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COEVOLUTION IN THE SLAVEMAKING ANT *PROTOMOGNATHUS AMERICANUS* AND ITS *TEMNOTHORAX* HOST SPECIES: INFLUENCE OF PARASITE PRESSURE, BEHAVIORAL ADAPTATIONS AND PATTERNS OF GENE FLOW

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GENERAL INTRODUCTION

Adaptation to abiotic conditions and biotic factors is certainly one of the main driving forces for the evolution of the enormous number of morphologies, behaviors and life history strategies in the animal kingdom (Futuyma 1986). This is especially true for the adaptation to and the interactions with other species. According to the geographic mosaic of coevolution theory (Thompson 1999b, 2005) the interactions between two species can be influenced by several parameters: The variation of ecological parameters, the composition and history of a local community and the evolutionary potential of the two opponents. In contrast to ecological parameters, species are capable and forced to change rapidly and frequently. Interacting species like competitors, predator and prey or parasite and host thus exert highly variable selection pressures (Thompson 1994, 1999a), which can potentially result in a continuous process of reciprocal adaptations: the so-called "coevolutionary arms race" (Dawkins & Krebs 1979). In the extreme case, this race can even end in the Darwinian extinction of one of its participants due to a time lack in the development of effective counter-adaptations (Darwin 1859).

The highly specialized relations of parasites and their hosts represent ideal model systems for the study of coevolution. Parasites were shown to influence their host species in various aspects (e.g. Anderson & May 1979, Freeland 1979, 1983; Price et al. 1986) and to dramatically reduce the fitness of infected host individuals up to complete sterilization. In the case of microparasites, this severe impact is due to the asymmetric evolutionary potentials of the opponents caused by differences in population sizes, mutations rates and generation times (e.g. viruses, bacteria, fungi). In contrast, macroparasites are much more similar to their host species and often show comparable evolutionary potentials (e.g. arthropods, helminthes). In the case of brood parasites the similarity between parasite and host is even greater since only closely related host species potentially fulfill both behavioral and nutritional needs of the parasitic young.

Most energy acquired during an animal's adult life is channeled into reproduction and it is thus not surprising that strategies evolved to lower the costs of brood care. This is especially true for species with extensive parental care, such as mammals, birds and social insects. One way to reduce these costs is brood parasitism, where the parasite exploits the brood care behavior of its host species. Brood parasitism has been found in a variety of different taxa (e.g. Boulton & Polis

2002, Sato 1986, Brooke & Davies 1988, Hölldobler & Wilson 1990). During the last decades, predominantly avian brood parasites, such as cuckoos and cowbirds, have received great attention and the potential occurrence of a coevolutionary arms race has been studied intensively in several species (Davies & Brooke 1989, Lotem & Rothstein 1995, Rothstein 1990, Soler & Soler 2000, Soler & Møller 1990). These avian brood parasites have been shown to lay their eggs in nests of other bird species and to thereby avoid the cost of parental care. Their avian host species thus suffer both the cost of brood care for the unrelated offspring and the loss of their own eggs due to the destructive behavior of the parasite young.

Social parasites represent a highly analogous system and reduce the cost of parental care by exploiting the brood care behavior and the entire social system of members of their own or another socially living species (Davies et al. 1989). Generally this form of brood parasitism can be found among social insects and, within the Hymenopterans, it is especially common in ants. Remarkably, in ants, more than 250 of the over 12,000 known ant species live a social parasitic life style (Buschinger 1986, Hölldobler & Wilson 1990) and these ants exploit their host species either temporarily or permanently. In ants, there are mainly two different forms of social parasites: inquilines, which have lost the worker caste and produce only sexuals to be raised by the hosts, and slavemakers, in which the queen produces her own workers that conduct slave raids on neighbouring host nests. Since its first detailed description in the year 1810 (Huber), the latter behavior in ants has fascinated both scientists and the general public.

Slavemaking ants are social parasites with an extremely interesting life style (Buschinger 1986, Hölldobler & Wilson 1990, Brandt et al. 2005a). Usually, these parasites obligatorily depend on related ant species for brood care, foraging, and nest defense and their specific morphology and behavior allows them to find and subdue colonies of their host species for their purposes. Slavemaking ants are characterized by a large body size, strong mandibles, thick cuticle, a broad postpetiole and antennal scrobes, which are used to protect the antennal scapes during fights. The life cycle of a slavemaking ant begins, when a mated parasite queen conquers a host nest, expels or kills the host queen(s) and adult workers and takes over the remaining worker brood (Fig. 1) (Alloway 1979, Alloway 1980, Wesson 1939, Wilson 1971). These first slave workers, which soon eclose from the usurped brood, learn to accept the slavemaker queen during the first days (Goodloe & Topoff 1987, Jaisson 1975, LeMoli & Mori 1982, LeMoli & Passetti 1977) and

subsequently fulfill all tasks of colony maintenance and brood care. Since enslaved host workers are unable to reproduce new slave workers, the steady supply of slaves has to be ensured in a different way. For this purpose, slavemaker workers raised during the following years, conduct recurrent slave raids on neighboring host colonies and steal new host worker brood (e.g. Alloway 1979). We consequently find a seemingly peaceful coexistence of species within a slavemaker colony but the colony survival often obligatorily depends on the collaboration of the enslaved host workers. These host workers rear both parasite and raided host brood and thus control the productivity of the social parasite nest.

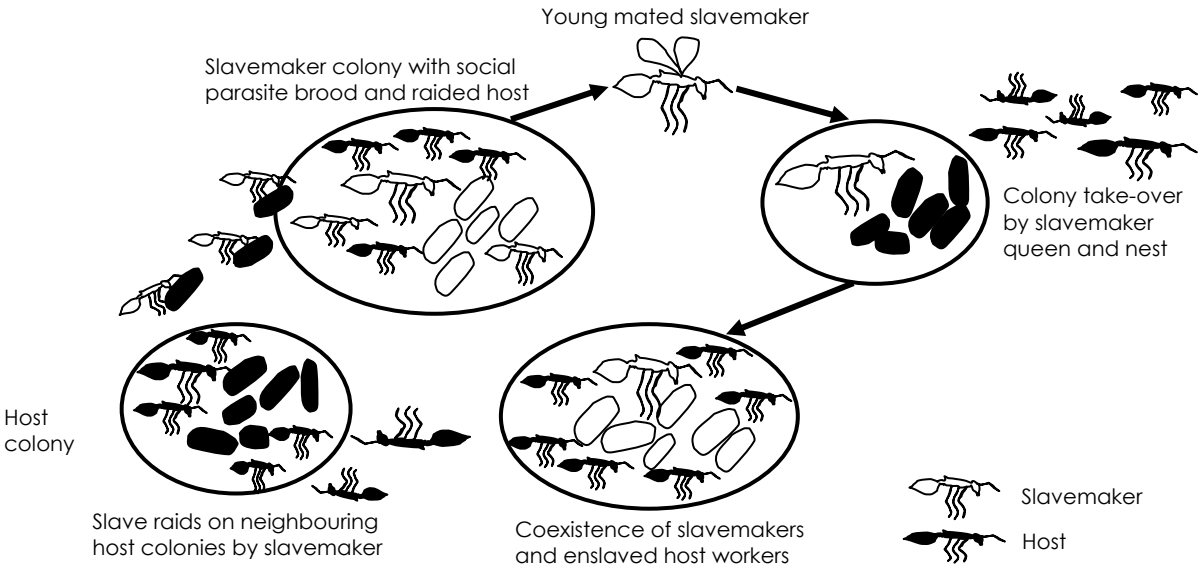

Figure 1: Life cycle of a slavemaking ant. Graphic by Susanne Foitzik

Only 50 ant species of the 200 ant social parasites are active slavemakers (D'Ettorre & Heinze 2001, Hölldobler & Wilson 1990) and this exceptional life style evolved several times independently among the subfamilies Myrmicinae and Formicinae (Buschinger 1990, Buschinger et al. 1980, Hölldobler & Wilson 1990, Stuart & Alloway 1983). Particularly the Myrmicinae tribe of the formicoxenine ants seems to be a hot spot in the evolution of slavemaking ants with at least six independent origins. Among this tribe, slavemakers were shown to originate from non-parasitic Formicoxenini at different points of times (Beibl et al. 2005).

The strength of reciprocal adaptations varies with the specificity of its participants and inevitably peaks in the relationship between obligate parasites and

their hosts (Futuyma & Slatkin 1983, Thompson 1994, Thompson 1999a). Despite of this and despite of the strong analogy to avian brood parasites, the fascinating relationship of insect social parasites and their hosts was not investigated under a coevolutionary perspective for a long time as it was assumed that social parasites have won the arms race against their hosts (Gladstone 1981, Grasso et al. 1992). Yet, recent studies on the association between slavemaker presence and host demographic and genetic structure have revealed strong evidence for an ongoing coevolutionary arms race (Foitzik & Herbers 2001a, Herbers & Foitzik 2002, Foitzik et al. 2003, Hare & Alloway 2001) and thus fiercely questioned this view.

In particular, empirical studies on the slavemaking ant *Protomognathus americanus* and its host species have demonstrated the strength of selection pressure, this social parasite exerts on its hosts. *P. americanus* is a tiny myrmicine ant, which obligatorily exploits three related species of the genus *Temnothorax*. These ants nest in hollow acorns, hickory nuts and small twigs on the ground of mixed deciduous forests throughout the north-eastern part of the American continent (Fig. 2).

Figure 2: *P. americanus* worker (left), *T. longispinosus* worker (left middle), *T.curvispinosus* worker (right middle), *T. ambiguus* worker (right). Pictures by Miriam Brandt

The strong impact of this social parasite on its host species largely stems from the frequent and destructive slave raids, with host colonies suffering from 2-10 successful raiding attacks per year and social parasite colony, and a low post- raid survival (Foitzik et al. 2001, Foitzik & Herbers 2001a, Blatrix & Herbers 2003). In addition, host colonies in the vicinity of the social parasite were shown to exhibit a changed demography and investment strategies. These host colonies were smaller, more frequently monogynous and their production rather focussed on dispersing sexuals than on colony maintenance (Foitzik & Herbers 2001b, Herbers & Foitzik 2002). Yet, a

direct and causal relationship between *P. americanus* presence and host nest density, demography and investment strategies was as yet lacking. Publication 1 is trying to fill this gap with empirical data on a large-scale and long-term field experiment, manipulating parasite density in field. According to the geographic mosaic theory (Thompson 1994, 1999b, 2005), the nature and outcome of species interactions can vary greatly across and between regions. Earlier studies have found evidence for local adaptation of the cuticular hydrocarbon profile of *P. americanus* (Foitzik et al. 2001, Brandt & Foitzik 2004, Brandt et al. 2005a), which matched the chemical odor of sympatric hosts. Therefore it was especially interesting, if the social parasite is also locally adapted on an ecological scale. Publication 1 is addressing this question by including a special cross-fostering design with two geographically separated ant communities into the set-up (e.g. Foitzik et al. 2001, Herbers & Foitzik 2002, Brandt & Foitzik 2004, Foitzik et al. 2004).

Within the parasite-host system of *P. americanus*, preliminary studies and Publication 1 have impressively demonstrated the strong selection pressure exerted by this social parasite. This strong parasite pressure has inevitably led to the evolution of host defenses, especially in the context of slave raids and thus proven an ongoing coevolutionary arms race between *P. americanus* and its host species (Alloway 1990, Mori et al. 1991, Foitzik & Herbers 2001a, Herbers & Foitzik 2002). Apart from the already mentioned changes in demography and investment strategy, host defenses include enemy recognition and better fighting abilities as well as the fast evacuation and escape of the attacked host colonies (e.g. Alloway 1990, Foitzik et al. 2001, Brandt et al. 2005a). All these adaptations are defense strategies that help to avoid falling victim to slave raids or the colony take-over by a mated social parasite queen. Yet, counter-parasite strategies after successful establishment of parasitism have been argued to be impossible to be selected for (Gladstone 1981), because enslaved host workers seemed to gain no fitness benefit by rebelling against their oppressors. Publication 2 intensely questions this view and documents the killing of social parasite brood by enslaved host workers.

The survival of a social parasite colony entirely depends on the collaboration of enslaved host workers. Apart from rearing social parasite brood, enslaved host workers are confronted with a steady supply of raided host brood representing the future slave workforce. Hence, the acceptance and brood care for alien raided brood is a critical point in the parasitic life style of slavemaking ants. Enslaved host workers could also develop defence strategies in this context. Publication 3 thus aims

to enlighten the potential evolution of a rejection behavior in the host and empirically investigates the acceptance of alien host and parasite pupae of different castes by *Temnothorax* workers in parasite colonies and in non-parasitized host colonies (Fig. 3). The rejection of raided alien host brood was so far excluded, since the brood acceptance of segregated host workers was shown to be either enhanced by the presence of the social parasite (Alloway 1982), or enforced by the exposure of enslaved host workers to allospecific odors during a critical learning period after their eclosion (Jaisson 1975, LeMoli & Passetti 1977, LeMoli & Mori 1982, Goodloe & Topoff 1987). Yet, behavioral studies on the brood acceptance in naturally composed field colonies, as in Publication 3, have as far been lacking.

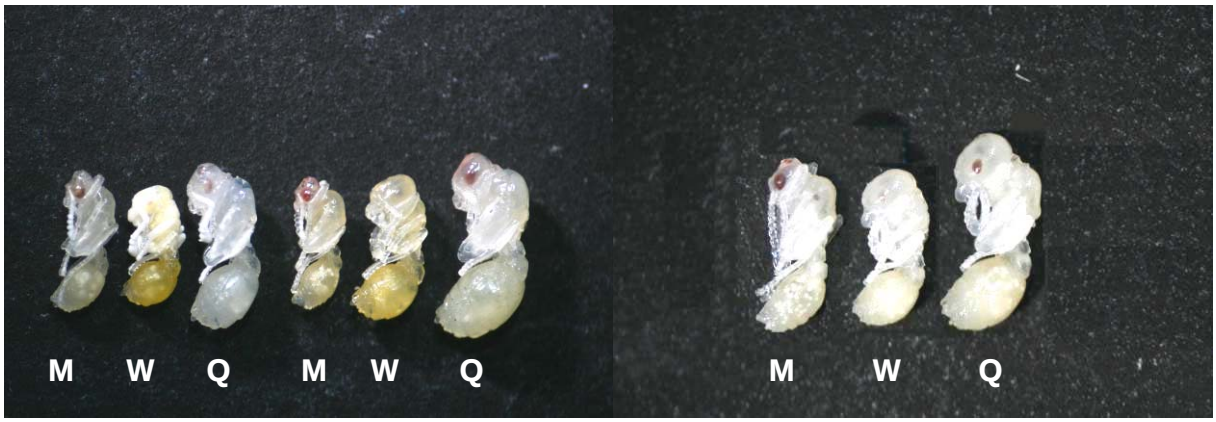

Figure 3: Male (M), worker (W) and queen (Q) pupae of the host species *T. longispinosus*, *T. curvispinosus* and the social parasite *P. americanus.*

Furthermore, Publication 3 investigates a potential chemical recognition mechanism, which *Temnothorax* workers might use to discriminate between pupae of different castes and species. In social insects, chemical signals e.g. cuticular hydrocarbons and gland secretions are known to be widely used in the recognition and discrimination of nestmates, sex and species (Howard 1993).

As demonstrated, *P. americanus* and its *Temnothorax* host species are caught in an ongoing coevolutionary arms race of reciprocal counter adaptations which were at least in part already shown to significantly vary across and between geographical regions (Foitzik et al. 2001, Brandt & Foitzik 2004, Brandt et al. 2005a, Foitzik et al. in press). Both the coevolutionary arms race and the local adaptation of *P. americanus* on a chemical and an ecological scale are clearly based on the evolutionary potentials of the interacting species. Unlike asymmetric host-parasite

systems, with a substantially higher evolutionary potential for the parasite (Hamilton et al. 1990), social parasites like the slavemaking ants *P. americanus* are very closely related to their host species and are thus characterized by similar generation times and mutation and recombination rates (Emery's rule; Emery 1909). In this evolutionary model, migration and gene flow can therefore be crucial for leading the coevolutionary arms race. Geographic population structure shapes the interspecific interactions between parasite and host (Gandon et al. 1996, Gandon & Michalakis 2002, Thompson 1994). Local adaptation is consequently found in populations with low to intermediate levels of gene flow and conspecific population diverge due to genetic drift (Brodie & Brodie 1991, Carroll et al. 1997, Wilkinson et al. 1996). High levels of gene flow can cause to homogenize species interactions due to the rapid spread of new evolutionary traits (Slatkin 1987). Excluding the latter extreme, gene flow will allow beneficial adaptations as a result of local selection pressure (Thompson 1994). With genetic variability being the limiting factor, gene flow among parasite populations is crucial for spreading adaptive traits (Thompson 1994) and gene flow among host populations enables the antagonist to keep up with its opponent by evolving anti-parasite adaptations (Dybdahl & Lively 1996, Ladle et al. 1993). Host populations which show a higher migration rate than their parasite, should consequently be able to locally adapt to their oppressor and vice versa (Gandon et al. 1996, Kaltz et al. 1999). Publication 4 thus represents a broad genetic microsatellite study on the amount of genetic variability and the pattern of gene flow between social parasite- and host populations.

SUMMARY: AIMS OF THIS THESIS

The aim of this thesis was to investigate the coevolutionary arms race between the slavemaker *P. americanus* and its *Temnothorax* host species from different perspectives. Previous studies on this obligate social parasite have already demonstrated the evolution of morphological, behavioral and chemical adaptations, and have given variable results on the strength of the selection pressure exerted by this parasite. Based on these results, Publication 1 investigates the direct and causal relationship of the parasite pressure exerted by *P. americanus* and the reaction of nest density, social structure and life history of its main host species *T. longispinosus* in two ant communities. Publication 2 also enlightens the effects of the substantial selection pressure of *P. americanus* on its host species, but with a focus on host behavior and defensive anti-parasite adaptations of *Temnothorax* workers, which are active after host workers are parasitized. Based on the finding of slave rebellion, Publication 3 further investigates the brood acceptance behavior of *Temnothorax* workers and a potential chemical, recognition mechanism to discriminate between pupae. Finally in Publication 4, a genetic study on the amount of genetic variability and the patterns of gene flow between social parasite- and host populations is presented.

PUBLICATION 1

LOCALLY-ADAPTED SOCIAL PARASITE AFFECTS DENSITY, SOCIAL STRUCTURE AND LIFE HISTORY OF ITS ANT HOSTS

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ABSTRACT

Selection and adaptation are important processes in the coevolution between parasites and their hosts. The slavemaking ant *Protomognathus americanus*, an obligate ant social parasite, has previously been shown to evolve morphological, behavioral and chemical adaptations in the coevolutionary arms race with its *Temnothorax* hosts. Yet, empirical studies have given variable results on the strength of the selection pressure this parasite exerts on its host populations. In this study, we directly investigated the pressure exerted by *P. americanus* and the reactions of the main host species, *T. longispinosus* in two ant communities by manipulating parasite density in the field over several years. In addition, a cross-fostering design with the exchange of parasites between host populations allowed us to investigate local adaptation of parasite or host. We demonstrate a severe impact of the social parasite on the two host populations in West Virginia (WV) and New York (NY), but also variation in host reactions between sites, as expected by the geographic mosaic theory of coevolution. Host density decreased at the WV site with the presence of local slavemakers, whereas at the ecologically favorable NY site, density was unaffected. Nevertheless, social organization, colony size and investment patterns of these host colonies at this site changed in response to our parasite manipulation. The release of *P. americanus* colonies led to a reduction in the number of resident queens and workers, an increase in intra-nest relatedness, a lower productivity, but also a higher investment in reproductives. In WV, colony demography did not change, but raiding activity by NY slavemakers caused different investment patterns of host colonies. In addition, the cross-fostering element revealed local adaptation of the parasite *P. americanus*: slavemaking colonies fared better in their sympatric host population, as they contained more slavemaking ant workers and slaves at the end of our 27 months experiment.

Keywords: Host-parasite interactions, geographic mosaic, coevolution, coevolutionary arms race, selection, social insects, slavemaking ants, *Protomognathus*, *Temnothorax*

INTRODUCTION

Natural selection as the driving force of evolution can be especially intense in the coevolutionary interactions between obligate parasites and their hosts. Parasites often dramatically lower the fitness of infected host individuals, up to complete sterilization. These strong selection pressures often cause rapid counter-adaptations in the host, leading to an evolutionary arms race between parasite and host (Dawkins & Krebs 1979). Furthermore, ecological interactions between antagonistic species can have strong effects on the community level, as is best known from the population oscillations in predator-prey interactions (Wade 2007). Besides this aspect, adaptations in other species can negatively alter the ecological environment for an interacting species to such an extent that it can go extinct if it does not develop counter-adaptations quickly enough (Darwinian extinction; Darwin 1859). In addition to changes in the coevolution over time, these interactions also vary over geographic landscapes. According to the geographic mosaic theory (Thompson 1994, 1999b, 2005), the nature and outcome of species interactions can differ greatly between locales. In coevolutionary "hot spots," predominant selection pressures on each species stem from its enemies, which results in each species being tightly coadapted. In contrast to these are "cold spots", where other selection pressures prevail and coevolution is less strong, or even absent if some species are lacking or occur at low densities.

Slavemaking ants are social parasites with a highly sophisticated lifestyle (Buschinger 1986, Hölldobler & Wilson 1990, Brandt et al. 2005b), most of which obligatorily depend on a heterospecific workforce for routine tasks such as brood care, foraging, and nest defense. These parasitic ants are behaviorally and morphologically well-equipped for one task: finding and subduing colonies of their host species. Parasite colonies are initiated in summer when mated young slavemaker queens take over host colonies. The first slave workers, which emerge from the usurped host brood accept the parasite queen and care for her and the brood. Slavemaker workers, raised during the following years, engage in frequent raids on neighboring host nests to steal host brood and to replenish their slave supply. The negative impact of these social parasites on their hosts largely stems from these frequent and destructive slave raids.

Genetic studies on the tiny formicoxenine slavemaking ant species *Protomognathus americanus* have shown between 2-10 successful attacks on host

colonies per year per parasite colony and a low post-raid survival rate of host nests (Foitzik et al. 2001, Foitzik & Herbers 2001a, Blatrix & Herbers 2003). In addition, demographic and ecological analyses have demonstrated the footprint of selection in these raids via a changed demography and investment pattern of host colonies in the vicinity of *P. americanus* nests. Host colonies within the raiding range of slavemaking nests were more frequently monogynous, smaller and reallocated resources from the production of new workers important for colony maintenance to dispersing sexuals (Foitzik & Herbers 2001b, Herbers & Foitzik 2002). However, as expected by the mosaic theory of coevolution (Thompson 1994, 1999b, 2005), the intensity of selection in the interaction between this obligate social parasite and its *Temnothorax* hosts differs greatly between ant communities. This variation has been attributed to pronounced geographic differences in parasite prevalence, raiding frequency, post-raid host survival and community composition (Foitzik et al. 2001, Herbers & Foitzik 2002, Blatrix & Herbers 2003, Brandt & Foitzik 2004, Johnson & Herbers 2006). The observed variation in the coevolutionary process can also explain the results of two experimental manipulations of *P. americanus* density in *Temnothorax* populations in Ohio and Ontario (Hare & Alloway 2001, Johnson & Herbers 2006). These small scale, short-term experiments compared parasite pressure by *P. americanus* to selection exerted by a second slavemaker, *T. duloticus*. In contrast to the latter species, *P. americanus* was found to have only a minor impact on its hosts, and was accordingly described as prudent. Yet, this contradicts correlative results on Northeastern populations of *T. longispinosus*, where parasite pressure by *P. americanus* was found to be intense and appeared to greatly influence host demography and allocation (Foitzik & Herbers 2001a, Herbers & Foitzik 2002).

In our large-scale field manipulation, we focus on the interaction between *P. americanus* and its main host species, *T. longispinosus*, which is a widely distributed ant species, with a highly variable ecology, social organization and life history (Herbers 1989, Herbers & Banschbach 1999, Herbers & Foitzik 2002, Foitzik et al. 2004). We directly manipulated slavemaker density in a long-term experiment in two ant communities in the States of New York (NY) and West Virginia (WV). Behavioral, chemical, genetic and ecological analyses agree that an ant community in upstate NY represents a coevolutionary "hot spot," with strong reciprocal selection pressures (Foitzik et al. 2001, Foitzik & Herbers 2001a, Herbers & Foitzik 2002, Brandt & Foitzik 2004, Foitzik et al. 2004, Brandt et al. 2005a). At this densely populated site, *T. longispinosus* hosts apparently experience optimal ecological conditions, with

large and frequently polygynous colonies. Host nest composition and allocation patterns strongly varied with the presence of *P. americanus*. In the vicinity of slavemaking nests, host colonies were generally smaller, contained fewer queens and showed higher intra-nest relatedness. In contrast, host nest demography was not associated with social parasite presence in the WV community, where genetic data indicate less frequent and less destructive raids by *P. americanus* (Foitzik et al. 2001, Herbers & Foitzik 2002). Moreover, the density, demography and social organization differed strongly between *T. longispinosus* populations with less dense, smaller and predominantly monogynous host nests located in WV (Herbers & Stuart 1996b).

Based on these site-specific differences, we hypothesize that the experimental addition of *P. americanus* nests will strongly decrease host nest density in WV, but not in NY. Despite the fact that frequent slave raids are expected to destroy host colonies at both sites, the host colonies in NY are expected to quickly repopulate vacant nest sites. In addition, the large, polygynous colonies in NY are anticipated to split up, as has been demonstrated for small, monogynous *Temnothorax* nests in this population when empty nests sites are experimentally added (Herbers 1986). Therefore, our second hypothesis states that in the polygynous population in NY, an increase in parasite pressure should lead to small, monogynous host nests with altered allocation strategies. However, these changes in demography and social organization are not expected in WV.

Our hypotheses are based on population comparisons and correlations between parasite presence and host demography in two ant communities. Unfortunately, these correlative approaches lack the power to disentangle cause and effect. Consequently, it remains unclear whether the recorded variation in life-history in the NY *T. longispinosus* population is directly caused by its social parasite or whether *P. americanus* preferentially settles in habitats with small, monogynous host colonies (Herbers & Foitzik 2002). Nevertheless, our first hypothesis invokes a direct and causal relationship, which our experimental approach will permit us to directly examine. Our approach will also allow us to differentiate the two hypotheses that we have proposed.

We included a cross-fostering element in our field manipulation with the exchange of *P. americanus* colonies between ant communities to investigate the potential occurrence of local adaptation in this social parasite system on an ecological scale. Local adaptation describes a situation where the mean fitness of a population is higher in its home locality than in any other environment (Kaltz & Shykoff

1998). While behavioral cross-fostering studies on *P. americanus* and its hosts did not indicate local adaptation, the cuticular hydrocarbon profile of this obligate parasite was found to be adapted to its sympatric host populations (Foitzik et al. 2001, Brandt & Foitzik 2004, Brandt et al. 2005a). If the social parasite is also locally adapted on an ecological scale, we would expect that the released *P. americanus* colonies fare better in their sympatric host populations. Maladaptation, in contrast, would indicate local adaptation of the *T. longispinosus* populations. However, an alternative hypothesis could suggest that low host density in WV might lead to lower parasite success independent of the origin of *P. americanus* colonies. Alternatively, host density could be is less important for the parasite than defensive behaviors of the host, which were found to be more effective in NY (Foitzik et al. 2001).

MATERIAL AND METHODS

Study system

The obligate social parasite, *Protomognathus americanus* (Tribus Formicoxenini), is widely distributed throughout the deciduous forests of the northeastern United States and Canada. This slavemaking ant parasitizes three *Temnothorax* species, with *T. longispinosus* being its main host (Creighton 1927, Wesson 1939, Creighton 1950). These tiny ants inhabit cavities in acorns, nuts and sticks in the leaf litter.

Our field manipulations were conducted at two well-studied ant communities in the eastern United States (Culver 1974, Herbers 1989, Herbers & Stuart 1996b): The study site at the Edmund Niles Huyck Preserve near Albany in New York State (NY) is situated at 600m above sea level, while the more Southern site in the State of West Virginia (WV) was at a slightly higher elevation (1,000 m above sea level) at Watoga State Park in Pocahontas County. In NY the host species *T. longispinosus* constitutes more than 95% of all host nests, while in WV this host species is slightly less dominant, comprising only about 84% of the local *Temnothorax* community. Secondary hosts are *T. ambiguus* in NY and *T. curvispinosus* in WV. Parasite prevalence in both communities varies between 7-10%, but slavemaking ant colonies are generally smaller in WV (Herbers & Foitzik 2002).

Field manipulations

In spring 2001, we started a 27-month, large scale, cross-fostering field experiment at the NY and WV field sites. To keep the experimental conditions for the ants as natural as possible we marked plots, but did not prohibit movement of host or parasite colonies. For Temnothorax ants, nest movement is regular part of a colony's life because their nest sites e.g. acorns quickly decompose. Our decision to not restrict colony movement was a risky one as migration in and out of our study plots could potentially equalize any effects of our manipulation. Due to the breadth of our experiment, involving over 1400 m² of forest floor and more than 950 ant colonies, we still felt confident we would be able to show, if present in the field, an influence of *P. americanus* on its hosts.

In our experiment, we used a hierarchical design with site (NY vs. WV), block (six-seven sub-sites within each site) and plot (three plots per block, each with a different treatment). In May of 2001, we collected and mapped all ant colonies of the study species in 39 plots of 5m x 5m by carefully searching the forest floor and opening all potential nest sites. We set up 18 plots in NY, located in six different sub-sites (blocks), and 21 plots in WV in seven blocks. Within each site, blocks were separated by a minimum of 10m and a maximum of 500m and each block contained three plots, which lay between 4 and 6 m apart. The block design was used to measure small scale ecological differences between areas, which are known to affect the biology of the host *T. longispinosus* (Foitzik et al. 2004). Ant colonies were transferred to the field station where they were censused and allowed to move into artificial nest sites. These nest sites were made of cylindrical beech dowels (10 cm long, 1.5 cm in diameter, with a longitudinal 4-mm hole), closely resemble natural nest sites and are readily accepted by *Temnothorax* ants (Herbers 1986, Foitzik et al. 2004). Ant colonies were returned to the exact position in the forest within two weeks. In each block, plots were randomly subjected to one of the following three treatments:

I. In six NY plots and seven WV plots we refrained from returning *P. americanus* colonies. These plots, which were free of slavemaking ant colonies at the onset of the experiments, are called "parasite-free plots", though *P. americanus* colonies could immigrate during the experiment. As parasite colonies only moved in over time, a long-term reduction in parasite pressure was expected.

II. In six plots in NY and seven plots in WV, we released *P. americanus* colonies from NY ("NY parasite plots"). *P. americanus* colonies were placed close to the center of our plots, so that parasite emigration out of the plot boundaries was less likely for these tiny ants with a body size of less than 4 mm.
III. In six plots in NY and seven plots in WV plots we transferred and released *P. americanus* colonies from WV ("WV parasite plots").

