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DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN
(DR. RER. NAT.) DER NATURWISSENSCHAFTLICHEN FAKULTÄT III –
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GENERAL INTRODUCTION

The principles of sexual selection

The principle of sexual selection was already defined by Darwin (1871) as depending "on the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction". While adaptations that arise by natural selection enhance the survival of their bearers, the adaptations created by sexual selection help their carriers to propagate their genes in the next generation. The fundamental issue of sexual selection are the two sexes and how they evolve in the footrace of mating, fertilization and parental care. The selective force of sexual selection can be divided into intra-sexual selection and inter-sexual selection (Darwin 1871; Fisher 1958; Huxley 1938). Intra-sexual selection is characterised by the competition within one sex for individuals of the opposite sex. In contrast to this, inter-sexual selection is the preference of one sex for certain mating partners over others. While intra-sexual selection takes place mainly in males (male sexual selection), inter-sexual selection in the sense of mate choice is executed prevalently by females (female sexual selection). This role allocation is caused by the difference in parental investment of the two sexes (Bateman 1948; Trivers 1972). The sex which makes the higher parental investment becomes a limiting resource for the opposite sex. Thus, the higher investing sex can choose among the less investing sex, which has to compete for mating opportunities. Female investment is generally higher and starts already with the production of large eggs compared to the small male sperm cells. Male investment often already ends directly after mating with the successful fertilization. Typically, female reproductive success is therefore limited by parental investment, while male reproductive success is limited by the access to females (Thornhill 1979). Where this role allocation does not apply, sex role reversal might be observed (Gwynne and Simmons 1990). Males should become choosy as soon as their reproductive success is no more limited by access to females but any other factors.

The act of mating is the picot of sexual selection. The outcome of sexual selection can therefore be influenced by mechanisms that act prior to mating and subsequent mechanisms that act after mating. Females manipulate the process prior to mating by their differential

investment into eggs and mate choice. After mating, females might perform cryptic female choice by choosing which sperm to use for fertilization (Eberhard 1997). Furthermore, they can provide differential parental care to their offspring. Males may influence female reproductive decisions by transfer of resources to females, elaborate courtship, frequent copulation with the same female, as well as sexual harassment and forced copulation. After mating, the application of mating plugs, manipulative seminal fluids, mate guarding and also infanticide and selective paternal investment secure male paternity and further enhance male reproductive success (e.g. Alcock 1998).

Sexual selection – the neglected topic in the research on social Hymenoptera

While sexual selection has been studied extensively in various species as different as peacock and spiders (Darwin 1871), this topic has been neglected in social Hymenoptera for a long time. This is not surprising because differential investment into sexual offspring is not as eye-catching as in other organisms. The sterile worker caste, the major component of a colony in social Hymenoptera and their interesting eusocial lifestyle, which concentrates on raising sisters instead of reproducing on their own, distracted the focus of research from issues of sexual selection to issues of social interaction (e.g. Hölldobler and Wilson 1990; Bourke and Franks 1995). In social Hymenoptera, reproduction in form of sexual offspring is realized only after the queen has established a prospering colony and therefore she has to produce several generations of sterile workers first. Furthermore, males in social Hymenoptera are short-lived and only produced for a short time period, when they are necessary for mating. They generally die directly after the mating period and only survive in form of stored sperm, which they left in the spermatheca of queens they mated with (Hölldobler and Bartz 1985; Hölldobler and Wilson 1990; Baer 2005; Boomsma et al. 2005). Nevertheless, males in social Hymenoptera have an essential role for reproduction and are therefore interesting study objects for hot topics of sexual selection (Heinze 2004).

Special constellations in life history patterns, genetics and physiology of ant sexuals

Sexual selection in bees (*Apis spp.*) (reviewed in Baer 2005) and bumble bees (*Bombus spp.*) (reviewed in Baer 2003) has been under investigation for quite a while, probably because of the agricultural importance of these species. Details of the sex life of ants, however, are still scarce (Bourke and Franks 1995), although ants form a major group of social Hymenoptera. The variety of life history patterns observed in the different ant species makes them a promising study object for important details of sexual selection. Theoretical predictions should be easy to study by choosing the adequate species. However, more insight in the general sexual life of ants must be gained first.

Interesting constellations arising from life history patterns, genetics and physiology of ants are therefore of major importance. Ant queens mate only at the beginning of their lives, store the sperm in their spermatheca and depend on this storage until they die, since they do not re-mate later. This results in a life-long pair-bonding between the mating partners (Boomsma et al. 2005), which is quite unusual among animals. Queens should be interested to receive enough sperm for fertilizing all female destined eggs throughout their lives, which can be a considerable amount when queens manage to live longer than one decade. Although queens in the majority of ant species are singly mated (Strassmann 2001), some evolved to be polyandrous. Not surprisingly, these are mainly species that form huge colonies, like army ants and leaf-cutters. Additionally, obligate multiple mating is to the present knowledge always associated with monogyny (Boomsma et al. 2008), so that the availability of sperm is crucial for the whole colony. Furthermore, multiple queen-mating can be a strategy for minimizing the negative impact of diploid male load (Kronauer et al. 2007). Haplodiploidy paired with complementary single locus sex allocation bears the risk in singly mated ant queens to produce 50% sterile diploid males instead of females (Page and Metcalf 1982; Crozier and Page 1985; Cook and Crozier 1995). This percentage is much reduced, when queens are multiply mated. All the more, singly mated queens should be very careful in their mate choice and particularly are expected to avoid mating with brothers because this increases extremely the risk of producing diploid male offspring. We, therefore, predict that the majority of ant queens should be choosy.

Ant males on the other hand might not be able to distribute their sperm arbitrarily to all queens they can get hold on, since they also might be sperm-limited. As it is the rule for social Hymenoptera, the testes of male ants degenerate by the time males reach sexual maturity and therefore sperm supply cannot be replenished once depleted. The limited sperm

supply might be restricted to suffice only for one or few copulations (Hölldobler and Bartz 1985). Thus, ant males should be choosier than males with replenishable sperm supply (Hölldobler and Bartz 1985; Heinze and Hölldobler 1993). This leaves us with the notion that in ants – depending on the particular circumstances – both sexes should be choosy.

Ant communication is based on chemical substances

The relevant characters for mate choice might not always be fixed values, like e.g. body size, but should also depend on the interacting individuals. Whether mating with close relatives like brothers can be prevented is constrained mainly by the ability to realize such constellations. Communication between the partners is therefore an important factor for mating decisions. Chemical substances play a central role in ant communication (Hölldobler and Wilson 1990). Cuticular hydrocarbons and sexual pheromones might carry important information for the opposite sex (e.g. Singer 1998; Ayasse et al. 2001; Bleibl et al. 2007; Johansson and Jones 2007). Knowledge about relatedness in form of an individual's colony or origin and furthermore information on the mating state of an individual should be communicated, if it is relevant for reproductive success. Sexual selection should work in a manner that maximises the fitness of the mating partners, but whether this means that available information is actually applied has to be checked independently. Behavioural observations and mating experiments are therefore indispensable.

Reproductive biology of sperm transfer in ants

Once the choice is done, ants copulate and transfer the sperm. Unfortunately, information on the mechanisms of the transfer is so far only available for some few ant species (Robertson 1995; Allard et al. 2002, 2006, 2007). In this, it is important whether sperm is transferred directly into the spermatheca, as it is observed in the dwarf bee *Apis florea* (Koeniger et al. 1989), or whether it has to migrate from its deposition point to the spermatheca after the copulation. The difference affects mainly sperm competition and sperm replacement. As long as the sperm has not yet arrived in the spermatheca, it can still be expelled by the female or hindered by other means. In honey bee (*Apis mellifera)* queens, only

a small percentage of the sperm from the different matings actually makes it into the spermatheca (Ruttner 1956). In this situation, cryptic female choice of sperm between the different ejaculates could therefore significantly influence the reproductive success of males. Thus, it is important for the male reproductive success that sperm is not only transferred to the queen but actually reaches its destiny – the spermatheca. As a consequence, the mechanisms involved in this process are critical for the outcome of sexual selection and require further investigation.

Seminal fluids as mediator of sexual interaction

An important impact on sperm competition – the competition that arises among sperm of different males for the fertilization of a single female's eggs (Parker 1970) – is attributed to seminal fluids, which males transfer with the sperm cells to the females. The male accessory glands are known to produce an essential part of the seminal fluids, especially in the well studied species *Drosophila melanogaster*. Proteins are known to be the major components of the biologically active male accessory gland products (Gillott 2003). In *Drosophila*, these proteins are proven to be responsible for reduced female receptivity (Chen et al. 1988; Kalb et al. 1993; Chapman et al. 2003b; Liu and Kubli 2003), stimulation of the female immune system (McGraw et al. 2004; Peng et al. 2005), antibacterial protein transfer (Lung et al. 2001), efficient sperm storage (Neubaum and Wolfner 1999; Tram and Wolfner 1999) and also for the stimulation of ovulation and egg maturation (Chen et al. 1988; Chapman et al. 2003b; Liu and Kubli 2003). Male accessory gland proteins obviously influence the outcome of sexual selection by manipulating the female physiology in a way that promotes the success of the sperm cells. However, almost nothing is known about accessory gland proteins in ants. The life history of ants promises interesting new insights into the role of accessory gland proteins for sexual selection.

Sexual conflict and sexual cooperation

In *D. melanogaster*, the male accessory gland proteins are so thoroughly investigated that it is known as the best-studied system of sexual conflict (Tregenza et al. 2006). As sexual

selection always requires interaction between the two sexes, this consequently creates room for conflict. Sexual conflict is defined as conflict between the evolutionary interests of individuals of the two sexes (Parker 1979). Sexual selection and sexual conflict are not equivalent but sexual selection has the potential to generate sexual conflict. In case of sexual conflict, the optimal result cannot be achieved simultaneously by both sexes (Parker 2006). The primordial sexual conflict might have been the origin for the evolution of anisogamy. While males invested less and less into their gametes, females were forced to spend more and more. It seems that males won this primordial sexual conflict (Parker 1979).

An important characteristic of sexual selection is, however, that it can lead to complex "evolutionary chases" – sexually antagonistic coevolution between the sexes. The process of sexually antagonistic coevolution has been metaphorized as "evolutionary dance" (Rice 1998). Each of the two sexes tries to drag the partner into the direction of its own optimum. Remarkably, the couple does not move simply across the dance floor but leaves a "trail of destruction" as a result of its fight (Tregenza et al. 2006). The sexes participate in an arms race that is creating costs to the population. In *Drosophila*, male seminal fluid intoxicates the females, which reduces their lifespan (Chapman et al. 1995; Lung et al. 2002) as a side effect of sexual conflict (Chapman et al. 2003a). That sexual conflict can be constricted has been shown by Holland and Rice (1999). They assigned an enforced monogamous lifestyle with random mate assignment to two *D. melanogaster* populations. As a result of this treatment, males became less harmful and females less resistant to male-induced harm. Furthermore, the net reproductive rate of the monogamous populations was higher than in promiscuous populations.

