-2) at the three time points (early, mid and late summer), between 1.1% of the variance was accounted for in Jena Nord-1 and 6.8% in Burgau-2 'among populations', between 21.3% in Jena Nord-2 and 39.4% at Jena Nord-1 'among individuals within populations', and between 57.4% in Jena West-Bhf and 76.4% in Jena Nord-2 'within individuals' (Table 3).

Alleles and allele frequencies

Based on the 610 individuals sampled over time (early, mid and late summer), the number of alleles was 82 in early summer (N=179), 82 in mid-summer (N=279) and 76 in late summer (N=152). Allelic richness based on the minimum sample of 152 diploid individuals shows a decline over time, *viz*. 80.6, 77.8 and 76 in early, mid and late summer respectively. New alleles appeared, others were lost, while others appeared and disappeared within the single annual season (appendix 2).

For the five sites over time, the total number of alleles and allelic richness (*R*) differed among the five sites over the three time points (Table 4). From the 24 parasitoids analyzed from Jena Nord-1, the total number of alleles increased slightly from 47 in early summer to 48 in mid-summer and dropped to 42 in late summer. A similar trend was observed at Burgau-2, Göschwitz-Bhf and Jena West-Bhf. Samples from Jena Nord-2 showed a decrease from 57 in 'early summer' to 50 in mid-summer and then increased slightly again to 51 in late summer. Allelic richness based on the minimum 11 parasitoid samples revealed similar trends as described above for total number of alleles i.e., in four of the five populations (Jena Nord-1, Burgau-2, Göschwitz-Bhf, and Jena West-Bhf), it increased from 'early summer' to 'midsummer' and thereafter decreased (Figure 4). There was decreased allelic richness from 'early summer' to 'midsummer' before an increase in 'late summer' at Jena Nord-2 (Figure 4).

Table 2. Diversity indices of the 11 microsatellite loci used in genotyping of *L. hirticornis* obtained from 610 individuals. HE=expected heterozygosity; HO=Observed heterozygosity

Locus	Alleles	HE	HO	Number of significant HWE deviations and overall deviation from HWE	Number of significant heterozygote deficits
Lhirt01	23	0.821	0.369	9***	9
Lhirt02	8	0.395	0.221	7***	7
Lhirt03	10	0.794	0.144	11***	11
Lhirt04	19	0.828	0.482	10***	8
Lhirt06	11	0.615	0.328	8***	7
Lhirt08	2	0.358	0.300	2***	2
Lhirt10	3	0.582	0.382	10***	10
Lhirt15	4	0.513	0.390	6***	6
Lhirt23	6	0.617	0.474	10***	7
Lysi08	2	0.215	0.185	1^NS	1
Lysi6b12	3	0.314	0.303	1^NS	1

***=$p<0.001$, **=$p<0.01$, *=$p<0.05$, NS= Not significant ($p>0.05$)

Table 3. Analysis of molecular variance table for five populations, each analysed as three temporal subpopulations (early summer, mid-summer and late summer)

Population	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Jena-nord-1	Among populations	2	11.38	0.032	1.13
	Among individuals within populations	81	316.05	1.112	39.39
	Within individuals	84	141.00	1.679	59.48
	Total	167	468.43	2.822	
Burgau-2	Among populations	2	25.44	0.197	6.84
	Among individuals within populations	66	242.17	0.990	34.44
	Within individuals	69	116.50	1.688	58.72
	Total	137	384.11		
Göschwitz-Bhf	Among populations	2	11.63	0.076	2.60
	Among individuals within populations	33	131.33	1.115	37.91
	Within individuals	36	63.00	1.750	59.50
	Total	71	205.96	2.941	

Population	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Jena-west-Bhf	Among populations	2	11.14	0.090	3.41
	Among individuals within populations	30	107.59	1.036	39.22
	Within individuals	33	50.00	1.515	57.37
	Total	65	168.73	2.641	
Jena-nord-2	Among populations	2	19.05	0.068	2.36
	Among individuals within populations	132	451.66	0.613	21.30
	Within individuals	135	296.50	2.196	76.35
	Total	269	767.20	2.877	
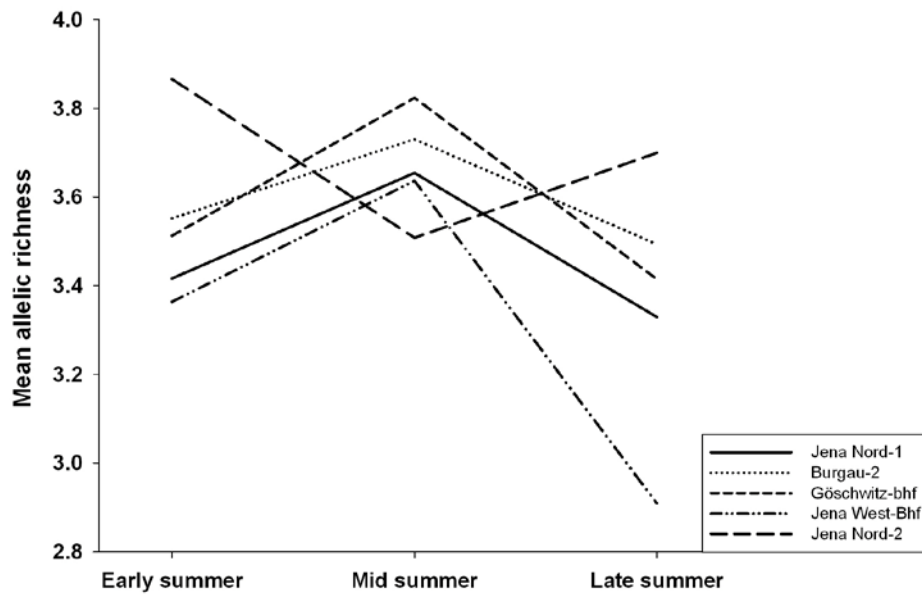
Figure 4. Mean allelic richness based on 11 parasitoid samples for five populations at three time points. In four populations, allelic richness increased in mid-summer before declining in late summer

Gene diversity

Based on the 610 individuals sampled over time, there was a decrease in levels of observed heterozygosity (H_O) with time. In early summer H_O was 0.333±0.126 (SD) that decreased to 0.328±0.116 in mid-summer and 0.311±0.111 (SD) in late summer. Expected heterozygosity (H_E) based on the 610 genotyped parasitoids decreased from 0.544±0.210 (SD) in early summer to 0.542±0.217 (SD) in mid-summer and eventually increased to 0.560±0.207 (SD) in late summer.

At the five sites, H_O ranged from 0.263±0.144 (SD) in samples from Jena Nord-1 in late summer to 0.408± 0.131 (SD) at Jena-Nord-2 in early summer (Table 4). H_O was observed to increase from early summer to mid-summer and later decreased in late summer in three populations, i.e., Jena Nord-1, Burgau-2 and Jena West-Bhf. At Göschwitz-Bhf, H_O increased throughout the season, whereas at Jena Nord-2 H_O decreased throughout the season (Figure 5).

Table 4. Descriptive statistics of *Lysiphlebus hirticornis* collected at five sites in Jena analyzed at 11 microsatellite loci. Number of samples genotyped (*N*), total number of alleles overall loci (Total_*A*), mean number of alleles over all loci (Mean_A), total allelic richness (Total_R) and mean allelic richness (Mean_R) based on the minimum 11 parasitoid samples. Observed (H_O) and expected (H_E) gene diversity, inbreeding coefficient (F_{IS}), and number of loci deviating from HWE (*HW*)

Sites	Jena-nord-1_early	Jena-nord-1_mid	Jena-nord-1_Late	Burgau-2_early	Burgau-2_mid	Burgau-2_late	Göschwitz-Bhf_early	Göschwitz-Bhf_mid	Göschwitz-Bhf_late	Jena-west-Bhf_early	Jena-west-Bhf_mid	Jena-west-Bhf_late	Jena-nord-2_early	Jena-nord-2_mid	Jena-nord-2_late
N	28	28	28	23	23	23	12	12	12	11	11	11	45	45	45
Total_A	47	48	42	44	47	44	39	43	38	37	40	32	57	50	51
Mean_A	4.27	4.36	3.82	4.00	4.27	4.00	3.55	3.91	3.70	3.89	3.64	2.91	5.18	4.55	4.64
Total_R	38	40	37	39	41	38	39	42	38	37	40	32	43	39	41
Mean_R	3.42	3.65	3.33	3.55	3.73	3.49	3.51	3.82	3.41	3.36	3.64	2.91	3.87	3.51	3.70
HO	0.315	0.338	0.263	0.300	0.320	0.300	0.273	0.333	0.383	0.273	0.331	0.273	0.408	0.398	0.392
HE	0.465	0.530	0.516	0.482	0.482	0.486	0.538	0.481	0.569	0.520	0.477	0.462	0.516	0.465	0.547
F_{IS}	0.327	0.367	0.495	0.381	0.341	0.387	0.504	0.317	0.336	0.488	0.317	0.421	0.212	0.146	0.286
Polymorphic loci (N)	11	11	11	11	11	11	11	11	10	9	11	11	11	11	11
HW	4	8	8	7	6	5	6	4	4	5	4	5	6	6	8

Early= early summer, mid= mid-summer and late= late summer
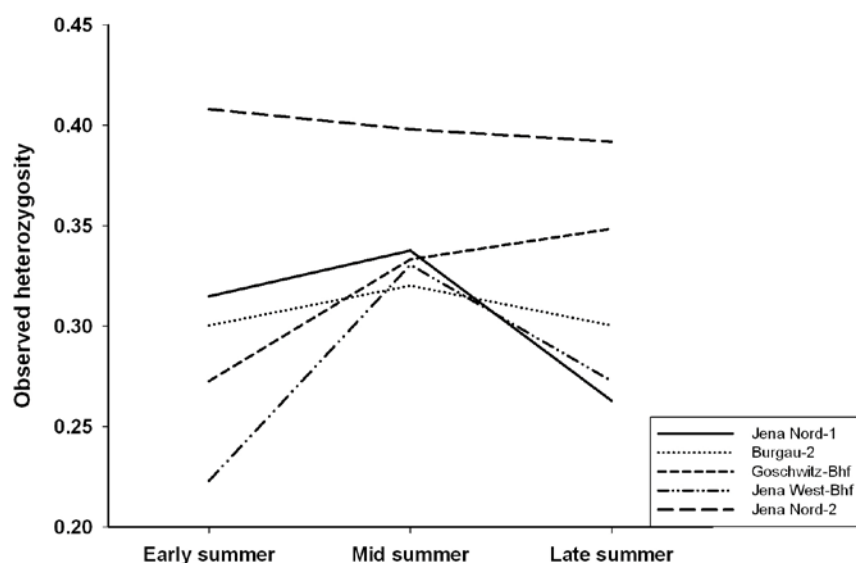
Figure 5. Observed heterozygosity (H_O) for five populations at three time points each. In three populations H_O increased in mid-summer and dropped by late summer. In Jena Nord-2, H_O decreased throughout the season while at Göschwitz-Bhf, H_O increased throughout the season

Inbreeding coefficients (F_{IS}) and relatedness

Average F_{IS} increased from 0.390 in early summer to 0.394 in mid-summer and to a high of 0.446 in late summer based on the 610 parasitoid samples over time. At the five sites over time, average F_{IS} values ranged from 0.218 at Jena Nord-2 to 0.40 at Jena West-Bhf (Table 4). The general trend showed an increase in F_{IS} at Jena Nord-1 throughout the growing season, whereas the other four sites displayed an initial decrease in 'midsummer' followed by an increase in 'late summer' (Figure 6).
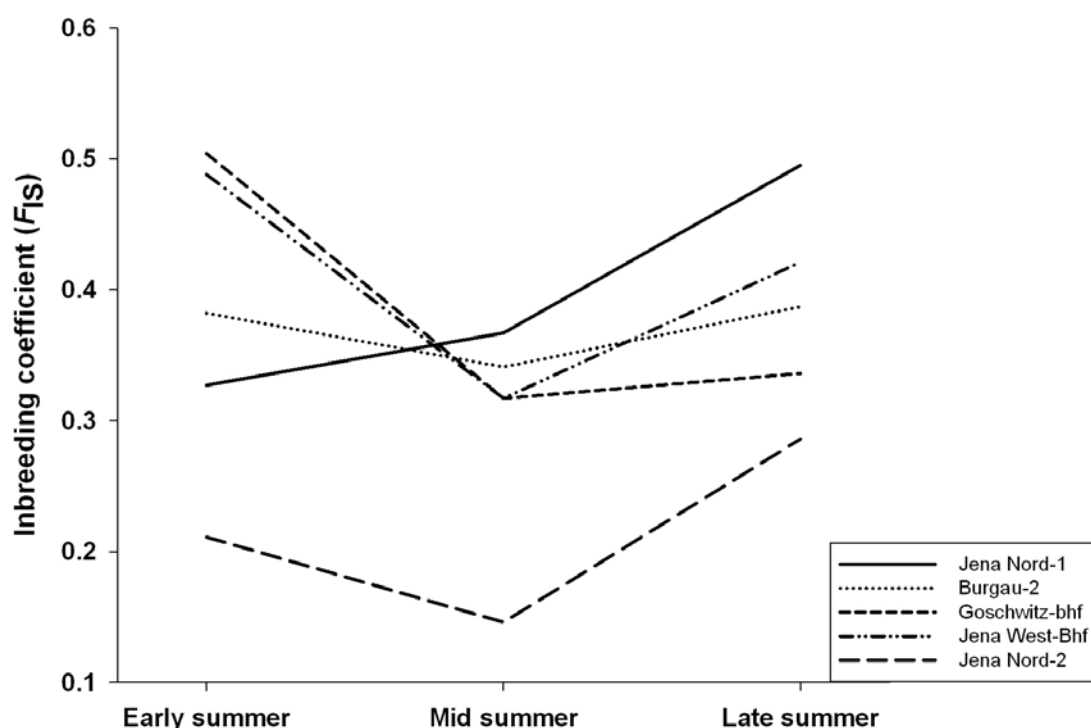
Figure 6. At four of the five populations, the inbreeding coefficient (F_{IS}) declined in mid-summer before increasing in late summer. At Jena Nord-1, F_{IS} increased all though the season

Genetic differentiation among and within populations

The reduction in levels of heterozygosity at sub-population relative to the whole population, F_{ST} was 0.00877 in early summer, 0.00878 in mid-summer and 0.00870 in late summer based on the 610 parasitoid samples. Pair-wise F_{ST} (Table 5) revealed that there is significant population differentiation at all the three time points.

Pair-wise F_{ST} values for the five populations over the three time points analyzed as 15 subpopulations, showed significant and non-significant 'within population differentiation' values over time (Table 6). In Jena Nord-1, values for 'early summer' to 'mid-summer' and 'mid-summer' to 'late summer' were not significantly different, but there was significant differentiation between 'early summer' and 'late summer'. All temporal comparisons of Jena Nord-1 with other populations at the various time points showed significant differentiation.

At Burgau-2, 'early summer' to 'mid-summer' samples were not differentiated, whereas 'early summer' to 'late summer' and 'mid-summer' to 'late summer' were significantly differentiated. All temporal comparisons of Burgau-2 to other populations at the various time

points showed significant differentiation. In Göschwitz-Bhf, 'midsummer' and 'late summer' were differentiated but not between 'early' and 'midsummer' and between 'early' and 'late summer'. Between temporal points of Göschwitz-Bhf and other populations and their time points, all were significant except between 'Göschwitz-Bhf -*early summer*' and 'Jena West-Bhf-*late summer*'. In Jena West-Bhf, significant differentiation was observed between 'early summer' and 'late summer'. Temporal points for Jena West-Bhf to all other populations at the various time points were significantly differentiated except between 'Jena West-Bhf -late summer' and 'Göschwitz-Bhf -early summer'. Significant differentiation was observed for all within Jena Nord-2 temporal variation and between temporal points of Jena Nord-2 to other populations over time.

Table 5. Pair wise F_{ST} test for the 610 parasitoid samples over the three time points. Below diagonal is the F_{ST} values while above diagonal is the p-values.

Time point	Early summer	Mid- summer	Late summer
Early summer		0.00089	0.00000
Mid- summer	0.00519		0.00000
Late summer	0.01237	0.01057	

Table 6. Pair wise F_{ST} test for each of the five populations, each at three time points analyzed as 15 populations. Lower diagonal shows FST values. Upper diagonal with + indicate significantly different populations (p<0.05) and -=not significant i.e., P>0.05.

Sites	Jena-nord-1_early	Jena-nord-1_mid	Jena-nord-1_Late	Burgau-2_early	Burgau-2_mid	Burgau-2_late	Göschwitz-Bhf_early	Göschwitz-Bhf_mid	Göschwitz-Bhf_late	Jena-west-Bhf_early	Jena-west-Bhf_mid	Jena-west-Bhf_late	Jena-nord-2_early	Jena-nord-2_mid	Jena-nord-2_late
Jena-nord-1_early		-	+	+	+	+	+	+	+	+	+	+	+	+	+
Jena-nord-1_mid	0.006		-	+	+	+	+	+	+	+	+	+	+	+	+
Jena-nord-1_Late	0.037	0.013		+	+	+	+	+	+	+	+	+	+	+	+
Burgau-2_early	0.116	0.120	0.139		-	+	+	+	+	+	+	+	+	+	+
Burgau-2_mid	0.119	0.122	0.139	-0.003		+	+	+	+	+	+	+	+	+	+
Burgau-2_late	0.159	0.158	0.169	0.111	0.110		+	+	+	+	+	+	+	+	+
Göschwitz-Bhf_early	0.119	0.118	0.138	0.066	0.065	0.082		-	-	+	+	-	+	+	+
Göschwitz-Bhf_mid	0.148	0.155	0.182	0.161	0.155	0.135	0.027		+	+	+	+	+	+	+
Göschwitz-Bhf_late	0.136	0.128	0.147	0.123	0.127	0.136	0.024	0.075		+	+	+	+	+	+
Jena-west-Bhf_early	0.105	0.106	0.125	0.098	0.093	0.169	0.127	0.174	0.137		-	+	+	+	+
Jena-west-Bhf_mid	0.105	0.103	0.144	0.107	0.128	0.172	0.124	0.162	0.125	0.042		-	+	+	+
Jena-west-Bhf_late	0.117	0.122	0.149	0.053	0.062	0.134	0.053	0.142	0.105	0.072	0.045		+	+	+
Jena-nord-	0.098	0.094	0.110	0.088	0.086	0.183	0.054	0.123	0.084	0.109	0.096	0.054		+	+

Sites	Jena-nord-1_early	Jena-nord-1_mid	Jena-nord-1_Late	Burgau-2_early	Burgau-2_mid	Burgau-2_late	Göschwitz-Bhf_early	Göschwitz-Bhf_mid	Göschwitz-Bhf_late	Jena-west-Bhf_early	Jena-west-Bhf_mid	Jena-west-Bhf_late	Jena-nord-2_early	Jena-nord-2_mid	Jena-nord-2_late
2_early															
Jena-nord-2_mid	0.107	0.108	0.126	0.124	0.127	0.193	0.067	0.141	0.083	0.128	0.121	0.071	0.014		+
Jena-nord-2_late	0.147	0.126	0.123	0.124	0.126	0.183	0.070	0.139	0.095	0.136	0.123	0.074	0.019	0.044	

Discussion

Within a tri-trophic system involving the plant, aphid and parasitoid, there is a close co-evolutionary relationship (Thompson, 1994). The parasitoids and aphids are not free-agents; rather, their ecologies and evolution are tightly and inexorably linked in a life and death struggle ('arms race') which impacts on the parasitoid population genetics (allele diversity and richness, etc.). As here shown, the specialist tansy aphid, *M. fuscoviride* and in consequence, the specialist parasitoid, *L. hirticornis*, both show local extinction and recolonization and phases on tansy (Weisser, 2000; Weisser & Härri, 2005; Zheng *et al.*, 2009). This study also highlights differences in the levels of genetic diversity over time within parasitoid metapopulations, and genetic divergence among such populations, i.e. there was both statistically significant as well as insignificant genetic structural changes among temporal samples taken from the same locality. These results reveal that regional genetic structure in *L. hirticornis* can be highly pronounced and very unstable. Genetic differentiation at some sites over time, but not others, showed that the sites themselves are actually isolated in space (and some sites also over time) and that the events unfolding at each of the resource patch (sites) are largely independent of each other.

In three of the five populations analyzed as a function of time, there was a lack of genetic differentiation between early summer and mid-summer subpopulations, indicative of temporary stability. In contrast, instability became evident only later on – in late summer (significant genetic differentiation). The genetic homogenization of sub-populations at some sites did not result in a general loss of genetic diversity among populations comprising the metapopulation, which as here demonstrated appears stable in some populations while increasing or decreasing in others. Such differences are consistent with the effects of different micro-evolutionary processes affecting both effectively neutral as well as adaptive genetic variation in natural populations.

The strong patterns of genetic differentiation at the level of site, and temporally within site, suggest a small population size (low number of mummified aphids as here shown) and reduced contemporary dispersal (see also Nyabuga *et al.*, 2010 [Article II]). At Jena Nord-2, there was a significant genetic differentiation at all three time points, showing there to be a lack inter-colony gene flow with time or a revolution in terms of gene/genotype frequencies. Significant population structure and genetic differences detected at the time points on a local level are likely the result of aphid population density fluctuations at the local spatial scales tested. The persistence of differentiation among populations over all three time points in the season shows that the spatial genetic structure may not be directly connected to seasonal or density dependent factors. In fact, our results showed that parasitoid abundance was not dependent on aphid abundance. Variance effective population size is affected by the sex ratio

in the breeding population, interindividual variation in offspring number, generation time and the mating system (Amos & Harwood, 1998). *Lysiphlebus hirticornis* is characterized by female biased sex ratios in the field, short and overlapping lifecycles and is thought to inbreed on the natal patches (Mackauer & Völkl, 2002; Nyabuga *et al.*, 2010 [Article II]).