With our manipulation, we imitated natural prevalence of *P. americanus* in the two ant communities. We standardized both the number of *P. americanus* colonies and the number of slavemaking workers per host colony in each plot: one *P. americanus* colony was released for ten *T. longispinosus* host colonies, with a minimum of two *P. americanus* colonies per plot. In addition, we selected *P. americanus* colonies according to the number of slavemaking ant workers. Careful colony selection allowed us to release *P. americanus* colonies in such a way that for every three *T. longispinosus* colonies in a plot at least one *P. americanus* worker was released (in its colony). For example, in a plot with 20 host nests, we released two *P. americanus* colonies, with a total of seven workers. The distribution of these seven workers among the two *P. americanus* nests could vary and depended on the natural composition of the available nests. We did not manipulate the natural composition of *P. americanus* colonies used in this experiment. Slavemaking nests were positioned close to the center of the study plots, with at least one meter between them and the boundaries of the plot. The demography of the released *P. americanus* colonies (N of queens and workers) did not vary with their origin or release site (Factorial ANOVA: Parasite origin $F_{2, 65} = 0.27$, $p = 0.76$; Release site: $F_{2, 65} = 0.89$, $p = 0.41$; Parasite origin x Release site $F_{2, 65} = 0.09$, $p = 0.92$).

We completely excavated all plots in July to August 2003, shortly before the emergence of pupae in ant nests. As the exact raiding range of *P. americanus* colonies is currently unknown, we additionally surveyed a 50 cm strip surrounding each plot, thus plot size in 2003 increased to 6m x 6m (36m²). We examined all potential nest sites (artificial and natural ones), mapped the position of all colonies and transported them to the laboratory in Germany where each colony was censused and frozen for genetic analysis. To estimate annual investment, we combined census data with data on the average dry masses of adult *T. longispinosus* males, new queens and workers (Foitzik et al. 2004), but adjusted the investment in female sexuals with the energetic cost ratio (Boomsma 1989). We calculated the sex

allocation ratio for each nest as the relative proportion of energy allocated to male versus female propagules (Crozier & Pamilo 1996). We also computed the reproductive allocation ratio, which is the relative proportion of energy invested in sexual reproduction compared to new workers (Sundström 1995, Herbers et al. 2001, Reuter & Keller 2001).

Influence on the genetic colony structure

Demographic data from this experiment and earlier studies have showed a strong effect of *P. americanus* on the social organization of *T. longispinosus* nests at the NY study site, but not in WV (Herbers & Foitzik 2002). To investigate whether the intracolonial relatedness was also affected by our manipulation, we investigated worker-worker relatedness in *T. longispinosus* colonies from NY. We analyzed three workers each from all *T. longispinosus* nests in a subsample of nine plots, three plots from each treatment. In total we genotyped 564 *T. longispinosus* workers from 188 different colonies. Ants were preserved in 100% ethanol and frozen at -20°C until extraction. DNA from individual ants was isolated with the Puregene® DNA isolation kit (Gentra systems). We genotyped all samples at four microsatellite loci: LXA GT1 (Bourke et al. 1997), L-18, L-5 (Foitzik et al. 1997) and Myrt3 (Evans 1993). PCR was conducted in a 20µl reaction volume using the Q biogene TaqCoreKit containing 1x incubation buffer, 2.2mM $MgCl_2$, 0.4mM each dNTP, 0.5µM labeled forward, 0.5µM unlabeled reverse primer, 0.13U of *Taq* DNA polymerase (Q biogene) and 1-2 µl of template. Initial denaturation for 5min at 94 °C was followed by 28 amplification cycles (denaturation at 92°C for 1min 30sec; annealing at 54°C for 45sec; and extension at 72°C for 30sec), and a final extension at 72°C for 7 min, in a Thermo Electron Corporation Thermocycler (PxE 0.2 Thermo). PCR products were precipitated with a mixture of 0.5µl of 3M Ammonium acetate and 10µl of ethanol (100%) at -60°C for 60 min, dehydrated with a series of different ethanol concentrations (100%, 70%) and dried. The thus purified PCR products were dissolved in 5.75 µl dd H_2O and 0.25 µl MegaBACE™ ET400-R size standard (Amersham Bioscience) and analyzed in a capillary sequencer (Amersham Bioscience MegaBACE™ 1000). Data analysis was performed using the software program MegaBACE™ Fragment Profiler 1.2 (Amersham Bioscience). Relatedness values, allele frequencies and allele numbers were calculated with the software program Relatedness 5.0 (Queller & Goodnight 1989). We calculated the population-level

estimates and their standard errors obtained by jack-knifing over colonies, and report them below.

Data analysis and statistics

Data analysis was complicated by the fact that host density and demography varies on a fine scale at the NY study site (Foitzik et al. 2004). Therefore, we included the factor 'block' in our analytical methods, which indicates the respective sub-site within each site. The raiding range of P. americanus colonies is unknown, but potentially extends outside of the study plots. Microsatellite analyses revealed an average distance between polydomous P. americanus nest units of 1.19 ± 0.92m, indicating that migrations and raids are conducted over a range of about 1 meter (Foitzik & Herbers 2001b). For this reason, analyses of the 2003 Temnothorax data set were generally based on the larger 36m² plots, with a few exceptions: first, we used the original 25m² when we directly compared the number of ant nests in 2001 and 2003 plots. Second, analyses of the density and demography of P. americanus colonies were also based on the original quadrat, because parasite nests found outside of this range most likely represent naturally occurring P. americanus colonies of the local ant community and not colonies released by us. We examined nest frequency data with a log-linear analysis (Sokal & Rohlf 1995), which allowed us to study the impact of our parasite manipulation and of ecological parameters (i.e. differences between blocks) and their interaction simultaneously. To analyze the impact of slave raids on host nest demography we used two different approaches. First, we compared social structure (i.e. N of queens per nest) and colony size (N of workers) for colonies found in the same plot in 2001 and 2003. We tested whether changes in social organization or colony size were associated with our manipulation of parasite density. Furthermore, we compared the demography of host colonies in 2003 between plots that experienced different treatments. The latter analyses additionally allowed us to contrast productivity and investment patterns, which are highly variable between years. We used a hierarchical approach and first analyzed the entire data set with factorial ANOVAs using as independent factors treatment and study site (NY vs. WV). Then we focused on each study site separately and in these factorial ANOVAS we included the treatment and block (sub-sites within each site) as independent factors. Data distributions were tested for deviation from normality and heteroscedasticity. As expected for large data sets such as ours (e.g. the analysis on host nest demography was based on 797 host colonies) tests

often revealed significant deviations from normality. Consequently, a number of transformations (log, arcsine, square root etc.) were used to normalize data or else we used non-parametric statistics. We generally report two sided p-values. All statistical tests were performed with the program Statistica 6.0.

RESULTS

Composition of ant communities and nest densities

In 2001, we found a total of 397 *T. longispinosus*, 13 *T. ambiguus* and 33 *P. americanus* nests within the 450m² of mapped forest floor at the NY study site, resulting in an average density of 0.909 ± 0.081 host nests / m² and a parasite prevalence of one *P. americanus* colony per 12.4 *Temnothorax* nests. In WV with a total study area of 525 m² (21 plots x 25m²), we collected 312 *T. longispinosus*, 3 *T. curvispinosus* and 34 *P. americanus* nests, resulting in a mean density of 0.598 ± 0.081 host nests / m² and a parasite prevalence of one per 9.3 host nests.

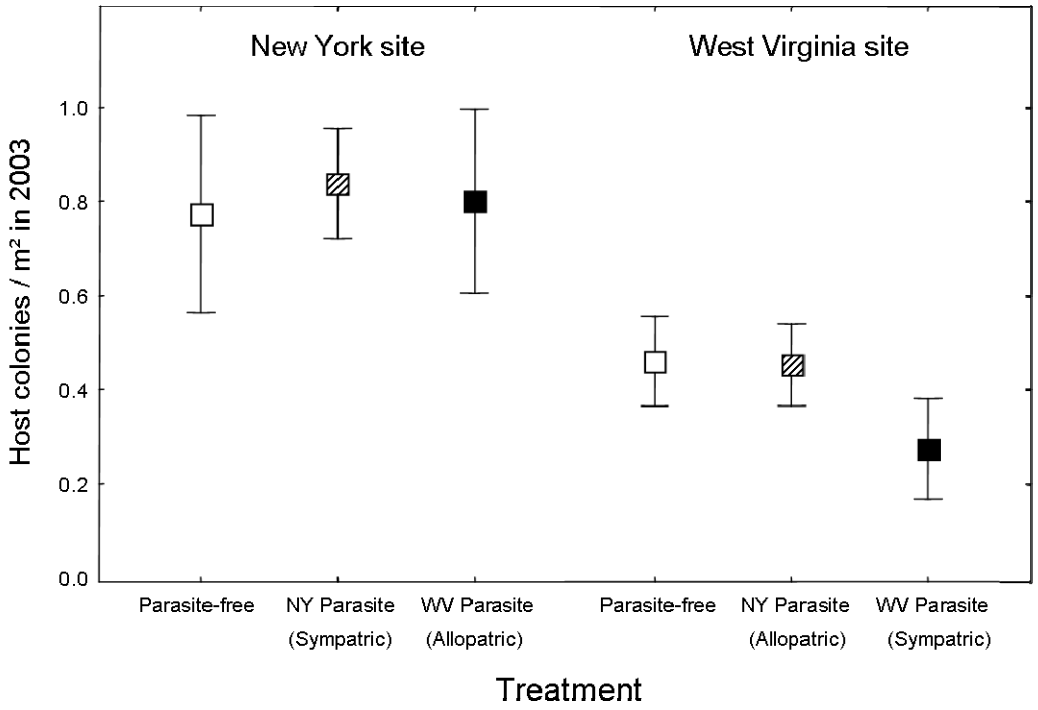

Figure 1 Influence of site (NY vs. WV) and parasite treatment on host nest density. In 2001, all *P. americanus* colonies were removed from the study plots and either no social parasite nests (parasite-free) or *P. americanus* colonies from New York (NY parasite) or West Virginia (WV parasite) were released. We show mean density of *Temnothorax* host nests ± SE in our study plots in 2003.

In 2003, we collected 499 *T. longispinosus*, 22 *T. ambiguus* and 86 *P. americanus* nests in NY in an area of 648 m² (18 plots x 36m²), yielding an average density of 0.804 ± 0.097 host nests / m² and a parasite prevalence of one *P. americanus* colony per 6.1 *Temnothorax* nests (Fig. 1). In WV (mapped area 756 m²), we collected 298 *T. longispinosus*, one *T. curvispinosus* and 45 *P. americanus* colonies, resulting in a mean density of 0.396 ± 0.056 host nests / m² and a parasite prevalence of one slavemaking colony per 6.6 host nests.

A between-years comparison at the NY site revealed a slight decrease in host density over time (Wilcoxon Matched Pairs test: N = 18; Z = 1.94; p = 0.053). In contrast, we uncovered a higher number of *P. americanus* nests in the inner 25m² in 2003 than we released in 2001 (Wilcoxon Matched Pairs test: N = 18; Z = 2.43; p < 0.015; median 2001: 2; median 2003: 3). In contrast, for the WV site we observed no significant change in the number of parasite nests per plot over time (Wilcoxon Matched Pairs test: N = 21; Z = 0.37; p = 0.71, median 2001: 2; median 2003: 1), but a strong reduction in *Temnothorax* nest density (Wilcoxon Matched Pairs test: N = 21; Z = 3.15; p = 0.002).

During the 27 months of our experiment, slavemaking colonies apparently moved around considerably, as indicated by the presence of *P. americanus* colonies in "parasite-free" plots in 2003. Nevertheless, in both study sites the number of parasite colonies was still higher in the 25m² plots where we released slavemaking colonies than in plots which were set-up parasite-free in 2001 (NY parasite plots: mean = 4.17 ± SE 0.74; NY parasite-free plots: mean = 2.50 ± SE 0.62; WV parasite plots: mean = 1.86 ± SE 0.56; WV parasite-free plots: mean = 0.86 ± SE 0.46). A general linear model demonstrated that the number of slavemaking colonies per plot in 2003 depended on the number of parasite colonies released in 2001 and the study site (GLM: Number of parasite nests released in 2001: $F_{1, 36}$= 9.15; p < 0.005, Site: $F_{1, 36}$= 9.18; p < 0.005).

Log linear analysis over the entire data set demonstrated that host nest density in the 36m² study plots in 2003 varied between study sites, sub-sites (blocks) and in reaction to our parasite manipulation (χ^2-tests of partial association : Site: χ^2_1= 58.0, p < 0.001; Block: χ^2_{12}= 210.9, p < 0.001; Treatment: χ^2_2= 5.5, p = 0.06). In NY, the host nest density strongly varied between blocks, but did not change in response to our treatments (Block: χ^2_5= 52.2, p < 0.001; Treatment: χ^2_2= 0.6, p = 0.75; Fig. 1). In contrast, the WV host density was over 40% lower in plots with sympatric *P. americanus* nests compared to parasite-free control plots and those with NY parasites (Block: χ^2_6= 66.2,

$p < 0.001$; Treatment: $\chi^2_2 = 14.5$, $p < 0.001$). Importantly, these differences in host density in WV were not present before our manipulation in the 2001 data set (Log linear analysis: Differences between plots later subjected to different treatments; $\chi^2_2 = 0.47$, $p = 0.79$).

Impact of treatments on *T. longispinosus* nest demography in 2003

At the onset of the experiment in 2001, the *T. longispinosus* nest demography (number of dealate queens and number of workers) did not differ between plots subjected to different parasite treatments, but as expected, we found strong variation between host populations (ANOVA: Site: $F_{2, 701} = 12.95$, $p < 0.0001$; Treatment: $F_{4, 402} = 0.47$, $p = 0.76$).

Factorial ANOVAs on the entire 2003 data set show strong differences between the two host populations, effects of our manipulation and interactions between site and treatments (Table 1, Fig. 2). In the NY site, *T. longispinosus* nests were larger, but less productive than host colonies in WV. In addition, NY *T. longispinosus* nests in 2003 produced a female-biased allocation ratio and invested more in sexuals than in new workers. At both sites, our parasite manipulation influenced investment patterns of host colonies, i.e. the male allocation ratio and reproductive allocation ratio. Site by treatment interactions indicated that our parasite manipulation differently affected the two host populations. We found this interaction in the social structure (number of dealate queens) and more strongly in the reproductive allocation ratio.

Table 1 Univariate results of factorial ANOVAs on the impact of our parasite manipulation on *T. longispinosus* nest demography and reproductive strategies over both sites (not including block effects), for NY and WV separately. Host nest demography varied strongly between sub-sites (blocks) within each study site and consequently we included block as an additional factor in the local analyses.

		Over both sites			New York		West Virginia	
Parameter	**Effect**	**$F_{df1, df2}$**	***P***	**Effect**	**$F_{df1, df2}$**	***P***	**$F_{df1, df2}$**	***P***
N of queens	Site	0.04 $_{1,787}$	0.84	Block	4.00 $_{5, 479}$	**0.001**	1.01 $_{6, 275}$	0.41
	Treatment	0.98 $_{2,787}$	0.38	Treatment	3.23 $_{2, 479}$	**0.04**	1.12 $_{2, 275}$	0.33
	Site × Treatment	3.35 $_{2,787}$	**0.03**	Block × Treatment	0.79 $_{10, 479}$	0.64	1.34 $_{12, 275}$	0.19
N of workers	Site	8.75 $_{1, 787}$	**0.003**	Block	0.91 $_{5, 479}$	0.47	1.56 $_{6, 275}$	0.16
	Treatment	0.45 $_{2, 787}$	0.64	Treatment	0.83 $_{2, 479}$	0.43	0.42 $_{2, 275}$	0.65
	Site × Treatment	1.00 $_{2, 787}$	0.37	Block × Treatment	2.05 $_{10, 479}$	**0.02**	1.19 $_{12, 275}$	0.29
Total annual production	Site	11.33 $_{1, 787}$	**< 0.001**	Block	4.19 $_{5, 479}$	**< 0.001**	1.81 $_{6, 275}$	0.09
	Treatment	1.49 $_{2, 787}$	0.23	Treatment	1.39 $_{2, 479}$	0.32	0.57 $_{2, 275}$	0.57
	Site × Treatment	0.02 $_{2, 787}$	0.98	Block × Treatment	1.89 $_{10, 479}$	**0.04**	1.02 $_{12, 275}$	0.43
Male allocation ratio	Site	37.69 $_{1, 457}$	**< 0.001**	Block	1.32 $_{5, 301}$	0.26	0.90 $_{6, 124}$	0.49
	Treatment	2.63 $_{2, 457}$	0.07	Treatment	1.21 $_{2, 301}$	0.30	0.56 $_{2, 124}$	0.45
	Site × Treatment	1.50 $_{2, 457}$	0.22	Block × Treatment	1.69 $_{10, 301}$	0.08	2.09 $_{11, 124}$	**0.02**
Reproductive allocation ratio	Site	53.53 $_{1, 732}$	**< 0.001**	Block	2.52 $_{5, 436}$	**0.02**	3.06 $_{6, 263}$	**0.007**
	Treatment	3.70 $_{2, 732}$	**0.02**	Treatment	2.38 $_{2, 436}$	0.09	15. 4 $_{2, 263}$	**< 0.001**
	Site × Treatment	9.74 $_{2, 732}$	**< 0.001**	Block × Treatment	2.56 $_{10, 436}$	**0.005**	1.99 $_{12, 263}$	**0.02**

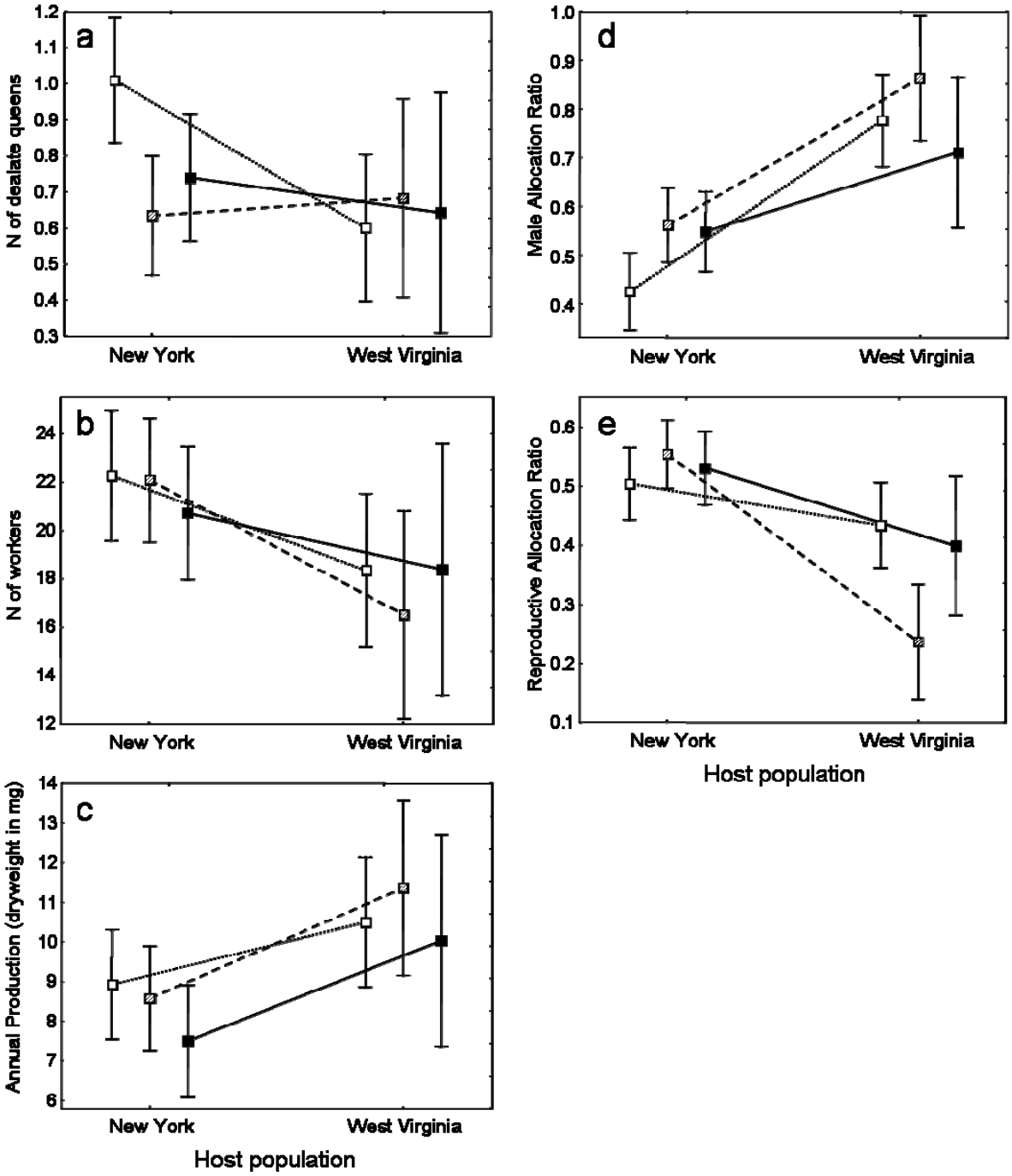

Figure 2 Social organization, demography, productivity and allocation patterns of *T. longispinosus* colonies varied between study sites and in response to our parasite treatments. We report weighted means ± SE for a) the number of dealate queens per nest, b) the number of workers per nest, c) the total annual production, d) male allocation ratio and e) reproductive allocation ratio. Open boxes represent parasite-free plots, hatched boxes NY parasite plots and filled boxes WV parasite plots. Note that NY *P. americanus* nests released in NY encountered sympatric host colonies, while those released in WV were confronted with allopatric hosts (and vice versa for WV parasites).

The site-specific factorial ANOVAs demonstrated that NY host colonies in parasite-free plots more frequently became polygynous (Fig 2a). These host colonies under reduced parasite pressure also invested correspondingly more in new workers than in reproductives (Fig 2e). However, the analysis also demonstrated different, but highly significant reactions to our manipulations in different sub-sites (blocks) (Table 1). These interactions could be shown for the number of workers, the total annual production, the reproductive allocation ratio and tentatively for the male allocation ratio. In the NY site, we further uncovered strong differences between sub-sites in the number of queens, total annual production and reproductive allocation ratio. These microgeographic differences, with the exception of the reproductive allocation ratio, were absent in the WV site.

In WV, our parasite treatment directly influenced the investment patterns of *T. longispinosus* colonies (Table 1; Fig. 2e). Here, the origin of the social parasite colonies was more important than the parasite pressure. Host colonies exposed to sympatric parasites generally invested more in reproductives than in new workers. The converse was true for *T. longispinosus* nests in allopatric NY parasite plots, whereas host nests from parasite-free plots showed intermediate investment patterns. We also found an interaction between treatment and block in respect to the male allocation ratio and the reproductive allocation ratio.

Comparison of host nest demography between 2001 and 2003

An alternative way to investigate how our treatments affected *T. longispinosus* nest demography is to compare the situation before and after our manipulation. To accomplish this, we contrasted the mean number of queens and workers in *T. longispinosus* nests from the same plots in relation to whether we removed or added *P. americanus* colonies from the study plot in 2001. An experimental increase in the number of slavemaking colonies led to a decrease in the average number of resident queens in free-living *T. longispinosus* nests in NY (Spearman Rank correlation: r_s= -0.63, $p < 0.005$, N = 18; Fig. 3), while we found no such change in social organization in WV (Spearman Rank correlation: r_s= -0.28, p = 0.22, N =21). There was no effect on the mean *T. longispinosus* colony size in NY and WV (Spearman Rank correlations; NY: r_s= -0.31, p = 0.19; WV: r_s= 0.17, p = 0.46).

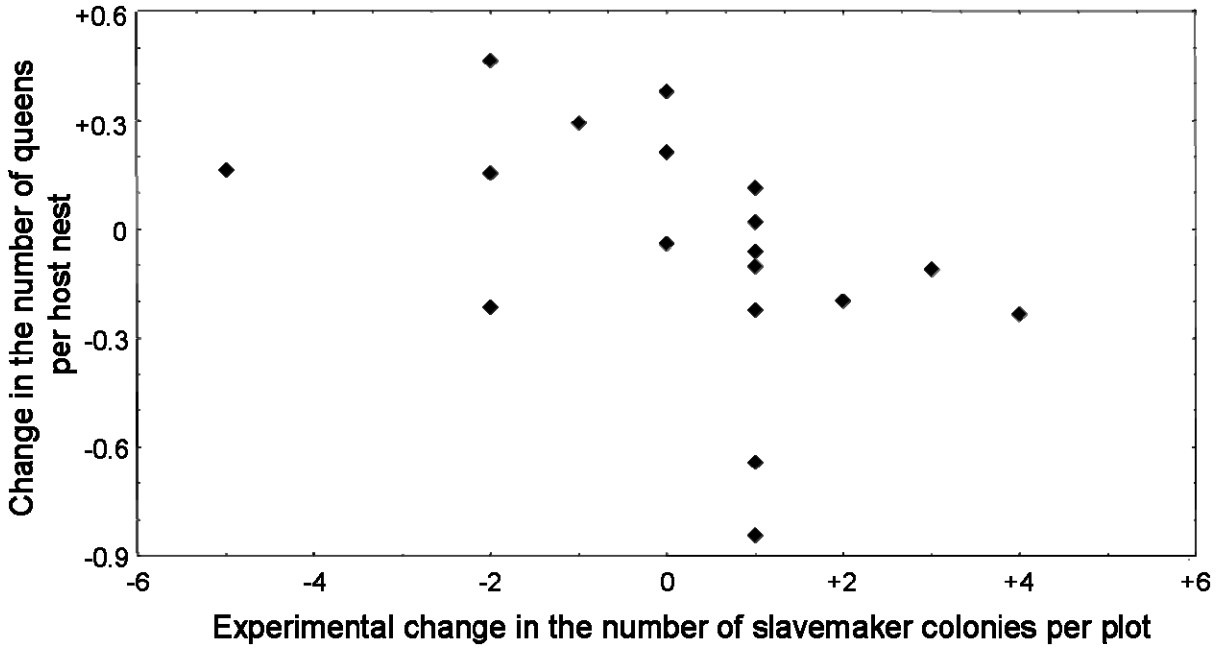

Figure 3 Change in the number of dealate queens per *T. longispinosus* nest in NY from the start of the experiment in May 2001 to the end in July 2003 in response to the experimental change in *P. americanus* nests per plot. Average queen number in host nests decreased with an experimental increase in the number of slavemaker colonies per plot.

Influence on the genetic colony structure

Intracolonial relatedness strongly responded to our treatments in the NY population (ANOVA: $F_{2, 185} = 6.50$, $p < 0.002$; Figure 4). *T. longispinosus* workers in colonies from plots without parasites were significantly less closely related than workers in colonies from social parasite plots (Fisher LSD post-hoc tests: Parasite-free / NY parasite: $p < 0.03$; Parasite-free / WV parasite: $p < 0.001$). Plots with either NY or WV parasites did not differ in worker-worker relatedness (Fisher LSD post-hoc test: $p = 0.19$).

Worker-worker relatedness in *T. longispinosus* colonies was not associated with any demographic or allocation parameter (Spearman Rank correlations: $p > 0.20$; $N = 188$). However, intracolonial relatedness was lower in polygynous *T. longispinosus* colonies than in monogynous or queenless nests (queenless / monogynous: mean $r = 0.509 \pm$ SE 0.020; polygynous: mean $r = 0.399 \pm$ SE 0.054; ANOVA: $F_{1, 180} = 3.92$, $p < 0.05$).

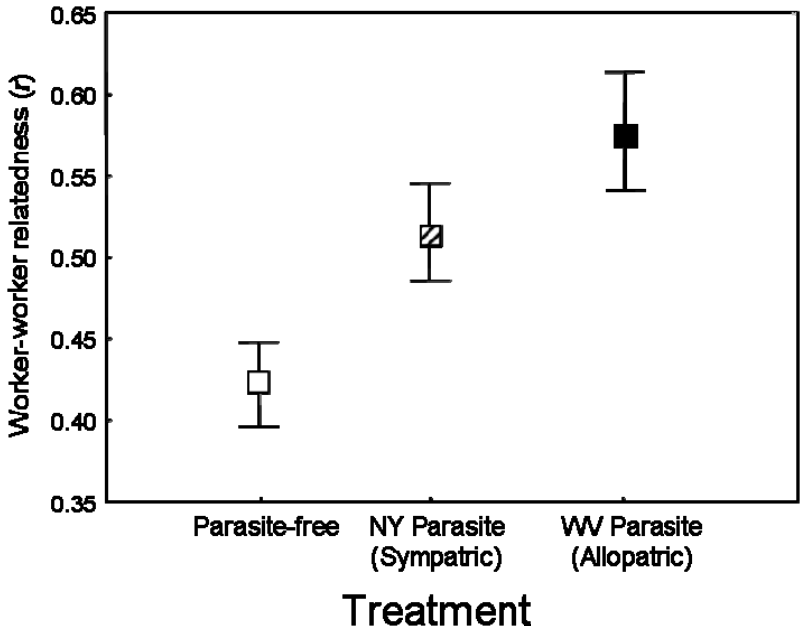

Figure 4 Intra-nest worker-worker relatedness in *T. longispinosus* colonies from the NY study site in parasite-free, NY parasite and WV parasite plots. Means ± SE are given over all colonies residing in three plots per treatment.

Local adaptation in the social parasite: the success of released *P. americanus* colonies

The survival rate of released *P. americanus* nests was higher in NY than in WV, but did not depend on the source population of the parasite or on an interaction between parasite origin and release site (General linear model: Site: $F_{1, 22} = 7.31$, $p < 0.015$; Treatment: $F_{1, 22} = 0.00$; $p = 1.00$; Site × Treatment: $F_{1, 22} = 0.01$, $p = 0.96$).

In contrast, the demography of slavemaking colonies strongly depended on an interaction between parasite origin and site, i.e. the host population the slavemaking ant colony was released in (Table 2, Fig. 5). In 2003, NY slavemaking colonies, which were released in NY, contained more slavemaking workers and slaves than the same parasite colonies that were released in WV. Likewise, WV *P. americanus* colonies were much larger after 27 months when living in their sympatric host population than in the NY ant community.

Table 2 Impact of source population of *P. americanus* colonies and release site on the demography of *P. americanus* nests. Univariate results of factorial ANOVAs are reported.