Thus, the life history seems to be a major factor, when it comes to determine the outcome of sexual selection. It is not always necessary that sexual conflict evolves (Parker 1979). A monogamous life style, as it is observed in the majority of ants (Strassmann 2001), rather restricts sexual conflict. The obligate partner commitment in social Hymenoptera combined with the late production of sexuals, only after several generations of workers, is assumed to prevent harmful traits like toxic male accessory gland products (Baer 2003; Boomsma et al. 2005). In case such traits evolved anyway, they are expected to show relatively mild effects (Boomsma 2007). Every damage to the queen results in a simultaneously reduced reproductive success of the male she has mated with. Since males survive only as stored sperm inside the female, both sexes stop their reproduction as soon as the queen dies (Boomsma et al. 2005). Sexually antagonistic coevolution should be replaced by beneficial coevolution in a constellation as it is observed in social Hymenoptera.

Recent findings on ants confirm this idea of sexual cooperation. In the ant *Cardiocondyla obscurior*, mating has a positive effect on a queen's lifetime. Mated queens lived longer and started egg-laying earlier than virgin queens and this indifferently of being mated to a fertile of sterilized male (Schrempf et al. 2005a). Therefore, more research in this field is promising to result in new important insights to questions of sexual conflict and sexual selection. Unfortunately, research on sexual conflict is rather challenging. This is the reason why Rowe and Day (2006) suggest that the study of the natural history of males and females remains the most direct way. I, therefore, have studied sexual selection of the ant species *Leptothorax gredleri* by focusing on important details of their mating biology.

Biology of the study object Leptothorax gredleri

Leptothorax gredleri Mayr 1855 (Hymenoptera: Formicidae) is a central European ant species. It can be found in deciduous forest but also in pine stands (Seifert 2007). The colony lives in dead branches and under the bark of the trees from where it can be easily collected. Nestmate queens establish a dominance hierarchy that results in a functional monogyny. Sexuals mate by female calling (Heinze et al. 1992). Virgin queens leave their maternal nest and climb up grass stems and branches, where they attract males by a droplet of a sexual pheromone (Hölldobler and Bartz 1985). Both sexes mate close to their maternal nest, so that gene flow is restricted (Oberstadt and Heinze 2003). Although mainly singly mated, queens sometimes mate multiply. Nonetheless, a genetic analysis of the offspring of multiply mated queens detected only offspring of a single father (Oberstadt and Heinze 2003). After mating, young queens may seek adoption in already established nests (Heinze et al. 1992). Hibernation in their mothers' colony and emigration only in spring might be a successful strategy of young queens in regions with harsh, cold winters (Heinze and Lipski 1990). In spring, queens often engage in aggressive fights (Heinze et al. 1992). Emigrating queens sometimes seem to be accompanied by some workers of the maternal colony (Heinze et al. 1992).

Aims of this thesis

In this thesis I tried to cover various aspects of sexual selection. As a parameter of premating sexual selection, I looked at the influence of cuticular hydrocarbons on the outcome of mate choice. Therefore, the first chapter deals with the male and female as well as colony specific cuticular hydrocarbon pattern of *L. gredleri* and its influence on mate choice with regard to incest avoidance. The second chapter addresses the question, whether females can avoid further harassment after mating by changing their cuticular hydrocarbon pattern in order to signal to males that it might be better to invest their sperm into another virgin queen. In chapter three I focused on the mechanisms of sperm transfer to detect indications of cryptic female choice or the application of mating plugs and similar devices to secure male paternity. Post-copulatory selection might also have a major impact on the process of sexual selection in this species. Thus, I investigated in chapter four the accessory gland products and assessed the variability of accessory gland proteins by comparing it to thorax protein variability. Furthermore, some few samples of the related ant species *Leptothorax muscorum*, *L. acervorum* and *Harpagoxenus subleavis* were investigated in order to get an impression of the variation in accessory gland proteins between these species and *L. gredleri*. Finally, I was interested in the genes expressed in male accessory glands of this species. Since the identification of genes expressed in these glands could give new interesting insights into the role of accessory glands for sexual conflict, I studied in chapter five gene expression of *L. gredleri* male accessory glands with differential gene expression analysis.

CHAPTER 1

The significance of intercolonial variation of cuticular hydrocarbons for inbreeding avoidance in ant sexuals

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Abstract

The reproductive biology of social Hymenoptera is characterized by lifelong sperm storage by queens and sperm limitation in males. Both sexes are therefore expected to be especially choosy about their mating partners. In particular, sexuals should avoid sib-mating because of the resulting risk of producing sterile diploid male offspring. Colonies of the ant *Leptothorax gredleri* are specialists of patchy habitats and often live in subpopulations of fewer than a few dozen colonies with restricted gene flow between patches. Sexuals therefore have a high probability of mating with a related partner. Using gas chromatography and mass spectrometry, we found that the blend of cuticular hydrocarbons, which is generally thought to be important in nestmate recognition in social insects, was colony specific in both sexes. In principle, this might provide sexuals with a chemical cue for the avoidance of sib-mating. When allowed to choose among equal numbers of mating partners from their own and another nest in flight cages, sexuals had only a weak although significant preference for mating with non-nestmates. Other characters might therefore be more important than relatedness in mate choice.

Introduction

Mating with the wrong partner is usually unfavourable (Pusey and Wolf 1996), but it is particularly detrimental in social insects because of their peculiar lifelong partner commitment (Boomsma et al. 2005; Boomsma 2007). Queens of social wasps, bees and ants mate with one or a few males during a short period shortly after adult eclosion and subsequently rely on the sperm obtained during this period for the fertilization of their eggs throughout the rest of their life (e.g. Hölldobler and Wilson 1990). Similarly, the mating opportunities of males are limited because their testes have degenerated by the time they have reached sexual maturity, leaving them with a fixed amount of sperm sufficient for only one or a few matings (e.g. Hölldobler and Wilson 1990). For example, under laboratory conditions males of *Leptothorax* and related taxa are capable of inseminating up to 10 female sexuals (Winter and Buschinger 1983). Both sexes thus necessarily stick to the mate(s) they have originally chosen without the option of changing partners in the future (Boomsma et al. 2005). Sexuals of social insects are therefore expected to be even pickier about their mates than other animals. They should in particular avoid mating with relatives, because sib-mating in haplodiploid Hymenoptera with single-locus complementary sex determination causes half of the fertilized eggs to develop into diploid males (Cook and Crozier 1995). Diploid males are usually sterile and, because males generally do not work, constitute a considerable cost for queens during colony foundation (e.g. Ross and Fletcher 1985; Bourke and Franks 1995; Gerloff and Schmid-Hempel 2005). Although multiple mating may reduce diploid male load (Crozier and Page 1985; Bourke and Franks 1995; Cook and Crozier 1995), in the majority of social Hymenoptera the effective mating frequency of queens is not much higher than one (Strassmann 2001). Despite these intriguing facets of sexual selection, little is known about the actual mating biology of social Hymenoptera and whether sib-mating is avoided or not (Hölldobler and Bartz 1985; Hölldobler and Wilson 1990; Foster 1992; Boomsma et al. 2005).

Inbreeding in ants has been thought to be uncommon because in many species mating involves sexuals from large numbers of colonies during highly synchronized “nuptial flights” (e.g. Hölldobler and Bartz 1985). Where mating occurs in smaller aggregations, diploid male load might instead be minimized by individual colonies focusing on the production of single-sex broods, sex-biased dispersal, multiple mating and/or the active avoidance of sib-mating (Crozier and Page 1985; Helms and Rissing 1990; Schrempf et al. 2005b). Male and female sexuals often seem to mate indiscriminately (Woyciechowski 1990), and the moderate to high

levels of inbreeding in species in which mating occurs in or close to the nest or in small leks indirectly suggest that mechanisms of inbreeding avoidance are absent or inefficient (e.g. Buschinger 1989; Hasegawa and Yamaguchi 1995; Cole and Wiernasz 1997; Schrempf et al. 2005b; Lenoir et al. 2007). Female sexuals of *Linepithema humile* are apparently incapable of differentiating between brothers and less closely related males from their own multiqueen colony (Keller and Fournier 2002) and those of *Gnamptogenys striatula* similarly fail to distinguish between unfamiliar related and unrelated males (Blatrix and Jaisson 2002). This is probably not surprising, given that the evidence for true kin recognition in social Hymenoptera is weak at best and that their discriminatory capabilities are usually limited to the differentiation between nestmates and non-nestmates, regardless of their actual degree of kinship (Carlin 1989; Grafen 1990). In species where mating may involve sexuals from different nests, nestmate discrimination may be sufficient to avoid incest. For example, female sexuals of *L. humile* appear to prefer mating with non-nestmates to mating with brothers (Keller and Passera 1993).

Sexuals of *Leptothorax gredleri* and related species neither engage in large nuptial flights nor mate in the maternal nest. Instead they show a mating syndrome referred to as "female calling" (Buschinger 1968a, 1971; Franks et al. 1991; Heinze et al. 1992; Oberstadt and Heinze 2003): female sexuals leave the nest and after some limited flight activity climb onto a grass stem or stick, raise their abdomen and attract males by secreting a droplet of sexual pheromone from their poison glands. As *L. gredleri* lives predominantly in patchy habitats, such as sun-exposed forest edges or small oak-pine stands, populations are often fragmented into small subpopulations of fewer than 60 colonies each with considerably restricted gene flow between subpopulations (Oberstadt and Heinze 2003). Female sexuals therefore risk encountering nestmate males when searching for mating opportunities. Indeed, inbreeding coefficients of approximately 0.2-0.3 (Oberstadt and Heinze 2003; I. Merten, unpublished data) suggest that mating regularly involves kin, but whether this is due to the lack of alternative mating partners or the lack of discriminatory capabilities has not been investigated.

In this study, we used gas chromatography (GC) and mass spectrometry (MS) to investigate whether the blend of cuticular hydrocarbons on the surface of male and female sexuals is colony specific and might in principle allow nestmate discrimination. We also studied whether sexuals of the ant *L. gredleri* show a preference for mating with non-nestmates when given the opportunity to choose between nestmates and non-nestmates in flight cages.

Materials and methods

Sampling

We collected colonies of *L. gredleri* for both set-ups (the GC analyses and the mating experiment) in spring 2005 and 2006 from their nests in rotting branches in small stands of pine and oak trees at the edge of an abandoned army drill ground in Erlangen, Germany (49°35'09"N, 11°02'02"E). Adult ants and brood were transferred into three-chamber plastic boxes (9.5 x 9.5 cm and 3 cm high) with a cavity between two microscope slides serving as a nest and kept under standard rearing conditions (Buschinger 1974; Heinze and Ortius 1991) until they produced sexuals 2-3 months later. The colonies were fed twice a week with diluted honey and small pieces of cockroach. Humidity was maintained by regularly watering the plaster. Temperatures were 0 °C night/10 °C day (winter), 10 °C night/20 °C day (spring), 15 °C night/25 °C day (late spring) and 17 °C night/28 °C day (summer). As males tended to leave the nests earlier under artificial summer conditions, male-producing colonies were kept under colder conditions (12 °C night/17 °C day) after sexuals had eclosed. Sexuals were used for GC analysis and for the mating experiment only after they had spontaneously left their nest sites, indicating that they were ready to engage in mating activities. As *L. gredleri* colonies usually contain only a single reproductive queen (Buschinger 1968b; Heinze et al. 1992), co-occurring sexuals are likely to be brothers and sisters. Unfortunately it was not possible to use the same individual for both the GC analysis and the mating experiment, since the small ants had to be killed for the GC-MS analysis and the paint marks used in the mating experiment might have affected the results of the GC analysis. Therefore, sexuals were analysed by GC in summer 2005 and the mating experiment was conducted in summer 2006 with sexuals from some of the 2005 colonies and from a large number of newly collected colonies.