The results of the field study showed that the population dynamics of the aphid, *M. fuscoviride* are subject to large fluctuations at the site/ deme level over time. Aphid survival at the genet level was highly variable with a mean of about nine weeks, although high numbers of extinctions were observed within approximately three weeks. The causes of aphid extinction were not only due to hymenopterous parasitoids, as shown by low rates of mummified aphids, but also predators including hoverfly larvae (Diptera: Syrphidae), ladybird larvae and adults (Coleoptera: Coccinelidae) and lacewing larvae (Neuroptera: Chrysopidae) (Weisser & Härri, 2005; Loxdale *et al.*, 2008). Obviously, aphid host extinction affects the parasitoid population. The tansy plant itself could also be a likely cause of aphid extinction: plants at different growth stages were observed within a site and even on a single genet, ramets at different stages of growth were common, i.e. while other genets and ramets were wilting and dying, others were sprouting or flowering etc. (Figure 4). Extinctions followed by recolonization events, as here observed, may explain the loss of alleles and appearance of new ones over time (Appendix 2). Parasitoid allelic richness and genetic diversity trends showed similar patterns of fluctuations to population dynamic trends, i.e. reduced genetic diversity with increase in extinction events. Loss of allelic diversity is also an indicator of historical bottlenecks (Leberg, 1992; Brookes *et al.*, 1997) and in *L. hirticornis* within a single season, extinctions of aphid colonies accounts for those loses. Hitherto, micro-geographic genetic differences had only been reported in a number of studies of small winged insects such as aphids (De Barro *et al.*, 1995; Sunnucks *et al.*, 1997; Haack *et al.*, 2000; Vorburger, 2006), where it was suggested that stochastic genetic drift or founder events were mainly responsible for the changes observed in population gene and genotype frequencies.

The tansy plant provides a resource with a patchy distribution at various spatial scales: aphid colonies form on single tansy ramets=shoots; due to clonal growth, tansy genets= whole plant, provide the next spatial level in the system, and finally, individual genets in field sites can be easily delineated (Weisser, 2000). A single female of *L. hirticornis* lays between 40 and 70 eggs on a single aphid colony on a ramet (Mackauer & Völkl, 2002). Large numbers of *L. hirticornis* mummies were found on a single ramet and even on several ramets on a single genet. Such a system undoubtedly creates a good breeding ground involving sib-mating for eclosing adult parasitoids which in turn results in local inbreeding. Inbreeding may create fine-scale genetic structuring (Chesser, 1991b) and indeed, analysis of

molecular variance showed that the genet rather than the site represents the level of genetic sub-structuring in *L. hirticornis*.

Positive inbreeding coefficients and deficiency of heterozygotes were also observed at particular sites and temporally within some sites. The deficiency of heterozygotes appears to be biologically founded, rather than due to purely stochastic influences, i.e. drift. *Lysiphlebus hirticornis* has an arrhenotokous mode of reproduction (parthenogenetic production of haploid males from unfertilized eggs) and as aforementioned, females have been reported to lay many eggs on the aggregated aphid colonies (Mackauer & Völkl, 2002). In cases where the parasitoid hatch and no suitable aphid hosts are found, either due to the unavailability of aphid hosts or because those available have already been parasitized, the adult wasps have no choice but to disperse in search of new resources. Such dispersal might create 'temporal Wahlund effects' (e.g., Van Rossum & Triest, 2006) and thus positive F_{IS} values due to homozygous excess as here found or insignificant pair-wise population differentiation as here established in some populations over time.

Departures from HWE always involved heterozygote deficiency, which could have been as a result of the presence of null alleles, Wahlund effects and/or inbreeding as discussed above. Null alleles occur when a mutation prevents annealing of one of the oligonucleotide primers at a given locus and hence prevents amplification of the products, resulting in underestimation of the number of heterozygotes (e.g., Pemberton *et al.*, 1995). Our earlier evidence showed that such null alleles were rare at the loci screened in *L. hirticornis* (Nyabuga *et al.*, 2009 [Article I]).

In conclusion, this study provides a unique demonstration of genetic variation and population sub-structuring in a specialist primary parasitoid attacking an aphid metapopulation over a single growing season. This metapopulation is characterized by aphid extinction and recolonization, and parasitoid inbreeding at the genet level, which we believe explains the disappearance and appearance of particular parasitoid alleles, enhances parasitoid population differentiation and reduced genetic diversity over the season. In *L. hirticornis*, a high molecular variance is explained at the level of genet compared to the level of site which implies that population differentiation occur at the fine level of the genet. More sampling, at the level of genet may be necessary to confirm with this population differentiation.

Appendices

Appendix 1. Sample sizes and their distribution within the sites on genets, and on genets over time

Population deme	Number of genets	Genets with two or more time points	Total samples	Sample range per genet per time point
Burgau-1	2	1 (2)	24	5-10
Burgau-2	15	6 (2*5, 3)	114	4-6
Dornburg-Bhf	2	1 (3)	36	7-10
Fritz-Winkler-straße	4	1 (3)	39	4-9
Göschwitz-Bhf	8	1 (2)	55	4-6
Jena Nord-1	8	2 (3,4)	78	6
Jena Nord-2	15	9 (2*8, 3)	137	4-8
Jena West-Bhf	9	1 (3)	67	4-8
Porstendorf-Bhf	4	1 (2)	27	4-10
Saalbahnhof	1	0	8	8
Zwätzen	4	0	25	4-8
		Number in bracket shows times repeated, and *followed by number indicate number of genets with such repetitions		

Appendix 2. Allele frequencies of the 610 genotyped parasitoids grouped into three time points

Locus	Allele length (bp)	Early summer N=179	Mid-summer N=279	Late summer N=152	Remark	
Lhirt01	182	0.000	0.002	0.000	Appeared and disappeared	
	192	0.042	0.050	0.092		
	194	0.014	0.014	0.000	disappeared	
	196	0.003	0.000	0.007	Disappeared and appeared	
	198	0.003	0.002	0.007		
	216	0.011	0.004	0.049		
	220	0.000	0.005	0.000	Appeared and disappeared	
	222	0.182	0.118	0.063		
	224	0.369	0.375	0.339		
	226	0.095	0.147	0.089		
	228	0.011	0.029	0.023		
	230	0.000	0.007	0.000	Appeared and disappeared	
	232	0.067	0.081	0.102		
	234	0.042	0.056	0.092		
	236	0.006	0.007	0.013		
	238	0.014	0.000	0.000	disappeared	
	252	0.000	0.000	0.007	appeared	
	254	0.045	0.034	0.023		
	256	0.028	0.025	0.049		
	258	0.008	0.018	0.020		
	260	0.045	0.020	0.000	disappeared	

Locus	Allele length (bp)	Early summer N=179	Mid-summer N=279	Late summer N=152	Remark	
	262	0.008	0.007	0.026		
	270	0.008	0.000	0.000	disappeared	
Lhirt02	126	0.020	0.014	0.030		
	128	0.014	0.009	0.026		
	130	0.025	0.022	0.010		
	132	0.000	0.016	0.053	appeared	
	134	0.774	0.806	0.701		
	136	0.109	0.052	0.092		
	138	0.042	0.052	0.086		
	140	0.017	0.029	0.003		
Lhirt03	192	0.008	0.005	0.007		
	208	0.117	0.129	0.138		
	212	0.358	0.249	0.214		
	214	0.249	0.337	0.342		
	218	0.095	0.082	0.039		
	222	0.017	0.088	0.184		
	224	0.064	0.038	0.063		
	228	0.075	0.045	0.010		
	230	0.006	0.011	0.003		
	232	0.011	0.016	0.000	disappeared	

Locus	Allele length (bp)	Early summer N=179	Mid-summer N=279	Late summer N=152	Remark	
Lhirt04	160	0.003	0.000	0.000	disappeared	
	244	0.022	0.045	0.023		
	246	0.014	0.002	0.007		
	248	0.050	0.047	0.039		
	250	0.008	0.011	0.003		
	252	0.154	0.246	0.112		
	254	0.321	0.263	0.368		
	256	0.182	0.149	0.201		
	260	0.101	0.052	0.046		
	262	0.034	0.011	0.020		
	264	0.039	0.063	0.020		
	266	0.011	0.036	0.053		
	268	0.053	0.054	0.076		
	270	0.003	0.009	0.010		
	272	0.000	0.002	0.000	Appeared and disappeared	
	274	0.003	0.002	0.010		
	278	0.003	0.005	0.003		
	280	0.000	0.005	0.003		
	286	0.000	0.000	0.007	appeared	
Lhirt06	246	0.017	0.013	0.000	disappeared	
	248	0.017	0.000	0.000	disappeared	
	250	0.059	0.052	0.089		

Locus	Allele length (bp)	Early summer N=179	Mid-summer N=279	Late summer N=152	Remark	
	252	0.073	0.032	0.016		
	254	0.003	0.002	0.003		
	256	0.022	0.061	0.063		
	260	0.084	0.029	0.020		
	262	0.039	0.093	0.122		
	266	0.601	0.634	0.530		
	268	0.000	0.002	0.000	disappeared	
	270	0.087	0.082	0.158		
Lhirt08	218	0.243	0.199	0.286		
	221	0.757	0.801	0.714		
Lhirt10	295	0.176	0.140	0.207		
	298	0.531	0.566	0.602		
	300	0.293	0.294	0.191		
Lhirt15	300	0.003	0.000	0.000	disappeared	
	320	0.031	0.036	0.046		
	326	0.341	0.385	0.388		
	330	0.626	0.579	0.566		
Lhirt23	246	0.006	0.004	0.007		
	250	0.059	0.099	0.109		

Locus	Allele length (bp)	Early summer N=179	Mid-summer N=279	Late summer N=152	Remark	
	252	0.003	0.000	0.000	disappeared	
	259	0.525	0.468	0.589		
	266	0.059	0.082	0.039		
	274	0.349	0.348	0.257		
Lysi08	117	0.137	0.125	0.099		
	119	0.863	0.875	0.901		
Lysi6b12	157	0.039	0.030	0.036		
	163	0.117	0.170	0.158		
	165	0.844	0.799	0.806		

*Article IV***:** Population structure of the tansy aphid, *Metopeurum fuscoviride* and its specialist parasitoid *Lysiphlebus hirticornis*

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Abstract

The interaction between two ecologically associated species may depend on the geographical scale and this in turn may well affect the co-evolution between them. The present study examines the population structures of an aphid host, *Metopeurum fuscoviride* Stroyan and its specialist hymenopterous parasitoid, *Lysiphlebus hirticornis*, both characterized by multivoltine life histories and a classical metapopulation structure. The aphid host was genotyped at five and the parasitoid at 11 microsatellite markers. The aphid host was greatly differentiated in terms of its spatial population genetic patterning, while the parasitoid was, in comparison, only moderately so differentiated. Positive Mantel correlations between pairwise F_{ST} for both aphid and parasitoid and between the insects shared allele distance (*DAS*) were calculated. We argue that the differences in the levels of differentiation could be due to the differences in the biology of the species and their interaction with ants. The parasitoid is tracking behind the aphid host, which could be the reason behind the persistence of the aphid population in the wake of the perceived very high parasitism pressures.

Introduction

The geographic structure of interacting species is a major component of evolving interactions and coevolutionary processes (Thompson, 1994). Evolutionary processes of differential selection, mating structure, and gene flow in subdivided populations all contribute in creating genetic variance among geographic mosaics of populations. Consequently, understanding the geographic structure of species helps to elucidate species interactions and levels of adaptation that may not be identical over entire species ranges (e.g., Kraaijeveld & Van Der Wel, 1994; Kraaijeveld & Van Alphen, 1995).

Population genetics of insects may be influenced by interactions among species and one of the strongest interactions is between hosts and their parasitoids. Kankare *et al.* (2005) noted that comparing the interacting species inhabiting the same landscape was a powerful approach to studying spatial population structures. This is because in such cases, possible differences are due to biological properties of the species rather than to particular features of the environment. A few studies have to date compared the spatial genetic structures of closely interacting species, especially between a host and its parasitoid (but see Althoff & Thompson, 1999; Johannesen & Seitz, 2003; Anton *et al.*, 2007), only the latter two in a metapopulation context.

Anton *et al.* (2007) found higher overall genetic differentiation in the specialist univoltine parasitoid *Neotypus melanocephalus* Gmelin (Hymenoptera: Ichneumonidae) compared with its univoltine host, the Dusky large blue butterfly, *Maculinea nausithous* (Bergsträsser) (Lepidoptera: Lycaenidae) and concluded that the differences are due to the differences in breeding system, i.e., diploid host *vs*. a haplodiploid parasitoid. Johannesen & Seitz (2003) examined the population genetic structures of the univoltine gall forming fly *Urophora cardui* (L.) (Diptera: Tephritidae) and its primary univoltine oligophagous parasitoid *Eurytoma robusta* Mayr (Hymenoptera: Eurytomidae). They found that the populations of *E. robusta* displayed more genetic structuring than the host populations. There is paucity of information on multivoltine highly specific and prolific haplodiploid systems.

We used the host-parasitoid system comprising the specialist wasp parasitoid, *Lysiphlebus hirticornis* Mackauer (Hymenoptera: Braconidae: Aphidiinae) attacking the specialist aphid, *Metopeurum fuscoviride* Stroyan (Hemiptera: Aphididae), which feeds exclusively on tansy, *Tanacetum vulgare* L. (Family Asteracae). The system has a classic metapopulation structure (Weisser, 2000; Zheng *et al.*, 2009). In a field study, Weisser (2000) reported frequent extinction events for the aphid, *M. fuscoviride*, and as a direct consequence, the parasitoid *L. hirticornis*, at the level of tansy plants (=genets) and shoots (=ramets). At the local (site) spatial scale, (Weisser, 2000; Nyabuga *et al.*, 2010 [Article II]) found that many tansy plants were not

colonized by the aphid and many tansy genets with the aphid were not colonized by the parasitoid. In support of the view that *M. fuscoviride* indeed showed a metapopulation structure, Massonnet (2002), using six microsatellite markers on seven subpopulations (five from the Alsace region of France and two from Germany), reported high population differentiation: Pairwise F_{ST} values ranged from 0.029 to 0.416, mean=0.222, and of the 21 pairwise F_{ST} comparisons, 18 were significant (p<0.05). Nyabuga *et al.* (2010 [*Article II*]), using 11 microsatellite markers on 11 populations of the parasitoid, *L. hirticornis* collected from a small spatial scale, furthest sites being approximately 15km apart, found significant pairwise genetic differentiation between all populations and F_{ST} values ranged from 0.065 to 0.236, mean=0.148.

The aphid, *M. fuscoviride* and the parasitoid *L. hirticornis* have short and overlapping lifecycles termed multivoltine. The aphid produces overwintering eggs that hatch in spring as temperatures rise and day length increases. Thereafter, adult females give birth asexually to young females (apomictic (=mitotic) parthenogenic reproduction) that develop to adulthood and start producing young in less than 10 days. As the growing season continues and plants senesce, day length reduces and temperatures fall towards autumn, wingless sexual females and males are produced that mate and lay the overwintering eggs, probably mostly on the same plant or nearby. Due to this type of reproduction, positive inbreeding coefficients (F_{IS}) in spatial units and high genetic diversity at start of annual season (in spring) that reduces to their lowest in autumn are reported (Massonnet, 2002). The parasitoid, *L. hirticornis* has a haplodiploid system whereby unfertilized eggs give rise to male offspring, while the mated and fertilized females produce females. *Lysiphlebus hirticornis* attacks aggregated aphids and will oviposit eggs in the same aphid colony until virtually all available hosts are parasitized or it becomes egg limited (Mackauer & Völkl, 2002). This sort of behaviour is thought to encourage inbreeding as inferred from microsatellite results and behavioral experiments (Nyabuga *et al.*, 2010 [Article II]). In field conditions, egg oviposition to adult emergence takes less than 16 days. Parasitized adult aphids continue to produce young for some time until 'total castration' occurs (Polaszek, 1986).

We hypothesize that the genetic differentiation in the aphid, *M. fuscoviride* has a direct and congruent effect on its parasitoid *L. hirticornis*. Specifically we ask; 1) how does the level of population genetic differentiation in the parasitoid compare to that of its aphid host? And 2) is there a relationship between the level of parasitoid population genetic differentiation and that of its aphid host?

Materials and methods

Field sites: The population genetics of *M. fuscoviride* and its parasitoid, *L. hirticornis* were studied between early June and late October, 2007 from 11 sites in and around Jena, Germany (50° 54' N, 11° 35' E; see Figure 1 and Appendix 1 in Nyabuga *et al.*, 2010 [Article II] for site details). Aphid and parasitoid samples in the form of aphid mummies were collected. Due to constraints in the number of individuals genotyped for either the aphid or the parasitoid, eight sites were compared in this study and the full data from the 11 sites is reported elsewhere.

Field surveys and population sampling

Genets at all sites were visited and inspected for *M. fuscoviride* every ten days, starting June 2007 and ending October 2007. If colonized by aphids, the genet was hand labeled and phenology noted; the numbers of ramets per genet were counted along with the number of ramets colonized by the aphid (data to be reported elsewhere.). Each aphid colony was estimated for size and further inspected for aphid mummies. The *L. hirticornis* aphid mummies' dark brownish coloration easily distinguished them from other parasitoid species attacking *M. fuscoviride*, in particular *Aphidius tanacetarius* Mackauer and *Ephedrus* Haliday spp. (Hymenoptera: Braconidae: Aphidiinae). During subsequent visits, the fate of marked genets with aphid colonies was followed and any new genet colonization marked and recorded. The study continued for the entire growing season, until the shoots senesced and the aphid colonies collapsed.

Sample collection and microsatellite analysis

Aphids

Aphids were killed in 100% ethanol in 1.0ml Eppendorfs and later stored refrigerated until electrophoretic testing. To maximise genetic heterogeneity, usually only 1-5 aphids were tested per ramet. Aphids were visually checked during sorting (also in 100% ethanol) and those obviously parasitized by braconid wasps or contaminated with entomopathogenic fungi were discarded.

Purified DNA was extracted from individual aphids (100-200 ng) using the 'Salting out' procedure of Sunnucks & Hales (1996). Microsatellite banding profiles were obtained using the six primers and protocols essentially as detailed by Massonnet *et al.* (2002a), with a 1 0 µl reaction mixture volume (with a final Mg^{2+} concentration of 1.5 mM). PCR products were detected on a Licor 4300 sequencer (Licor 6.5% polyacrylamide gels; 0.25 mm thick run on 25 x 26 cm glass plates) using fluorescently labeled forward primers (IRD700 and -800) and

fluorescently-labeled size markers (Licor; IRD700 and 800; 50-350 bp) were used to determine the product size from electronic gel photographs. PCR reactions were performed on Eppendorf thermocyclers (Mastercycler®).

Parasitoids

A maximum of ten *L. hirticornis* aphid mummies were sampled per genet. These were collected in 1.5ml Eppendorfs for transport to the laboratory where they were left to emerge and the sex of the winged adults determined under a stereo binocular microscope. Males and females from a single genet were stored separately in Eppendorfs in 100% ethanol. Both aphid and parasitoid samples were stored at 4°C prior to DNA extraction.

For genetic analyses, only female parasitoids were used. As with the aphids, DNA was extracted using the 'salting-out' method. Eleven microsatellite loci were identified as follows: 9 (*Lhirt 01, Lhirt02, Lhirt03, Lhirt04, Lhirt06, Lhirt08, Lhirt10, Lhirt15, Lhirt23*) developed from *L. hirticornis*, and two others derived from published primer sequence results for other *Lysiphlebus* species, i.e. *Lysi08* from *L. fabarum* (Marshall), and *Lysi6b12* from *L. testaceipes* (Cresson) (Nyabuga *et al.*, 2009 [Article I]).

Data analysis

In order to gather a uniform number of individuals from the eight sites, the lower total number of individuals analysed was used as the bench mark, that is, site Burgau-2=61, Fritz-Winkler-Straße=42, Göschwitz-Bhf=66, Jena Nord-1=93, Jena West-Bhf=27, Saalbahnhof=25, Porstendorf-Bhf=34, and Zwätzen=28. Randomization was performed in R- statistical software to establish individuals for analysis in each of the insect species.

Both *M. fuscoviride* and *L. hirticornis* were analysed as eight populations according to the site of collection. The number of alleles, inbreeding coefficient (F_{IS}), global and pairwise F_{ST} according to Weir & Cockerham (1984) and relatedness according to Queller & Goodnight (1989) were calculated in FSTAT version 2.9.3.2 (Goudet, 2002). Observed and expected heterozygosity was calculated in ARLEQUIN (v.3.1; 2006, Excoffier *et al.*, 2005). Shared allele distance (*DAS*) for populations was performed in POPULATIONS V1.2.30 (Langella, 1999). Mantel correlation test for pairwise F_{ST} and *DAS* for the two insects was performed in Isolation by distance web service (IBDWS Version 3.15; Jensen *et al.*, 2005). Independent sample t-tests to test for statistical differences and other correlations between *M. fuscoviride* and *L. hirticornis* were performed in SPSS v. 16 (SPSS Inc, 2007).

Results

Global population genetic differentiation showed *M. fuscoviride* (F_{ST}=0.167) to be marginally more genetically differentiated (t=10.52, p=0.060) compared to its parasitoid, *L. hirticornis* (F_{ST}=0.138). Pairwise F_{ST} Mantel correlations revealed an increase in aphid host, *M. fuscoviride*, population sub-structuring was positively and marginally correlated to increased parasitoid, *L. hirticornis* sub-structuring (R=0.538, p=0.059; Figure 2).

All aphid and parasitoid populations had inbred population structures (Positive F_{IS}, Table 1). Though higher, the global *M. fuscoviride* F_{IS} (0.503) was not statistically different (t=8.063, p=0.079) from that of *L. hirticornis* (0.392). The relationship between aphid level of inbreeding (F_{IS}) and parasitoid F_{IS} was not significant (R=+0.079, $F_{1,\,7}$=0.038, p=0.852).