	Parameter	$F_{df1, df2}$	*P*
N of *P. americanus* queens	Site	$1.07_{1, 72}$	0.30
	Parasite origin	$1.07_{1, 72}$	0.30
	Parasite origin × site	$0.52_{1, 72}$	0.47
N of *P. americanus* workers	Site	$1.67_{1, 72}$	0.20
	Parasite origin	$1.95_{1, 72}$	0.17
	Parasite origin × site	$6.99_{1, 72}$	**0.01**
Total annual production	Site	$0.00_{1, 72}$	0.97
	Parasite origin	$0.16_{1, 72}$	0.69
	Parasite origin × site	$2.63_{1, 72}$	0.11
N of *Temnothorax* slave workers	Site	$0.54_{1, 71}$	0.46
	Parasite origin	$5.46_{1, 71}$	**0.02**
	Parasite origin × site	$6.71_{1, 71}$	**0.01**

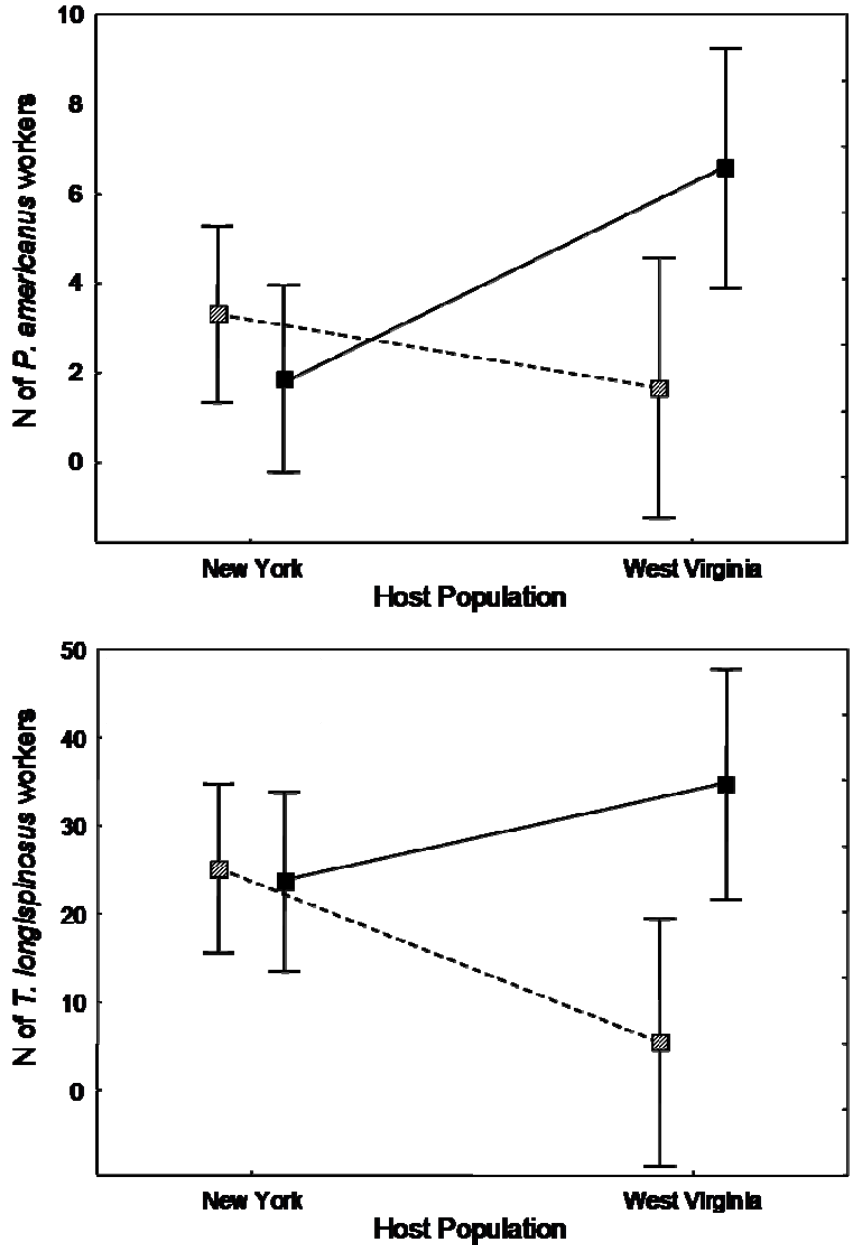

Figure 5 Demography of *P. americanus* colonies in respect to their origin and their release site. Weighted means ± SE are given for the N of slavemaking workers and the N of enslaved *Temnothorax* workers present in slavemaking ant colonies. Hatched boxes represent NY parasite plots, filled boxes WV parasite plots.

DISCUSSION

Our field manipulation clearly demonstrates exceptionally strong selective forces on *T. longispinosus* caused by the frequent and destructive raids of its main obligate social parasite *P. americanus*. These findings reflect earlier results of correlative approaches, but we can now confirm a direct and causal relationship between *P. americanus* presence and host nest density, demography and investment strategies (Foitzik & Herbers 2001a, Herbers & Foitzik 2002). As expected, we found a strong negative impact of the slavemaker *P. americanus* on host nest density in WV, while host density did not respond to our treatments at the high density NY study site. However, especially in NY, we uncovered strong reactions of *Temnothorax* colonies to our parasite manipulation in their social organization, intranest relatedness, demography and allocation patterns. The observed variation in the species interaction between the two communities is one of the requirements for a geographic mosaic of coevolution (Gomulkiewicz et al. 2007, Thompson 1994, 1999b). Moreover, we found preliminary indications for local adaptation in the social parasite as *P. americanus* colonies generally fared better in their local host population.

At the WV study site, where the *T. longispinosus* population is characterized by a low nest density, predominantly monogynous social organization and an independent colony foundation strategy (Herbers & Stuart 1996b, Herbers & Foitzik 2002), we uncovered a negative impact of local *P. americanus* colonies. *Temnothorax* nest density was reduced by over 40% as a consequence of slave raids by the local parasite, while we found no reduction in host density with the allopatric *P. americanus* colonies from NY. This difference in parasite impact can be explained by NY *P. americanus* colonies performing rather poorly in WV and containing less than a third of slavemaking and slave workers than WV social parasite nests after 27 months. Interestingly, *T. longispinosus* colonies in plots with NY parasites were slightly more often polygynous, showed a male-biased allocation ratio and invested more in reproductives compared to the situation in plots with WV parasites. This shift towards a more polygynous social organization with its typical allocation pattern (Bourke & Franks 1995) might be due to the recovery of the host population as the result of a reduced impact of NY parasites during the final months of the experiment. Demographic analysis on the destructive NY *P. americanus* nests released in WV

indicates a collapse of these nests presumably caused by local overexploitation of host colonies.

As predicted and in contrast to the situation in WV, we found no host density reduction in NY, but much more pronounced changes in the demographic composition and social structure of *T. longispinosus* nests as a reaction to the presence of *P. americanus* colonies, independent of their origin. This is in accordance with earlier studies, which found strong associations between host demography and social organization in NY, but not in WV (Foitzik & Herbers 2001a, Herbers & Foitzik 2002). Indeed, social parasite nests from both populations fared similarly well at this high density site, and significantly better than in WV. This indicates that host density is more important for the parasite than host defenses, which were found to be more effective in NY (Foitzik et al. 2001). At this study site, the origin of released parasite colonies did not matter and the strongest differences were found between parasite-free and parasitized plots. Host colonies in plots that we supplemented with social parasites were more likely to be monogynous, highly related, contain fewer workers, have a lower annual production, and show different allocation patterns. The observed changes in host demography and allocation patterns presumably reflect a transition in the age structure of host colonies: nests in parasitized plots appeared to be in earlier developmental stages (Herbers & Stuart 1996a). Under the high nest density situation in NY, destroyed host colonies are quickly replaced by young founding nests and budding or immigrating colonies from the vicinity, which all tend to be small, monogynous nests with a high relatedness.

We also found that host nests in areas with experimentally increased social parasite density showed different investment patterns: parasite presence was correlated with low allocation to new workers and high allocation to sexuals. Since high investment in sexuals is generally associated with later stages of colony development (Oster & Wilson 1978, Bourke & Franks 1995), this observation is inconsistent with the shifts in nest size discussed above. Theoretical work in epidemiology has shown that under sufficiently high virulence, two alternative strategies can coexist in a host population. The first is to develop rapidly and reproduce before being infected, at the cost of reduced fecundity (Hochberg et al. 1992). For ant colonies, this strategy is equivalent with allocating their resources to early production of sexual offspring, which may ensure at least some reproductive output before the colony is destroyed in a slave raid. Polygynous ants are often characterized by high differences in dispersal rates between the sexes, with young

queens showing higher philopatry than males (Pamilo 1990, Chapuisat et al. 1997). Since males are thus likely to disperse far by flight, they have a chance of escaping parasitism by mating with young queens residing in parasite-free patches. The alternative strategy is to develop slowly, thereby risking infection and death before reproduction, but benefiting from high fecundity (Restif et al. 2001). Our results indicate that *T. longispinosus* colonies preferentially follow the first strategy: when host nests perceive that they live in a high-risk, parasite-infested area, an investment in the highly mobile sexuals appears to provide greater fitness returns than investment in growth.

The cross-fostering element of our field manipulation revealed local adaptation in the social parasite *P. americanus* on an ecological scale. In both study sites, slavemaking ant colonies fared better in their home locality than in an allopatric environment. As climatic conditions and ant community composition are roughly similar between the two sites (Herbers & Foitzik 2002) and this social parasite obligatorily depends on a heterospecific workforce, we conclude that *P. americanus* is locally adapted to its host *T. longispinosus* and/or its density. The occurrence of local adaptation is generally thought to be the result of a balance between selection and gene flow (Fisher 1950, Endler 1973, Slatkin 1973). In the case of adaptation to a coevolving enemy, local adaptation or maladaptation also depends on the evolutionary speed with which the opponents in a species interaction are adapting. In our study, the observed local adaptation of *P. americanus* means that the host species *T. longispinosus* is maladapted, as it suffers more from its local parasite than from *P. americanus* colonies from a different location. This is especially evident in the WV host population. Population genetics also demonstrate higher gene flow between populations of *P. americanus* than between *T. longispinosus* populations, indicating the importance of migration for the evolutionary potential of these species (Brandt et al. 2007). In our study, local adaptation supported the findings of previous work on chemical integration strategies (Brandt et al. 2005a). However, it stands in contrast to earlier laboratory studies of slave raids, which demonstrated strong differences between slavemaking and host populations in raiding and defense efficiency, despite uncovering no evidence for adaptation to the sympatric host populations (Foitzik et al. 2001, Brandt & Foitzik 2004). Since NY hosts were known to have sound defenses against slave raids, we expected WV parasites to fare less well at the NY site than in their home locale, which we have indeed found in the current study. The fact that

P. americanus colonies from NY did not do well in the WV host population cannot be explained in terms of behavioral interactions during raids. In laboratory trials, NY slavemakers were easily able to overcome the defenses of WV *Temnothorax* colonies (Foitzik et al. 2001). In the field, factors other than behavioral adaptations must come into play. One possible explanation is that the NY parasites were negatively affected by the low host density in WV. This interpretation implies that, concordant with the geographic mosaic of coevolution theory, local communities differ in traits of the interacting species that are selected for during the arms race. Whichever characters are affected, our field manipulation indicates local adaptation of *P. americanus* populations.

Local adaptation has been described commonly in parasites or pathogens of non-social parasite systems (Parker 1985, Ebert 1994). For example the trematode, *Microphallus*, was found be adapted to common local clones of its molluscan host (Lively & Dybdahl 2000) and the rust fungus *Melampsora amygdalinae* was adapted to sympatric populations of its plant host *Salix triandra* (Niemi et al. 2006). However, other studies have uncovered no local adaptation (Davelos et al. 1996, Roy 1998) or maladaptation of the parasite species to its sympatric host (Kaltz et al. 1999, Zhan et al. 2002). This latter finding indicates maladaptation might be the consequence of local adaptation of the host to the parasite.

Lastly, we would like to address potential problems caused by allowing movement of host and parasite colonies during our experiments. Movement was evident by the presence of *P. americanus* colonies in "parasite-free" plots. Mean densities in these plots were still only half of *P. americanus* densities in plots where we originally released slavemaking ant colonies and parasite densities. Colony migration could potentially have made it difficult to show effects of our manipulation making our findings of complex changes in host biology even more remarkable. Thus, although slavemaking ant colonies slowly moved into originally parasite-free areas, parasite pressure over the duration of the 27 months was clearly lower in these plots causing different host demography, social organization and investment patterns. In addition, we cannot be sure that all *P. americanus* colonies collected at the end of the manipulation are the same colonies we originally released. However, as similarly-sized *P. americanus* colonies from two populations released at the same study sites showed a different demographic composition at the end of the experiment, parasite colony movement and mistaking local parasites for released colonies should have made it more difficult to show local adaptation. Clearly colony movement, a regular

part of the colony life in these small ants, led us to underestimate rather than overestimate both the impact of these social parasites on their hosts and the strength of local adaptation in the social parasite *P. americanus*.

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PUBLICATION 2

FIRST EVIDENCE FOR SLAVE REBELLION: ENSLAVED ANT WORKERS SYSTEMATICALLY KILL THE BROOD OF THEIR SOCIAL PARASITE *PROTOMOGNATHUS AMERICANUS*

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ABSTRACT

During the process of coevolution, social parasites have evolved sophisticated strategies to exploit the brood care behavior of their social hosts. Slavemaking ant queens invade host colonies and kill or eject all adult host ants. Host workers, which eclose from the remaining brood, are tricked into caring for the parasite brood. Due to their high prevalence and frequent raids, following which stolen host broods are similarly enslaved, slavemaking ants exert substantial selection upon their hosts, leading to the evolution of anti-parasite adaptations. However, all host defenses shown to date are active before host workers are parasitized, while selection was thought to be unable to act on traits of already enslaved hosts. Yet, here we demonstrate the rebellion of enslaved *Temnothorax* workers, which kill two thirds of the female pupae of the slavemaking ant *Protomognathus americanus*. Thereby, slaves decrease the long-term parasite impact on surrounding related host colonies. This novel anti-parasite strategy of enslaved workers constitutes a new level in the coevolutionary battle after host colony defense has failed. Our discovery is analogous to recent findings in hosts of avian brood parasites where perfect mimicry of parasite eggs leads to the evolution of chick recognition as a second line of defense.

Keywords: Coevolution, arms race, host defenses, brood parasites, slavemaking ants

INTRODUCTION

Social brood parasites reduce the cost of parental care by exploiting the brood care behavior of other social insect species in an analogous manner to avian brood parasites such as cuckoos and cowbirds (Davies & Bourke 1989). Social parasitism evokes counter-adaptations in host species, eventually resulting in a coevolutionary arms race between these parasites and their hosts (Foitzik et al. 2001, Als et al. 2004, Brandt & Foitzik 2004, Brandt et al. 2005b, Brandt et al. 2007, Martin et al. 2007, Nash et al. 2008).

Slavemaking ants are social parasites, which depend on enslaved allospecific host workers for routine tasks such as brood care, foraging, and nest defense (Buschinger 1986, Hölldobler & Wilson 1990). These ants are behaviorally and morphologically well-equipped for one task: finding and subduing colonies of their host species. A mated parasite queen conquers a host nest, expels or kills the host queen(s) and adult workers and usurps the remaining worker brood (Stuart 1984, Hölldobler & Wilson 1990, Schumann & Buschinger 1994). Slave workers, which eclose from this host brood, imprint on the slavemaking queen during the first few days (Jaisson 1975, LeMoli & Mori 1982, Goodloe & Topoff 1987) and they subsequently take over colony maintenance and care for the allospecific parasite brood. Slavemaking workers raised during the following years, attack and raid neighboring host colonies and steal the brood to replenish the slave labor force (Wilson 1971, Alloway 1979). Yet, within the nests of slavemaking ants, social parasites and workers from up to two different host species seemingly coexist peacefully.

Slavemaking ants exert substantial selection on host populations due to their high abundances and their repeated, destructive raids (e.g. Cool-Kwait & Topoff 1984). Field studies have shown a significant reduction in productivity and average life expectancy of host colonies in parasitized populations (Foitzik & Herbers 2001a, Fischer-Blass et al. 2006, Johnson & Herbers 2006, Foitzik et al. in press). As expected during host-parasite coevolution, this strong parasite pressure has led to the evolution of host defenses, especially in the context of slave raids (Alloway 1990, Mori et al. 1991, Foitzik & Herbers 2001a, Herbers & Foitzik 2002).

The slavemaking ant *Protomognathus americanus* is an evolutionarily old, obligate social parasite that exerts exceptionally high parasite pressure upon its *Temnothorax* hosts (Foitzik & Herbers 2001a, Blatrix & Herbers 2003, Beibl et al. 2005, Johnson & Herbers 2006). *Protomognathus americanus* colonies successfully raid

between 2-10 host colonies per year with higher raiding frequencies recorded for larger slavemaking colonies (Foitzik et al. 2001). Although raiding success appears to increase with size, *P. americanus* colonies are unexpectedly small with a mean of two to five slavemaking workers per nest depending on population (Herbers & Foitzik 2002). The behavior, ecology, chemistry and genetics of the coevolutionary interactions between this tiny social parasite and its three host species have been studied intensively (Herbers & Stuart 1998, Foitzik & Herbers 2001a, Herbers & Foitzik 2002, Brandt et al. 2005a, Johnson & Herbers 2006, Brandt et al. 2007). These studies have demonstrated that the effectiveness of host defenses against slave raids depends on parasite pressure and the composition of the local ant community. Yet, all anti-parasite strategies of its *Temnothorax* hosts described so far are effective only before host workers are parasitized.

Generally within host-parasite systems, selection can evoke two lines of host defenses, which are either effective before or after parasitism has been established. In vertebrate–microparasite interactions, an infection barrier preventing the entry of the parasite into the host body is the first line of defense. Once this line has been broken and parasites have entered the host, an effective host immune system can shorten the duration of the infection and can minimize its detrimental effects. Two such defense lines have been shown in hosts of avian brood parasites as well. Many cuckoo hosts reject parasitic eggs and thus preclude brood parasitism (Rothstein 1982, Lyon 2003). Recently a second line of defense has been demonstrated in the superb fairy wren, the host of the Australian Bronze cuckoo, which discriminates and rejects parasitic young (Langmore et al. 2003). By this point, the host has already suffered severe fitness costs through the parasite's ejection of host chicks and the investment in allospecific brood care.

In slavemaking ant systems, host adaptations that provide protection against enslavement include enemy recognition, adjustment of the recognition threshold, better fighting abilities or fast evacuation and escape from the attacked host colonies (e.g. Alloway 1990, Foitzik et al. 2001, Brandt et al. 2005a). Yet, it has been argued that selection could not alter the behavior of already enslaved hosts (Gladstone 1981). In this context, three behavioral options of how slaves could benefit from rebelling against their oppressors have been either rejected for theoretical reasons or were eliminated on empirical grounds. The return of slaves to their mother host colony has been discussed as one potential strategy. This scenario appears highly unlikely, because enslaved workers are raided during the pupal or

larval stage and with their emergence as adult ants they adopt the parasitic colony odor (Kaib et al. 1993). Consequently, they would neither find their mother colony, nor would their relatives accept these escaped slave workers due to their deviant colony odor. A second option, the refusal to work or desertion of the parasite colony, could potentially force slavemakers to conduct new slave raids earlier, to compensate for the loss of workforce. Thereby slaves' indirect fitness could be reduced due to the enhanced raiding risk on related neighboring host colonies (Gladstone 1981). Only in the long run slave desertion or strike would result in the reduced growth of parasite colonies and lower parasite pressure on the host population. The third option, the possibility of slave reproduction, has been rejected on empirical grounds, because behavioral and genetic studies have demonstrated that slavemakers successfully prevent host workers from reproducing (Heinze et al. 1994, Foitzik & Herbers 2001b). All in all, slaves are thought to be caught in an evolutionary trap where no behavioral strategy could increase their direct or indirect fitness.

Yet, we think that one potential slave rebellion strategy has been completely missed: We argue that enslaved host workers could increase their inclusive fitness by killing the brood of their social parasite, providing populations are strongly structured and neighboring host colonies are related. If so, then destruction or negligence of slavemaking brood would reduce parasite colony growth, and thereby reduce the frequency or efficacy of raids on uninfected, but related host colonies (Foitzik & Herbers 2001a). Selection would then favor this defense trait via its effect on inclusive fitness, even though it is expressed in forced-to-be-sterile, enslaved host workers.

In addition to these theoretical considerations, several empirical findings led us to investigate the potential occurrence of slave rebellion in hosts of *P. americanus*. Colonies of the slavemaker *P. americanus* consistently show a high production of slavemaker brood early in the season, yet only few adult slavemakers emerge per nest in summer (Herbers & Foitzik 2002). The resulting small colony sizes could potentially thus be explained by the fact that enslaved *Temnothorax* host workers are less effective in their care for the social parasite brood compared to the productivity of host workers in their unperturbed nests (Foitzik & Herbers 2001b). Enslaved host workers rear fewer workers and queens per capita than their unparasitized conspecifics, while the production of males is no different between slaves and unparasitized host ants. However, the mechanism(s) behind this reduced productivity of enslaved host workers in colonies of the slavemaker, *P. americanus*,

has remained unclear. For these reasons, we compared the brood care behavior of enslaved host workers in colonies of the social parasite *P. americanus* with the brood care of host workers in unparasitized *Temnothorax* colonies and followed the development of parasite and host brood.

MATERIALS AND METHODS

Study system, ant collection and housing

The slavemaking ant *P. americanus* and its host species *T. longispinosus*, *T. ambiguus* and *T. curvispinosus* occur in the mixed deciduous forests of northeastern North America. They nest in hollow acorns, sticks and hickory nuts on the forest floor. Ant colonies were collected at the Huyck Preserve in Albany County, New York (N 42° 31`35.3`` W 74° 9`30.1``) and in Harpersfield, Ashtabula County, Ohio (N 41° 45`34.2`` W 80° 57`55.7``) during May and June of 2005 and 2006. Two ant communities were analyzed to elucidate potential inter-population differences in brood care. Colonies were transported to the laboratory in Munich in their natural nesting sites. Ants were counted and transferred to artificial plastic boxes (10 cm x 10 cm x 1.5 cm) with three chambers, an artificial nest and moistened plaster floor (Heinze and Ortius 1991). Subsequently, the ant colonies were kept in an incubator (20°C for 14 h light, 15°C for 10 h dark) and fed water, pieces of cricket and honey twice a week.

Standardized observations

We compared the brood-rearing success of *Temnothorax* workers in slavemaking *P. americanus* colonies to the situation in unparasitized host colonies of *T. longispinosus* and *T. curvispinosus*. Enslaved *Temnothorax* workers in parasite colonies care for allospecific brood, while non enslaved host workers raise only their close relatives. We surveyed brood development in a total of 141 ant colonies and directly observed brood care behavior of *Temnothorax* workers in host and parasite colonies. We could not monitor ant colonies over the entire brood developmental time continuously, however we did survey each ant colony once per day for about 5 min (total observation time >150h). This allowed us to directly observe the behavior of *Temnothorax* workers towards the brood and to determine how their behavior affects their fate.

In total, we monitored the development of queen, worker and male pupae in 88 *P. americanus*, 36 *T. longispinosus* and 17 *T. curvispinosus* colonies. Brood development was checked daily and the fate of larvae and pupae was followed until seven days after eclosion from the pupal stage (Table 1). When we collected *P. americanus* colonies in the field in May and June, they exclusively contained the slavemaking brood. Slave raids, where host worker pupae are stolen, occur only later in the season (July – September). *Protomognathus americanus* and *Temnothorax* pupae can be easily distinguished based on their external morphology, e.g. head shape and size and the absence or presence of ocelli.

Table 1 Number of slavemaker and host pupae observed in ant colonies. Individuals were monitored until the 7th day after eclosion

Species	Caste of pupae	N pupae	N colonies	N pupae per nest Mean ± SE
P. americanus	Queen	113	47	2.4 ± 0.44
	Worker	191	65	2.9 ± 0.23
	Male	169	37	4.6 ± 0.69
T. longispinosus	Queen	13	10	1.3 ± 0.15
	Worker	75	31	2.4 ± 0.27
	Male	72	21	3.4 ± 0.63
T. curvispinosus	Queen	3	3	1.0 ± 0.00
	Worker	34	13	2.6 ± 0.50
	Male	18	8	2.3 ± 0.45

The development of pupae was observed in colonies of all three species. Larval development was monitored only in *P. americanus* colonies. In these 26 nests, social parasite larvae were cared for by *Temnothorax* slaves (n= 86; 3.3 ± 0.57 larvae per nest). Larval development was observed in *P. americanus* colonies as a control for potential artifacts caused by laboratory housing and/or artificial nutrition. All *T. longispinosus* and *T. curvispinosus* colonies contained at a least one queen and had 30 ± 5 workers. *Protomognathus americanus* colonies invariably contained a single *P. americanus* queen, at least two *P. americanus* workers and 10 ± 3 enslaved *Temnothorax* host workers. 59.1% of these 88 *P. americanus* colonies contained only

T. longispinosus slave workers, 26.1% *T. curvispinosus* workers, 4.5% *T. ambiguus* and the remaining social parasite colonies enslaved either *T. longispinosus* and *T. curvispinosus* (5.6%) or *T. longispinosus* and *T. ambiguus* (4.5%) slaves. We did not alter the slavemaking or host colony demography.

Data analysis

Brood development and mortality rates of pupae did not differ between populations of each species (Mann-Whitney U tests: $p > 0.05$). Consequently, we combined data from New York and Ohio colonies for further analysis. We compared pupal mortality rates between castes (queen, worker, male) and species (*P. americanus*, *T. longispinosus*, *T. curvispinosus*) on a colony basis using non-parametric tests (Mann-Whitney U,- Kruskal-Wallis tests) as our data were far from a normal distribution. When several tests were performed on the same dataset, p-values were corrected with the sequential Bonferroni correction (Rice 1989). Statistics were carried out using the program Statistica 6.0 (StatSoft).

RESULTS

We recorded the fate of 473 *P. americanus* pupae (113 queens, 191 workers, 169 males) in 88 slavemaking colonies. In general, slavemaking larvae were successfully reared to the pupal stage (97.7% ± 1.3; n = 86; 3.3 ± 0.57 larvae per nest), but many of these *P. americanus* pupae were unable to complete their development and did not reach adulthood (Mann-Whitney U test, n = 149, 26; U = 695.0; p = 0.0001).

On average 66.9% of slavemaking worker pupae, 83.2% of the queen pupae, but only 2.8% of the male pupae died during this last developmental stage or shortly after eclosion (Figure 1). The mortality rate clearly depended on caste in that male pupae showed a much lower mortality than female pupae (Mann-Whitney U test, n = 112, 37; U = 332.5; p = 0.0001). Among the latter, slavemaking queen pupae less frequently reached adulthood than worker pupae (Mann-Whitney U test, n = 47, 65; U = 1065.0; p = 0.006). Mortality was independent of the slave species (*T. longispinosus* /*T. curvispinosus*, *T. ambiguus*) present in the *P. americanus* nests (Kruskal Wallis test, H (4, n = 49) = 1.12; p = 0.88).

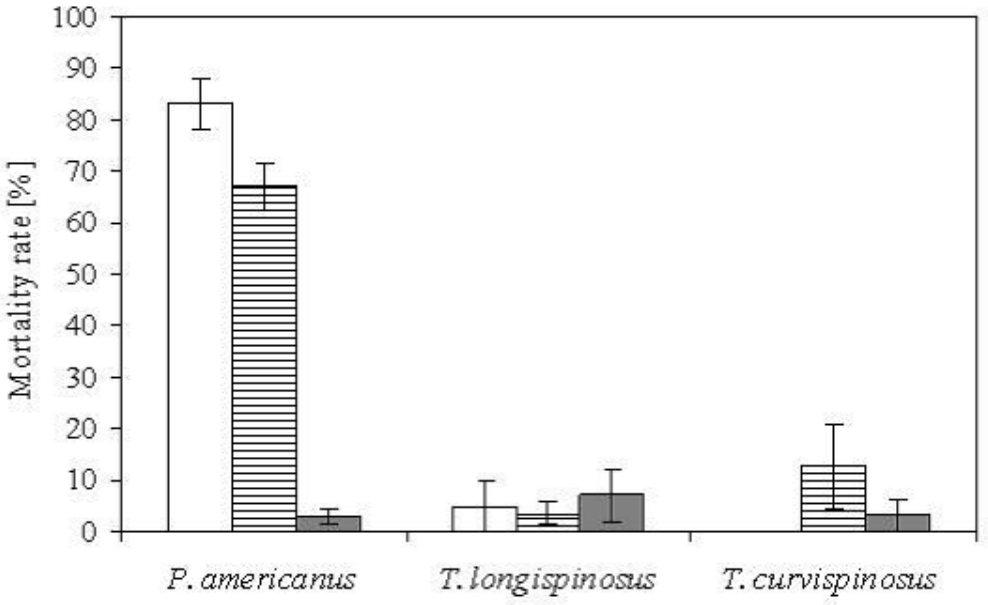

Figure 1 Mortality of queen (white bars), worker (striped bars) and male (grey bars) pupae of the slavemaker *Protomognathus americanus* (n Q = 113; n W = 191; n M = 169) and its two *Temnothorax* host species (*T. longispinosus*: n Q = 13; n W = 75; n M = 72; *T. curvispinosus*: n Q = 3; n W = 34; n M = 18). Individuals were monitored until the 7th day after eclosion. Given are mean ± SE.

Behavioral observations revealed that enslaved workers of both *Temnothorax* host species actively killed the seemingly healthy slavemaking pupae. Specifically, we observed the removal of slavemaker pupae from the nest chamber by enslaved host workers and their frequent death due to negligence. Of all slavemaking pupae, which failed in development (n = 27), we directly observed the killing of 29.5% (n = 67) by enslaved host workers (Figure 2). In addition, 52.8% (n = 120) of the parasite pupae were selectively removed from the nest chamber and died due to negligence.

Figure 2 Enslaved *Temnothorax longispinosus* workers attacking and tearing apart a *Protomognathus americanus* worker pupa in a slavemaker colony.

In contrast to the high mortality rates of *P. americanus* pupae in slavemaking nests, the vast majority of *Temnothorax* pupae of both host species successfully eclosed in their mother colonies. We monitored the development of 160 *T. longispinosus* (13 queens, 75 workers, 72 males) and 55 *T. curvispinosus* pupae (3 queens, 34 workers, 18 males). The average mortality rates of *T. longispinosus* queen, worker and male pupae were 5.0%, 3.5% and 7.1%, respectively and did not differ from each other (Kruskal-Wallis-test: H (2, n = 62) = 0.27, p = 0.87). Similar low mortalities were observed in colonies of the second host species *T. curvispinosus*, where less than 10% died during the pupal stage (differences between castes: Kruskal-Wallis-test: H (2, n = 24) = 1.09, p= 0.58; differences between host species: Mann-Whitney U test, n = 24, 62; U = 703.5; p = 0.69).

A comparison of pupae mortality rates between unparasitized *Temnothorax* colonies and *P. americanus* colonies demonstrated dramatically higher mortalities for slavemaking worker (66.9% versus 6.4%) and queen pupae (83.2% versus 5.6%; differences within caste, but between species: worker pupae: Kruskal-Wallis-Test: H (2, n =109) = 52.72, p = 0.0001; queen pupae: Kruskal-Wallis-Test: H (2, n = 60) = 30.4, p =0.0001). In contrast, *P. americanus* male pupae developed as successfully as

T. longispinosus and *T. curvispinosus* male pupae (2.8% versus 6.2%) Kruskal-Wallis-test: $H (2, n = 66) = 0.03$, $p = 0.99$). We never observed killing of host pupae or their removal by conspecific nest mates.

DISCUSSION

Here, we have demonstrated rebellion of enslaved *Temnothorax* workers, which revolt against their oppressors by attacking slavemaking pupae under their care. The active destruction of parasitic pupae by slaves can explain the low productivity and ultimately the extraordinary small nest size of *P. americanus* colonies (Foitzik & Herbers 2001b, Herbers & Foitzik 2002). While *Temnothorax* workers in unparasitized colonies successfully rear conspecific pupae, enslaved *Temnothorax* workers selectively eliminate parasite queen and worker pupae. Direct observations show that these parasite pupae were either actively killed or removed from the nest chamber and neglected. The strong discrepancy between the high production of slavemaking larvae (Foitzik & Herbers 2001b) and the low number of adult slavemakers (two to five) per nest (Herbers & Foitzik 2002) is consequently not due to a low efficiency of allospecific brood care. The behavior of enslaved *Temnothorax* workers instead dramatically switches from nurturing care for parasitic larvae to aggressive and detrimental attacks on pupae.

The elimination of parasite brood by enslaved *Temnothorax* workers can be seen as a second line of defense, when host nest defense has failed. Our finding is thus analogous to chick recognition and rejection in hosts of the bronze cuckoo (Langmore et al. 2003). In this Australian host-brood parasite system, strong egg resemblance of parasite eggs and the consequent failure of egg discrimination has led to an escalation of the coevolutionary arms race: the host, the superb fairy wren, was shown to recognize and eliminate parasitic young. But, unlike the situation in avian brood parasites evidence for anti-parasite strategies after parasitic colony usurpation has so far been lacking in social parasite systems and enslaved host workers have been thought to be unable to rebel against their oppressors (Gladstone 1981, Heinze et al. 1994, Foitzik & Herbers 2001b).