Chemical analysis

Female sexuals (N = 32), workers (N = 26) and males (N = 31) from six different colonies (three to six individuals per category and colony) were removed from their nestboxes with clean forceps, individually placed into labelled glass vials with inserts, and stored at -23 °C. Surface chemicals were extracted by soaking individuals for 15 min in 25 µl of pentane. Thereafter, the ants were removed from the inserts, the pentane was allowed to evaporate, and the cuticular substances were resolved in 10 µl of pentane. Of this extract, 2 µl were injected in the splitless mode into an Agilent Technologies (Böblingen, Germany) 6890N gas

chromatograph with HP-5 capillary column (30 m x 0.32 mm x 0.25 μm, J&W Scientific, Folsom, CA, U.S.A.). Helium with a flow rate of 1 ml/min was used as the carrier gas. The oven temperature increased from 70 °C to 180 °C at 30 °C/min, from 180 °C to 310 °C at 5 °C/min and finally kept at 310 °C for an additional 5 min.

We identified cuticular substances by GC-MS using an Agilent 6890N gas chromatograph coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with an RH-5ms+ capillary column (30 m x 0.25 mm x 0.25 μm; J&W Scientific) and the temperature profile was adjusted as described above. Helium was used as the carrier gas with a constant flow of 1 ml/min. A split/splitless injector was used (250 °C) with the purge valve opened after 60 s. The electron impact mass spectra were recorded with an ionization voltage of 70 eV, a source temperature of 230 °C and an interface temperature of 315 °C.We used MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, U.S.A.) for Windows for data acquisition. For the identification of the cuticular substances, we used pooled extracts of 10 individuals for each category. We identified n-alkanes and alkenes by comparing mass spectra with data from a commercial MS library (NIST, Gaithersburg, MD, U.S.A.) and methyl and dimethyl alkanes by diagnostic ions and standard MS databases (see above), and by determining Kovats indexes by the method of Carlson et al. (1998).We identified cholesterol by comparing retention time and mass spectrum with a synthetic standard. A total of 28 peaks were identified (Appendix). Our identification of substances matches that by Tentschert et al. (2002) only for the first few peaks eluting before C_{25} and 13-methyl C_{25}, but thereafter differs in a systematic way. For example, the peak we identified as C_{27} is given as C_{28} by Tentschert et al. (2002). We double-checked our identifications and conclude that the list of substance names in Tentschert et al. (2002) is shifted relative to the peaks.

For the statistical analysis we used the peak areas of 28 substances identified by GC-MS. The resulting peak areas were standardized to 100% for each individual. We chose not to transform the data to compensate for the non-independence of data, since transformation introduces additional background noise into the data when values of zero have to be replaced to make transformation possible. However, a reanalysis of the data after transformation according to Reyment (1989) gave similar results, but with a slightly inferior separation of groups.

The number of variables was reduced by principal components analysis and the data were analysed by discriminant analysis using the predefined groups “colony” and “category” (female sexuals, workers, males). In a separate analysis, only the profiles of males were

investigated to determine colony specificity. Peak 24 (C_{29}) did not appear in sufficient quantity in males and therefore only 27 peaks were used in this analysis.

Mating experiment

As sexuals of *L. gredleri* (Oberstadt and Heinze 2003) show mating activity in the morning (1000-1200 hours), our mating experiment focused on this time interval. Between 1 and 2 h before the experiment, sexuals that had left their nests were individually marked with colour dots and then placed into flight cages consisting of a Perspex frame with fly screens (approximately 15 x 15 cm and 23 cm high) and a thin plaster layer that was regularly moistened. In most trials, flight cages contained five males and five female sexuals from each of two colonies. In trials in which fewer than five male or female sexuals were available, we chose the number of sexuals in a way that each individual could potentially mate with an equal number of mating partners from its own and another colony. During the 2 h observation period, the flight cage was kept at room temperature (approximately 25 °C) and exposed to bright sunlight, if possible. All ants remained in the flight cage for the same amount of time and were not used again in later trials. For statistical analysis we used Statistica 6.0 (Statsoft, Tulsa, OK, U.S.A.). Trials without any matings were excluded from the analysis as we could not completely rule out that the failure was due to environmental/light conditions. Because of the large number of individuals, we could not monitor all interactions among female and male sexuals and focused only on copulations and copulation attempts. Mated female sexuals were not dissected in this study, but in a previous study almost all female sexuals that had mated in flight cages had a filled spermatheca (Oppelt and Heinze 2007 - chapter three of this thesis).

Results

Chemical analysis

Cuticular compounds were mostly linear and (di)-methyl-branched alkanes with chain lengths between C_{23} and C_{32}. In addition, cholesterol was present in considerable quantities on the cuticle of males, but only in small amounts on the cuticle of females. Cholesterol may be a contamination from a gland. The peak areas of 28 identified compounds (Appendix) were reduced to nine principal components with eigenvalues above 0.9, explaining more than 80% of the total variance. We decided to include two factors with eigenvalues of 0.996 and 0.930,

since together they explained 6.9% of the total variance. Discriminant analysis revealed a significant separation of the individuals according to colonies (Wilk's $\lambda = 0.045$, $F_{45.338} = 7.494$, $p < 0.001$; Fig. 1a), and 74.2% of all individuals were correctly classified to their respective colonies. This means that the cuticular profiles of workers, female sexuals and males had a colony-specific component.

In addition, the cuticular profile provided information about caste or sex. Discriminant analysis classified 94.4% of all individuals correctly according to sex or caste (Wilk's $\lambda = 0.111$, $F_{18.156} = 17.340$, $p < 0.001$; Fig. 1b). All males were correctly identified.

The PCA with only the male profiles extracted eight principal components with eigenvalues above 1.0, which explained more than 86% of the total variance. Discriminant analysis significantly separated males from different colonies (Wilk's $\lambda = 0.002$, $F_{40.81} = 6.428$, $p < 0.001$) and correctly classified all individuals.

Mating experiment

During 32 trials in flight cages, we observed a total of 111 matings, which were generally short and lasted between 30 s and 120 s (median 50 s). A total of 66 matings involved partners from different colonies and 45 copulations occurred between nestmates. This gives a slightly, but significantly lower frequency of mating among nestmates than expected under random mating (chi-square test: $\chi^2 = 3.973$, $p = 0.046$). Of the 302 female sexuals and 270 males used in the experiment, 92 (30.5%) and 61 (22.6%), respectively, mated at least once. Nine (9.8%) of the 92 sexually active female sexuals copulated repeatedly (six queens twice, two queens three times, one queen 10 times), and 26 (42.6%) of the 61 active males mated more than once (11 males twice, 10 males three times, three males four times, two males six times). Excluding multiply mating queens from the analysis gave a nonsignificant trend for the avoidance of mating with nestmates (49 versus. 34; chi-square test: $\chi^2 = 2.711$, $p = 0.100$). No pattern was apparent in the mate selection behaviour of multiply mating queens.

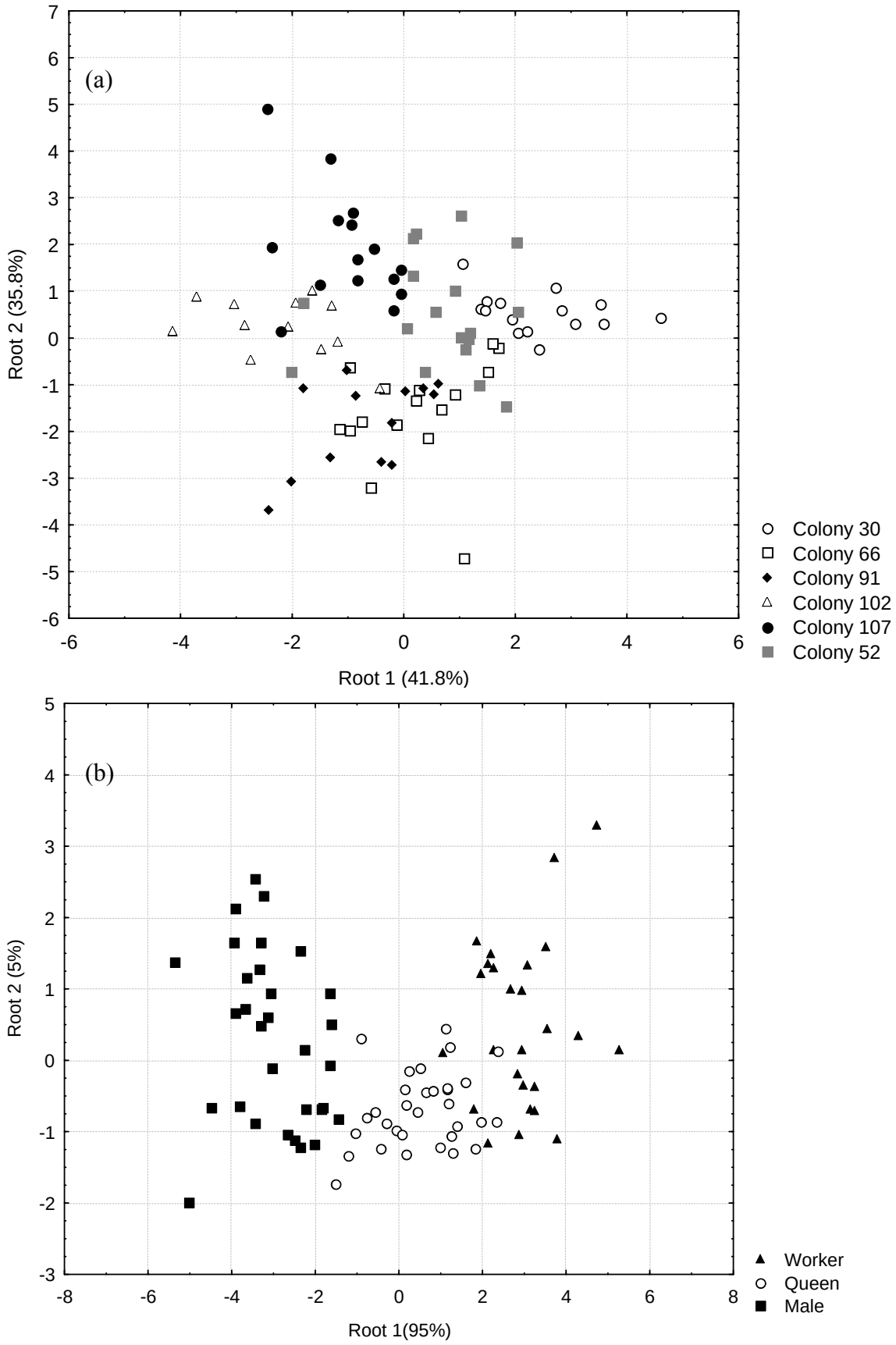

Fig. 1 Plot of the first two functions of the discriminant analysis of cuticular hydrocarbon profiles of workers, female sexuals and males from six colonies of *Leptothorax gredleri*, showing separation of individuals by (a) colony and (b) caste.

