Coevolutionary dynamics and geographic mosaics in the Social Parasite *Harpagoxenus sublaevis* and its two Host Species

Sabine Maria Veronika Bauer

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Sabine Maria Veronika Bauer
(geb. Demel)
aus Rosenheim
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Prüfungsausschuss: Vorsitzender:	Prof. Dr. Susanne Foitzik
1. Prüfer:	Prof. Dr. Susanne Renner
2. Prüfer:	Prof. Dr. Dirk Metzler (Protokoll)
3. Prüfer:	Prof. Dr. Matthias Starck
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GENERAL INTRODUCTION

"Nothing in biology makes sense except in the light of evolution," --Theodosius Dobzhansky, 1973

The natural history of parasitism has been an immensely fascinating topic for evolutionary biologists in the last couple of centuries. Parasitism represents the most common lifestyle on earth (Thompson 1994), and can be found in all modes of life in the animal kingdom, from simple plathelminthes to vertebrates such as fish and birds (Anderson and May 1982). Moreover, according to most estimates, more than 50% of all known species are parasitic at certain stages of their life cycle (Price 1980). Because of the close relationship between host and parasite, interactions between both organisms are important and have ultimately shaped the organisation of communities and influenced the diversification of life (Thompson 1999b).

In all cases, this relationship is asymmetric, because the parasite lives at the expense of its host, causing a fitness reduction in the host and sometimes even its death. Because of its serious implications, the processes and dynamics of this interaction has been studied theoretically and empirically in many scientific sectors (Anderson and May 1982, Thompson and Burdon 1992, Frank 1993, 1996, Ewald 1994, 1996; Ebert and Herre 1996, Ebert 1998).

The two most important challenges for a parasite are transferring itself or its progeny from one host to another (transmission) and overcoming the defenses of its host (Futuyama 1998). Thus, it is not surprising that there is a strong association between parasite transmission and virulence (Anderson and May 1982; Dunn and Smith 2001). Some parasites are transmitted vertically, e.g. from a host parent to her offspring. However, most parasites are transmitted horizontally among host individuals in a population through the external environment, vectors or contact between hosts.

In general, parasitism has to be differentiated from mutualism, commensalism, parasitoid-host and predator-prey interactions as parasites increase their fitness while lowering the fitness of its host drastically which leads however not involuntary to host´s death. In mutualisms, both interactants derive a fitness benefit from their relationship. Commensalism describes a symbiotic relationship between two organisms, where one benefits and the other is not significantly harmed, while parasitoids live part of their life on or

within a single host organism, which they ultimately kill. Finally predators feed on another living organism.

Evolution of e.g. life history traits in hosts is often driven by selection exerted through their parasites (Gandon 2002, Gandon et al 1996a, 1998, Thompson 1999a). Two main forms of parasites can be distinguished: Micro-parasites such as viruses, bacteria and fungi exploit the physiology of multicellular hosts that are phylogenetically distant with radically different life histories. These hosts are often at a disadvantage, because microparasites have markedly larger population sizes and significantly shorter generation times compared to their hosts and can thus react with a higher evolutionary speed to selection pressures exerted by their hosts. In macroparasites such as arthropods, these discrepancies in life history and population traits are much less pronounced. They are even less marked in brood parasites, such as cuckoos or cowbirds, where brood care behaviour of other bird species is exploited to raise the parasite offspring, often at the expense of host offspring (Brooke and Davies 1988, Davies and Brooke 1989a,b, Lotem et al. 1992, Rothstein 1990, 2001). Life histories of parasites and host differ less, because brood parasites are often phylogenetically closely related to the species that they parasitize, possessing similar nutritional requirements and behavioural attributes. This brood parasitism has been intensely studied, and has served as the ideal system for the study of coevolution (Davies and Brooke 1989a,b; Rothstein 1990; Soler and Moller 1990). Coevolution has been defined as the interaction between two or more species, in which evolutionary changes in one species reciprocally influences the evolution of the other species (Ridley 2003).

Recently, attention has been drawn to the social parasites of the ants, wasps and bees. Social parasites are frequently even more closely related to their host species – host and social parasite often being sister species (Emery 1909). In contrast to brood parasites, social parasites exploit not only the brood care behaviour, but the whole social system of other social insect species (e.g. Johnson 2008). In addition, they have very similar population sizes, generation times and supposedly also similar migration, recombination and mutation rates. Hosts are, therefore, expected to be able to keep up in the arms race with their parasites. Considering only two counter interactants, one host and one obligate parasite, coevolution might lead to different outcomes. It could lead to the extinction of one ore both of these antagonistic species or to a coexistence of the two species. If these species continue to interact, their associations can evolve towards mutualism, where both individuals derive a fitness benefit or finally, both sides could engage in an ever-escalating arms race, where no party wins (Dawkins and Krebs 1979; Futuyma 1998). The latter model is supposed to be

most applicable for obligate social parasites, especially slavemaking ants, which are often highly specialized. Social parasites that would become too virulent drive themselves to their own extinction by depleting the host population.

In general there are different forms of social parasitism, varying in form and level of integration into host life cycle. One of these is xenobiosis, which refers to a more integrated association, in which the xenobiotic species lives and forages inside the host colony but keeps its brood apart. These species are dependent on their host. Xenobiotic species either exploit other species only temporarily during colony foundation, or they are dependent on their hosts throughout all stages of their lifecycle. Such parasites include species whose colony founding queens invade the nests of other species, killing the resident queen or not, and using the workers as their labour force. Temporary parasites produce their own workers over time, which eventually completely replace the host workers. The colony, thus, slowly develops from a mixed parasite-host colony to a pure society that does not need the host. In this case, the social parasitism is called facultative, where the parasitic species is still able to maintain its colonies without a heterospecific work force. Obligate social parasites, however, such as many slave-making ants (also called dulotic ants), which cannot complete the colony life cycle by themselves must continually raid other ant nests for the brood to refresh their workforce. Another form of obligate social parasitism is inquilinism, which is a permanent parasite-host association without slave raiding. The most extreme form shows an invading queen, which produces sexual offspring only, while killing the host queen (queen intolerant) or letting the host queen survive (queen tolerant) to furnish a continuous supply of workers. This is the ultimate, degenerate stage of parasitism as this kind of parasite relies completely on its host (Bourke and Franks 1991, Aron et al 1999) due to the reduction or complete loss of its own worker caste. In cases, where the host queen is kept alive, they are true “social” parasites, which exploit a single super-organism and may allow host reproduction to some extent. Queen intolerant inquilines, however, annihilate the fitness of the host colony, and are thus best described as social parasitoids (Lafferty and Kuris 2002).

Dulosis or slavery in ants is one of the most intriguing forms of social parasitism and has fascinated scientists and the general public since the first detailed description almost 200 years ago by Huber (Huber 1810). Darwin (1859) described the raiding behaviour of the slave making ant, *Formica sanguinea*, in his famous book On the Origin of Species. Slavery in ants evolved several times independently within the two large ant subfamilies, Myrmicinae and Formicinae (Buschinger 1990; Hölldobler and Wilson 1990; Stuart and Alloway 1982)

Inquilinism is a convergent phenomenon which follows one of at least two available

pathway in evolution. These two main pathways of parasitic evolution are shown here: slavemaking and temporary parasitism which may lead to inquilinism or permanent parasitism (Fig.1).

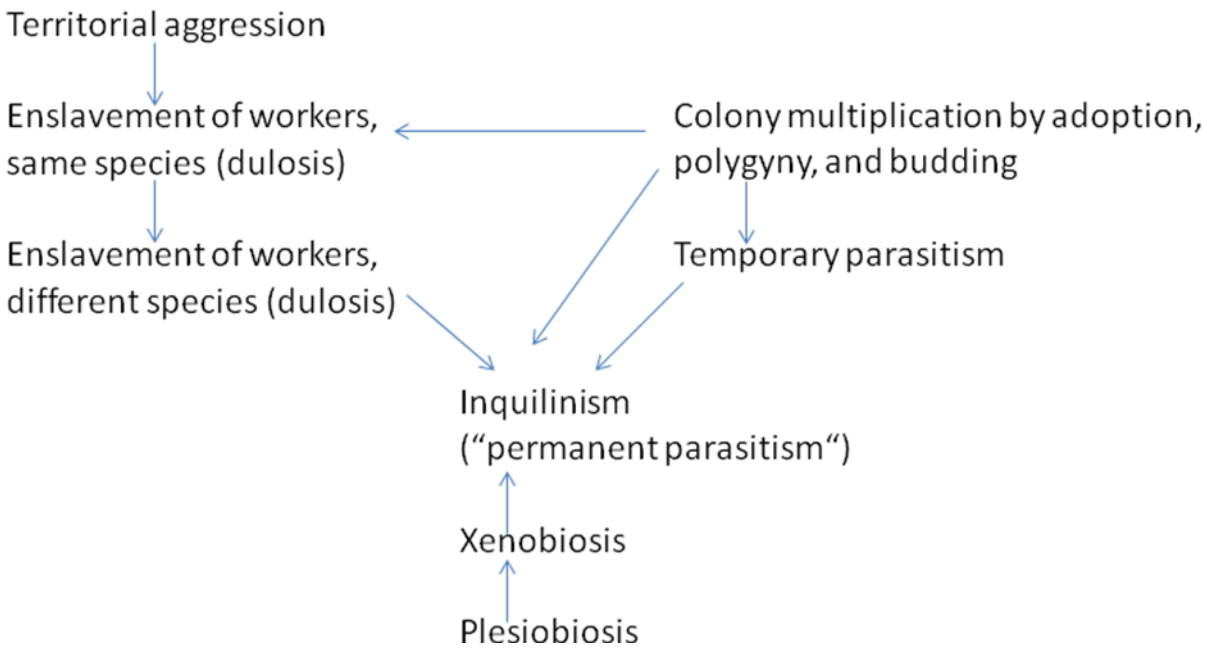

Figure 1: Evolution of slavery in ants (Hölldobler and Wilson 1990: The ants)

In the current study, we focus on the European slave-making ant *Harpagoxenus sublaevis*, which is an obligate social parasite, commonly utilizing two host species, *Leptothorax acervorum* Fabricius (1793) and *Leptothorax muscorum* Nylander (1852, Fig. 2).

Figure 2: Slavemaking ant nest of *H. sublaevis* and its two host species *L. acervorum* and *L. muscorum*

Workers of *H. sublaevis* are incapable of carrying out routine colony tasks such as brood care, foraging and nest maintenance (Buschinger 1974; Buschinger et al. 1980), but instead specialize on exploiting colonies of *Leptothorax* ants. To found a new colony, a mated slave-making queen has to invade a host colony and kill or drive away all resident adult ants to appropriate host brood (Fig. 3). When the new host workers emerge from the stolen brood they are imprinted on the odour of their parasite and thus accept the slave-making ant colony as their own and take over all routine tasks such as foraging, brood care and nest building (Alloway 1979, Buschinger 1966a,b; Buschinger et al. 1980).

The success rate of colony usurpations is usually very low. Yet if successful, a parasite queen lays eggs, which are raised by enslaved host workers. Adult *H. sublaevis* workers do not forage or care for brood, but they are still able to feed themselves (Buschinger 1966a, 1968b; Hölldobler und Wilson 1990). They regularly conduct slave raids on neighboring host colonies and steal their brood to replenish the enslaved workforce (Buschinger 1974; Buschinger et al. 1980).

Figure 3: A queen pupa, eggs and larvae of the host species *L. acervorum*

These slave raids are characterized by a sequence of four behaviours: scouting, recruiting, fighting and transporting brood (Buschinger 1968b, Buschinger et al. 1980, Buschinger and Winter 1977, Stuart and Alloway 1983). *H. sublaevis*, as well as other slave-making ants such as *Chalepoxenus muellerianus*, the North American parasite *Protomognathus americanus* and *Polyergus rufescens*, recruit nestmates by tandem-running (Buschinger and Winter 1977). Simply described, during tandem-running a parasite scout ant performs an invitation behaviour and then leads nestmates one by one to the target nest (Buschinger and Winter 1977). Slave raids impose high selection pressures on host populations, lowering the mean

life expectancy of host colonies considerably (Fischer-Blass et al. 2006). Ecological field data indicate that *H. sublaevis* shows a slight preference for its smaller host species *L. muscorum*, and consequently has a stronger impact upon it than on its other host (Bauer et al. 2009a,b; Böhm et al submitted; Fischer-Blass, Heinze et al. 2006). *L. muscorum* is also less competitive compared to the larger host *L. acervorum* and it shows a more restricted distribution (Brandt et al. 2007; Bauer et al. 2009a,b). Nevertheless, in most sites the social parasite and its two host species co-occur, such as in pine forest habitats throughout the boreal regions of Eurasia where these ants nest in slightly rotten logs and twigs (Collingwood 1971; Ratschenko et al. 1999).

The vast majority of *H. sublaevis* queens are wingless, and can only attract mates on foot by female calling (Buschinger 1966; Buschinger 1971; Hölldobler and Wilson 1990; Heinze 1993). Once mated, queens attack host colonies in walking distance from their mother nest to establish new colonies. The smaller of the two hosts, *L. muscorum*, also exhibits female calling, although its queens are winged and can disperse by flying after mating (Brandt et al. 2007). Winged queens and males of the larger host *L. acervorum* frequently mate in large mating swarms. In general, *L. acervorum* shows very high nest densities of up to 50 nests per 100m² and the widest distribution (Fischer-Blass et al. 2006). In contrast to the parasite, where each colony invariably contains a single reproductive queen, both host species are facultatively polygynous with queen numbers varying between 1-10 (Buschinger 1968; Heinze and Buschinger 1988; Lipski et al. 1994). These differences in reproductive biology and population sizes lead to strongly structured populations in *H. sublaevis* and *L. muscorum*, while *L. acervorum* is characterized by high levels of gene flow and genetic variability (Heinze et al. 1995; Brandt et al. 2007).

Generally, gene flow and genetic variability influences selection pressure of the parasite on its host and vice versa. Moreover, the strength of this reciprocal selection pressure often differs between local populations and thus creates a a coevolutionary mosaics (Gandon et al. 1996a, Thompson 1999a,b). The geographic mosaic theory of coevolution states that the interactions between host and parasites can differ locally in the coevolutionary outcome and intensity, and therefore result in coevolutionary cold and hot spots (Thompson 1994). In addition, the geographic mosaic theory argues that the overall coevolutionary dynamics of species interactions depend to a large extent on the amount of genetic variability, which is available within a population (Gandon et al. 1996b; Gomulkiewicz et al. 2000; Hochberg and van Baalen 1998; Thompson 1994). This, in turn, is determined by the rates of mutation, recombination and migration.

In this study we examine the coevolutionary interactions between the slave-making ant *H. sublaevis* and its two hosts at different locales. We analyse the genetic, chemical and behavioural variability and geographic structure in the obligate social parasite and its two *Leptothorax* host species.

In the first chapter we discuss the genetic structure and variability at three different localities. The population genetic patterns are strongly affected by current gene flow and drift processes (Hutchison and Templeton 1999). Genetic drift largely depends on effective population sizes, whereas gene flow is affected by dispersal capabilities of species, the geographical distances between populations and physical and nutritional barriers. Species, which obligatorily rely on certain plants for food or nest sites, are restricted in their distribution by the ranges of their host plants. For instance, the geographic patterns of the butterfly *Lycaena dispar batavus* mirrors the recolonization routes in its host *Rumex hydrolapathum*, the great water dock (Martin and Pullin 2004). Besides this the dispersal capabilities also influence population genetic patterns in our study organisms. Migration rates have a strong impact on current gene flow; species with low dispersal exhibit more strongly structured and less genetically diverse populations. The reproductive biology of our study organisms indicates strong differences in dispersal capabilities, as well as their mating systems. With polymorphic microsatellite and mitochondrial markers, we want to investigate gene flow in both sexes and genetic variation in different communities in Europe in more detail. We hypothesize that the biology of these ants, with their varying dispersal capabilities, can explain differences in the genetic structure and variation in populations. This is of special interest in these coevolving species, as the time of co-occurrence at certain sites can determine the stage in the coevolutionary arms race.

These different levels in the arms race are not only characterized by genetic patterns but additionally in the chemical traits. One of the chemical key innovations in slave-making ants is the development of the hypertrophied Dufour gland (Fig. 4), whose secretions are known to be used as an interspecific “propaganda substance” when raiding host nests to induce fights amoung defending host workers (Buschinger 1974a, Allies et al 1986, Regnier and Wilson 1971). Studies on *Temnothorax* and *Leptothorax* host colonies revealed that this secretion is involved in fights over reproductive dominance in these non-parasitic species (Heinze et al. 1998). Brandt et al. (2006) suggested that in the North American system, the Dufour gland of *Protomognathus americanus* may contain a fertility signal, which could provoke excitement and disorganisation amongst host workers during a slave raid. This “propaganda substance” is also used in *H. sublaevis* against its hosts.

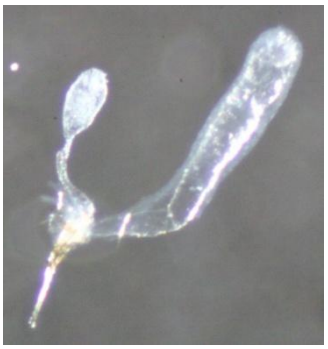

Figure 4: Dufour gland and poisson gland of *H. sublaevis*

H. sublaevis can induce intra-colonial fights among *L. muscorum* or *L. acervorum* nestmates, by applying the Dufour gland secretion onto the gaster of a host worker, and thus facilitates the successful capture of a brood by the slavemaker (Allies et al. 1986; Foitzik et al. 2003). The Dufour gland secretion can also be released in the host nest without contact, causing similar outbreaks of panic and disorganisation among defending workers (Regnier and Wilson 1971). In chapter II, we investigate whether local adaptation in this chemical trait occurs and examined in behavioural tests whether there was evidence for universal or localized coevolution. To distinguish between local or general adaptation we investigated behaviour of host and parasite species from different communities. This allowed us to test whether the social parasite was specialized on its local hosts and vice versa. In addition, it allowed us to investigate if host populations differ in their reaction to the parasite's chemical weapon and if host evolved resistance to this chemical manipulation. When there are indeed differences, it could be due to genetic and geographic variations, such as habitat conditions and host-parasite density. Variation in host-parasite interactions between sites would lend support for the geographic mosaic of coevolution (Blatrix and Herbers 2003; Brandt and Foitzik 2004).

Furthermore, we were interested if the parasite is able to manipulate host behaviour in both species to the same extent. The sympatric occurrence of *L. acervorum* and *L. muscorum* in close proximity in Germany, Russia and Italy might allow host oscillations as shown in avian brood parasites (Davies and Brooke 1989a,b). Analyses via gas chromatography and mass spectrometry (GC-MS) of the Dufour gland secretion, should give us insight in the variation of the chemical compositions of the Dufour gland secretion in different *H. sublaevis* populations. The chemical weapon of the Dufour gland secretion is one of many fascinating traits of this social parasitic ant.

Besides chemical weaponary, parasites can adjust their cuticular profile to overcome host defenses. Indeed, social parasites have evolved various strategies to bypass the recognition system in order to either successfully usurp a host nest or to integrate themselves into the host society. Slave-making queens of the genus *Polyergus* use chemical insignificance (D'Ettorre and Errard 1998) to avoid host aggression during colony takeover. The cuticule of these young queens harbour few, if any hydrocarbons, hence their detection is hampered by the absence of chemical cues. In contrast, chemical mimicry (Howard and Blomqvist 2005) and chemical camouflage (Allan et al. 2002) describe strategies, in which a social parasite attempts to imitate host recognition cues, either by actively producing the host cuticular hydrocarbons or by passively acquiring these chemicals through direct contact with its host.

Leptothorax kutteri, an inquiline ant species, relies on a behavioural strategy best described as sneaking. In this tactic, queens try to invade host colonies without being detected. In addition, to guarantee a smooth functioning of a slave-making ant colony, it is necessary for parasite and workers to share the same colony odour (Lenoir et al. 2001). This is exactly the case in *Polyergus* slave-making workers, which were able to adjust their profile to that of their host (Yamaoka 1990; D'Ettorre et al. 2002). Chemical adaptation to host odour is especially important for young queens of socially parasitic species, which frequently enter an ant host colony on their own, relying on not being recognized as an intruder.

As social parasites can have a very negative influence on host populations (Foitzik et al. 2001a,b; Fischer-Blass et al. 2006), hosts are forced to devise strategies to avoid or lower the impact of the social parasitism. One approach could be by evolving defences against intruding parasitic ants, since enslaved host workers have little possibility to rebel (Gladstone 1981). These defence mechanisms (Foitzik et al. 2001a,b) have to include an efficient recognition system, and indeed there is evidence that *Temnothorax* host species show enemy recognition (Alloway 1980). These counter-adaptations can further include immunity to chemical weapons of social parasites and better fighting abilities. Another defense is the flexible adjustment of the recognition threshold, i.e. lowering of the threshold during raiding season, which was shown in the Formicine slave-making ant *Polyergus rufescens* and its host *Formica rufibarbis* (Brunner et al. 2002). Reactions of parasitic species to the described host counter-adaptations can include a close resemblance of the cuticular hydrocarbon profile to both or one of its hosts, which has been suggested for the European slave-making ant *H. sublaevis* (Kaib 1993).

In chapter III, we were interested in how far chemical adaptation occurs in *H. sublaevis* and analysed adaptation strategies to its two host species *L. acervorum* and

L. muscorum using gas chromatography and mass spectrometry. In particular, we were interested in whether this social parasite actively produces host hydrocarbons or only passively acquires these compounds from its hosts. We studied two different ant communities to investigate potential local adaptation, which was indicated in a previous behavioural study (Fischer and Foitzik 2004).

In chapter IV community and colony composition data were used to investigate whether *H. sublaevis* specializes on one of its two host species. As the success of raids of *H. sublaevis* strongly depends on the original combination of parasite and host population, we were interested about the outcome between the interacting hosts and the sympatric and allopatric released parasites. The presence of a parasite can indeed influence social organisation and reproductive strategies of hosts (Savolainen et al. 1996, Foitzik et al. 2009).

Recent studies on birds already showed that brood parasites can drastically reduce the reproductive output during lifetime of their host, or can even threaten host populations (Rothstein and Robinson 1994). In ants, there may be a trade off between early reproduction and lower fecundity to evade parasites, or developing slowly with higher fecundity, but thereby risking infection (Hochberg et al. 1992, Restif et al. 2001). For that, we examined in a field manipulation experiment the impact of the parasite on host populations and potentially parasite induced changes in social structure, productivity and demography of hosts in a German and an Italian community.

CHAPTER I

Genetic diversity, population structure and sex-biased dispersal in three ant species of a host-parasite system

(Susanne Foitzik, Sabine Bauer, Stefan Laurent, Pleuni Pennings)

I.I ABSTRACT

Genetic diversity and spatial structure of populations are important for antagonistic coevolution. We investigated genetic population structure of three European ant species: the social parasite *Harpagoxenus sublaevis* and its two host species *Leptothorax acervorum* and *Leptothorax muscorum*. We sampled populations in 12 countries and analyzed eight microsatellite loci and an mtDNA sequence. We found that levels of genetic variation are high in all three species, only slightly lower in the host *L. muscorum*. Using a newly introduced measure of differentiation (Jost's D), we detected strong population structuring in all species and we found that dispersal is much less male-biased than previously thought. We found no phylogeographic patterns that could give information on post-glacial colonization routes - Northern populations are as variable as more southern populations. We conclude that conditions for Thompson's geographic mosaic of coevolution are ideal in this system: all three species show ample genetic variation and population structure.

I.II INTRODUCTION

To understand the course of evolution of a species or a group of interacting species, we need to know not only the selective forces that work on them, but also their adaptive potential (Gandon and Nuismer 2009). It is hard to study the evolutionary potential of natural populations directly. Yet as it is influenced by their demographic history, effective population size, standing genetic variation, population structure and patterns of gene flow, we can study these factors instead. In particular, population genetics can be used to analyze neutral genetic variation and geographic population structure - in the hope that these parameters tell us something about the adaptive potential of the species we study. The same data can also give us information on the recent history of the species, for example the impact of the last ice age (Hewitt, 2004). Finally, by studying both biparentally inherited nuclear microsatellite loci and mitochondrial markers, which are only maternally transmitted, we can indirectly examine male and female dispersal (e.g. Holzer et al. 2009).

In this paper, we analyze eight nuclear microsatellite loci and MtDNA sequences to investigate neutral genetic variation and population structure of three ecologically and behaviorally well studied ant species, which engage in coevolutionary arms races (Foitzik et al. 2003; Fischer and Foitzik, 2004; Fischer-Blass et al. 2006; Bauer et al. in press a, b). *Harpagoxenus sublaevis* (Nylander 1852) is an obligate social parasite, which needs enslaved hosts to take care of its brood. *Leptothorax muscorum* is the preferred host species of this parasite. *L. acervorum*, the second host species, is slightly larger, more common and more widespread than *L. muscorum*. The slavemaker *H. sublaevis* is completely dependent on its hosts throughout its life cycle. To found a new colony it kills host queens and other adults and takes over a host nest, the brood that is left in the nest will grow up to be the first generation of slaves. Workers of *H. sublaevis* are incapable of carrying out routine colony tasks such as brood care, foraging, and nest maintenance (Buschinger 1974; Buschinger et al. 1980). Slavemaker brood is raised by enslaved hosts. Slavemaker workers regularly conduct slave raids on neighboring host colonies and steal their brood to replenish their labor force (Buschinger 1974; Buschinger et al. 1980). Colonies can survive for 10-15 years. Because of its parasitic life style, *H. sublaevis* has an approximately 10-fold lower density (0.06-0.1 nests/m^2) than the host species (Fischer-Blass et al. 2006). It also has a smaller range, likely because some regions have too low host densities to sustain parasite populations. Its reproductive strategy probably also makes it a relatively slow disperser: the vast majority of *H. sublaevis* queens are wingless and attract winged males by emitting pheromones (female

calling) (Buschinger 1966abc; Buschinger 1971; Heinze 1993; Hölldobler and Wilson 1990). The mated queens attack host colonies in walking distance from their mother nest to establish new colonies.