M. fuscoviride (0.211) samples were more related (t=16.583, p=0.038) compared to those of *L. hirticornis* (0.187). A Mantel test performed for shared allele distance (*DAS*) of the aphid to that of the parasitoid, revealed that an increase in shared alleles in the aphid was positively correlated with that in the parasitoid (R=0.667, p=0.044; Figure 3).

Globally, the parasitoid showed a significantly larger level of observed heterozygosity (H_O; 0.297) compared to the aphid (0.161) (t=3.368, p=0.184) although the relationship (correlation) between *M. fuscoviride* H_O at each population to that of *L. hirticornis* in the congruent population (Table 1) was not statistically significant (R=-0.559, $F_{1,\,7}$=2.728, p=0.150). Expected heterozygosity (H_E) levels in *L. hirticornis* (0.561) compared with the aphid host, *M. fuscoviride* (0.323) were not significantly different (t=3.714, p=0.167), whilst the correlation between H_E in the aphid populations with that of the parasitoid populations was non-significant (R=-0.602, $F_{1,\,7}$=3.409, p=0.114).

The total number of alleles in the *M. fuscoviride* populations was not correlated with the total number of alleles in the parasitoid populations (R=+0.022, $F_{1,\,7}$=0.003, p=0.959; Table 1).
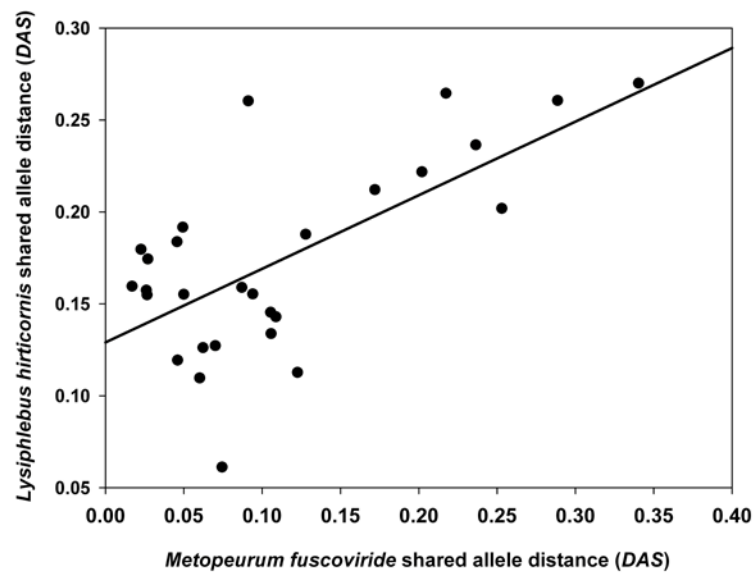
Figure 1 An increase in population shared allele distance (DAS) in *Metopeurum fuscoviride* was in congruent with an increase in shared allele distance in the parasitoid, *Lysiphlebus hirticornis* (R=0.667, p=0.044)

Table 1 Localities investigated for *Metopeurum fuscoviride* and *Lysiphlebus hirticornis*; N $_{aphids}$ and N $_{parasitoids}$ is the number of individuals genotyped and used in analysis, total number of alleles over all loci (A), inbreeding coefficient (F_{IS}), observed heterozygosity (H_O) and expected heterozygosity (H_E).

Site	*Metopeurum fuscoviride*					*Lysiphlebus hirticornis*				
	N aphids	A	F_{IS}	H_O	H_E	N Parasitoids	A	F_{IS}	H_O	H_E
Jena Nord-1	93	19	0.506	0.127	0.257	93	55	0.401	0.309	0.514
Burgau-2	61	18	0.693	0.112	0.362	61	51	0.420	0.288	0.494
Göschitz-Bhf	66	14	0.766	0.023	0.097	66	53	0.363	0.336	0.526
Saalbahnhof	25	17	0.351	0.313	0.480	25	40	0.285	0.313	0.435
Fritz-winkler-Str.	42	14	0.296	0.190	0.270	42	46	0.452	0.286	0.519
Porstendorf-Bhf	34	22	0.310	0.225	0.325	34	39	0.384	0.262	0.423
Jena West-Bhf	27	15	0.447	0.222	0.399	27	48	0.360	0.286	0.519
Zwätzen	28	14	0.672	0.071	0.215	28	32	0.392	0.336	0.526

Discussion

Using the qualitative guidelines for genetic differentiation (F_{ST}) of Wright (1978), our data show an overall 'greater genetic differentiation' in the aphid host, *M. fuscoviride* compared with 'moderate genetic differentiation' in the parasitoid, *L. hirticornis*. This result may be expected because of the differences in the biology of the insects: *M. fuscoviride* are resident sap sucking herbivores (plant parasites), i.e. they insert their proboscis into the plant phloem and if undisturbed, continue to feed and reproduce young, which in turn will start to feed and produce on the same plant. Winged morphs (alate exules; Dixon, 1998) have been observed early in the season as populations buildup (Nyabuga personal abservation), but as to whether it is a response to overcrowding or other stresses as shown in other aphids (Dixon, 1998; Sloggett & Weisser, 2002) is yet to be explored. The parasitoid, *L. hirticornis* on the other hand, has fully developed wings and although active fanning of wings and direct flight was not observed, walking between and among aphid colonies is reported (Weisser, 2000). Our results indicate that *L. hirticornis* has a higher dispersal ability compared to that of its aphid host. Given these factors, high gene flow and therefore reduced differentiation is expected in the parasitoid compared to the aphid host. These factors could also be the reason for the difference between the present results and those of Anton *et al*. (2007) who found moderate differentiation in the butterfly host, *M. nausithous* and high differentiation in the parasitoid, *N. melanocephalus* and explained this difference as due to the haplodiploid breeding system. Predators have also been found to have greater dispersal abilities compared to their hosts (Jones *et al.*, 1996; Brodmann *et al.*, 1997; Ellner *et al.*, 2001).

For specialist invertebrate herbivores feeding on plants and where host plants are aggregated and therefore providing a concentration of food resource in patches, the resource concentration hypothesis by Root (1973) suggests that such specialist herbivores should attain high densities; dispersing herbivores will locate and reproduce in those patches and will less likely leave the patches. The tansy plant is recorded to grow in patches and therefore provides a suitable breeding ground on which the specialist aphid, *M. fuscoviride* can reproduce and develop population structures. The aphid population structure certainly has a role in the parasitoid, *L. hirticornis* population structure (see Pacala *et al.*, 1990; Hassell *et al.*, 1991; Umbanhowar *et al.*, 2003). Pairwise F_{ST} correlations showed that an increase in aphid host, *M. fuscoviride* population sub structuring increased the parasitoid, *L. hirticornis*. The implication is that the parasitoid is tracking behind the aphid host. Weisser (2000) and Nyabuga *et al*. (2010 [*Article II*]) established that at a local (site) spatial scale, many tansy plants genets with the aphid were not colonized by

the parasitoid. If the local aphid population persists, then, in consequence, the parasitoid population will persist. If the aphid population is driven to extinction, the parasitoid in turn will also be driven to extinction.

M. fuscoviride populations were more related compared to the parasitoid and this could be explained by the parthenogenic mode of reproduction in the former. Aphids, during the season, produce young with their young within them (telescoping of generations; Dixon, 1998) and this mode of reproduction may be assumed to select for persistent genes and therefore reduced genetic diversity. The parasitoid on the other hand will only produce females when mated. Genetic diversity is reduced because few males are produced (Local mate competition) and one male mates with many females (Menken, 1991). We also report higher total heterozygosity (H_T) in *L. hirticornis* compared to *M. fuscoviride*. Since some variables are dependent on the number of microsatellite loci: here five loci were used for the aphid compared to 11 in the parasitoid. It is therefore not meaningful to compare the mean number of alleles for genetic diversity.

Metopeurum fuscoviride is obligately ant attended; the ants not only provide hygienic services but also protect them against predators and hymenopterous parasitoids. Ant attended aphid colonies are always bigger than non attended (Flatt & Weisser, 2000). The dependence on ants implies that dispersing aphids, in addition to finding suitable host plants, have to settle in the presence of ants (see Stadler & Dixon, 2005) and therefore perhaps another reason for the great genetic differentiation in this species. The parasitoid, *L. hirticornis* is thought to mimic the cuticular hydrocarbons of its aphid host and deceives the ants while attacking the aphids (Dettner & Liepert, 1994; Liepert, 1996). Ant attacks on the parasitoid have been observed (Nyabuga, pers. obs.). It is surely amazing that the aphid survives from season to season, let alone its specialized parasitoid. Besides the fact that no predator or parasitoid is likely to be 100% efficient in locating all available aphid patches, another reason why the aphid persists is because their subpopulations are guarded by aggressive ant species such as *Lasius niger* L. and *Formica rufa* L. that keep would-be predators (especially spiders, coccinellids, syrphid lacewing larvae, etc.) and other wasp parasitoids at bay. The exclusion of other players gives the chemically adapted *L. hirticornis* an edge to propagation. Furthermore, even in the face of high parasitoid mortality, aphids can still continue to propagate parthenogenetically, for a while at least.

In conclusion, in a trophic system where one trophic level is fully dependent on another –as here in the case of *L. hirticornis*, a specialist parasitizing *M. fuscoviride* – close co-evolutions are bound to occur. Such co-evolution will, however, almost always be determined by the lower trophic level, in this case the aphid *M. fuscoviride*. We have shown that the parasitoid has lower differentiation compared to the aphid host and population sub-structuring to track that of its host.

That the parasitoid tracks behind the aphid host could be the mechanism for *M. fuscoviride- L. hirticornis* persistence.

Article V: Mating strategies in solitary aphid parasitoids: effect of patch residence time and ant attendance

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Abstract

We compared the mating behavior of three solitary aphid parasitoids, *Aphidius ervi* Haliday, *Lysiphlebus hirticornis* Mackauer and *Pauesia pini* Haliday (Hymenoptera: Braconidae: Aphidiinae) that differ in resource exploitation and ant mutualism. Results showed that *L. hirticornis,* which is obligately ant-attended, had a patch residence time of approximately 4hours compared with less than 2hours for the facultatively ant-attended *P. pini*; the sexes did not differ in residence time. Females of *A. ervi,* which is not attended by ants, stayed for slightly more than 2hours on the natal patch while their male siblings remained for only 1hour. In *L. hirticornis*, 90% of all siblings in a clutch mated on the natal patch but only 13% in *A. ervi* and 42% in *P. pini* did so. Off-patch mating (23%) was observed only in *A. ervi*. Males and females of *L. hirticornis* were 12-times more likely to mate on the natal patch when aphids and ants were present than when either of the latter species was removed; and patch residence time declined from approximately 4hours to approximately 2.5 hours in the absence of either aphids or ants. We propose that, in aphidiine wasps and perhaps other pseudogregarious parasitoids, the mating behavior is influenced by the availability of resources on the natal patch and the presence or absence of trophobiotic ants. Partial sib mating is expected in species producing large clutches and having a long patch residence time.

Introduction

In hymenopteran parasitoids, behavior on the natal patch has a significant influence on mating opportunities and hence fitness returns to females and males. Gregarious parasitoid species show sib mating on the natal patch and local mate competition (LMC) (Godfray, 1994; Gu & Dorn, 2003). Solitary parasitoid species, in contrast, are expected to disperse from the natal patch and to mate with non-siblings (Nadel & Luck, 1992), but solitary parasitoids of colony-forming hosts such as aphids and coccids often produce pseudogregarious broods (van den Assem *et al.*, 1980) that allow partial sib mating and local mate competition (Loch & Walter, 2002; Mackauer & Völkl, 2002). In theory, variation in mating systems can have profound effects on important evolutionary processes such as sexual selection. Because mating systems determine the genetic population structure, it is important to define the mating system for each species if management strategies are to be applied.

A large body of literature on sex determination and sex ratio variation in the parasitoid Hymenoptera (King, 1987; Wrensch & Ebbert, 1993; Hardy, 2002) suggests that many species produce a female-biased offspring sex ratio, both in the laboratory and in the field. Inferences drawn from the population sex ratio regarding the underlying mating structure, however, may not be reliable and hence can be argued. For example, studies of parasitoids attacking hosts on their natal patches have shown different mating systems (Suzuki & Hiehata, 1985; Kazmer & Luck, 1991; Nadel & Luck, 1992; Kazmer & Luck, 1995; Loch & Walter, 2002; Gu & Dorn, 2003). In *Cotesia glomerata* L. (Hymenoptera: Braconidae), a gregarious parasitoid of first- to third-instar caterpillars of pierid butterflies, about 70% of the mating occurred on the natal patch between resident females and immigrant males (Loch & Walter, 2002; Gu & Dorn, 2003). Loch & Walter (2002) reported that, in *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae), a pseudogregarious egg parasitoid of *Nezara viridula* L. (Hemiptera: Pentatomidae), more than 18% of the F1-females were not mated by the dominant protandrous male upon emergence, 13% of the females left their natal site as virgins, and 25% of the females were mated multiple times and sometimes by multiple males; females remained near the natal site for up to several hours after emergence before emigrating, and males dispersed away from the natal site during female emergence.

In Aphidiinae wasps (Hymenoptera: Braconidae), solitary and koinobiont endoparasitoids which only parasitize aphids (Hemiptera: Aphididae), the population sex ratio is generally female-biased (Mackauer, 1976a; Sequeira & Mackauer, 1993; Singh & Pandey, 1997). With few exceptions (Starý, 1999; Belshaw & Quicke, 2003), reproduction is by facultative arrhenotoky, with females developing from fertilized (=diploid) and males from unfertilized

(=haploid) eggs (Cook, 1993). Examination of females captured in the field showed that virtually all were mated (Mackauer, 1976a; Mishra & Singh, 1991), with only a small proportion being constrained and capable of producing only male offspring. A female may be constrained because she has found no suitable mating partner; however, even a mated female may be constrained during a variable period after insemination when sperm is unavailable (Mackauer, 1976b). Because a female's receptiveness of a potential mate declines with her age (Srivastava & Singh, 1995), she can gain in fitness in terms of the number of daughters produced if she mates early. Especially in pseudogregarious parasitoids which produce clutches consisting of many males and females, a female's optimal strategy is to mate on the natal patch after emergence from the mummy. We tested this hypothesis by comparing the mating behavior on the natal patch in *Aphidius ervi* Haliday, *Lysiphlebus hirticornis* Mackauer and *Pauesia pini* Haliday, three species of aphid parasitoids differing in average clutch size and resource use.

Mating opportunities on the natal patch may be influenced by several factors. First, clutch size and hence the number of siblings emerging at the same time is determined by the pattern of oviposition, which is species-specific (Mackauer & Völkl, 1993). In species with low resource utilization per patch, such as many *Aphidius* species (Mackauer & Völkl, 1993; Weisser, 1995; Schwörer & Völkl, 2001), the number of potential mates emerging simultaneously is generally lower than in species with high resource utilization, such as *L. hirticornis* (Völkl, 1994; Weisser, 2000). Therefore, the probability of sib mating should be lower in species producing only few offspring per patch. Second, the offspring sex ratio and any differences between the sexes in the time of eclosion will influence the number of potential mates on the natal patch (Mackauer & Henkelman, 1975; Mackauer & Völkl, 2002). Male parasitoids eclosing from the mummy before females are available are likely to leave the natal patch and search for suitable mates elsewhere. Third, mutualistic ants collecting honeydew from the aphid hosts may influence the behavior of newly emerged parasitoids. Ants attack most aphid parasitoid species (Völkl, 1997) and often cause adult wasps to rapidly leave the patch upon emergence without mating. Exceptions include *L. hirticornis* and related species, which rely on chemical camouflage to escape ant detection and attack (Liepert & Dettner, 1993); mating on the natal patch in the presence of ants is possible in these species (Mackauer & Völkl, 2002). Last, many hymenopteran parasitoids (Quicke, 1997), including aphidiine species (McNeil & Brodeur, 1995; Nazzi *et al.*, 1996; Battaglia *et al.*, 2002; McClure *et al.*, 2007) produce a sex pheromone in order to locate mating partners. Therefore, the presence of other males and females and off-patch matings will vary with each species' dispersal behavior and the distribution of suitable hosts in the environment.

In this paper, we examine the mating strategies of three species of aphidiine species that differ in resource use and interactions with trophobiotic ants. We hypothesize that *A. ervi* (a 'low resource user' of aphids not attended by ants), *L. hirticornis* (a 'high resource user' of aphids obligately attended by ants) and *P. pini* (a 'low resource user' of aphids either attended or not attended by ants) have evolved different mating systems on their natal patches and, furthermore, that differences in patch behavior are sex-specific. We predicted that sib mating should be more common in species with a long patch residence time and producing large clutches than in species with a short residence time and producing small clutches. In *L. hirticornis,* we used exclusion tests to examine the influence of tritrophic interactions on mating behavior on the natal patch.

Material and Methods

Parasitoid biology

We selected three species with different ecological and behavioral attributes including habitat preference, host and host-plant range, foraging pattern, mean clutch size, sex ratio, and interactions with trophobiotic ants.

Aphidius ervi Haliday attacks a broad range of species in the subfamily Macrosiphinae (Aphidoidea: Aphididae) on various host plants. Its principal host is the pea aphid, *Acyrthosiphon pisum* (Harris), a common aphid on various species of Fabaceae including *Medicago sativa*, *Trifolium pratense*, *Pisum sativum*, *Ononis* species and *Vicia* species (Mackauer & Starý, 1967; Starý, 2006). It was also recorded as a parasitoid of *Macrosiphum euphorbiae* (Thomas) on tomato, *Aulacorthum solani* (Kaltenbach) on sweet pepper, and of *Sitobion avenae* (F.) on cereals (Cameron *et al.*, 1984; Takada & Tada, 2000). Aphids attacked by *A. ervi* are generally not tended by ants (Schwörer & Völkl, 2001).

Lysiphlebus hirticornis Mackauer exclusively parasitizes *Metopeurum fuscoviride* Stroyan (Aphidoidea: Aphididae) on common tansy, *Tanacetum vulgare* (Starý, 1973). Females of *L. hirticornis* mimic the epicuticular hydrocarbon pattern of their hosts which allows them to forage successfully in colonies guarded by honeydew-collecting ants (Liepert & Dettner, 1993; Mackauer & Völkl, 2002). Typically, rates of parasitism on a single tansy genet or ramet increase rapidly during the season and sometimes approach 100% during mid-summer (Weisser, 2000; Mackauer & Völkl, 2002).

Pauesia pini (Haliday) is a common parasitoid of *Cinara* species (Aphidoidea: Lachnidae) on conifers. Its main host is *C. pilicornis* (Hartig) feeding on the young branches of Norway spruce, *Picea abies,* throughout the summer season; the aphid is usually not attended by ants (Mackauer & Völkl, 2002; Völkl *et al.*, 2007). Other hosts are *C. piceicola* (Cholodkovsky) and *C. pruinosa* (Hartig), which feed on 2- to 5-year-old shoots of Norway spruce but, in contrast to *C. pilicornis*, are both heavily attended by honeydew-collecting workers of wood ants (*Formica spp.)*. Mackauer & Völkl (2002) observed that ants attacked and eventually killed newly eclosed parasitoids. However, females of *P. pini* have evolved behavioral adaptations to counter capture by ants. For example, females projected the antennae out of the mummy casing but did not emerge as long as ants were nearby. In spite of the ant interference, females were more successful laying eggs in the presence than in the absence of guarding ants (Völkl & Novak, 1997). Females also searched more intensively for hosts after contact with *Formica polyctena* (Hymenoptera: Formicidae) ants as compared to contact with honeydew alone in the absence of ants (Völkl *et al.*, 2007). *Cinara* spp. on pine were accepted by *P. pini* as alternate hosts during the summer when the preferred hosts on spruce were not available (Mackauer & Völkl, 2002; Völkl *et al.*, 2007).

Experimental design

All experiments were carried out in a greenhouse (4m x 7m x 4m) at 20° ± 2°C, 65-75% R.H. and approximately 3000 Lx (daylight). Plants were arranged so as to simulate, as much as possible, conditions found in the field. The "natal patch" consisted of a single experimental plant, which was surrounded by a circle of 4 - 6 other plants termed "other patches". The average distance between all plants was approximately 30cm. All "natal patch" plants were infested with parasitized aphids or mummies (see below) containing the offspring of a single mated female, i.e., all offspring were siblings. One half of the "other patch" plants were infested with unparasitized aphids of the same species, while the second half of the plants was left free of aphids and mummies. After each observation, the "natal patch" plant was replaced by a new plant infested with the same number of parasitized aphids or mummies.

For each parasitoid species, we established a stock culture with individuals obtained from the field. The stock cultures were maintained in a climate chamber at 21±1°C, 70-80% R.H., 3000 Lux, and a 16:8h light/dark cycle.

Aphidius ervi - Third-instar nymphs of pea aphids, *A. pisum*, were parasitized in a Petri dish by a single mated female and then transferred to broad bean plants (*Vicia* faba) until mummies

formed. To form the "natal patch", we selected from each clutch 4-6 mummies together with approximately 15 healthy aphids differing in age and placed them on a clean bean plant. Earlier observations had shown that several wasps would emerge within a short period of each other from clutches of this size and hence could mate.

Pauesia pini - A single mated female of *P. pini* was released on a potted 7-year-old Norway spruce infested with a mixed-age colony of 15-25 individuals of the fundatrigeniae generation *of C. piceicola*. Females usually deposited between 5 and 8 eggs per host colony (Völkl & Novak, 1997). Aphids were left on the plant until mummies had formed. Eclosing parasitoids were observed on their natal plant, which also served as the natal patch, as defined above. All *C. piceicola*-colonies were provided with *F. polyctena* ants until mummies formed. Because newly emerged *P. pini* left the natal host plant within a very short time in response to ant attacks (Mackauer & Völkl, 2002), the latter were excluded after mummy formation. Thus, the mating behavior of *P.* pini was observed only in the absence of ants.