In contrast to these earlier considerations, we argue that enslaved ant workers can increase their inclusive fitness by killing the social parasite brood. The elimination of parasite brood by enslaved *Temnothorax* workers decreases the workforce of the

parasite. Furthermore, genetic analyses have indicated that smaller *P. americanus* colonies conduct fewer and less destructive slave raids (Foitzik et al. 2001). Enslaved ants can benefit from a decline in the number and destructive power of future slave raids if this reduction directly benefits relatives in neighboring colonies. This indirect fitness benefit ultimately depends on the relatedness of slaves to host ants residing in surrounding nests, which itself is a consequence of three different processes. First, host colonies and their queens occasionally survive a slave raid, which has been demonstrated for a Vermont *Protomognathus* and *Temnothorax* community (Blatrix & Herbers 2003). Second, *Temnothorax* species are known to be polydomous, meaning a single colony can occupy several nests concurrently, so that slaves taken from one nest have close relatives in nearby nests (Herbers 1986, Foitzik and Herbers 2001a). For example, in the New York population one fifth of all *T. longispinosus* nests belong to polydomous colonies, with an average distance between subunits of 78cm (Foitzik et al. 2004). Finally, the facultative polygynous *Temnothorax* colonies frequently reproduce by budding, such that daughter colonies are established in close vicinity to mother colonies. The polygynous and polydomous social organization of *Temnothorax* host populations has led to a strong genetic structure on a microgeographic scale (Foitzik et al. 2004). However, variation in post-raid survival, social organization and therefore genetic structure of host populations (Herbers & Stuart 1996b) should lead to differential selection for the evolution of slave rebellion. We would therefore expect variation in the expression of this defensive trait between various host populations and species, which indeed is indicated in preliminary follow-up studies.

Variation in the level of host defenses has also been reported for hosts of avian brood parasites (Davies & Brooke 1989, Soler & Møller 1990, Lotem et al. 1995, Servedio & Hauber 2006). Two main hypotheses – evolutionary lag or equilibrium - have been put forward to explain why some hosts do not evolve defenses to prevent brood parasitism (Davies & Brooke 1989¸ Krüger 2007). The evolutionary lag hypothesis states that it would be advantageous for hosts to counteract brood parasitism but they do not evolve defenses, because of a lack of time or due to insufficient genetic variation in host populations (Rothstein 1975). In contrast, the evolutionary equilibrium hypothesis suggests that it can be adaptive for a host to accept brood parasitism if rejection costs are high. Empirical studies on the evolution of host defenses have shown various outcomes, from hosts that have apparently evolved counter-adaptations that prevent parasitism (Davies 2000, Rothstein 2001) to

oscillatory systems, where brood parasite prevalence and the levels of host defense fluctuate around an evolutionary equilibrium and to systems where continuous exploitation of defenseless hosts is commonplace (Krüger 2007).

In our social parasite system, selection must currently be tremendous on *P. americanus* to develop chemical or behavioral adaptations to counter the killing of nearly two-thirds of its female pupae. A recent phylogeny has shown that *P. americanus* is an evolutionarily old parasite with a long coevolutionary history with its hosts (Beibl et al. 2005). Thus, this parasite should have had enough time to adapt to the exploitation of slave behavior, and its obvious failure has to be explained by the recent evolution of this novel defense trait in the host species. To stay in the arms race with its hosts, *P. americanus* has to develop counter-adaptations or else host defenses will rise to a level that could preclude persistent brood parasitism.

Our study shows that slaves do not destroy all pupal castes: rather parasite males were spared and frequently reached adulthood. *Temnothorax* workers can thus discriminate brood of different developmental stages and/or different castes. Social insects generally recognize their nestmates by chemical recognition cues on their cuticle (Singer 1998) and social parasites, therefore, mainly use chemical strategies to manipulate host behavior (Allies et al. 1986, Martin et al. 2007, Nash et al. 2008). A lack of chemical recognition cues on parasite larvae might explain why enslaved *Temnothorax* workers are unable to recognize and eliminate them. At this early stage in development, the chemical profile on the body surface might still be insufficiently developed or chemical compounds of larvae might actively induce brood care behavior (Alloway 1982, Zimmerli & Mori 1993). Chemical analyses on more advanced developmental stages revealed caste- and species-specific differences in cuticular hydrocarbon profiles of parasite and host pupae (Achenbach and Foitzik in preparation). Parasite male pupae, which were only rarely killed, showed a characteristic chemical profile, which was distinct both from host male profiles and from the profiles of parasite queen and worker pupae. So, if parasite males exhibit a distinctive chemical profile, why were they not killed by enslaved host workers? In addition to chemical cues, slaves could use differences in morphology to distinguish pupae. *Protomognathus* and *Temnothorax* males show a very similar morphology, whereas the female castes of the parasite with their characteristic large heads are clearly distinct from *Temnothorax* queen and worker pupae. Similarly, to escape host recognition and elimination, males of the inquiline social parasite *Plagiolepis xene* were proposed to have first evolved chemical

mimicry and only later morphological changes – in this case miniaturization - to prevent host workers from secondarily using size as a recognition cue (Aron et al. 2004).

Ultimately, male pupae survival could also be explained by insufficient fitness benefits from killing parasite males. The selective aggression of enslaved *Temnothorax* workers against female parasite pupae can be due to the strong selection that female slavemakers exert on their host. Adult male slavemakers eclose, participate in mating flights, copulate and subsequently die. In contrast, adult parasite queens and workers take part in slave raids and the destructive usurpation of *Temnothorax* host colonies (Wilson 1971, Alloway 1979). As a result, the evolution of counter-parasite adaptations in the host especially against female slavemaking castes should be under strong selection. The low percentage of female slavemaking pupae that survived the systematic killings may then be explained by rare discrimination errors. Our discovery of enslaved host workers selectively killing female parasite pupae suggests that this behavioral trait evolved as a host defense in the context of host-parasite coevolution.

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PUBLICATION 3

EVOLUTIONARY ARMS RACES WITHIN SOCIAL PARASITE COLONIES: BEHAVIORAL AND CHEMICAL STRATEGIES OF SLAVES AND SLAVEMAKERS

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ABSTRACT

Parasite and hosts frequently engage in a coevolutionary arms race, in which newly developed host defenses will be counteracted by parasite adaptations. Slavemaking ants are virulent social parasites that trick enslaved host workers into caring for their brood. These slaves also have to accept kidnapped host pupae, which slavemakers retrieve during raids. Until recently, selection was thought to be unable to act on traits of these enslaved host ants. Yet, a novel study demonstrated rebellion of enslaved *Temnothorax* workers, which selectively killed female pupae of the slavemaking ant *Protomognathus americanus*. This defensive trait could lower the costs of parasitism, because slowed growth of parasite nests reduces the raiding impact on related neighboring host colonies. In cross-fostering experiments, we investigated the acceptance of host and parasite pupae by *Temnothorax* workers in parasitized and unparasitized colonies. Host workers killed a large fraction of the transferred pupae, and the social parasite was only able to increase the acceptance of parasite pupae in its major host and of heterospecific host pupae in its minor host. Parasite pupae survived much better when transferred to sympatric host colonies than to allopatric ones, indicating local adaptation in the parasite *P. americanus*. Cuticular hydrocarbon analyses explain this with chemical differences in pupae profiles between communities. Overall, parasite and host pupae have highly divergent profiles, hence cuticular hydrocarbons can be used by host workers to identify and selectively destroy parasite pupae. The parasite *P. americanus* is now under strong selection to adapt its pupal recognition cues to those of its hosts to counteract slave rebellion.

Keywords: Coevolution, arms race, chemical communication, host defenses, brood parasites, slavemaking ants

INTRODUCTION

Brood parasites, which exploit the brood care behavior of other animal species, have evolved sophisticated strategies to trick their hosts into caring for their young. Many avian brood parasites, for example cuckoos, actively mimic visual or acoustic host cues, such as the color pattern of host eggs (Davies 2000) or the calls of host young (Langmore et al. 2003). In contrast, communication is mainly chemical in social insects, and as such, hymenopteran parasites have developed chemical strategies to secure an exploitative relationship with their hosts (Lenoir et al. 2001; Sledge et al. 2001). Ant social parasites can manipulate host behavior by using glandular secretions, such as chemical weapons (Allies et al. 1986, Foitzik et al. 2003) or they can deceive their hosts, either by actively producing (Dettner & Liepert, 1994) or passively adopting (Johnson et al. 2008, Kaib et al. 1993, Vander Meer et al. 1989) host species-specific cuticular hydrocarbon cues. Long-chained hydrocarbons on the cuticle are known not only to serve as desiccation barriers, but are widely used in social insects for the recognition of nestmates, sex partners and conspecifics (Howard 1993).

Slavemaking ants are highly specialized social parasites with an extraordinary life cycle. They are able to circumvent the time and energy that brood care requires by concurrently exploiting a slave work force of several host colonies (Buschinger 1986, Davies et al. 1989). The life cycle of the tiny slavemaking ant *Protomognathus americanus* starts when a young mated parasite queen successfully conquers a nest of *Temnothorax* host ants (Wesson 1939). She kills or expels the host queen(s) and the adult workers and usurps the remaining host brood. Workers that hatch from this brood become her first generation of slaves (Hölldobler & Wilson, 1990). Subsequently, all tasks of colony maintenance and brood care are taken over by these enslaved host workers, which can belong to up to three different *Temnothorax* species. To maintain a steady supply of slaves, slavemaker workers raised during the following years conduct raids on host colonies in the vicinity of their mother nest, stealing host larvae and pupae of these colonies (Alloway 1979, Brandt et al. 2005b). This remarkable parasitic life style entirely depends, therefore, on the behavioral exploitation of these enslaved host workers. The recognition and destruction of the social parasite brood can be avoided by chemical mimicry of host brood profiles (Akino et al. 1999) or by the absence of detectable recognition cues (Cervo et al. 2008). The ability to manipulate the behavior of enslaved workers into caring for

parasite brood can also be linked to the exposure of slave workers to allospecific odors during a critical period of chemical learning shortly after their eclosion from the pupae (Goodloe & Topoff 1987, Hare & Alloway 1987, Jaisson 1975). Early exposure to a wider range of chemical cues could extend the brood acceptance threshold of slaves and thereby lower aggression of the enslaved workers towards the alien brood, though recognition and destruction of the social parasite brood has been demonstrated repeatedly. This reduction in aggression is not only important in slave interactions with parasite brood, but also in their relationship with the stolen host brood that slavemakers kidnap on raids against neighboring host colonies. Enslaved workers must rear these larvae and pupae to adulthood and integrate them into the slave workforce of the parasite nest. In addition to influencing the natal environment, the physical presence of slavemaker workers can enhance slave acceptance of alien host pupae either through behavioral or chemical interference (Alloway 1982). Slavemaking ant workers of the species *Harpagoxenus sublaevis* aggressively attack enslaved host workers, which start to develop their ovaries (Heinze et al. 1994). Hence, direct behavioral control of slave worker behavior also occurs in social parasite nests.

During host-parasite coevolution, (social) parasites are continuously adapting to succeed in host exploitation, whereas hosts are expected to evolve defenses if parasite pressure is sufficiently strong. Indeed, slavemaking ants exert substantial selection on host populations due to their high prevalences and repeated destructive raids (Bono et al. 2006, Cool-Kwait & Topoff 1984, Fischer-Blass et al. 2006, Foitzik et al. in press, Herbers & Foitzik 2002) and this leads to the evolution of defensive adaptations in their hosts. These host defenses can provide protection against enslavement and include enemy recognition, adjustment of the recognition threshold, better fighting abilities or fast evacuation and escape from the attacked host colonies (Alloway 1990, Brandt et al. 2005a, Foitzik et al. 2001). Selection could, in theory, also alter the behavior of already enslaved hosts (Gladstone 1981). However, most behavioral options of how slaves could benefit from rebelling against their oppressors - through own reproduction, strike or desertion - have been rejected, either because they do not increase slave fitness or were eliminated on empirical grounds such as the possibility of slave reproduction (Foitzik & Herbers 2001a, Heinze et al. 1994). Therefore, slaves were thought to be caught in an evolutionary trap where no behavioral strategy could increase their direct or indirect fitness.

Nonetheless, we recently demonstrated that enslaved *Temnothorax* workers regularly destroy the brood of their slavemaker *P. americanus*. After successfully raising parasitic larvae to the pupal stage, enslaved workers killed two thirds of the queen and worker pupae of the slavemaking ant *P. americanus* (Achenbach & Foitzik in press). This active destruction of parasite pupae, described as slave rebellion, leads to a slower growth of *P. americanus* colonies and consequently to a lower raiding frequency (Foitzik & Herbers 2001a). The murderous slaves, therefore, actively decrease the long-term parasite impact on surrounding host colonies to which they are frequently related. This behavioral trait could consequently increase their indirect fitness. A molecular phylogeny showed that *P. americanus* is an evolutionarily old parasite with a long co-evolutionary history (Beibl et al. 2005), which had enough time to adapt to the exploitation of its hosts. Systematic killing of parasite brood by enslaved host workers appears therefore to be a novel resistance trait in *Temnothorax* hosts to which *P. americanus* is now under strong selection to react.

In this study, we investigate the acceptance of transferred queen and worker pupae in *P. americanus* and *Temnothorax* host colonies. We test several hypotheses: First, if the slavemaker *P. americanus* is leading the evolutionary arms race with its hosts (Dawkins & Krebs 1979), it should increase the acceptance of raided host or parasite pupae by *Temnothorax* slave workers. Acceptance of transferred brood should then be higher in the slavemaker nests rather than in the unparasitized host nests. Slavemakers are only able to exploit two host species concurrently (mixed nests), if they can also persuade their slaves to accept raided pupae of a different species. Second, if host workers developed resistance against slavemaker exploitation, then host workers should be able to recognize and selectively kill parasite pupae more often than pupae of other host species (enemy recognition; Alloway 1990).

Coevolution is not a uniform process over broad geographic ranges (Thompson 1999). Therefore, our third hypothesis states that if pupal traits of the parasite and host rejection behavior are coevolving on a local scale, brood acceptance should depend on the geographic origin of the experimental colonies. If parasites are well-adapted to their local hosts, transferred parasite pupae from sympatric colonies should be killed less often than *P. americanus* pupae from allopatric nests. To investigate these geographic patterns, we included parasite and host colonies from communities in New York and Ohio.

In the darkness of ant nests, chemical and tactile signals are the most important communication cues (Hölldobler & Wilson 1990). Enslaved *Temnothorax* workers selectively kill *P. americanus* queen and worker pupae, while unparasitized host workers raise nearly 100% of the conspecific brood to adulthood (Achenbach & Foitzik in press). This selective destruction of parasite brood by enslaved *Temnothorax* workers demonstrates that slaves are able to discriminate between pupae of different species, as well as castes. We hypothesize that enslaved host workers recognize parasite pupae by their distinct cuticular hydrocarbon profile. Finally, our fifth hypothesis states that parasite populations respond to slave rebellion by closely mimicking the pupae profiles of their local hosts. We therefore analyzed the cuticular hydrocarbon profiles of pupae of different castes, populations and species by gas chromatography and mass spectrometry to investigate the chemical side of this coevolutionary arms race between the social parasite *P. americanus* and its ant hosts.

MATERIAL AND METHODS

Study system, ant collection and housing

The tiny slavemaking ant *P. americanus* parasitizes three host species of the genus *Temnothorax*: *T. longispinosus*, *T. curvispinosus* and infrequently *T. ambiguus*. These ant species occur in mixed deciduous forests throughout northeastern North America, where they nest in hollow acorns, twigs and hickory nuts on the forest floor. Experimental colonies were collected at two locations, at the Huyck Preserve, Albany County, New York (NY, N 42° 31`35.3`` W 74° 9`30.1``), and in Harpersfield, Ashtabula County, Ohio (OH, N 41° 45`34.2`` W 80° 57`55.7``) in May - June of 2005 and 2006. The host community in New York is composed of 95% *T. longispinosus* and 5% *T. ambiguus*; while in Ohio *T. curvispinosus* contributes 71% and *T. longispinosus* 29% to the host community (Brandt & Foitzik 2004). We therefore focused our analyses on *P. americanus* and *T. longispinosus* colonies both from New York and Ohio and *T. curvispinosus* colonies from Ohio. Colonies were transported to our laboratory in Munich in their natural nesting sites, censused and transferred to artificial nest sites in three chambered plastic boxes (10 cm x 10 cm x 1.5 cm) with a moistened plaster floor (Heinze & Ortius 1991). The ants were kept in an incubator

(22°C for 14 h light, 18°C for 10 h dark) and fed water, pieces of cricket and honey twice weekly.

Transfer experiment

In this experiment, we observed the developmental success of transferred queen and worker pupae of the slavemaker *P. americanus*, of its host species *T. longispinosus* and of worker pupae of *T. curvispinosus*. Lightly colored, freshly pupated individuals were relocated to alien con- or heterospecific nests where either enslaved or non-parasitized *Temnothorax* workers cared for them. Queen and worker pupae of all three species were very carefully removed from their native nests and relocated to either alien con- or heterospecific adoptive nests and gently positioned among the brood. Between one and two pupae were transferred per source nest. Transferred pupae in colonies of a different species could be followed, because *P. americanus* and *Temnothorax* pupae can be distinguished based on their external morphology, e.g. head shape and size. For the supervision of transferred conspecific pupae, we used visible developmental differences such as color changes due to the hardening cuticular of pupae. Brood development was monitored daily and the fate of pupae was followed until seven days after eclosion from the pupal stage. Besides the daily surveillance of brood development in a total of 222 colonies, we directly observed brood care behavior of *Temnothorax* workers. Ant colonies could not be observed over the entire time of brood development, but we surveyed each ant colony at least every other day for 5 min (Total observation time >350h). This allowed us to monitor the behavior of *Temnothorax* workers towards alien brood and to determine how their behavior affected the pupal fate.

For the transfer experiments, we removed queen and worker pupae from 52 *P. americanus* colonies (20 from NY and 33 from OH), 42 and 31 *T. longispinosus* colonies, from NY and OH respectively, and 10 OH *T. curvispinosus* colonies. Ninety percent of the 20 *P. americanus* colonies from NY contained only *T. longispinosus* slaves, the remaining two colonies also contained *T. ambiguus* slaves. From the OH *P. americanus* colonies only 36.3% had solely *T. longispinosus* slaves, 58.7% had only *T. curvispinosus* slaves and 6.0% had slaves of both species.

Pupae were transferred to 92 *P. americanus* (45 from NY and 47 from OH), 88 *T. longispinosus* (49 from NY and 39 from OH) and 50 *T. curvispinosus* from OH adoptive colonies. All *T. longispinosus* and *T. curvispinosus* adoptive colonies contained a queen and had 30 ± 10 workers. *Protomognathus americanus* adoptive

colonies were monogynous, contained at least two *P. americanus* workers and 15 ± 5 slave workers. Of these 92 *P. americanus* colonies 59.1% contained only *T. longispinosus* slave workers, 26.1% *T. curvispinosus* slaves, 4.5% *T. ambiguus* slaves and the remaining social parasite colonies enslaved either *T. longispinosus* and *T. curvispinosus* (5.6%) or *T. longispinosus* and *T. ambiguus* (4.5%) slaves.

Chemical analyses

To study the recognition mechanism behind the species- and caste-specific killings (Achenbach & Foitzik, in press), we analysed the hydrocarbon profiles of 37 *P. americanus* pupae from 18 colonies (18 workers, 13 queens and 6 males), 55 *T. longispinosus* pupae from 24 colonies (18 workers, 20 queens and 17 males) and 19 *T. curvispinosus* pupae from nine colonies (9 workers, 3 queens and 7 males) by gas chromatography and mass spectrometry. Hydrocarbons on the cuticle of pupae were extracted by individually immersing the ant pupae in 200 µl of pentane (HPLC grade) for five minutes in 2 ml glass vials. Extracts were then analyzed by coupled gas chromatography (GC) and mass spectrometry (MS) (Agilent Technologies 6890N GC and 5975 MSD) equipped with a Restek Rxi-5MS column (30m length, 0.25 mm ID, 0.25 µm film thickness). Sample injection was splitless over 1 minute at 280°C. Helium was used as carrier gas at a constant flow of 1ml/min. The oven program started at 150°C for 3 minutes, followed by a temperature increase to 300°C in two steps (150°C - 250°C with 30°C/min; 250°C - 300°C with 3°C/min). The final temperature of 300°C was held for two minutes. After an initial solvent delay of 3.8 minutes a mass range of 50 to 500 amu was scanned. The transfer line was held constant at 310°C.

Data analysis

Statistical tests were performed on the mortality rate of all pupae of a single caste, which were transferred from the same source nest into the same adoptive nest. Data distributions were tested for deviation from normality and heteroscedasticity. As expected for a large dataset such as ours (e.g. the transfer experiment included 511 exchanges), these tests often revealed significant deviations from normality. Consequently, we tried to normalize the data with a number of transformations (log, arcsine, square root etc.). We compared mortality rates, depending on the caste (queen, worker), species (*P. americanus*, *T. longispinosus*, *T. curvispinosus*) and geographic origin of pupae and the species and source population of the adoptive nest using MANOVAs, ANOVAs and factorial ANOVAs. To differentiate between

various groups, we used Fisher LSD post-hoc tests. Calculations were carried out with the program Statistica 6.0 (StatSoft).

Chemical data were processed by integrating peak areas with the software MSD ChemStation D.03.00.611 and exported to the program Primer 6 (Version 6.1.6 Primer-E Ltd.). Peak areas were fourth root transformed and standardized by the maximum of each sample. Similarity percent (SIMPER) procedures were used to calculate the relative contributions of chemical compounds to the similarity of a within-caste and species group. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances was used to visualize the chemical distances between the samples and the proximities between sample groups were statistically evaluated by analysis of similarities (ANOSIM).

RESULTS

Transfer experiments

To follow up on the first hypothesis, which assumed that *P. americanus* can influence the acceptance of host pupae, we contrasted the survival of introduced host pupae in different types of adoptive colonies. The mortality of transferred *T. longispinosus* host pupae depended both on the species present in the adoptive nest and the caste of the pupae (Fig. 1; ANOVA: Species in adoptive nest: $F_{3,299} = 6.86$, $p < 0.0001$; caste of pupae: $F_{1,299} = 16.46$, $p < 0.0001$). Worker pupae were less often killed (mean mortality: 48.33 % ± SE 0.03) than transferred queen pupae (mean mortality: 70.31 % ± SE 0.05). *T. longispinosus* pupae introduced into conspecific host nests survived more often than those transferred to *T. curvispinosus* host colonies (LSD test: $p < 0.006$) or *P. americanus* colonies with *T. curvispinosus* slaves (LSD test: $p < 0.05$). The mortality rate did not differ between *T. longispinosus* pupae transferred into *P. americanus* colonies with *T. longispinosus* slaves and unparasitized *T. longispinosus* nests (LSD test: $p = 0.73$), nor from *P. americanus* colonies with *T. curvispinosus* slaves (LSD test: $p = 0.09$). However, *T. longispinosus* pupae, which were introduced to *P. americanus* colonies with *T. curvispinosus* slaves had a lower mortality than those transferred to unparasitized *T. curvispinosus* nests (LSD test: $p < 0.0005$).

Survival of transferred *T. curvispinosus* worker pupae clearly depended on the species of the adoptive colony (Fig. 1; MANOVA: Species of adoptive nest: $F_{1,91} = 12.96$, $p < 0.000001$). *T. curvispinosus* pupae had a lower mortality in conspecific

colonies than in *T. longispinosus* host colonies (LSD test: $p < 0.00001$) or in *P. americanus* colonies with either *T. curvispinosus* slaves (LSD test: $p < 0.00001$) or enslaved *T. longispinosus* workers (LSD test: $p < 0.00001$). The mortality of *T. curvispinosus* pupae did not differ between unparasitized *T. longispinosus* host colonies and *P. americanus* colonies with *T. longispinosus* slaves (LSD test: $p = 0.40$).

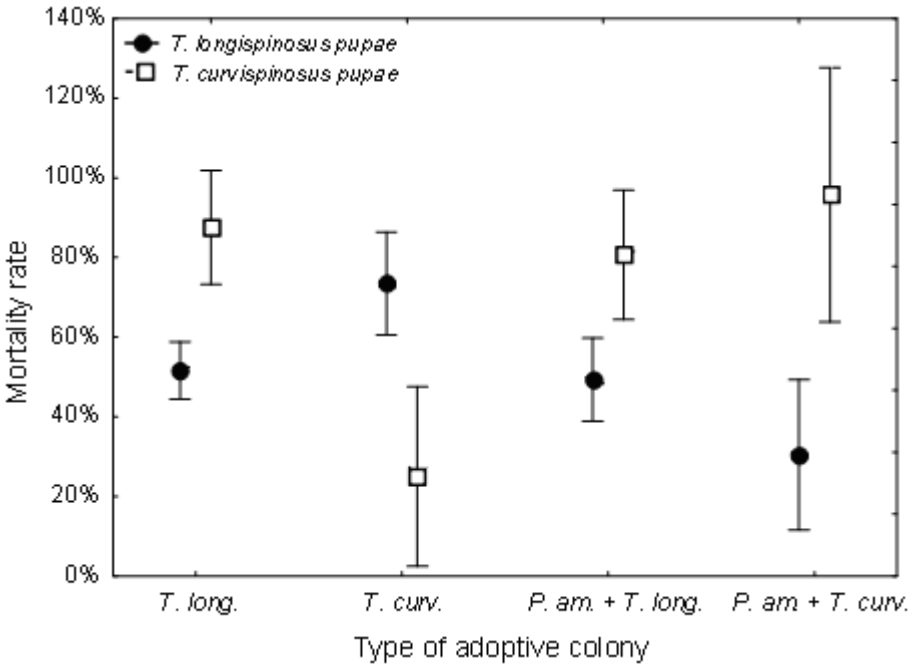

Figure 1 Mortality rate of transferred *Temnothorax* host pupae in different types of colonies, i.e. host colonies and *P. americanus* colonies with slaves of either host species. Results for *T. longispinosus* pupae are shown in filled circles, for *T. curvispinosus* pupae are in open squares. Weighted mean ± SE is given.

We also compared mortality rates of queen and worker pupae of the slavemaker species *P. americanus*, which were transferred either into conspecific slavemaker or *T. longispinosus* host colonies. Slavemaker pupae survived better when relocated into other slavemaker colonies (mean mortality: 79.00 % ± SE 6.81) than when brought into unparasitized *T. longispinosus* host colonies (mean mortality: 92.09 % ± SE 2.85), while the caste (worker / queen) of the pupae had no influence on its survival (Factorial ANOVA: Species of adoptive nest: $F_{1,95} = 3.83$, $p < 0.05$; caste of pupae: $F_{1,95} = 1.73$, $p = 0.19$).

To address the second hypothesis, which stated that resistant host colonies should accept parasite pupae less often than pupae of other host species, we tested whether *T. longispinosus* host colonies accepted *P. americanus* pupae less

often than *T. curvispinosus* pupae. Survival of worker pupae in *T. longispinosus* colonies clearly depended on the species of the pupae, with *T. longispinosus* pupae (mean mortality: 43.11 % ± SE 4.77) surviving at much higher rates (MANOVA: Species of pupae: $F_{1,182} = 25.05$, $p < 0.00001$). However, there was no difference in survival between *P. americanus* (mean mortality: 90.28 % ± SE 4.81) and *T. curvispinosus* pupae (mean mortality: 87.50 % ± SE 5.30; LSD test: $p = 0.78$).

Next, we compared the mortality of *P. americanus* worker pupae, which were transferred to a conspecific nest, with the mortality of *T. longispinosus* or *T. curvispinosus* host pupae that were also relocated into conspecific nests. *P. americanus* pupae (mean mortality: 71.67 % ± SE 10.15) survived less well than host pupae of either *Temnothorax* species, while there was no difference between *T. longispinosus* (mean mortality: 43.12 % ± SE 4.77) and *T. curvispinosus* (mean mortality: 25.00 % ± SE 11.18; MANOVA: $F_{2,137} = 3.72$, $p < 0.03$; LSD tests: *P. am. – T. long:* $p < 0.04$; *P. am. – T. curv.*: $p < 0.008$; *T. long – T. curv.*: $p = 0.16$).

In slave raids, *P. americanus* retrieve *Temnothorax* and occasionally also *P. americanus* (Foitzik & Herbers, 2001b) worker pupae and integrate them into their societies. In the following analysis, we explicitly tested how the species of slaves in *P. americanus* colonies influenced the acceptance of pupae. We tested whether the survival of worker pupae transferred into *P. americanus* colonies depend on their species and / or on the species of the slaves residing in the slavemaker colony (*T. longispinosus* or *T. curvispinosus*) or in an interaction between these two factors. Of the three species, *T. longispinosus* pupae survived best, independent of the slave species present (Fig. 2; Factorial ANOVA: Slave species: $F_{1,129} = 0.79$, $p = 0.37$; slave species *x* pupae species $F_{2,129} = 2.31$, $p = 0.10$; Pupae species $F_{2,129} = 15.73$, $p < 0.00001$; LSD tests: *P. am. – T. long:* $p < 0.008$; *P. am. – T. curv.*: $p = 0.48$; *T. long – T. curv.*: $p < 0.00001$).

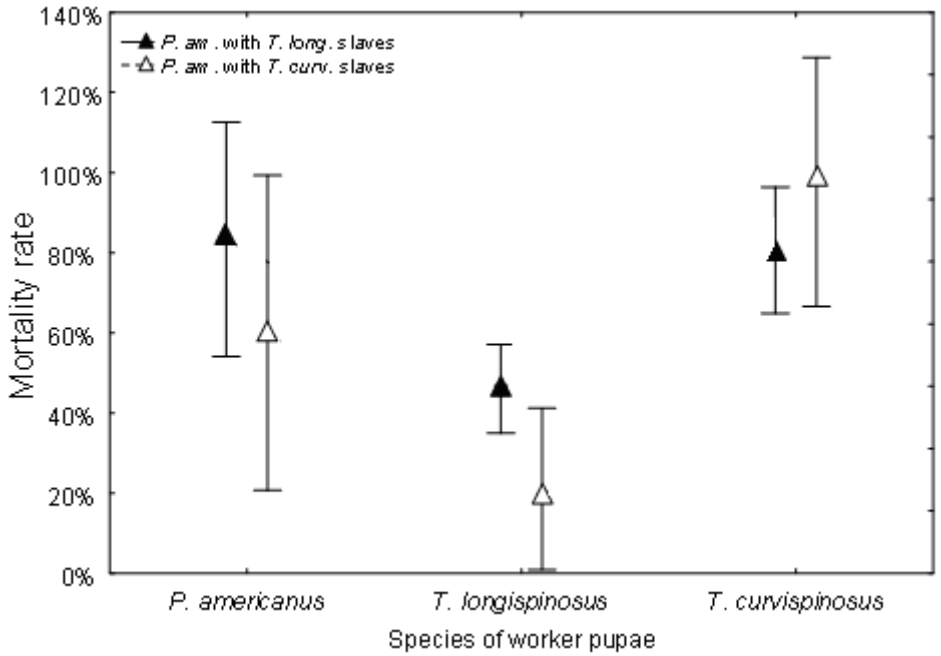

Figure 2 Mortality rate of pupae in *P. americanus* colonies depending on the species of the pupae and the slave species present. Results for *P. americanus* colonies with *T. longispinosus* slaves are shown in filled triangles, for *P. americanus* colonies with *T. curvispinosus* slaves in open triangles. Weighted mean ± SE is given.