Both of the host species, *Leptothorax acervorum* (Fabricius 1793) and *Leptothorax muscorum* (Nylander 1846), inhabit the leaf litter layer of pine forests of the boreal regions of Eurasia (Collingwood, 1971; Ratschenko et al. 1999), where they nest in pine twigs and logs. *L. acervorum* can also be found in deciduous forests. A typical colony consists of 10-200 workers and one to several queens. Life expectancy for queens is estimated to be around 10 years. Slave raids impose severe selection on host populations lowering the mean life expectancy of host colonies considerably (Fischer-Blass et al. 2006). Ecological field data indicate that the slavemaker *H. sublaevis* shows a slight preference for the smaller host species *L. muscorum,* and consequently has a stronger impact upon it (Fischer-Blass et al. 2006; Bauer et al. 2009 a, b). In many sites the social parasite co-occurs with the two host species and mixed parasite nests containing slaves of both species are frequently found. *L. muscorum* has higher densities than the parasite, but slightly lower than the other host (*L. muscorum* up to 0.8 nests /m^2, *L. acervorum* 1 nest /m^2). The reproductive biology of *L. muscorum* is similar to that of its parasite, only that both sexes are winged. Virgin queens attract mates by emitting pheromones (female calling), and there are no mating flights in this species. After mating, *L. muscorum* queens can return into the mother nest or start a colony in the vicinity on their own. Of the three species, *L. acervorum* shows the highest nest densities and the widest distribution (Ratschenko et al. 1999; Fischer-Blass et al. 2006). This species has winged queens and winged males that mate in large mating swarms. It is therefore expected to be most dispersive.

A recent population genetic study, based mainly on mitochondrial gene sequences revealed higher genetic variation in the larger host species *L. acervorum* than in the parasite and the smaller host *L. muscorum*. In the same study, it was found that *L. acervorum*, which is the species that performs mating flights, showed much lower Φst values than both *H. sublaevis* and *L. muscorum* (Brandt et al. 2007). Both findings qualitatively fit to what we know about nest densities and reproductive strategies in these species. However, MtDNA is inherited through the female line only and stochastic effects can have a huge effect on sequence variation found in an MtDNA locus. For example, a recent selective sweep in one of the species on the MtDNA could have removed a large part of the genetic variation - making it impossible to determine whether differences in genetic variation or population structuring

reflect the real situation for most of the genome. For that reason, Brandt et al. (2007) studied also a small number of microsatellites in the three species.

In this study, we considerably extend the microsatellite study of Brandt et al. (2007) to more populations and eight microsatellite loci for each species and each population. With this larger dataset, we will focus on three questions. First, we are interested in the amount of genetic variation each of the three species harbor, because this is relevant for the adaptive potential of the species.

Second, we want to know whether there is population structuring and whether there is evidence for isolation by distance. For this we used a newly introduced measure of differentiation (Jost's D) which is not sensitive to levels of heterozygosity. We are also interested in the effects of the last ice age - with large glaciers over Northern Europe and the alpine regions- on the distribution of genetic variation in the species we study. For example, we are interested whether more northern populations, which were resettled after the ice ages, are genetically less diverse compared to areas, which could have served as refuges. We therefore tested whether there was any effect of latitude or longitude on the amount of genetic variation. We also used the program Structure (Pritchard et al. 2000) to see whether any hidden population structure could be identified, which could help us to reconstruct potential resettlement routes.

Third, we will compare male and female migration patterns. This is possible, because we have data for MtDNA, which is only inherited through the female line, and nuclear microsatellites which are inherited by males and females. This comparison, however, is not straightforward, mainly because of the large differences in population wide mutation rates (N*mu) and the resulting differences in heterozygosities. It was noted by several authors that standard F statistics and their relatives, such as Gst, are not good measures of differentiation (Hedrick 2005; Jost 2008). Specifically, Fst and relatives greatly underestimate differentiation when heterozygosity is high as it is commonly found with microsatellite markers. We therefore used a measure of differentiation, D, which was recently introduced by Jost (2008) and which is independent of heterozygosity.

We will interpret the findings of our study in terms of adaptive potential in the coevolutionary arms race between the obligate social parasite *H. sublaevis* and its *Leptothorax* hosts.

I.III MATERIAL AND METHODS

Sampling sites

Colonies of the parasite *H. sublaevis* and its hosts *L. muscorum* and *L. acervorum* were collected in pine forests throughout Europe, where they were found mainly in logs and twigs on the forest floor. In the summers of 2004-2008, ant colonies were collected in twelve countries (Fig.1; Table 1).

Figure 1: Collection sites. The geographic position of these study sites are: Germany R (Regensburg: N 48° 48´59.62´´; E 11° 50´45.40´´), Germany B (Berlin: N 52° 17´39.72´´; E 13° 37´31.18´´), Russia (N 59° 56´20.54´´; E 30° 18´47.55´´) Sweden (N 56° 39´41.23´´; E 16° 21´45.78´´), Austria (N 47° 19´56.19´´; E 11° 11´03.13´´), Italy (N 46° 43´33.66´´; E 12° 17´46.88´´), Poland (N 54° 03´36.48´´; E 14° 56´14.99´´), Spain (N 42° 42´16.81´´; E 0° 47´31.37´´), Switzerland (N 46° 22´03.55´´; E 8° 10´54.54´´), England (N 52° 27´31.32´´; E 0° 13´22.26´´). Circles symbolise species found at the different sites; in black: *L. acervorum*, in white: *L. muscorum*, in grey: *H. sublaevis*

DNA extraction for microsatellite analyses and MtDNA sequencing

For microsatellite analysis, DNA was extracted from a total of 78 *H. sublaevis*, 284 *L. acervorum* and 221 *L. muscorum* workers. Each worker came from a different colony, for sample sizes per location, see Table 1. DNA was extracted using Puregene DNA extraction kit, Gentra System. In total, the same eight highly variable microsatellite loci were amplified with PCR using the primers LXA GA1 (Bourke et al. 1997), L-18 (Foitzik et al. 1997), LX GT 223 (Hamaguchi et al. 1993) LXA GA2 (Bourke et al. 1997), LXA GT2 (Bourke et al. 1997), LXA GT1 (Bourke et al. 1997), LX GT 218 (Hamaguchi et al. 1993) and Myrt 3 (Evans 1993). These polymerase chain reactions were performed in a 20 µl volume containing 2.0 µl 10x *Taq* polymerase buffer (*Taq* Core Kit 10, MP Biomedicals), between 2.0 – 2.2 mM, depending on loci $MgCl_2$ (*Taq* Core Kit 10, MP Biomedicals), 4 mM of each dNTP, 0.5 µM of labelled forward primer, 0.5 µM of reverse primer and 1 U of *Taq* DNA polymerase (*Taq* Core Kit 10, MP Biomedicals). The following amplification conditions were used in a thermocycler (Thermal Cycler PxE 0.2) LXA GA1, L-18, LX GT 223: one cycle of 94 °C for 1 min 30 s, 54 C° for 45 s and 72 °C for 30 s. 28 cycles 92 °C for 45 s, 54 °C for 45 s, 72 °C for 30 s and followed by a final extension at 72 °C for 7 min and hold at 4 °C. Annealing temperature varied LXA GA2, LXA GT2: 48 °C, for Myrt 3: 45 °C, for LXA GT1: 42 °C and for LX GT 218: 44 °C. For control we transferred part of our product on a TBE agarose gel (1.5%). Fragments were then analysed in a 96 capillary sequencer (Megabace, Amersham Biosciences) and evaluated using the software Fragment Profiler (Amersham Biosciences).

For the MtDNA analysis, we used the sequences from Brandt et al. (2007), and added new sequences to enlarge the dataset. DNA extraction and sequencing was done as described in Brandt et al. (2007) for a total of 51 (34) *H. sublaevis*, 113 (55) *L. acervorum* and 79 (30) *L. muscorum* individuals (in brackets are the number of individuals that were already used Brandt et al. (2007)). For the sample sizes per location, see Table 1.

Table 1: Number of colonies samples per species at each of the study sites. In brackets are the number of individuals that were also used in Brandt et al (2007).

Sampling Location	*L. acervorum*		*L. muscorum*		*H. sublaevis*	
	Microsat.	MtDNA	Microsat.	MtDNA	Microsat.	MtDNA
Germany	57	12 (10)	56	20 (10)	33	19 (11)
Italy	84	15 (10)	86	14 (10)	30	11 (8)
Russia	30	14 (10)	21	15 (10)	10	12 (8)
Sweden	25	12 (2)	30	12	5	0
Poland	10	4	7	6	0	1
Czech Republic	25	15	11	7	0	0
Austria	6	5	10	5	0	0
Switzerland	14	13 (8)	0	0	0	2 (2)
Finland	0	8 (7)	0	0	0	6 (5)
England	30	10 (5)	0	0	0	0
Spain	3	1	0	0	0	0
Estonia	0	4 (3)	0	0	0	0

Data Analysis

Summary statistics for the microsatellite data were calculated using Microsatellite-Analyzer 4.05, and GENEPOP 3.4 (web version). The data were tested for heterozygote deficiency (test for H-W equilibrium, using a U test (Raymond and Rousset 1995)) and the program GENEPOP, option 1 sub-option 4. Expected heterozygosities (H_{exp}) and observed heterozygosities (H_{obs}) were calculated for each locus, in each population for each species using MSA. The data were tested for linkage disequilibrium between loci (Fisher exact test for each pair of loci across all populations) using GENEPOP (option 2 sub-option 1).

To compare levels of genetic variation between species, H_{exp} was used, because it is less sensitive to null-alleles than H_{obs}. We fitted a linear model with H_{exp} as response variable and population, species and locus as explanatory variables using R (version 2.5.1,

{http://www.R-project.org}). We did not allow for interactions. We repeated the same analysis with explanatory variables latitude and longitude instead of population name. For the linear model we had to make the assumption that populations of a species are independent, which is not strictly the case as they are related by genealogy.

For the MtDNA sequences, summary statistics were calculated using DNASP. With the MtDNA data, we repeated the same analysis (linear model in R) as with the microsatellite data. We used a web version of Phylip dnapars (v3.66, http://bioweb2.pasteur.fr/docs/phylip/doc/dnapars.html, Felsenstein 2004) to find the most parsimonious tree and used this to make a haplotype network by hand.

Global and pairwise Gst_est values for the microsatellite data and the MtDNA sequences were calculated with R, following Nei and Chesser (1983). Gst values are greatly influenced by the amount of heterozygosity in the populations (Hedrick 2005; Jost 2008). When heterozygosity is high, Gst is automatically low, even when populations carry completely distinct sets of alleles. We therefore also calculated the D statistic, which was proposed by Jost (2008). For comparison:

$$\text{Gst\_est} = \frac{Ht\_est - Hs\_est}{Ht\_est}, \quad \text{D\_est} = \frac{n}{n-1} * \frac{Ht\_est - Hs\_est}{1 - Hs\_est};$$

(n is the number of populations that were sampled, Ht is heterozygosity over all populations, Hs is mean heterozygosity within the populations). We calculated D_est according to formula 12 in Jost (2008).

In addition to the Gst analysis, we analysed our data with the programs Structure 2.2 (Pritchard et al. 2000, Falush et al. 2003). This program uses a Bayesian MCMC approach and multi-locus genotype data to infer the presence of distinct populations, without using information on where individuals were sampled. For Structure we did the standard analysis with admixture, burn in period of 50,000 steps, then 100,000 steps. K values (number of clusters) ranged from 1 to 12 for all species and we did 4 repeats per K value.

To test for isolation by distance a Mantel test was performed over the whole data set using IBDWS (Jensen et al. 2005) for both microsatellites and MtDNA, using both pairwise G_est values and pairwise D_est values.

I.IV RESULTS

Microsatellites

The eight microsatellite loci were highly variable for all three species with between 14 and 76 (mean 31) alleles for *L. acervorum*, between 9 and 65 (mean 26) alleles for *L. muscorum* and between 9 and 36 (mean 21) alleles per locus for *H. sublaevis*. H-W equilibrium over all populations and loci could be rejected due to heterozygote deficiency for all three species ($p < 0.0001$). The difference between expected heterozygosity (Nei) and observed heterozygosity indicates how "far" a population is from H-W equilibrium. In *H. sublaevis* the difference was largest (H_{obs} 0.70 and H_{exp} 0.80), in *L. acervorum* smaller (H_{obs} 0.77 and H_{exp} 0.82) and in *L. muscorum* smallest (H_{obs} 0.73 and H_{exp} 0.72) (averages over loci and populations). The underlying reason could be the presence of null alleles and/or inbreeding. No significant linkage disequilibrium could be detected in any of the species (in *L. acervorum* 2 locus pairs out of 28 had p-values < 0.05, *L. muscorum*: 2 significant locus pairs out of 28 and *H. sublaevis*: 2 significant locus pairs out of 28).

Microsatellites genetic variability

We tested whether the amount of genetic variation, as measured by Nei's expected heterozygosity (arcsine transformed), depends on location, species, and locus. There was a significant effect of species (*L. muscorum* populations had 10% lower heterozygosity than the other two species, $p<0.005$; Fig. 2a) and of locus ($p<0.0001$), but there was no effect of location ($p=0.83$). We repeated the same Anova using geographic location (coordinates) instead of the name of the location. There was no significant effect of latitude or longitude ($p=0.68$) on the amount of genetic variation as measured by microsatellites. There was a clear effect of locus: Ga2 and GT1 were more variable than the other loci ($p<0.0001$ in both cases).

MtDNA genetic variability

Genetic variability, calculated as mean pairwise differences (Pi) within populations and then averaged over populations was (mean ± standard error): 0.0037 ± 0.00063 for *L. acervorum*, 0.0036 ± 0.00196 for *L. muscorum* and 0.0021 ± 0.00066 for *H. sublaevis* (Fig 2b). Genetic variability within populations, was not significantly different in the different species (p=0.66) nor in the different locations (p=0.78). However, this could be due to the fact that we have only one data point per species and population (for the microsatellites, we have eight data points per species and population). Table 2 shows main summary statistics for each of the species for microsatellite and mtDNA analyses.

Table 2: Main summary statistics for each of the species.

	Microsatellites				**MtDNA**		
	Number of alleles per locus (min, max)	**H_{exp}** averaged over loci and populations ± SE	**Gst** ± SE	**D_est** ± SE (Jost 08)	**Mean pairwise differences (Pi)** ± SE	**Gst**	**D_est** (Jost 08)
L. acervorum	31 (14 - 76)	0.82 ± 0.013	0.030 ± 0.007	0.23 ± 0.073	0.0037 ± 0.00063	0.16	0.50
L. muscorum	26 (9 - 65)	0.72 ± 0.030	0.066 ± 0.012	0.28 ± 0.085	0.0036 ± 0.00196	0.34	0.82
H. sublaevis	21 (9 - 36)	0.80 ± 0.034	0.043 ± 0.015	0.35 ± 0.073	0.0021 ± 0.00066	0.19	0.86

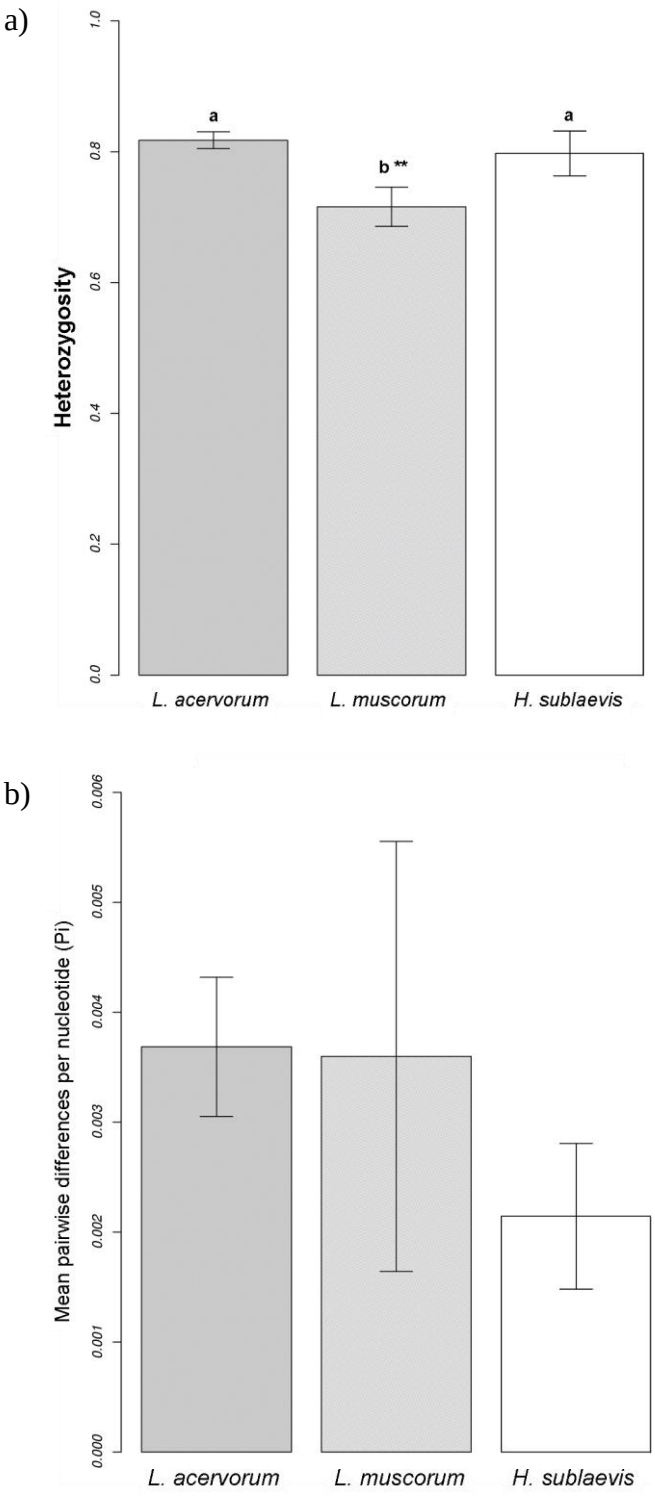

Figure 2: a) Heterozygosity (microsatellites) averaged over populations and loci. **b)** Mean pairwise differences per nucleotide, calculated within each population and averaged over all populations.

Haplotype networks

For *L. acervorum* and *H. sublaevis*, the haplotype network of the MtDNA sequences looked very similar to the ones published in Brandt et al. (2007) (not shown). However, we found a large discrepancy for *L. muscorum* between our current results (Fig. 3) and previous results. In the Brandt et al. (2007) study, only three populations were analyzed for *L. muscorum*: Germany, Italy and Russia. These three populations formed clear clusters in the haplotype network and we found a correspondingly high Gst value. In this study four more populations were added. Two of these populations, Austria and Czech Republic, have haplotypes that do not cluster like the ones from Italy, Russia and Germany. Austrian individuals cluster with both Germany and Italy. Czech individuals cluster with both Russia and Germany. In addition, both Austria and Czech Republic carry haplotypes that are relatively distant from the clusters originally found. Individuals from Sweden and Poland cluster with the Germany haplotypes.

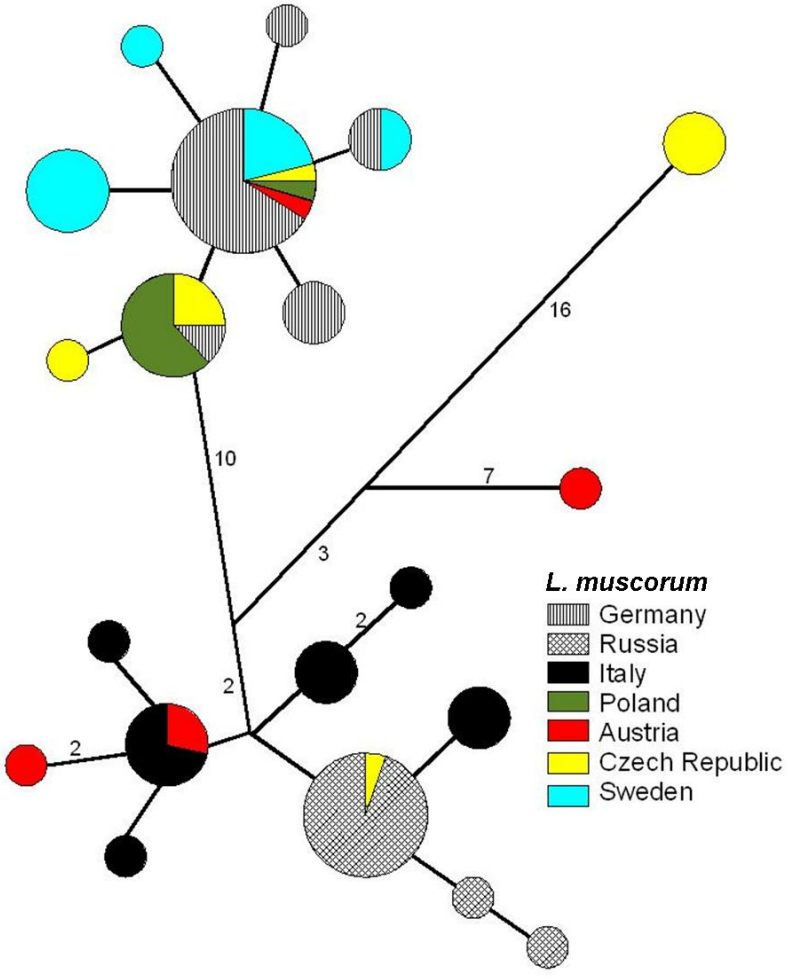

Figure 3: Haplotype network for *L. muscorum* (the numbers along the branches are distances of more than one mutation).

Genetic differentiation

Global Gst_est values (from microsatellites) were small (estimates ± standard errors: *L. acervorum* 0.030 ± 0.007, *L. muscorum* 0.066 ± 0.012, *H. sublaevis* 0.043 ± 0.015. However, Gst values are greatly influenced by the amount of heterozygosity in the populations (Hedrick 2005; Jost 2008). We therefore also calculated Jost's D statistic (Jost 2008), which is independent of the level of heterozygostity. D values are much higher than the Gst values: *L. acervorum* 0.23 ± 0.073, *L. muscorum* 0.28 ± 0.085, *H. sublaevis* 0.35 ± 0.073 (see Fig. 4). D values are not significantly different between species ($p>0.05$).

We also calculated Gst_est values for MtDNA and found 0.16 for *L. acervorum*, 0.34 for *L. muscorum* and 0.19 for *H. sublaevis* (based on a single locus, so no standard errors can be given). Also for MtDNA data it is possible to calculate Jost's D statistic, which allows a direct comparison of the MtDNA data and the microsatellite data. D_est values were 0.50 for *L. acervorum*, 0.82 for *L. muscorum* and 0.86 for *H. sublaevis* (see Fig. 4).

We found no evidence for isolation by distance (IBDWS) for either MtDNA or microsatellites using G_est values (p-values microsatellites: *L. acervorum* 0.10, *L. muscorum* 0.13, *H. sublaevis* 0.36; p-values MtDNA: *L. acervorum* 0.62, *L. muscorum* 0.10, *H. sublaevis* 0.54). When using D_est values we found a significant effect of isolation by distance only in *L. muscorum* when using MtDNA sequence data, which would no longer be significant after a Bonferroni correction (p-values microsatellites: *L. acervorum* 0.22, *L. muscorum* 0.27, *H. sublaevis* 0.54; p-values MtDNA *L. acervorum* 0.14, *L. muscorum* 0.02, *H. sublaevis* 0.14). No evidence for population substructure was found using the software Structure. Structure calculates the likelihood of the dataset given a number of clusters, K. When there is detectable population structure, the likelihood of the data goes up when the number of clusters is increased. We did not find evidence for population structure with this method; for all three species, when K was increased, the likelihood stayed the same or even decreased.

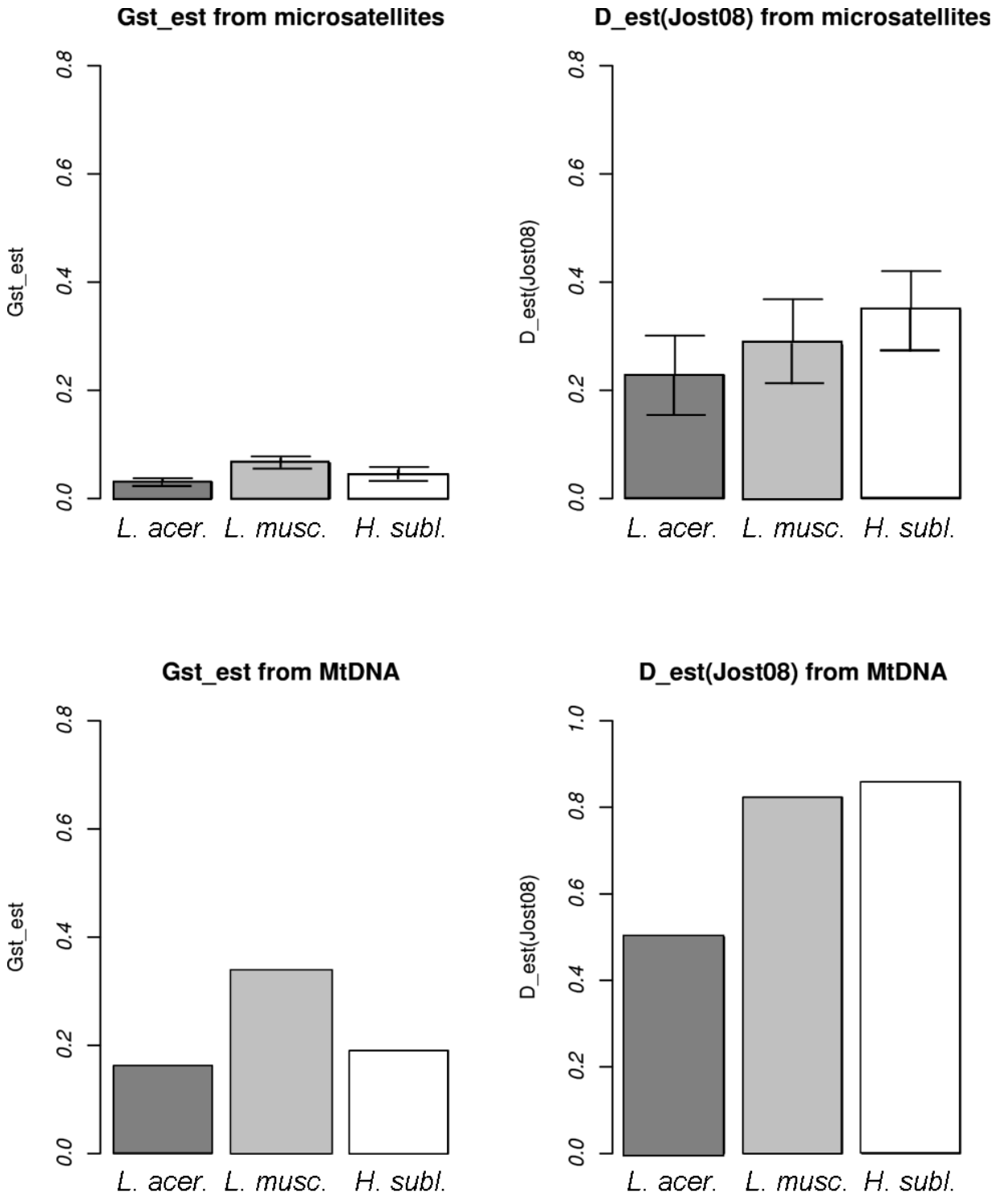

Figure 4: Different measures of genetic differentiation for microsatellites (upper two panels) and MtDNA (lower two panels).