Lysiphlebus hirticornis - We tested the mating behavior of *L. hirticornis* under three different conditions: in the presence and absence of trophobiotic ants and in the presence and absence of healthy aphids. A single mated female was released on a potted tansy plant infested with a mixed-age colony of 50 - 80 *M. fuscoviride* aphids attended by a colony of *Lasius niger* ants kept in a terrarium. Females usually parasitized a high proportion of the available hosts and laid between 40 and 70 eggs, independent of aphid colony size (Mackauer & Völkl, 2002). Aphids were kept on the plant until mummies formed; the rearing plant was used as the "natal patch", as defined above.

   In the first treatment (=control), ants as well as any remaining living and presumably unparasitized aphids were left on the plant ('ants and aphids' treatment). In the second treatment, all living aphids were removed but mummies and ants were left on the plant ('ants only' treatment). And in third treatment, ants were excluded but mummies and any remaining living aphids were left on the plant ('aphids only' treatment).

Mating behavior

The behavior of individual male and female parasitoids was observed from emergence until they left the natal plant. The time of emergence was estimated from the developmental stage of pupae found in dissected mummies. Observations began before dawn. Preliminary experiments indicated that a majority of adults in the three species do not leave the mummy before dawn

although they may have emerged from the pupal case within the mummy during the night (Mackauer & Henkelman, 1975; Loch & Walter, 2002). This early-morning eclosion peak after light-on increased the probability that males and females were found together on the natal patch. Within 2h after dawn, approximately 95% of *L. hirticornis* and 90% of *P. pini* had emerged; this peak was less pronounced in *A. ervi*.

In *A. ervi* and *P. pini*, it was possible to observe all emerging parasitoids until they left the natal patch since three or fewer parasitoids eclosed simultaneously. In *L. hirticornis*, however, the number of parasitoids eclosing at the same time exceeded the number that could be monitored. Therefore, we selected the first three individuals emerging on the patch for continuous observation; if one of these left the patch, it was replaced with the next individual which eclosed from a mummy. In addition, all matings that occurred on the natal plant were noted.

For continuously observed individuals only, we recorded the following variables: (1) residence time, which is the time between eclosion and an individual wasp leaving the natal plant; (2) mating on the natal plant, which is the proportion of individuals mating among those eclosing on the natal patch, and mating among siblings; (3) the time until first mating on the natal patch or on other patches; observations were continued on neighboring plants if the wasp under observation did not mate on the natal plant; (4) the number of oviposition attempts and successful ovipositions (in females only); ovipositions were counted as successful if an attacked aphid subsequently resulted in a mummy.

Data analysis

For the analysis, we considered only observations made when at least one male and one female were searching simultaneously on the natal plant and hence had a chance of mating. If an individual wasp left the natal patch before a potential mate emerged, the data were not included in the analysis except in cases of another potential mate (i.e., a non-sib individual) arriving on the same plant from one of the other patches as was observed in *A. ervi*.

We used the program R (R Development Core Team, 2008) to analyze the data. For residence time and time until first copulation, generalized linear models (GLM) with gamma distributions for time data and log transformations were fitted. GLM takes into account imbalances in the data. Mating success, attacks and oviposition success were analyzed as proportional data with binomial errors. First, we compared the three parasitoid species, and the sexes within each species, by fitting an interaction model to each variable (residence time, time until first copulation, mating success, oviposition attempts and successes). For *L. hirticornis*, we used the

data of the 'ants + aphids' treatment (=control) for the among-species comparison. Second, we examined the effect of trophobiotic ants and/or aphids on the mating behavior of *L. hirticornis* by fitting an interaction model to the data for the three treatments (ants only, aphids only, and aphids + ants) × sex (males and females). Statistical significance was assessed by F- and Chi-square tests, as appropriate. In case the interaction was not significant ($p>0.05$), the interaction term was excluded from the model and only the main factors considered. For *L. hirticornis,* we also calculated the odds ratio of a female mating under the three treatments (Sokal & Rohlf, 1995, pages 761-763). Linear regressions were performed on SigmaPlot Version 11.0 (SigmaPlot, 2008). Original values of means and the standard errors of the means are reported.

Results

Residence times on the natal patch

The residence times on the natal patch were species- and sex-specific as indicated by a significant interaction term ($F_{2,167}=8.47$, $p<0.001$) (Figure 1). Newly emerged males and females of *L. hirticornis* remained longer on the natal patch than individuals of *A. ervi* and *P. pini*; however, only females of *A. ervi* stayed longer on the patch than their male siblings (Figure 1).

In *L. hirticornis*, the treatment × sex interaction term was not significant ($F_{2,188}=0.60$, $p=0.552$) but only main factor effects. Males (192.1 ± 10.5 min; n=97) remained significantly longer on the natal patch than their female counterparts (165.0 ± 10.0 min; n=97) ($F_{1,190}=6.53$, $p=0.011$). Removal of either ants or aphids significantly reduced the patch residence time of newly eclosed individuals (females=134.7 ± 10.9; males=163.6 ± 12.1 minutes) ($F_{2,191}=120.28$, $p<0.001$) (Figure 2).
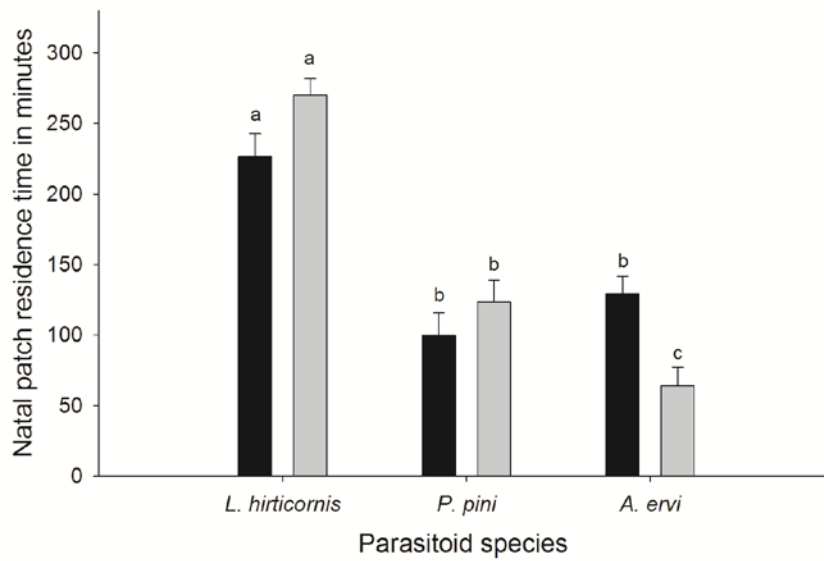
Figure 1 Residence times (mean ± SE in minutes) on the natal patch in three aphid species in relation to parasitoid sex. Black bars= females, gray bars=males. Means sharing the same letter do not differ significantly at p<0.05
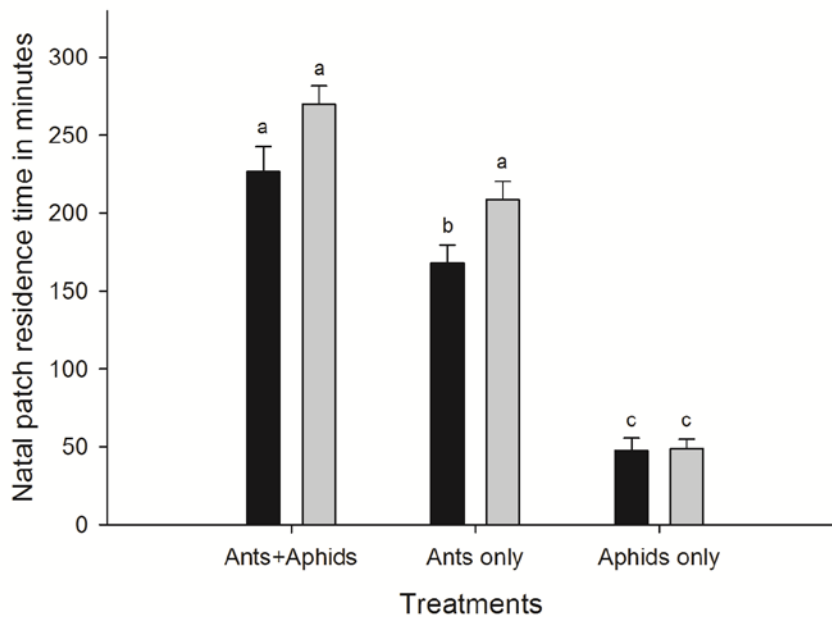
Figure 2 Residence times (mean ± SE in minutes) of *L. hirticornis* on the natal patch in three different situations in relation to sex. Black bars= females, gray bars=males; means sharing the same letter do not differ significantly at p<0.05

Mating on the natal patch

The proportion of siblings that mated on the natal patch was highest in *L. hirticornis* followed by *P. pini* and *A. ervi*, in that order (χ^2=76.01, n=6, p<0.001) (Figure 3). In *L. hirticornis* and *P. pini*, all matings occurred among siblings on the natal patch, while only 61.1% of the females of *A. ervi* mated on the natal patch and the rest on "other patches" in the experimental universe (Table 1). Neither the *s*pecies × sex interaction term (χ^2=1.33, n=6, p=0.515) nor the main effect of sex were significant (χ^2=0.582, n=6, p=0.445) for mating success.

In the three parasitoid species, all the males that mated on the natal patch mated with their siblings as did all the females of *L. hirticornis* (Table 1). In contrast, only 5 (45.5%) and 4 (44.4%) of females of *A. ervi* and *P. pini*, respectively, mated with male siblings on the natal patch.

In *L. hirticornis*, the interaction between treatment × sex on mating success on the natal patch was not significant (χ^2=1.32, N=6, p=0.517). The percentage of individuals mating on the natal patch was highest when both trophobiotic ants and aphids were present. In females, it declined from 93.8% to 46.8% and 38.9%, respectively, when either the aphids or the ants were removed from the patch while, in males, the percentage of individuals mating declined from 84.6% to 39.2% and 45.0%, respectively (Table 1) (χ^2=41.29, N=6, p<0.001; Figure 4). Because the two exclusion treatments did not differ significantly from each other (χ^2=0.72, p=0.937), the odds ratio was calculated from data pooled between sexes and between exclusion treatments, yielding a value of 11.66 (95% CI, 4.69-28.98), i.e., males and females of *L. hirticornis* were 12-times more likely to mate on the natal patch when both aphids and ants were present than when either species was removed from the system.
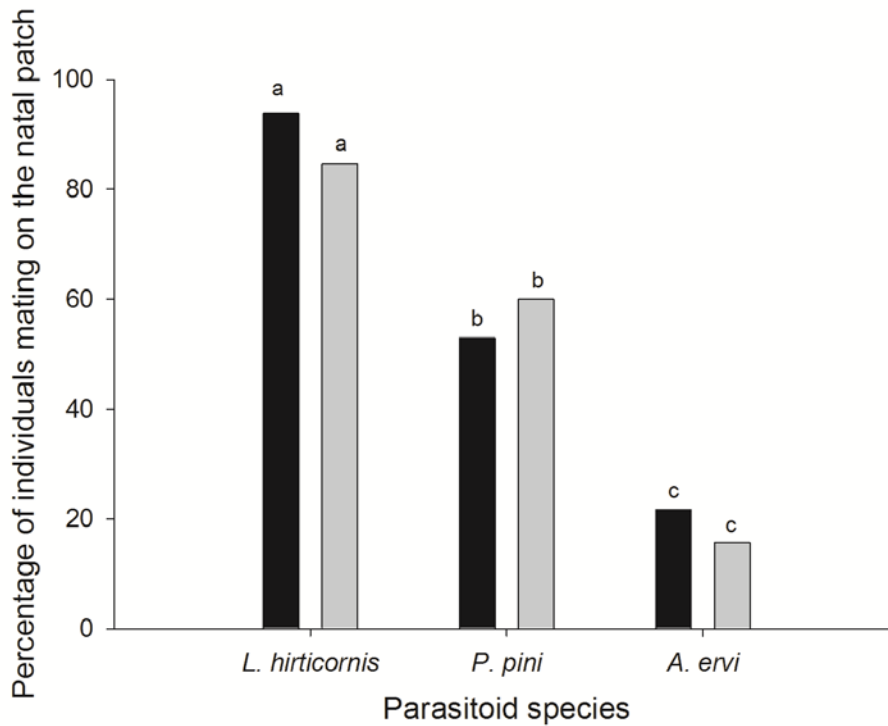
Figure 3 Proportion of parasitoids mating on the natal patch in relation to parasitoid species and sex. Black bars= females, gray bars=males. Within and among species, means sharing the same letter do not differ significantly at p <0.05

Time until first mating on the natal patch

The time between emergence and first mating varied between the three parasitoid species, with *L. hirticornis* having the shortest time ($F_{2,83}$=12.96, p<0.001) (Figure 5). The species × sex interaction term was not significant ($F_{2,80}$=1.86, p=0.162). In *A. ervi* (χ^2=0.101, p=0.683) and *P. pini* (χ^2=0.172, p=0.589), the sexes did not differ in the time to first mating. In contrast, in *L. hirticornis*, the time to first mating was significantly shorter in females (5.43±2.1 min) than in males (23.95±8.7 min) (χ^2=27.58, p=0.007). The time to first mating was not correlated with the residence time on the natal patch in *A. ervi* (R=0.379, $F_{1,14}$=2.34, p=0.148) and *L. hirticornis* (R=0.152; $F_{1,51}$=1.18, p=0.282). However, in *P. pini*, the time until first mating was positively correlated with residence time (R=0.817, $F_{1,16}$=32.19, p<0.001).

In *L. hirticornis*, the treatment × sex interaction term was significant ($F_{2,\ 104}$=3.58, p=0.031). The removal of aphids (='ants only' treatment) resulted in a longer time to first mating in both sexes whereas the removal of ants (='aphids only' treatment) resulted in a shorter time to first

mating in males but had no measurable effect in females (Figure 6).

Table 1 Summaries of number of individuals observed in each of the three parasitoid species. For *L. hirticornis*, the number of individuals observed at the exclusion of either ants or aphids treatments is also given.

Parasitoid species and treatments	sexes	Total (N) observed	Unmated (N)	Mated (N)	Mating Patch	
					Natal (N)	Other (N)
Aphidius ervi	Female	51	33	18	11	7
	male	32	15	17	5	12
Pauesia pini	Female	17	8	9	9	…
	male	15	6	9	9	…
Lysiphlebus hirticornis (controls='Aphids+ants' treatment)	Female	32	2	30	30	…
	male	26	4	22	22	…
Lysiphlebus hirticornis ('Aphids only'Treatment)	Female	18	11	7	7	…
	male	20	11	9	9	…
Lysiphlebus hirticornis ('Ants only'Treatment)	Female	47	25	22	22	…
	male	51	31	20	20	…
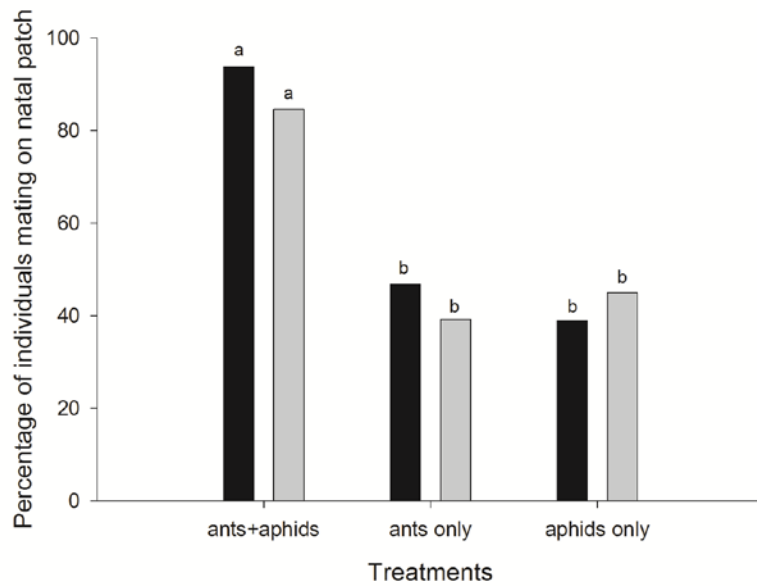
Figure 4 Proportion of *L. hirticornis* mating on the natal patch in three different treatments in relation to parasitoid sex. Black bars= females, gray bars=males; means sharing the same letter do not differ significantly at p < 0.05.
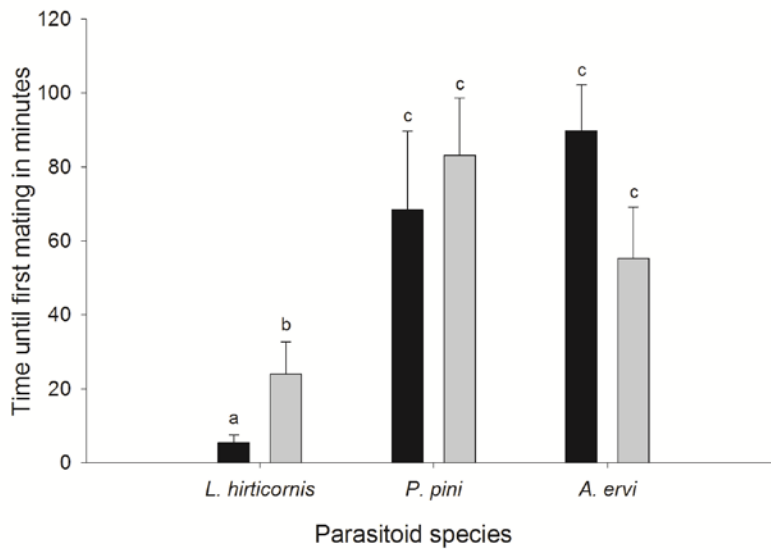
Figure 5 Time interval between eclosion and first mating (mean ±SE in minutes) in three parasitoid species. Black bars= females, gray bars=males; means sharing the same letter do not differ significantly at p < 0.05.
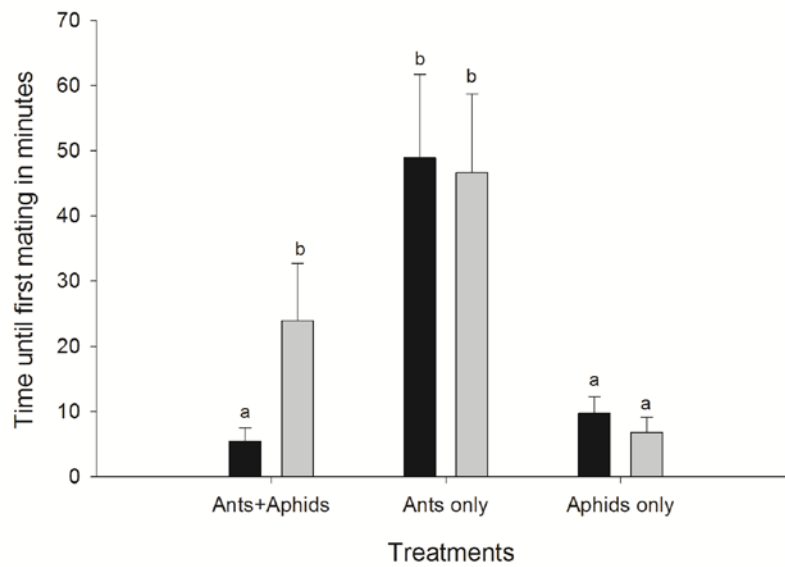
Figure 6 Time interval between eclosion and first mating (mean ±SE in minutes) in *L. hirticornis* in three different situations. Black bars= females, gray bars=males; means sharing the same letter do not differ significantly at p < 0.05.

Oviposition attempts and success on the natal patch

The percentage of female parasitoids attacking their aphid hosts on the natal patch among those emerging varied among the three species (χ^2=54.38, DF=2, p<0.001). In *A. ervi*, only 35.3% (n=51) of the emerging females attacked healthy aphids on the natal patch compared with 100% (n=32) and 94.1% (n=17) in *L. hirticornis* and *P. pini,* respectively. None of the attacks by *A. ervi* and *P. pini* resulted in parasitism, while nearly all (96.9%, n=31) oviposition attempts by *L. hirticornis* were successful.

Discussion

Aphidiine wasps parasitize colony-forming aphids in patchy environments. Although offspring develop as solitary larvae, many species produce pseudogregarious broods during a single oviposition bout; brood size can vary from a few individuals to several hundred. Females of *L. hirticornis,* which may produce broods of over 200 individuals (Mackauer & Völkl, 2002), had a mean patch residence time of approximately 4hours while *A. ervi* females left after 2hours, which is consistent with its low level of resource use (Schwörer & Völkl, 2001). Residence time was >2hours in *P. pini* although females generally attack many more aphids per patch as compared to *A. ervi* (Völkl & Novak, 1997; Schwörer & Völkl, 2001). The residence time of

males of *L. hirticornis* and *P. pini* did not differ from that of their female counterparts; however, males of *A. ervi* generally left the natal patch before females (Figure 1).   Differences between the sexes in dispersal behavior after emergence were also observed in other parasitoids. In species of *Leptopilina, Asobara* and *Tanycarpa,* all parasitoids of *Drosophila* larvae, males are thought not to disperse from the natal site (Hardy, 1994). Similarly, only females of *Mellitobia australica* Girault (Hymenoptera: Eulophidae), a gregarious ectoparasitoid of prepupae and pupae of solitary wasps and bees, are reported to disperse while males are assumed to spend their entire life inside the host cocoon (Abe *et al.*, 2003). In *Trichogramma turkestanica* Meyer (Hymenoptera: Trichogrammatidae), a facultative gregarious species attacking lepidopteran eggs, both males and females dispersed within one hour after sunrise but females dispersed earlier (Martel & Boivin, 2007). Because a male can gain fitness only if it can find a mate, males are expected to disperse from the natal site in the absence of females. In contrast, even a constrained female can gain fitness if her sons can mate with the daughters of other females. Therefore, a female's strategy should be based on the optimal balance between finding suitable hosts for her offspring and finding a mate.