Under a geographic mosaic of coevolution, parasites are expected to adapt their pupal traits to those of their sympatric host to avoid recognition and killing. Please, note that only *P. americanus* and *T. longispinosus* occur in both communities, so that we restricted the following analyses to those two species. To reveal potential local adaptation in this context, we analyzed whether the survival of parasite pupae in our transfer experiments depended on the source population of the pupae or on the geographic origin of the adoptive *T. longispinosus* host nest or on an interaction between the two parameters. Indeed, transferred *P. americanus* pupae were accepted at higher rates in sympatric *T. longispinosus* host colonies than in allopatric ones (Fig 3; Factorial ANOVA: Population of parasite pupae: $F_{1,69} = 3.13$, $p = 0.08$; Population of adoptive host nest: $F_{1,69} = 5.56$, $p < 0.02$; Pupae population *x* host population: $F_{1,69} = 8.19$, $p < 0.006$). In a second analysis, we investigated whether this local pattern would also be detectable in a more comprehensive data set, including all transfer experiments with *P. americanus* and *T. longispinosus* pupae into colonies with parasitized or unparasitized *T. longispinosus* workers. Indeed, ant pupae were generally better accepted in colonies of the same community (Factorial ANOVA:

Community of pupae: $F_{1,442} = 10.86$, $p < 0.001$; Community of adoptive nest: $F_{1,442} = 1.02$, $p = 0.31$; Pupae community *x* Adoptive nest community: $F_{1,442} = 4.10$, $p < 0.04$).

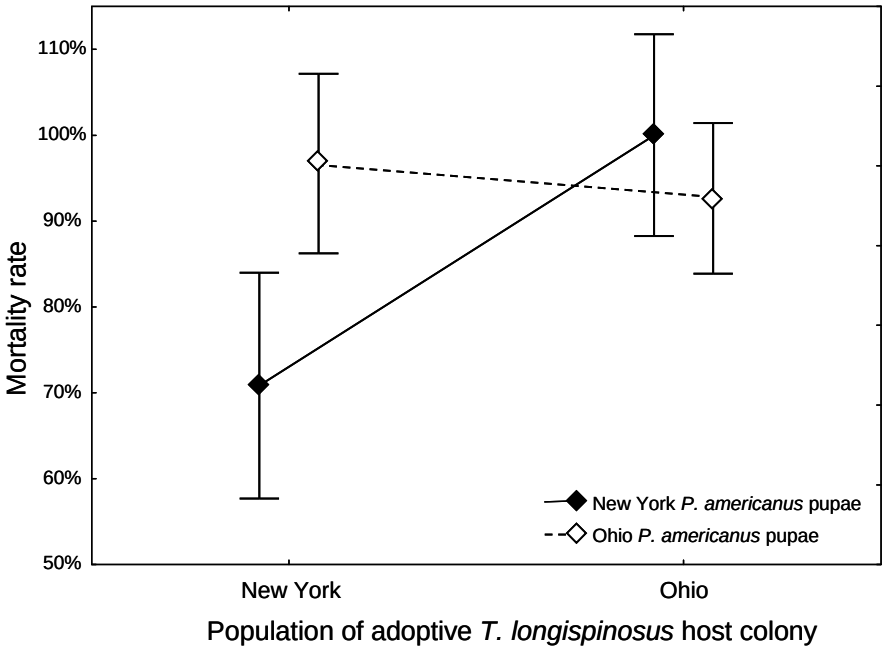

Figure 3 Mortality of *P. americanus* parasite pupae in *T. longispinosus* host colonies depending on the geographic origin of pupae and adoptive colonies. Results for *P. americanus* pupae from New York are shown in filled diamonds, Ohio *P. americanus* pupae in open diamonds. Weighted mean ± SE is given.

We also compared pupae mortality in our transfer experiments with data on brood development in undisturbed ant colonies of all three species from an earlier study (Achenbach & Foitzik in press). Queen and worker *P. americanus* pupae were destroyed by *Temnothorax* slaves at similarly high rates in their undisturbed native colony than when transferred to an alien conspecific colony (MANOVA: $F_{1,131} = 0.75$, $p = 0.39$), whereas *T. longispinosus* pupae showed a much higher survival when left undisturbed in their native nest (MANOVA: $F_{1,194} = 37.64$, $p < 0.000001$). In contrast, *T. curvispinosus* pupae survived equally well in their native nests than when transferred to alien conspecific nests (MANOVA: $F_{1,38} = 2.55$, $p = 0.12$).

Behavioral observations revealed the causes for the frequent death of transferred ant pupae. Enslaved and unparasitized *Temnothorax* workers of both host species actively killed introduced ant pupae. In addition, we observed host workers removing pupae from the nest chamber and pupal death due to negligence. At the same time, *P. americanus* larvae, *Temnothorax* larvae and pupae, which were not

transferred, but remained in their native nests, showed nearly 100% survival (Achenbach & Foitzik in press), indicating that laboratory conditions were not responsible for the observed low survival rates of pupae.

Chemical analyses

Cuticular hydrocarbon profiles of ant pupae were comparatively simply structured. They were composed of more than 90% of only eight hydrocarbons: tricosane, tetracosane, pentacosane, hexacosane, heptacosane, 5-methyl-pentacosene, 3-methyl-pentacosene and nonacosane (Table 1, Fig. 4). Multivariate analysis based on the comparison of the relative proportions of cuticular hydrocarbons showed distinct chemical differences depending both on species and caste across all pupae (Fig. 4, 5; Anosim: N = 111; Species: $R = 0.378$, $p < 0.001$; Caste: $R = 0.118$, $p < 0.001$). Slavemaker worker pupae could be separated very clearly from host worker pupae, while worker pupae of the two *Temnothorax* species showed only marginal differences (Anosim, Global: $R = 0.584$, $p < 0.001$, $N_{Pa, Tl, Tc} = 18, 18, 9$; *P. am. – T. long.*: $R = 0.789$, $P < 0.001$; *P. am. – T. curv.*: $R = 0.577$, $P < 0.001$; *T. long. – T. curv.*: $R = 0.158$, $P < 0.04$). *P. americanus* queen pupae could be differentiated statistically from queen pupae of either of the two *Temnothorax* species, while these host pupae could not be separated with our multivariate analysis (Anosim: Global: $R = 0.546$, $p < 0.001$, $N_{Pa, Tl, Tc} = 13, 20, 3$; *P. am. – T. long.*: $R = 0.630$, $P < 0.001$; *P. am. – T. curv.*: $R = 0.476$, $P < 0.03$; *P. am. – T. curv.*: $R = 0.256$, $P = 0.13$). Finally, *P. americanus* male pupae differed chemically from host male pupae, but again host pupae did not vary in chemical profile (Anosim: Global: $R = 0.539$, $p < 0.001$, $N_{Pa, Tl, Tc} = 6, 17, 7$; *P. am. – T. long.*: $R = 0.957$, $P < 0.001$; *P. am. – T. curv.*: $R = 0.75$, $P < 0.02$; *T. long. – T. curv.*: $R = 0.095$, $P = 0.17$).

Table 1 Contribution (%) of each compound to the cuticular hydrocarbon profiles of queen, worker and male pupae of the slavemaker *P. americanus* and its two *Temnothorax* host species as calculated by SIMPER analyses. Eight hydrocarbons accounted for at least 90% of the group similarity. Missing values represent chemical substances of less than 0.001% contribution.

Species	Caste	Tricosane	Tetracosane	Pentacosane	Hexacosane	Heptacosane	5-Methyl-Pentacosene	3-Methyl-Pentacosene	Nonacosane
P. americanus	Queen	0.22	0.87	38.20	1.28	46.98	0.24	6.53	5.67
	Worker	0.00	0.00	28.61	4.67	57.05	0.00	6.57	3.10
	Male	0.00	0.00	38.04	1.70	55.46	0.00	3.59	1.21
T. longispinosus	Queen	0.00	0.00	23.17	5.86	56.98	0.36	5.18	8.44
	Worker	0.00	0.00	8.39	0.36	78.32	0.00	6.01	6.55
	Male	0.00	0.00	6.01	1.46	73.92	0.32	9.05	9.25
T. curvispinosus	Queen	0.00	0.00	15.68	7.51	57.24	2.41	9.90	7.26
	Worker	0.00	0.00	16.21	3.06	73.76	0.00	4.73	2.24
	Male	0.00	0.00	10.65	2.39	73.63	0.00	5.59	7.75

Within species, we could distinguish all three castes in *P. americanus*, except male from queen pupae (Anosim; Global: $R = 0.331$, $p < 0.001$, $N_{w,q,m} = 18, 13, 6$; worker – queen: $R = 0.435$, $P < 0.001$, worker - male: $R = 0.321$, $P < 0.02$, queen - male: $R = -0.038$, $P = 0.58$). In *T. longispinosus* only the combination worker - male pupae were indistinguishable (Anosim; Global: $R = 0.369$, $p < 0.001$, $N_{w,q,m} = 18, 20, 17$; worker – queen: $R = 0.502$, $P < 0.001$, worker - male: $R = 0.03$, $P = 0.20$, queen - male: $R = 0.486$, $P < 0.001$). Yet, we could not detect differences in the chemical profile of pupae of different castes in *T. curvispinosus*, for which we had the lowest sample size (Anosim: Global: $R = 0.055$, $p = 0.25$, $N_{w,q,m} = 9, 3, 7$).

To test our fifth hypothesis of chemical local adaptation we analysed a data set including *P. americanus* and *T. longispinosus* pupae from New York and Ohio colonies. We investigated whether the chemical profile of a pupa depended on its species and / or on the ant community, in which its colony had been found. Albeit differences between species were much more pronounced than those between communities, we nevertheless detected a geographic pattern in the chemical profile of the ant pupae (Anosim: N = 92; Species: R = 0.541, p < 0.001; Community: R = 0.064, p < 0.025).

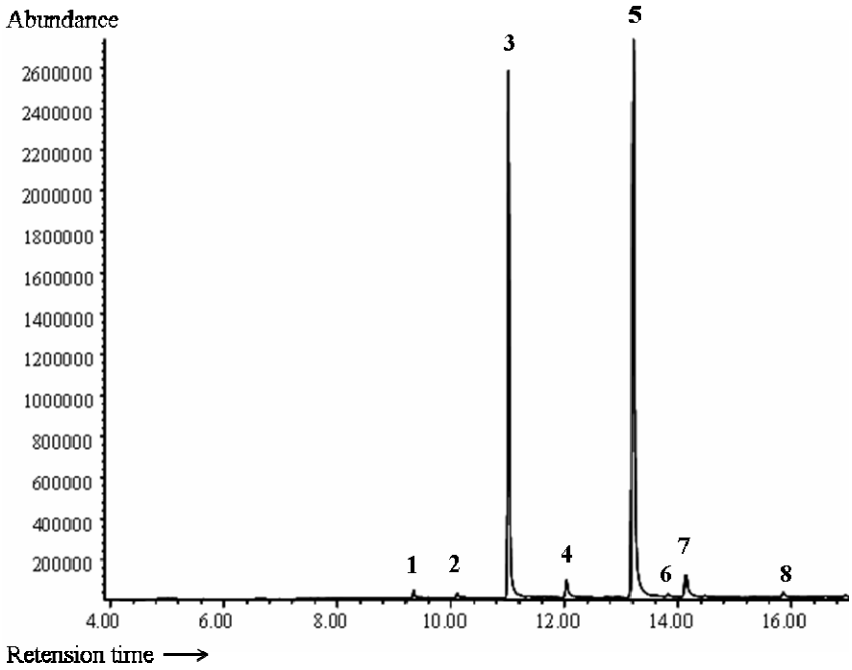

Fig. 4 Gas chromatogram of the cuticular hydrocarbon profile of a *P. americanus* queen pupae. 1 = Tricosane, 2 = Tetracosane, 3 = Pentacosane, 4 = Hexacosane, 5 = Heptacosane, 6 = 5-Methyl-Pentacosene, 7 = 3-Methyl-Pentacosene, 8 = Nonacosane.

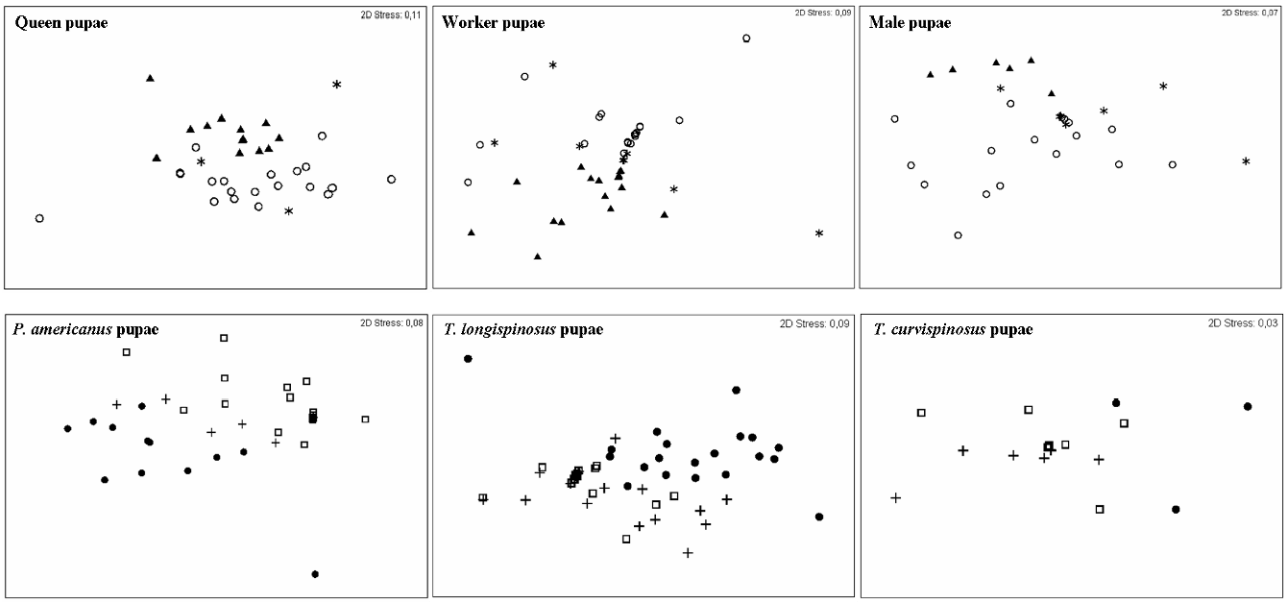

Fig. 5 Non-metric, multidimensional scaling plots (NMDS) of chemical distances based on cuticular hydrocarbon components extracted from queen, worker and male pupae of the slavemaker *P. americanus* and its two *Temnothorax* host species. **a) Species-specific differences**: *P. americanus* pupae are symbolized by black triangles, *T. longispinosus* pupae by open circles and *T. curvispinosus* pupae by black stars. **b) Caste-specific differences:** Queen pupae are symbolized by black circles, worker pupae by open squares and male pupae by black crosses.

DISCUSSION

Our transfer experiments clearly demonstrate that *Temnothorax* workers are able to recognize and selectively destroy pupae of different species and castes, but also that the slavemaker *P. americanus* partly induces enslaved *T. longispinosus* workers to accept alien host and parasite pupae. The GC-MS analyses show consistent differences in chemical profiles of host and parasite pupae, such that these cuticular hydrocarbons can be used by host workers as recognition cues. The apparent chemical discrepancy between parasite and host pupae profiles indicates that the social parasite is running behind its hosts at least on the chemical side of this coevolutionary arms race (Dawkins & Krebs 1979). This was also readily apparent in the high destruction rate of parasite pupae in undisturbed *P. americanus* colonies (Achenbach & Foitzik in press). On the other hand, both the transfer experiments and the chemical analyses reveal that the parasite tries to mimic the profile of its local hosts, indicating that these coevolutionary processes are occurring on a local scale.

Our data give mixed support of our first hypothesis that *P. americanus*, when leading the coevolutionary arms race, should be able to induce enslaved *Temnothorax* workers to accept alien pupae. Enslaved *T. longispinosus* workers accepted transferred *P. americanus* pupae more often, than unparasitized *T. longispinosus* workers. Interestingly, this was not the case for host pupae, which were equally well accepted by parasitized and unparasitized *T. longispinosus* workers. In addition, a different pattern was shown for enslaved *T. curvispinosus* workers, which accepted alien conspecific pupae less, but *T. longispinosus* pupae more often than unparasitized workers of the same species. Overall, *T. longispinosus* pupae were clearly best accepted by enslaved host workers in *P. americanus* colonies, irrespective of the slave species present in slavemaker nests. This indicates a tighter adaptation of the parasite *P. americanus* to its major host *T. longispinosus*, while the secondary host *T. curvispinosus* is largely resistant to this parasite manipulation. This result is in accordance with earlier studies on the behavior during raids, which also found closer adaptation of *P. americanus* to its host *T. longispinosus* and partial resistance of its secondary host *T. curvispinosus* at the same communities (Brandt & Foitzik 2004).

How can parasites influence slave worker behavior, in particular the acceptance of alien brood? Observations have shown that slavemaking ants force slave workers to do what they want by physical aggression (Heinze et al. 1994).

Social parasites could also manipulate enslaved *Temnothorax* workers by using chemicals, such as an 'acceptance pheromone' which, applied to pupae or transmitted via trophallaxis to enslaved host workers could induce them the accept alien brood (Alloway 1982). In addition, the brood acceptance of parasitized workers can be influenced by early experience of slaves, which emerge in parasite nests with a wider range of odor sources (Goodloe & Topoff 1987, Hare & Alloway 1987, Jaisson 1975). If the behavior of enslaved workers is under selection, as suggested by the behavioral trait "slave rebellion" (Achenbach & Foitzik in press), than it is also possible that hosts are under selection to evolve a less flexible, more innate odor template. Such an intrinsic odor template would enable enslaved host workers to recognize parasites reliably. Similarly, cuckoo chicks do not learn their song from their foster parents, as is typical for passerine birds, but rather, they have an innate template (Davies 2000). A learned component in the development of an odor template certainly has its advantages (fast adaptation to environmentally-induced changes in the colony odor), but these have to be contrasted with fitness costs caused by frequent social parasitism. Parasite pressure could thus influence the development of an odor template, a hypothesis, which is testable by comparing host populations under variable social parasite pressure.

The second hypothesis stated that host workers which developed resistance against its social parasite, should be able to recognize and selectively kill parasite pupae more often than pupae of other host species (enemy recognition; Alloway 1990). We could only test this for *T. longispinosus* where we introduced both *P. americanus* and *T. curvispinosus* host pupae. Pupae of both species rarely, if ever, survived the transfer, but mortality rates did not differ between the parasite pupae and pupae of the alternative host *T. curvispinosus*. It is difficult to draw conclusions from this finding, as over 90% of the transferred parasite pupae were killed. Host defenses against alien brood appear to be generally well-developed, albeit not only focused on the enemy *P. americanus*. On the other hand, enslaved host workers regularly come into the situation that alien heterospecific pupae are added to the colony, so that rejection of these pupae could also be under selection.

Generation times of ant social parasites and their hosts are long (approx. 10 years), so it is impossible to directly observe evolutionary changes in the field (Brandt et al. 2005b). However, as host and parasite populations are genetically structured (Brandt et al. 2007), we can compare coevolutionary interactions at sites, which can be at different stages of the interaction or where different outcomes are caused by

variation in community composition (Brandt & Foitzik 2004, Thompson 1999). In addition, the investigation of coevolution in a distinct geographic setting allows us to study local adaptation. Local adaptation frequently occurs in host-parasite interactions and describes a situation when the mean fitness of a population is higher in its home locality than in any other environment (Hoeksema & Forde 2008, Kaltz & Shykoff 1998). And, this is exactly what we observe in *P. americanus* for our transfer experiments: Social parasite pupae, which were transferred into unparasitized sympatric host nests survived much better than those relocated into host colonies of the same species from a different community. This finding supports results from a large scale field manipulation, where *P. americanus* colonies fared much better in their local host population, than when transferred to a distant host population (Foitzik et al. in press). Parasite local adaptation also means that the hosts have to be mal-adapted to their local parasites. This was obvious in the field manipulation, where a reduction in host nest density was only observed in interactions with the sympatric parasite. Our cuticular hydrocarbon analyses explain why parasitic pupae are less frequently killed by their local hosts: Surface chemicals of the parasite appeared to be adapted to the local host population.

In addition, our chemical analysis of the cuticular hydrocarbons of pupae revealed strong species and caste specific differences, which should enable *Temnothorax* workers to discriminate between pupae. Cuticular hydrocarbons are the most important recognition cues in social insects (Howard 1993), such that cuticular chemicals are the most likely basis for pupae recognition. The distinct differences between the chemical profile of parasite and host pupae, which were much more pronounced than those between the two host species, raises the question of why *P. americanus* is not able to mimic the host profiles better. Indeed, the high rate of parasite pupae destruction by slaves even in undisturbed *P. americanus* colonies demonstrates that these mal-adapted chemical profiles entail large fitness costs (Achenbach & Foitzik in press). *P. americanus* colonies are known for their very low productivity and extraordinary small nest size with a mean of only 2 - 4 slavemaker workers per nest (Foitzik & Herbers 2001b, Herbers & Foitzik 2002). Possibly, phylogenetic constraints hinder *P. americanus* from adapting better to its hosts, though we see the first indications of local adaptation in chemical profiles. It is even more likely that pupae detection and destruction – that is the trait "slave rebellion" – only evolved recently and there has not been enough time for the parasite to respond.

Our experiments and chemical analyses clearly show that *P. americanus* is ahead in certain aspects of the coevolutionary arms race, while in others, the *Temnothorax* hosts, with their effective defenses, are leading. This coevolutionary struggle in the leaf litter remains fascinating.

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PUBLICATION 4

COMPARATIVE POPULATION STRUCTURE, GENE FLOW AND POST-GLACIAL COLONIZATION OF THE SOCIAL PARASITE *PROTOMOGNATHUS AMERICANUS* AND ITS HOST SPECIES *TEMNOTHORAX LONGISPINOSUS* AND *TEMNOTHORAX CURVISPINOSUS*

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Manuscript in preparation

ABSTRACT

Coevolution of hosts and parasites is strongly affected by the evolutionary potential of the antagonists, which in turn depends on population sizes as well as on levels of recombination, mutation, and gene flow. Using six to eight microsatellite markers, we investigated population structure, gene flow and post-glacial migration routes of the obligate social parasite *Protomognathus americanus* and its two main host species of the genus *Temnothorax* across their natural range. The polymorphic nuclear markers displayed high levels of genetic diversity in all three species with variation across loci and populations. Genetic differentiation was significant, but moderate to low in *T. curvispinosus*, *P. americanus and T. longispinosus* with F_{ST} values of 0.086, 0.070, *0.052*, for the three species *respectively. Along with high levels of genetic diversity*, *this pattern of host and parasite populations connected by limited gene flow can lead to a geographic mosaic of coevolution and accelerated arms races between species. The main host species T. longispinosus showed the weakest genetic differentiation*, which can be explained by its high effective population size with retained ancient lineages and gradual lineage sorting. Genetic distances between populations did not correlate with geographic distances and this absence of isolation-by-distance could in part be explained by the Pleistocene history of these species, which might have survived in different refugia. In the main host *T. longispinosus*, eastern populations were genetically separated from western populations and cluster analysis revealed one to two clusters and additional unclustered populations in all three species. Glacial history thus might still influence population genetic structure in these ants until today.

Keywords: Gene flow, microsatellites, genetic differentiation, slavemaking ants

INTRODUCTION

Genetic variation in natural populations is a fundamental parameter in evolution and has fascinated scientists for the last century. The development of the polymerase chain reaction, allowed to swiftly analysing genetic variation especially by using highly polymorphic neutral markers, such as microsatellites. Microsatellite mutation rates are believed to be substantial compared to normal rates of point mutations (Jarne & Lagoda 1996, Queller *et al.* 1993) resulting in a positive correlation of effective population size and number of alleles. Consequently, microsatellite variation of current populations might on the one hand correlate directly with the effective population size, and might on the other hand reflect the size of past populations and the process of post-glacial dispersal (Jarne & Lagoda 1996).

Patterns of genetic variation reflects the potential of a species to respond to selection and to adapt to changing environmental conditions but also delivers insights into the history of past populations. Since species vary in their dispersal capacity, population size and in life history parameters (such as their mating system), different species show different levels of genetic structuring, which determine the effects of selection and genetic drift (Avise 1994). Yet also historical processes and environmental barriers may have shaped the genetic structure of a species.

In temperate North-America, Pleistocene glaciations predominantly influenced biological communities. Species were restricted to ice free region south of the Laurentide Ice Sheet or to glacial refugia, areas that persisted within, or adjacent to the ice sheets. Subsequently, the dispersal and migration of species was affected and limited substantially affecting ecology, geographic distribution of species and genetics (Hewitt 2000). Refugial populations are generally expected to harbour higher levels of genetic diversity compared to populations in areas that have been colonized since the retreat of glaciers (Hewitt 1996). This hypothesis was supported by recent studies that demonstrated the identification of refugia and post-glacial colonization routes using nuclear markers, such as microsatellites (Comps *et al.* 2001, Koskinen *et al.* 2000).

However, the evolution of species is not only influenced by the geographic population structure and past colonization, but also by interspecific interactions (Gandon *et al.* 1996, Gandon & Michalakis 2002). Especially host-parasite systems show close coevolutionary relationships with strong reciprocal selection and adaptation (Dydahl & Storfer 2003), and are thus regarded as perfect model systems

of coevolution (Kaltz & Shykoff 1998). In the coevolutionary arms race, the ability of a species to keep up with its opponent strongly depends on selection pressure and the evolutionary potential, e.g. the amount of genetic variation of a species. Intermediate levels of genetic structuring, allowing the exchange of alleles between populations (gene flow) without a complete admixture facilitates local adaptation to the antagonists (Barton & Hewitt 1985), and thus to be ahead in the arms race.

Here we examine the population structure, gene flow and post-glacial colonization of the North American slavemaking ant *Protomognathus americanus* and two of its *Temnothorax* host species, *T. longispinosus* and *T. curvispinosus*. *P. americanus* is an obligate social parasite, which depends on its hosts to carry out all tasks of colony maintenance (Wesson 1939). Instead, slavemaking ants exploit their host species by raiding neighboring host colonies. During raids host brood is stolen and carried back to the slavemakers' nest to restock slave supplies and thus to obtain the slavemaker colony's work force (Alloway 1980, Hölldobler & Wilson 1990). All three interacting species are widely distributed in mixed deciduous forests throughout the northeastern American continent and are closely related to each other (Emery's rule, (Emery 1909)) and consequently characterized by similar generation times and comparable rates of recombination and mutation. Since a recent phylogeny could show that *P. americanus* is an evolutionary old parasite with a long coevolutionary history with its hosts (Beibl *et al.* 2005), over time, the interacting species are expected not only to be influenced by climate changes, but also by parasite-host interactions and coevolution.

MATERIAL AND METHODS

Sampling and DNA extraction

Genetic specimens were collected from 10 spatially separated populations in the summers of 2001-2007 (Figure 1) and were preserved in 100% ethanol and frozen at -20°C until extraction. The DNA was isolated from individual ant workers using liquid nitrogen and the Puregene® DNA isolation kit (Gentra systems), according to Foitzik & Herbers (Foitzik & Herbers 2001b).

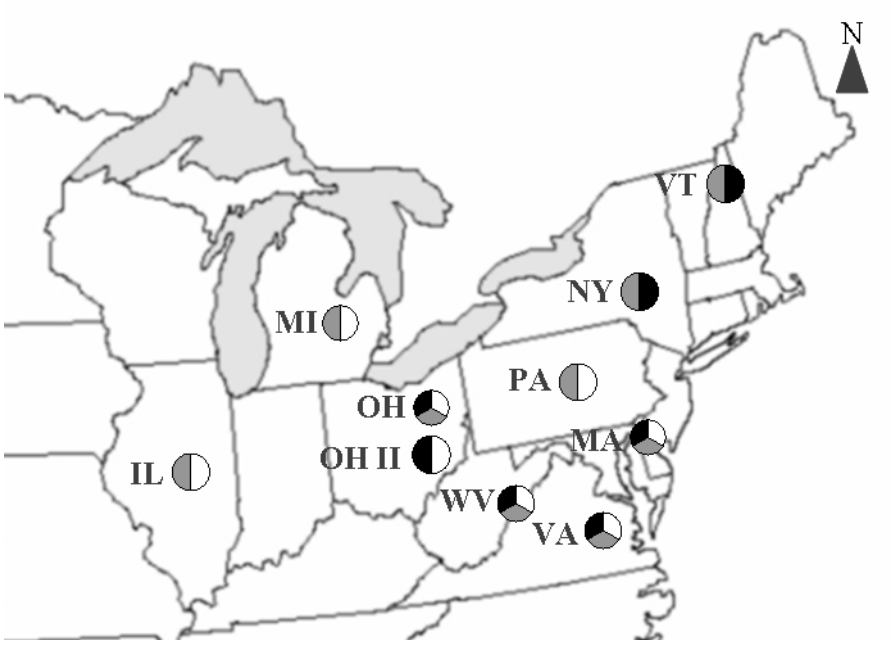

Figure 1 Collecting sites of *P. americanus* (black), *T. longispinosus* (grey) and *T. curvispinosus* (white): IL (Ottawa, Illinois, N 41°22'366'' W 88°50'279''), MA (Maryland, N 39°08'64.6'' W 77°14'4''), MI (Hell, Michigan, N 42°25'754'' W 83°58'956''), NY (Huyck Preserve Rensselaerville, New York, N 42°31'35.3'' W 74°9'30.1''), OH (Harpersfield, Ohio, N 41°45'34.2'' W 80°57'55.7''), OH II (Kraus Wilderness Preserve, N 40°7'31.2'' W 82°57'92.3''), PA (Elliot State Park, Pennsylvania, N 41°6'30'' W 78°31'59''), VT (East Middlebury, Vermont, N 43°58'182'' W 73°5''01''), VA (Shenandoah NP, Virginia, N 38°53'25.4'' W 78°12'12.3''), WV (Watoga SP, West Virginia, N 38°6'25.1'' W 80°7'48.7'')

Amplification and scoring

We genotyped one or two ant workers per colony at six *(P. americanus)* to eight *(T. longispinosus, T. curvispinosus)* specific polymorphic microsatellite loci (Table 1). Depending on the community composition, a maximum of three different ant species and a maximal number of 50 colonies per population was analysed (Table 2).