I.V DISCUSSION

In this study we analyzed genetic variation and population structure of three European ant species: two hosts and one social parasite. Compared to our previous study (Brandt et al. 2007) we have looked at eight microsatellite loci (3 in previous study) and MtDNA sequences in more individuals and in more populations per species. In addition, for the analysis of population structure we have used a newly introduced measure of genetic differentiation, which allows a better comparison between nuclear and MtDNA data.

When looking at the microsatellite data, we find that the three species each harbor a lot of genetic variation. The smaller host species *L. muscorum*, which has less dense populations and a more restricted range (Ratschenko et al. 1999; Fischer-Blass et al. 2006) exhibits less genetic variability than the larger host, *L. acervorum***,** and the slavemaker, *H. sublaevis*. It is surprising that the parasite, *H. sublaevis,* has similarly high levels of genetic variation as *L. acervorum,* given that populations of this species have approximately 10-fold lower densities and therefore a much lower census population size. In a previous paper (Brandt et al. 2007) it was found that *H. sublaevis* had higher genetic variation than *L. acervorum*, but with our larger dataset we could not confirm this finding. Our results show again that census population size does not necessarily correlate with effective population size even among closely related species (Bazin et al. 2006). It is currently unclear why this is the case, but we suppose that populations of these two species are so large that genetic variability at these highly variable microsatellite loci is not limited by the population size.

We also find similar levels of genetic diversity in all species when we use MtDNA, though the slavemaker seems to have a slightly lower level of genetic variation than the two host species. Unfortunately, analyses of MtDNA have the disadvantage that this marker behaves like a single locus. MtDNA sequences are linked by a single genealogical tree, because there is no recombination. Therefore large differences may occur by chance.

We found low Gst values for the microsatellite data, which is the typical pattern found in many ant species. However, Gst is strongly influenced by levels of heterozygosity and if heterozygosity is high as it is typical for microsatellite loci, Gst is invariably low. Our low Gst values may therefore simply reflect the high values of within population heterozygosity. We therefore also looked at the D statistic, which was recently proposed by Jost (2008) and which is independent of levels of heterozygosity and therefore allows a direct comparison of different marker systems. D values calculated from microsatellites are relatively similar for all species and are four to eight times larger than the Gst values (*L. acervorum* Gst: 0.03, D: 0.23,

L. muscorum Gst: 0.07, D: 0.28, *H. sublaevis* Gst: 0.04, D: 0.35). The D values show that our populations are much more differentiated than would have been suggested by the Gst values. The fact that *L. acervorum* has the lowest value of D (even though the difference is not significant) fits our expectations, because it is the only species of the three that has mating flights so it is likely to be the most dispersive.

We also calculated Gst and D values from the MtDNA data (*L. acervorum* Gst: 0.16 D: 0.50, *L. muscorum* Gst: 0.34, D: 0.82 and *H. sublaevis* Gst: 0.19, D: 0.86) and found that the D values for MtDNA were between 2.1 and 2.9 times larger than D values based on microsatellites. This can be explained by male biased dispersal, which was commonly found in ants. Especially in *L. muscorum* and *H. sublaevis*, where females do not participate in mating flights, male biased dispersal is expected. Females of *L. acervorum* however do fly, so the two fold difference between D from microsatellites and MtDNA is somewhat surprising in this species. We think it can be explained by a facultative polygyny. Some of the young queens return to the mother nest after mating and are readopted. Polygynous colonies and readoption of daughter queens can also occur in *L. muscorum*, but are strictly absent in *H. sublaevis*, where queens mainly disperse on foot.

Gst values from MtDNA were much higher (roughly five fold) than Gst values from microsatellites. Most of this difference disappears when we convert to D values. Many studies on ant population genetics compare the Gst values directly and find up to 20 times higher values in the MtDNA based calculations (for example, Doums et al. 2002; Clemencet et al. 2005; Brandt, et al. 2007; Goropashnaya et al. 2007;). They conclude that male dispersal is much more pronounced in ants than female dispersal. We think that this pattern may at least partly be caused by the differences in variability, rather than a real difference in differentiation. It is good that there are now other statistics available (Gst' by Hedrick, 2005 and D by Jost 2008), which do not have this bias. In addition, it would be very useful if nuclear sequences would become available for ants so that they can be compared to the other two marker systems. This would give us a much better insight in male and female dispersal in ants.

We found no evidence for population structure with *Structure* analysis. This could be due to the relatively high number of rare alleles. Rare alleles contribute to differentiation (when D is used to estimate differentiation), but do not have a large influence on the likelihood estimates for *Structure*, which are based on H-W proportions of genotypes. For both marker systems, we find no clear pattern of evidence for isolation by distance, nor do we find that northern populations have lower genetic variation. This is different in some other ant

species (e.g., Viginier 2004; Clémencet et al. 2005; Schluns et al. 2009). The three ant species we study depend largely on pine forests. Pine (*Pinus sylvestris*) occupied several refugia in Central and Eastern Europe in addition to the "standard" ones in southern Europe (Cheddadi et al. 2006). Even a small forest can harbor a large population of ants. We therefore expect that also relatively large ant populations survived the last ice age in several refugia in Europe. As the forest moved back up North, the ants moved along. Ants can move much faster than trees, but because they depend on the trees they will have moved at exactly the same speed as the trees. For the ants, this meant a rather slow dispersal, allowing for a lot of individuals to contribute to the dispersal. We therefore expect that there were large (effective) population sizes all along and not much genetic variability was lost during migration north or in the high alpine sites. Large population sizes in the past and now, combined with relatively slow migration could explain why the species have high variability even in the northern populations.

We conclude that in this host-parasite system we expect to find evidence for the Thompson's geographic mosaic of coevolution (Thompson 2005) because a) there is abundant genetic variation and population sizes are large in all three species. This means that adaptation should not be hindered by lack of variation. In addition b) the populations of all three species strongly are structured. Coevolutionary processes in one community are therefore somewhat independent of other communities and we would expect parasite and host to adapt to their local opponent. This was indeed found in behavioral studies, which investigated the crucial host – parasite encounter – the slave raids (Fischer and Foitzik 2004). A species comparison suggests high levels of genetic variation and population structure in all of the three species, only the smaller host, *L. muscorum*, might be less genetically variable in comparison to the larger host. The smaller species is also the preferred host for the parasite (Fischer-Blass et al. 2006, Bauer, et al. 2009 a, b). It is possible that the smaller host's population size is low because of the preferred exploitation by the parasite. It could also be that the parasite is ahead in the arms race against the smaller host, because *L. muscorum* has lower adaptive potential and therefore the parasite prefers this species.

I.VI ACKNOWLEDGEMENT

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CHAPTER II

Fight or flight? A geographic mosaic in host reaction and potency of a chemical weapon in the social parasite *Harpagoxenus sublaevis*

(Sabine Bauer, Volker Witte, Melanie Böhm, Susanne Foitzik)

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II.I ABSTRACT

Ant social parasites use chemical warfare to facilitate host colony takeover, which is a critical, but recurring step in their life cycle. Many slave-making ants use the secretion of the Dufour gland to manipulate host behaviour during parasitic nest foundation and slave raids. *Harpagoxenus sublaevis* applies this chemical weapon onto defending *Leptothorax* host workers, which elicits deadly fights amongst them. Host species are expected to evolve counter-adaptations against this behavioural manipulation and in this study we investigated the geographic structure of this co-evolving trait. We compared the effectiveness of the parasitic gland secretion from different *H. sublaevis* populations in host colonies from various sites and analysed the occurrence of local adaptation. The two host species *L. muscorum* and *L. acervorum* generally showed different responses to the parasites' chemical weapon: *L. acervorum* attacked nestmates treated with Dufour-gland secretion, while *L. muscorum* workers fled. Flight, instead of intraspecific fights, is an adaptive host reaction as it results in fewer host fatalities during raids. Beside interspecific host differences, we found a geographic mosaic of host resistance: Parasites from a German population strongly manipulated the behaviour of both sympatric *Leptothorax* populations. Russian or Italian hosts instead did not react with intracolonial aggression, but fled when confronted with the gland secretion of their sympatric parasite. Not only variation in host resistance explains differences in the effectiveness of the parasitic gland secretion, but also interpopulational differences in its chemical composition, which were revealed by gas chromatography and mass spectrometry.

II.II INTRODUCTION

Chemical communication was the primary form of communication in evolution and continues to be important today (Oldham and Boland 1996, Johansson and Jones 2007). Information can be efficiently transferred between individuals through the temporary emission of chemicals, for example alarm cues. In ants, many tasks such as reproduction, brood care and foraging are regulated via chemical signals (Hölldobler and Carlin 1987). A single ant worker can contain more than 20 major glands in its body (Billen and Morgan 1998). These glandular depots facilitate a very sophisticated communication system in these social insects. However, intraspecific chemical communication signals can be intercepted by other species and insect societies are hereby especially threatened by parasites. For example, the varroa mite exploits honey bee societies by using chemical signals to achieve hive integration (Martin 2001). The socially parasitic ant *Formicoxenus nitidulus* employs a general chemical deterrent to circumvent host aggression, which allows its exploitation of eleven different host species (Martin et al. 2008).

Social parasitism, common among the ants, includes the exploitation of brood care behaviour of one social species by another. Slave-making ants live in their own nests, in contrast to avian brood parasites, but conduct periodic slave raids on neighbouring host colonies to acquire slaves, which are stolen as pupae. Host workers that emerge from the stolen brood take over all routine tasks in slavemaker nests. The lifelong dependency of these parasites on their hosts and especially their frequent and destructive raids lead to strong selection on host populations, which is the driving force of co-evolutionary arms races (Brandt et al. 2005a,b, Fischer-Blass et al. 2006; Foitzik and Herbers 2001). The slave-making niche evolved repeatedly in ants, with six origins among the Formicoxenine ants alone, the taxonomic group to which our study species, *Harpagoxenus sublaevis*, (Nylander 1852) belongs (Beibl et al. 2005).

Obligate parasites have to overcome host barriers to start a successful parasitic life. In many slavemaking ants, the entry of young parasite queens into host nests is facilitated by sophisticated chemical strategies. One of these is to bypass the nestmate recognition code through chemical insignificance. Before colony usurpation, young *Polyergus* queens are characterized by the nearly complete absence of hydrocarbons on their cuticle. These slavemaker queens quickly acquire host colony specific cues from the killed host queen after takeover (Topoff and Zimmerli 1993; Johnson et al. 2001; D'Ettorre et al. 2002). Other slavemaking ants mimic the chemical profiles of their hosts before host colony entry (Brandt

et al. 2005ab). Another strategy is the active production of specific allomones - chemical weapons - that manipulate host behaviour to the advantage of the parasite (Topoff et al. 1988; D'Ettorre et al. 2000; Mori et al. 2000a,b). These chemical arms include appeasement or repellent substances and "propaganda pheromones", which protect parasite queens from host aggression.

Social parasites frequently use secretions from the Dufour gland for behavioural manipulation of their hosts. The Dufour gland is a common abdominal exocrine gland found in social Hymenoptera with very diverse functions. In ants, secretions of the Dufour gland are employed as trail or recruitment pheromones (Blatrix et al. 2002; Coll et al. 1987; Witte et al. 2007). More importantly, this secretion was found to play a role as a "propaganda substance" that induces attacks against ants, which were marked with it (Regnier and Wilson 1971; Buschinger 1974; Allies et al. 1986). This was demonstrated in intraspecific fights among *Leptothorax gredleri* queens over reproductive dominance (Heinze et al. 1998), but also in the social parasites *L. kutteri* and *Harpagoxenus sublaevis*, which thereby manipulate host worker aggression (Allies et al. 1986). An experimental study by Brandt et al. (2006) suggested that a similarly used propaganda substance of the slave-making ant *Protomognathus americanus* evolved as a fertility signal. The Dufour gland secretion of fertile host workers likewise provoked aggression and disorder amongst host workers.

The obligate social parasite and slave-making ant *H. sublaevis* uses this "propaganda substance" during colony takeovers and slave raids against its hosts *Leptothorax acervorum*, Fabricius 1793 and *Leptothorax muscorum*, Nylander 1852 (Allies et al. 1986; Foitzik et al. 2003). Host workers marked with the Dufour gland secretion are attacked by their nestmates and these often deadly host fights facilitate parasite nest entrance and brood raiding. This chemical weapon can also be sprayed into host nests without contact to ants, then eliciting panic and disorganisation among defending workers (Regnier and Wilson 1971). Comparative analyses of Dufour gland chemistry have revealed heptadecadiene and heptadecene as major constituents of the secretion of both the parasite and its main host *L. acervorum* (Ali et al. 1987; Ollett et al. 1987). This again suggests that *H. sublaevis* is intercepting a host communication signal and employs it in its own interest.

Harpagoxenus sublaevis exhibits unusually high prevalences and very destructive slave raids, which lead to a significant reduction in the life expectancies of host colonies in parasitized populations (Fischer-Blass et al. 2006). Selection is therefore strong enough to drive a coevolutionary arms race between *Harpagoxenus* and its *Leptothorax* hosts. Indeed, experimental studies in the context of slave raids have demonstrated behavioural local

adaptation of *H. sublaevis* to populations of its larger host *L. acervorum* (Fischer and Foitzik 2004). In a different study, *L. acervorum* workers from unparasitized populations from the British Isles reacted more aggressively towards the secretion of the parasitic Dufour gland than *L. acervorum* workers from parasitized populations, indicating partial immunity against behavioural manipulation in the latter populations (Foitzik et al. 2003).

Our findings of a spatial structure in parasite-host interactions or of a geographic mosaic of coevolution (Thompson 1999) are supported by population genetic analyses, which showed that host and parasite populations are connected via limited gene flow (Brandt et al. 2007). The larger host species, *L. acervorum* exhibited the highest levels of both genetic variability and gene flow, and previous field data showed that it is less affected by its social parasite *H. sublaevis* than the smaller host *L. muscorum* with genetically depleted and very isolated populations (Fischer-Blass et al. 2006).

Here, we investigated the coevolutionary dynamics of the chemical weapon "Dufour gland secretion" of *H. sublaevis* and *Leptothorax* host defenses against this trait. We analysed variation in the chemical composition of the Dufour gland's secretion in three European *H. sublaevis* populations using gas chromatography and mass spectrometry. As chemical differences could be explained by local adaptation, drift or differences in ecology, we investigated the effectiveness of this secretion in eliciting favourable host responses, using colonies of both host species from various populations. This experimental approach allowed us to test whether the effectiveness of the parasites' chemical weapon depends on the geographic origin of parasite and host populations, which would indicate local adaptation. Additionally, we studied whether the secretion of the parasite is more effective against one of its two host species. All analysed *H. sublaevis* populations have to deal with two host species and adaptation to any one host might result in a lower manipulation efficiency of the other host. To shed light onto this potential trade-off, we compared the effectiveness of the chemical weapons between sympatric populations of the two host species and analysed the species composition of host communities.

II.III MATERIAL AND METHODS

Study sites and species

Ant colonies of the social parasite *H. sublaevis* and its hosts *L. muscorum* and *L. acervorum* were collected in summer 2005 and 2006 at two study sites in Bavaria, Germany (Abensberg N 48° 49', E 11° 50'; Nuremberg N 49° 27', E 11° 4') and at an high-alpine site in South Tyrol, Italy (San Candido N 46° 44', E 12° 16'). Additional ant colonies were collected in Russia in summer 2005 (St. Petersburg N 59° 56', E 30° 16'). The community composition was analysed at each of the study sites using Chi-Square-tests. We noted the number of host and parasite colonies found at each site and examined parasite prevalence and host infection rates in the three communities.

Colony Maintenance and Behavioural Experiments

Ant colonies were brought to the laboratory in Munich and transferred into artificial nests inside small plastic boxes with a moistened plaster floor (Buschinger 1974; Heinze and Ortius 1991). During the experimental period, the ants were kept in an incubator at 20°C for 12h light, and 10°C for 12h dark. Colonies were fed twice weekly with diluted honey and crickets.

To analyse the impact of the Dufour gland secretion on the behaviour of host workers, a *Leptothorax* worker was marked with an Edding paint dot on the thorax approximately 30min prior to the control and Dufour experiment. For each gland trial, a *H. sublaevis* worker was dissected under the stereomicroscope. Ant workers were killed by freezing and placed on a clean microscope slide with a droplet of distilled water to prevent the gland from desiccating. The Dufour gland was obtained by slowly pulling at the sting of the ant, and subsequently separating the gland from the sting and the poison gland. The gland was then placed on the tip of an insect pin, and carefully transferred onto a host worker's gaster. Immediately afterwards, the worker was placed back into its mother colony, and the nest entrance was sealed with cotton wool. In control experiments, which were conducted 24 h prior to the Dufour gland experiments, a droplet of water instead of the contents of the Dufour gland was used. Parasite glands were chosen at random, as were the experimental host colonies. We did not perform experimental and control trials in a randomised fashion, because the effect of the parasitic Dufour's gland per se was already shown in earlier experiments (Allies et al. 1986, Foitzik et al. 2003). Instead, we focussed on potential differences between host and parasite populations and potentially local adaptation in this chemical trait. Detection of the parasite by the host colony through chemical perception of the gland secretion might

also potentially lead to long-term behavioural changes. This could potentially influence all trials following including the control experiments. We waited at least 24h after the control trials, to ensure that host colonies had enough time to calm down, which was also apparent in our behavioural observations. For each colony, trials with Dufour gland secretions from different populations were conduced at least 48h apart.

The reaction of the nestmates to the treated worker was monitored by scan-sampling in 30 second intervals for 15 minutes. Host workers either reacted with aggression, flight or peaceful behaviours such as antennation. Aggressive interactions include stinging, biting and dragging. Flight described a fast stampede after contact with the focal worker. To control the frequency of interactions between the focal worker and its nestmates, we calculated the proportion of aggression, flight and antennation in relation to the total of interactions.

We conducted 57 control experiments and 73 Dufour gland trials with colonies of the smaller host species *L. muscorum* in fall of 2006. We used 19 host colonies from Germany and 19 from Italy. Host nests contained between 15 and 30 host workers, and host nest size did not vary between populations (MWU test: $U = 332.5$, $N_{1,2}= 19$, $P > 0.05$). In addition to the control trials, each colony was tested twice where the focal ants was treated with a gland secretion. One Dufour trial was conducted each with a gland from a German and Italian *H. sublaevis* worker, respectively. Three colonies were not tested against the Italian gland secretion, due to a lack of Italian *H. sublaevis* workers. For the extraction of the Dufour glands, we used *H. sublaevis* workers derived from 19 different parasite colonies from Germany and 15 colonies from Italy. Whenever possible two workers from each *H. sublaevis* colony were used: One gland was tested against a German host colony and one against an Italian one. For the Italian slavemaker colonies we could not follow this experimental design entirely, because we did not have enough colonies. Therefore we had to use more than two *H. sublaevis* workers from two colonies, with a maximum of six workers used from a single colony.

The reaction of *L. acervorum* colonies to the Dufour gland secretion of *H. sublaevis* was analysed in a trial series in fall 2005 with 25 control trials and 59 experiments with parasitic Dufour glands. Eight host colonies each from Germany and Italy and nine Russian *L. acervorum* colonies were tested. Host nest size varied between 60 and 75 workers and did differ between populations (Kruskal Wallis test: $N_{1,2,3}= 8,8,9$; $P > 0.05$). Next to the control trials, each colony was tested once with a nestmate treated with a parasite gland from a German and from a Russian *H. sublaevis* worker. Three host colonies from Russia and Germany and two colonies from Italy were additionally treated with an Italian Dufour gland

(N=8). Glands of slave-making workers from six different German colonies, nine different Russian and six different Italian colonies were used.

The behavioural experiments were analysed with the program STATISTICA (StatSoft Inc., Version 6.0). To evaluate host responses to the parasite Dufour glands, we first compared the control experiments between populations using Mann-Whitney-U test (MWU) and Kruskal-Wallis test (KW). Then, we compared the control experiments with the gland trials using Wilcoxon matched-pair tests (WIL). These comparisons were conducted independently for each host species, host population and gland origin and we report the number of tests or comparisons. Interactions between host population and parasite population (gland origin), which would indicate local adaptation, could only be examined with parametric multivariate tests. We conducted repeated measure analyses (RMA) based on behavioural data, which were arcsine transformed to approach normality. Normality was not achieved in all cases. Repeated measure analyses analysis were hampered by our low sample size for experiments with the Italian gland secretion (N=8) and therefore we decided to exclude these few trials. Thus, we analysed whether the behaviour of *L. acervorum* workers in the experimental situation depended on the origin of the gland (Germany / Russia) or on the origin of the *L. acervorum* colonies (Germany / Russia / Italy) or on an interaction of host and gland origin. Finally, we contrasted the behaviours of *L. muscorum* and *L. acervorum* colonies in the treatment and control, using Mann-Whitney-U (MWU) tests.

Chemical Analyses of the Dufour Gland Secretion of *H. sublaevis*

To investigate potential interpopulation variation in the chemical composition of the Dufour's gland secretion, we investigated the secretion of eight *H. sublaevis* workers from Germany, six from Italy and two from Russia. All workers were taken from different colonies. *H. sublaevis* Dufour glands were dissected as described above, directly transferred to the partition of a glass vial, and immediately dissolved in 20 µl of hexane solvent. 1 µl of sample was then transferred to coupled gas chromatography and mass spectrometry (GC-MS) on an Agilent Technologies 6890N GC and 5975 MSD equipped with a Restek Rxi-5 MS column (30 m length, 0.25 mm ID, 0.25 µm film thickness). Injection was splittless at 280 °C with helium as the carrier gas. Initial oven temperature was 50 °C for 1.5 minutes, which subsequently increased by 15°C/min up to 140 °C, followed by a gentle temperature ramp of 3 °C/min until 200 °C and increased by 20 °C/min up to 290 °C and finally stayed isothermal for 3 minutes. Initial solvent delay was 3.8 minutes. Peaks in the GC-MS analyses were identified by fragmentation pattern and by comparison with Wiley7N database spectra.

We compared the gland composition of all *H. sublaevis* populations with the software Primer 6 (Version 6.1.6, Primer-E), which offers non-parametric statistical procedures robust to data type and distribution. Before importing the data into Primer 6™, the peaks in the total ion chromatograms were integrated with the software Chemstation (Agilent™). Only chromatograms of reasonable total concentration (i.e. > 30 x 106 detector counts) were included in the subsequent multivariate analysis to ensure a reliable comparison, because otherwise differences in the multivariate analysis are likely due to minor gland constituents that fall below the detection threshold of the instrument in lower concentrated runs. Peak areas were log- transformed to achieve equality of variances and subsequently standardized for each sample relative to the total area. To determine which compounds contribute most to the similarities within groups we performed a Similarity percent (SIMPER) analysis. Compounds that add up to at least 90% similarity were included. Then, we calculated a resemblance matrix, based on Bray Curtis similarities, which was used for the following non-parametric statistical analyses. Differences between populations were tested with an analysis of similarities (ANOSIM). A canonical analysis of principle coordinates (CAP) was used to visualize data distribution. This is a nonparametric method that allows visualizing group differences (analogous to a parametric discriminant analysis).

II.IV RESULTS

Community Composition

The Formicoxenine ant communities generally showed a similar species composition at the three study sites (Table 1). At each location, we found the two host species *L. acervorum* and *L. muscorum* and their social parasite *H. sublaevis*. In addition, this slave-making ant parasitized both host species at all three sites.

Table 1: Composition of the Formicoxenine ant community at the three study sites. Shown are the number of colonies, which were collected in 2005 in Germany, Italy and Russia and in 2006 in Germany and Italy.

Community	*L. muscorum*	*L. acervorum*	***H. sublaevis***			**Parasite prevalence**	
			L. muscorum slaves	*L. acervorum* slaves	Both slave species	*L. muscorum*	*L. acervorum*
Germany	81	79	11	2	4	1 : 5.4	1 : 13.2
Italy	53	127	10	9	5	1 : 3.5	1 : 9.1
Russia	28	78	1	6	2	1 : 9.3	1 : 9.8

In Italy and Russia *L. acervorum* was clearly more common while in Germany the two host species occurred at comparable frequencies. Parasite prevalence was similarly high at all three study sites (all χ^2 tests: $p > 0.05$) and ranged from 1 *H. sublaevis* nest per 7.5 host colonies in Italy to 1 : 11.8 in Russia. *L. muscorum* was clearly the preferred host for the German and Italian parasite population (Germany $\chi^2_1 = 5.86$, $P < 0.02$; Italy $\chi^2_1 = 5.91$, $P < 0.02$). We did not detect an overexploitation of a host species in Russia, probably due to the low sample size and the resulting low statistical power for this site.