Discussion

The blend of hydrocarbons extracted from the cuticle of *L. gredleri* ants varied with both sex and colony of origin and thus in principle provides sexuals in small populations with the information needed to avoid mating with a nestmate. When confronted with equal numbers of nestmate and non-nestmate mating partners, sexuals showed slight preferences for unrelated mates and thus to some extent avoided sib-mating. However, the discrimination against nestmates was surprisingly weak.

Female sexuals of *Leptothorax* and related genera use alkaloid pheromones, in particular 3-methyl pyrrolidines, from their poison glands to attract males over long distances (Reder et al. 1995). The significant but weak association between mating decisions and variation in the cuticular hydrocarbon blends in our study corroborates previous suggestions that these substances play a role not only in nestmate recognition among workers of social Hymenoptera (e.g. Singer 1998) but also in mate choice and mating behaviour (Ayasse et al. 2001; Cremer et al. 2002; Beibl et al. 2007; Hora et al. 2008), including incest avoidance (e.g. Ryan and Gamboa 1986). At the same time, however, our data clearly reflect the ambiguity of previous studies, for example in bumblebees, in which inbreeding appeared to be avoided in some species but not in others (Foster 1992). The deviation from random mating observed in our experiment is rather weak and more than 40% of all matings involved nestmates. Laboratory rearing is often associated with a harmonization of colony odours because of the loss of environment-derived, colony-specific cues (Heinze et al. 1996) and the discriminatory capability of sexuals might therefore be better in the field. However, the considerable inbreeding coefficients found in previous genetic studies suggest that mechanisms for the avoidance of sib-mating are not particularly pronounced (Oberstadt and Heinze 2003; I. Merten, unpublished data). Observations suggest that both female and male sexuals are choosy and do not mate with the first partner they encounter, but obviously neither body size and fluctuating asymmetry (Oberstadt and Heinze 2003) nor, as we have shown here, familiarity and relatedness appear to play a major role in mate choice.

Inbreeding in Hymenoptera with single-locus complementary sex determination leads to homozygosity at the sex determination locus, and queens that have mated with a male carrying a matching allele at this locus produce diploid males from half of their fertilized eggs. Diploid males are usually sterile and do not work and thus pose considerable costs, in particular during the establishment of new colonies. Karyological studies have revealed a surprisingly high diploid load in Nearctic ants closely related to *L. gredleri* (Loiselle et al.

1990), but the incidence of diploid males in the latter is as yet not known. Multiple mating has been interpreted as a strategy for decreasing the risk of colony failure through the production of diploid males (e.g. Crozier and Page 1985). Indeed, a considerable number of female sexuals of *L. gredleri* were observed copulating with up to 10 males. However, previous genetic studies have documented that both queens from natural colonies and queens that had mated with multiple partners use sperm from only a single male (Oberstadt and Heinze 2003; I. Merten, unpublished data). Mating order does not appear to be pivotal in the context of paternity and at present it is unknown how it is chosen whose sperm migrates from the vagina to the sperm-storing receptacle (Oppelt and Heinze 2007 - chapter three of this thesis). In any case, multiple mating does not help to counterbalance the negative effects of inbreeding. Instead, diploid male load might be minimized through the particular life history of this species: most young queens either return to their maternal nests after mating or probably enter another nest, where they engage in dominance interactions with the resident queen. Dominant queens inherit the nest and subordinates may emigrate together with workers and found new colonies by budding (Heinze et al. 1992). Sib-mating might therefore be less critical for this species than for species with solitarily founding queens.

Acknowledgements

This study was supported by DFG (He 1623/19). We thank two anonymous referees for their helpful comments on our manuscript.

Appendix

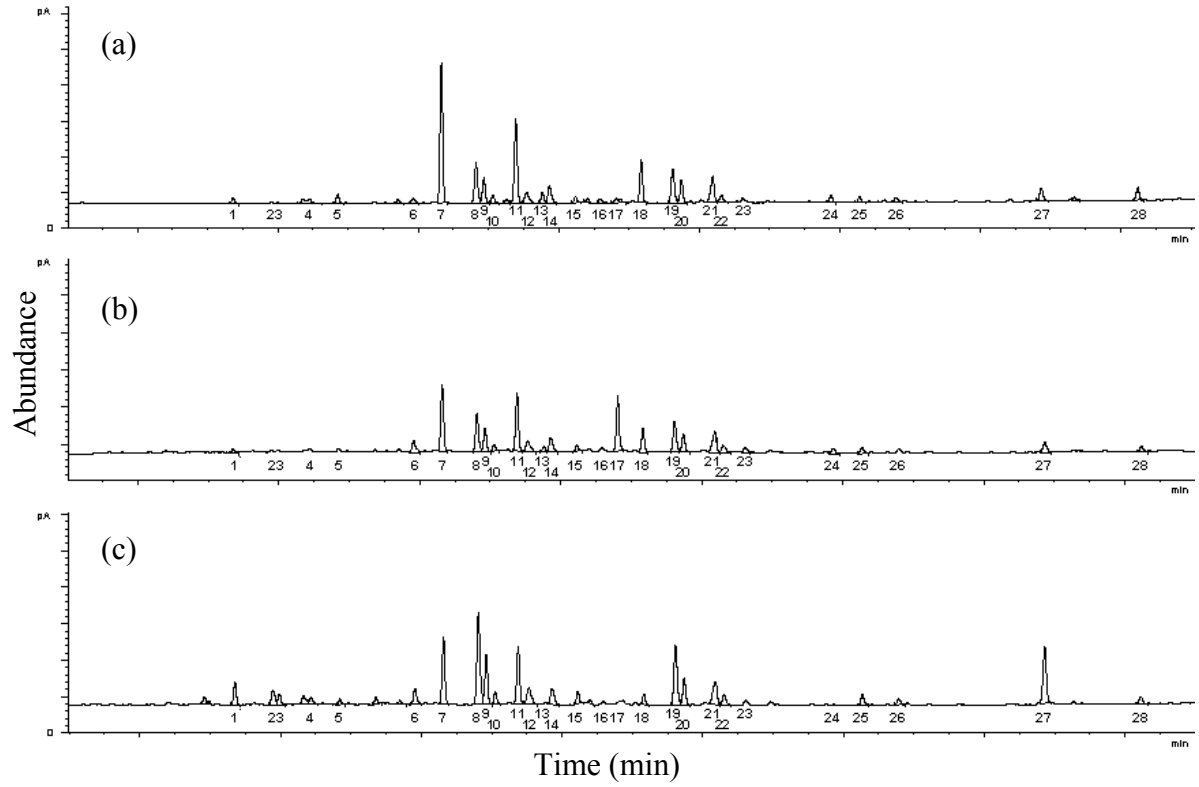

Fig. A1 Chromatogram of cuticular hydrocarbons in (a) workers, (b) virgin queens and (c) males of *Leptothorax gredleri*. 1: C_{23}; 2: 11- and 9-methyl C_{23}; 3: 7-methyl C_{23}; 4: 3-methyl C_{23}; 5: C_{24}; 6: $C_{25:1}$; 7: C_{25}; 8: 13- and 11- and 9-methyl C_{25}; 9: 7-methyl C_{25}; 10: 5-methyl C_{25}; 11: 3-methyl C_{25}; 12: 5-x-dimethyl C_{25}; 13: C_{26}; 14: 3-x-dimethyl C_{25}; 15: 13- and 12- and 11-methyl C_{26}; 16: 4-methyl C_{26}; 17: 6-x-dimethyl C_{26}; 18: C_{27}; 19: 13- and 11- and 9-methyl C_{27}; 20: 7-methyl C_{27}; 21: 3-methyl C_{27}; 22: 5-15- and 5-19-dimethyl C_{27}; 23: 3-x-dimethyl C_{27}; 24: C_{29}; 25: 15- and 13- and 11- and 9-methyl C_{29}; 26: 3-methyl C_{29}; 27: cholesterol; 28: 3-methyl C_{32}.

CHAPTER 2

Mating is associated with immediate changes of the hydrocarbon profile of *Leptothorax gredleri* ant queens

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Keywords: Hymenoptera, sexual communication, chemical signalling, cuticular hydrocarbons

Abstract

While sexual communication is often characterized by attempted manipulation, both sexes agree about females reliably signalling their receptivity. Female sexuals of the ant *Leptothorax gredleri* quickly became unattractive to males after their first copulation. This loss of attractiveness coincided with almost immediate changes in their cuticular hydrocarbon (CHC) profiles. Already 30 min after mating, the CHC profiles of female sexuals had significantly lower relative amounts of branched alkanes and higher amounts of linear alkanes than those of unmated and freshly mated female sexuals. Discriminant analysis did not distinguish between the profiles of freshly mated and unmated female sexuals, suggesting that the extremely rapid modification of CHC profiles is not caused by males marking females with anti-aphrodisiac CHCs. Instead, the new profile is produced by the female sexuals themselves. In addition to making them unattractive to males, this change may also help mated female sexuals when seeking adoption into established colonies.

Introduction

Males and females may have conflicting interests about mating and reproduction, but both benefit equally from signals that reliably communicate the female's receptivity. Female sexuals in this way secure matings and thereafter reduce the costs from additional male courtship and harassment, while males can adjust their sperm expenditure to the likely degree of sperm competition (e.g., Maynard Smith and Harper 2003; Johansson and Jones 2007). Sexuals of social Hymenoptera probably benefit particularly from honest signalling of female mating status because of the peculiar features of their reproductive biology. Female sexuals mate only during early adulthood and fertilize their eggs with the then received sperm throughout their lives without ever mating again. In males, testes usually have degenerated by the time they reach sexual maturity, and their fixed sperm supply suffices only for one or a few copulations, after which they die (Hölldobler and Bartz 1985; Boomsma et al. 2005). For both sexes it is therefore critically important to find a suitable mating partner during an often extremely narrow time window.