Table 1 Microsatellites used for the three different ant species

Species	Microsatelites used
P. americanus	L4 (Giraud *et al.*, 1999); L5, L18 (Foitzik *et al.*, 1997); LXAGT1 (Bourke *et al.*, 1997); Myrt 3 (Evans, 1993); LXGT218 (Hamaguchi *et al.*, 1993);
T. longispinosus	L4 (Giraud *et al.*, 1999); L5, L18 (Foitzik *et al.*, 1997); MS 86 (Azuma *et al.*, 2005); LXAGT1 (Bourke *et al.*, 1997); Myrt 3 (Evans, 1993); LXGT223, LXGT218 (Hamaguchi *et al.*, 1993);
T. curvispinosus	L4 (Giraud *et al.*, 1999); L5, L18 (Foitzik *et al.*, 1997); MS 86 (Azuma *et al.*, 2005); LXAGT1 (Bourke *et al.*, 1997); Myrt 3 (Evans, 1993); LXGT223, LXGT218 (Hamaguchi *et al.*, 1993);

Table 2 Number of workers genotyped per nest and population

Population (Code)	No. of workers / nests genotyped		
	P. americanus	*T. curvispinosus*	*T. longispinosus*
Illinois (IL)	-	50/50	50/50
Maryland (MA)	13/8	50/50	3/3
Michigan (MI)	-	50/50	6/3
New York (NY)	50/50	-	50/50
Pennsylvania (PA)	-	18/10	50/33
Ohio (OH)	50/50	50/50	50/50
Ohio II (OH II)	17/12	50/50	-
Vermont (VT)	2/2	-	32/32
Virginia (VA)	1/1	44/44	2/2
West Virginia (WV)	50/50	50/50	50/50

The PCR was accomplished in a 20µl reaction volume using the Q biogene TaqCoreKit containing 1x incubation buffer, 2,2mM $MgCl_2$, 0,4mM of each dNTP, 0,5µM of labelled (TET, FAM and HEX dyes) forward, 0,5µM unlabeled reverse primer and 0,13U of *Taq* DNA polymerase (Q biogene). The PCR was performed in a thermocycler (PxE 0.2 Thermo) using the following program: polymerase activation at 94°C (5 min), initial denaturation at 92°C (1min 30s), annealing at 54°C (45s) and extension at 72°C (30s), followed by 28 cycles at 92°C (45s), 54°C (45s) and 72°C (30s). The last step was a final extension at 72°C (7min). PCR products were precipitated with a mixture of 0,5µl of 3M Ammonium acetate and 10µl of ethanol (100%) at -60°C (60 min), dehydrated with a series of different ethanol concentrations (100%, 70%) and dried. The purified PCR products were visualized on a capillary sequencer (Amersham Bioscience MegaBACE™ 1000) using MegaBACE™ ET400-R

size standard (Amersham Bioscience) and the MegaBACE™ Fragment Profiler 1.2 software (Amersham Bioscience).

Data Analysis

Genetic diversity was evaluated for each population by the number of alleles per locus (A_N), allelic frequencies (A_F), observed heterozygosity (H_O) and expected heterozygosity (H_E). H_E is equivalent to the proportion of heterozygous loci per individual under Hardy–Weinberg expectations. A locus was considered polymorphic if the frequency of its most common allele did not exceed 0.95 (the 95% criterion). Calculations were assessed for each locus using the program MICROSATELLITE ANALYSER (MSA, (Dieringer & Schlötterer 2002). We also calculated the number of private alleles (alleles present exclusively in one of the populations) and allelic richness (number of alleles corrected for sample size) by the method of El Mousadik and Petit (El Mousadik & Petit 1996), which is based on the rarefaction method of Hurlbert (Hurlbert 1971). Allelic richness and linkage disequilibrium was tested with the software FSTAT version 2.9.3 (Goudet 2001).

Tests for Hardy-Weinberg equilibrium were calculated for each locus and population using the software package GENEPOP 3.2 (Raymond & Rousset 1995). The significance of departures from Hardy-Weinberg expectations was tested by the Markov chain exact test (Guo & Thompson 1992) and by the inbreeding coefficient F_{IS} (Wright 1951). As null alleles may also cause heterozygosity deficiency, we estimated the frequency of null alleles as:

$$r = H_E - H_O / 1 + H_E \text{ (Brookfield 1996)}$$

Genetic differentiation between and across all populations and degree of inbreeding was quantified using Wright's (Wright 1965) *F*-statistics. F_{ST} and F_{IS} estimates (Weir & Cockerham 1984) were computed using the software MSA and FSTAT 2.9.3 (Goudet 2001). There were no qualitative differences between the two calculations. F_{ST} can theoretically range from 0 (no genetic divergence) to 1 (complete fixation of alternative alleles). Values above 0.15 are suggested to indicate great genetic differentiation (Wright 1978). Mean and 95% confidence intervals (CI) for *F*-statistics were estimated by jackknifing and bootstrapping over loci, respectively. Genetic viscosity of a population is expressed as the increase of genetic differentiation between geographically distant groups and consequently as the increase of

relatedness among neighbours. Genetic distances were calculated between pairs of populations (pairwise F_{ST} values (Goudet *et al.* 1996)) with the software MSA (Dieringer & Schlötterer 2002) and correlation between metric and genetic distance was tested by the Mantel test (Mantel 1967) using the software XLSTAT 2006.5 (Addinsoft).

During the time following a genetic bottleneck event, populations are out mutation-drift equilibrium conditions (Cornuet & Luikart 1996, Kimmel *et al.* 1998) and alleles at low frequency (< 0.1) are expected to become less abundant in a population than alleles with intermediate frequencies. To reveal possible recent bottlenecks, we analysed the distributions of allele frequencies within populations with the software BOTTLENECK 1.2.02 (Cornuet & Luikart 1996, Luikart *et al.* 1998).

Associations between populations were visualized using the proportion of shared alleles between populations (MSA Dieringer & Schlötterer 2002), in a non-metric multi-dimensional scaling plot (Primer 6 Version 6.1.6 Primer-E Ltd.). Proximities between hypothetical refugia were statistically evaluated by analysis of similarities (ANOSIM). Additionally a Neighbour-Joining phylogram based on Nei's genetic distance (Nei 1978) (Populations 1.2.30, (Langella 1999) was constructed using the software TreeView (Page 1996) to depict the pattern of genetic relationships among populations. Support for the topology was estimated using 1000 bootstrap replicates.

The degree of gene flow among the populations was calculated using Wright's indirect method (Wright 1978) as $N_m = (1/F_{ST} - 1)/4$ (Slatkin & Barton 1989).

RESULTS

Pattern of genetic diversity and variation among microsatellite loci and among populations

We genotyped 183 *P. americanus* workers from seven populations at six microsatellite loci, 293 *T. longispinosus* workers from nine populations and 362 *T. curvispinosus* workers from eight populations at eight microsatellite loci each. All loci were polymorphic in all populations (Table 3, 4, 5). Genotypic disequilibrium was not apparent for any pair of loci according to a global test for each of the 15 *(P. americanus)* and 28 *(T. longispinosus, T. curvispinosus)* different pairs of loci across all populations based on 300 and 500 permutations. Therefore we proceeded under the assumption of statistical independence between loci.

In total 22 - 45 alleles per microsatellite locus were detected in the species *P. americanus*, 17 – 39 alleles per locus in *T. longispinosus* and 23 - 58 alleles per locus in *T. curvispinosus* (Table 3, 4, 5). Among loci, the highest genetic diversity was shown in *T. curvispinosus* with a mean number of alleles of 35.4, followed by *P. americanus* with a mean of 27.9 and *T. longispinosus* with a mean number of 26 different alleles. The mean expected heterozygosity H_E among loci ranged from 0.78 to 0.95 in *P. americanus*, from 0.32 to 0.94 in *T. longispinosus* and from 0.62 to 0.97 in the species *T. curvispinosus*.

Genetic diversity also varied among populations (Table 6, 7, 8). Mean allele numbers ranged from 2.0 (Virginia) to 19.5 (New York) in *P. americanus*, from 2.5 (Virginia) to 17.1 (Ohio) in *T. longispinosus* and from 11.1 (Virginia) to 18.6 (Michigan) in *T. curvispinosus*. Allelic richness showed less variation, but still values differed among populations. A test for correlation between allelic richness and longitude and/or latitude in all three species showed no statistically significant correlations (Spearman correlations: $p \geq 0.19$). Nevertheless, there seemed to be a positive association between allelic richness and both longitude and latitude in *P. americanus* (Spearman: r= 0.40, p= 0.37; r= 0.55, p= 0.20), between allelic richness and longitude in *T. longispinosus* (Spearman: r= 0.48, p= 0.19) and between allelic richness and latitude in *T. curvispinosus* (Spearman: r= 0.36, p= 0.39).

Mean expected heterozygosity H_E among populations varied from 0.72 (Maryland) to 1.00 (Virginia) in *P. americanus*, from 0.71 (Virginia) to 0.82 (Maryland) in *T. longispinosus* and from 0.75 (Ohio) to 0.87 (Michigan, Ohio 2, Pennsylvania) in *T. curvispinosus*. Alleles present exclusively in one of the populations (private alleles) were found at each species and each locus, except in the locus LXGT 223 in *P. americanus*. All populations, except *P. americanus* and *T. longispinosus* from Virginia (VA), contained unique alleles, suggesting limited current levels of genetic exchange (Barton & Slatkin 1986). Their numbers ranged from two to 15 in *P. americanus*, from one to 15 in *T. longispinosus* and from one to seven in *T. curvispinosus* and generally occurred in low mean frequencies from 0.021 in *T. curvispinosus* to 0.045 in *P. americanus*. The main host species *T. longispinosus* had an intermediate mean frequency of private alleles of 0.030.

Significant deviation from Hardy-Weinberg equilibrium was observed in most of the loci analysed in all three study species (*P. americanus*: 4 out of 6; *T. longispinosus*: 5 out of 8; *T. curvispinosus*: 7 out of 8) and a global test for Hardy-Weinberg expectations across all loci and populations in the three study species

showed a *P* value (Hardy-Weinberg equilibrium) of 0.001. This deviation may be due to an excess or a deficiency of heterozygotes from expected values. The Markov chain exact test for significance of deviations from Hardy-Weinberg-expectations revealed deficiency of heterozygotes in 15 out of 42 locus-population combinations in *P. americanus*. In *T. longispinosus* 24 out of 72 and in *T. curvispinosus* 36 out of 64 locus-population combinations had less heterozygotes than expected. Multilocus F_{IS} analysis showed a significant multilocus heterozygote deficiency in four out of seven sampling localities for *P. americanus*, in six out of nine localities for *T. longispinosus* and in eight out of eight sampling localities for *T. curvispinosus* indicating significant levels of inbreeding in the latter species (Table 6, 7, 8). Besides inbreeding, the deficiencies of heterozygotes observed may also be caused by the presence of null alleles. Frequencies of null alleles (NA_F) varied from -0.021 to 0.206 in *P. americanus*, from -0.035 to 0.156 in *T. longispinosus* and from -0.006 to 0.149 in *T. curvispinosus*.

Since genetic bottlenecks cause an excess of heterozygosity we thus found no evidence for this event in the study species *P. americanus* and *T. curvispinosus* under the Stepwise Mutation Model (SMM) or the Infinite Alleles Model (IAM) (Wilcoxon test: *P. americanus*: $p_{SSM} \geq 0.34$, $p_{IAM} \geq 0.42$; *T. curvispinosus*: $p_{SSM} \geq 0.98$, $p_{IAM} \geq 0.16$). In *T. longispinosus* we detected evidence for bottleneck events in two populations (Wilcoxon test: Maryland $p_{SSM} = 0.001$, $p_{IAM} = 0.45$; Michigan $p_{SSM} = 0.04$, $p_{IAM} = 0.004$), even though sample sizes were rather small in these locations ($n_{MA} = 3$, $n_{MI} = 6$). For the remaining seven populations we could not significantly show the experience of a genetic bottleneck (Wilcoxon test: $p_{SSM} \geq 0.93$, $p_{IAM} \geq 0.31$). The some populations displayed discrepancy of p-values between the IAM test and the SMM test is the consequence of different heterozygosity expectations at mutation equilibrium (Shriver *et al.* 1993, Valdes & Slatkin 1993). Since microsatellite mutation is generally thought to occur through a stepwise process, the best estimates of heterozygosity for a bottleneck analysis may be expected by using a combination of both models.

Table 3 Genetic variation at 6 microsatellite loci for *P. americanus;* A_N = number of alleles per locus, H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = inbreeding coefficient, NA_F = null allele frequency

Population		Locus					
		L4	L5	L18	LXAGT1	Myrt3	LXGT218
MA	A_N	9	14	12	14	12	8
	H_O	0.500	1.0	0.923	0.727	1.0	0.923
	H_E	0.866	0.957	0.911	0.957	0.935	0.825
NY	A_N	22	14	15	26	28	12
	H_O	0.679	0.940	0.889	0.741	0.920	0.260
	H_E	0.923	0.906	0.869	0.968	0.930	0.501
OH	A_N	14	22	15	14	22	15
	H_O	0.783	1.0	1.0	0.952	0.875	0.460
	H_E	0.906	0.900	0.888	0.850	0.949	0.785
OH II	A_N	12	13	14	11	12	9
	H_O	0.643	0.882	1.0	0.667	0.692	0.750
	H_E	0.857	0.900	0.923	0.902	0.935	0.879
VT	A_N	2	4	2	4	3	2
	H_O	0.500	1.0	0.500	1.0	1.0	0.500
	H_E	0.500	1.0	0.500	1.0	0.833	0.500
VA	A_N	2	2	2	2	2	2
	H_O	1.0	1.0	1.0	1.0	1.0	1.0
	H_E	1.0	1.0	1.0	1.0	1.0	1.0
WV	A_N	21	17	16	24	14	8
	H_O	0.536	0.980	0.958	0.854	0.85	0.255
	H_E	0.921	0.919	0.873	0.928	0.874	0.490
Total A_N		42	27	28	45	31	22
Mean H_O		0.639	0.967	0.953	0.837	0.879	0.413
Mean H_E		0.953	0.934	0.913	0.950	0.945	0.780
NA_F		0.161	-0.017	-0.021	0.060	0.034	0.206

Table 4 Genetic variation at 6 microsatellite loci for *T. longispinosus;* A_N = number of alleles per locus, H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = inbreeding coefficient, NA_F = null allele frequency

Population		**Locus**							
		L4	L5	L18	MS86	LXAGT1	Myrt3	LXGT223	LXGT218
IL	A_N	10	27	12	12	18	13	4	8
	H_O	0.857	0.800	0.860	0.207	0.979	0.932	0.404	0.333
	H_E	0.880	0.935	0.874	0.584	0.908	0.858	0.576	0.654
MA	A_N	5	5	4	2	3	5	2	5
	H_O	1.0	1.0	1.0	0.500	0.667	1.0	0.500	1.0
	H_E	0.933	0.933	1.0	0.500	0.800	0.933	0.500	0.933
MI	A_N	6	6	6	2	5	4	3	5
	H_O	1.0	0.500	0.833	0.250	1.0	1.0	0.833	0.800
	H_E	0.818	0.879	0.864	0.250	0.848	0.758	0.591	0.844
NY	A_N	21	19	18	7	19	15	4	8
	H_O	0.956	0.604	0.940	0.194	1.0	0.878	0.375	0.563
	H_E	0.923	0.894	0.926	0.372	0.918	0.829	0.326	0.704
OH	A_N	24	21	25	3	21	16	11	16
	H_O	0.829	0.629	0.917	0.049	0.932	0.784	0.471	0.646
	H_E	0.921	0.930	0.935	0.072	0.940	0.863	0.604	0.821
PA	A_N	19	22	20	5	22	11	6	12
	H_O	0.900	0.960	0.979	0.161	1.0	0.937	0.261	0.540
	H_E	0.924	0.857	0.929	0.495	0.932	0.838	0.360	0.684
VT	A_N	12	17	15	3	17	8	7	9
	H_O	0.591	0.933	0.933	0.500	1.0	0.933	0.455	0.581
	H_E	0.877	0.939	0.903	0.500	0.903	0.815	0.874	0.546
VA	A_N	3	2	3	2	4	2	2	2
	H_O	0.500	0.500	1.0	0.500	1.0	1.0	0.0	0.0
	H_E	0.833	0.500	0.833	0.500	1.0	0.667	0.667	0.667
WV	A_N	24	16	18	7	19	11	8	11
	H_O	0.824	0.891	0.979	0.086	0.979	0.933	0.353	0.318
	H_E	0.946	0.738	0.927	0.265	0.912	0.795	0.680	0.651
Total A_N		35	39	31	17	35	30	21	26
Mean H_O		0.853	0.796	0.935	0.123	0.978	0.905	0.378	0.502
Mean H_E		0.930	0.933	0.939	0.323	0.932	0.840	0.568	0.780
NA_F		0.040	0.071	0.002	0.151	-0.024	-0.035	0.121	0.156

Table 5 Genetic variation at 6 microsatellite loci for *T. curvispinosus;* A_N = number of alleles per locus, H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = inbreeding coefficient, NA_F = null allele frequency

Population		Locus							
		L4	L5	L18	MS86	LXAGT1	Myrt3	LXGT223	LXGT218
IL	A_N	31	17	17	9	25	14	16	10
	H_O	0.891	0.958	0.755	0.286	0.980	0.980	0.745	0.634
	H_E	0.958	0.877	0.879	0.545	0.943	0.906	0.883	0.766
MA	A_N	17	12	19	3	31	17	15	19
	H_O	0.400	0.563	0.714	0.0	0.957	0.771	0.405	0.735
	H_E	0.912	0.684	0.794	0.329	0.967	0.921	0.925	0.820
MI	A_N	31	17	17	12	22	22	16	12
	H_O	0.792	0.837	0.688	0.829	1.0	1.0	0.750	0.605
	H_E	0.955	0.815	0.839	0.859	0.943	0.928	0.898	0.734
OH	A_N	14	13	22	8	30	20	14	14
	H_O	0.290	0.604	0.612	0.138	0.978	0.891	0.775	0.511
	H_E	0.599	0.672	0.860	0.344	0.965	0.928	0.825	0.764
OH II	A_N	7	9	20	5	36	18	19	22
	H_O	0.375	0.432	0.740	0.333	0.957	0.841	0.619	0.980
	H_E	0.867	0.545	0.870	0.405	0.962	0.869	0.927	0.916
PA	A_N	18	12	16	9	16	13	8	12
	H_O	0.944	0.667	0.889	0.529	0.944	0.611	0.455	0.611
	H_E	0.943	0.849	0.944	0.613	0.946	0.906	0.879	0.889
VA	A_N	12	12	11	4	15	15	10	10
	H_O	0.444	0.651	0.433	0.333	0.971	0.700	0.360	0.667
	H_E	0.941	0.859	0.870	0.867	0.927	0.898	0.733	0.580
WV	A_N	34	16	19	2	20	17	21	18
	H_O	0.830	0.640	0.796	0.025	1.0	0.791	0.800	0.735
	H_E	0.968	0.813	0.867	0.025	0.941	0.901	0.943	0.828
Total A_N		48	28	30	23	58	32	30	34
Mean H_O		0.694	0.677	0.702	0.341	0.976	0.851	0.654	0.696
Mean H_E		0.963	0.824	0.901	0.623	0.965	0.926	0.943	0.899
NA_F		0.137	0.081	0.105	0.174	-0.006	0.039	0.149	0.107

Table 6 Genetic variation among populations for *P. americanus;* A_N = number of alleles per locus, A_R= allelic richness, PA_N= number of private alleles, H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = inbreeding coefficient

Population	A_N Mean	H_O Mean	H_E Mean	A_R	PA_N	F_{IS}
MA	11.5	0.846	0.909	1.90	2	0.072
NY	19.5	0.738	0.850	1.85	15	0.133
OH	17	0.845	0.880	1.88	5	0.039
OH II	11.8	0.772	0.899	1.90	7	0.146
VT	2.8	0.750	0.722	1.80	2	-0.067
VA	2.0	1.00	1.00	1.91	0	-
WV	16.7	0.739	0.834	1.83	9	0.115

Table 7 Genetic variation among populations for *T. longispinosus*; A_N = number of alleles per locus, A_R= allelic richness, PA_N= number of private alleles, H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = inbreeding coefficient

Population	A_N Mean	H_O Mean	H_E Mean	A_R	PA_N	F_{IS}
IL	13	0.672	0.784	1.78	9	0.145
MA	3.9	0.833	0.817	1.69	2	-0.030
MI	4.6	0.777	0.732	1.73	1	-0.069
NY	13.9	0.689	0.737	1.74	7	0.066
OH	17.1	0.657	0.761	1.76	15	0.138
PA	14.6	0.717	0.752	1.75	10	0.047
VT	11	0.741	0.795	1.73	2	0.077
VA	2.5	0.563	0.708	1.65	0	0.304
WV	14.3	0.670	0.739	1.74	7	0.095

Table 8 Genetic variation among populations for *T. curvispinosus*; A_N = number of alleles per locus, A_R= allelic richness, PA_N= number of private alleles, H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = inbreeding coefficient

Population	A_N Mean	H_O Mean	H_E Mean	A_R	PA_N	F_{IS}
IL	17.4	0.779	0.845	4.44	5	0.080
MA	16.6	0.568	0.794	4.22	5	0.289
MI	18.6	0.813	0.871	4.57	2	0.068
OH	16.9	0.600	0.745	3.92	5	0.196
OH II	17.0	0.660	0.871	4.21	7	0.176
PA	13.0	0.706	0.871	4.63	1	0.195
VA	11.1	0.570	0.834	4.24	2	0.335
WV	18.4	0.702	0.786	4.29	4	0.107

Genetic structure and degree of inbreeding of the populations

Data on *F*-Statistics is summarized in Table 9. F_{ST} values, which can be interpreted as a measure of the level of differentiation among populations relative to the limiting amount under complete fixation (Wright 1978), showed a highly significant differentiation between populations in all three study species. We found the highest structuring in one of the host species *T. curvispinosus* (Global F_{ST} = 0.086), followed by the slavemaker *P. americanus* (Global F_{ST} = 0.070) and its main host species *T. longispinosus* (Global F_{ST} = 0.052). Since confidence intervals of all three species

largely overlap, F_{ST} estimates are not qualitatively different (Mann-Whitney U test: $p > 0.05$) (Table 6).

The global inbreeding coefficients F_{IS} were positive in all three study species (Table 9) but in *P. americanus* and *T. longispinosus* confidence intervals extended beyond zero, indicating no significant inbreeding. In contrast, the second host species *T. curvispinosus* showed the highest global F_{IS} value of 0.141 with a confidence interval clearly above zero. Additional, multilocus F_{IS} analysis for each population indicated significant multilocus heterozygote deficiency in eight out of eight sampling localities for *T. curvispinosus* (Table 8) and consequently a significant degree of inbreeding.

Table 9 *F*-Statistics for microsatellite date of *P. americanus*, *T. longispinosus* and *T. curvispinosus*

Species	locus	F_{IS} Mean ± SE	Global F_{IS} Mean ± SE (95% CI)	F_{ST} Mean ± SE	Global F_{ST} Mean ± SE (95% CI)
P. am.	L 4	0.297 ± 0.062	0.092 ± 0.069 (-0.022-0.228)	0.053 ± 0.021 ***	0.070 ± 0.027 (0.038-0.129) ***
	L 5	-0.064 ± 0.021		0.028 ± 0.016 ***	
	L 18	-0.081 ± 0.026		0.042 ± 0.009 ***	
	LXAGT 1	0.073 ± 0.089		0.059 ± 0.024 ***	
	Myrt 3	0.044 ± 0.027		0.036 ± 0.011 ***	
	LXGT 218	0.370 ± 0.075		0.227 ± 0.124 ***	
T. long.	L 4	0.062 ± 0.036	0.079 ± 0.057 (-0.008-0.211)	0.022 ± 0.010 ***	0.052 ± 0.016 (0.027-0.084) ***
	L 5	0.092 ± 0.092		0.072 ± 0.039 ***	
	L 18	-0.021 ± 0.014		0.027 ± 0.009 ***	
	MS 86	0.613 ± 0.048		0.048 ± 0.032 **	
	LXAGT 1	-0.067 ± 0.016		0.017 ± 0.005 ***	
	Myrt 3	-0.090 ± 0.036		0.015 ± 0.008 ***	
	LXGT 223	0.267 ± 0.086		0.100 ± 0.057 ***	
	LXGT 218	0.278 ± 0.069		0.128 ± 0.023 ***	
T. curv.	L 4	0.223 ± 0.072	0.141 ± 0.039 (0.071-0.209)	0.065 ± 0.059 ***	0.086 ± 0.027 (0.045-0.147) ***
	L 5	0.111 ± 0.053		0.084 ± 0.038 ***	
	L 18	0.183 ± 0.041		0.053 ± 0.018 ***	
	MS 86	0.247 ± 0.188		0.426 ± 0.192***	
	LXAGT 1	-0.028 ± 0.011		0.018 ± 0.005 ***	
	Myrt 3	0.062 ± 0.044		0.020 ± 0.007 ***	
	LXGT 223	0.264 ± 0.065		0.064 ± 0.023 ***	
	LXGT 218	0.112 ± 0.057		0.143 ± 0.035 ***	

Differentiation between populations and gene flow

Genetic differentiation among populations was examined using a pairwise comparison of F_{ST} values (Table 10, 11, 12). The pairwise test revealed significant differences in allele frequencies between most populations in all three study species. Non-significant results were most likely due to small sample sizes. Pairwise F_{ST} values ranged from 0.023 to 0.205 in *P. americanus*, from 0.007 to 0.165 in *T. longispinosus* and from 0.042 to 0.189 in *T. curvispinosus*.

There was no significant correlation between genetic differentiation (pairwise F_{ST}) and geographical distance between populations for *P. americanus* (Mantel test: r= 0.308; p = 0.17) and its host species *T. curvispinosus* (Mantel test: r= -0.262; p = 0.18). However, low p-values indicated a tendency for an association of both factors in both species. The non-significance of results might be due to small sample sizes in Virginia and Vermont und thus low statistical power. The Mantel test for the main host species *T. longispinosus* (Mantel test: r= 0.063; p = 0.72) revealed no significant correlation and no tendency for an association. Consequently, isolation by distance certainly played no important role in the genetic structure of the main host species *T. longispinosus*.

The proportion of shared alleles between populations revealed no significant clustering of populations for *P. americanus* and *T. curvispinosus* (ANOSIM: p> 0.05). However in the host species *T. longispinosus*, western populations were clearly separated from populations originating from a hypothetical eastern refugia (ANOSIM: r= 0.33, p= 0.012). The genetic distance between pairs of populations ranged from 0.32 to 0.85 in *P. americanus*, from 0.16 to 0.76 in *T. longispinosus* and from 0.30 to 0.61 in *T. curvispinosus*. The Neighbour-Joining clustering analysis, based on Nei's genetic distance (Nei 1978) between populations, showed the existence of one cluster and two ungrouped populations in *P. americanus*, one cluster and two ungrouped populations in *T. longispinosus* and two clusters and one ungrouped population in *T. curvispinosus*. In *T. longispinosus*, populations mostly deriving from a hypothetical western refugia (except Vermont) formed one large cluster (Illinois, West Virginia, Michigan, Ohio, Vermont, Virginia), whereas New York and the ungrouped populations (except Maryland) presumably represent populations colonized from the hypothetical eastern refugia. However, *P. americanus* and *T. curvispinosus* showed no clear relationship between genetic distances and geographical location based on the phylogram of genetic distance generated by the Neighbour-Joining method (Figure 2).

Gene flow (N_m) between populations ranged from 2.66 in *T. curvispinosus* to 4.56 in *T. longispinosus* (Table 13). The slavemaker *P. americanus* showed an intermediate number of 3.32 migrants per generation.

Table 10 Pairwise matrix of genetic differentiation (F_{ST} lower diagonal) and geographical distance (in km, upper diagonal) for *P. americanus;* ns = not significant

Population	MA	NY	OH	OH II	VT	VA	WV
MA	-	456.2	428.6	503.3	641.6	88.6	277.3
NY	0.080 **	-	566.3	782.7	182.7	528.8	704.6
OH	0.060 **	0.043 **	-	248.0	689.1	395.7	412.3
OH II	0.057 **	0.097 **	0.065 **	-	923.2	431.7	332.6
VT	0.093 ns	0.139 ns	0.086 ns	0.061 ns	-	711.7	883.0
VA	0.027 ns	0.075 ns	0.031 ns	0.023 ns	0.205 ns	-	189.0
WV	0.055 **	0.089 **	0.065 **	0.079 **	0.070 ns	0.081 ns	-

Table 11 Pairwise matrix of genetic differentiation (F_{ST} lower diagonal) and geographical distance (in km, upper diagonal) for *T. longispinosus*; ns = not significant

Population	IL	MA	MI	NY	OH	PA	VT	VA	WV
IL	-	1022.3	407.1	1223.2	661.1	867.5	1321.0	953.0	837.6
MA	0.034 ns	-	703.4	456.2	428.6	244.0	641.6	88.6	277.3
MI	0.066 ns	0.059 ns	-	824.4	285.8	501.1	914.3	655.7	610.8
NY	0.044 **	0.012 ns	0.057 ns	-	566.3	395.3	182.7	528,8	704.6
OH	0.040 **	0.007 ns	0.047 ns	0.041 **	-	215.3	689.1	395.7	412.3
PA	0.049 **	0.014 ns	0.044 ns	0.021 **	0.050 **	-	914.3	248.2	360.7
VT	0.070 **	0.120 **	0.121 **	0.116 **	0.053 **	0.118 **	-	711.7	883.0
VA	0.122 ns	0.165 **	0.131 ns	0.119 ns	0.112 ns	0.114 ns	0.102 ns	-	189.0
WV	0.047 **	0.038 ns	0.081 *	0.044 **	0.055 **	0.047 **	0.088 **	0.110 ns	-

Table 12 Pairwise matrix of genetic differentiation (F_{ST} lower diagonal) and geographical distance (in km, upper diagonal) for *T. curvispinosus*

Population	IL	MA	MI	OH	OH II	PA	VA	WV
IL	-	1022.3	407.1	661.1	521.3	867.5	953.0	837.6
MA	0.036 **	-	703.4	428.6	503.3	244.0	88.6	277.3
MI	0.081 **	0.096 **	-	285.8	297.2	501.1	655.7	610.8
OH	0.063 **	0.053 **	0.124 **	-	248.0	215.3	395.7	412.3
OH II	0.069 **	0.043 **	0.087 **	0.086 **	-	390.5	431.7	332.6
PA	0.042 **	0.042 *	0.059 **	0.079 **	0.049 **	-	248.2	360.7
VA	0.123 **	0.140 **	0.054 **	0.189 **	0.138 **	0.095 **	-	189.0
WV	0.047 **	0.040 **	0.120 **	0.059 **	0.064 **	0.044 **	0.173 **	-

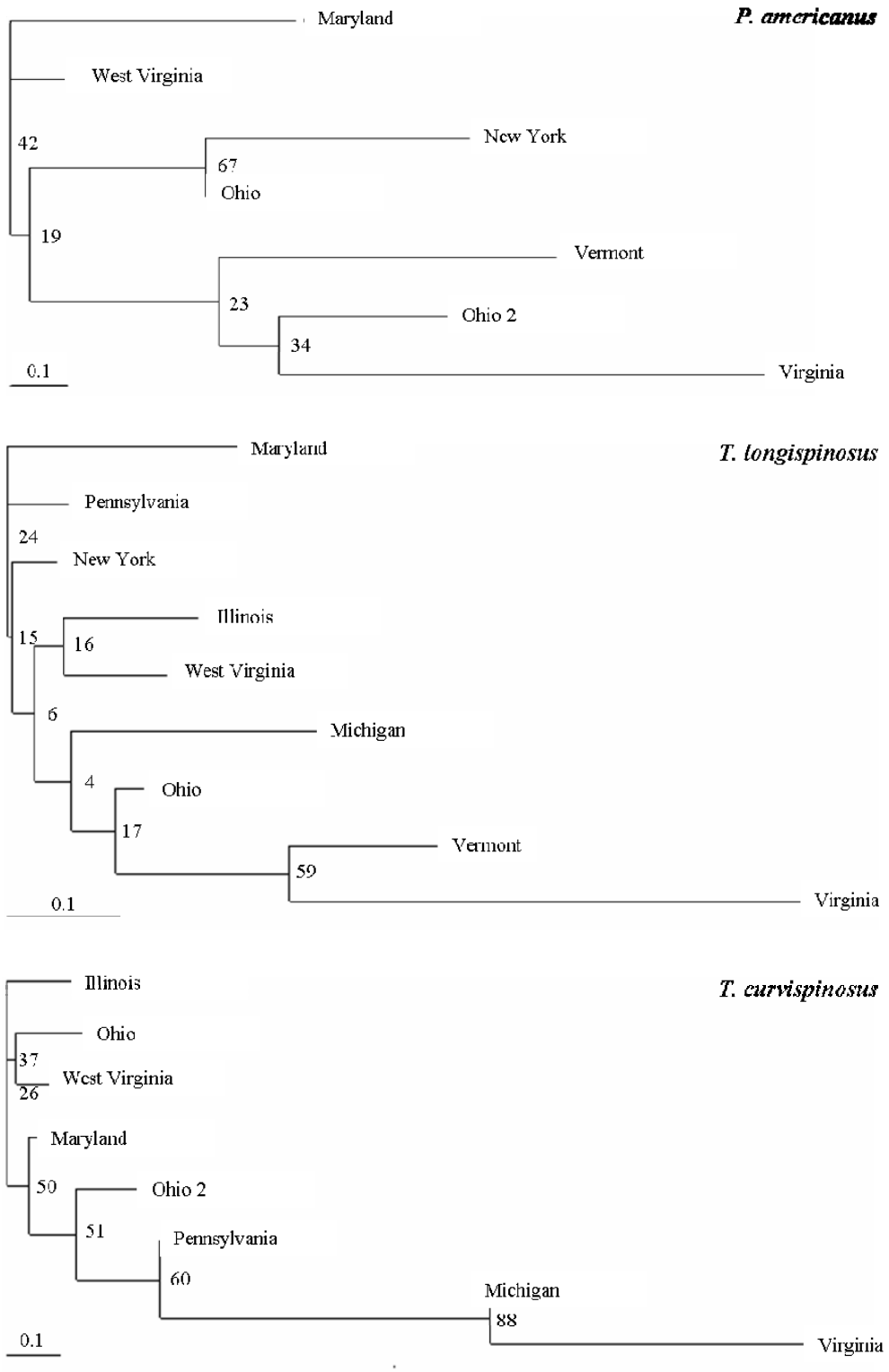

Figure 2 Phylogram of Neighbour-Joining method based on genetic distance (Nei, 1978) among populations of *P. americanus*, *T. longispinosus* and *T. curvispinosus*. Confidence values for nodes are percentages over 1000 bootstrap replications.