Behavioural Reactions of the Host *L. muscorum* to the Parasite Dufour Gland Secretion

Workers from German and Italian *L. muscorum* colonies did not differ in their behaviour during the control situation (Mann Whitney U tests: $p > 0.60$), but strongly reacted to nestmate workers treated with the Dufour gland secretion of the social parasite (Fig. 1).

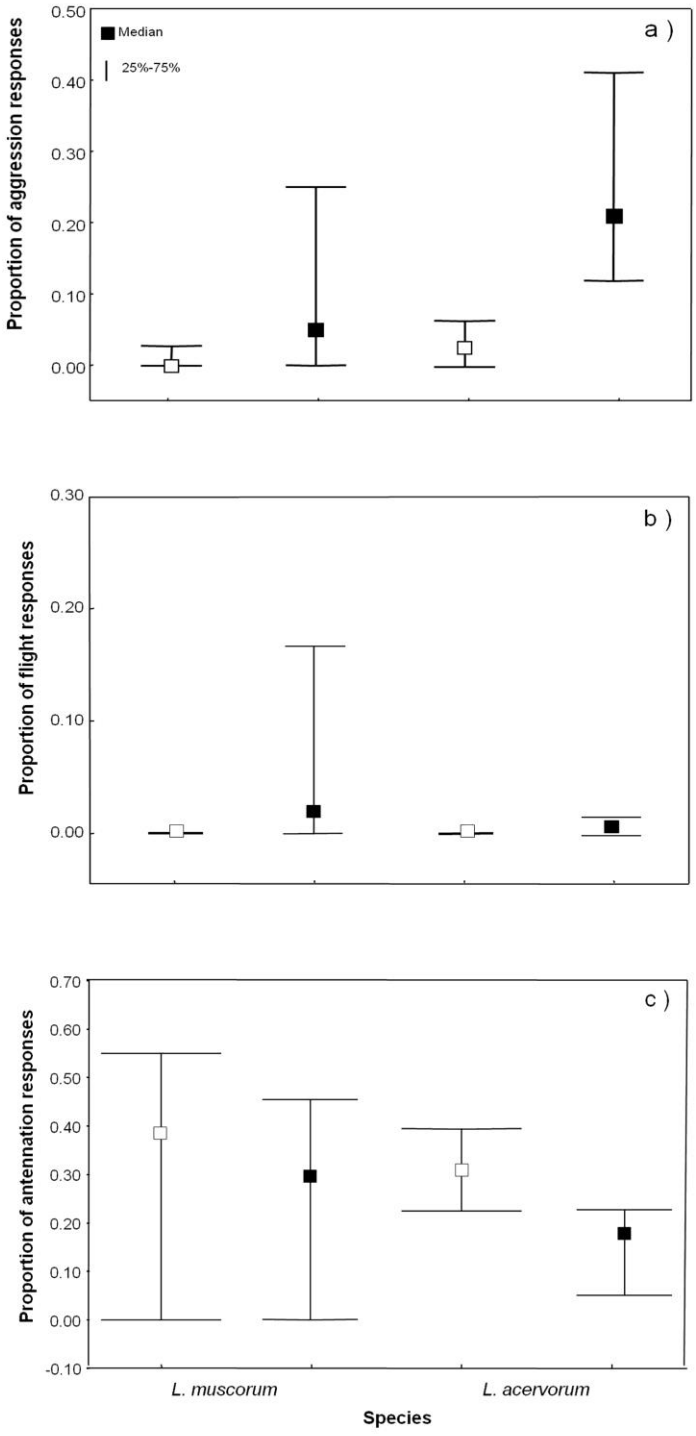

Figure 1: Aggression (A), flight responses (B) and antennation (C) of *L. muscorum* and *L. acervorum* colonies in the control situation with water (white squares) and in the experimental treatment (black squares) with the parasitic Dufour gland.

We compared the control trials with water to the trials with the Dufour gland treatment for each host population separately. *L. muscorum* colonies from Germany reacted to nestmates treated with the parasite Dufour gland secretions from either of the two populations with an increase in aggression and flight behaviour, while peaceful interactions such as antennation decreased (WIL N = 28, aggression T = 13, $P < 0.01$; flight T = 0, $P < 0.01$; antennation T = 74, $P < 0.02$). On the other hand, Italian colonies did not show an increased aggressive response or a decrease in antennation rate, yet a strong increase in flight behaviour (WIL N = 29, aggression T = 29, $P = 0.25$; flight T = 0, $P < 0.001$; antennation T = 85, $P = 0.20$).

Furthermore, we analysed whether glands of the two parasite populations elicited different changes in host colony behaviour. Host colonies from both populations reacted to the German gland secretion with remarkable increase in aggression and flight behaviour along with a strong reduction in antennation (WIL N = 37, aggression T = 16, $P < 0.0005$; flight T = 0, $P < 0.0001$; antennation T = 77, $P < 0.0001$). Yet, no changes in aggression and antennation could be detected in response to the Italian gland secretion, only a raise in flight behaviour (WIL N = 20, aggression T = 11, $P = 0.17$; flight T = 0, $P < 0.005$; antennation T = 20, $P = 0.25$).

Finally we compared the behaviour of the two experimental treatments (German / Italian gland) to detect potential interactions between host colony and parasite gland origin. We found a strong geographic effect by comparing the three behaviours (aggression, flight and antennation) with the origin of the Dufour gland (multivariate RMA: gland origin, $F_{1, 60} = 10.8$, $P < 0.003$; *L. m.* population: $F_{1, 60} = 0.60$, $P = 0.44$; Gland origin × *L. m.* population: $F_{1, 60} = 1.15$, $P = 0.29$, Fig. 2a, b, c). However, when we analysed the behaviours separately, the German host populations was shown to react more aggressively (RMA: gland origin, $F_{1, 33} = 0.85$, $P = 0.36$; *L. m.* population: $F_{1, 33} = 4.37$, $P < 0.04$; gland origin × *L. m.* population: $F_{1, 33} = 0.79$, $P = 0.38$), while flight behaviour did not vary with origin of colony or gland ($P > 0.16$). Finally, we found that antennation depended on an interaction between host population and gland origin (RMA gland origin: $F_{1, 33} = 0.03$, $P = 0.87$; *L. m.* population: $F_{1, 33} = 0.35$, $P = 0.56$; Gland origin × *L. m.* population: $F_{1, 33} = 6.91$, $P < 0.013$).

Behavioural Reactions of the Host *L. acervorum* to the Parasitic Dufour Gland Secretion

We uncovered no interpopulation behavioural differences of *L. acervorum* colonies in the control situation (Kruskal-Wallis tests: $p > 0.27$), but the ants exhibited distinct behavioural changes when confronted with the parasite Dufour gland secretion (Fig. 2).

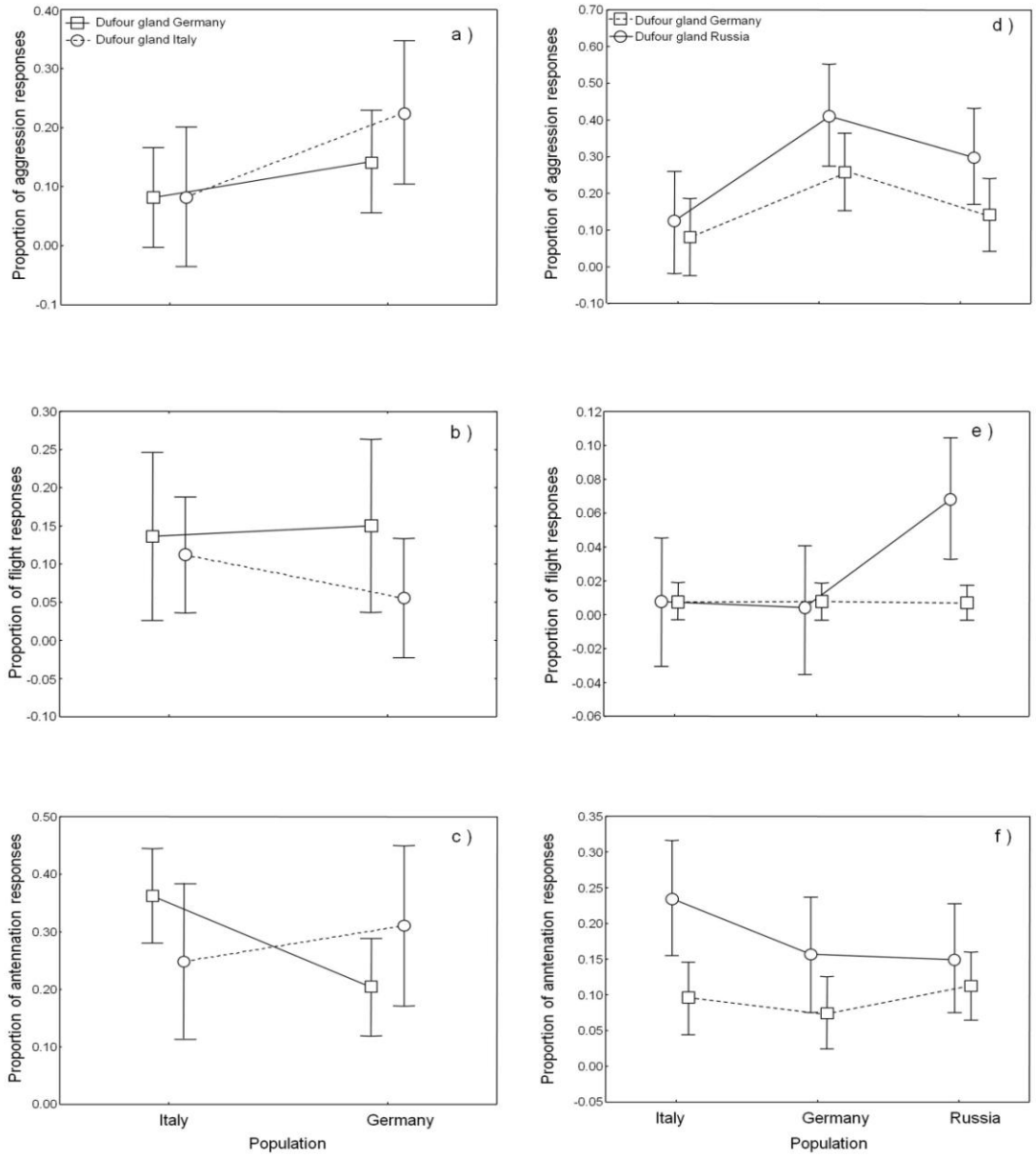

Figure 2: Aggression, flight and antennation responses of *L. muscorum* (a-c) and *L. acervorum* (d-f) colonies from various populations in response to nestmate host workers treated with the secretion of *H. sublaevis* Dufour glands from different parasite populations.

First, we compared the behaviour in the water and parasite gland treatment independent of gland origin for each host population separately. Both German and Italian *L. acervorum* colonies showed more aggression when encountering a nestmate with the Dufour gland secretion than in the control trials, while an increase in aggression was absent in *L. acervorum* colonies from Russia (WIL, Germany: T = 22, N = 19, P < 0.003; Italy: T = 26, N = 18, P < 0.009, Russia: T = 90, N = 21, P = 0.58). Yet, workers from all three populations showed less antennation towards nestmates treated with the parasite Dufour gland secretion than to those coated with water (WIL, Germany: T = 19, N = 19, P < 0.002; Italy: T = 23, N = 18, P < 0.006; Russia: T = 27, N = 21, P < 0.002). Flight reactions increased in Italian and Russian nests in the Dufour trials, while no changes in flight behaviour were detected in German colonies (WIL, Germany: T = 13, N = 19, P = 0.86; Italy: T = 1.0, N = 18, P < 0.02; Russia: T = 4, N = 21, P < 0.01).

Next, we analysed whether glands of the three parasite populations elicited different reactions in host worker behaviour. *Leptothorax acervorum* colonies reacted to the German gland secretion with a strong decrease in antennation rate, but aggressive and flight behaviour did not change (WIL: N = 25, aggression T = 112, P = 0.28; flight: T = 18, P = 0.33; antennation T = 15, P < 0.0001). In contrast, changes in flight behaviour could be reported in response to the Italian Dufour gland secretion, while the other behaviours did not change in frequency. However, this could be due to the lower sample size for trials with the Italian gland (WIL: N = 8, aggression T = 8, P = 0.16; flight: T = 0.0, P < 0.04; antennation T = 8, P = 0.16). The strongest responses in *L. acervorum* colonies were found when confronted with the Dufour gland secretion from Russian *H. sublaevis*. Flight and aggressive behaviours strongly increased, whereas less antennation could be observed compared to control trials (WIL: N = 25, aggression T = 47, P < 0.002; flight: T = 8, P < 0.03; antennation T = 62, P < 0.007).

Finally, we examined our dataset for evidence of local adaptation using a repeated measure design. By examining all three behaviours (aggression, flight and antennation), we found a strong effect of host colony origin and a trend for an interaction between host and gland origin (multivariate RMA: gland origin: $F_{2,\ 22} = 2.64$, P = 0.12; *L. a.* population: $F_{2,\ 22} = 8.40$, P < 0.002; Gland origin × *L. a.* population: $F_{1,\ 22} = 3.11$, P = 0.06, Fig. 2d, e, f). Subsequently, we analysed each behaviour separately, and found that host worker aggression depend on both host and gland origin, but the interaction between both factors was not significant (RMA: gland origin: $F_{1,\ 22} = 20.8$, P < 0.0002; *L. a.* population: $F_{2,\ 22} = 4.5$, P < 0.02; gland origin × *L. a.* population: $F_{2,\ 22} = 2.4$, P = 0.12). Flight behaviour was shown

to be influenced by host origin, an interaction between host and gland origin, yet only marginally by the origin of the Dufour gland (RMA: gland origin: $F_{1, 22} = 3.1$, $P = 0.09$; *L. a.* population: $F_{2, 22} = 3.8$, $P < 0.04$; gland origin × *L. a.* population: $F_{2, 22} = 4.2$, $P < 0.03$). Finally, the antennation rate in Dufour trials varied with the origin of the Dufour gland (Repeated measure analysis: gland origin: $F_{1, 22} = 16.9$, $P < 0.0005$; *L. a.* population: $F_{2, 2} = 0.93$, $P = 0.41$; gland origin × *L. a.* population: $F_{2, 22} = 2.00$, $P = 0.16$).

Comparison of the Responses of *L. muscorum* and *L. acervorum* colonies to the Parasite Dufour Gland Secretion

Colonies of the two host species, *L. muscorum* and *L. acervorum* behaved differently during the control situation encountering nestmates treated with water. Aggression occurred less frequently in *L. muscorum* colonies than in *L. acervorum* colonies (MWU, $N_{1,2} = 57, 21$; $U = 370$, $P < 0.009$), while no differences were found in flight and antennation behaviour (MWU, $N_{1,2} = 57, 21$; flight: $U = 552$, $P = 0.61$, antennation $U = 546$, $P = 0.56$).

We also compared the behaviours between the different host species when they encountered a nestmate treated with the gland secretion of Italian and German parasite individuals. Again, *L. acervorum* colonies showed much more aggressive responses than *L. muscorum* colonies, while the frequency of flight and antennation did not differ between species (MWU $N_{1,2} = 73, 21$; aggression: $U = 400$, $P < 0.0007$; flight: $U = 580.5$, $P = 0.09$, antennation: $U = 571.5$, $P = 0.08$).

Indeed, we were most interested in whether the parasite managed to manipulate the behaviour of the host species to different degrees in response to the parasite's chemical weapon. Thus, we analysed behavioural differences between the control and Dufour trials for each colony. *L. acervorum* colonies showed a greater elevation in aggression when confronted with the parasite gland secretion, while *L. muscorum* colonies responded with a stronger increase in flight behaviour (MWU Difference between control-Dufour trials: $N_{1,2} = 57, 21$, aggression: $U = 334$, $P < 0.002$; flight: $U = 422$, $P < 0.05$, antennation: $U = 534$, $P = 0.47$).

Interpopulation Differences in the Chemical Composition of Parasitic Dufour Gland Secretions

We detected a total of 48 compounds in the Dufour gland secretion of *H. sublaevis* workers, which were all included in the multivariate analyses. The SIMPER analysis identified eight major compounds, which contributed together to more than 90 % similarity of each population (Table 2, Fig. 3a).

Table 2: Average similarity within groups according to a SIMPER analysis. Compounds that add up to at least 90% similarity are included (RI = retention index, SD = standard deviation).

		Germany (N=8)		**Italy (N=6)**		**Russia (N=2)**
Compound	**RI**	**Av.Similarity**	**SD**	**Av.Similarity**	**SD**	**Av.Similarity**
1 Homofarnesene	1565	2.27	0.87	2.28	1.03	2.46
2 Heptadecadiene	1669	19.01	3.06	14.62	2.69	27.09
3 Heptadecene	1678	34.77	3.13	29.38	4.16	36.17
4 Heptadecane	1700	2.6	3.99	2.19	3.77	3.01
5 Nonadecane	1900	4.04	3.15	3.58	1.04	3.6
6 Heneicosane	2100	2.57	2.31	2.79	1.58	1.47
7 Tricosane	2300	2.83	1.98	4.27	1.84	1.44
8 Pentacosane	2500	0.76	1.94	1.54	1.55	0.37

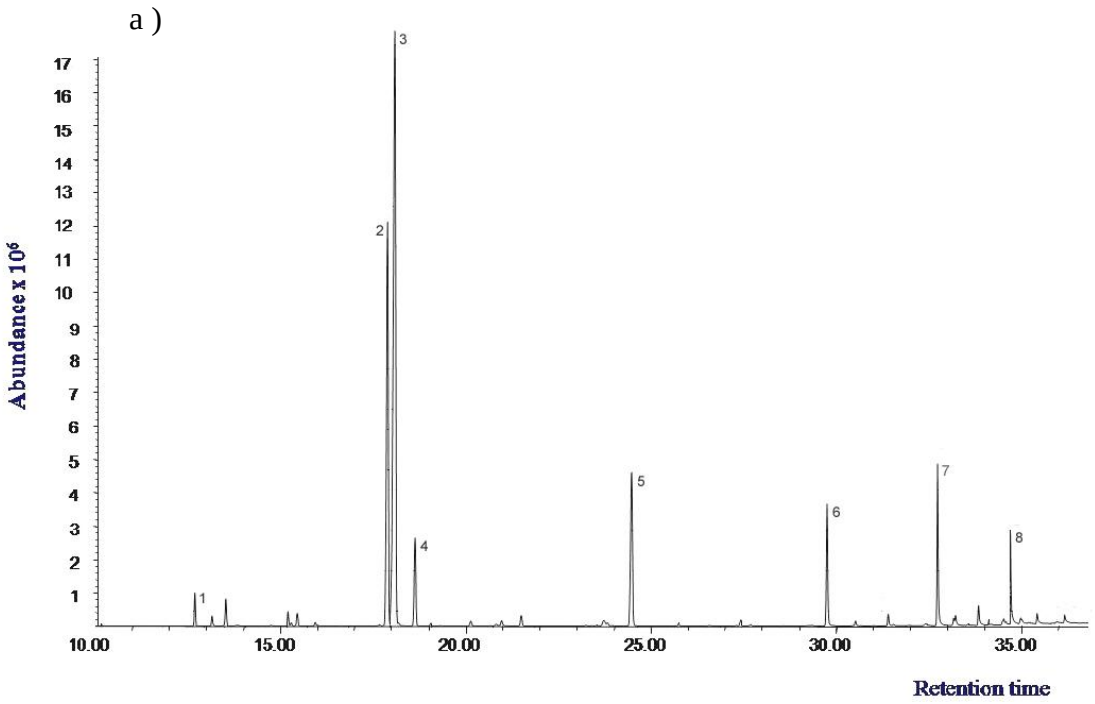

Figure 3a) Chemical profile of the Dufour gland of an Italian *H. sublaevis* worker as analysed with GC-MS. Numbers denote compounds that contribute to more than 90% of similarity of each population according to a SIMPER analysis

Based on the full data set including all 48 hydrocarbons, we uncovered differences in the chemical composition of the Dufour gland secretion from different *H. sublaevis* populations (ANOSIM Global R = 0.27; $p < 0.02$, CAP plot, Fig 3b). In particular, chemical differences in the secretion of the Dufour gland could be found between the German and Italian parasite population ($R = 0.21$, $P < 0.04$). Although sample sizes were very low, the permutation test suggested chemical differences between the Russian and Italian ($R = 0.48$, $P < 0.07$) and the Russian and German parasite populations ($R = 0.36$, $P < 0.13$).

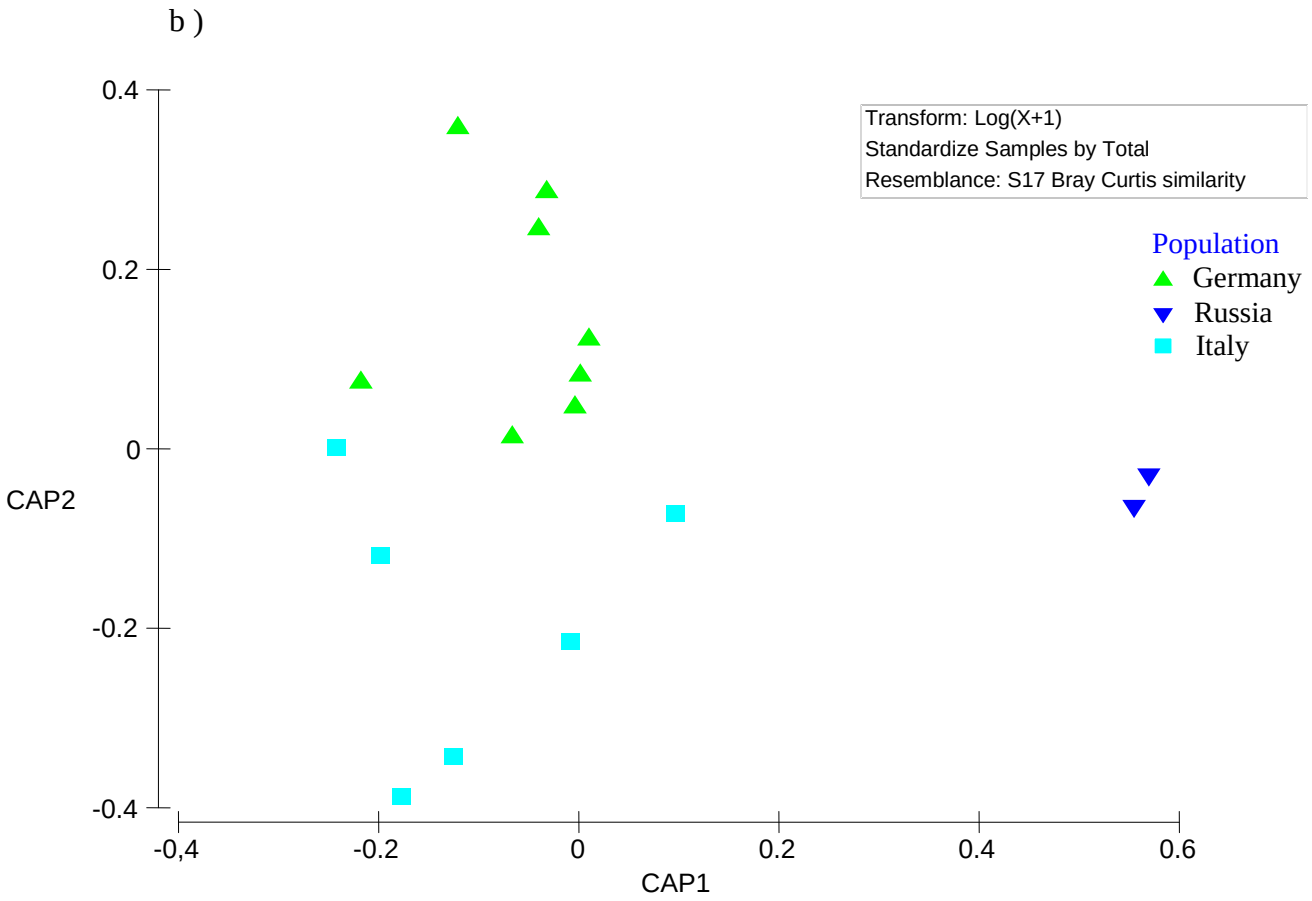

Figure 3b) Canonical analysis of principle coordinates of the chemical composition of Dufour glands from German (green rectangle), Russian (blue rectangle) and Italian (turquoise square) *H. sublaevis* workers. 62.5% of samples were allocated correctly to their group in the cross validation test.

II.V DISCUSSION

Our experiments support earlier findings of strong responses of the host *L. acervorum* to the Dufour gland secretion of its social parasite *H. sublaevis* (Allies et al. 1986; Ollett et al. 1987; Heinze et al. 1998; Foitzik et al. 2003). This glandular secretion is used by the parasite to distract host workers during colony usurpation and slave raids by eliciting flights among defending host workers, which facilitate parasite entry. This induced aggression is costly for the host, as it causes injury and death (Foitzik et al. 2003, Fischer and Foitzik 2004). Earlier studies already showed geographic differences in the interaction between *L. acervorum* and its social parasite, and this project was designed to compare the reactions of the two host species *L. acervorum* and *L. muscorum*, which are commonly used by *H. sublaevis*. Furthermore we explicitly included Dufour gland secretions from social parasites from different sites, which allowed us to reveal local adaptation in this chemical trait. In addition, we are the first to report interpopulational differences in the chemical composition of a social parasites' manipulative secretion.

Three common reactions are shown by the hosts to the parasites' glandular secretion: aggression, flight and antennation. If aggression is elicited amongst host workers, the parasite will be more successful in entering and raiding the host colony. Nestmate fights reduce host fitness not only because host workers frequently die in these fights, but also because the parasite can steal more brood (Foitzik et al. 2003). In contrast, flight behaviour of hosts could be beneficial for both opponents. The parasite is less confronted when the host flees, and host survival increases. *H. sublaevis* workers try to prohibit the flight of host workers with brood, so that fleeing host workers might save less brood. Antennation is a neutral response to the parasite chemical weapon, and was mainly observed in situations, where host workers neither fought nor fled. We interpret that host colonies, which reacted to nestmates coated with the parasite gland secretion with antennation as being immune to the parasite's chemical weapon (see also Foitzik et al. 2003).