Our results suggest that the value of the natal patch as a rendezvous site for potential mates varies with clutch size and the presence of suitable hosts, among other factors. In *A. ervi,* a species that parasitizes only few aphids in each colony, mate finding is mediated by a sex pheromone (McClure *et al.*, 2007). Many males left the natal patch before females; they also mated off-patch with non-sibling females while newly emerged females mated with immigrant males (Table 1). In contrast, in *L. hirticornis*, which produces large clutches containing many males and females, almost all matings occurred on the natal site between siblings.

Völkl (1994) reported that, in *Lysiphlebus cardui* (Marshall), patch residence time was correlated with colony size. Using exclusion experiments, we also found that the patch residence time of females of *L. hirticornis* was influenced by the presence of suitable hosts rather than the presence of males. Females left the natal patch earlier when aphids and/or ants were absent than when both species were present (Figure 2). Interestingly, in both sexes, giving-up time was shorter when only aphids were present. Although unattended colonies of *M. fuscoviride* can be found in the field (Mackauer & Völkl, 1993), laboratory experiments showed that the aphids persist only when attended by trophobiotic ants (Flatt & Weisser, 2000; Fischer *et al.*, 2001). Therefore, it is possible that females of *L hirticornis* use ants as an index of a colony's quality and avoid colonies that may not survive sufficiently long for offspring to complete development. Kaneko (2003) reported similar observations for *Lysiphlebus japonicus* Ashmead, a parasitoid of *Aphis gossypii* Glover (Homoptera: Aphididae); he found that the number of mummies were

lower in aphid colonies that were not attended by ants than in colonies attended by *Lasius niger* L. and *Pristomyrmex pungens* Mayr (Hymenoptera: Formicidae). Females of *P. pini* foraged longer for *C. piceicola* in colonies that were attended by ants than in colonies without trophobiotic ants (Völkl & Novak, 1997).

We did not record differences in eclosion sequence between males and females. If males eclose before females (=protandry), females can mate on the natal patch before dispersing. In *L. hirticornis,* the time to first mating was considerably shorter in males than females (Figure 4), which is consistent which males emerging from the mummy, on the average, earlier then female counterparts developing under similar conditions. Evidence of protandry in aphidiine wasps is problematic, however. While some males tend to emerge before the majority of females, eclosion rhythms are gated by light-dark cycles and hence vary with environmental conditions (Mackauer & Henkelman, 1975).

Our results support Mackauer & Völkl (2002) who concluded from the analysis of brood-size and sex-ratio variation that *P. pini* is largely out breeding while the mating system of *L. hirticornis* is determined by sib mating and LMC. Two systems, termed Type 1 and Type 2 for convenience, can be distinguished that differ in dispersal behavior and sib mating. The Type 1 mating system is characterized by dispersal from the natal patch and out breeding; it is typical of species parasitizing only one or few aphids in each colony such as *A. ervi*. In comparison, the Type 2 mating system is characterized by a high degree of sib mating and LMC on the natal patch; it is typical of species producing large clutches such as *L. hirticornis*. The two systems are not mutually exclusive but represent the extremes of a continuum; the position of each species on this continuum depends on its pattern of resource utilization and average clutch size. Females of *L. hirticornis* responded flexibly by dispersing from the natal patch when deprived of suitable hosts for their offspring or when the absence of trophobiotic ants may have indicated low host quality. Also, the population mating structure of *P. pini* is intermediate between Type 1 and Type 2, being characterized by facultative inbreeding in females and mainly sib mating in males. Males may use the natal patch as a rendezvous site and remain until females emerge, as we observed in *L. hirticornis*. By contrast, males of *A. ervi* departed even when female siblings were present, with the latter often mating with immigrant males. While the relatively "open" mating system of Type 1 species will favor out breeding, the extent of inbreeding in the relatively "closed" mating system of Type 2 species cannot be assessed without information on the genetic population structure and the mechanism by which sex is determined. Nevertheless, inbreeding in *L. hirticornis* and similar species may be less severe than indicated by sib mating on the natal patch. In the field, several parasitoid females are commonly observed searching for hosts in large

aphid colonies at the same time. On-patch matings therefore would be among non-siblings and not distinguishable from off-patch matings between unrelated males and females in terms of the genetic population structure.

Article VI: Effects of pea aphid secondary endosymbionts on aphid resistance and development of the aphid parasitoid, *Aphidius ervi*: a correlative study

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Abstract

In order to reduce parasite-induced mortality, hosts may be involved in mutualistic interactions where the partner contributes to resistance against the parasite. The pea aphid, *Acyrthosiphon pisum*, harbours secondary bacterial endosymbionts, some of which have been reported to confer resistance against aphid parasitoids. While this resistance often results in death of the developing parasitoid larvae, some parasitoid individuals develop to an eclosing adult. Whether these individuals suffer from fitness reduction compared to parasitoids developing in pea aphid clones without symbionts has not been tested so far. Using 30 pea aphid clones that differed in their endosymbiont complement, we studied the effects of these endosymbionts on aphid resistance against the parasitoid *Aphidius ervi*, host-parasitoid physiological interactions and fitness of emerging adult parasitoids. The number of symbiont species in an aphid clone was positively correlated with a number of resistance measures but there were clear symbiont-specific effects on the host-parasitoid interaction. As in previous studies, pea aphid clones infected with *Hamiltonella defensa* showed resistance against the parasitoid. In addition, pea aphid clones infected with *Regiella insecticola* and co-infections of *H. defensa-Spiroplasma*, *R. insecticola-Spiroplasma* and *R. insecticola- H. defensa* showed reduced levels of parasitism and mummification. Parasitoids emerging from symbiont-infected aphid clones often showed a longer developmental time and reduced mass. The number of teratocytes was generally lower when parasitoids oviposited in aphid clones with a symbiont complement. Interestingly, unparasitized aphids infected with *Serratia symbiotica* and *R. insecticola* had a higher fecundity than unparasitized aphids of uninfected pea aphid clones. Our study concludes that in addition to conferring resistance, pea aphid symbionts also negatively affect parasitoids that successfully hatch from aphid mummies. Because of the link between aphid resistance and the number of teratocytes, the mechanism underlying resistance by symbiont infection may also involve interference with teratocyte development.

Introduction

The existence of parasitoids is dependent on successful parasitism of their hosts. Given that parasitoids often kill their hosts as part of their lifecycle, there is intense selection pressure on the host to evolve defenses against parasitoid attack, and the parasitoid itself is selected to develop counter resistance mechanisms against these host's defenses (Godfray, 1994; Kraaijeveld & Godfray, 2009). A suitable host allows all or nearly all immature parasitoids to develop into adults, whereas marginal hosts allow only a small proportion to develop and unsuitable hosts allow no parasitoid development (Firlej *et al.*, 2007). Host suitability depends on factors such as the host immune system and host toxins (Lavine & Strand, 2002).

While encapsulation has been reported as the main physiological mechanism for defense against parasitoids in insects (Godfray, 1994), it has not been observed or recorded in some insect taxa such as aphids (Homoptera: Aphididae). Nevertheless, there is ample evidence for resistance against parasitoids in the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) (Henter & Via, 1995; Hufbauer & Via, 1999; Ferrari *et al.*, 2001; Li *et al.*, 2002; Stacey & Fellowes, 2002), the peach potato aphid, *Myzus persicae* (von Burg *et al.*, 2008) and in the black bean aphid, *Aphis fabae* (Vorburger *et al.*, 2009). In *A. pisum*, a number of recent studies show that various facultative secondary bacterial symbionts including *Hamiltonella defensa, Serratia symbiotica* and co-infections of *S. symbiotica- H. defensa* and *H. defensa- pea aphid X-type symbiont* (*PAXS*) are involved in host resistance against the parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae; Aphidiinae) (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Bensadia *et al.*, 2006; Oliver *et al.*, 2006; Guay *et al.*, 2009). While the majority of these studies were correlative, comparing pea aphid clones differing in their symbiont complement, the seminal study by Oliver *et al.* (2003) showed by manipulating the presence of *H. defensa* that symbiont infection leads to aphid resistance. More recently, it has been established that *H. defensa* contains a lysogenic lamdoid bacteriophage called *Acyrthosiphon pisum secondary endosymbiont* (*APSE*) whose toxin- encoding genes target and destroy the cells of attacking parasitoids or other eukaryotic enemies thereby protecting their aphid host (Moran *et al.*, 2005a; Degnan & Moran, 2008b, a; Oliver *et al.*, 2009).

Other bacterial symbionts in pea aphid include a *Rickettsia* species (Pea Aphid *Rickettsia, PAR*) and an alpha-Proteobacteria, *Spiroplasma* species (Chen *et al.*, 1996; Chen & Purcell, 1997; Fukatsu *et al.*, 2000; Tsuchida *et al.*, 2002). While *Rickettsia* and *Spiroplasma* are reported to induce female biased sex ratios by killing males in other insects (Williamson *et al.*, 1999; Lawson *et al.*, 2001; Tinsley & Majerus, 2006), their effects on pea aphids or against parasitoids developing in pea aphid viscera have not been explored. Further, pea aphid clones

may be co-infected with up to three facultative endosymbionts (Frantz *et al.*, 2009), and the effects of these symbionts either as single or multiple infections have not been exhaustively explored. Studies by von Burg *et al.* (2008) and Vorburger *et al.* (2009) in *M. persicae* and *A. fabae* respectively indicated that resistance is not solely due to the presence of symbionts but is also due to some innate heritable clonal variability. In pea aphids, symbionts are thought to be fully responsible for the resistance (Oliver *et al.*, 2005).

In *A. ervi* as is in many other wasp parasitoid species, a female parasitoid injects alecithal eggs (i.e., without nutrients) into the aphid's viscera where they absorb nutrients for development. After successful hatching the parasitoid embryo releases specialized teratocyte cells from the serosa membrane that are involved in successful parasitoid development through immunosuppression, secretion and nutrition (Vinson, 1990; Pennacchio *et al.*, 1994). The teratocyte diameter increases with time but their number decreases progressively when the parasitoid larva ingests them. Li *et al*. (2002) showed that in a resistant strain of *A. pisum* both the number and the growth of functional teratocytes released by the developing parasitoid embryo was drastically reduced compared to parasitism of a susceptible aphid strain. Cloutier and Douglas (2003) counted low numbers of teratocytes in a pea aphid clone infected with *H. defensa* and *Spiroplasma* compared to a clone that bore *R. insecticola* and another that harboured *S. symbiotica*, *H. defensa* and *Pea aphid Rickettsia (PAR)*. Because teratocytes mediate the host-parasitoid relationship, teratocyte growth patterns and numbers are thought to reflect host suitability (Firlej *et al.*, 2007). Together with alecithal eggs, aphid parasitoids may inject a venom that stops their hosts from continued reproduction, a "behaviour" termed parasitic castration or sterilization (Polaszek, 1986). There is paucity of information on the extent to which the secondary symbionts protect their hosts against castration.

Resistance of pea aphid clones to parasitoids, is rarely complete, i.e. the parasitoid larva is often able to survive (Hufbauer & Via, 1999; Li *et al.*, 2002; Oliver *et al.*, 2003; Oliver *et al.*, 2005, 2006) but see (Guay *et al.*, 2009). In the successfully developing parasitoid individuals, developmental time may be prolonged (Li *et al.*, 2002). Other effects on the parasitoid may be expected, as parasitoid development in marginal hosts is generally associated with reductions in body size, fecundity, and longevity (e.g., Arakawa *et al.*, 2004). There may also be effects on parasitoid sex ratio as reported for the eulophid wasp *Neochrysocharis Formosa* Westwood parasitizing the leafminer *Liriomyza trifolii* Burgess in Japan where an undescribed *Rickettsia* species in the host is responsible for parthenogenetic reproduction in the parasitoid (Hagimori *et al.*, 2006). Such sublethal effects have not been studied in adult parasitoids emerging from aphids infected with secondary symbionts.

Here, we screened 30 genetically distinct pea aphid clones, that differed in their complement of secondary symbionts (Simon *et al.*, 2003; Frantz *et al.*, 2009) and asked the following questions: (1) Are other symbionts besides *H. defensa*, *S. symbiotica* and co-infections of *H. defensa- S. symbiotica* associated with resistance against *A. ervi* and to what extent? (2) What is the effect of symbiont complement on aphid reproduction? (3) Do the symbionts influence teratocyte numbers? (4) Are there sub lethal effects of secondary symbionts on emerging adult parasitoids?

Material and Methods

Pea aphid culture

Thirty pea aphid clones (Table 1), all green, collected from different plant species belonging to the family Fabaceae, were established on broad bean, *Vicia faba*, at the INRA research station in Le Rheu, France. All the pea aphid clones were distinct at seven microsatellite loci. Secondary symbiont complements were determined using PCR assays based on the 16S rDNA of the bacteria (Simon *et al.*, 2007). The number of symbionts per aphid ranged from zero (uninfected) to three symbionts (see Frantz *et al.*, 2009 for clonal symbiont details) although only up to two symbiont co-infections and 10 different levels of symbiont complements were used in this study (Table 1). Adult aphids for use in the experiments were collected from these cultures.

***Aphidius ervi* culture**

An *A. ervi* culture was established and maintained on a green clone of pea aphid (SG) described in Weisser & Braendle (2001). An initial *A. ervi* culture was obtained from Katz Biotech, Germany. The mummies were singly placed into gelatin capsules, at emergence the adults were put into micro cages as a male and female pair, provided with honey and water *ad libitum* until their use in parasitization experiments. Adults were between two and five days old when used.

Preparation of aphids for parasitism

Ten wingless adult aphids from the stem culture of a particular pea aphid clone were introduced onto two clean broad bean plants (five aphids per plant) and each plant was covered with air permeable cellophane bag (30 cm height). The aphid-inoculated plants were put back onto trays and transferred into the climate chamber. The adult aphids were then allowed to feed and reproduce for 24 hours after which the mother aphids were removed to leave only the offspring (L1) to continue feeding for a further five days in the climate chamber. The six day old offspring from the two plants were mixed and subjected to parasitism in glass Petri dishes in groups of 10.

Table 1 Pea aphid, *Acyrthosiphon pisum* clone identities and background information on host plant of collection, region of collection, number of secondary symbionts and identity of the secondary symbionts found on the clones

clone	host plant	region	Symbiont present in clone
CS05	*Cytisus scoparius*	Le Rheu 35, Western France	Uninfected
lOR09	*Ononis repens*	Buget 01, Eastern France	Uninfected
lOS07	*Ononis spinosa*	Buget 01, Eastern France	Uninfected
lOS11	*Ononis spinosa*	Buget 01, Eastern France	Uninfected
LL01-VF	*Medicago sativa*	Lusignan 86, Central France	Uninfected
jVC08	*Vicia cracca*	Jena, Eastern Germany	*Spiroplasma*
P123	*Pisum sativum*	Mauzé Le Mignon 86, Central France	Pea aphid *Ricketssia*
CS10	*Cytisus scoparius*	Le Rheu 35, Western France	*Serratia symbiotica*
jPS07	*Pisum sativum*	Jena, Eastern Germany	*Serratia symbiotica*
jVC06	*Vicia cracca*	Jena, Eastern Germany	*Serratia symbiotica*
jVC07	*Vicia cracca*	Jena, Eastern Germany	*Serratia symbiotica*
jVC09	*Vicia cracca*	Jena, Eastern Germany	*Serratia symbiotica*
L7c	*Medicago sativa*	Chile	*Serratia symbiotica*
P1c	*Pisum sativum*	Chile	*Serratia symbiotica*
F19	*Vicia faba*	Le Rheu 35, Western France	*Serratia symbiotica*
P136	*Pisum sativum*	Mauzé Le Mignon 86, Central France	*Serratia symbiotica*
T6c	*Trifolium pratense*	Chile	*Regiella insecticola*
TC02	*Trifolium campestre*	Le Rheu 35, Western France	*Regiella insecticola*
TD07	*Trifolium dubium*	Le Rheu 35, Western France	*Regiella insecticola*
VH02	*Vicia hirsuta*	Le Rheu 35, Western France	*Regiella insecticola*

T3-8V1	*Trifolium*	Domagné, 35, Western France	*Regiella insecticola*
L2-2V1	*Medicago sativa*	Domagné, 35, Western France	*Hamiltonella defensa*
L2-4V1	*Medicago sativa*	Domagné, 35, Western France	*Hamiltonella defensa*
L84	*Medicago sativa*	Le Rheu 35, Western France	*Hamiltonella defensa*
lVC07	*Vicia cracca*	Buget 01, Eastern France	*Serratia symbiotica + Spiroplasma*
L1-22	*Medicago sativa*	Domagné, 35, Western France	*Regiella insecticola + Hamiltonella defensa*
L2-1V1	*Medicago sativa*	Domagné, 35, Western France	*Regiella insecticola + Hamiltonella defensa*
jMS04	*Medicago sativa*	Jena, Eastern Germany	*Hamiltonella defensa + Spiroplasma*
L2-5V1	*Medicago sativa*	Domagné, 35, Western France	*Hamiltonella defensa + Spiroplasma*
T2-14V1	*Trifolium*	Domagné, 35, Western France	*Regiella insecticola + Spiroplasma*

Parasitization and data collection

Filter papers were put into glass Petri dishes (Ø 40 mm) and moistened. Leaves from broad bean plants were then excised and 2× 15mm diameter leaf discs introduced into the Petri dishes. For a single group, 10 six day old aphids were introduced into a single Petri dish, using a fine camel brush (size 0100), and let to settle and start feeding for approximately 20 minutes. One mated female *Aphidius ervi* was then introduced into the Petri dish and observed for the commencement of oviposition. In cases where the parasitoid did not start to forage and oviposit within 3 minutes after introduction, it was removed and replaced by another. After the start of oviposition, the parasitoid was left in the Petri dish for 20 minutes. Exposure of aphids to parasitoids was repeated in 3-4 replicates (denoted as group B, C, D and/or E) while group "A" was retained as a control i.e. was not exposed to parasitoid. The 10 aphids of a group were then moved onto new, 2-3 week old bean plants, covered by a cellophane bag, placed on a water tray

and moved into the climate chamber. For each pea aphid clone, this was repeated 4-5 times on different days creating temporal "blocks". In total there were 30 clones ×10 aphids per replicate × 4-5 replicates/ groups ×4-5 "temporal blocks" in the experiment. All culturing and experiments took place in the climate chamber at 20°C, 16h: 8h, L: D and 75% relative humidity. Five days after parasitism, groups (D, E) were dissected to determine percent parasitism (see below). After six days, aphids of the remaining two parasitized groups (B, C) and the control (group A) were transferred to new plants and the number of nymphs produced counted. After six more days (12days post parasitization) the mummified aphids were counted (groups B, C) and mummies carefully collected from the plant using forceps and put singly into gelatine capsules. The number of nymphs was once more counted in all groups (A-C). The mummies were observed daily in the morning and evening for emergence of adult parasitoids (percent emergence and development time) until 22 days post parasitism. For each emerging individual the sex was noted. The emerging adults were preserved in 95% alcohol in 1.5ml Eppendorfs until time of weight measurement. Individual weights were taken from at least five individuals per group (B, C) where more than five individuals had emerged otherwise the entire sample was measured. For weight measurements, parasitoids were taken out of the alcohol by use of hand brush, placed on clean tissue paper, allowed to air dry, and weight measurements taken on a Mettler (XS105 Dual Range; 0.01mg precision) balance.

Dissection to determine parasitism and number of teratocytes

Aphids of groups D and/ or E were dissected under a stereomicroscope to confirm the presence or absence of parasitoid larvae and teratocytes, if present, their numbers were counted following the method described by Cloutier & Douglas (2003). Briefly, individual aphids were dissected on a glass slide with a drop of Ringers saline solution. The aphid was held by the head on a drop of Ringers saline solution by use of a fine forceps and with another forceps, the posterior end was slowly pulled off. The larvae swam out of the aphid viscera and wiggled on the saline solution at which point a long fine pin was used to liberate all teratocytes which were then counted.

Variables measured

Secondary symbionts association with resistance and or ability to inhibit parasitoid virulence was measured by: (i) *Percentage parasitism* i.e. the fraction of aphids with parasitoid larvae divided by the total number of aphids exposed to parasitoid multiplied by 100. (ii) *Percentage of mummification,* calculated as the fraction of aphids that formed the characteristic yellow

coloured hardened mummy divided by the total number of aphids that were transferred to new plants at day six in the parasitism treatment, multiplied by 100 (iii) *Number of larvae* (iv) *Number of teratocytes* (mean number of larvae and teratocytes was calculated by dividing the total numbers found in a group (also Petri dish as unit of parasitism) by the total number of aphids dissected from that group) (v) *Aphid fecundity*, sum of the total of number of nymphs between day 0 and day 6 post parasitism (*Fecundity per aphid between day 0-6*=total number of nymphs on each group/ number of aphids introduced to plant on day 0) and between day 6 and day 12 post parasitism (*Fecundity per aphid between day 6-12*=total number of nymphs on each group/ number of aphids transferred to new plant on day 6).

Percentage *adult parasitoid emergence* (the fraction of adult parasitoids that broke out of the mummy casing) as measure of progeny survival, *development time* of parasitoid, *parasitoid weight* and secondary *sex ratio* as measures of secondary symbiont sub-lethal effects were also taken (groups B, C).