Female sexuals may quickly become unattractive after mating, in general because they change their pheromonal signal or are made unattractive by their mating partners by marking with anti-aphrodisiacs (e.g., Scott 1986; Andersson et al. 2000; Johansson and Jones 2007). Cuticular hydrocarbons (CHCs) appear to be involved in the communication of female mating status in a number of solitary insects, regardless of the origin of the post-mating changes (Scott 1986; Ayasse et al. 1999; Schiestl and Ayasse 2000; Simmons et al. 2003). CHCs are also known to serve in near-range communication among social Hymenoptera (Singer 1998; Hefetz 2007; Le Conte and Hefetz 2008) and appear to be involved in fertility signalling (e.g., Heinze 2004; Monnin 2006; Hefetz 2007; Le Conte and Hefetz 2008). Unmated egg layers often achieve similar CHC signatures as mated egg layers (Peeters et al. 1999; Monnin et al. 1998; Liebig et al. 2000; Heinze et al. 2002; D'Ettorre et al. 2004). This suggests that in the context of the reproductive division of labour fertility signalling is more important than communicating mating status (Lommelen et al. 2006). Differences between the CHC profiles of female sexuals and mated queens have been reported (e.g., Hora et al. 2008), but whether and how quickly CHC profiles of female sexuals change after mating remains largely unexplored (but see Johnson and Gibbs 2004).

The ant *Leptothorax gredleri* is a suitable model system for investigating post-mating odour changes. Female sexuals exhibit "female calling," i.e., they climb up grass stems or other vegetation near their maternal nests, extrude their stings and release poison gland

pheromone, which attracts males from a distance (Heinze et al. 1992; Oberstadt and Heinze 2003; Oppelt et al. 2008 - chapter one of this thesis). Mated female sexuals immediately stop "calling" but continue to elicit courtship from nearby males for a few minutes, which occasionally results in additional mating (10 second and third matings, median 19 min, range 3–37 min after first copulation, N. Spitzenpfeil and A. Oppelt, unpublished). After this time span, mated female sexuals have become fully unattractive, which suggests rapid post-mating changes of those signals that are involved in near-range communication between the sexes. A simple stop in the release of sex pheromones cannot explain this pattern sufficiently, because mating experiments took place in rather small flight cages with several calling female sexuals. Sex pheromones are long-distance attractants, and the behaviour of males shows that they cannot reliably distinguish between calling and non-calling sexuals when they are surrounded by a single pheromone cloud (J. Heinze, unpublished). Instead, the loss of female attractiveness after mating appears to be based on substances involved in short-range communication.

To elucidate the chemical basis of this quick loss in attractiveness, we compared the CHC profiles among virgin and freshly mated female sexuals. We show that the profiles of female sexuals change within 30 min after mating, but that these changes are not due to anti-aphrodisiacs, but instead produced by the female sexuals themselves.

Materials and methods

Animal collection

Colonies of *L. gredleri* Mayr 1855 (Hymenoptera: Formicidae) were collected from their nests in rotting wood in small stands of pine and oak trees at an abandoned army drill ground in Erlangen, Germany (49°35'09"N, 11°02'02"E) in spring 2005. Colonies were kept in three-chamber plastic nest boxes (9.5 cm x 9.5 cm x 3 cm) with a plaster floor under standard conditions in incubators (Buschinger 1974; Heinze and Ortius 1991). We provided diluted honey and small pieces of cockroach twice a week and controlled for humidity in the nest box through regularly moistening the plaster during the feeding sessions. Incubator temperatures were kept at 10 °C night/20 °C day (spring), 15 °C night/25 °C day (late spring) and 17 °C night/28 °C day (summer). Sexuals developed 2-3 months after collection. They

were used in the mating experiments only after they had left their nest sites voluntarily and had hereby indicated that they were ready to mate.

Mating experiments

Mating experiments were conducted between 1000 and 1200, when sexuals of *L. gredleri* (Oberstadt and Heinze 2003) show the highest mating activity. Ten or 15 female and 5 or 10 male sexuals were transferred into a Perspex flight cage (approximately 15 cm x 15 cm x 23 cm) with fly screens and a plaster floor to keep moisture. Experiments were conducted at room temperature (approximately 25 °C) in bright sunlight. In each experiment, we used sexuals from two colonies, one as source of the female sexuals and the other as source of the males. Over the 5 days of the experiment we used female sexuals from four different colonies and males from five different colonies. A total of 28 matings were observed. Mated queens were removed with clean forceps from the flight cage and shock frozen in liquid nitrogen in individual clean glass vials with insert at different time intervals after mating (five queens immediately; five queens 10 min; four queens 30 min; three queens 2 h; four queens 7 h; five queens 3 days; and two queens 10 days). Mated queens were kept in a separate new nest box until they were transferred to the glass vials immediately before shock freezing them. Six virgin female sexuals from the mating experiments and two additional female sexuals, which had not been part of any experiment, were similarly frozen. Vials were stored at -23 °C for subsequent analysis. Queens from different colonies were evenly assigned to the different intervals until shock freezing counterbalancing in this manner against possible influence of colony odour.

GC analyses

For GC profile analyses, single individuals were soaked in 25 µl of pentane for 15 min. Afterwards, they were removed from the glass insert and the pentane extracts were placed under an extractor hood for total evaporation. The residuals were resolved in 10 µl of pentane, of which 2 µl was used for splitless mode injection into an Agilent Technologies (Böblingen, Germany) 6890N gas chromatograph.

Helium at 1 ml/min was used as carrier gas with a HP-5 capillary column (30 m x 0.32 mm x 0.25 µm, J&W Scientific, Folsom, CA, U.S.A.). Oven temperature was programmed from 70 to 180 °C at 30°C min^{-1}, from 180 to 310 °C at 5°C min^{-1}. Temperature was kept on

310 °C for 5 min. Peaks were identified as before (Oppelt et al. 2008 - chapter one of this thesis).

Statistical analysis

Statistical analyses were performed using Statistica 6.0 (Statsoft, Tulsa, OK, U.S.A.) and SPSS 16.0 (SPSS Inc., 2008). We used the standardized peak areas of 28 previously identified peaks (Oppelt et al. 2008 - chapter one of this thesis). As before, we chose not to apply any transformation to the data to compensate for non-independency, as this would have introduced an additional source of "noise" into the data, in particular when values of zero have to be replaced to make transformation possible. Nevertheless, an analysis of the data set transformed according to Reyment (1989) gave similar results, but an inferior separation of groups as consequence of this additional background noise. We reduced the number of variables by principal components analysis and used the extracted factors in a discriminant analysis with "time since mating" as pre-defined group character. As cuticular changes do presumably not occur abruptly but continuously after mating, a significant separation of the eight, rather small groups was not expected and indeed not found (Wilk's $\lambda = 0.176$, $F_{49.116} = 0.966$, $p > 0.5$). First, we created only two groups, putting in the first unmated queens, queens immediately and 10 min after mating. The second group consisted of queens 30 min, 2 h, 7 h, 3 days and 10 days after mating. In the second analysis we pre-defined three groups and separated therefore the queens 30 min, 2 h and 7 h after mating from the queens 3 days and 10 days after mating. To determine which substances are responsible for presumed differences between groups, we compared the proportions of individual substances by exact tests for two independent groups and corrected for multiple testing following Hochberg and Benjamini (1995).

Results

A factor analysis applied to the proportional peak areas of 28 identified cuticular compounds of *L. gredleri* queens resulted in seven principal components with eigenvalues above 1.0, explaining more than 87% of the total variance. A discriminant analysis significantly separated two pre-defined groups, i.e., unmated female sexuals and mated female sexuals frozen within 10 min after mating and mated female sexuals frozen after longer time

spans (Wilk's $\lambda = 0.534$, $F_{7.28} = 3.492$, $p < 0.008$). Similarly, discriminant analysis also allowed to distinguish among three pre-defined groups (before 10 min, 30 min to 7 h, after 3 days, Wilk's $\lambda = 0.359$, $F_{14.54} = 2.581$, $p < 0.006$, Fig. 1). Discriminant analysis did not allow to separate unmated and mated individuals (Wilk's $\lambda = 0.751$, $F_{7.28} = 1.329$, $p < 0.274$).

Comparing the proportions of individual cuticular compounds between female sexuals from the two groups (virgin and up to 10 min after mating vs. 30 min and more after mating) showed that the proportions of several linear hydrocarbons (C_{24}, C_{26}, C_{27} and C_{29}) increase after mating, while those of branched hydrocarbons (13- and 11- and 9-methyl C_{25}, 7-methyl C_{25}, 5-x-dimethyl C_{25}, 3-x-dimethyl C_{25}, 11- and 13-methyl C_{27}, 3-methyl C_{27}) decrease (see Table 1). Only one branched hydrocarbon, 4-methyl C_{26}, shows the opposite trend. Correcting for multiple tests following Hochberg and Benjamini (1995) still gives significant values for six substances (see Table 1). Averaged over all individuals, the percentage of alkanes increased from 19.1 ± 5.3% to 23.9 ± 4.2% while that of branched alkanes and alkenes decreased from 75.4 ± 6.3% to 71.0 ± 5.7%.

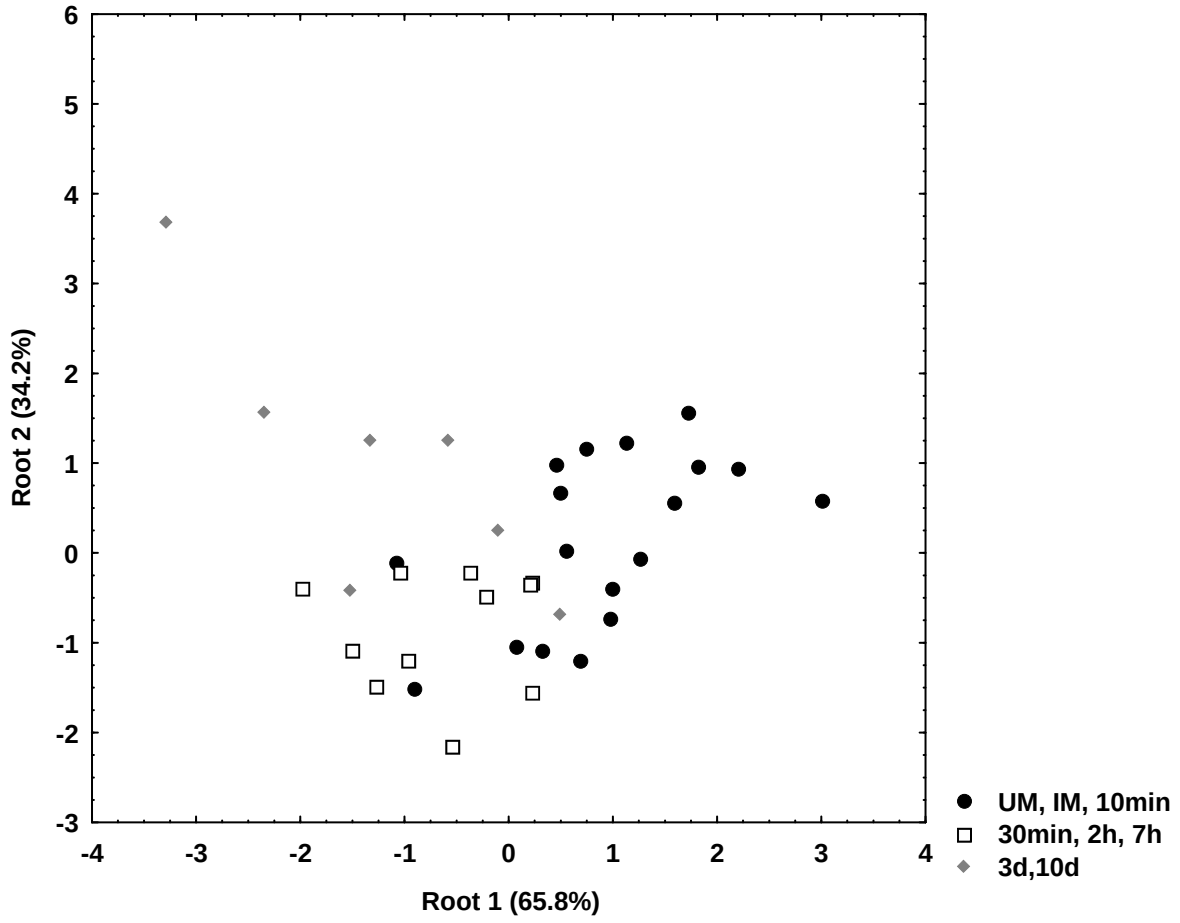

Fig. 1 Plot of the two functions of the discriminant analysis of cuticular hydrocarbon profiles of *Leptothorax gredleri* queens that were shock frozen at different times after mating. (1) UM (unmated), IM (immediately after mating) and 10 min after mating shock frozen, (2) 30 min, 2 and 7 h after mating shock frozen and (3) 3 days and 10 days after mating shock frozen individuals.