L. acervorum is the more aggressive species of the two hosts, as shown in the control trials and in previous studies both in con- and allospecific encounters (Foitzik et al. 2003). *L. acervorum* occurs in higher densities and has a larger range and thus appears to be ecologically the more competitive species (Radchenko et al. 1999). More importantly due to their larger body size, *L. acervorum* workers are better able to fight off *H. sublaevis* attacks. They were shown to kill about 10% of the parasite workers during raids (Fischer and Foitzik 2004). In contrast to that, the smaller host *L. muscorum* reacted mainly with flight and thus

appears to focus on a fast escape. Laboratory raiding experiments and analyses of post-raid host colony survival in the field indicate that *L. muscorum* colonies were more often successfully raided, but also survived these raids generally better (Fischer-Blass et al. 2006, Böhm et al. submitted). Aggression can be an adaptive response towards social parasites, but it is clearly mal-adaptive when directed towards nestmates. Yet, our Dufour gland trials show that *L. acervorum* workers vigorously attack nestmates coated with the parasite secretion. Hence, the behaviour of the larger host is easily manipulated by the parasites' secretion, in contrast to the smaller host, which more often fled. This might be due to the parasite adapting its chemical weapon mainly to the larger host *L. acervorum*, whose nests are better defended.

We also found differences between *L. acervorum* populations in the aggressive responses towards the parasitic gland secretion, as indicated by earlier work (Foitzik et al 2003). Russian *L. acervorum* did not attack nestmates coated with the parasites Dufour gland secretion, while we found highly aggressive reactions in German and Italian colonies. Furthermore, the Russian and Italian host colonies reacted with flight, a response absent from *L. acervorum* colonies from Germany. Attacks among nestmates were most pronounced in German host colonies in response to the parasite's chemical weapon, possibly indicating mal-adaptation of this host population. In contrast, Russian hosts did not show nestmate attacks, but generally fled - a more adaptive reaction to parasite attacks. Interestingly, similar interpopulational differences were found for the smaller host *L. muscorum*. Again, the German host populations reacted more aggressively towards treated nestmates than the Italian hosts. Taken together the parasite was well able to manipulate the behaviour of both German host populations, indicating that in this trait the hosts were lagging behind in the coevolutionary arms race.

Local adaptation, the situation where the mean fitness of a population is higher in its home locality than in any other environment (Kaltz and Shykoff 1998), is a common phenomenon in geographically structured antagonistic species interactions (e.g. Springer 2007, Rieder et al. 2008). The cross-fostering design of our experiments allowed us to investigate local adaptation, i.e. whether host behaviour was influenced by an interaction between the origin of the host colony and the parasitic gland. Such local adaptation was evident in the Russian *L. acervorum* population, which reacted with flight only to the gland of the sympatric parasite. As flight is considered an adaptive host response, we conclude that the Russian *L. acervorum* population is well adapted to its local opponent. Our analysis of the *L. muscorum* Dufour trials revealed that antennation behaviour was influenced by an interaction between origin of host and parasite, indicating local effects also in the smaller

host. Here, sympatric parasites elicited less neutral responses, that is local hosts reacted more with either flight or fight than with antennation, suggesting that the parasite is well-adapted to its local *L. muscorum* host. The different degrees of local adaptation in this host-parasite system matched the predictions from earlier population genetic analyses (Brandt et al. 2007). High genetic variability and migration rates lead to the expectation that the larger host *L. acervorum* should be locally adapted to its parasite, whereas *H. sublaevis* with its higher evolutionary potential should be able to adapt to the genetically impoverished populations of the smaller host *L. muscorum* and this is precisely what we found in this behavioural study.

In addition to these local effects, we uncovered strong differences in the potency of the Dufour gland secretion between different *H. sublaevis* populations and against different host species. In particular, the secretion from German *H. sublaevis* workers elicited highly aggressive responses in the host *L. muscorum*, while the larger host *L. acervorum* was less manipulated to attack nestmates. Hence the German social parasite population appears to focus on the smaller host *L. muscorum*, which was also evident from our community composition analysis. Italian parasitic glands caused flight responses in both host species, yet this chemical weapon was less able to manipulate host workers to attack nestmates. Furthermore, while the *H. sublaevis* gland secretion from Germany elicited aggression in *L. muscorum* colonies, the Italian gland failed to manipulate behaviour of this host. In contrast, gland secretions from either parasite population did not induce nestmate attacks in the larger host *L. acervorum*. Yet, aggressive reactions in *L. acervorum* colonies were caused by the gland secretion for Russian slavemakers. In conclusion, the potency of the parasitic weapon as indicated by host responses was influenced by specialization of the parasite to a particular host or its sympatric host population. The evolution of host counter-defences was shown in the flight reaction of Russian *L. acervorum* to the secretion of its local parasite.

The main finding of the chemical analyses was that we uncovered interpopulational differences in the composition of the Dufour gland secretion. The glandular secretion from German *H. sublaevis* workers showed a different composition from Italian ones and indeed glands from the two populations elicited different behavioural responses especially in the host *L. muscorum*. The comparison to the Russian population was hampered by a very low sample size, but our preliminary analysis already indicates chemical differences to the two other populations.

Eight alkanes and alkenes were responsible for population differences. Homofarnesene was among those hydrocarbons, which was assumed to be biologically active in the context of host manipulation (Ollet et al. 1987). In contrast, Heinze et al. (1998) suggested that the

activity of the "propaganda substance" in *H. sublaevis* results from species and colony specificity rather than from a single toxic or repellent agent. Ants exhibit an extreme diversity of chemical compounds in the Dufour gland both within and among species indicating that glandular secretions are flexible and can undergo rapid evolutionary changes (Ali et al. 1987; Ollett et al. 1987; Ali et al. 1989; Bagnères et al. 1991; Bestmann et al. 1995; Visicchio et al. 2000; Morgan et al. 2003). Population genetic analyses demonstrated a restriction in gene flow between different *H. sublaevis* populations (Brandt et al. 2007). Hence chemical differences can be due to different ecology (unlikely as ants were kept in the laboratory under standardized conditions), genetic drift or adaptation. The behavioural tests revealed variation in host responses to the secretion of different parasite populations, pointing to adaptation in the context of an evolutionary arms race as the cause for these differences. The development of this chemical weapon is part of a remarkable suite of adaptations in the social parasite *H. sublaevis* to facilitate host exploitation. Restrictions in gene flow between host and parasite populations lead to an independence of different geographic sites, so that local adaptation in the parasite and hosts lead to a complex evolutionary arms race in this fascinating trait of a parasitic "propaganda substance".

II.VI ACKNOWLEDGEMENT

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CHAPTER III

An ant social parasite in-between two chemical disparate host species

(Sabine Bauer, Melanie Böhm, Volker Witte, Susanne Foitzik)

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III.I ABSTRACT

Host-parasite coevolution shapes the structure of communities and simultaneously the traits of the interacting species. Social parasites developed sophisticated chemical integration strategies to circumvent host defences. Here, we show that the two *Leptothorax* host species of the obligate social parasite *Harpagoxenus sublaevis* exhibit extremely divergent chemical profiles, making it nearly impossible for this parasite to closely adapt to both hosts at once. Our cuticular hydrocarbon analyses demonstrate that *H. sublaevis* acquires some host chemicals passively, but additionally, actively biosyntheses some host hydrocarbons. The parasite adjusts thereby more closely to its smaller host, *L. muscorum*, because it actively produces two of its cuticular substances and also more easily acquires the short-chained hydrocarbons of this host. Community composition analyses indicate that the social parasite overexploits this chemical closer host species and, albeit costly for the parasite, frequently enslaves workers of the second host concurrently.

III.II INTRODUCTION

Social insects have evolved highly sophisticated recognition systems, which enables them to behave altruistically towards relatives, but to reject alien individuals. The ability to recognize nestmates impedes the exploitation of their societies from parasites and predators (Wilson 1971). Cuticular hydrocarbon profiles, a diverse blend of surface chemicals are responsible for nestmate recognition and communication in social insects (Howard and Blomquist 2005; Lahav et al. 1999; Vander Meer and Morel 1998). This hydrocarbon signature of a colony is maintained by individual production and storage of chemical compounds in the post-pharyngeal gland and through exchange via active or passive contact (Kaib et al. 2000). Recent studies indicate that ants and other social hymenopterans recognize intruders by the presence of undesirable odours on their cuticle rather than by the absence of colony or species specific recognition cues (Couvillon and Ratnieks 2008; Guerrieri et al. unpublished ms). This explains why many social parasites e.g. *Polyergus* slavemakers use a strategy called chemical insignificance, where they carry almost no detectable recognition cues on their cuticle (D'Ettorre and Errard 1998; Johnson et al. 2001; Lambardi et al. 2007).

In general, social parasites, which exploit the social behaviours of other species, have evolved sophisticated strategies to bypass the recognition system of their hosts (Lenoir et al. 2001). As mentioned above, young *Polyergus* queens harbour few hydrocarbons on their cuticle. Hence their detection is hampered by the absence of detectable chemical cues. In contrast, chemical mimicry or camouflage (Dettner and Liepert 1994; Lenoir et al. 2001) describe strategies whereby a parasite attempts to imitate host recognition cues, either by actively producing the host cuticular hydrocarbons or by passively acquiring these chemicals through direct contact with its host. The parasitic large blue butterfly *Maculinea alcon* coevolved to mimic the cuticular hydrocarbon profile of its main host ant *Myrmica rubra* (Akino et al. 1999; Nash et al. 2008) and another species of lycaenid butterfly larvae even exhibits chemical mimicry to a certain caste of its host ant (Hojo et al. 2009). This imitation of host chemicals rather than chemical insignificance suggests that in some cases (host) ants are able to detect enemies by the absence of desirable cues.

Here, we investigate chemical adaptation of the Eurasian slavemaking ant *Harpagoxenus sublaevis* to its two main host species, *Leptothorax acervorum* Fabricius 1793 and *Leptothorax muscorum* Nylander 1846 (Collingwood 1971; Ratschenko et al. 1999). Slavemaking ants interact with their hosts in three different situations, which vary in the degree by which chemical resemblance is important: colony usurpation by mated queens,

slave raids by slavemaking workers and interaction between slavemakers and their slaves in established parasite colonies (Brandt et al. 2005a). Slave-making workers frequently encounter host colonies during raids with open aggression, suggesting that chemical resemblance is of less importance. However, behavioural experiments with *H. sublaevis* colonies indicate that matching host profiles also lowers host defences in the context of slave raids (Fischer and Foitzik 2004). Chemical adaptation to host odour is especially important for young Formicoxine slavemaker queens, which enter an ant host colony on their own and which often rely on not being recognized as an intruder. In addition, to guarantee a smooth functioning of the slave-making ant colony, the parasite should ensure that parasite and host workers mutually adapt their profiles to create a common colony odour (Lenoir et al. 2001). *Polyergus* slave-making workers adjust their profiles to that of their enslaved host workers (D'Ettorre et al. 2002), while reciprocal adaptation of the chemical profile was shown for the slave-making ant *Protomognathus americanus* and its co-inhabiting *Temnothorax* slave workers (Brandt et al. 2005b). Similarly, the presence of the social parasite *Rossomyrmex minuchae* influenced the chemical profile of their *Proformica longiseta* slave workers, which are clearly chemically distinct from conspecific non-enslaved workers (Errard et al. 2006).

Gaschromatographical analyses revealed that workers of our study species *H. sublaevis* workers adopt the odour of their *L. acervorum* or *L. muscorum* slaves to an extent that they are undistinguishable from their hosts (Kaib et al. 1993). Yet, *H. sublaevis* colonies regularly contain slaves of both host species, resulting in a heterogeneous colony odour, as the parasite can not match the odours of both hosts concurrently. In these mixed parasite colonies interactions among workers of the three species were frequently aggressive, indicating that slave and slavemaker workers are able to differentiate between the different species despite mutual chemical adaptation. This intracolonial aggression should be costly for the parasite as it lowers colony productivity (Heinze et al. 1994). Here, the down-side of exploiting two host species concurrently becomes apparent. Other problems arise for slavemaking workers or queens, when attacking unparasitized host colonies. If indeed, alien intruders are recognized by their undesirable cues, than slavemakers from mixed nests – which adopt cues from both hosts - are recognized as foreign by colonies of either host species. In addition, parasites with slaves of one species should have great difficulties attacking the respective other host.

Yet, in all *H. sublaevis* populations analysed so far, the parasite uses both host species and about a third of all slavemaking colonies contain slaves of both hosts (Brandt et al. 2007; Fischer and Foitzik 2004; Foitzik et al. 2003). Many slavemaking species (e.g. *Polyergus* and

Protomognathus) exploit two or three host species in a community (Bono et al. 2006; Foitzik et al. 2001), while other, especially the Mediterranean slave-making ants of the genus *Chalepoxenus* and *Myrmecoxenus* invariably use only a single host species at a given site (Buschinger et al. 1988). *Chalepoxenus muellerianus* (Finzi 1922) was found to enslave up to 12 or more *Temnothorax* species, but parasite populations regularly specialize on a sole host. *C. muellerianus* queens are imprinted on the host odour and selectively parasitize host colonies of the species they were raised with (Beibl et al. 2007).

Here, we analyse the chemical basis of how the slavemaking ant *H. sublaevis* exploits the two different host species, *L. acervorum* and *L. muscorum*, using gas chromatography and mass spectrometry. In particular, we were interested in whether the social parasite actively synthesizes host hydrocarbons or passively acquires these compounds from its slaves. Active production of host hydrocarbons would only be adaptive if both hosts show similar chemical profiles. On the other hand, host species might benefit from divergent chemical cues, as this would make concurrent exploitation of both hosts difficult. In addition, we studied two different ant communities to investigate potential local adaptation to host populations, which was indicated in a behavioural study (Fischer and Foitzik 2004). Finally, we used community and colony composition data to investigate whether *H. sublaevis* more frequently exploits one of its two host species. If yes, we would expect a closer chemical resemblance of the cuticular hydrocarbon profile of the parasite to this overexploited host. In addition, we analysed whether enslaved host workers exhibit a chemical profile distinct from non-enslaved host workers, which would indicate that the parasite also influences host profiles.

III.III MATERIAL AND METHODS

Study sites and colonies

The ant colonies were collected in the summer of 2006 at one study site in Bavaria, Germany (Abensberg N 48° 49’ 0.52’’, E 11° 50’ 46.25’’; Nuremberg N 49° 27’ 1.78’’, E 11° 4’ 50.67’’) and at an alpine site in South Tyrol, Italy (San Candido / Innichen N 46° 44’ 2.11’’, E 12° 16’ 48.81’’). Ant colonies were transported in their natural nesting site (pine logs or twigs) to our laboratory in Munich and stored in clean glass vials at -20°C.

We investigated the cuticular hydrocarbon profiles of all three species from the two different communities and invariably analysed only a single individual of each colony (Table 1).

Table 1: Sample sizes (N of workers) for our gas chromatographical analyses of cuticular hydrocarbons. We invariably analysed only a single worker of each species per colony. *L. muscorum* and *L. acervorum* slaves were taken from the same mixed *H. sublaevis* colonies.

Species		Slave species	Germany	Italy	Total per species
H. sublaevis		*L. muscorum*	3	3	26
		L. acervorum	3	4	
		mixed	12	1	
L. muscorum	non-enslaved		37	23	89
	enslaved	*L. muscorum*	7	5	
		mixed	15	2	
L. acervorum	non-enslaved		31	17	74
	enslaved	*L. acervorum*	7	2	
		mixed	14	3	

Our gas chromatographic analyses were based on hexane extractions of 189 ant workers. In particular, we analysed the cuticular hydrocarbon profiles of 18 German and eight Italian *H. sublaevis* workers. Furthermore, we examined the chemical profiles of a total of 89 *L. muscorum* workers (59 from Germany and 30 from Italy). 29 of these workers were found enslaved in *H. sublaevis* nests. Additionally, we analysed the chemical profiles of 74 *L. acervorum* workers (52 from Germany and 22 from Italy), of which 26 lived as slaves in *H. sublaevis* colonies.

H. sublaevis colonies can either contain slaves of a single host species (*L. muscorum* or *L. acervorum*) or both species and we noted for each slave and *H. sublaevis* worker, which we analysed, the composition of the slave workforce from its native nest. Consequently, we could investigate how the presence of each slave species influences slave and parasite profiles. In addition, we contrasted the chemical profiles of non-enslaved and enslaved host workers to examine whether slaves exhibit an altered profile due to the acquisition of chemical substances from *H. sublaevis* or allospecific slaves.

Beside our chemical analyses, we investigated the community composition of the two study sites to explore preferences of the slave-making ant for a particular host species and parasite pressure on either host. These composition data were based on ant collections in both communities from 2000 to 2006 (Fischer and Foitzik 2004) and were analysed using χ^2 - tests.

We contrasted in each community the number of host colonies of either species with the number of parasite nests containing slaves of the respective host. About a third of all *H. sublaevis* colonies exploited two host species concurrently that is we found slaves of both hosts in their nests in varying proportions. To include these frequent mixed *H. sublaevis* colonies in our analyses, we calculated the sum of slaves of the two species over all mixed nests. Consequently these mixed colonies were allocated to one of the two host species according to the overall ratio of slaves in mixed colonies. For example, if 70% of all slaves from mixed parasite colonies belonged to the species *L. acervorum*, 70 % of these mixed nests were grouped to single host *L. acervorum* slave-making ant nests, the remaining to pure *L. muscorum* parasite nests. Host specialization could also be explained by the level of chemical adjustment to either host species, as the profiles of the social parasite might resemble the cuticular hydrocarbon composition of one host species better than that of the other.

Chemical analyses

Ants were individually immersed in 20µl hexane for three minutes, to dissociate hydrocarbons from the cuticle. To gain optimal yields we placed glass vials in a hot water bath at 50 °C at constant agitation. For the chemical analyses of our extractions we used coupled gas chromatography and mass spectrometry (GC-MS) on an Agilent Technologies 6890N GC and 5975 MSD equipped with a Restek Rxi-5 MS column (30 m length, 0.25 mm ID, 0.25 µm film thickness). Injection was splittless over 1 minute at 280 °C under a pressure pulse of 10.52 psi for 0.5 minutes followed by automatic flow control at 1.0 ml/min with helium as carrier gas. The oven program started isothermally at 100 °C for 3 minutes, subsequently increased rapidly by 30 °C/min up to 220 °C, followed by a moderate temperature ramp of 3 °C/min until 300 °C and finally stayed isothermal for 3 minutes. The transfer line was held at 310 °C. A range of 50-500 u was scanned after an initial solvent delay of 3.8 minutes.

We then statistically compared the hydrocarbon profiles of all three species with the software Primer 6 (Version 6.1.6, Primer-E). Before importing data into Primer 6, profile peak areas were integrated using Chemstation Version G1701 DA D.03.00.611 (Agilent Technologies). Since extraction yields varied markedly between the individual ant samples, we equalized the samples for multivariate analyses by focussing on high abundance compounds only. Therefore, peaks with an area below 20% of the largest peak in each sample were omitted. We then identified the hydrocarbon peaks to be included in analyses by their

characteristic mass spectra and by comparison with Wiley 7N mass spectral library. Peak areas were standardized afterwards to the sample total and fourth root transformed. Hereupon, we calculated a resemblance matrix, based on Bray Curtis similarities, which we used for further statistical analyses. We tested our dataset for differences between groups using an ANOSIM, a non-parametric test of similarities.

Inter-specific comparisons were subsequently performed including only the most characteristic host peaks. These peaks were identified with a SIMPER analysis, determining those which together contributed to 90% of the species group similarity. Next, we compared the occurrences of these characteristic compounds between non-enslaved *L. acervorum* and *L. muscorum* host workers. In a second step, we analysed how frequently these characteristic host substances occurred in *H. sublaevis* profiles from single slave species nests. These presence / absence comparisons were conducted with Fisher-Exact-Tests based on the raw data set including all minor peaks (in contrast to the 20% filter criterion used for multivariate comparisons described above). Hereby we investigated whether characteristic substances of one host species were also present in the profiles of the slavemaking ant workers living with the respective other host species. The latter comparison reveals whether the *H. sublaevis* actively produces distinctive host substances.

Chemical analyses uncovered also additional groups of chemicals such as octadecenoic acid and linoleic acid ethyl ester on the cuticle of ant workers. These substances were mainly found on *L. acervorum*, in low amounts on *L. muscorum* and never on *H. sublaevis*. We did not include these non-hydrocarbon substances in our multivariate analyses, because they are ubiquitous natural compounds. Cuticular profiles of insects are generally composed of long-chained hydrocarbons and we therefore analysed only hydrocarbons with chain lengths of C_{21} or more (Carlson et al. 1998). Albeit we also detected short chained hydrocarbons such as heptadecene and heptadecadiene in the ant profiles, we did not include these substances in our analyses, because they are known as components of the Dufour's gland secretion (Ollett et al. 1987).

III.IV RESULTS

Community composition data

At the German study sites, the host community was composed of *L. acervorum* (409 colonies, 54 %) and *L. muscorum* (347 colonies, 46 %) in roughly similar proportions. In the Italian community the larger host *L. acervorum* was with 70% (187 colonies) more common than *L. muscorum* with 30% (80 colonies; Comparison between communities: $\chi^2_1 = 20.61$, $P < 0.0001$). Parasite abundances were very high with one social parasite colony per 6.1 host colonies in Germany and 1 : 5.9 host colonies in Italy ($\chi^2_1 = 0.02$, $P = 0.89$).

In both ant communities, *H. sublaevis* colonies frequently contained slaves of both host species. These three-species mixed colonies made up about one third in Germany (35 %, N = 44) and Italy (27 %, N= 12, $\chi^2_1 = 1.16$, $P = 0.28$). The remaining parasite colonies contained only slaves of a single host species. *H. sublaevis* nests with only *L. acervorum* slaves occurred in Germany with 30 % and in Italy with 35 %. As frequent were parasite colonies with only *L. muscorum* slaves (Germany 35 %, Italy 38 %). Moreover, the two communities did not differ in the frequency of either pure *L. muscorum* or *L. acervorum H. sublaevis* nests ($\chi^2_1 = 0.11$, $P = 0.74$).

Both host communities contained a smaller proportion of *L. muscorum* nests, yet absolute numbers of single slave species *L. muscorum* and *L. acervorum* slave-making nests were similar. This indicates an overexploitation of the smaller host *L. muscorum* and which was much more pronounced in Italy (Germany $\chi^2_1 = 2.83$, $P = 0.09$; Italy $\chi^2_1 = 1.30$, $P < 0.001$). For the smaller host *L. muscorum* parasite-host ratios were 1 : 5.2 in Germany and 1 : 3.2 in Italy and for *L. acervorum* these ratios were 1 : 7.2 in Germany and 1 : 9.4 in Italy.

General findings of chemical analysis

To investigate general differences between the slave-making ant *H. sublaevis* and its hosts *L. muscorum* and *L. acervorum*, we compared the cuticular hydrocarbon profiles between species (independently of their origin or status, i.e. parasite and enslaved / non-enslaved hosts from Italy or Germany). We found that all three species exhibited distinct profiles, that could be clearly separated statistically (Anosim: N = 189, Global R = 0.738, P < 0.001; *Hs.* – *L. m.*: R = 0.446; P < 0.001; *Hs.* – *L. a.*: R = 0.580; P < 0.001; *L. m.* – *L. a.*: R = 0.887; P < 0.001). The profiles of the two host species differed most, while the profiles of the social parasite lay in-between its two hosts (Fig. 1). Multidimensional scaling illustrates that *H. sublaevis* profiles were closer to those of its smaller host, *L. muscorum* (Fig. 2).

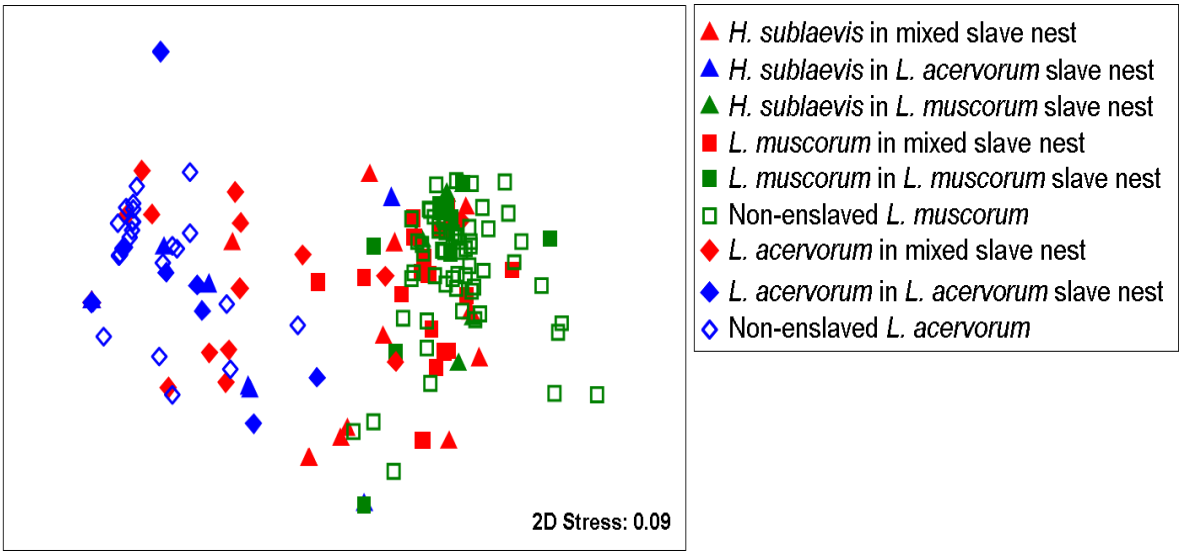

Figure 2: Species-specific differences visualized in a non-metric, multidimensional scaling (NMDS) plot based on the cuticular hydrocarbon components of workers from the slavemaker *H. sublaevis* (red symbols), and its hosts *L. muscorum* (green symbols) and *L. acervorum* (blue symbols).