Data analysis

Analyses were performed using the program R (R Ver. 2.8.1. R Development Core Team, 2008). For each dependent variable statistical modeling was used to test the following questions: a) what is the effect of number of symbiont species (0, 1 & 2) that are present in an aphid clone? b) What are the effects of the different symbiont complements that infect an aphid clone? And c) is there significant variation between pea aphid clones, in addition to the effect of symbiont infection? Because the total number of clones tested was 30, five uninfected and 25 infected with five different symbionts in different single and double co-infections, it was not possible to test for the effects of each symbiont separately or interactions between these symbionts, and simultaneously test for the effect of clone and the number of symbionts in a clone. The following procedure was therefore adopted: First, to test for effects of the number of symbionts (0, 1 & 2), symbiont complement was considered a random effect and a linear mixed effect (LME) model to estimate the appropriate variance components used. The LME model accounts for the fact that the level of replication for each symbiont combination is unbalanced. The significance of number of symbionts term was assessed by comparing a model with the term "number of symbionts" fitted and a model without the term using a Chi-square (χ^2) test (Crawley, 2007). Contrasts between the different levels of numbers of symbionts (0, 1, 2) was then assessed by factor level reduction and model comparison (Crawley, 2007). In R, the procedure for factor level reduction involves lumping together levels with similar means then fitting a new "simpler" model with fewer levels of the factor. The "simple" model is then compared to the initial model

(model having all levels of factor of interest) using anova. If the value of P >0.05 then it is justified to perform factor level reduction, if not, i.e. p<0.05 then lumping together the levels is not justified (see supplementary material or Crawley, 2007 pages 374-377 for an example). Factor level reduction was continued until "the minimal adequate model" was attained. Since a model with "number of symbiont" term and another without the term are compared, χ^2 test values and changes in degrees of freedom (Δ d.f.) are reported. Changes in degrees of freedom could be defined as the difference in degrees of freedom for a model fitted with factor of interest and second model without the factor but parameter estimate represented with one (1) (Crawley, 2007).

Second, a GLM was used to test for the effect of symbiont complement (10 levels) on variables measured. The significance of symbiont complement was assessed by comparing a model with the term "symbiont complement" fitted and a model without the term using a χ^2 test (Crawley, 2007). If P<0.05 indicating significant effect, factor level reduction and model comparison ensued to contrast the 10 levels of symbiont complement. Factor level reduction and model comparison was performed as described above (see also supplementary material for an example). An F-test was performed for the minimal adequate model. For symbiont complement, χ^2-values and changes in degrees of freedom for initial model comparison and an F-test for minimal adequate model after factor level reduction are therefore reported.

Third, to test if there was significant variation between clones in resistance, in addition to the effect of symbiont complement; nested ANOVA models with both symbiont complement and clone (nested within symbiont complement) were fitted to the data set containing all replicates. F-tests were performed using "among pea aphid clones" mean sum of squares divided by "within pea aphid clones" mean sum of squares (see supplementary material for an example).

In the analysis of reproduction, "Treatment" (parasitized or control) was added to the models. Regression analyses were also performed where appropriate. For the effect of number of symbionts and specific symbionts, percentages were fit as binary variables with binomial errors while count data were fit with Poisson errors. Parasitoid weight was fit as normally distributed data with Gaussian family of errors while development time was fit with gamma errors (Crawley 2007).

Because of the amount of work involved in screening 30 aphid clones, all the clones could not be tested at the same time. The experiment was therefore performed with temporal "blocks" to increase the number of replicates and increase reliability of the results. These temporal blocks were thereafter aggregated to clone level, i.e., 30 pea aphid clonal means, and used in effect of "number of symbionts" and "symbiont complement" analysis. For effect of "clone", all

replicates were used in the analysis. Therefore, the reported means and standard errors for "number of symbiont" and "symbiont complement" were calculated over clonal means, while those for "clone" effects were calculated over all replicates.

Results

Effect of symbionts on aphid resistance against parasitoids

Parasitism and mummification: While the mean percentage parasitism at five days tended to be lower when more symbionts were present in an aphid clone (uninfected clones 88±3%, single infected clones 62±7% and double infected clones 26±15%), the relationship was not significant (χ^2=4.57, Δ d.f.=2, p=0.102). Bacterial complement influenced parasitism (χ^2=566.0, Δ d.f.=9, p<0.001). The minimal adequate model showed that clones infected with *H. defensa and* co-infections of *H. defensa- Spiroplasma* and *R. insecticola- Spiroplasma* differed from the uninfected clones ($F_{1,28}$=34.5, p<0.001, Figure 1). In addition to bacterial complement, there was a significant pea aphid clonal variation ($F_{20,105}$=5.4, p<0.001).

Percentage mummification was not influenced significantly by the number of symbionts (χ^2=5.42, Δ d.f.=2, p=0.067; uninfected 88±3%, single infection 64±7%, double infections 21±13%). Mummification was influenced by the bacterial complement (χ^2=1157.3, Δ d.f.=9, p<0.001) and model simplification showed five symbiont complements to be significantly different from the uninfected ($F_{2,27}$=27.1, p<0.001). These were *H. defensa, H. defensa- Spiroplasma* co-infection and *R. insecticola- Spiroplasma* co-infection, *R. insecticola* and *R. insecticola- H. defensa* co-infection. The latter two were also significantly different from the former three (Figure 1). In addition to bacterial complement, there was significant pea aphid clonal variation on percentage mummification ($F_{20,220}$=8.7, p<0.001).

Parasitoid larvae and teratocytes: The mean number of parasitoid larvae (per aphid) decreased with increasing number of symbionts (χ^2=6.93, Δ d.f.=2, p=0.031). Model simplification showed that doubly infected aphid clones (0.4±0.2) had significantly lower mean number of larvae compared with single infections (1.2±0.2) and uninfected clones (1.9±0.2) (χ^2=5.54, Δ d.f.=1, p=0.019). Bacterial complement influenced the number of parasitoid larvae (χ^2=15.2, Δ d.f.=9, p<0.001). The minimal adequate model showed that aphid clones infected with *H. defensa* and co-infections of *R. insecticola- H. defensa, H. defensa- Spiroplasma* and *R. insecticola-Spiroplasma* had significantly reduced number of larvae relative to the uninfected (Figure 2, $F_{1,28}$=25.3, p<0.001). Again, in addition to the symbiont effect, there was significant pea aphid clonal variation in the number of parasitoid larvae ($F_{20,105}$=3.4, p<0.001).

The mean number of teratocytes found per aphid decreased with increasing number of symbionts (χ^2=8.46, Δ d.f.=2, p=0.015). Model simplification (χ^2=7.83, Δ d.f.=1, p=0.005) showed that doubly infected clones (2.9±2.2) had significantly lower mean number of teratocytes than the singly (22.0±3.9) and the uninfected (35.3±2.4) clones. Bacterial complement influenced teratocyte numbers (χ^2=332.4, Δ d.f.=9, p<0.001) and clones infected with *H. defensa,* co-infections of *H. defensa- Spiroplasma, R. insecticola- H. defensa, R. insecticola- Spiroplasma* had fewer teratocytes than uninfected clones ($F_{1,28}$=35.8, p<0.001, Figure 3). Teratocyte count differed among aphid clones ($F_{20,105}$=3.3, p<0.001). Positive and significant correlations were calculated between number of teratocytes and percentage mummification (R=0.737, $F_{1,28}$=33.3, p<0.001), and between number of teratocytes and number of parasitoid larvae per aphid (R=0.839, $F_{1,28}$=66.4, p<0.001).
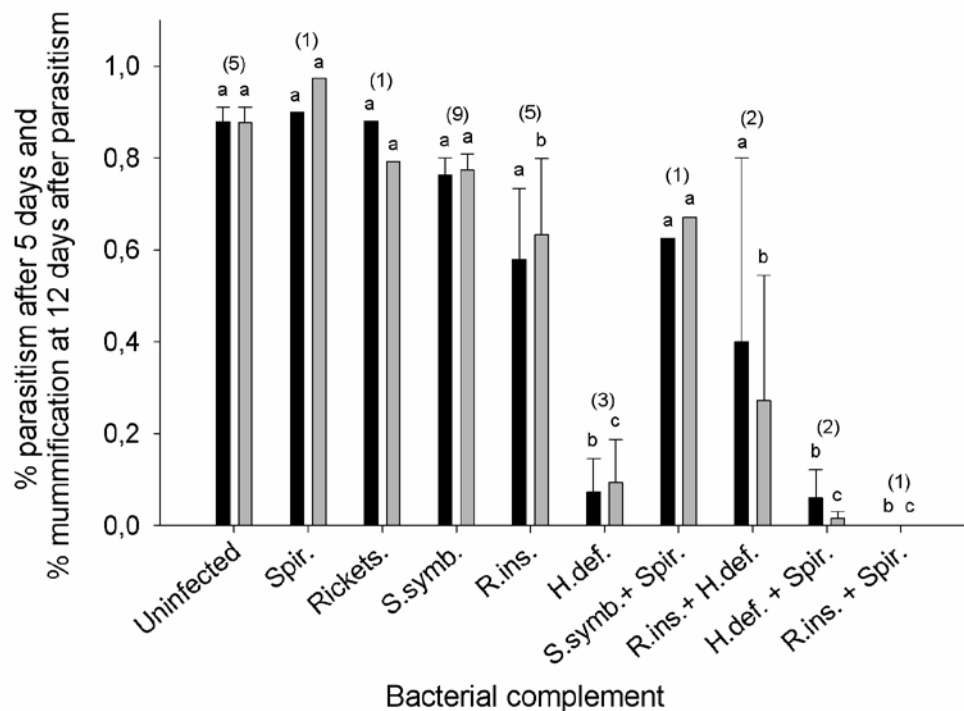
Figure 1 Proportion of aphids parasitized after five days (Black bars) and percent mummification (Gray bars) for aphid clones infected with different complements of secondary symbionts. Bars (mean ± se over N clonal means, number above the bars) of the same colour with same letter are not significantly different after model simplification (p>0.05).
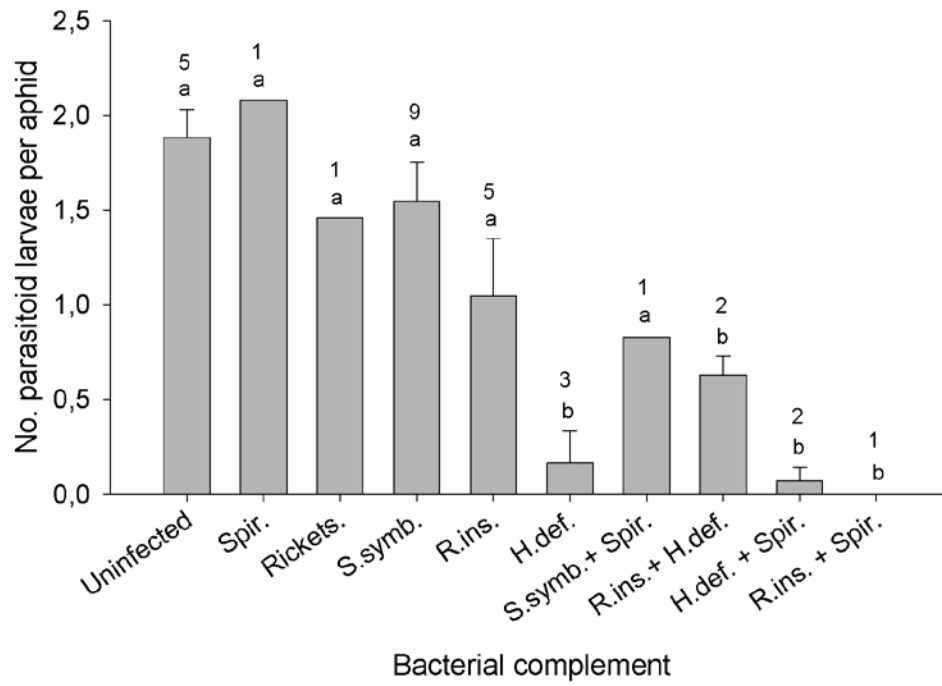
Figure 2 Number of parasitoid larvae found in aphid viscera five days after parasitism for aphid clones infected with different complements of secondary symbionts. Bars (mean ± se over N clonal means, number above the bars) with same letter are not significantly different after model simplification (p>0.05).
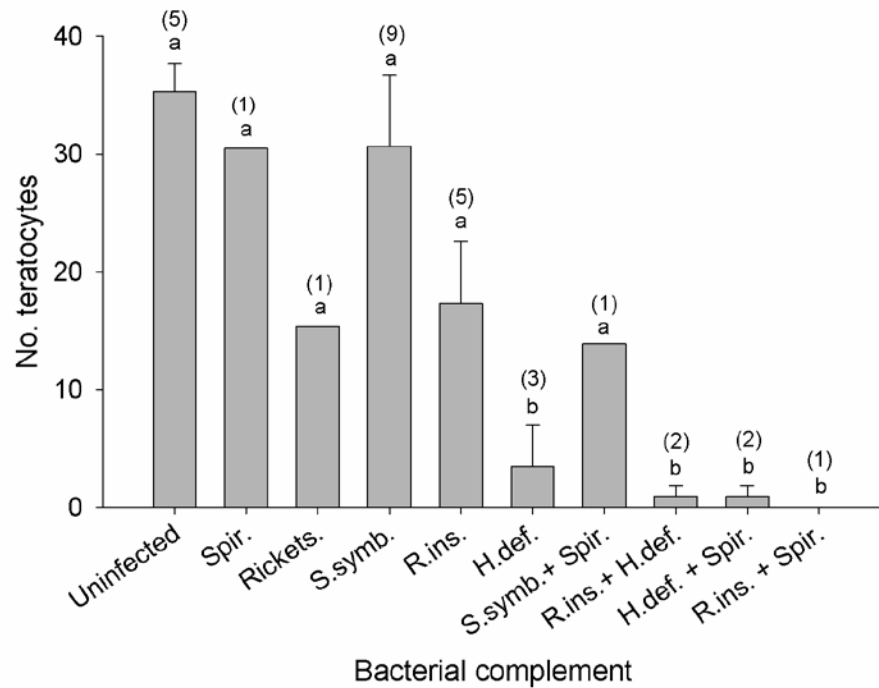
Figure 3 Number of teratocytes found in aphid haemolymph five days after parasitism for aphid clones infected with different complements of secondary symbionts. Bars (mean ± se over N clonal means, number above the bars) with same letter are not significantly different after model simplification (p>0.05).

Effect of secondary symbionts and parasitism on aphid reproduction

In addition to number of symbionts (uninfected, single and double infection), the treatments (parasitized and control) had an effect on 12 days cumulative aphid fecundity (χ^2=407.7, Δ d.f.=5, p<0.001). Model simplification showed that fecundity was significantly lower in parasitized treatments compared with control treatments at each of the symbiont numbers (Figure 4, χ^2=406.3, Δ d.f.=3, p<0.001). Symbiont complement in addition to treatments affected 12 days cumulative aphid fecundity (χ^2=564.7, Δ d.f.=19, p<0.001). A simplified model for symbiont complements × treatments ($F_{3,56}$=51.6, p<0.001, Figure 5) showed that parasitized clones infected with *H. defensa* and *H. defensa- Spiroplasma* co-infection were not different from their unparasitized control counterparts. Figure 5 also shows that compared with the uninfected, *S. symbiotica, R. insecticola, Rickettsia, R. insecticola- H. defensa* and *R. insecticola- Spiroplasma* tended to have higher fecundity while *H. defensa and S. symbiotica- Spiroplasma* co-infection had lower fecundity. In presence of symbionts and parasitism, the aphid clones also showed significant variability in their level of reproduction ($F_{20,335}$=12.5,

p<0.001). There was no general relationship between cumulative fecundity (days 0-12) and mummification (R=0.2832, $F_{1,19}$=1.63 p=0.217).
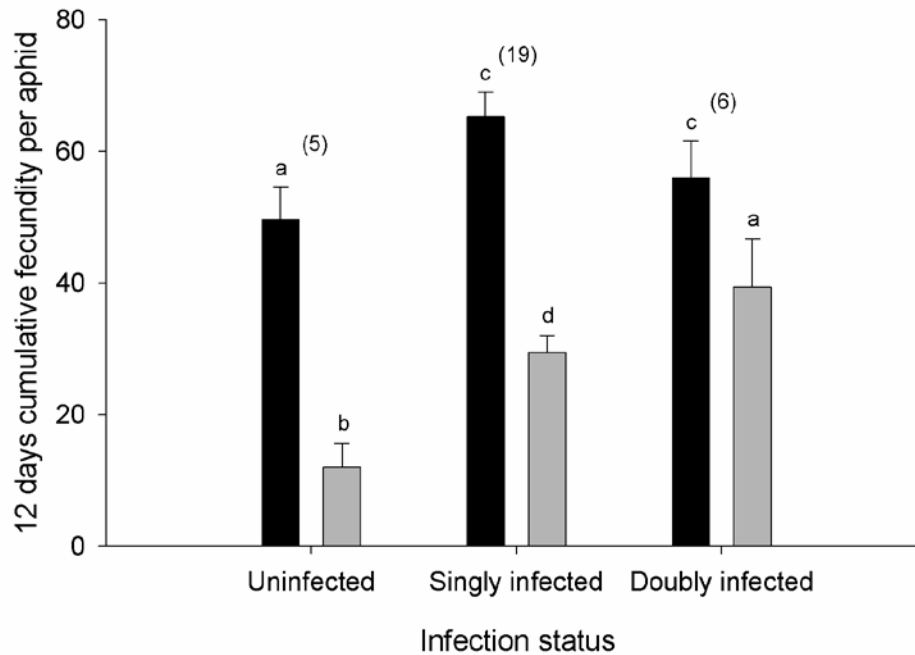
Figure 4 Cumulative fecundity per aphid for 12 days (Black bars=control group; Gray bars=Parasitized group) for uninfected, singly and doubly infected aphid clones. Bars (mean ± se over N clonal means, number above the bars) with same letter within and among infection status are not significantly different after model simplification (p>0.05).
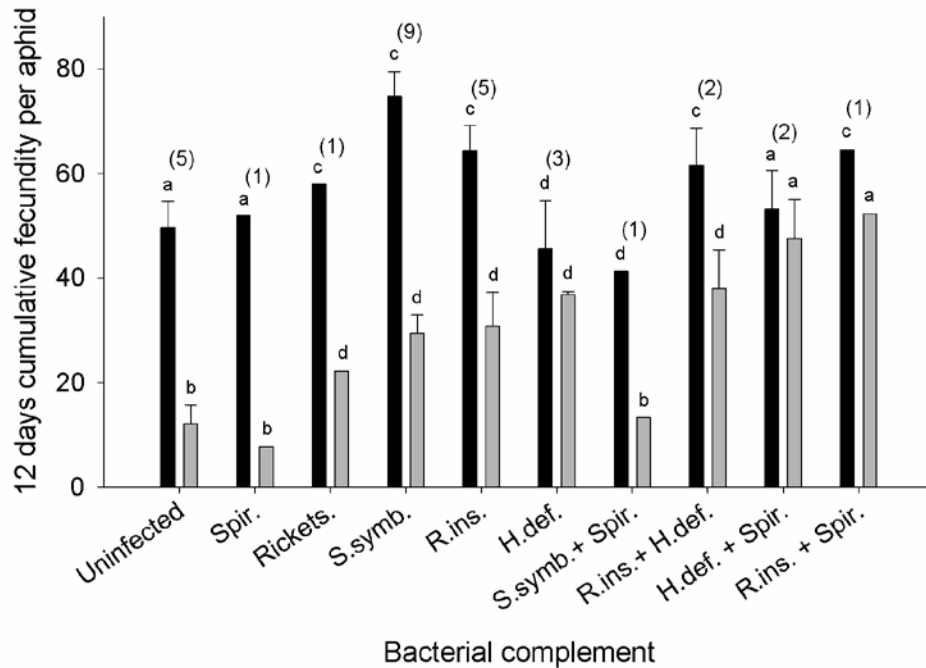
Figure 5 Cumulative fecundity per aphid for 12 days (Black bars=control group; Gray bars=Parasitized group) for aphid clones infected with different complements of secondary symbionts. Fecundity was influenced by bacterial complement × parasitism. Bars (mean ± se over N clonal means, number above the bars) with same letter within and among symbiont complements are not significantly different after model simplification (p>0.05, see Methods section for details).

Effects of symbionts on parasitoid developmental time, sex ratio and size

Cumulatively, over 90% of both male and female parasitoids (of those that did emerge) had emerged by the 16th day post parasitism. On average, female parasitoid wasps emerged at 14.82±0.14 days and males at 14.94±0.17 days. The number of symbionts did not influence the level of emergence (χ^2=0.68, Δ d.f.=2, p=0.711). The proportion of parasitoids emerging from the mummified aphids differed between the different bacterial complements (χ^2=74.1, Δ d.f.=8, p=0.002). Low levels of parasitoid emergence were recorded on pea aphid clones co-infected with *R. insecticola- H. defensa* ($F_{1,22}$= 17.1, p<0.001, Table 2). Parasitoid emergence also differed between pea aphid clones, in addition to the effects of bacterial complement ($F_{15,160}$=2.2, p=0.008). There was no correlation between percent emergence and the number of teratocytes (R=0.323, $F_{1,23}$=2.6, p=0.124) and the number of parasitoid larvae per aphid (R=0.056, $F_{1,23}$=0.1, p=0.796).

The number of symbionts had no influence on parasitoid development time (χ^2=2.65, Δ d.f.=2, p=0.266). Bacterial complements, however, affected parasitoid development time (χ^2=0.1, Δ d.f.=8, p=0.001) and model simplification ($F_{1,22}$=27.0, p<0.001) showed that parasitoids emerging from pea aphids co-infected with *R. insecticola- H. defensa* had significantly longer development time than those emerging from aphids infected with other bacterial complements (Table 2). There was significant variation in parasitoid development time between pea aphid clones in addition to the effect of bacterial complement ($F_{15,157}$=5.9, p<0.001). Parasitoid development time was neither correlated to the number of teratocytes (R=0.37, $F_{1,23}$=3.6, p=0.072) nor to the number of parasitoid larvae (R=0.08, $F_{1,23}$=0.1, p=0.705).

Parasitoid sex ratio was not influenced by the number of symbionts (χ^2=0.60, Δ d.f.=2, p=0.740). Although *Spiroplasma* was associated with female biased sex ratio (0.25±0.06, Table 2) there was neither an effect due to bacterial complements (χ^2=29.5, Δ d.f.=8, p=0.237) nor due to pea aphid clones ($F_{15,157}$=1.5, p=0.123).