Table 1 Median proportions with upper and lower quartile of substances from the cuticula of female sexuals of *Leptothorax gredleri* shock-frozen either UM (unmated), IM (immediately) and 10 min after mating or 30 min, 2 h, 7 h, 3 days and 10 days after mating. Percentages were compared by exact tests for two independent groups and significant p-values are given in bold. In addition, we give p-values corrected for multiple testing following the method by Hochberg and Benjamini (1995) correction for multiple testing.

	Compound	Median (%) UM, IM, 10min	Lower quartile (%)	Upper quartile (%)	Median (%) 30min, 2h, 7h, 3days, 10days	Lower quartile (%)	Upper quartile (%)	p values	p values corrected
1	C_{23}	1.74	1.14	2.83	1.56	0.89	2.48	0.521	0.810
2	11- and 9-methyl C_{23}	0.00	0.00	0.00	0.00	0.00	0.00	0.466	0.768
3	7-methyl C_{23}	0.00	0.00	0.00	0.00	0.00	0.00	0.744	0.947
4	3-methyl C_{23}	4.23	3.98	7.06	6.46	3.71	8.13	0.372	0.651
5	C_{24}	0.00	0.00	0.66	0.77	0.48	0.82	**0.003**	**0.021**
6	$C_{25:1}$	3.30	2.56	4.77	2.70	2.50	3.40	0.118	0.275
7	C_{25}	11.88	9.03	14.99	13.90	12.73	15.28	0.126	0.271
8	13- and 11- and 9-methyl C_{25}	7.74	6.93	8.61	6.21	5.53	7.43	**0.004**	**0.022**
9	7-methyl C_{25}	5.63	5.13	6.04	4.83	4.14	5.54	**0.008**	**0.037**
10	5-methyl C_{25}	1.56	1.39	1.73	1.61	1.48	1.75	0.650	0.910
11	3-methyl C_{25}	8.98	7.95	9.94	9.71	8.41	11.65	0.265	0.495
12	5-x-dimethyl C_{25}	2.48	1.80	3.01	1.79	1.60	2.20	**0.045**	0.115
13	C_{26}	0.00	0.00	0.64	0.94	0.84	1.02	**<0.001**	**<0.025**
14	3-x-dimethyl C_{25}	3.24	2.50	4.11	2.55	2.13	3.19	**0.022**	0.068
15	13- and 12- and 11-methyl C_{26}	1.65	1.31	1.89	1.64	1.40	1.76	0.982	1.000
16	4-methyl C_{26}	0.00	0.00	1.03	1.05	0.00	1.73	**0.025**	0.070
17	6-x-dimethyl C_{26}	4.89	1.88	7.69	4.23	1.41	6.24	0.673	0.897
18	C_{27}	4.23	2.59	5.00	4.80	4.27	5.87	**0.019**	0.067
19	13- and 11- and 9-methyl C_{27}	10.61	9.02	11.32	9.02	7.27	9.74	**0.013**	0.052
20	7-methyl C_{27}	4.58	4.21	4.90	4.69	3.96	5.19	0.767	0.9347
21	3-methyl C_{27}	5.13	4.89	6.48	4.27	3.67	4.87	**0.001**	**0.009**
22	5-15- and 5-19-dimethyl C_{27}	4.87	4.10	5.34	4.88	3.64	5.79	0.988	1.000
23	3-x dimethyl C_{27}	0.00	0.00	1.61	1.00	0.00	1.32	0.820	0.957
24	C_{29}	0.00	0.00	0.93	1.07	0.87	1.49	**0.001**	**0.014**
25	15- and 13- and 11- and 9-methyl C_{29}	2.65	2.24	3.06	2.55	2.29	2.71	0.542	0.799
26	3-methyl C_{29}	1.07	0.00	1.78	0.00	0.00	1.14	0.140	0.280
27	Cholesterol	5.64	3.06	6.28	4.45	3.12	7.14	0.864	0.968
28	3-methyl C_{32}	0.00	0.00	0.63	0.00	0.00	0.81	1.000	1.000

Discussion

Female sexuals of *L. gredleri* quickly become unattractive to males after mating. This loss of attractiveness coincides with extremely rapid changes in the cuticular hydrocarbon profiles of female sexuals, which already 30 min after mating are significantly different from the pattern of unmated and freshly mated female sexuals. The odour change appears to be a continuous process,which starts immediately after mating but with time becomes more pronounced and detectable by statistic analysis.With the exception of one queen that mated 10 times within 1:32 h, all second and third matings observed in a previous study (Oppelt et al. 2008 - chapter one of this thesis) occurred within 37 min after the first copulation, suggesting that males are usually capable of reliably distinguishing between virgin and mated female sexuals after this time period. The extremely rapid modification of cuticular hydrocarbons might provide males with the necessary information for this discrimination.

In contrast to *Drosophila* and a number of other solitary insects (e.g., Scott 1986; Scott et al. 1988; Andersson et al. 2000), postmating CHC changes do not result from the transfer of anti-aphrodisiacs from males onto the female cuticula during mating, since the CHC profiles did not change immediately with the act of mating itself. A comparison of the profiles of virgin female sexuals and female sexuals immediately after copulation did not yield a significant discrimination between these two groups (details not shown, Wilk's $\lambda = 0.680$, $F_{7.5} = 0.336$, $p = 0.906$). This matches observations in solitary Hymenoptera, where differences between virgin and mated females also appeared to result from changes in the females' pheromonal signals rather than from the application of male-derived substances (Ayasse et al. 1999; Schiestl and Ayasse 2000; Simmons et al. 2003).

Mating is known to trigger immediate changes in the physiology and behaviour of females. In ants this includes phenomena such as the loss of wings at pre-determined breaking points within minutes after mating. Nevertheless, the promptness of cuticular changes is striking. Previously reported "rapid" changes in gland compounds or the CHC profiles of Hymenoptera are usually detectable only much later, i.e., several days after mating (Ayasse et al. 1993) or, in the contexts of fertility signalling and colony odour, at least 20 h after a change in colony composition (Lenoir et al. 2001a; Cuvillier-Hot et al. 2005). The almost immediate change in *L. gredleri* therefore indicates that given the appropriate physiological changes, the complex CHC profile of social insects may change much faster.

Proximately, these changes are not simply caused by the female sexual ceasing to release sex pheromones or other glandular substances. Poison gland secretions, which in

Leptothorax serve as sex pheromones (e.g., Buschinger 1972, 1976), are hydrophilic and contain alkaloids (Reder et al. 1995), and Dufour gland secretions are dominated by alkanes and alkenes with chain lengths between C_{15} and C_{23} (Heinze et al. 1998), i.e., substances very different from the CHCs reported in our study. Female sexuals usually groom themselves intensively after mating and in this way might apply a changed CHC pattern from their postpharyngeal gland (e.g., Soroker et al. 1995; Lenoir et al. 2001a). Similarly, a recent analysis suggests that in *Drosophila* CHCs may change within hours due to decay and synthesis (Kent et al. 2007).

After mating, the proportion of linear hydrocarbons on the cuticula of *L. gredleri* increased, while that of most branched hydrocarbons decreased. This stands in contrast to observations in the desert harvester ant, *Pogonomyrmex barbatus*, in which over a 2-day period from unmated to mated queens the proportions of linear alkanes decreased and those of branched alkanes increased (Johnson and Gibbs 2004). Replacing linear alkanes by branched alkanes is generally associated with increased cuticular permeability, but the magnitude of the change in *P. barbatus* was too small to fully account for the observed changes in water loss rate (Johnson and Gibbs 2004). Similarly, it is unlikely that the similarly small CHC changes in *L. gredleri* females are an adaptation to variation in humidity. Most young queens of *L. gredleri* return into their maternal nests or seek adoption into other conspecific colonies immediately after mating (Heinze et al. 1992) and are thus only shortly exposed to external environmental conditions.

It is often assumed that branched alkanes and alkenes are used as signals, while linear alkanes mainly serve for preventing desiccation and as “solvents” for other substances (e.g., Dani et al. 2001; Krokos et al. 2001; Gibbs 1998, 2002; Monnin 2006; but see Akino et al. 2004). One might therefore hypothesize that replacing branched alkanes by linear alkanes makes mated female sexuals of *L. gredleri* inconspicuous for males. Furthermore, the modification of CHC profiles might help young queens of *L. gredleri* to become adopted into established colonies in a similar way as “chemical insignificance” through very low quantities of CHCs allows freshly eclosed workers (Lenoir et al. 1999) and social parasites to integrate themselves into insect societies (Lenoir et al. 2001b). *L. gredleri* is one of the very few ant species with “functional monogyny”, i.e., only the top-ranking queen in a dominance hierarchy lays eggs. Freshly adopted queens usually refrain from reproduction in the presence of a fertile queen, but attempt to replace it in often violent dominance interactions after hibernation. To become adopted into a queenright colony and to avoid being attacked by workers and the dominant queen, freshly mated female sexuals might replace branched by

linear alkanes. We did not quantify the amount of hydrocarbons on the surface of female sexuals, but the qualitative change might be associated with similar changes in quantity.