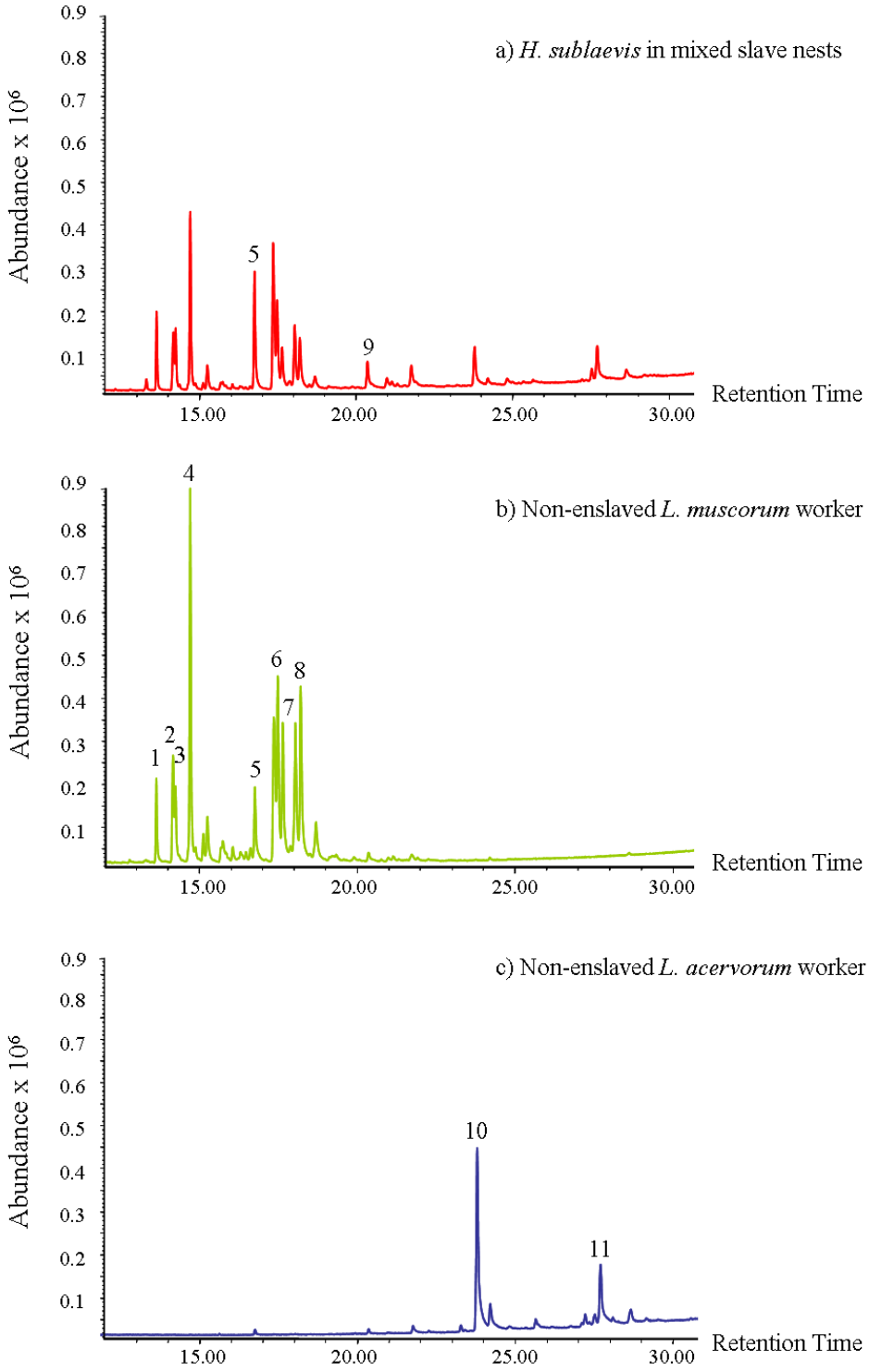

Figure 1: Cuticular hydrocarbon profiles of a *H. sublaevis* worker living in a mixed slave nest and of non – enslaved *L. muscorum* and *L. acervorum* host workers. Characteristic substances for each species are marked with a number, which correspond to hydrocarbons listed in Table 2.

Table 2: Characteristic compounds in the cuticular profiles of non-enslaved *L. muscorum* and *L. acervorum* as identified by a 90% SIMPER analysis. For each substance and host species, the frequency of occurrence in worker profiles is given. Presence / absence of peaks were compared with Fisher-exact test, p –values are reported. Substances in bold are characteristic for *H. sublaevis* profiles.

				L. muscorum	***L. acervorum***
Substance		**Retention time**	**Fisher exact test (df=1)**	(%) N = 59	(%) N = 48
1	(C_{23}) Tricosane	13.7	0.0001	97	2
2	(C_{23})11-Methyl Tricosane	14.2	0.0001	73	13
3	(C_{23}) 9-Methyl Tricosane	14.3	0.0001	86	0
4	(C_{24})3-Methyl Tetracosane	14.8	0.0001	98	0
5	**(C_{25}) Pentacosane**	16.8	0.0001	88	6
6	(C_{25})11-Methyl Pentacosane	17.4	0.0001	93	6
7	(C_{25}) 7-Methyl Pentacosane	17.5	0.0001	88	0
8	(C_{25}) Multi branched	18.3	0.0001	68	0
9	**(C_{27}) Heptacosane**	20.4	0.53	27	33
10	(C_{29}) Nonacosene	23.8	0.0001	0	96
11	(C_{31}) Hentriacontene	27.8	0.0001	0	67

Influence of slave species on *H. sublaevis* profiles

In addition, our analyses clearly demonstrate that slave species influenced the odour of the social parasite. Slave-making workers from colonies with only *L. muscorum* slaves differed in their profile from slave-making workers with only *L. acervorum* as slaves (N = 13, Global R = 0.614, P < 0.003). *H. sublaevis* workers from colonies with both slave species lay in-between the profiles of workers of single slave species nests. Consequently, they could statistically not be distinguished from parasite workers with only one slave species (*H. sublaevis* with *L. muscorum* only vs. mixed: N = 19, Global R = -0.017, P = 0.485; *H. sublaevis* with *L. acervorum* only vs. mixed: N = 20, Global R = 0.104, P = 0.138). Profile shift of the parasite living in a one-slave species nest or in a mixed slave species nest is shown in Fig. 3.

Comparison of *H. sublaevis* hydrocarbon profiles to enslaved host workers

Next we investigated chemical differences between slave-making workers and their slaves. The profiles of *H. sublaevis* workers did not differ from their slaves in parasite nests with a single host species, while the parasite was unable to adapt its odour to two co-occurring slave species (Table 3).

Table 3: Non-parametric ANOSIM comparisons between the relative proportions of cuticular hydrocarbons of the profiles of *H. sublaevis* workers and enslaved host workers from single or mixed species colonies. Significant results are shown in bold. For sample sizes please refer to Table 1.

	***L. acervorum* (single)**		***L. acervorum* (mixed)**		***L. muscorum* (single)**		***L. muscorum* (mixed)**	
	R	P	R	P	R	P	R	P
***H. sublaevis* (*L. a.* slave)**	0.033	0.298	0.046	0.277	**0.652**	**0.002**	**0.765**	**0.001**
***H. sublaevis* (*L. m.* slave)**	**0.837**	**0.001**	**0.503**	**0.001**	-0.068	0.654	0.03	0.338
***H.sublaevis* (mixed slaves)**	**0.431**	**0.002**	**0.316**	**0.001**	**0.195**	**0.012**	**0.296**	**0.001**

Comparison of *H. sublaevis* hydrocarbon profiles to non-enslaved host workers

H. sublaevis queens and raiding parties are frequently confronted with non-enslaved host colonies and here we analyse how well these slave-making ants match the profiles of non-enslaved hosts. *H. sublaevis* workers from mixed parasite colonies differed from non-enslaved host workers of both species (for *L. muscorum*: N = 73, Global R = 0.547, P < 0.001 and for *L. acervorum*: N = 61, Global R = 0.797, P < 0.001), whereas slave-making workers from colonies with only a single slave species were closer to their respective non-enslaved hosts. In particular, we could not statistically separate *H. sublaevis* workers from nests with only *L. muscorum* slaves and non-enslaved *L. muscorum* workers (N = 66, Global R = -0.009, P = 0.473; Fig. 3). In contrast, *H. sublaevis* workers from colonies with only *L. acervorum* slaves differed significantly in their cuticular hydrocarbon profile from non-enslaved *L. acervorum* workers (N = 55, Global R = 0.483, P < 0.004).

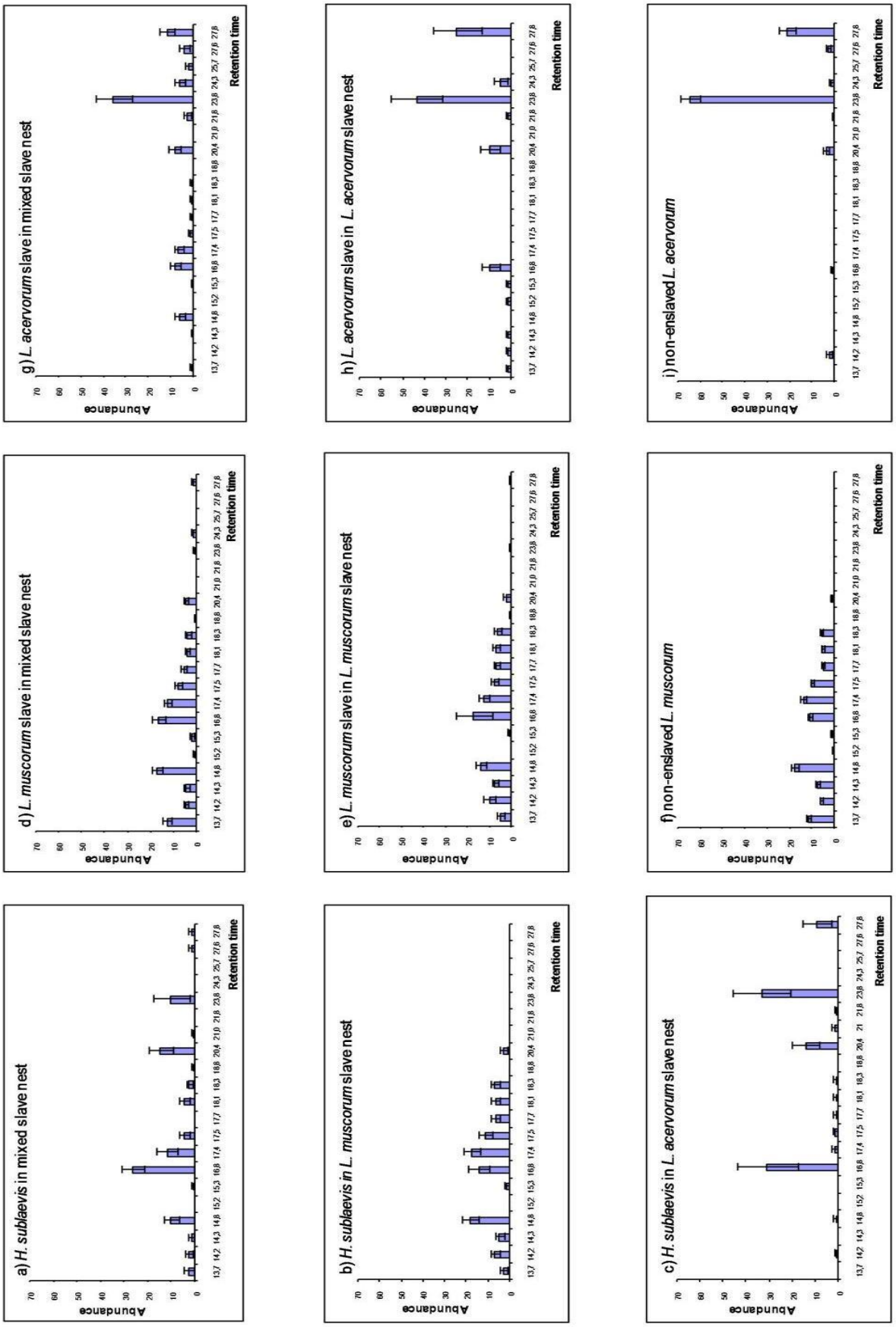

Figure 3: Mean values of peak abundance for characteristic peaks of *H. sublaevis* in mixed or single slave species nest, and enslaved and non-enslaved host species *L. muscorum* and *L. acervorum*.

In a cross-study of profiles we compared profiles of the parasite with only one host species either *L. muscorum* or *L. acervorum* as slaves with non-enslaved host profiles of the respective other host species, within one population and between both populations. All these profiles differed significantly from each other (for all comparisons: $P < 0.005$).

Comparison of hydrocarbon profiles of enslaved and non-enslaved host workers

Enslaved workers from colonies with only a single slave species could not be distinguished from enslaved workers of the same species living in colonies with both host species as slaves (for *L. muscorum*: N = 29, Global R = 0.078, P = 0.10, for *L. acervorum*: N = 26, Global R = -0.043, P = 0.655).

Next, we compared enslaved host workers with non-enslaved host workers. Our results showed, that non-enslaved *L. muscorum* workers exhibit a similar hydrocarbon profile to *L. muscorum* slaves from single (N = 72, Global R = 0.015, P = 0.372) or mixed slave species *H. sublaevis* nests (N = 77, Global R = 0.076, P = 0.155). In contrast, *L. acervorum* slaves clearly differed from non-enslaved *L. acervorum*, when these slaves lived in a mixed slave species nest (N = 65, Global R = 0.353, $P < 0.001$). Yet, when living in a single slave species nest, *L. acervorum* slaves differed less, but still significantly from non-enslaved *L. acervorum* (N = 57, Global R = 0.246, $P < 0.025$).

Comparison between the profiles of the different host species

Furthermore, we were interested in hydrocarbon profile differences between the two host species (Fig. 1, 3). Comparisons showed that the profiles of the two non-enslaved host species differed strongly from each other, and also when living together as slaves in one colony (for non-enslaved host workers: N = 108, Global R = 0.972, $P < 0.001$; for enslaved host workers in one colony: N = 34, Global R = 0.594, $P < 0.001$).

Interpopulation variation in hydrocarbon profiles of the three species

The profiles of non-enslaved *L. muscorum* workers from Germany and Italy differed strongly (N = 60, Global R = 0.222, $P < 0.001$). Yet, the profiles of slave-making workers, enslaved and non-enslaved *L. acervorum* workers as well as of enslaved *L. muscorum* workers did not vary with geographic origin (for all comparisons: $P > 0.22$).

Analysis of characteristic peaks in cuticular hydrocarbon profiles of the three species

Detailed analyses of the cuticular hydrocarbons demonstrated that some chemical compounds occur on the cuticle of workers of all three species, while others were clearly species-specific (Table 3, Fig. 3).

A simper analyses revealed eleven characteristic substances for either of the two host species and univariate comparisons supported the species specificity of these hydrocarbons (Fisher-Exact-tests: $N_{1,1}$= 59, $N_{2,1}$ = 48, $P_{global} < 0.001$). In particular, two long-chained hydrocarbons nonacosene (C_{29}) and hentriacontene (C_{31}) are characteristic for *L. acervorum*, but were never found in *L. muscorum* profiles. These substances were also absent on the cuticule of *H. sublaevis* workers, which enslaved only *L. muscorum* slaves, indicating that they were either never actively produced by the parasite or only in the presence of *L. acervorum* slaves (Fisher-Exact-test: $N_{1,1}$= 6, $N_{2,1}$ = 48, $P_{global} < 0.03$). The characteristic profiles of *L. muscorum* workers were composed of eight short-chained hydrocarbons from tricosane (C_{23}) to branched pentacosane (C_{25}). Four of these short-chained hydrocarbons were completely absent in the profiles of non-enslaved *L. acervorum* and four occurred in less than 15% of *L. acervorum* profiles. One of these hydrocarbons, n-pentacosane was as frequently present on the cuticle of non-enslaved *L. muscorum* workers as on parasite workers from colonies with only *L. acervorum* slaves. As *H. sublaevis* can not obtain this C_{25}-hydrocarbon from its *L. acervorum* slave workers, whose profiles generally do not exhibit this compound, it must be actively synthesized by *H. sublaevis*. The remaining characteristic compounds of *L. muscorum* were not regularly found on *H. sublaevis* workers from *L. acervorum* slave nests (Fisher-Exact-test: $N_{1,1}$= 6, $N_{2,1}$ = 48, $P_{seven\ substances} < 0.05$, Pentacosane: P = 1.0).

In addition to the eleven mentioned host-specific hydrocarbons, heptacosane (C_{27}) was found more regularly on the cuticle of *H. sublaevis* workers (59%), than on non-enslaved *L. muscorum* (27 %) or *L. acervorum* (33%) workers (Fisher-Exact-tests: *H. sublaevis vs. L. muscorum* $N_{1,1}$= 27, $N_{2,1}$ = 59, P < 0.008*; H. sublaevis vs. L. acervorum* $N_{1,1}$= 27, $N_{2,1}$ = 48, P < 0.05; *L. muscorum* vs. *L. acervorum* $N_{1,1}$= 59, $N_{2,1}$ = 48, P = 0.53). This indicates that the parasite does not obtain this hydrocarbon from its hosts, but can actively synthesise it.

III.V DISCUSSION

Many parasites specialize on a single host species and consequently closely adapt their behaviour, morphology and chemistry to this host e.g. (Akino et al. 1999; Davies 2000). In contrast, parasites using several hosts face a trade-off, as adaptation to one host frequently entails costs for the interactions with its other host(s) e.g. (Brandt and Foitzik 2004). This scenario clearly applies to the interaction between the obligate social parasite *H. sublaevis* and its hosts. Our long-term data on community composition reveals the regular concurrent usage of the two main host species in two communities, with a clear overexploitation of the less frequent, smaller host *L. muscorum*. Our chemical analysis demonstrated stronger cuticular hydrocarbon profile resemblance of *H. sublaevis* to non-enslaved workers of the overexploited host, *L. muscorum*. Chemical similarity is especially important for founding queens and raiding parties, which have to successfully attack and overtake host colonies (Buschinger 1974; Herbers and Foitzik 2002). The mimicry of host profiles was shown in several social parasite systems to avoid or lower host aggression (Howard et al. 1990a,b; Dettner and Liepert 1994; Lenoir et al. 1997). To attain this congruency two possible scenarios were proposed: The parasite can actively biosynthesize host cues and / or acquire cues from their host either actively via allogrooming or trophallaxis or passively through direct contact with host or nest material (Franks et al. 1990; Yamaoka 1990; Dettner and Liepert 1994; Bonavita-Cougourdan et al. 1997; D'Ettorre et al. 2002). Most studies on social parasite interactions demonstrated or proposed that host cues are obtained via contact rather than active synthesis of host compounds (Lenoir et al. 2001). Instead, our study reveals that the parasite *H. sublaevis* actively produces two hydrocarbons exhibited in host profiles and in addition passively adopts of several host substances, especially the short-chained hydrocarbons of the smaller host *L. muscorum*.

Although general chemical resemblance of parasite profiles to both hosts was evident, our in-depth analyses revealed, in contrast to earlier studies (Kaib et al. 1993; Heinze et al. 1994), that the profiles of *H. sublaevis* are statistically clearly separable from host profiles. Parasite profiles are distinct from their hosts possibly because of incomplete acquisition of host hydrocarbons and because of active production of two hydrocarbons. One of these synthesized substances, heptacosane, is less frequently found on host workers of both species, while the other, pentacosane, is a characteristic hydrocarbon in the profiles of *L. muscorum* workers. For a parasite to produce characteristic recognition cues entails the risk that hosts are able to identify their enemy thereby. Enemy recognition was indeed demonstrated for the host

L. acervorum and in a comparable social parasite system from North America (Franks et al. 1990; Alloway 1990). So, why does *H. sublaevis* synthesize cuticular hydrocarbons, which are present on host cuticles only in minor amounts or are characteristic only for a single host? This would be especially surprising, if nestmate recognition in social hymenoptera would be indeed invariably based on an undesirable cue-absent mechanism (Couvillon and Ratnieks 2008; Guerrieri et al. unpublished ms). Than chemical insignificance should be the much better strategy. Active biosynthesis of hydrocarbons in the parasite can be explained either by phylogenetic constraints, a need for physical protection or to facilitate intraspecific communication and interactions within slave-making nests. A consistent colony odour in a slave-making nest and the imprinting on freshly hatched callows might enhance an effective colony functioning especially in nests with slaves of two species. Behavioural observations indicate frequent aggression between *L. acervorum* and *L. muscorum* slaves that can be possibly reduced by chemical profile adjustment (Heinze et al. 1994).

Although the two *Leptothorax* hosts are closely related (Beibl et al. 2005), their cuticular hydrocarbon profiles are vastly dissimilar. The profile of the smaller host, *L. muscorum* is characterized by a great diversity of short chained hydrocarbons, while the only few, but long-chained hydrocarbons were found on the cuticle of *L. acervorum* workers. Possibly shorter chains are cheaper to produce or easier to modify, allowing these ants to spend less energy and to produce a more complex and possibly more effective recognition system. At the same time the usage of easily transferable short chained hydrocarbons makes *L. muscorum* possibly more susceptible to *H. sublaevis*. The strong discrepancy in chemical profiles of these two related host species is very unusual, as comparisons of cuticular profiles of congeneric ants often show a high chemical congruency (Brandt et al. 2005b, Foitzik et al. 2007). Indeed, only two out of 33 hydrocarbons were not shared among three different *Temnothorax* species (Foitzik et al 2007). In contrast, over half of the cuticular hydrocarbons detected in this study are only expressed in a single host species. This causes a great difficulty for the parasite, which cannot adapt to both hosts simultaneously, as active production of hydrocarbons of one host, makes him at the same time more dissimilar to the other. Interestingly, the only hydrocarbon exhibited in similar frequencies in the profiles of both host species, heptacosane, is actively produced by the parasite. In addition to this chemical cue, *H. sublaevis* apparently biosynthesizes a second host cue, pentacosane, which is characteristic for the smaller host *L. muscorum*. Possibly this is due to a higher degree of specialization to this host, which is suggested by the overexploitation of this host as shown in the community composition analyses. In addition to active biosynthesis, our chemical

analyses demonstrate that *H. sublaevis* workers effectively adopt host hydrocarbons of both slave species. The adjustment to slave species is most effective for parasites living with *L. muscorum* slaves, as these slave-making workers could not be statistically separated from non-enslaved *L. muscorum* host workers. The reverse was not true, that is *H. sublaevis* workers, no matter with which slaves they live, were always distinct from non-enslaved *L. acervorum.* This difference is presumably not only due to the active production of two hydrocarbons of the *L. muscorum* profiles, but rather due to easier acquisition of the more volatile shorter hydrocarbons of this host. Moreover, the stronger chemical disparity between *H. sublaevis* and its host *L. acervorum* was also apparent in the influence of the parasite on slave profiles. Enslaved *L. acervorum* workers exhibit a profile distinct from their non-enslaved counterparts, while the profiles of enslaved *L. muscorum* workers did not differ from non-enslaved conspecifics.

Both pentacosane and heptacosane are linear alkanes, which are thought to play a minor role in chemical communication (Howard and Blomquist 2005; Dani et al. 2005; Monnin 2006; Martin et al. 2008). Possibly the social parasite uses these linear alkanes to prohibit desiccation, because they are easily synthesized and difficult to perceive for the hosts.

This study was also set up to investigate potential local adaptation of the parasite to host profiles. Indeed, we could show chemical differences in cuticular hydrocarbon composition of non-enslaved *L. muscorum* between the two communities north and south of the Alps. Yet, *H. sublaevis* and *L. acervorum* populations did not show interpopulational differences in their profiles. The disparity between these species can be explained by variation in population structure. Population genetic analyses demonstrated clearly distinct *L. muscorum* populations, while the more variable *L. acervorum* populations are well connected by gene flow (Brandt et al. 2007). *H. sublaevis* was found to show intermediate structure and we suspect that underlying chemical differences were difficult to reveal with our sample size given the strong impact of slave species on parasite profiles.

Remarkably, a third of the *H. sublaevis* colonies in Germany and Italy exploit two host species simultaneously. Yet, our analyses indicate that this ability of the social parasite to utilize two host species might be costly for the parasite suggesting a trade-off between specific adaptations to either host (Futuyma and Moreno 1988; Ward 1992). A major difficulty for mixed nests arises when slave-making queens or raiding parties attack host colonies, because parasite workers from three-species nests do not match host profiles as well as parasite workers from single slave species nests, resulting in more fierce host defenses.

Indeed, raiding experiments by Böhm et al. (submitted) showed that *H. sublaevis* workers were more often killed by *L. acervorum* colonies in raids when the social parasite came from colonies with *L. muscorum* slaves. Beside a higher mortality rate, *H. sublaevis* workers from mixed or *L. muscorum* parasite nests obtained less brood from attacked *L. acervorum* colonies. A second problem emerges as slave-making ant workers from colonies with both slave species were less capable of gaining chemical uniformity within their three-species nests, which is the basis for a peaceful co-existence and efficient cooperation (Heinze et al. 1994). The observed intra-colonial animosities should entail fitness costs for the parasite such as reduced colony growth and reproduction.

H. sublaevis workers from mixed colonies are apparently more easily recognized by colonies of either host species compared to parasite workers raised in a colony with only a single slave species when attacking the host species they have grown up with. In addition, intra-colonial problems with host aggression occur in mixed slave species nests. So why does this social parasite frequently exploit two host species at once? As already suggested in (Heinze et al. 1994) it may be costly for the parasite to increase the duration of scouting activities by specializing on one of the two potential host species. Furthermore, community composition analyses show that both host species occur frequently in the two study sites, and microgeographic separation was not apparent. This would mean that for a given raiding party of *H. sublaevis* it is as likely to discover a *L. muscorum* as it is to encounter a *L. acervorum* host nest. Whether it should attack a host colony, although it is not of the same species as the one already present as slaves in its nest, should depend on the absolute density of the preferred host. The latter conclusion was drawn from the classical optimality models on prey choice (Mac Arthur and Pianka 1966). Host densities are also not independent of the social parasite itself. A recent field analysis of raiding frequencies and post-raid survival rates indicated that *L. muscorum* populations suffer more strongly from raids by *H. sublaevis* (Fischer-Blass et al. 2006). This would result in a feed-back mechanism as specialization on this smaller host would lower its nest density and thus shift the threshold towards accepting *L. acervorum* colonies as the search time for the preferred host would increase.