In weight analyses, because female parasitoids are known to be larger and therefore expected to have higher weights, sex ratio was fit as a covariate. The mass of adult parasitoid decreased with an increase in number of symbionts (uninfected 0.75±0.02mg, single infection 0.74±0.02mg and double infection 0.6±0.03mg) and the relationship was significant (χ^2=9.39, Δ d.f.=2, p=0.009). The minimal adequate model (χ^2=9.38, Δ d.f.=1, p=0.002) showed that the effect was due to the reduced mass at the double infection. Bacterial complements also influenced parasitoid mass (χ^2=0.1, Δ d.f.=8, p=0.046) and model simplification showed that parasitoids emerging from pea aphid clones co-infected with *S. symbiotica- Spiroplasma and R. insecticola- H. defensa* had reduced weights in relation to those emerging from uninfected pea aphid clones (Table 2, $F_{1,21}$=12.5, p=0.002). In addition to the effects of bacterial complements, parasitoid adult mass was influenced by pea aphid clones ($F_{14,156}$=7.4, p<0.001). Parasitoid mass was not correlated to number of larvae (R=0.28, $F_{1,23}$=1.8, p=0.190) but positively to the number of teratocytes (R=0.46, $F_{1,23}$=5.9, p=0.024).

Table 2 Summary statistics of secondary symbiont effects on percentage adult parasitoid emergence, parasitoid development time in days, sex ratio of emerging adult parasitoids and adult parasitoid mass in milligrams.

symbionts	Frequency of symbiont complement	Percent emergence	development time (days)	Sex ratio (males : total emerged)	Parasitoid mass (mg)
Uninfected	5	94±1a	14.48±0.19b	0.46±0.03a	0.75±0.02a
Spiroplasma	1	90a	14.25b	0.25a	0.68a
Rickettsia	1	97a	14.81b	0.54a	0.72a
S. symbiotica	9	89±3a	14.13±0.26b	0.43±0.04a	0.77±0.03a
R. insecticola	4	90±1a	13.83±0.21b	0.47±0.06a	0.75±0.03a
H. defensa	1	82a	13.85b	0.61a	0.62a
S. symbiotica. + *Spiroplasma.*	1	98a	13.92b	0.29a	0.57b
R. insecticola + *H. defensa*	1	45b	17.62a	0.42a	0.57b
H. defensa + *Spiroplasma*	1	100a	14.67b	1.00a	0.66a

Means ± se (over N clonal means= Frequency of symbiont complement) on the same column followed by the same letter are not significantly different (p<0.05).

Discussion

Our results show that it is not the number of symbionts infecting the aphid, but particular bacterial complements that are associated with resistance against the parasitoid wasp, *A. ervi*. As in previous studies (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Bensadia *et al.*, 2006; Guay *et al.*, 2009), clones infected with *H. defensa* showed some resistance against the parasitoid. In addition, reduced levels of parasitism and mummification and therefore enhanced

resistance for pea aphid clones harbouring *R. insecticola, H. defensa- Spiroplasma* co-infection, *R. insecticola- Spiroplasma* co-infection and *R. insecticola- H. defensa* co-infection were established. Interestingly, no significant effect of *S. symbiotica* on resistance was found, an observation also documented by Guay *et al*. (2009). *Serratia symbiotica- H. defensa* co-infection has also been reported to increase resistance (Oliver *et al.*, 2006) but did not occur among our clones and therefore could not be tested. There was significant variation in resistance between clones harbouring the same symbiont emphasizing that there is variation in resistance that can not solely be attributed to the presence of a particular species of symbiont (cf. von Burg *et al.*, 2008; Vorburger *et al.*, 2009). A limitation however, is that the 30 aphid clones tested in this study could not allow for a test of each particular symbiont with full statistical power. For example, full resistance was recorded on *R. insecticola- Spiroplasma* co-infection but only one clone was analysed.

Resistance of aphids against a particular parasitoid is usually incomplete (but see Guay *et al.*, 2009 in the case of pea aphids). A certain fraction of parasitoid larvae is able to develop successfully despite possible decrease in overall survival of the parasitoid larvae. Indeed, there was reduced emergence even after successful mummification. Besides, sublethal effects on emerging adult parasitoids were observed. These included: reduced weights of adult parasitoids emerging from aphid clones co-infected with *R. insecticola- H. defensa* and *S. symbiotica- Spiroplasma*; prolonged developmental time for parasitoids emerging from aphid clones co-infected with *R. insecticola- H. defensa*; a negative correlation between weight of emerging parasitoids and development time. Parasitoid sex ratio was neither influenced by the number of symbionts nor the bacterial complements, even though, *Spiroplasma* recorded a highly female biased sex ratio. This result on *Spiroplasma* is inconclusive since only one clone was analysed.

No parasitoid larvae and no teratocytes were recorded in *R. insecticola - Spiroplasma* co-infected aphid clones, and indeed no mummification was observed. We hypothesize that *R. insecticola - Spiroplasma* co-infection inhibits parasitoid egg development and eventual hatching, resulting in no larval hatchlings (number of larvae) and no teratocytes, perhaps aborted parasitoid eggs due to disorganized early cellularization (cf. Li *et al.*, 2002; Firlej *et al.*, 2007; Guay *et al.*, 2009). Low numbers of larvae and reduced numbers of teratocytes were observed in aphid clones harbouring *H. defensa* and co-infections of *R. insecticola- H. defensa*, *H. defensa- Spiroplasma* and *R. insecticola- Spiroplasma*, indicating, at least, parasitoid egg development to hatching. Low numbers of teratocytes could be due to constrained parasitoid egg development. Teratocytes are necessary for the breakdown of food substrates and therefore with low numbers of teratocytes, the young growing parasitoids struggle and emerge as light weighted individuals

or starve to death, as shown by low parasitoid emergence on *R. insecticola- H. defensa* co-infection. Parasitoid weight was positively correlated to the number of teratocytes. In cases where the egg develops and manages to release a few teratocytes they might become non-functional (Li *et al.*, 2002).

The ability of some symbionts to protect their aphid hosts against castration was observed. Nymph production in the early stages of parasitism, i.e. between day zero and day six after parasitism in parasitized aphid clones harbouring *H. defensa, S. symbiotica* and co-infections *of H. defensa- Spiroplasma, R. insecticola- Spiroplasma* and *R. insecticola- H. defensa* were not significantly different from unparasitized counterparts for the symbionts listed. For instance, parasitized aphid clones harbouring *H. defensa* and *H. defensa– Spiroplasma* co-infection produced <1% lower number of nymphs compared to their unparasitized counterparts in the first six days. Between day six and day 12 post parasitism, however, parasitized aphid clones harbouring *H. defensa* and *H. defensa– Spiroplasma* co-infection produced 25% and 16% fewer nymphs respectively, compared to their unparasitized counterparts. Although total resistance was established in aphid clones co-infected with *R. insecticola- Spiroplasma,* parasitized treatments had up to 11% lower nymphal production at days 0 to day 6, and up to 26% lower nymphal production at days 6 and day 12 post parasitism, compared to unparasitized controls. In some reports, aphids with symbionts survived but had severe impediment on reproduction (Ferrari *et al.*, 2004).

Oliver *et al*. (2003) argue that vertically transmitted endosymbionts must increase host fitness or manipulate host reproduction in ways that increase their own transmission. Indeed, unparasitized aphids that harbored *S. symbiotica* had almost 50% higher reproduction than uninfected and unparasitized aphids. High reproduction was also observed in aphid clones harbouring *R. insecticola* compared with clones harbouring other symbionts. This could a reason as to why *S. symbiotica* and *R. insecticola* seem to be the prevalent symbionts whenever pea aphid clones have been surveyed or screened. In general, there was no evidence for a trade-off between resistance and fecundity, but differences between bacterial complements on their effect on fecundity.

In summary, besides *H. defensa* other symbionts including *R. insecticola,* co-infections of *H. defensa- Spiroplasma*, *R. insecticola- Spiroplasma* and *R. insecticola- H. defensa* also confer some resistance to pea aphids against parasitism by the braconid wasp *Aphidius ervi*. Partial and full resistance instances were established. Parasitoids that successfully developed and emerged from symbiont infected pea aphid clones had their fitness-related traits such as weight and development time negatively affected. There was no evidence for a general trade-off between

aphid fecundity and resistance against parasitism. In some symbiont infected aphid clones, fecundity was higher, and in some, fecundity was lower compared to the uninfected aphid clones. *S. symbiotica* and *R. insecticola* infected aphid clones showed significant enhanced reproduction compared to aphid clones without symbionts. Secondary symbionts influence development of teratocytes in the aphid hemolymph which is likely to be a proximate factor for resistance. The variability between the symbiont effects, in particular the significant variations among pea aphid clones infected with the same bacterial complement deserve further attention.

General discussion

Studies were undertaken on hymenopteran parasitoids and/or their aphid insect hosts for: (1) microsatellite markers isolation and identification in *L. hirticornis* together with cross species amplification; (2) the population dynamics and genetics of *L. hirticornis* and its host aphid, *M. fuscoviride,* specifically how the aphid metapopulation structure influences that of the parasitoid and also how the system is able to persist; (3) the mating opportunities of three solitary parasitoids, *A. ervi*, *P. pini* and *L. hirticornis* and their sexes in their natal patches, including a manipulative experiment where either the *Lasius niger* ants or living *M. fuscoviride* aphids were excluded from the mutualistic association; and (4) the interaction between *A. ervi* and its host pea aphid, *A. pisum*, infected by different combinations of secondary endobacterial symbionts in a correlative laboratory experiment. It was established that parasitoid dynamics and genetics are influenced by the metapopulation structure of the aphid hosts. Through limited dispersal, asynchrony and heterogeneity, the parasitoid-aphid host system persists. Aphid hosts and their ant myrmecophiles affect parasitoid mating structure. While resistance is mainly conferred by bacterial endosymbionts, pea aphid clones were found to have an innate resistance. Parasitoid fitness was affected by host resistance.

Parasitoid - host metapopulation genetic structure and mating systems

Nine polymorphic microsatellite markers were directly isolated from *L. hirticornis,* and two others were established, one from *L. fabarum* and another from *L. testaceipes* in a cross species amplification (*Article I*). Microsatellites have been isolated and characterised in many insect species including aphid wasp parasitoids (Hufbauer *et al.*, 2001; Baker *et al.*, 2003; MacDonald *et al.*, 2003; Fauvergue *et al.*, 2005; Sandrock *et al.*, 2007; Tentelier *et al.*, 2008) and used to test various aspects of molecular ecology. A problem encountered in attempting to isolate microsatellites from wasp parasitoids is related to their haplo-diploidy, whereby males, being hemizygous and bearing single allele copies on chromosomes, are liable to be of restricted genetic variability since any deleterious influence of such alleles is likely to be selected against (Menken, 1991). However, where polymorphic loci are found, allelic diversity per locus may be considerable, e.g. 17 alleles in *Lysiphlebus testaceipes* (Hymenoptera: Braconidae) (Fauvergue *et al.*, 2005) to 23 alleles in *Diaretiella rapae* (Loxdale & MacDonald, 2004).

Like its aphid host, *M. fuscoviride* (Massonnet, 2002), *L. hirticornis* is characterized by a metapopulation structure and sub population differentiation (*Article II*). Dispersal rates calculated for *L. hirticornis* were low to enhance population differentiation and high enough to

prevent isolation by distance (*Article II*). Likewise, little gene flow was found between introduced and native *A. ervi* (Hufbauer *et al.*, 2004). This result indicates that *L. hirticornis* does not have a single panmictic population, but populations perhaps brought about by habitat discontinuities. The pattern of population differentiation could also be explained by spatially correlated selection pressures because genotype frequencies were not consistent with Hardy-Weinberg equilibrium. If selection favours the different alleles in different local populations, then despite gene flow that homogenizes allele frequencies and prevents isolation by distance, population differentiation occurs. Genetic based studies provide dispersal estimates similar in quality to long-term mark-and–recapture studies (Rousset, 2001; Berry *et al.*, 2004). Genetic assignment methods allow the identification of dispersers in populations (Manel *et al.*, 2005) and additionally, the genetic structure among populations through time can provide estimates for the effectiveness of dispersal.

At each of the parasitoid subpopulations, low levels of heterozygosity and positive inbreeding coefficients were recorded (*Article II*) indicating non-random mating within sites. Solitary parasitoids tend to have partial sib-mating on the natal patch (*Article V*). We observed that 90% of *L. hirticornis* mated on the natal patch, the rest of the individuals did not mate, implying that a small percentage of mating might have taken place off the natal patch. In *P. pini* and *A. ervi*, more than 40% and 80% of the individuals, respectively, did not mate on the natal patch. We observed high levels of sib-mating for *L. hirticornis* unlike in *A. ervi* where a female mating on the natal patch was not necessarily sib-mating; migration from the "natal patch" to "other patch" was observed (*Article V*).

Expected heterozygosity (H_E) was positively correlated with total number of parasitoids as determined by mummified aphid counts, an indication that the higher the population size, the higher the size of the effective population. Because low rates of mummification were observed, then perhaps a small effective population size was present on the sites sampled. Heterozygote deficiency could result from a subdivision of the local population into isolated and differentiated reproductive units (Wahlund effect). Indeed, the plant (genet) was established to be the metapopulation unit for this wasp parasitoid (*Article III*). Mating of close relatives could also bring about heterozygote deficiency. *L. hirticornis* attacks many aphid hosts on a single ramet colony and emerging adult wasps mate on their emergence sites before starting to attack hosts on their natal patch (*Article V*). Due to these observations, I conclude that *L. hirticornis* has philopatric tendencies (*Article II*).

In a study involving univoltine moth hosts, *Greya subalba* Braun and *G. enchrysa* and their respective parasitoids *Agathis thompsoni* and *Agathis n.* sp, Althoff and Thompson (1999) found

incongruent geographical genetic structures. Subsequently, the same researchers established that the patchy distribution of the host food plant and variation in flowering times contributed to the genetic structure of the *Aganthis* parasitoids (Althoff & Thompson, 2001). Where the host plants of specialist invertebrate herbivores are aggregated, providing a concentration of food resource in patches, the resource concentration hypothesis (Root, 1973) suggests that such specialist herbivores should attain high densities. Dispersing herbivores will locate and reproduce in those patches and will less likely leave the patches. The tansy plant is known to grow in patches and therefore provides a suitable breeding ground on which the specialist aphid, *M. fuscoviride* can reproduce and develop population structures. The aphid in turn, provides a reproduction resource for the specialist parasitoid, *L. hirticornis* and hence affects its genetic population structure (see Pacala *et al.*, 1990; Hassell *et al.*, 1991; Umbanhowar *et al.*, 2003).

Studies have shown that ants inhibit myrmecophile dispersal (Kindlmann *et al.*, 2007; Oliver *et al.*, 2007) and since this study has shown that parasitoid dispersal is to an extent influenced by its host aphid dispersal, then the ants may indirectly influence parasitoid gene flow and differentiation. Colonies of *M. fuscoviride* are obligatorily attended by ants, especially *Lasius niger* (Mackauer & Völkl, 1993). From the aphid, ants obtain an important source of nutrients in the form of honeydew; in return, they act as guards, warding off predators and parasitoids (Völkl, 1997). The parasitoid is apparently able to evade ant attacks by chemical mimicry (Liepert & Dettner, 1996). As in many *Lysiphlebus* species, chemical signals on the epicuticular surface of *L. hirticornis* mimic those of its aphid host, hence making the parasitoid chemically undetectable to ants (Dettner & Liepert, 1994; Liepert, 1996). As a consequence, the parasitoid is able to exploit ant attended aphid colonies and the offspring obtain protection from attack by even higher trophic levels, i.e. hyperparasitoids (Völkl, 1992). Ant myrmecophiles affect the mating system of parasitoids on the natal patch (*Article V*). Exclusion of ants drastically reduced the natal patch residence times and the mating success of *L. hirticornis* from approximately 90% to about 42% (*Article V*).

An overall large genetic differentiation in the aphid host, *M. fuscoviride* and moderate genetic differentiation in the parasitoid, *L. hirticornis* was calculated (*Article IV*). This result was expected because of the differences in the biology and life histories of the insects: *M. fuscoviride* are resident feeders, i.e. they insert their proboscis into the plant and if not disturbed, they continue to feed and reproduce on a single plant; the young in turn will start to feed and reproduce on the same plant. The parasitoid, *L. hirticornis* on the other hand unlike the aphid host, has fully developed wings and movement between and among aphid colonies has been observed (Weisser, 2000; Article II). This result suggests that *L. hirticornis* has higher dispersal

abilities compared to the aphid host which is in line with lower differentiation in *L. hirticornis* compared to the aphid host (*Article IV*). Similarly, studies have shown that predators have greater dispersal abilities compared to their hosts (Jones *et al.*, 1996; Brodmann *et al.*, 1997; Ellner *et al.*, 2001). In contrast, observations have been made for specialist parasitoids attacking hosts characterized by metapopulation structures: Johannesen & Seitz (2003) established higher sub-population differentiation in *Eurytoma robusta* compared to the gall forming fly host, *Urophora cardui*, and Anton *et al.* (2007) found higher genetic differentiation in *Neotypus melanocephalus* compared to its host, the Dusky large blue butterfly, *Maculinea nausithohous*.

An increase in aphid host, *M. fuscoviride* population sub structuring was positively correlated with increased population sub structuring in the parasitoid, *L. hirticornis*, which implied that the parasitoid was tracking behind the aphid host. Alternatively, there could also be a time lag between the aphid colonizing a host plant patch and the parasitoid colonizing the aphid host on its new patch (*Article IV*).

Extinctions and recolonization events were recorded on the sites sampled and these events could explain the loss of alleles and appearance of new ones at the level of genets (*Article III*). Parasitoid allelic richness and genetic diversity showed similar patterns of fluctuations to population dynamic trends, i.e., reduced genetic diversity with increased extinction events. At low dispersal rates of both host and parasitoid species, parasitoids go extinct from local patches more frequently than they recolonized those patches, causing regional parasitoid extinction. Either low or high dispersal rates may synchronize dynamics across all patches and allow predators to drive their prey to extinction at a regional level (Blasius *et al.*, 1999; Jansen, 1999). At intermediate dispersal rates, both species may be able to persist through rescue effects (Brown & Kodric-Brown, 1977) and create a balance between patch extinction and recolonization (Crowley, 1981; Kareiva, 1990; Taylor, 1990).

Population densities of the aphid host and parasitoids as established by mummy counts were highly variable among the sites sampled indicating asynchrony in aphid and parasitoid populations (*Article III*). Within site heterogeneity was observed, genets, and ramets within genets of different growth stages (*Article III*), the number of habitats (genets) and amount of habitat (number of ramets in genets) (*Article II*) was variable. Further, allele and genotype frequencies were variable among the sites sampled (*Article II*). I conclude that asynchrony in plant growth, aphid host colonization of host plants, and parasitoid colonization of aphid hosts on those host plants as well as heterogeneity of alleles in both parasitoid and aphid host, and the parasitoid tracking behind the aphid host "time lagging" are some of the mechanisms for population persistence in this aphid host-parasitoid metapopulation.

Resistance in aphid hosts and effects on parasitoids

Resistance of pea aphid, *A. pisum* to the parasitoid, *A. ervi* is mediated by secondary endobacterial symbionts. (*Article VI*). As in previous studies (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005), clones infected with *H. defensa* or *R. insecticola* showed some resistance against the parasitoid. In addition, aphid clones co-infected with *H. defensa-Spiroplasma*, *R. insecticola-Spiroplasma* and *R. insecticola- H. defensa* showed low levels of parasitism and mummification and therefore enhanced resistance. Interestingly, no significant effect of *S. symbiotica* on resistance was found, a result also documented by Guay *et al*. (2009). Full resistance was recorded on *R. insecticola-Spiroplasma* co-infection.

There was significant variation in resistance between clones harbouring the same symbiont emphasizing that there is variation in resistance that can not solely be attributed to the presence of a particular species of symbiont, and I conclude that the aphid clones possess an innate resistance (cf. Vorburger *et al.*, 2009). Also important, is a finding that the number of symbionts infecting the aphid did not confer resistance but only particular symbiont species did.

This study established that symbiont infection decreased a number of fitness-related parameters in successfully emerged parasitoid individuals: parasitoids eclosing from pea aphid clones co-infected with *R. insecticola- H. defensa* and *S. symbiotica-Spiroplasma* showed reduced weights; parasitoids emerging from pea aphid clones co-infected with *R. insecticola- H. defensa* had prolonged development time. Overall, there was a negative correlation between weight of emerging parasitoids and the time to emergence. Parasitoid sex ratio was neither influenced by the number of symbionts nor the specific symbionts.

No larvae and no teratocytes were recorded on *R. insecticola - Spiroplasma* co-infected pea aphid clones and indeed neither parasitism nor mummification was recorded on this particular symbiont. I hypothesize that this symbiont co-infection, *R. insecticola – Spiroplasma,* inhibits parasitoid egg development or enhances egg abortion, perhaps the reason to absence of parasitoid larva and teratocytes during dissection experiments (cf. Li *et al.*, 2002; Firlej *et al.*, 2007). On the other hand, a low number of larvae and reduced number of teratocytes relative to the uninfected pea aphid clones were counted in pea aphid clones infected with *H. defensa*, and co-infections of *R. insecticola- H. defensa*, *H. defensa-Spiroplasma* and *R. insecticola-Spiroplasma*. A low number of teratocytes could be due to constrained egg development. Teratocytes are necessary for the breakdown of food substrates and therefore with low numbers of teratocytes, the young growing parasitoid might starve to death as shown by low parasitoid emergence on *R. insecticola- H. defensa* co-infection or light-weighted parasitoids that emerged

from the same symbiont background. Parasitoid weight was dependent on number of teratocytes. In cases where the egg develops and manages to release a few teratocytes, then they might also be made non-functional (Li *et al.*, 2002).