Acknowledgements

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CHAPTER 3

Dynamics of sperm transfer in the ant *Leptothorax gredleri*

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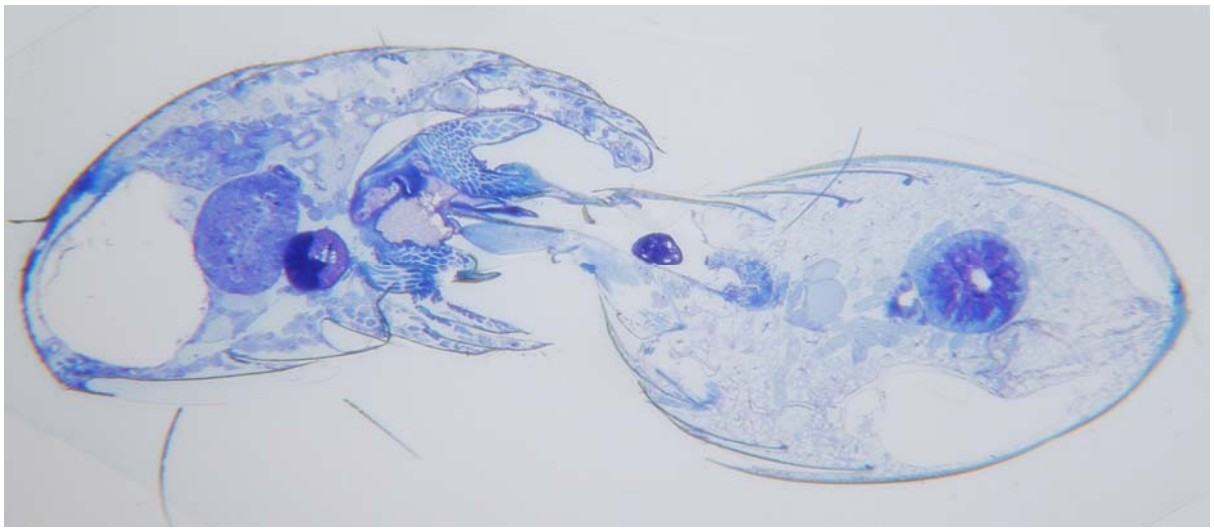

Keywords: Reproductive biology, mating, spermatophore, spermatheca, spermathecal duct, Formicidae

Abstract

Mating tactics differ remarkably between and within species of social Hymenoptera (bees, wasps, ants) concerning, e.g., mating frequencies, sperm competition, and the degree of male sperm limitation. Although social Hymenoptera might, therefore, potentially be ideal model systems for testing sexual selection theory, the dynamics of mating and sperm transfer have rarely been studied in species other than social bees, and basic information needed to draw conclusions about possible sperm competition and female choice is lacking. We investigated sperm transfer in the ant *Leptothorax gredleri*, a species in which female sexuals attract males by "female calling." The analysis of 38 female sexuals fixed immediately or up to 7 days after copulation with a single male each revealed that the sperm is transferred into the female *bursa copulatrix* embedded in a gelatinous mass, presumably a spermatophore. Sperm cells rapidly start to migrate from the tip of the spermatophore towards the spermatheca, but transfer is drastically slowed down by an extreme constriction of the spermathecal duct, through which sperm cells have to pass virtually one by one. This results in the spermatheca being filled only between one and several hours after mating. During this time, the posterior part of the spermatophore seals the junction between *bursa copulatrix* and spermathecal duct and prevents sperm loss. The prolonged duration of sperm transfer might allow female sexuals to chose between ejaculates and explain previously reported patterns of single paternity of the offspring of multiply mated queens.

Introduction

Social Hymenoptera are exciting models for investigating the mechanisms of sexual conflict and sperm competition (Simmons 2001). Ants, bees, and wasps show peculiar lifelong partner commitment (Boomsma et al. 2005), and because their testes degenerate early in adult life, Hymenopteran males have a limited sperm supply, which should make them choosier than males of other taxa. Mating systems vary considerably among different species of social Hymenoptera, in traits such as pre-mating dispersal distance and density of female sexuals, mating frequency of male and female sexuals, and the occurrence of sib-mating (e.g., Hölldobler and Bartz 1985; Hölldobler and Wilson 1990; Boomsma et al. 2005; Schrempf et al. 2005a), and the reproductive physiology of both sexes might, therefore, show manifold adaptations to the varying strength of sperm competition.

Despite of these promising features, inter- and intra-sexual selection has only recently found the interest of social insect researchers (Hölldobler and Bartz 1985; Baer 2005; Boomsma et al. 2005; Koeniger 2005). This is because male Hymenoptera are comparatively short-lived, and in particular in ants, mating often takes place during a very short period of frantic activity high up in the sky. Consequently, the details of copulation and sperm transfer are still not well known for species other than commercially important honeybees, *Apis* (Koeniger and Koeniger 1991; Koeniger et al. 2000; Baer 2005), stingless bees (Melo et al. 2001; Martins and Serrão 2004), bumble bees, *Bombus* (Duvoisin et al. 1999; Baer et al. 2000, 2001, 2003), and a few wasps (e.g., Martins et al. 2005). Considerable variation in mating tactics exists already in this limited sample. For example, in the Western honeybee, *Apis mellifera*, sperm is transferred to the lateral oviducts during or shortly after ejaculation, from where it is moved back to the *bursa copulatrix* and finally stored in the spermatheca during a process that takes about 40 h (Woyke 1983; Baer 2005 and references therein). In contrast, males of the dwarf honeybee *Apis florea* inject sperm directly into the spermathecal duct (Koeniger et al. 1989), which presumably gives queens considerably less power in cryptic female choice.

The few histological analyses conducted in ants suggest that sperm may be transferred during copulation to the female *bursa copulatrix* covered by a gelatinous spermatophore (Robertson 1995; Allard et al. 2002, 2006). From the *bursa*, it is slowly shifted into the spermatheca, suggesting that multiply inseminated queens have the opportunity to choose among individual ejaculates. In the leaf-cutter ant *Acromyrmex versicolor*, the spermatheca

was found to be completely filled with sperm only 5 h after the mating flight (Reichardt and Wheeler 1996).

To better understand the dynamics of sperm transfer, we allowed males and female sexuals of the ant *Leptothorax gredleri* to mate under controlled conditions in flight cages and investigated the location of sperm in the female genital tract at different time periods after mating. In *L. gredleri*, female sexuals leave their maternal nests in the late morning, climb up on grass stems or branches, and attract males with droplets of a sexual pheromone secreted from their poison gland ("female calling," Buschinger 1968; Heinze et al. 1992). The copulation takes approximately 1 min (mean 66±SD 25 s, Oberstadt and Heinze 2003). Previous analyses had further shown that female sexuals may occasionally mate multiply, but genotyping the offspring of multiply mated queens never revealed multiple paternity (Oberstadt and Heinze 2003).

We investigated whether sperm is transferred into the *bursa copulatrix* or directly into the spermathecal duct and how long it takes until the spermatheca is completely filled. We describe the anatomy of the female genital tract, including a hitherto unknown, extremely narrow section of the spermathecal duct through which sperm cells have to migrate, report on the occurrence of a spermatophore and estimate the duration of sperm transfer to the spermatheca.

Materials and methods

Colonies of *L. gredleri* Mayr 1855 (Hymenoptera: Formicidae) were collected from dead branches in small oak and pine stands on an abandoned army drill ground in Erlangen, Germany. In the laboratory, adult ants and brood were carefully removed from their nests and kept under standard conditions in three-chambered plastic boxes with a nest site consisting of two object slides separated by a plastic spacer (Buschinger 1974; Heinze and Ortius 1991).

Sexuals developing in the colonies were used in mating experiments only after they had left their nests voluntarily. Mating was induced by placing 10 to 20 males and 10 to 20 female sexuals into a flight cage consisting of a Perspex frame with fly screens (approx. 15×15×23 cm) at 1000 $_{A.M.}$ The flight cage was exposed to sunlight either outside the university building or in a greenhouse on its roof. Most female sexuals soon showed the typical posture of sexual calling, with slightly raised gaster and extruded sting. Copulation is characterized by the male tilting backwards and becoming immobile (Oberstadt and Heinze

2003). We noted the time of the copulation and quick-froze female sexuals (in total N = 38) immediately or up to 7 days after copulation (see Table 1) by placing them into liquid nitrogen.

Frozen gasters were fixed in Bouin's fixative for at least 24 h, embedded in Durcopan, and serially sectioned at a thickness of 2 µm with a Reichert–Jung microtome. Sections were stained with Mallory's solution following Richardson et al. (1960) and inspected for the location of sperm, etc. using a Zeiss Axiophot M45 light microscope. During the early stages of spermatheca filling, sperm remained clumped in the outer parts of the spermathecal lumen and was not evenly dispersed. The filling level could, therefore, be only crudely estimated. Compression of the spermathecal reservoir during sectioning might occasionally have led to erroneous estimates. Estimates are always based on multiple sections per individual to avoid artifacts from disruptions in single sections.

Series of 2-µm sections of the gaster of a queen fixed 5 min after copulation were digitally photographed using a Nikon Coolpix 990 camera to reconstruct the anatomy of the female genital tract and the spermatophore in three dimensions. Digital images were manually aligned utilizing the 3D visualization software Amira® (Mercury Computer Systems, Berlin), and in each slide spermatophore, *bursa copulatrix*, and spermatheca including spermathecal duct were manually marked in specific colors.

Results

The genital tracts of all mated female sexuals (listed in Table 1) contained sperm either in the *bursa copulatrix* or the spermatheca and spermathecal duct. Sperm was apparently transferred into the female genital tract embedded by a gelatinous mass measuring approximately 300 µm in length and 40 µm in width and almost completely filling the *bursa copulatrix*. This substance apparently functioned as a spermatophore (Fig. 1). Immediately after copulation, sperm cells were located in the upper third to half of the spermatophore, close to the entrance of the spermathecal duct. Sperm storage started immediately after copulation, and already 2 min after copulation, a small amount of sperm was found in most parts of the 200-µm long spermathecal duct. However, the migration of sperm cells appeared to be blocked by a 40-µm long and approximately 1-µm wide constriction close to the entrance of the spermathecal lumen (Fig. 2a), through which the 100-µm long sperm cells had to pass virtually one by one. Due to this bottleneck, it took several hours until the spermatheca

was completely filled. Considerable quantities of sperm were found in the spermatheca only between 45 min and 2 h after copulation, and it took approximately 5 h until all sperm was transferred (see Table 1 for discrete estimates of spermatheca filling). We could not detect any muscular tissue that might serve as "sperm pump" in the spermathecal duct. The spermathecal gland was situated dorsally of the spermatheca, with its duct opening next to the constriction of the spermathecal duct (Fig. 2b).

A correctly positioned spermatophore might prevent the loss of sperm during the long process of sperm transfer. This is corroborated by the observation that sperm apparently leaked out of the *bursa copulatrix* in one female sexual, in which the spermatophore was incorrectly placed and covered only the bottom of the *bursa copulatrix* instead of sealing it completely. The spermatophore dissolved and was partly expelled 3 h or longer after mating (see Table 1 for the presence of spermatophores). While it was still present in one individual 24 h after copulation, no trace of a spermatophore was found in another female sexual 1 day after mating, and all sperm had been transferred into the spermatheca.