However, there are also potential advantages of having two slave species present in a single parasite nest. Slave workers of two host species and therefore also of two sizes might allow the social parasite to exploit a greater variety of food resources. Indeed, temperate and boreal *Leptothorax* ant communities predominantly consist of two species, which exhibit a distinct difference in worker size, presumably to reduce interspecific competition (Foitzik and Heinze 1999). Furthermore, three-species colonies might be less prone to micro-parasitic

infections similar to polyandrous hymenopteran colonies, which are thought to profit from the presence of several patrilines (Brown and Schmid-Hempel 2003; Mattila and Seeley 2007; Fournier et al. 2008). It is currently unclear whether the costs or benefits of exploiting two ant hosts at once preponderate. Careful field analyses of the microgeographic distribution of host nests and the composition of the slave force of *H. sublaevis* colonies might reveal in future whether three-species parasite nests are more or less common than expected at random.

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CHAPTER IV

Influence of a social parasite on the social structure and the investment patterns of its *Leptothorax* hosts

IV.I ABSTRACT

Social parasites can exert tremendous selection on their host species, driving coevolutionary arms races between slavemaking ants and their hosts. However, parasite prevalence and raiding frequency can vary between sites, leading to selection mosaics. Consequently geographic mosaics of coevolution can arise with different evolutionary outcomes at different sites. Here we report results from a large scale field manipulation in two ant communities, where we experimentally manipulated social parasite density and exchanged nests of the obligate social parasite and slavemaking ant *Harpagoxenus sublaevis* between sites. Overall, we found changes in the social structure, demography and investment patterns of host colonies as a result of our manipulation. *Leptothorax muscorum* colonies from the German host community reacted to experimental addition of parasites with a higher number of new workers and a higher productivity, independently of the origin of the parasite. In the Italian ant community sympatric parasites caused a decrease in new queen production and productivity of the larger host *L. acervorum*, which shifted to a male-biased allocation ratio. The changes in social organisation in *L. muscorum* and *L. acervorum* could be directly caused by the slavemaker or a response of the host to the high raiding frequency. According to demographic data and host species usage the smaller *L. muscorum* was clearly the preferred host species. In conclusion, both *Leptothorax* host communities responded strongly, but variably to the presence of the social parasite *H. sublaevis*.

IV.II INTRODUCTION

Parasites negatively influence host fitness, resulting in either host mortality or a reduction in the host reproductive rate. Avian brood parasites can reduce the life time reproductive output of their hosts drastically and can even threaten host populations (Rothstein and Robinson 1994). The same drastic consequences were shown for the ichneumonid parasitoid *Venturia canescens*, when living in a three-species system, which can drive one host to extinction (Bonsall and Hassell 1997). Parasites however, often vary in their virulence level and in the degree of adaptation to local hosts. Additionally local adaptation can be influenced through geographical variation, e.g. habitat conditions, temperature, parasite prevalence and nesting abilities. It can lead to different host-parasite interactions, e.g. which was already shown in the interactions between a North-American social parasite and its host (Blatrix and Herbers 2003; Brandt and Foitzik 2004). These interactions reflects Thompson´s theory of the geographic mosaic of coevolution, which states that the interactions between host and parasites can differ locally in the coevolutionary outcome and intensity, and therefore result in cold and hot spots (Thompson 1994).

If the parasite has more than one host to exploit, two options are given. It can expand its niche and utilize both host species or it could specialize on the most profitable or less well-defended host species. Specialisation of the parasite however can sometimes lead to host alternations over time, as specialisation of the parasite can exert stronger selection pressure on the used host and therefore can promote a higher rate of evolutionary response and the evolution of effective defenses. As a possible consequence the parasite could switch between its hosts, preferential always exploiting the host with the lower defensive abilities. The latter szenario was suggested for the North American social parasite *Protomognathus americanus* (Brandt et al. 2005).

Co-evolution is especially strong in slavemaking ants and their hosts where parasite pressure aroused through frequent and destructive slave raids can be extremely strong (Foitzik et al. 2001; Foitzik and Herbers 2001; Foitzik et al. 2003; Fischer and Foitzik 2004). Slavemaking ants frequently conduct slave raids to ensure a stable work force of allospecific slaves on surrounding host colonies. They steal host brood, mainly pupae, and take it back to their own nest. When the raided host brood ecloses, they carry out all routine tasks in the parasite colony such as brood care, grooming and foraging (Alloway 1979; Schumann 1992; D'Ettorre and Heinze 2001). This life style forces the slavemaker to be in a lifelong dependency with its hosts, which makes them to obligate parasites.

Beside raiding pressure the presence of a parasite also influences social organisation and reproductive strategies of its hosts by sharing the same ecological niche (Savolainen et al. 1996). As a consequence to that hosts undergo a trade-off between early reproduction to evade parasites and reduced fecundity, developing slowly and thus risking infection and death before reproduction (Hochberg et al. 1992).

In the European slavemaking ant *Harpagoxenus sublaevis* (Nylander 1852) empirical field data on parasite pressure and host reponses in different populations are still lacking although the behaviour and life cycle of this social parasite was well studied (Buschinger 1966; Buschinger et al. 1975; Buschinger 1978; Bourke et al. 1988) and behavioural data demonstrated coevolutionary interactions including the occurence of local adaptation (Fischer-Blass and Foitzik 2004). Ecological data of a German community indicated that the impact of *H. sublaevis* was much more severe on the smaller host *Leptothorax muscorum* (Fischer-Blass et al. 2006) than on the larger host *L. acervorum*.

Hence, *H. sublaevis* is expected to be generally more adapted to its smaller host *L. muscorum*. Based on this, we assumed that the experimental addition or removal of social parasite colonies in plots with both hosts would lead to different host reactions. To analyze the impact of this social parasite on its two host species *L. acervorum* and *L. muscorum* we conducted a 15-months cross – fostering field manipulation in two different locales. The study site in Italy was located in the South Tirolan Alps with longer and colder winters compared to the second site in Abensberg, Germany, which was situated at lower elevation. Both ant communites differ in host occurrence and composition. For example a non-parasitized nestsite competitor, *Temnothorax crassispinus* occurs only in Germany (Foitzik and Heinze 1999). Besides, Italian *L. acervorum* colonies are sometimes parasitized by the workerless inquiline ant *Leptothorax kutteri*. Italian host colonies were found to contain more often several queens (polygyny), which is typical for ecologically unfavourable sites, in which independent nest foundations are difficult (Heinze et al. 1995). As the success of slave raids of *H. sublaevis* strongly depends on the origin of parasite and host (Fischer and Foitzik 2004), we examined host preferences and changes in social structure, productivity and demography of hosts associated with parasite density in the German and Italian community and to investigate the geographic mosaic of coevolution in more depths.

IV.III MATERIAL AND METHODS

Study system

The slavemaking ant *H. sublaevis*, an obligate social parasite and its two host species *Leptothorax acervorum* (Fabricius 1793) and *Leptothorax muscorum* (Nylander 1846) of the tribe Formicoxenini inhabit decaying tree stumps, sticks or logs on the forest floor (Fig. 1). They are widely distributed throughout the pine forests of the boreal regions of Eurasia in lowlands from 400 NN to alpine sites around 1200 NN (Collingwood 1971; Ratschenko et al. 1999). Colonies of the social slavemaking parasite *H. sublaevis* always contain a single founding queen (Buschinger 1966a,b,c; Hölldobler and Wilson 1990). Its two hosts are facultatively polygynous with one to several queens per colony (Buschinger 1968a,b; Heinze and Buschinger 1988; Lipski et al. 1994). Our non-manipulated field data from 2005 showed that in both communities colonies of the larger host species, *L. acervorum* contained more queens per nest than *L. muscorum* (Germany df = 1, $\chi^2 = 20.21$, $p < 0.001$; Italy df = 1, $\chi^2 = 3.80$, $p = 0.05$).

Field manipulation 2005-2006

In a 15 months large scale experiment we mapped 21 study plots in the lowland community in Abensberg, Germany (400 NN) and additional 21 study plots in the alpine community in Innichen (1200 NN), Italy. We used a cross-fostering design, in which slavemaker colonies from two different populations were exchanged. The forest floor of all these 10 x 10 m plots was completely searched to ensure that all ant colonies were collected and transported to the laboratory in Munich. In Germany, beside the host colonies, the nest site competitor, *Temnothorax crassispinus* was also collected and brought to our laboratory. Plots were located in areas, in which at least a single slavemaker colony could be found and/or several hosts, mostly 5-8 colonies per plot. Plots with similar habitat conditions were selected. We mapped the position of all ant colonies within each plot and GPS measurements were taken to note the current position within the forest.

Figure 1: Habitat and wooden nest site of a *Leptothorax* ant colony in Italy, Innichen.

In the laboratory, all colonies were removed from their nest site and the number of queens, workers, males and brood (larvae and pupae castes) determined. Afterwards, we allowed the ants to move into artificial nest sites. For this we used cylindric wooden beech dowels (around 8 cm long and 2 cm high and longitudinal holes between 2 to 4 mm) which were known to be generally accepted by Formica ants (Herbers 1986; Herbers and Banschbach 1995; Foitzik et al. 2004). All host colonies were released again to the community from which they were collected. As some plots had only two host colonies, we added additional host colonies of both species to adjust host density in these manipulated plots to all other plots. Hereby we paid special attention in providing natural nest density by releasing ant colonies with artificial nest sites. Host colonies were taken from plots with exceptional high nest density or else taken from areas adjacent to the study plots. The nest site competitor *T. crassispinus*, which is not parasitized by *H. sublaevis* was not released. We replaced these nests with host colonies if the required number of hosts in one plot was not reached. *L. acervorum* colonies, which were parasitized by an inquiline ant *L. kutteri* were not released in the field.

The three treatments were randomly assigned to the plots (design is shown in Table 1). Thus some non - parasitized plots contained parasites later on and vice versa. Plots with no slavemaker are called "parasite-free plots" in the following. Slavemaker colonies were released in the centre of the plot. As raiding intensity increases with parasite nest size, we released additional slavemaker colonies, when their nest size was low. For onset a total of 33 *H. sublaevis* colonies, 116 *L. muscorum* and 166 *L. acervorum* were released in spring 2005.

Table 1: Number of manipulated and unmanipulated plots in the two communities

Treatment	**Population Innichen (Italy)**	**Population Abensberg (Germany)**
I	7 plots with *H. s.* from Italy	7 plots with *H .s.* from Italy
II	7 plots without *H .s.*	7 plots without *H. s.*
III	7 plots with *H.s.* from Germany	7 plots with *H .s.* from Italy
	All hosts stem from Italy	All hosts stem from Germany

The relative slavemaker density at the onset of the manipulation not including the control plots was set to 0.17 ± SE 0.01 slavemaker colonies per host colony in Abensberg, Germany and 0.22 ± SE 0.03 slavemaker colonies per host colony in Innichen, Italy. This reflects the range of parasite frequency in the wild (Fischer and Foitzik 2004).
In July and August 2006 all nest sites, natural and artificial ones, within the 42 plots were collected and transported to the laboratory. Here all colonies were completely counted again and afterwards frozen for subsequent analysis.

Investment in weights of different castes

For further calculations in male allocation ratio, total production and productivity of hosts, we measured the dry weight of winged queens, founding queens, males and workers of both host species *L. acervorum* and *L. muscorum* from Innichen and compared it to the German *L. acervorum* and *L. muscorum* dry weight of the same castes. For that they were killed by freezing and dried for 70 h at 65 °C (Foitzik and Heinze 2000). The dry weight of all castes and sexes was measured with a Sartorius Supermicro and Lüdi A6 boats.

Statistical Analysis of Field Data

To investigate the influence of *H. sublaevis* on its hosts in two communities, Germany and Italy, we compared parasite densities as well as host densities in the years 2005 and 2006 in the manipulated and non-manipulated plots. To test for correlations between relative *H. sublaevis* density and host density we used a multiple regression (MR). Moreover we calculated parasitation rate and looked for host species preference using the Chi - square test, indicated by the χ^2 - value. For the latter we counted parasite nests containing only *L. acervorum* or *L. muscorum* as slaves. To study differences between nest sizes of both host species at different collecting sites, we used Wilcoxon Matched Pair tests (WMP), Mann -

Whitney U tests (MWU test). Data sets were tested for normal distribution and as the data mostly did not conform to a normal distribution we used non-parametric tests for this data analysis.

Furthermore we examined the social structure of hosts in plots with parasite presence from Italy and Germany and in plots without parasite presence. For this purpose we chose five parameters which would reflect our manipulation best: new workers, new queens, male allocation ratio, total production and productivity. As adult workers and queens already exist before manipulation, differences in reproductive strategies (new males, new queens, new workers) due to manipulation can be best shown with offspring. All statistical tests were performed with the program STATISTICA 6.0 where we used non-parametric tests for this data analysis: Kruskal - Wallis test (KW test) and Mann - Whitney U test (MWU test). All variables analysed in the manipulation study with the KW test were then sequential Bonferroni corrected after Rice (Rice 1989). Although sequential Bonferroni correction has become the primary method of addressing the problem of multiple statistical tests in ecological research it shows some mathematical problems. Major problem with this method is that it only examines individual p - values of each test, while ignoring the number of statistical tests that are significant (Moran 2003). Hence several relatively high p - values are stronger evidence against a null hypothesis than one moderately corrected low value (Rosenthal 1978).

IV.IV RESULTS

Community composition and host species usage

In 2005 we collected within 2100 m² (21 x 100 m²) in Germany a total of 12 *H. sublaevis* colonies, 56 *L. muscorum*, 63 *L. acervorum* and 62 T. crassispinus. In Italy, we found in the same total study area 25 *H. sublaevis*, 36 *L. muscorum* and 106 *L. acervorum*. In summer 2006, we collected 20 *H. sublaevis* colonies, 57 *L. muscorum*, 57 *L. acervorum* and 69 *T. crassispinus* colonies in our marked study plots in Germany. In Italy we found 17 *H. sublaevis* colonies, 70 *L. muscorum*, 157 *L. acervorum* colonies.

In Germany host nest density slightly decreased from 6.95 per 100 m² in 2005 onset to 5.43 per 100 m² in 2006 (WMP test: host $Z = 2.25$, $p < 0.02$). For each host species alone however we could not show a change in nest density (WMP tests: L. a.: $Z = 0.92$, $p < 0.36$; L. m.: $Z = 0.92$, $p = 0.36$). In the German community the mean parasitation rate in plots, where we released slavemaker colonies the year before, did not change in 2006 (WMP test: H.s.: $Z = 0.67$, $p = 0.50$). In plots, where we refrained from releasing parasite nests in 2005, *H. sublaevis* colonies could nevertheless be found in 2006 with a mean of parasitation rate of 0.31.

Host density of the Italian community increased during our experiment from 6.48 per 100 m² in 2005 to 10.81 per 100 m² in 2006 and this density increase was most pronounced for *L. acervorum* (WMP tests: hosts: $Z = 3.06$, $p < 0.002$; L. a.: $Z = 2.75$, $p < 0.006$).
In contrast, the mean parasitation rate decreased for both treatments, where we released sympatric (from 0.23 to 0.09 in 2006) or allopatric (from 0.22 to 0.06) slavemaker colonies (WMP test, sympatric *H.s.*: $Z = 2.37$, $p < 0.02$; allopatric *H.s.*: $Z = 2.37$, $p < 0.02$). In plots without any parasite at onset it increased from zero to 0.12 in 2006.

If *H. sublaevis* colonies tended to overexploit local host communities, they should be preferentially found in areas with low host density. Conversely, if the parasite can only survive in areas with high host nest density, we would have expected to find social parasite nests in areas with relative high host nest density. Hence, we investigated the relationship between relative *H. sublaevis* density and host density in a multiple regression. The non-manipulated field data from 2005 showed a negative correlation between relative parasite density and *Leptothorax* nest density in both populations (MR for Italy: $\beta = -0.53$, $t12 = -2.17$, $p < 0.05$; for Germany: $\beta = -0.55$, $t11 = -2.17$, $p < 0.05$) in that with increasing parasite density host density decreased (Fig. 2).

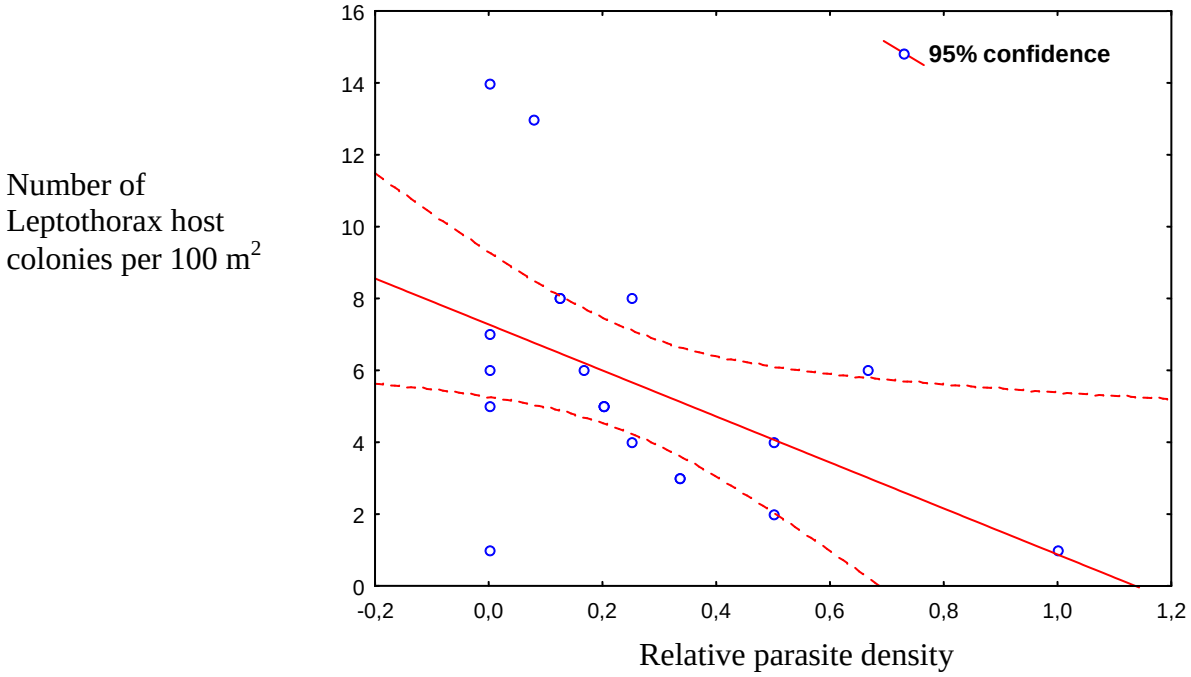

Figure 2: Negative correlation between relative parasite density and *Leptothorax* nest density in both populations

Nestsize of *L. muscorum* and *L. acervorum* in both communities

In 2005 non-enslaved *L. acervorum* colonies contained more workers than non-enslaved *L. muscorum* colonies in Abensberg (MWU - test: nest size: $p < 0.01$, $U = 1334.5$), whereas in Italy 2005 nest size of both non - enslaved host species did not differ. In both ant communities the social parasite and its two main host species were present, of which in Italy 2005 the larger host species *L. acervorum* was twice as common as the smaller host *L. muscorum* (Italy df = 1, $\chi^2(2005) = 13.4$, $p2005 \leq 0.0003$). In contrast, host frequency for both host species was similar in the German community. However, in both ant communities the smaller host species *L. muscorum* was clearly the preferred host for *H. sublaevis* (N(Germany) = 177, $\chi^2 = 5.86$, $p \leq 0.02$ and N(Italy) = 204, $\chi 2 = 5.91$, $p \leq 0.02$).

Field manipulation

We examined differences in reproductive outcome of both host species between parasitized and non-parasitized plots in Italy and Germany (Table 2).

Table 2: Impact of *H. sublaevis* colonies on the social structure and investment patterns of *L. acervorum* and *L. muscorum* host colonies in the two study sites. Results of the Kruskal Wallis Tests are given (p= p-value, N= number of colonies, H = parameter of KW-Test).

	Germany						**Italy**					
	L. acervorum			*L. muscorum*			*L. acervorum*			*L. muscorum*		
	H	N	P	H	N	P	H	N	P	H	N	P
N of queens	1.0	57	0.61	2.4	56	0.30	5.8	154	**0.05**	1.1	70	0.58
N of workers	4.6	57	0.10	8.6	56	**0.01**	3.5	154	0.18	2.9	70	0.24
Male allocation ratio	1.9	15	0.38	3.9	39	0.14	10.8	59	**0.005**	0.3	39	0.85
Productivity	4.4	52	0.11	8.1	54	**0.02**	7.0	151	**0.03**	0.9	68	0.63
Total Production	4.4	57	0.11	4.9	56	0.09	3.6	154	0.16	3.0	70	0.22

No effect of our manipulation was found on the German *L. acervorum* population (MWU – multiple tests: $p > 0.05$). However, in the German population of the smaller ant *L. muscorum*, our manipulation revealed significant differences in the number of new workers and in the productivity. Host colonies in plots with sympatric or allopatric parasites showed a higher number of new workers and a higher productivity compared to our control plots (MWU - test: new workers in sympatric and allopatric parasitized plots vs. control plots: $p < 0.01$, $U = 56.5$ and $p < 0.009$, $U = 103.0$ and productivity in sympatric and allopatric parasitized plots vs. control plots: $p < 0.03$, $U = 59.5$ and $p < 0.006$, $U = 86.5$). P – values of the number of new workers of the German *L. muscorum* population remained significant after sequential Bonferroni correction (corrected $p' = 0.01$, $\alpha = 0.05$, $N = 5$).

In the Italian population we could find effects of our manipulation on *L. acervorum* productivity, male allocation ratio and on the number of new queens that were raised. The productivity was lowest for plots where we released sympatric parasites compared with either the control plots or the plots with German parasites (MWU – test: sympatric plots vs. control plots: $p < 0.02$, $U = 1181.5$; sympatric plots vs. allopatric plots: $p < 0.04$, $U = 880.0$). A comparison of sympatric and allopatric parasitized plots could show that the allocation ratio became more male-biased with the presence of the sympatric slavemaker (MWU – test: $p < 0.005$, $U = 75.0$). The comparison of the control plots with those where German parasites were released revealed a more female-biased allocation ratio in the presence of *H. sublaevis*

(MWU – test: $p < 0.005$, $U = 102.0$). Number of new queens was again lower in sympatric parasitized plots than in allopatric parasitized plots (MWU – test: $p = 0.07$, $U = 947.0$). After sequential Bonferroni correction changes in the male allocation ratio in the Italian *L. acervorum* population was still significant (corrected $p' = 0.01$, $\alpha = 0.05$, $N = 5$). However no effect of our manipulation could be found on the smaller host ant *L. muscorum* (MWU – multiple tests: $p > 0.05$).

In a second analysis we investigated the effect of the social parasite on both hosts together to increase our sample size for each site, Italy and Germany. In general in both host communities we could find effects on the number of new workers, the male allocation ratio, the productivity and the total production (Table 3). Furthermore, the number of new queens was significant in the Italian host community which is described as followed.

	Germany			**Italy**		
	H	N	P	H	N	P
N of queens	0.81	113	0.67	7.64	224	**0.02**
N of workers	8.98	113	**0.01**	6.36	224	**0.04**
Male allocation ratio	7.23	54	**0.03**	6.81	98	**0.03**
Productivity	9.02	106	**0.01**	8.21	219	**0.02**
Total production	7.07	113	**0.03**	6.2	224	**0.05**

Table 3: Impact of *H. sublaevis* colonies on the social structure and investment patterns of its *Leptothorax* hosts in the two study sites. Results of the Kruskal Wallis Tests are given (p= p-value, N= number of colonies, H = parameter of KW-Test).

Comparing the significant parameters of the German host community between allopatric parasitized plots and control plots, we found that all parameters significantly increased in plots with parasite presence (MWU – test: new workers $p < 0.003$, $U = 392.0$; male allocation ratio $p < 0.007$, $U= 60.5$; productivity $p < 0.003$, $U = 300.0$; total production $p < 0.009$, $U = 420.0$). When we compared the sympatric parasitized plots with our control plots, we found that the number of new workers were significantly higher in plots with local parasite presence (MWU – test: new workers $p < 0.04$, $U = 299.0$). However, no differences could be found between manipulated plots with allopatric and local parasites (MWU - test multiple: $p > 0.05$). After sequential Bonferroni correction, the impact of parasite presence on the number of new workers and the productivity remained significant (new worker $p'= 0.01$; productivity $p' = 0.01$; $\alpha = 0.05$, $N = 5$).

In the Italian host community, a comparison between plots with sympatric parasites and control plots revealed that the productivity in parasitzed plots was significantly lower (MWU – test: productivity $p < 0.01$, $U = 2359.5$). Furthermore the number of new workers and new queens, total production and productivity was significantly lower in sympatric parasitized plots compared to allopatric parasitized plots. However the allocation ratio became more male-biased in the presence of the sympatric slavemaker compared to allopatric manipulated plots (MWU – test: new workers $p < 0.02$, $U = 2086.5$; new queens $p < 0.006$, $U = 2117.0$; total production $p < 0.01$, $U = 2069.5$; productivity $p < 0.01$, $U = 1974.5$; male allocation ratio $p < 0.01$, $U = 292.5$). However no differences could be found between allopatric manipulated plots and control plots (MWU - test multiple: $p > 0.05$). After sequential Bonferroni correction none of these tests remained significant.

IV.V DISCUSSION

Ecological parameters such as nest availability and food resources can vary between sites and can result in variable outcomes of species interactions in different communities (Stireman III and Singer 2002). For example the North-American obligate social parasite *Protomognathus americanus* has a negative impact on host nest density in some sites, but not in those with high host nest densities (Foitzik et al. 2009). The aim of our study was to understand host - parasite dynamics in different European communites including the social parasite *H. sublaevis* and its *Leptothorax* hosts. We could show changes in investment patterns and reproductive strategies under varying parasite presence and origin in both hosts *L. acervorum* and *L. muscorum*.