In summary, the results from this study are as follows:
1. Microsatellites were found in *L. hirticornis*. Although up to 23 microsatellites were isolated, only nine were polymorphic. Allele number ranged from two to 21. Cross species amplifications were found in *L. fabarum* and *L. testaceipes*. Of all markers tested from these two *Lysiphlebus* species, only a single marker from each of the species was found to be polymorphic in *L. hirticornis*.
2. The analyzed *L. hirticornis* population demes were spatially differentiated with diverse allele and genotype frequencies and were therefore heterogeneous. The mean number of alleles and expected heterozygosities at each parasitoid deme was positively related to the total number of aphid mummies counted on the sites in the entire sampling season.
3. Parasitoid genetic variability tended to increase with increased aphid host genetic variability and, overall, the aphid host was highly genetically differentiated compared to the parasitoid. This indicated that the parasitoid was tracking behind the host and the 'time lags' could explain their continued persistence.
4. The plant genet was the scale at which the populations persisted rather than at the site level. Plants in which extinction and recolonization occurred were recorded. Within genets, allele diversity changed over time; in some cases alleles were lost, in other plants allelic richness increased over time, and in some new alleles appeared. This, therefore, showed the importance of dispersal, and more importantly at the genet level. At site level, limited dispersal was detected.
5. Solitary parasitoid species differed in their mating opportunities within their natal patches, and some level of off patch mating occurred even where quasi-gregarious parasitoid broods are produced. Ant myrmecophily influenced the mating system.
6. While secondary symbionts played a role in pea aphid resistance to parasitoids, not all variability was explained by the symbionts. Some level of innate resistance was detected among the aphid clones. Parasitoids that overcame the resistance or those that developed in the presence of symbionts had reduced fitness compared to those that emerged from hosts with no symbionts. Parasitoid weight and development times were negatively affected.

Implications of these results and future research

In conservation strategies, insects of the higher trophic level, e.g., parasitoids are considered more endangered compared to their hosts. This is especially more pronounced in specialist insects. It is amazing that the *M. fuscoviride* aphid survives from season to season, let alone its specialized parasitoid, *L. hirticornis*. That it does so, despite the swings in the level of genetic variability and involving a high level of inbreeding, reveals another aspect of the system: that the parasitoid, like its aphid host, have high levels of tolerance to inbreeding depression. This may well be an adaptation - or rather a co-adaptation - to a life where resources are highly spatially distributed, and to some large extent temporally fragmented so that finding them at all –both in the case of aphid host and parasitoid – is a considerable challenge. That the aphid persists is because: its subpopulations are guarded by aggressive ant species, which keep would-be predators (spiders, coccinellids, syrphid, lace wing larvae, etc.) and other parasitoids at bay; or production of lush ramets through the vegetative/asexual and sexual means of reproduction of the tansy plant and therefore new food resources for the aphid. Truly, it is a bizarre, but successfully co-evolved system that works for the principal players.

Several facultative endosymbionts in single or co-infections provide the pea aphid with protection against the parasitoid *A. ervi*, and parasitoids that manage to debilitate the resistance have some reduced fitness. This may give rise to genotype specificity in the host's interaction with parasitoids. Considering that the pea aphid has biotypes and is thought to be undergoing speciation according to host races, speciation in *A. ervi* parasitoids may be in the offing.

Off patch mating, though very subtle, seemed to be a common phenomenon in solitary parasitoids. These varying levels of off patch mating and/or out breeding may be responsible for curtailing effects of inbreeding due to simultaneous eclosion of related parasitoid individuals on the natal patch.

During this study, a number of questions were proposed but unsuccessfully attended to due to unforeseen reasons. Other questions came up during the write-up and may form a basis for further interesting research. *Lysiphlebus hirticornis* was found to be highly structured within a small spatial scale, at least at the site level. AMOVA showed high variation at the genet level. More sampling at the genet level is necessary to determine whether genetic differentiation occurs at that level too. Experimental set ups whereby other natural enemies are excluded may be necessary to determine the mechanisms of persistence in *L. hirticornis- M. fuscoviride* system. *L. hirticornis* was detected to have high levels of sib-mating on the natal patch. Genetic studies also detected high levels of inbreeding on the resource sites. A study on inbreeding depression if such exists would be interesting.

In addition to symbionts, pea aphid clones harbour innate resistance. Through crossing experiments, the genetical mechanism and probably genes involved or up regulated by the symbionts could be unravelled.

In conclusion, the intimate interactions between hosts and parasitoids, influences parasitoid population dynamics, genetics, fitness and mating systems.

Summary

Understanding multitrophic interactions between parasitoids and their aphid hosts is of theoretical and practical interest. Here, investigations were performed on: 1) metapopulation dynamics and genetics of a specialized parasitoid and its aphid host; 2), mating systems of solitary parasitoids on their natal patches, and how they may be influenced by aphid attending ants; and 3) host resistance and its effect on parasitoid fitness.

Lysiphlebus hirticornis is a specialist solitary parasitoid attacking the specialist aphid *Metopeurum fuscoviride* which feeds exclusively on tansy, *Tanacetum vulgare*. Tansy plants are usually found in a patchy distribution. *L. hirticornis* has a classic metapopulation structure characterized by extinction and recolonization dynamics. I used the system to determine: i) genetic differentiation of *L. hirticornis* at a small spatial scale and infer levels of dispersal; ii) determine how allele frequencies change in a single annual season; and iii) how the system persists in the wake of high parasitism pressures. Field sampling was done between June and October 2007 and *L. hirticornis* aphid mummies were collected every two weeks from different tansy plants in 11 sites. Distances between sites varied from 217m to 15000m. Plant phenology was recorded and population dynamics of *M. fuscoviride* and *L. hirticornis* were noted. In the laboratory, parasitoid mummies were allowed to eclose for DNA extraction and genotyping. Nine polymorphic microsatellite markers from *L. hirticornis* were isolated and two others were found in a cross amplification screening of already known microsatellite markers from two other *Lysiphlebus* species. In total, 11 polymorphic microsatellites were used in the population genetics study. *L. hirticornis* mummies were found at all sites. In a number of genets, all aphids in a single colony were found to be parasitized. In some aphid occupied genets, no *L. hirticornis* mummies were found throughout this period of sampling, an indication that the parasitoid did not colonize them. Variations on plant phenology on the sites as well as *M. fuscoviride* extinctions at genet level and later recolonization events were recorded. Microsatellite analysis results showed that allelic richness varied from one site to another and was not dependent on the sample size. All parasitoid populations from different sites were significantly differentiated from one another. The genets compared to sites explained a high percentage of allelic variation, indicating that the unit of differentiation for *L. hirticornis* could be the genet rather than the site. These results confirmed that *L. hirticornis* has low powers of dispersal and has high levels of inbreeding. There was no isolation by distance. Over the season in a temporal analysis, allelic richness decreased over the season and observed heterozygosity increased in midsummer and declined by end of the season. Parasitoid differentiation tended to increase with host aphid

differentiation and on average the aphid host was more differentiated compared to the parasitoid. These results indicate that there is a time lag in parasitoid colonization of aphid hosts. In addition, asynchrony in plant phenology and heterogeneity of alleles could explain the mechanism of persistence for *M. fuscoviride* and its parasitoid, *L. hirticornis*.

Aphids are resident feeders and are considered gregarious. Depending on solitary parasitoid resource use behaviour, an attack of aphids may produce quasi-gregarious parasitoid broods, which might have an effect on mating opportunities. Males and females of three solitary parasitoids species, *A. ervi*, *Pauesia pini* both known for "low resource use" and *L. hirticornis*, a "high resource user" were studied and compared for mating opportunities on the natal patch. Mummified aphids parasitized by a single female parasitoid were placed on host plants of the respective aphid species. Thereafter, newly eclosing adult parasitoids were monitored for mating behaviour. *Lysiphlebus hirticornis* was observed to stay for more than 4 hours on the natal patch with up to 90% sib mating on those patches. *Pauesia pini* and *A. ervi* on the other hand, stayed for about two hours on the natal patch but only ~60% and ~20% mated on the natal patch for *P. pini* and *A. ervi*, respectively. Important to note is that in all the three species, not all mating occurred on the natal patch. In *A. ervi*, females stayed longer on the natal patch compared with males. I conclude that partial sib-mating and off-patch mating are common phenomenon in solitary parasitoids of gregarious hosts.

Some aphids have developed mutualistic associations with ants. The ants protect the aphids from enemies and in turn profit from the aphid's honeydew. The *L. hirticornis - M. fuscoviride*-system was used to observe the effect of the ant *Lasius niger* on the mating behaviour of *L. hirticornis*. *Lysiphlebus hirticornis* is obligatorily ant attended. Mating opportunities were observed when either ants or healthy aphids were excluded from the system and were compared with a control including mummified aphids, healthy aphids and ants. Removal of ants or aphids from the *M. fuscoviride* mutualism affects *L. hirticornis* natal patch residence time negatively and reduced mating on the natal patch from 90% in the control to less than 42% when ants were excluded and 43% when healthy aphids were excluded.

The pea aphid, *Acyrthosiphon pisum* and its parasitoid *Aphidius ervi* is another system characterised by resistance of the aphid against the parasitoid. The resistance has mainly been attributed to bacterial endosymbionts, including *Hamiltonella defensa*, *Serratia symbiotica*, *pea aphid X-type symbiont* (*PAXS*) and *H. defensa- S. symbiotica* co-infection. Up to six symbionts occur either in single infections or up to three co-infections. In a correlative study, I sought to establish: i) whether any other symbionts besides the aforementioned conferred resistance to pea aphid against *A. ervi*; ii) whether resistance is solely due to secondary symbionts, or also due to

innate clonal differences; and iii) the effect of symbionts on eclosing parasitoid fitness. 30 genetically distinct pea aphid clones, 25 of these infected with five symbionts in various single and double complements, were parasitized in Petri dish experiments in the laboratory. The bacterial endosymbionts were responsible for resistance against *A. ervi*. Relative to the uninfected clones, mummification was reduced by 78% in *H. defensa*, 24% in *Regiella insecticola*, 85% in *H. defensa-Spiroplasma* co-infection, 87% in *R. insecticola- Spiroplasma* co-infection and 63% in *R. insecticola- H. defensa* co-infection. Mummification did not differ in *S. symbiotica* infected pea aphid clones compared to uninfected clones. There was also significant clonal variation in resistance that cannot be explained by the secondary symbionts, indicating the existence of innate resistance. Low numbers of teratocytes were counted in pea aphid clones that eventually turned out to be resistant. Therefore, I suggest that the mechanism underlying resistance by symbionts may also involve interference on teratocyte development. Reproduction in pea aphid clones infected with *S. symbiotica* was higher relative to the uninfected clones, an indication of increased fitness of the aphid hosts when infected with certain symbiont species. Adult parasitoids that emerged from *R. insecticola- H. defensa* co-infection and *S. symbiotica- Spiroplasma* sp. co-infection showed reduced weights and those that emerged from *R. insecticola- H. defensa* co-infected pea aphid clones showed increased development time.

This thesis discusses how one trophic level shapes the dependent upper trophic level. The parasitoid cannot exist without its host and this intimate relationship affects the ecology, genetics and behaviour of the parasitoid. Through asynchrony and heterogeneity in the first and second trophic level, and parasitoid time lag in colonization, the aphid host- parasitoid system persists. Parasitism pressures may, however, force hosts to develop resistance that affects parasitoid fitness, or to evolve mutualistic associations with ants that offer protection to aphids. The aggregation behaviour of aphid hosts and the mutualistic associations affect the mating system.

Zusammenfassung

Das Verstehen von multitrophischen Interaktionen zwischen Parasitoiden und Blattläusen ist von theoretischem und praktischem Interesse. In dieser Arbeit wurden folgende Aspekte untersucht: 1) Metapopulationsdynamik und Genetik eines spezialisierten Parasitoiden und dessen Blattlaus-Wirtes, 2) Paarungsverhalten von Parasitoiden am Schlupfort und deren Beeinflussung durch Interaktionen mit Ameisen, und 3) Resistenzentwicklung von Blattläuse und deren Auswirkung auf die Fitness von Parasitoiden.

Lysiphlebus hirticornis ist ein solitärer Parasitoid der die Blattlaus *Metopeurum fuscoviride* parasitiert, die ihrerseits auf Rheinfarn, *Tanacetum vulgare*, spezialisiert ist. *T. vulgare* kommt typischerweise in einer sehr lückenhaften Verteilung vor. Populationen von *L. hirticornis* folgen einer klassischen Metapopulationsdynamik, geprägt durch Prozesse des Aussterbens und der Wiederbesiedlung. Dieses tritrophische System wird verwendet um i) die genetische Differenzierung von *L. hirticornis* auf einer kleinen räumlichen Skala zu überprüfen und daraus das Ausbreitungspotenzial abzuleiten, ii) die Veränderung von Allelhäufigkeiten während einer Saison zu bestimmen und um iii) zu analysieren, wie das System unter hohem Parasitoidendruck fortbestehen kann. Dazu wurden *L. hirticornis* infizierte Blattlausmumien alle zwei Wochen zwischen Juni und Oktober 2007 an 11 verschiedenen Standorten von unterschiedlichen *T. vulgare* Pflanzen im Feld gesammelt. Die Standorte lagen in Distanzen von 217m bis 15000m voneinander entfernt. Die Phänologie der Pflanzen und die Populationsdynamik von *M. fuscoviride* und *L. hirticornis* wurden jeweils aufgenommen. Die DNA, von im Labor geschlüpften Parasitoiden, wurde zur Bestimmung des Genotyps extrahiert. Neun polymorphische Mikrosatelliten-Marker konnten von *L. hirticornis* isoliert werden und zwei weitere wurden in einem Cross-Amplification-Screening von bereits bekannten Markern zweier anderer *Lysiphlebus* Arten gefunden. Insgesamt wurden 11 polymorphische Mikrosatelliten für die populationsgenetische Arbeit verwendet. *L. hirticornis* Blattlausmumien konnten im Feld an allen Sandorten gefunden werden. Auf einigen Pflanzen-Geneten wurden ausschließlich parasitierte Blattläuse gefunden, wohingegen Blattlauskolonien anderer Geneten gänzlich von Parasitierungen verschont blieben. Die Bestimmung der Genotypen zeigt, dass sich die Anzahl der Allele der verschiedenen Standorte, unabhängig von der Stichprobengröße unterscheidet. Parasitoiden-Populationen verschiedener Standorte grenzten sich genetisch signifikant voneinander ab. Ein hoher Prozentsatz der Allel-Variation konnte durch den Pflanzen-Geneten erklärt werden, was darauf hindeutet dass die Einheit der Differenzierung eher der Genet als der Standort sein könnte. Diese Resultate bestätigen, dass *L. hirticornis* nur eine geringe

Ausbreitung und einen hohen Grad an Inzucht aufweist. Eine räumliche Isolierung der Populationen konnte nicht nachgewiesen werden. Während der Saison nahm die Allelvielfalt ab und die Heterozygotie war im Sommer zunehmend und gegen das Ende der Saison abnehmend. Die Differenzierung der Parasitoiden-Populationen nahm tendenziell mit jener der Blattläuse zu, wobei die Blattlaus-Populationen jeweils stärker differenziert waren. Dies deutet darauf hin, dass der Differenzierungsprozess der Parasitoiden dem der Blattläuse hinterherhinkt. Somit könnte in Kombination mit Variationen in der Phänologie der Pflanzen und Heterogenität der Allele das gemeinsame Fortbestehen von Parasitoiden und Blattläusen erklärt werden.

Ein weiterer interessanter Aspekt, der durch ein enges Parasitoid-Wirt Verhältnis beeinflusst wird, ist das Paarungsverhalten der Parasitoiden. Blattläuse sind sehr sesshaft und kommen meist in grösseren Aggregationen vor. Wird eine Blattlaus-Kolonie intensiv von einem solitären Parasitoiden genutzt, können sich quasi-gregäre Bruten von Parasitoiden bilden, die einen Einfluss auf das Paarungsverhalten haben können. Drei Blattlaus-Parasitoid-Systeme wurden ausgewählt, um die Auswirkung der Wirtsnutzung der solitären Parasitoiden auf ihr Paarungsverhalten am Schlupfort zu untersuchen. Die Parasitoiden *Aphidius ervi* und *Pauesia pini* sind beide bekannt für eine geringe Ressourcennutzung, wohingegen *L. hirticornis* vorhandene Wirte sehr intensiv nutzt. Mumifizierte Blattläuse, parasitiert von einem einzelnen Weibchen, wurden auf die Wirtspflanzen der entsprechenden Blattläuse gesetzt. In der Folge wurden die Paarungsoptionen frisch geschlüpfter Parasitoiden (Weibchen und Männchen) beobachtet und verglichen. *L. hirticornis* Individuen hielten sich während mehr als 4 Stunden am Schlupfort auf, wo sich bis zu 90% der Individuen mit Geschwistern verpaarten. *P. pini* und *A. ervi* hingegen, verweilten ca. 2 Stunden am Ort und nur ca. 60% bzw. 20% der Individuen verpaarten sich mit Geschwistern. Weibchen der Art *A. ervi* verweilten verglichen mit Männchen länger am Ort des Schlupfes. Paarungen von solitären Parasitoiden gregärer Wirte, sowohl zwischen Geschwistern am Schlupfort aber auch erst nach Abwanderung vom Schlupfort ist ein häufig beobachtetes Phänomen.

Einige Blattlausarten leben in mutualistischen Gemeinschaften mit Ameisenarten. Die Ameisen bieten den Blattläusen Schutz vor Feinden und profitieren ihrerseits vom Honigtau der Blattläuse. Das *L. hirticornis – M. fuscoviride* System wurde verwendet, um den Einfluss der Ameise *Lasius niger* auf das Paarungsverhalten von *L. hirticornis* zu beobachten. *M. fuscoviride* ist zwingend abhängig von *L. niger*. Das Paarungsverhalten von *L. hirticornis* wurde unter Ausschluss von Ameisen oder unter Ausschluss von nicht parasitierten Blattläusen untersucht. Als Kontrolle diente das Gesamtsystem mit unparasitierten Blattläusen und Ameisen. Der Ausschluss von Ameisen oder unparasitierten Blattläusen beeinflusste die Verweildauer der

frisch geschlüpften Parasitoiden am Ort des Schlupfes negativ und reduzierte Paarungen von 90% der Individuen auf weniger als 42% unter Ausschluss der Ameisen und auf 43% unter Ausschluss unparasitierter Blattläuse.

Der Parasitoid *A. ervi* und die Erbsenblattlaus *Acyrthosiphon pisum* sind Bestandteil eines weiteren trophischen Systems, das durch die Entwicklung von Resistenz der Erbsenblattlaus gegenüber einer Parasitierung durch den Parasitoiden geprägt wird. Die Resistenzwirkung basiert auf bakteriellen Endosymbionten (*Hamiltonella defensa*, *Serratia symbiotica*, Symbiont der Erbsenblattlaus Typ X (*PAXS*) und eine Koinfektion von *H. defensa* und *S. symbiotica*). Bis zu sechs verschiedene Symbionten treten auf und können bisweilen einen Organismus gleichzeitig infizieren. Dieses System wurde verwendet, um i) die Rolle verschiedener bakterieller Symbionten in der Resistenzentwicklung von *A. pisum* gegen *A. ervi* zu untersuchen, ii) herauszufinden ob auch angeborene Resistenzmechanismen beteiligt sind und iii) um die Auswirkungen der Symbionten auf die Fitness der Parasitoiden zu analysieren. Dazu wurden 30 genetisch verschiedene Blattlaus-Kolonien verwendet. Davon waren 25 mit fünf unterschiedlichen Symbionten oder Kombinationen von jeweils zwei Symbionten infiziert. Die Blattläuse wurden in Petrischalen durch *A. ervi* parasitiert. Verglichen mit uninfizierten Blattlauskolonien war die Mumifizierung bei einer Infektion mit *H. defensa*, *Regiella insecticola*, *H. defensa* kombiniert mit *Spiroplasma* sp. und *R. insecticola* kombiniert mit *Spiroplasma* sp., um 78%, 24%, 85%, 87% bzw. 63% reduziert. Keinen Unterschied in der Mumifizierung konnte zwischen *S. symbiotica* infizierten und uninfizierten Klonen festgestellt werden. Zwischen den verschiedenen Blattlaus-Kolonien wurden signifikante Abweichungen bezüglich der Resistenzwirkung gegen *A. ervi* festgestellt, die nicht durch die Infektion mit sekundären Symbionten erklärt werden können, was darauf hindeutet, dass auch angeborene Mechanismen für die Resistenzentwicklung verantwortlich sein könnten. Nur eine geringe Anzahl Teratozyten konnte in resistenten Blattlaus-Kolonien gefunden werden. Deshalb wird angenommen, dass der Resistenzmechanismus durch die Symbionten auch die Entwicklung der Teratozyten beeinflusst. Blattlausklone, die mit *S. symbiotica* infiziert waren, produzierten mehr Nachkommen als uninfizierte Klone. Dies deutet darauf hin, dass eine Infektion mit bestimmten Symbionten die Fitness der Blattläuse erhöht. Parasitoiden, die sich auf *R. insecticola- H. defensa* und *S. symbiotica- Spiroplasma* sp. infizierten Blattläusen entwickelt hatten, hatten ein reduziertes Körpergewicht, jene die sich auf *R. insecticola- H. defensa* infizierten Blattläusen entwickelt hatten, hatten eine längere Entwicklungszeit.

Zusammenfassend wird in dieser Arbeit diskutiert, wie ein Wirt die trophisch höhere Ebene in einem System beeinflussen kann. Die Abhängigkeit der Parasitoiden von ihren Wirten

beeinflusst die Ökologie, Genetik sowie das Verhalten des Parasitoiden. Asynchronität, sowie Heterogenität der ersten und zweiten Ebene der tritrophischen Systeme und die zeitliche Verzögerung, mit der Parasitoiden Wirtspopulationen kolonisieren, verhilft dem Blattlaus-Parasitoid-System zu bestehen. Zu großer Parasitierungsdruck kann jedoch die Resistenzentwicklung der Wirte oder mutualistische Interaktionen mit Ameisen, die die Blattäuse vor Feinden schützen, fördern, was wiederum die Fitness der Parasitoiden oder deren Paarungsverhalten verändern kann.

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Declaration

I declare that in accordance with the conferral of the Doctorate degree from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance of literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

This thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

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