Table 13 Summary of genetic variation at 6 to 8 microsatellite loci, *F*-Statistics and estimates of the approximate number of migrants per generation N_m for *P. americanus* and its host species *T. longispinosus* and *T. curvispinosus*

	No. of workers/ nests/ populations	locus	A_N	H_O	H_E	Global F_{ST} Mean ± SE (95% CI)	Global F_{IS} Mean ± SE (95% CI)	N_m from F_{ST}
P. am.	183/173/7	L 4	42	0.64	0.95	0.070 ± 0.027 (0.038-0.129) ***	0.092 ± 0.069 (-0.022-0.228)	3.32
		L 5	27	0.97	0.93			
		L 18	28	0.95	0.91			
		LXAGT 1	45	0.84	0.95			
		Myrt 3	31	0.88	0.95			
		LXGT 218	22	0.41	0.78			
T. long.	293/273/9	L 4	35	0.85	0.93	0.052 ± 0.016 (0.027-0.084) ***	0.079 ± 0.057 (-0.008-0.211)	4.56
		L 5	39	0.80	0.93			
		L 18	31	0.94	0.94			
		MS 86	17	0.12	0.32			
		LXAGT 1	35	0.98	0.93			
		Myrt 3	30	0.91	0.84			
		LXGT 223	21	0.38	0.57			
		LXGT 218	26	0.50	0.78			
T. curv.	362/354/8	L 4	48	0.69	0.96	0.086 ± 0.027 (0.045-0.147) ***	0.141 ± 0.039 (0.071-0.209)	2.66
		L 5	28	0.68	0.82			
		L 18	30	0.70	0.90			
		MS 86	23	0.34	0.62			
		LXAGT 1	58	0.98	0.97			
		Myrt 3	32	0.85	0.93			
		LXGT 223	30	0.65	0.94			
		LXGT 218	34	0.70	0.90			

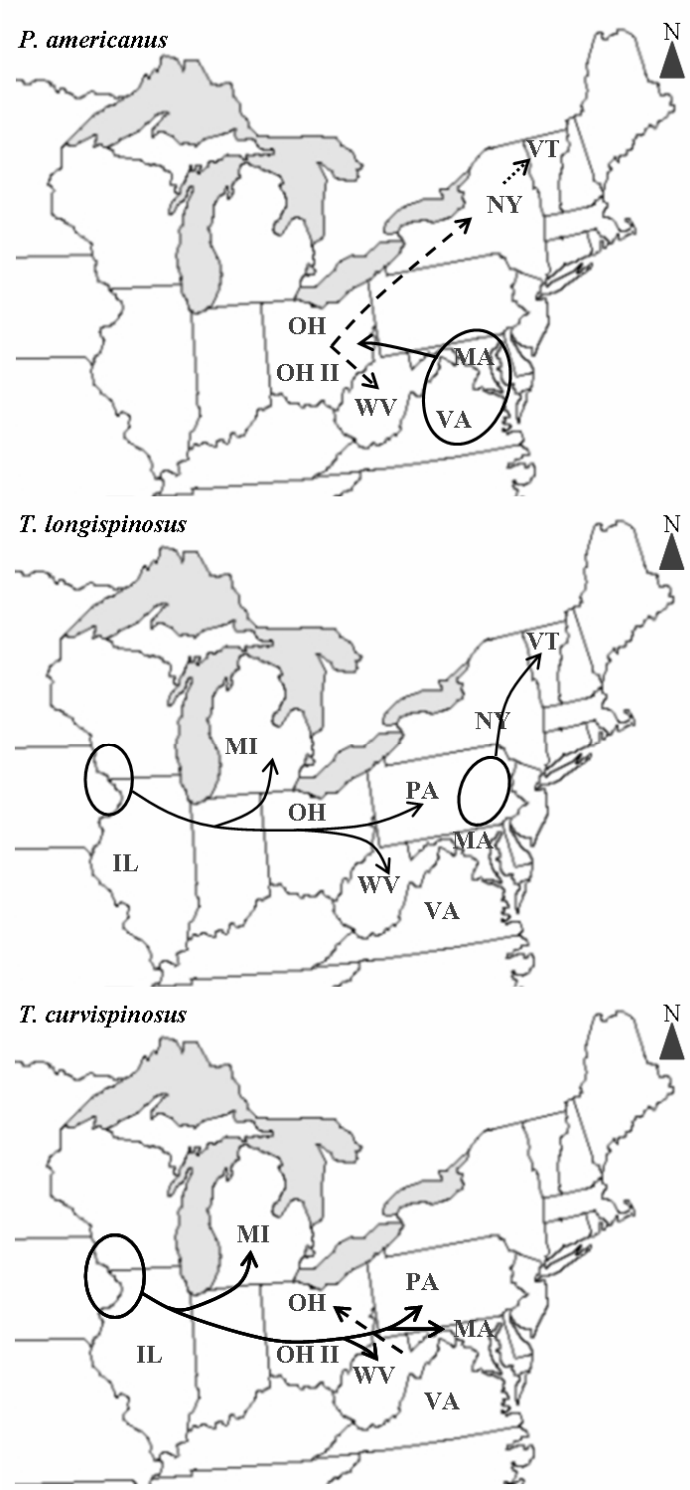

Figure 3 Hypothetical post-glacial colonization routes (arrows) of the slavemaker *P. americanus* and its host species *T. longispinosus* and *T. curvispinosus*. Ovals indicate hypothetical refugia. Black line first migration movement, broken line second migration movement, dotted line third migration movement

DISCUSSION

In the present study, microsatellite markers revealed high levels of genetic diversity in the slavemaker *Protomognathus americanus* and its two *Temnothorax* host species suggesting a high evolutionary potential of the species to adapt to changing environmental conditions and to respond to selection. The levels of genetic differentiation among populations, as indicated by global F_{ST} values (Hartl & Clark 1997, Wright 1978), documented moderate, but statistically significant genetic differentiation in the slavemaker *Protomognathus americanus* and its host species *T. curvispinosus* and little significant genetic differentiation in its main host species *T. longispinosus*. Our findings are thus consistent with previous studies on the nuclear and the mitochondrial level (Brandt *et al.* 2007), and demonstrated weak to moderate nuclear genetic structure in all three study species. Under the assumption of the island model of migration (Wright 1931), the degree of genetic structuring correlates negatively with gene flow among populations ($N_m = (1/F_{ST} - 1)/4$ (Slatkin & Barton 1989)). However in our three study species, predominantly in the main host species *T. longispinosus*, gene flow (mostly via males) among populations was shown to be at a sufficient level to compensate genetic drift ($N_m > 1$) and thus to counteract isolation by distance as also indicated by our Mantel tests. The estimation whether gene flow is more strongly biased by one particular sex remains somewhat unclear, since high genetic diversities may have restricted the range of F_{ST} values thus leading to an overestimation of male dispersal to some extent.

Altogether, the slavemaker *P. americanus* and its two host species *T. longispinosus* and *T. curvispinosus* demonstrated high evolutionary potentials with high levels of genetic variability and gene flow leading to new adaptations developing faster and an accelerated arms race between the interacting species. The main host species *T. longispinosus* had the highest migration, e.g. gene flow, and thus the highest potential for local adaptations (Gandon *et al.* 1996). Along with little genetic differentiation between populations, we suppose a high effective population size with retained anciently separated lineages and gradual lineage sorting (Avise 2000). This seems reasonable since *T. longispinosus* can reach extremely high nest densities throughout its range (Herbers 1985). In the slavemaker *P. americanus* and its host species *T. curvispinosus* restricted migration increased differentiation among populations.

Glacial refugia populations are generally expected to harbour higher levels of genetic diversity than populations in areas that have been colonized since the retreat of glaciers (colonization mostly involved only a few individuals) (Hewitt 1996) and differences in allelic diversity can help to reveal geographic range shifts (Figure 3). Consequently, recent studies have shown that refugia and postglacial migration routes can be identified using nuclear markers by a steady decline in the number of alleles and subsequently allelic richness (Comps *et al.* 2001, Koskinen *et al.* 2000). In our three study species, the reconstruction of potential postglacial migration routes is rather speculative since differences in allele numbers and allelic richness are not of great significance. Still the following part will attempt a hypothetical interpretation of our data.

In *T. curvispinosus* the mean number of alleles and allelic richness was substantially high and the frequency of private alleles was lowest compared to *P. americanus* and *T. longispinosus*, indicating one large refugia population in Pleistocene history that may have been less affected by genetic drift. This scenario is also supported by recent sequencing data indicating one recent population expansion into the present geographical extension (Brandt *et al.* 2007). With the exception of West Virginia, *T. curvispinosus* allelic richness was highest in the north-western populations followed by eastern populations. This pattern was mostly consistent with genetic distances (Nei 1978) between pairs of populations and partly consistent with different degrees of genetic differentiation (pairwise F_{ST} values), that both also point to a hypothetical population expansion from a refugia around Illinois in east- and north-eastward direction. Interestingly Virginia seemed to be colonized rather via a northern route (Michigan, Pennsylvania) than via West Virginia, explaining that we could neither find a significant correlation between geographic distance and pairwise F_{ST} values, nor between allelic richness and longitude and/or latitude. The hypothetical migration pattern found in our dataset is in accordance with knowledge about glacial movements in North America. During Pleistocene most of north-eastern and north-central North America was covered by the Laurentide Ice Sheet, leaving only a few glacial refugia mostly along mountain ranges, coastlines or between adjacent ice sheets ice-free. The most famous non-glaciated region was the so called "driftless area" in Wisconsin and neighbouring Illinois and Iowa which was bypassed by the glacial fronts (Lomolino *et al.*, 2006).

In contrast the slavemaker *P. americanus* and its main host species *T. longispinosus*, which are assumed to have occupied multiple Pleistocene refugia

(Brandt *et al.* 2007, Rogers & Harpending 1992), were genetically less diverse and more distinct e.g. showed more private alleles. The estimation of migration routes using pairwise F_{ST} values and genetic diversity parameters is thus much more speculative. Especially, the large effective population size and high contemporary gene flow between populations in *T. longispinosus* might obscure historical signals, making an interpretation more difficult. A plausible scenario for *T. longispinosus* is the population expansion from a refugia around Illinois and a second one along the Appalachian Mountains around Pennsylvania in east- and north-eastward direction. This hypothetical expansion in mostly eastward direction is supported by a positive association between allelic richness and longitude suggesting a movement from west to east. The expansion from two hypothetical refugia is to some extent supported by our neighbour-joining clustering based on genetic distances (Nei 1978) and the clustering of populations based on the proportion of shared alleles. Both methods also revealed two distinct groups of populations, one group originating from a hypothetical western refugia and a second one originating from a hypothetical eastern refugia. This clustering might also explain the missing correlation between genetic differentiation (pairwise F_{ST}) and geographical distance between populations of the host species *T. longispinosus*.

Our findings for the slavemaker *P. americanus* did not confirm the assumption of multiple Pleistocene refugia (Brandt *et al.* 2007, Rogers & Harpending 1992). In contrast *P. americanus* might have survived Pleistocene in one southern refugia around Virginia and Maryland, and than have expanded north-west- and north-eastward following the glacial retreat.

The different post-glacial migration routes of the three interacting species might have resulted in different durations of coexistence and thus varying potentials for the evolution of adaptations to each other. Consequently, we would expect different levels of reciprocal selection resulting in a geographic mosaic of coevolutionary adaptations (Thompson 1994). Values of allelic diversity parameters, e.g. allelic richness was higher for populations close to assumed refugia, but expected heterozygosity values showed no such distinct pattern. A possible reason for this is that H_E is less sensitive to the presence of rare alleles.

Several factors can hamper the correct interpretation of microsatellite data and make our interpretations speculative. On the one hand, mutation processes underlying the evolution of microsatellite loci are yet not fully understood (Jarne & Lagoda 1996, Slatkin 1995, Valdes & Slatkin 1993) and the selective neutrality of

microsatellites is not undisputed (Jarne & Lagoda 1996). On the other hand, microsatellite data can deviate from Hardy-Weinberg equilibrium due to excess or deficit of heterozygotes.

The deficiency of heterozygotes observed in our study species *P. americanus*, *T. longispinosus* and *T. curvispinosus* may be due to three factors: the presence of null alleles due to mismatching primers (Callen *et al.* 1993, Koorey *et al.* 1993), the Wahlund effect or inbreeding (Lade *et al.* 1996, Paxton *et al.* 1996). The existence of null alleles can not be excluded, since the primers used in this study were not specially designed for *P. americanus* and its *Temnothorax* host species and nucleotide sequence variation in the primer annealing sites thus seem feasible. Moreover, we detected relatively high, locus dependent null allele frequencies, which were mainly consistent with heterozygote deficient loci. The Wahlund effect arises from subdivision of populations into groups, differing in allele frequencies. Since different dispersal strategies, linked with the level of polygyny, can lead to different genetic structure within populations, polygyny can potentially forward breeding subunits within a population (Pamilo & Rosengren 1984, Seppä & Pamilo 1995). Consequently, population subdivision may be more likely in the facultatively polygynous *Temnothorax* host species, where young mated queens can be re-adopted or disperse by budding at limited distances (Alloway *et al.* 1982), compared to the strictly monogynous slavemaker *P. americanus* (Herbers & Stuart 1998). Inbreeding, as a third cause for deficiency of heterozygotes, seemed to be unlikely in *P. americanus* and *T. longispinosus*, and was only suggested in *T. curvispinosus* by its substantial global and consistent multilocus F_{IS} (inbreeding coefficient) values.

Consequently for our dataset, we propose null alleles as the most likely explanation for heterozygote deficiency in the slavemaker *P. americanus*, and a combination of null alleles and population subdivision in its polygynous host species *T. longispinosus* and *T. curvispinosus*. Inbreeding should only be considered in *T. curvispinosus* as additional cause for less heterozygotes than expected. Thus it is also not surprising, that there was an absence of any detectable large, recent genetic bottleneck in *P. americanus* and *T. curvispinosus*. We could only show that *T. longispinosus* has undergone a population bottleneck in Maryland and Michigan, but this finding remains doubtful, because of the small sample sizes at these locations.

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SYNOPSIS

The host-parasite interactions of the tiny myrmicine slavemaking ant *Protomognathus americanus* and its *Temnothorax* host species have shown itself to be ideal models for the study of antagonistic coevolution. The frequent and highly destructive slave raids of this obligate social parasite exert substantial selection pressure on its host species and hence set base for the ongoing coevolutionary arms race between the opponents (Foitzik et al. 2001, Foitzik & Herbers 2001a, Blatrix & Herbers 2003).

Previous correlative approaches on the association between slavemaker presence and host demographic and genetic structure suggested a very strong parasite impact leading to dramatic changes in host demography and investment patterns (Foitzik & Herbers 2001a, Herbers & Foitzik 2002). Yet these earlier studies lacked the power to disentangle cause and effect. The question whether the slavemakers actively causes host changes or whether it preferentially settles in patches with small monogynous and unproductive host colonies so far kept unsolved. The unique large-scale and long-term field manipulation in two different study sites (Publication 1) finally confirms the causality and demonstrates that social parasite presence causes the observed changes in host demography and investment strategies. In both study sites, the social parasite was shown to exert a severe impact on its host populations. In addition, parasite colonies fared better in their home locality than in an allopatric environment, consequently leading to the conclusion that *P. americanus* colonies are locally adapted to their local host community and/or its density. In response to the parasite manipulation, host colonies were shown to change their investment patterns and to focus on the production of highly mobile sexuals, which appear to provide greater fitness returns than the investment in colony growth. As expected by the geographic mosaic theory of coevolution (Thompson 1994, 1999b), we also found a variation in host reactions between sites. Whereas the presence of local social parasites did not affect host density at the NY site, it significantly reduced host density in WV. Furthermore, parasite pressure led to a reduction in the number of resident queens and workers, an increase in intra-nest relatedness and a lower productivity in NY, while colony demography did not change at the WV site. Since climatic conditions and ant community composition are roughly similar between the two study sites (Herbers & Foitzik 2002), these documented changes were supposedly due to characteristic site specific differences with NY being a densely populated evolutionary "hot spot"

(Foitzik et al. 2001, Foitzik & Herbers 2001a, Herbers & Foitzik 2002, Brandt & Foitzik 2004, Foitzik et al. 2004, Brandt et al. 2005a). In contrast, *T. longispinosus* populations at the WV site are less dense, smaller and predominantly monogynous (Herbers & Stuart 1996b). Hence, Prof. Dr. Foitzik's field manipulation and my sociogenetic analyses of the experiment are finally able to show a causal and direct relationship between parasite presence and changes of host demography and relatedness and investment patterns. The social parasite *Protomognathus americanus* thus exerts a strong, negative impact on its host species.

Based on these findings and the high evolutionary potential (Publication 4) of both *Temnothorax* host species, selection pressure exerted by the parasite should enforce the evolution of anti-parasite adaptations in the host. In fact, studies could already demonstrate the existence of such reciprocal adaptations in slavemaking ant systems. These strategies mainly provide protection against slave raids and include offensive mechanisms like the active recognition of and effective fighting against enemies, but also more defensive behaviors like the evacuation and escape of the attacked host colonies (e.g. Alloway 1990, Foitzik et al. 2001, Brandt et al. 2005a). Yet, all these host defenses are only active before host workers are parasitized, whereas enslaved host workers were though to lack possibilities to rebel against their oppressors out of evolutionary reasons (Gladstone 1981).

Publication 2 contradicts this view and reveals a novel potential anti-parasite strategy after successful enslavement of host workers by the social parasite: Enslaved *Temnothorax* workers actively destroyed two thirds of parasite queen and worker pupae resulting in a decreasing productivity and consequently smaller nest sizes of *P. americanus* colonies. In theory this might reduce the long-term parasite impact and hence the raiding risk on related neighboring host colonies (Foitzik & Herbers 2001b, Herbers & Foitzik 2002) but this hypothesis has to be tested in future studies. Nevertheless the destructive behavior stands in strong contrast to the brood care of unparasitized host colonies, where *Temnothorax* workers successfully reared the vast majority of their conspecific pupae. *Temnothorax* workers were obviously able to discriminate between pupae of different species and castes leading to a selective behavioral reaction.

Besides the active killing of social parasite pupae, the acceptance and brood care for alien raided brood in social parasite colonies is an additional critical point for effective counter-parasite strategies. The survival of a parasite colony thus obligatorily depends on the collaboration of enslaved host workers, because raided

host pupae represent the future supply of slaves and thus an essential part in the life cycle of a social parasite colony. Publication 3 demonstrates that enslaved *Temnothorax* workers kill high proportions of alien introduced host pupae and thus exert substantial selection pressure on the social parasite. Despite of this strong influence, slaves reared a higher percentage of introduced pupae than *Temnothorax* workers in unparasitized host colonies. This consequently points to an active manipulation of brood acceptance of enslaved host workers by the parasite. Nevertheless, the observed and documented killings of both social parasite and alien introduced host pupae in colonies of the social parasite *Protomognathus americanus* indicates that *Temnothorax* hosts are leading the arms race in the coevolutionary battle in this respect.

Chemical signals were well known to be of utmost importance for communication in social insects. Ants recognize nestmates, mating partners and conspecifics on the basis of cuticular hydrocarbons (Singer 1998, Lahav et al. 1999, Peeters & Tsuji 1993). Publication 3 thus analyzed the cuticular hydrocarbon profiles of pupae. Parasite and host pupae showed distinct species and caste specific differences in their profiles, which potentially enables *Temnothorax* workers to discriminate between pupae of different species and sex. Besides the recognition and discrimination using chemical profile dissimilarities, tactile cues, mostly due the characteristic interspecific variation in head width, seem likely.

The basic requirement and key factor for the coevolutionary arms race between the social parasite *P. americanus* and its hosts and for the local adaptation, which has already been documented in various aspects, lies in the evolutionary potential of the interacting species. Social parasites and their host are closely related species (Emery 1909) with comparable population sizes, mutations rates and generation times. It is therefore expected that their evolutionary potential is similar and strongly influenced by the levels of gene flow and the amount of genetic variation present in parasite and host.

My population genetic analyses of *Protomognathus americanus* and its two host species (Publication 4) revealed high levels of genetic variability and gene flow between populations. Consequently all these species exhibit high evolutionary potentials, which are important in the coevolutionary arms races between the parasite with its hosts. Genetic differentiation showed a gradient from very low levels in the main host species *Temnothorax longispinosus* to moderate levels in the second host *T. curvispinosus* with the parasite *P. americanus* in-between its hosts. None of the

three species showed a clear pattern of isolation-by-distance, which can be in part explained by the Pleisocene history of these species, which survived in different refugia. In the main host *T. longispinosus*, eastern populations were genetically separated from western populations and cluster analysis also revealed one to two clusters and unclustered populations in all three species. Glacial history thus might still influence population genetic structure in these ant species today.

CONCLUSION

The complex relationship of the social parasite *Protomognathus americanus* and its *Temnothorax* host species represent a fascinating study system for coevolutionary interactions. The parasite and its two host species exhibit high evolutionary potentials to engage in an escalating coevolutionary arms race. The substantial selection pressure exerted by the parasite coerces the hosts to evolve anti-parasite adaptations such as the novel trait "slave rebellion", which now forces the parasite to react. The evolutionary battle will continue.

ZUSAMMENFASSUNG

Die Koevolution von Arten kann entweder auf mutualistischen oder antagonistischen Interaktionen der Gegenspieler basieren. Vor allem letztere Interaktionen, zwischen Räuber und Beute oder Parasit und Wirt, prägen die Evolution des Lebens entscheidend und gipfeln oft in einem endlosen Prozess des koevolutiven Wettrüstens. Aufgrund der oft hohen Spezifität gelten besonders die Wechselwirkungen zwischen Parasit und Wirt als ideales Studienmodel und wurden bereits in zahlreichen Systemen und Aspekten untersucht. Im Gegensatz zu Mikroparasiten, die aufgrund von großen Populationen, schnellen Mutationsraten und kurzen Generationszeiten in starker Asymmetrie zu ihren Wirten stehen und die Physiologie ihres Wirtsorganismus ausbeuten, sind Makroparasiten ihren Wirtsarten meist vielfach deutlich ähnlicher und zeichnen sich meist durch ein Gleichgewicht des evolutionären Potentials von Parasit und Wirt aus.

Ein weithin bekanntes Beispiel hierfür, stellt der Brutparasitismus des Kuckucks dar, bei dem der Parasit seine Eier in das Nest anderer Vogelwirtsarten legt und so die oft erheblichen Kosten von Brutpflege vermeidet. Gleich den Brutparasiten der Vögel, beuten Sozialparasiten in analoger Weise das Brutpflegeverhalten ihrer sozial lebenden Wirtsarten aus. Die Gegenspieler sind in der Regel phylogenetisch sehr nah verwandt, und deren ähnliches evolutionäres Potential macht sie zu idealen Modelsystemen zur Entschlüsselung gegenseitiger koevolutiver Anpassungen.

Bei der nordamerikanischen sklavenhaltenden Ameisenart *Protomognathus americanus* handelt es sich um einen obligaten Sozialparasiten, der drei nah verwandte Arten der Gattung *Temnothorax* parasitiert. Die Parasitierung beginnt mit der Nestgründung, bei der eine verpaarte Sklavenhalter-Jungkönigin in ein Wirtsnest eindringt, alle adulten Wirtsameisen tötet oder vertreibt, und die Wirtsbrut übernimmt. Diese Wirtsbrut wird im Folgenden versorgt und nach dem Schlupf auf den artfremden Nestgeruch geprägt. Die adulten Wirtsarbeiterinnen stellen die erste Generation an Sklaven und übernehmen alle anfallenden Arbeiten des Kolonieerhalts. Nach dieser kritischen Gründungsphase beginnt die Sklavenhalterkönigin eigene Eier zu legen und die sich daraus entwickelnden Sklavenhalterarbeiterinnen sorgen durch regelmäßige Raubzüge auf die Brut benachbarter Wirtsnester für stetigen Nachschub an Sklaven.

Studien zeigen deutlich, dass der durch die Koloniegründung und die Raubzüge entstehende Selektionsdruck des lokal angepassten Sozialparasiten auf

seine Wirte erheblich ist. Dies führt bei den Wirtsnestern unter bestimmten Voraussetzungen sowohl zu einer Reduktion der Dichte, als auch zu einer Reduzierung der Königinnen und Arbeiterinnen, geringerer Nestproduktivität und einer Erhöhung der Verwandtschaft innerhalb der Wirtsnester. Unter Parasitendruck zeigen Wirtsnester zudem eine Veränderung ihrer Investmentstrategie, und produzieren deutlich mehr in Geschlechtstiere.

Diese Ergebnisse zeigen deutlich den starken Einfluss des Sozialparasiten *Protomognathus americanus* auf seine Wirte, wobei der ausgeübte Selektionsdruck auch direkt die Evolution von Anti-Parasit-Strategien bewirken kann. Neben bereits dokumentierten Feinderkennungs- und Feindabwehrstrategien zur Vermeidung von Raubzügen und Versklavung, haben bereits versklavte *Temnothorax* Arbeiterinnen eine effektive Verhaltensweise entwickelt um ihre indirekte Fitness theoretisch zu erhöhen. Während dieser Sklavenrebellion werden ca. zwei Drittel aller Königinnen und Arbeiterinnen des Sozialparasiten im Puppenstadium getötet und so würde auf lange Sicht der Parasitdruck auf benachbarte, mit den Sklaven verwandte Wirtkolonien reduziert. Sowohl art- als auch kastenspezifische Kohlenwasserstoffprofile auf der Kutikula ermöglichen den Wirtsarbeiterinnen die Erkennung und vor allem die Unterscheidung von Parasit- und Wirtspuppen. Ein tatsächlicher Beweis für den theoretischen Fitnessgewinn durch das Töten von Parasitenbrut muss jedoch noch durch weitere Studien erbracht werden.

Versklavte *Temnothorax* Arbeiterinnen werden in den heterogenen Sozialparasitenkolonien aber nicht nur mit Parasitenbrut konfrontiert, sondern auch mit geraubter Wirtsbrut. Trotz der Fähigkeit art- und nestfremdes zu erkennen, ziehen versklavte *Temnothorax* Arbeiterinnen diese Puppen mit größerer Wahrscheinlichkeit auf, als unversklavte Arbeiterinnen in homogenen Nestern. Dies legt eine Einflussnahme des Sozialparasiten auf die Puppenakzeptanz nahe.

Die Grundlage dieses faszinierenden koevolutiven Wettrüstens in all seinen bisher offengelegten Fassetten, liegt im evolutionären Potential des Sozialparasiten *Protomognathus americanus* und zwei seiner Wirtsarten. Alle drei untersuchten Arten zeichnen sich durch ein hohes Maß an Genfluss aus, das koevolutive Anpassungen der Gegenspieler stark begünstigt.

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08 / 2004 – 01 / 2008	**Promotion an der Ludwig-Maximilians–Universität (LMU) München** Drittmittelfinanziert durch die Deutsche Forschungsgemeinschaft (DFG)
07 / 2003 – 07 / 2004	**Deutscher Tierschutzbund** Fachreferentin für Tierversuche und Alternativ-methoden in der Wissenschaftsabteilung des Deutschen Tierschutzbunds (befristet auf 12 Monate)
04 / 2003 – 06 / 2003	**Bund Naturschutz München** Praktikum mit Fortbildung im Bereich Umwelt-pädagogik und Erste Hilfe für Kinder

STUDIUM

Bis 03 / 2003	**Diplomarbeit an der TU München** Titel: „Entwicklung der Haarzellen und Stereovillibündel der Papilla basilaris der Schleiereule *(Tyto alba)* - Development of hair cell and Stereovilli of the papilla basilaris in the barn owl (*Tyto alba)*"
10 / 1997 – 03 / 2003	**Biologiestudium an der Technischen Universität (TU) München (Abschlussnote 1,6)** Fachrichtung: Zoologie, Tierökologie und Verhalten, Limnologie

SCHULBILDUNG

09 / 1988 – 07 / 1997	**Gymnasium Neubiberg** Leistungskurs Biologie und Englisch
09 / 1984 – 07 / 1988	**Grundschule Neubiberg**

AUSLANDSERFAHRUNG

04 / 2008 – 06 / 2008	**Bachpan Bachao Andolan (Indien)** Freiwilligenarbeit mit Kindern (6-16 Jahre) in einem Hilfsprojekt gegen Kinderarbeit Unterricht in Englisch, Hygiene- und Umweltbewusstsein

KONGRESSE UND DIENSTREISEN

09 / 2007	International Union for the Study of Social Insects (IUSSI) in Bochum (Posterpräsentation)
09 / 2007	Deutsche Zoologische Gesellschaft (DZG) in Köln (Fachvortrag)

02 / 2007	Graduiertentreffen der Deutschen Zoologischen Gesellschaft in Bayreuth
08 / 2005	International Union for the Study of Social Insects (IUSSI) in St. Petersburg
03 / 2005	International Union for the Study of Social Insects (IUSSI) in Halle
2001 - 2007	Forschungsaufenthalte in Indonesien, USA, Russland, Schweiz, Österreich, Italien, Schweden, Deutschland (Aalen, Regensburg, Nürnberg, Köln, Bochum, Halle, Bayreuth)

DRITTMITTELFÖRDERUNGEN UND STIPENDIEN

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REFERENCES

Achenbach A., S. Foitzik. in press. First evidence for slave rebellion: Enslaved ant workers systematically *kill the brood of their social parasite Protomognathus* americanus. Evolution.