Moreover we found changes in host density and parasitation rate between 2005 and 2006 in both communities, Germany and Italy. In the German community the relative parasite density correlated negatively with host density in 2005. This indicates a local host overexploitation of host populations through the parasite, which was also indeed be found in other animal taxa e.g. in caterpillars of the butterfly *Maculinea nausithous* which frequently overexploit ant resources (Anton et al. 2007).

The smaller host species *L. muscorum* was overexploitated by the parasite. Although this smaller ant occurred less frequently in Italy, *H. sublaevis* clearly preferred this host. Indeed, *L. muscorum* was significantly more often used as host in Italy and in Germany in 2005. Interestingly, former studies found no evidence for a preference of the parasite for one of the two main host species (Buschinger 1966a,b,c; Schumann and Buschinger 1991; Fischer

and Foitzik 2004; Foitzik et al. 2004).

We assume that the parasite prefers *L. muscorum* as host because of its smaller body size and less aggressive defence strategy. We could show in chapter III that the efficiency of *L. muscorum* to defend the nest against the social parasite differed from that of *L. acervorum* (Foitzik et al. 2003). The smaller host showed a risk-averse strategy, running away with brood, risking no life, but ensuring the survival of its offspring. In contrast, *L. acervorum* fought with the parasite and therefore suffered from a greater loss of lives and brood, but these hosts also killed more slavemakers. Thus this aggressive behaviour resulted in greater losses of workers and scouts of the parasite, when entering the host nest. This could be shown in a recent study on raiding behaviours of the slavemaking ant *H. sublaevis* and behavioural defenses of its host species (Böhm et al. submitted).

Another reason for the parasite to prefer *L. muscorum* in the German community is the smaller nest size compared to *L. acervorum* and associated with a smaller defense force. We found that *L. muscorum* nests were smaller and significantly more often monogynous in 2005 compared to *L. acervorum*, which showed a high number of polygynous nests and also a greater nest size.

Monogynous colonies are supposed to have a more uniform colony odour and show higher intercolonial aggression than polygynous ones, which can result in better enemy recognition (Buschinger 1974; Buschinger and Winter 1977; Hölldobler and Wilson 1977; Alloway 1980; Breed and Bennett 1987; Morel et al. 1990). Accordingly *L. muscorum* should more easily recognize intruding parasites. However, our chemical analysis of cuticular hydrocarbon profiles (Chapter III) demonstrated that the parasite chemically resembles *L. muscorum* more closely (Bauer et al. 2009a). Maybe monogyny is a strategy of this small host to be able to recognize a chemically well-adapted parasite.

It was hypothesized that the monogyny of host colonies in parasitized areas could be the consequence from stopping readopting daughter queens when parasites are in the vicinity. Instead of investing in colony growth, host colonies, which detected the presence of the parasite could try to mainly invest in queens and males (Foitzik et al. 2003). These would disperse from the high-risk areas and could potentially detect unparasitized spots (Foitzik et al. 2009).

Our manipulation in Germany showed that the smaller host *L. muscorum* produced more new workers and had a higher productivity in parasitized plots than in control plots. Here it seemed that *L. muscorum* tried to establish a larger workforce in plots with parasite presence. This would confirm the hypothesis that when less new queens were produced,

energy could be saved for a faster colony development and therefore a higher fecundity be reached (Restif et al. 2001; Foitzik et al. 2003). *L. muscorum* produced more new workers in parasite presence, independently of parasite´s origin whereas *L. acervorum* was not affected by parasite influence as the social structure remained the same in 2006.

The field experiment in Italy however revealed a longterm effect of *H. sublaevis* on the productivity and social structure of the larger host *L. acervorum*. Surprisingly no effect on the smaller host *L. muscorum* could be detected, albeit it was clearly more frequently parasitized. Host colonies of the larger host *L. acervorum* in the vicinity of local parasites produced fewer new queens and the allocation ratio was more male-biased. In addition, the productivity was decreased in plots with the experimental addition of sympatric parasites. Local parasites thus had a stronger effect on this *L. avervorum* population. Possibly German slavemaker colonies did not cope so well with this new alpine environment and consequently had a reduced influence on this host. Producing males could be beneficial, because ant males are winged and generally disperse more than ant queens (e.g. Hardy et al. 2008). Indeed, we could show male-biased dispersal for all three study species in Chapter I. Dispersal during mating flights allows males to distributes their genes in parasite-free patches.

Strong changes in investment patterns of host colonies with the presence of local parasites were also found in comparable studies of the North American slavemaker *Protomognathus americanus* where parasite pressure on hosts was correlated to low allocation into new workers, but high investment into sexuals (Foitzik and Herbers 2001; Herbers and Foitzik 2002; Foitzik et al. 2009).

To compare the investment pattern between the different sites, we combined both host species from one site in Germany and Italy. In both communities, allopatric parasites had no negative impact on hosts, whereas local parasites led to changes in investment patterns. At the German site more workers were produced, whereas at the Italian site a higher investment in male offspring was found. These differential findings reflect the different outcomes of host-parasite interactions at the two study sites. Under social parasite pressure, host colonies have to carefully choose how to allocate energy into workers, males and queens to secure the highest possible fitness. The Italian *L. acervorum* colonies invested more resources in reproduction (Brandt et al. 2007), whereas German *L. muscorum* focused on the production of new workers to ensure colony maintencance even under attack.

Social parasitism is not the only selective force exerted on *Leptothorax* host colonies. These small ants also suffer from nest site and food limitation and these ecological factors can influence colony growth and reproduction (Foitzik and Herbers, 2001). Studies on food limitation revealed variable findings, possibly due to annual variation in the selection regime experienced by these ants (Backus and Herbers 1992).

IV.VI ACKNOWLEGDEMENT

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GENERAL DISCUSSION

In this study on the European slave-making ant system of *H. sublaevis*, we investigated the impact of the slave-making ant at several geographically distant sites, which now allows us to form general conclusions on the virulence, the degree of local adaptation of host or parasite and the evolutionary trajectories within this host-parasite system.

According to the geographical mosaic theory of coevolution, the outcome of interactions and the degree of specialization vary among physical and biotic environments and depend on the community context, in which the interactions takes place. The composition and the strength of ecological selection pressures is especially shaped through competition and resource availability in different populations with different suites of adaptations (Thompson 1994; 1999b). Coevolutionary trajectories can be strongly shaped by the geographic distribution of coevolutionary hot and cold spots, and by the pattern of gene flow among populations (Gomulkiewicz et al 2000).

The geographic mosaic of coevolution was recently indicated in a laboratory raiding experiment of the European slavemaking ant system (Fischer and Foitzik 2004). Hence our interest in this thesis was to underline these behavioural observations with additional chemical, behavioural and genetic examinations and a much larger sample size.

Our genetic analyses in chapter I revealed high levels of genetic variation in the obligate slavemaking ant species *Harpagoxenus sublaevis* and its hosts *Leptothorax muscorum* and *L. acervorum*. In this host-parasite system we expected to find evidence for the Thompson's geographic mosaic of coevolution (Thompson, 2005), because there is abundant genetic variation and population sizes are large in all three species.. In addition the populations of all three species are strongly structured. Coevolutionary processes in one community are therefore somewhat independent of other communities and we would expect parasite and host populations to adapt to their local opponent. This was indeed found in behavioral studies, which investigated the crucial host – parasite encounter – the slave raids (Fischer and Foitzik 2004). A species comparison suggests high levels of genetic variation and population structure in all of the three species, only the smaller host, *L. muscorum,* might be less genetically variable in comparison to the larger host. However high genetic variability is especially crucial for species with limited dispersal (Schmid Hempel 1995).

Additionally Gst values from MtDNA were higher than Gst values from microsatellites. Many studies on ant population genetics compare the Gst values directly and find up to 20 times higher values in the MtDNA based calculations (for example, Doums et al.

2002; Clemencet et al. 2005; Brandt, et al. 2007; Goropashnaya et al. 2007). They conclude that male dispersal is much more pronounced in ants than female dispersal. We think that this pattern may at least partly be caused by the differences between the two marker systems with very high heterozygosities in microsatellites, rather than a real difference in differentiation.

Discrepancies between species in mitochondrial data and dispersal rate could indeed influence coevolutionary dynamics and produce spatially variable outcomes across the landscapes, over which these species interact. That coevolutionary interactions do not take place in isolation, and that their dynamics can be profoundly altered by the community context in which they occur was illustrated by the well-studied example of crossbills and the cone-producing tree species they feed on. Here the presence of red squirrels (*Tamiasciurus hudsonicus*) was hypothesized to affect the occurrence of coevolution between red crossbills (*Loxia curvirostra complex*) and Rocky Mountain lodgepole pine (*Pinus contorta ssp. latifolia*) in so far that when *Tamiasciurus* are absent, crossbills increase in abundance and coevolve in an evolutionary arms race with pine. Thereby it provides a mechanism giving rise to a geographic mosaic of selection (Benkman 1999; Benkman et al. 2001; Benkman et al. 2003; Parchman and Benkman 2002). Another example for effects of species-interaction and spatiotemporal host distribution is the butterfly genus *Maculinea*. *Maculinea* butterflies are parasites of *Myrmica* ants. The butterfly caterpillars infest host ant nest in the vicinity of their initial oviposition plant. Simulations could show that the spatial distribution of host ants adapts to the spatially distributed parasitism from *Maculinea* caterpillars. Areas without host plants (unexploited areas) are completely inhabited by host ants. In areas with host plants (exploited areas) mean density of host ants is lower (Singer 2006).

Beside these differences in host distribution we furthermore could find strong discrepancies in *L. muscorum* and *L. acervorum,* demonstrated in the Dufour gland trial series, which showed differences in the behaviour between host species and intraspecific differences between sites. In this experiment host workers of both species were coated with the host defense manipulating Dufour gland secretion of *H. sublaevis* and immediately released into their host nests. *L. acervorum* workers displayed a more aggressive behaviour amongst their manipulated nestmates than *L. muscorum,* indicating that *L. acervorum* is, in general, the more belligerent host species. Nevertheless, the parasite´s Dufour gland secretion manipulated the behaviour of both host species. The larger host, *L. acervorum*, showed a less well-adapted behaviour to the parasitic manipulation than the smaller host due to its strong intracolonial aggressive response. We assume that due to the high aggressiveness of the larger host, the parasite has to manipulate this host more to be successful in raiding whereas raiding

the smaller *L. muscorum* is much easier for the parasite. The smaller host, *L. muscorum*, reacted with flight behaviour, instead of aggression. The recognition of highly varying Dufour gland secretions of a parasite is extremely difficult for species which are genetic limited such as the *L. muscorum* host (see Chapter I). The different reaction of a host to a parasite was also found in cuckoo parasite host system. Unlike the Yellowhammer and Reed Bunting birds, Corn Bunting birds rejected only 42% of experimentally introduced nonmimetic model Common Cuckoo eggs and none of the experimentally introduced conspecific eggs. They stated that breeding habitat characteristics may explain the difference in egg discrimination abilities between Corn Buntings and other Old World Emberizinae (Antonov et al. 2006).

Further differences in our experiment were found between geographic distant populations and communities. The German population of both host species were effectively manipulated by the parasite and vigorously attacked nestmates, indicating that here the hosts were lagging behind in the coevolutionary arms race. The diverse behavioural reactions of the hosts to the gland secretion in different communities follow the geographic mosaic theory of coevolution which states that species interactions differ between locales due to the age of the interaction and varying species composition, community composition host density, resource availability or ecological pressures (Benkman et al. 2003; Cho et al. 2003; Nuismer et al. 2003; Thompson 1994, 1997, 1999b).

Under the assumption of the geographic mosaic theory, hosts that co-occur with their parasite for a long time should exhibit much stronger and most effective defences. In contrast host populations which co-occur with their parasite only for a short evolutionary time should exhibit a lower parasite defence. Yet little is known about the age of both host species and interactions with their parasite. In general, defences against enemies are costly for hosts and therefore the degree of parasite virulence is important for the amount of energy they invest in parasite defence. Considering the less well defended smaller *L. muscorum* host and the additional higher virulent behaviour of the parasite to this host it could be assumed that the interaction between these ants could be of younger age. This was found in avian brood parasites and their hosts that showed variable degrees of reciprocal adaptation depending on the duration of the evolutionary interaction (Payne et al. 2002; Soler and Moller 1990).

Furthermore, sympatric parasites to *L. muscorum* elicited less neutral responses, as local hosts reacted more with flight or fight than with antennation, indicating that the parasite is well-adapted to its local *L. muscorum* hosts in both communities. In contrast to that our study showed that the Italian parasite is maladapted to its hosts. These behavioural variations can also be influenced by differences in the compositions of the Dufour glands of *H. sublaevis*

populations. Dufour gland contents appear to be highly variable among ant species and can exhibit an extreme diversity of chemical compounds (Ali et al. 1987; Ollett and Morgan 1987; Ali et al. 1989; Bagnères et al. 1991; Bestmann et al. 1995; Visicchio et al. 2000; Morgan et al. 2003).

Further evidence of the higher virulence of the parasite to *L. muscorum* can be shown by comparing the cuticular hydrocarbon profiles of these three ant species although the parasite faces here a trade off in virulence when using more than one host species: adaptation to one host frequently entails costs for the interactions with its other host(s) (Brandt and Foitzik 2004). This scenario clearly applies to the interaction between the obligate social parasite *H. sublaevis* and its hosts. Although our long-term data on community composition revealed the regular concurrent usage of both host species *L. muscorum* and *L. acervorum* by the parasite, it shows a preference for the less frequent, smaller host *L. muscorum*. Our chemical analysis has demonstrated a stronger cuticular hydrocarbon profile resemblance of *H. sublaevis* to non-enslaved workers of the host, *L. muscorum*.

In general chemical resemblance is especially important for founding queens and raiding parties in this system. They have to successfully attack and overtake host colonies (Buschinger 1974; Herbers and Foitzik, 2002). This kind of mimicry was also shown in several other social parasite systems to avoid or lower host aggression (Howard et al. 1990a,b; Dettner and Liepert, 1994; Lenoir et al. 1997). In this study we demonstrate that the parasite *H. sublaevis* actively produces two hydrocarbons exhibited in host profiles, while passively acquiring several other host substances, especially short-chained hydrocarbons characteristic for the smaller host *L. muscorum*. The trade-off of the parasite is the chemical uniformity within a three-species nest, which is the basis for a peaceful co-existence and efficient cooperation (Heinze et al. 1994). A consistent colony odour, which is imprinted on freshly hatched callows, might enhance effective colony functioning, especially in nests with slaves of two species. Although both *Leptothorax* hosts are more closely related to each other than to *H. sublaevis* (Beibl et al. 2005), the cuticular hydrocarbon profiles of the two host species are extremely dissimilar. Indeed, only very few substances produced in minor amounts are exhibited in the profiles of both *L. muscorum* and *L. acervorum*. This causes great difficulties for the parasite, which cannot adapt to both hosts simultaneously. Active production of hydrocarbons of one host, makes the parasite more dissimilar to the other leading to difficulties in the exploitation of this host. Beside further discoveries of differences in both host species, this study was also set up to look for more evidence for a geographic mosaic by investigating potential local adaptation of the parasite to host profiles. Indeed, we could show

chemical differences in cuticular hydrocarbon composition of *L. muscorum* between the German and Italian communities. Yet, *H. sublaevis* and *L. acervorum* populations did not show interpopulation differences in their profiles. Both species showed a higher migration rate than *L. muscorum*, which is much more limited and in so far differences in profiles between sites is more likely to occur due to lower gene flow and higher structuring (Chapter I).

More disparities of both host species can be found in their behaviour in a field manipulation experiment. Here we intended to investigate host-parasite interactions in different communities. Our previous behavioural data suggested that the two host species *L. acervorum* and *L. muscorum* followed different strategies in anti-parasite defense. While the larger host *L. acervorum* uses a fighting strategy, the smaller *L. muscorum* host chooses the flight strategy, which resulted in a higher survival rate and a higher raiding risk. It is reasonable to expect, then, that parasite pressure may induce changes in host defenses, and therefore, in demographic parameters. Behavioural experiments in the North-American slavemaking system of *Protomognathus americanus* have demonstrated that the release of the parasite led to a reduction of the number of host queens and workers while intranest releatedness increased (Foitzik et al. 2009). These monogynous nests might therefore show a better nest defense against the parasite as the odour of each worker is very close to the overall nest odour. Indeed in this study both host species follow different investment pattern, when confronted with the parasite. Different behaviours of hosts when parasitized was experimentally tested in the screaming cowbird system using as alternative hosts two suitable but unparasitized species: house wrens (*Troglodytes aedon*) and chalk-browed mockingbirds (*Mimus saturninus*). They assessed host defences against parasitic females and eggs, and reproductive success of the parasite in current and alternative hosts. Alternative hosts did not discriminate against screaming cowbird females or eggs. Egg survival and hatching success were similarly high in current and alternative hosts, but the survival of parasitic chicks was significantly lower in alternative hosts. The results indicate that screaming cowbirds have the potential to colonize novel hosts, but higher reproductive success in the current host may favour host fidelity (De Marsico et al. 2008).

In our ant system several factors such as nest structure influences host defense mechanism. Monogynous colonies are supposed to have a more uniform colony odour and show higher intercolonial aggression than polygynous ones (Buschinger 1974; Buschinger and Winter 1977; Hölldobler and Wilson 1977; Alloway 1980; Breed and Bennett 1987; Morel et al. 1990). If monogynous colonies are better at recognizing invading social parasites,

then host colonies could be selected to stay monogynous and to refuse to adopt daughter queens. This could theoretically lead to a balance between ecological factors promoting monogyny (e.g. parasite pressure) to those which might promote polygyny, for example, the costs of solitary colony founding (Bourke and Heinze 1994; Herbers and Tucker 1986). We found that the preferred *L. muscorum* colonies were significantly more often monogynous (one queen per nest) in both German and Italian populations than *L. acervorum*, which showed a high number of polygynous nests with multiple queens. The rather monogynous structure in *L. muscorum* could have resulted from strong parasite pressure on this particular host (see chapters I, II, III). Monogynous colonies may arise from lower production of new queens during periods of higher parasite pressure to save energy for more worker development in order to ensure nest survival and so the survival of the colony when involved in fights or flight. This was indeed found for the German *L. muscorum* where the host focused on the production of new workers to maintain its workforce in parasitized plots. However nest size of *L. muscorum* was lower than that of *L. acervorum*. Here species competition, ecological limitations such as nest and food availability and parasite pressure may influence the nest size, indicating stronger raiding pressure on the smaller host.

Yet another hypothesis could be that ant colonies which are under strong raiding pressure will try to produce more winged sexuals than workers to ensure reproductive output before colony death. Winged virgin queens and males can escape parasitization and found colonies in unparasitized areas. Follwing this scenario, the colony size stays at a very low level and is not able to readopt daughter queens, because they are raided early in colony development (Foitzik et al. 2009). The Italian *L. acervorum* focused on producing more sexuals when the parasite was present, thereof lending only some support to the above hypothesis. In addition, *L.acervorum* nests were more polygynous and genetically more diverse than nests of *L. muscorum* in both communities. The stronger dispersal behaviour of *L. acervorum* also fits into the findings from the distribution and genetic structure of this species as it reveals a higher gene flow and is widely distributed, whereas populations of the smaller host *L. muscorum* are well structured and this species shows a more restricted distribution (Brandt et al. 2007).

Divergent outcomes in different populations and communities of the ongoing arms race lead to a geographic mosaic of coevolution, which could be found by using genetic, chemical and behavioural approaches. This could be best illustrated in this study of the interactions the two host species *Leptothorax muscorum* and *Leptothorax acervorum* and their social parasite *Harpagoxenus sublaevis*.

SUMMARY

Social parasites such as bees, wasps and ants parasitize complete insect societies. They take advantage of the brood care behaviour of other social insect species, and thus avoid the costs of parental care similar to avian brood parasites such as cuckoos and cowbirds. The European social parasite *Harpagoxenus sublaevis* is an obligate slavemaking ant species that exploits mainly two closely related host species of the genus *Leptothorax*. To found a new colony, a slavemaking queen invades a host colony, kills the resident queen and workers. The inseminated queen raises the alien brood and the later emerging host workers accept the parasite queen as their own and become slaves that carry out all necessary colony tasks. A year later, slavemaking workers emerge, which conduct regular slave raids on neighbouring host colonies for worker brood to replenish the labour force of the slavemaker nest. These slave raids can impose severe selection pressure on the hosts, as slavemaking colonies attack several host nests per year and raided host colonies often perish as a consequence of the attack.

According to the geographical mosaic theory of coevolution, differences in the advance or trajectory of the coevolutionray process between local communities are predicted due to their composition and the strength of ecological selection pressures through competition and resource availability. In our study system, investigations of the impact of the slave making ant *H. sublaevis* at several geographic distant sites allow general conclusions on the virulence, the degree of reciprocal adaptation and specialization of the species, and the evolutionary trajectories within this host-parasite system. The European slave making ant *H. sublaevis* and its host species are good examples as parasites and hosts are widely distributed throughout Eurasia whereas other social parasites use host species with small or patchy populations, e.g. *Myrmoxenus* or *Chalepoxenus*, where selection should be strong to decrease their virulence. Furthermore *H. sublaevis* produces a large army of slave making workers indicating that this species remains highly virulent. In accordance with the assumption of a geographic mosaic in the interaction of *H. sublaevis* and its hosts, these studies have shown that parasite prevalence is a good predictor of the strength of reciprocal adaptation in different communities.

In our genetic, chemical and behavioural studies we could show that *H. sublaevis* prefers the smaller host *L. muscorum,* which is more limited in dispersal than its larger competitor *L. acervorum*. Both hosts showed differences in defense strategies of which *L. acervorum* is the more aggressive host, while *L. muscorum* tend to flee when getting into

contact with its parasite. Moreover for the genetic more variable parasite the chemical profile of *L. muscorum* may be easier to imitate as this host is more limited in gene flow than its counter part. Further explanation of the better resemblance of the parasite to its smaller host could be the easier acquisition of the more volatile shorter hydrocarbons.

Also in the field manipulation study both host species showed different responses to the parasite pressure of *H. sublaevis* following the two strategies, investment in sexuals or in workforce. Moreover our crossfostering experiment indicated that the local parasite showed a greater impact on its hosts than the allopatric one. This led to the conclusion that coevolutionary trajectories differ between communities, asumed by different historical processes, community context and ecological conditons at each site which confirms the geographic mosaic theory of coevolution.

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PUBLICATIONS

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Bauer S, Witte V, Böhm M, Foitzik S (in press 2009) Fight or flight? A geographic mosaic in host reaction and potency of a chemical weapon in the social parasite *Harpagoxenus sublaevis*. Behavioral Ecology and Sociobiology published online

Böhm M, Bauer S, Foitzik S (submitted 2009) A jack of all trades is a master of none: Behavioural coevolution between an ant social parasite and its two host species. Animal Behaviour

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Zahn A, Bauer S, Kriner E, Holzhaider J (accepted 2009) Foraging habitats of the notched ear bat (*Myotis emarginatus*) in Central Europe. European Journal of Wildlife Research

Auszüge aus der Diplomarbeit „Untersuchungen zu Jagdhabitatswahl und Quartiernutzung der Wimperfledermaus *Myotis emarginatus* (Geoffroy, 1806) in Oberbayern in dem Buch „Fledermäuse in Bayern“, *Ulmer Verlag 2004*

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Ehrenwörtliche Versicherung

(Siehe Promotionsordnung vom 25.08.05, § 5, Abs. 1 Pkt. 6.)

Ich versichere hiermit ehrenwörtlich, dass die Dissertation von mir selbstständig, ohne unerlaubte Beihilfe angefertigt ist.

Stephanskirchen, 31.7.09 — S. Bauer

Ort, Datum — Unterschrift Doktorand/in

Erklärung

(Siehe Promotionsordnung vom 25.08.05, § 5, Abs. 1 Pkt. 9.)

Hiermit erkläre ich, dass ich mich **nicht** anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.*

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_____ Fakultät der _____

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dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.*

* Nichtzutreffendes bitte streichen

Stephanskirchen, 31.7.09 — S Bauer

Ort, Datum — Unterschrift Doktorand/in

Curriculum Vitae

PERSÖNLICHE DATEN

Name:	Sabine Bauer
	geb. Demel
Geburtsdatum:	09.03.1978
Geburtsort:	Rosenheim
Nationalität:	Deutsch
Familienstand:	Verheiratet

SCHULBILDUNG

09 / 1988 – 06 / 1997	Karolinen-Gymnasium Rosenheim, 1997 Leistungskurs Biologie und Englisch
09 / 1984 – 07 / 1988	Grundschule Rosenheim

AUSBILDUNG UND STUDIUM

08 / 2008 – ongoing	**Auftragsforschungsinstitut Kendle GmbH München**: Klinischer Monitor (CRA)
08 / 2004 – 10 / 2009	**Promotion an der Ludwig - Maximilian - Universität, München,** finanziert durch die Deutsche Forschungsgemeinschaft (DFG), Fachrichtung: Ökologie Thema: Wirt-Parasit-System
11 / 1998 – 04/ 2004	**Biologiestudium an der Technischen Universität, München,** (Abschlussnote 1,3) Fachrichtung: Zoologie, Medizinische Virologie, Biochemie Diplomarbeit finanziert durch die Bayerische Akademie für Naturschutz und Landschaftspflege Laufen (ANL) und den Landesbund für Vogelschutz (LBV)
10 / 1997 – 07 / 1998	**Studium der Mikro- und Feinwerktechnik an der Fachhochschule München,** Fachrichtung: Medizintechnik
03 / 1995 – 08 / 1995	**Ausbildung zur Schwesternhelferin** (Abschlussnote 1,0)

AUSLANDSERFAHRUNG

11 / 2001 – 02 / 2002	**Australian Institute for Marine Science (AIMS) Townsville, Australia** Teilfinanzierung durch den Deutschen Akademischen Austausch Dienst (DAAD) ○ DNA-Sequenzierung des tropischen Felsenhummers *Panulirus ornatus* ○ Mikroskopische Präparierung von Garnelen - Antennen zur Untersuchung olfaktorischer Rezeptor Neurone (ORN)

INTERESSEN

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Sabine Bauer