Achenbach A., S. Foitzik. Manuscript in preparation. The behavioral and chemical basis of the novel resistance trait in hosts of the social parasitic ant *Protomognathus americanus.*

Akino T., J.J. Knapp, J.A. Thomas, G.W. Elmes. 1999. Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of Myrmica ant colonies. Proceedings of the Royal Society Biological Sciences Series B 266:1419-1426.

Allies A. B., A. F. G. Bourke, N. R. Franks. 1986. Propaganda substances in the cuckoo ant *Leptothorax kutteri* and the slavemaker *Harpagoxenus sublaevis*. Journal of Chemical Ecology 12:1285-1293.

Alloway T. M. 1979. Raiding behaviour of two species of slavemaking ants, *Harpagoxenus americanus* (Emery) and *Leptothorax duloticus* Wesson (Hymenoptera: Formicidae). Animal Behavior 27:202-210.

Alloway T. M. 1980. The origins of slavery in leptothoracine ants (Hymenoptera: Formicidae). American Naturalist 115, 247-261.

Alloway T. M. 1982. How the slavemaking ant *Harpagoxenus americanus* (Emery) affects the pupa-acceptance behavior of its slaves. In: The Biology of social insects: Proceedings of the Ninth Congress of the International Union for the Study of Social Insects (Breed MD, Michener CD, Evans HE, eds). Boulder, Colorado: Westview Press; 261-265.

Alloway T. M. 1990. Slave-species ant colonies recognize slavemakers as enemies. Animal Behaviour 39: 1218-1220.

Alloway T. M., A. Buschinger, M. Talbot, R. J. Stuart, C. Thomas. 1982. Polygyny and polydomy in three North American species of the ant genus Leptothorax Mayr(Hymenoptera: Formicidae). Psyche 89, 249-274.

Als T. D., R. Vila, N. P. Kandul, D. R. Nash, S. H. Yen, Y. F. Hsu, A. A. Mignault, J. J. Boomsma, and N. E. Pierce. 2004. The evolution of alternative parasitic life histories in large blue butterflies. Nature 432:386-390.

Anderson R., R. May. 1979. Population biology of infectious diseases. I. Nature 280: 361-367.

Aron S, L. Passera, L. Keller. 2004. Evolution of miniaturisation in inquiline parasitic ants: Timing of male elimination in *Plagiolepis pygmaea*, the host of *Plagiolepis xene*. Insectes Sociaux 51:395-399.

Avise J. C. 1994. Molecular markers, natural history and evolution. Chapman&Hall, London.

Avise J. C. 2000. Phylogeography. The History and Foundation of Species. harvard University Press, Cambridge, Massachusetts.

Azuma N., J. Takahashi, T. Kikuchi. 2005. Microsatellite loci for *Myrmica kotokui* and their application in some congeneric ant species distributed in northern Japan. Molecular Ecology Notes 5: 118-120.

Barton H., G. M. Hewitt. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics 16: 113-148.

Barton H., M. Slatkin. 1986. A quasi-equilibrium theory of the distribution of rare alleles in a subdivided population. Heredity 56

Beibl J., R. Stuart, J. Heinze, S. Foitzik. 2005. Six origins of slavery in formicoxenine ants. Insectes Sociaux 52: 291-297.

Blatrix R., J. M. Herbers. 2003. Coevolution between slavemaking ants and their hosts: host specificity and geographical variation. Molecular Ecology 12: 2809-2816.

Bono J., M. Antolin, J. Herbers. 2006. Parasite virulence and host resistance in a slavemaking ant community. Evolutionary Ecology Research 8:1117-1128.

Boomsma J. J. 1989. Sex-investment ratios in ants: has female bias been systematically overestimated? American Naturalist 133: 517-532.

Boulton A. M., G. A. Polis. 2002. Brood parasitism among spiders: Interactions between salticids and *Diguetia mojavea*. Ecology 83: 282-287.

Bourke A. F. G. 1991. Queen behaviour, reproduction and egg cannibalism in multiple-queen colonies of the ant *Leptothorax acervorum*. Animal Behavior 42: 295-310.

Bourke A. F. G. 1994. Indiscriminate egg cannibalism and reproductive skew in a multiple-queen ant. Proceedings of the Royal Society London Series B Biological Science 255: 55-59.

Bourke A. F. G., N. R. Franks. 1995. Social evolution in ants. Princeton University Press, Princeton.

Bourke A., H. Green, M. Bruford. 1997. Parentage, reproductive skew and queen turnover in a multiple-queen ant analysed with microsatellites. Proceedings of the Royal Society London Series B 264: 277-283.

Brandt M., B. Fischer-Blass, J. Heinze, S. Foitzik. 2007. Population structure and the co-evolution between social parasites and their hosts. Molecular Ecololgy 16: 2063-2078.

Brandt M., J. Heinze, T. Schmitt, S. Foitzik. 2005a. A chemical level in the coevolutionary arms race between an ant social parasite and its hosts. Journal of Evolutionary Biology 18: 576-586.

Brandt M., S. Foitzik, B. Fischer-Blass, J. Heinze. 2005b. The coevolutionary dynamics of obligate ant social parasite systems--between prudence and antagonism. Biological Reviews of the Cambridge Philosophical Society 80: 251-267.

Brandt M., S. Foitzik. 2004. Community context and specialization influence coevolution between a slavemaking ant and its hosts. Ecology 85: 2997-3009.

Brodie E. D. III, E. D. Jr. Brodie. 1991. Evolutionary response of predators to dangerous prey: Reductions of toxicity of newts and resistance of garter snakes in island populations. Evolution 45: 221-224

Brooke M. d. L., N. B. Davies. 1988. Egg mimicry by cuckoos *Cuculus canorus* in relation to discrimination by hosts. Nature 335: 630-632.

Brookfield J. F. Y. 1996 A simple new method for estimating null allele frequency from heterozygote deficiency. Molecular Ecology 5: 453-455.

Buschinger A. 1986. Evolution of social parasitism in ants. Trends in Ecology & Evolution 1:155-160.

Buschinger A. 1990. Sympatric speciation and radiative evolution of socially parasitic ants. Heretic hypotheses and their facual background. Zeitschrift für zoologische Systematik und Evolutionsforschung 28: 241-260.

Buschinger A., W. Ehrhardt, U. Winter. 1980. The organization of slave raids in dulotic ants – a comparative study (Hymenoptera: *Formicidae*). Zeitschrift für Tierpsychologie 53: 245-264.

Callen D. E., A. D. Thompson, Y. Shen. 1993. Incidence and origin of 'null' alleles in the (AC)n microsatellite markers. American Journal of Human Genetics 52: 922-927.

Carroll S. P., H. Dingle, S. P. Klassen. 1997. Genetic differentiation of fitness-associated traits among rapidly evolving populations of the soapberry bug. Evolution 51: 1182-1188.

Cervo R., F. Dani, C. Cotoneschi, C. Scala, I. Lotti, J. Strassmann, D. Queller, S. Turillazzi. 2008. Why are larvae of the social parasite wasp *Polistes sulcifer* not

removed from the host nest? Behavioral Ecology and Sociobiology 62:1319-1331.

Chapuisat M., J. Goudet, L. Keller. 1997. Microsatellites reveal high population viscosity and limited dispersal in the ant *Formica paralugubris*. Evolution 51: 475-482.

Comps B., D. Gömöry, J. Letouzey, B. Thiebaut, R. J. Petit. 2001. Diverging trends between heterozygosity and allelic richness during postglacial colonization in the European beech. Genetics 157: 389-397.

Cool-Kwait E., H. Topoff. 1984. Raid organization and behavioral development in the slavemaking ant *Polyergus lucidus* Mayr. Insectes Sociaux 31: 361-374.

Cornuet J.-M., G. Luikart. 1996. Description and power analysis of two tests for detecting recent bottlenecks from allele frequency data. Genetics 144: 2001-2014.

Creighton W. S. 1927. The slave-raids of *Harpagoxenus americanus*. Psyche (Camb.) 34: 11-29.

Creighton W. S. 1950. The ants of North America. Bulletin of the Museum of Comparative Zoology 104: 1-585.

Crozier R. H., P. Pamilo. 1996. Evolution of social insect colonies. Oxford University Press, Oxford.

Culver D. C. 1974. Species packing in Caribbean and North Temperate ant communities. Ecology 55: 974-988.

D'Ettorre P., J. Heinze. 2001. Sociobiology of slavemaking ants. Acta Ethologica 3: 67-82.

Darwin C. 1859. On the Origin of Species by Means of Natural Selection. London: John Murray

Davelos A., H. Alexander, N. Slade. 1996. Ecological genetic interactions between a clonal host plant (*Spartina pectinata*) and associated rust fungi (*Puccinia seymouriana* and *Puccinia sparganioides*). Oecologia 105: 205–213.

Davies N. B. 2000. Cuckoos, cowbirds and other cheats. London: T. & A. D. Poyser.

Davies N. B., A. F. G. Bourke, M. d. L. Brooke. 1989. Cuckoos and parasitic ants: Interspecific brood parasitism as an evolutionary arms race. Trends in Ecology and Evolution 4: 274-278.

Davies N. B., M. d. L. Brooke. 1989. An experimental study of co-evolution between the cuckoo, *Cuculus canorus*, and its hosts. I. Host egg markings, chick discrimination and general discussion. Journal of Animal Ecology 58: 207-224.

Dawkins R., J. Krebs. 1979. Arms races between and within species. Proceedings of the Royal Society Biological Sciences Series B 202: 489-511.

Dettner K., C. Liepert. 1994. Chemical mimicry and camouflage. Annual Review of Entomology 39: 129-154.

Dieringer D., C. Schlötterer. 2002. Microsatellite Analyser (MSA): a platform independant tool for large micropatellite data sets. Molecular Ecology Notes 3: 167-169.

Dydahl M. F., A. Storfer. 2003. Parasite local adaptation: red Queen versus Suicide King. Trends in Ecology and Evolution 18: 523-530.

Dydahl M. F., C. M. Lively. 1996. The geography of coevolution: comparative population structures for a snail and its trematode parasite. Evolution 50: 2264-2275.

Ebert D. 1994. Virulence and local adaptation of a horizontally transmitted parasite. Science 265: 1084–1086.

El Mousadik A., R. J. Petit. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree (*Argania spinosa* (L.) Skeels) endemic to Morocco. Theoritical and applied Genetics 92: 832-839.

Emery C. 1909. Über den Ursprung der dulotischen, parasitischen und myrmekophilen Ameisen. Biologisches Centralblatt 29: 352-362.

Endler J. 1973. Gene flow and population differentiation. Science 179: 243–250.

Evans D. J. 1993. Parentage analysis in ant colonies using simple sequence repeat loci. Molecular Ecology 2: 393-397.

Fischer-Blass B., J. Heinze, S. Foitzik. 2006. Microsatellite analysis reveals strong but differential impact of a social parasite on its two host species. Molecular Ecology 15: 863-872.

Fisher R. 1950. Gene frequencies in a cline determined by selection and diffusion. Biometrics 6: 353–361.

Foitzik S., A. Achenbach, M. Brandt, in press. Locally-adapted social parasite affects density, social structure and life history of its ant hosts. Ecology.

Foitzik S., B. Fischer, J. Heinze. 2003. Arms-races between social parasites and their hosts: Geographic patterns of manipulation and resistance. Behavioral Ecology 14: 80-88.

Foitzik S., C. J. DeHeer, D. N. Hunjan, J. M. Herbers. 2001. Coevolution in host-parasite systems: behavioral strategies of slavemaking ants and their hosts. Proceedings of the Royal Society London Series B 168: 1139-1146.

Foitzik S., J. M. Herbers. 2001a. Colony structure of a slavemaking ant. II. Frequency of slave raids and impact on the host population. Evolution 55: 316-323.

Foitzik S., J. M. Herbers. 2001b. Colony structure of a slavemaking ant: I. Intra-colonial relatedness, worker reproduction and polydomy. Evolution 55: 307-315.

Foitzik S., M. Haberl, J. Gadau, J. Heinze. 1997. Mating frequency of *Leptothorax nylanderi* ant queens determined by microsatellite analysis. Insectes Sociaux 44: 219-227.

Foitzik S., M. Strätz, J. Heinze. 2003. Ecology, life history and resource allocation in the ant, *Leptothorax nylanderi*. Journal of Evolutionary Biology 16: 670-680.

Foitzik S., V. L. Backus, A. Trindl, J. M. Herbers. 2004. Ecology of *Leptothorax* ants: impact of food, nest sites and social parasites. Behavioral Ecology and Sociobiology 55: 484-493.

Freeland W. J. 1979. Primate social groups as biological islands. Ecology 60: 719-728.

Freeland W. J. 1983. Parasites and the coexistence of animal host species. American Naturalist 121: 223-236.

Futuyma D. J., M. Slatkin. 1983. Coevolution. Sinauer, Sunderland.

Futuyma, D. J. 1986. Evolutionary biology. Sinauer, Sunderland.

Gandon S., Y. Capowiez, Y. Dubois, Y. Michalakis, I. Olivieri. 1996. Local adaptations and gene for gene coevolution in a metapopulation model. Proceedings of the Royal Society London Series B 263: 1003-1009.

Gandon S., Y. Michalakis. 2002. Local adaptation , evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. Journal of Evolutionary Biology 15: 451-462.

Giraud T., R. Blatrix, M. Solignac, P. Jaisson. 1999. Polymorphic microsatellite DNA markers in the ant Gnamptogenys striatula. Molecular Ecology 8: 2143-2145.

Gladstone D. E. 1981. Why there are no ant slave rebellions. American Naturalist 117: 779-781.

Gomulkiewicz R., D. M. Drown, M. F. Dybdahl, W. Godsoe, S. L. Nuismer, K. M. Pepin, B., J. Ridenhour, C. I. Smith, J. B. Yoder. 2007. Dos and don'ts of testing the geographic mosaic theory of coevolution. Heredity 98: 249-258.

Goodloe L., H. Topoff. 1987. Pupa acceptance by slaves of the social-parasitic ant *Polyergus* (Hymenoptera: Formicidae). Psyche (Cambridge) 94: 293-302.

Goudet J. 2001. FSTAT: a program to estimate and test gene diversities and fixation indices; Version 2.9.3 Available from http://www.unil.ch/izea/softwares/fstat.html. updated from Goudet (1995).

Goudet J., M. Raymond, T. De Meeus, F. Rousset. 1996. Testing differentiation in diploid populations. Genetics 144: 933-1940.

Grasso D. A., A. Mori, F. Le Moli. 1992. Analysis of the aggression between slave and slavemaking (facultative and obligatory) ant species (Hymenoptera: *Formicidae*). Ethology, Ecology and Evolution 2: 81-84.

Guo S. W., E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics 48: 361-372.

Hamaguchi K., Y. Ito, O. Takenaka. 1993. Gt dinucleotide repeat polymorphisms in a polygynous ant, *Leptothorax spinosior*, and their use for measurement of relatedness. Naturwissenschaften 80: 179-181.

Hamilton W. R., R. Axelrod, R. Tanese. 1990. Sexual reproduction as an adaptation to resist parasites. Proceedings of the National Academy of Science 87: 3566-3573

Hare J. F., T. M. Alloway. 1987. Early learning and brood discrimination in *leptothoracine* ants (Hymenoptera: Formicidae). Animal Behaviour 35: 1720-1724.

Hare J. F., T. M. Alloway. 2001. Prudent *Protomognathus* and despotic *Leptothorax duloticus*: Differential costs of ant slavery. Proceedings of the National Academy of Science 98: 12093-12096.

Hartl D. L., A. G. Clark. 1997. Principles of population genetics. Sinauer Associates Inc. Sunderland, MA.

Heinze J., D. Ortius. 1991. Social organization of *Leptothorax acervorum* from Alaska (Hymenoptera: Formicidae). Psyche (Cambridge) 98: 227-240.

Heinze J., D. Ortius, M. Kaib, B. Hölldobler. 1994. Interspecific aggression in colonies of the slavemaking ant *Harpagoxenus sublaevis*. Behavioral Ecology and Sociobiology 35: 75-83.

Herbers J. M. 1985. Seasonal structuring of a north temperate ant community. Insectes Sociaux 32: 224-240.

Herbers J. M. 1986. Nest site limitation and facultative polygyny in the ant *Leptothorax longispinosus*. Behavioral Ecology and Sociobiology 19: 115-122.

Herbers J. M. 1989. Community structure in north temperate ants: temporal and spatial variation. Oecologia 81: 201-211.

Herbers J. M., R. J. Stuart. 1996a. Multiple queens in ant nests: impact on genetic structure and inclusive fitness. American Naturalist 147: 161-187.

Herbers J. M., C. J. deHeer, S. Foitzik. 2001. Conflict over sex allocation drives conflict over reproductive allocation in perennial social insect colonies. American Naturalist 158: 178-192.

Herbers J. M., R. J. Stuart. 1996(b). Patterns of reproduction in southern versus northern populations of *Leptothorax* ants (Hymenoptera: Formicidae). Annals of the Entomological Society of America 89: 354-360.

Herbers J. M., R. J. Stuart. 1998. Patterns of reproduction in slavemaking ants. Proceedings of the Royal Society London Series B 265: 875-887.

Herbers J. M., S. Foitzik. 2002. The ecology of slavemaking ants and their hosts in north temperate forests. Ecology 83: 148-163.

Herbers J. M., V. S. Banschbach. 1999. Plasticity of social organization in a forest ant species. Behavioral Ecology and Sociobiology 45: 451-465.

Hewitt G. M. 1996. Some genetic consequences of ice ages, and their rolein divergence and speiation. Biological Journal of the Linnean Society 58: 247-276.

Hewitt G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature 405: 907-913.

Hochberg M. E., Y. Michalakis, T. de Meeus. 1992. Parasitism as a constraint on the rate of life-history evolution. Journal of Evolutionary Biology 5: 491-504.

Hoeksema J., S. Forde. 2008. A meta-analysis of factors affecting local adaptation between interacting species. American Naturalist 171:275-290.

Hölldobler B., E. O. Wilson. 1990. The ants. Harvard University Press, Cambridge, Mass.

Howard R. W. 1993. Cuticular hydrocarbons and chemical communication. In Insect lipids: Chemistry, Biochemistry and Biology (ed. D. W. Stanley- Samuelson & D. R. Nelson). Lincoln, Nebraska : University of Nebraska Press. 179-226

Huber P. 1810. Recherches sur les moeurs des formis indigènes. J. J. Paschoud, Paris.

Hurlbert S. H. 1971. The nonconcept of species diversity: a critique and alternative parameter. Ecology 52 : 577-586.

Jaisson P. 1975. L'impregnation dans l'ontogenese des comportements de soins aux cocons chez la jeune fourmi rousse (Formica polyctena Forst.). Behaviour 52: 1-37.

Jarne P., P. J. L. Lagoda. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution 11: 424-429.

Johnson C. A., J. M. Herbers. 2006. Impact of parasite sympatry on the geographic mosaic of coevolution. Ecology 87: 382–394.

Johnson C. A., P. L. Phelan, J. M.Herbers. 2008. Stealth and reproductive dominance in a rare parasitic ant. Animal Behavior 76:1965-1976.

Kaib M., J. Heinze, D. Ortius. 1993. Cuticular hydrocarbons profiles in the slavemaking ant *Harpogoxenus sublaevis* and its hosts. Naturwissenschaften 80: 281-285.

Kaltz O., J. Shykoff. 1998. Local adaptation in host-parasite systems. Heredity 81: 361-370.

Kaltz O., S. Gandon, Y. Michalakis, J. Shykoff. 1999. Local maladaptation in the anther-smut fungus *Microbotryum violaceum* to its host plant *Silene latifolia*: evidence from a cross-inoculation experiment. Evolution 53: 395–407.

Kimmel M., R. Chakraborty, J. P. King. 1998. Signatures of population expansion in microsatellite repeat data. Genetics 148: 1921-1930.

Koorey D. J., G. W. M. Bishop. 1993. Allele non-amplification:a source of confusion in linkage studies employing microsatellite polymorphisms. Human Molekular Genetics 2: 289-291.

Koskinen M. T., E. Ranta, J. Piironen. 2000. Genetic lineages and postglacial colonization of grayling (*Thymallus thymallus*, Salmonidae) in Europe, as revealed by mitochandrial DNA analyses. Molecular Ecology 9: 1609-1624.

Krüger O. 2007. Cuckoos, cowbirds and hosts: adaptations, trade-offs and constraints. Philosophical transactions of the Royal Society London Series B 362: 1873–1886.

Lade J. A., N. D. Murray, C. A. Marks, N. A. Robinson. 1996. Microsatellite differentiation between Philipp Island and mainland Australian populations of the red fox *Vulpes vulpes*. Molecular Ecology 5: 81-87.

Ladle R. J., R. A. Johnston, O. P. Judson. 1993. Coevolutionary dynamics of sex in a metapopulation: Escaping the Red Queen. Proceedings of the Royal Society London Series B 253: 155-160.

Lahav S., V. Soroker, A. Hefetz, R. K. Vander Meer. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. Naturwissenschaften 86: 246-249.

Langella O. 1999. Populations. CNRS UPR9034.

Langmore N. E., S. Hunt, R. M. Kilner. 2003. Escalation of a coevolutionary arms race through host rejection of brood parasitic young. Nature 422: 157-160.

LeMoli F., A. Mori. 1982. Early learning and coccon nursing behaviour in the red wood ant *Formica lugubrious* Zett. (Hymentoptera: *Formicidae*). Bolletin Zoologica 49: 93-97.

LeMoli F., M. Passetti. 1977. The effect of early learning on recognition, acceptance and care of cocoons in the ant *Formica rufa*. L. Atti Soc Ital Sci nat 118: 49-64.

Lenoir A., P. D'Ettorre, C. Errard, A. Hefetz. 2001. Chemical ecology and social parasitism in ants. Annual Review of Entomology 46:573-599.

Lively C., M. Dybdahl. 2000. Parasite adaptation to locally common host genotypes. Nature 405: 679-681.

Lomolino M. V., B. R. Riddle, J. H. Brown. 2006. Biogeography. Sinauer Assosiates.

Lotem A., H. Nakamure, A. Zahavi. 1995. Constraints on egg discrimination and cuckoo-host co-evolution. Animal Behavior 49: 1185-1209.

Lotem A., S. I. Rothstein. 1995. Cuckoo-host coevolution: from snapshots of an arms race to the documentation of microevolution. Trends in Ecology and Evolution 10: 436-437

Luikart G., F. W. Allendorf, J.-M. Cornuet, W. B. Sherwin. 1998. Distortion of allele frequency distributions provided a test for recent population bottlenecks. Journal of Heredity 89: 238-247.

Lyon B. E. 2003. Egg recognition and counting reduce costs of avian conspecific brood parasitism. Nature 422: 495-499.

Mantel N. 1967. The detection of disease clustering and a generalised regression approach. Cancer Research 27: 209-220.

Martin S.J., E. A. Jenner, F. P. Drijfhout. 2007. Chemical deterrent enables a socially parasitic ant to invade multiple hosts. Proceedings of the Royal Society Biological Sciences Series B 274: 2717-2722.

Mori A., D. A. Grasso, F. Le Moli. 1991. Eco-ethological study on raiding behaviour of the European amazon ant, *Polyergus rufescens* Latr. (Hymenoptera: *Formicidae*). Ethology 88: 46-62.

Nash D. R., T. D. Als, R. Maile, G. R. Jones, J. J. Boomsma. 2008. A mosaic of chemical coevolution in a large blue butterfly. Science 319: 88-90.

Nei M. 1978. Eestimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Niemi L., A. Wenström, J. Hjältén, P. Waldmann, L. Ericson. 2006. Spatial variation in resistance and virulence in the host–pathogen system *Salix triandra*–*Melampsora amygdalinae*. Journal of Ecology 94: 915–921.

Oster G. F., E. O. Wilson. 1978. Caste and ecology in the social insects. Princeton University Press, Princeton.

Page R. D. M. 1996 treeView: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.

Pamilo P. 1990. Sex allocation and queen-worker conflict in polygynous ants. Behavioral Ecology and Sociobiology 27: 31-36.

Pamilo P., R. Rosengren. 1984. Evolution of nesting strategies of ants: genetic evidence from different population types of Formica ants. Biological Journal of the Linnean Society 21: 331-348.

Parker M. 1985. Local population differentiation for compatibility in an annual legume and its host-specific fungal pathogen. Evolution 39: 713–723.

Paxton R. J., P. A. Thoren, J. Tengö, A. Estroup, P. Pamilo.1996. Mating structure and nestmate relatedness in a communal bee, *Andrena jacobi* (Hymenoptera, Andrenidae), using microsatellites. Molecular Ecololgy 5: 511-519.

Peeters C., K. Tsuji. 1993. Reproductive conflict among ant workers in *Diacamma sp*. from Japan: dominance and oviposition in the absence of the gamergate. Insects Sociaux 40: 119-136.

Price P. W., M. Westoby, B. Rice, P. R. Atstatt, R. S. Fritz, J. N. Thompson, K. Mobley. 1986. Parasite mediation in ecological interactions. Annual Review of Ecology and Systematics. 17: 487-505.

Queller D. C., J. E. Strassmann, C. R. Hughes. 1993. Microsatellites and kinship. Trends in Ecology and Evolution 8: 285-288.

Queller D. C., K. F. Goodnight. 1989. Estimating relatedness using genetic markers. Evolution 43: 258-275.

Raymond M., F. Rousset.1995. GENEPOP (version1.2.): population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248-249.

Restif O., M. E. Hochberg, J. C. Koella. 2001. Virulence and age at reproduction: new insights into host-parasite coevolution. Journal of Evolutionary Biology 14: 967-979.

Reuter M., L. Keller. 2001. Sex ratio conflict and worker production in eusocial Hymenoptera. American Naturalist 158: 166-177.

Rice W. R. 1989. Analysing tables of statistical tests. Evolution 43: 223-225.

Rogers A. R., H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Molecular Ecololgy and Evolution 9: 552-569.

Rothstein S. I. 1975. Evolutionary rates and host defenses against avian brood parasitism. American Naturalist 109: 161-176.

Rothstein S. I. 1982. Mechanisms of avian egg recognition: Which egg parameters elicit responses by rejecter species? Behavioral Ecology and Sociobiology 11: 229-239.

Rothstein S. I. 1990. A model system for coevolution: avian brood parasitism. Annual Review of Ecology and Systematics 21: 481-508.

Rothstein S. I. 2001. Relic behaviours, coevolution and the retention versus loss of host defences after episodes of avian brood parasitism. Animal Behaviour 61: 95-107.

Roy B. 1998. Differentiating the aspects of origin and frequency in reciprocal transplant experiments used to test negative frequency-dependent selection hypotheses. Oecologia 115: 73–83.

Sato T. 1986. A brood parasitic catfish *Synodontis multipunctatus* of mouthbrooding cichlid fishes in Lake Tanganyika. Nature 323: 58-59.

Schumann R. D., A. Buschinger. 1994. Imprinting effects on host-selection behaviour of colony-founding *Chalepoxenus muellerianus* (Finzi) females (Hymenoptera, Formicidae). Ethology 97: 33-46.

Seppä P., P. Pamilo. 1995. Gene flow and population viscosity in Myrmica ants. Heredity 74: 200-209.

Servedio M. R., M. E. Hauber. 2006. To eject or to abandon? Life history traits of hosts and parasites interact to influence the fitness payoffs of alternative anti-parasite strategies. Journal of Evolutionary Biology 19: 1585-1594.

Shriver M. D., L. Jin, R. Chakraborty, I. Boerwinkle. 1993. VNTR allel frequency distributions under the stepwise mutation model- a computer simulation approach. Genetics 134: 983-993.

Singer T. L. 1998. Roles of hydrocarbons in the recognition system of insects. American Zoologist 38: 394-405.

Slatkin M. 1973. Gene flow and selection in a cline. Genetics 75: 733–756.

Slatkin M. 1987. Gene flow and geographic structure of natural populations. Science 236: 787-792.

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457-462.

Slatkin M., H. Barton. 1989. A comparison of three indirect methods for estimating average levels of geneflow. Evolution 43: 1349-1368.

Sledge M., F. Dani, R. Cervo, L. Dapporto, S. Turillazzi. 2001. Recognition of social parasites as nest-mates: adoption of colony-specific host cuticular odours by

the paper wasp parasite *Polistes sulcifer*. Proceedings of the Royal Society Biological Sciences Series B 268.

Sokal R. R., F. J. Rohlf. 1995. Biometry. W. H. Freeman and Company, New York.

Soler J., M. Soler. 2000. Brood-parasite interactions between great spotted cuckoos and magpies: a model system for studying coevolutionary relationships. Oecologia 125/3: 309-320.

Soler, M., A. Møller. 1990. Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. Nature 343: 748-750.

Stuart R. J. 1984. Experiments on colony foundation in the slavemaking ant *Harpagoxenus canadensis* M. R. Smith (Hymenoptera; Formicidae). Canadian Journal of Zoology 62:1995 - 2001.

Stuart R. J., T. M. Alloway. 1983. The slavemaking ant *Harpagoxenus canadensis* M. R. Smith, and its host-species, *Leptothorax muscorum* (Nylander): slave raiding and territoriality. Behavior 85: 58-90

Sundström L. 1995. Sex allocation and colony maintenance in monogyne and polygyne colonies of *Formica truncorum* (Hymenoptera: Formicidae): the impact of kinship and mating structure. American Naturalist 146: 182-201.

Thompson J. N. 1994. The coevolutionary process. The University of Chicago, Chicago, London.

Thompson J. N. 1999a. The evolution of species interactions. Science 284: 2116-2118.

Thompson J. N. 1999b. Specific hypotheses on the geographic mosaic of coevolution. The American Naturalist 153: S1-S14.

Thompson J. N. 2005. The Geographic Mosaic of Coevolution. University of Chicago Press, Chicago.

Valdes A. M., M. Slatkin. 1993. Allelefrequencies at microsatellite loci: the stepwise mutation model revisited. Genetics 133: 737-749.

Van Valen L. 1973. A new evolutionary law. Evolutionary Theory 1: 1-30.

Vander Meer R. K., D. P. Jouvenaz, D. P. Wojcik. 1989. Chemical mimicry in a parasitoid (Hymenoptera: Eucharitidae) of fire ants (Hymenoptera: Formicidae). Journal of Chemical Ecology 15: 2247-2261.

Wade M. 2007. The co-evolutionary genetics of ecological communities. Nature Reviews 8: 185-195.

Weir B. S., C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38: 1358-1370.

Wesson L. G. 1939. Contributions to the natural history of *Harpogoxenus americanus* Emery (Hymenoptera: Formicidae). Transactions of the American Entomological Society 65: 97-122.

Wilkinson H. H., J. M. Spoerke, M. A. Parker. 1996. Divergence in symbiotic compatibility in a legume- *Bradyrhizobium* mutualism. Evolution 50: 1470-1477

Wilson E. O. 1971. The insect societies. Harvard University Press, Cambridge, Mass.

Wright S. 1931. Evolution in Mendelian populations. Genetics 16: 97-159.

Wright S. 1951. The genetical structure of populations. Annals of Eugenics 15: 323-354.

Wright S. 1965. The interpretation of population structure by F-statistics with special regard to system of mating. Evolution 19: 395-420.

Wright S. 1978. Evolution and the genetics of populations. Variability within and among natural populations 4.

Zhan J., C. Mundt, M. Hoffer, B. McDonald. 2002. Local adaptation and effect of host genotype on the rate of pathogen evolution: an experimental test in a plant pathosystem. Journal of Evolutionary Biology 15: 634–647.

Zimmerli E. J., A. Mori. 1993. The role of an attractive brood pheromone in the obligatory, slavemaking ant, *Polyergus breviceps* (Hymenoptera: Formicidae). Journal of Insect Behavior 6: 761-770.

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