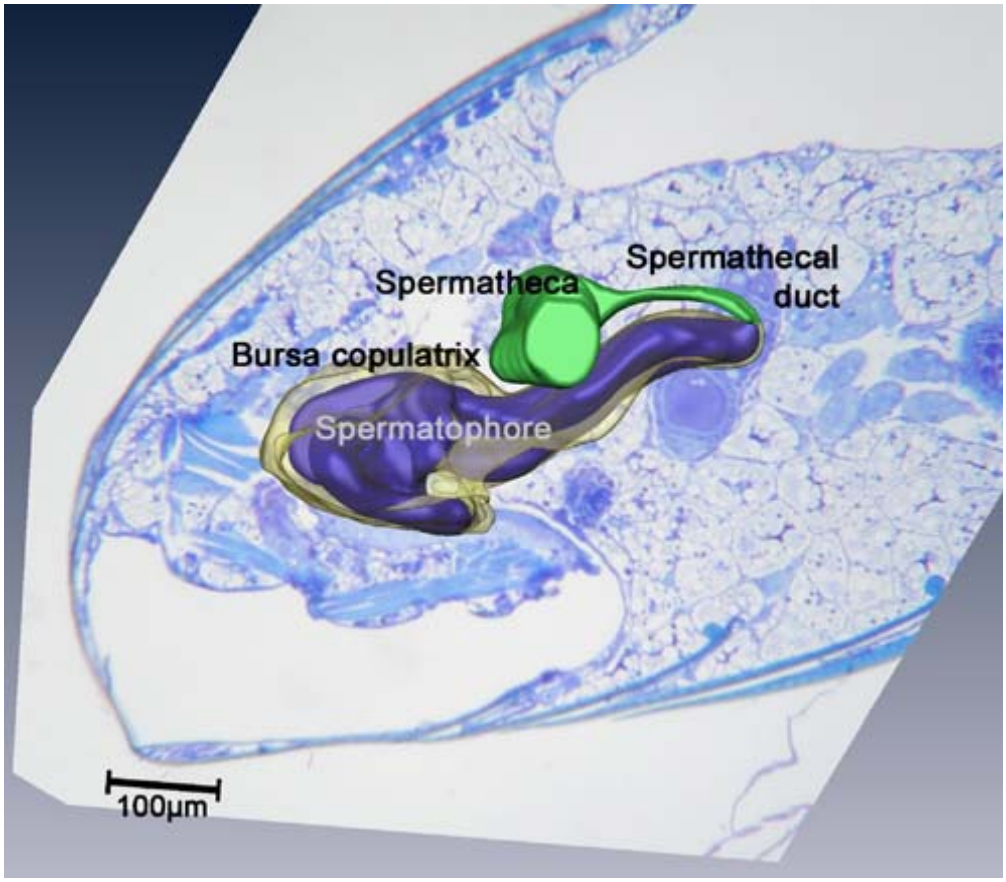

Fig. 1 3D reconstruction of the gaster of a *L. gredleri* queen shortly after mating. The spermatophore almost completely fills the *bursa copulatrix* and, hereby, seals the junction between *bursa copulatrix* and spermathecal duct. *Scale bar* 100 µm

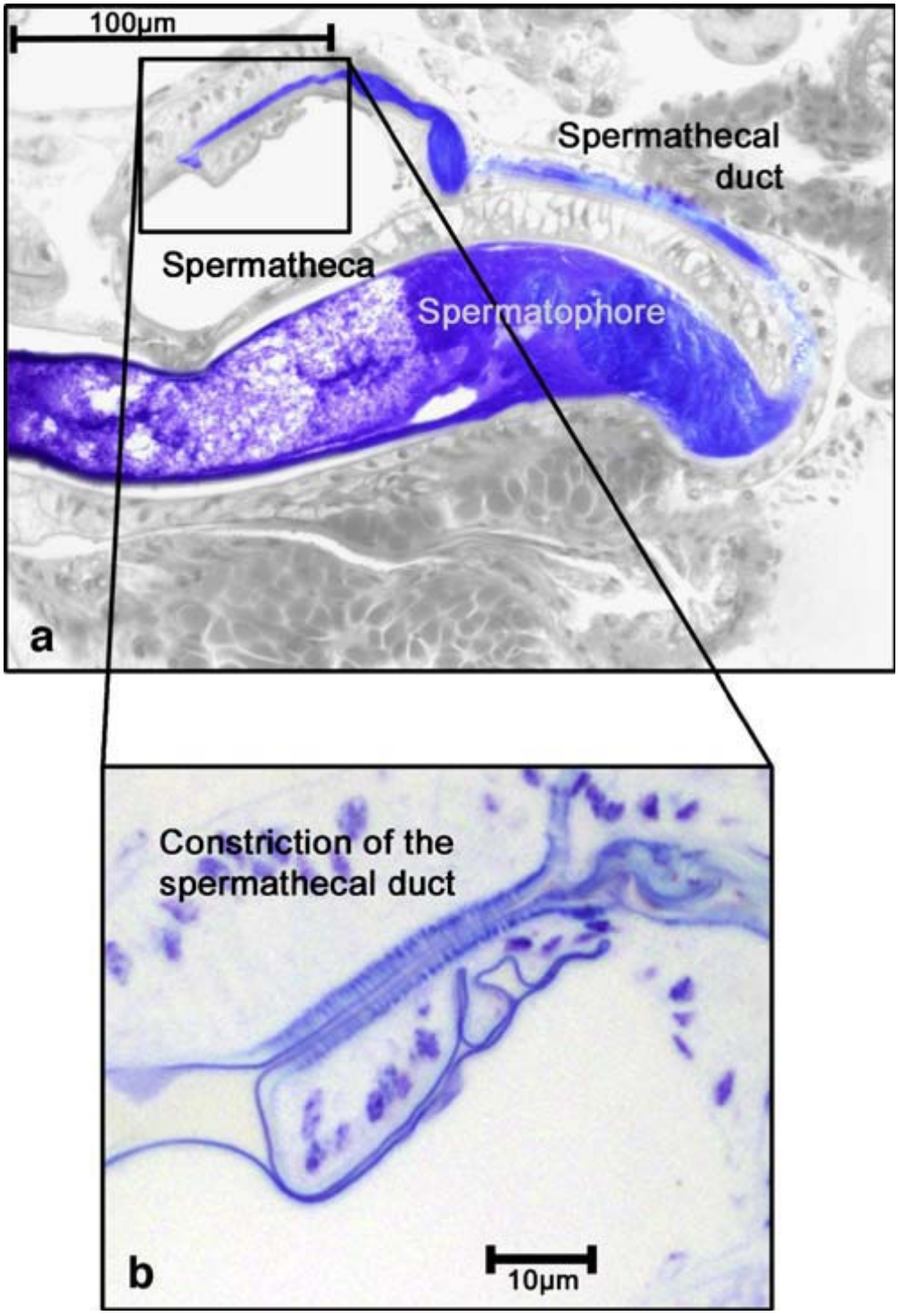

Fig. 2 a Spermatophore and spermatheca of a *L. gredleri* queen 2 min after mating. Sperm cells have already begun to migrate from the tip of the spermatophore into the spermathecal duct and accumulate in front of a constriction that impedes sperm flow into the spermatheca. *Scale bar* 100 µm. **b** Constriction of spermathecal duct, which appears to slow down the transfer of sperm into the spermatheca. *Scale bar* 10 µm

Table 1 Data from the dissection of the genital tract of 38 female sexuals of *Leptothorax gredleri* that were fixed in different time intervals after mating. The filling of the spermatheca was crudely estimated from the analysis of the semi-thin sections of the respective sample.

Number	Time interval	Presence of spermatophore	Sperm in spermatophore	Sperm in spermatheca
1	Immediately	Yes	Yes	Empty
2	Immediately	Yes	Yes	Empty
3	Immediately	Yes	Yes	Empty
4	Immediately	Yes	Yes	Empty
5	1 min	Yes	Yes	Empty
6	1 min	Yes	Yes	Empty
7	2 min	Yes	Yes	Empty
8	2 min	Yes	Yes	Empty
9	2 min	Yes	Yes	Empty
10	5 min	Yes	Yes	Empty
11	10 min	Yes	Yes	Empty
12	10 min	Yes	Yes	Some
13	20 min	Yes	Yes	Thin film along spermathecal wall
14	20 min	Yes	Yes	Single sperm cells
15	30 min	Yes	Yes	Thicker film along spermathecal wall
16	45 min	Yes	Yes	Half full
17	1 h	Yes	Yes	Thin film along spermathecal wall
18	1 h	Yes	Yes	Half full
19	1 h	Yes	Yes	Half full
20	1 h	Yes	Yes	Half full
21	1 1/2 h	Yes	Yes	Half full
22	2 h	Yes	Yes	Half full
23	2 h	Yes	Yes	Half full
24	2 1/2 h	Yes	Yes	Three quarter full
25	3 h	Yes	Yes	Full
26	3 h	Yes	Yes	Thicker film along spermathecal wall
27	3 1/2 h	Yes, remains	?	Half full
28	3 1/2 h	Yes	Yes	Three quarter full
29	4 h	Yes, remains	?	Full
30	4 h	Yes	Yes, remains	Full
31	5 h	Yes, remains	Yes, remains	Half full
32	5 h	Yes, remains	Yes, remains	Half full
33	5 h	Yes	Yes, remains	Half full
34	7 h	Yes	Yes	Full
35	7 h	Yes	Yes, remains	Half full
36	24 h	No	No	Half full
37	24 h	yes, remains	Yes	Full
38	7 days	No	No	Half full

Discussion

The mechanisms of sperm transfer in the ant *L. gredleri* resemble that reported from other species of social Hymenoptera in that males do not inject their sperm directly into the spermathecal duct but, embedded in a gelatinous spermatophore, into the female *bursa copulatrix*. Similar spermatophores, which presumably consist of secretions of the males' accessory glands, have previously been described also from other ants (Robertson 1995; Allard et al. 2002, 2006).

Sperm is transferred to the spermatheca via the 200-µm long spermathecal duct, which in *L. gredleri* has a 40-µm long constriction that slows down sperm migration. Such a constriction has, to our knowledge, not yet been reported from other social Hymenoptera. Consequently, it takes 1 h and longer until a considerable amount of sperm has been transferred into the spermatheca. According to previous studies, the spermatheca of Western honeybees, *A. mellifera*, is completely filled only 24 to 40 h after copulation (Woyke 1983; Koeniger et al. 2000),while those of *Apis andreniformis*, with direct injection of sperm into the spermathecal duct (Koeniger et al. 2000), and of *Bombus terrestris* are filled within 30 to 90 min (Duvoisin et al. 1999).

A correctly placed spermatophore might serve to prevent sperm loss during the prolonged period of sperm uptake in *L. gredleri*. Indeed, in the dwarf honeybee *A. florea*, where drones also transfer their sperm directly into the spermathecal duct (Koeniger et al. 1989), and two species of *Atta* leaf-cutting ants, where immediate sperm transfer is also assumed (Baer and Boomsma 2006), male accessory glands are relatively small (Baer and Boomsma 2004; Mikheyev 2004). In addition, spermatophores might function as mating plugs and, thus, serve in the context of sperm competition (Duvoisin et al. 1999; Baer et al. 2000, 2001; Mikheyev 2004). Queens of *L. gredleri* mate only once, but multiple copulations have occasionally been observed (Oberstadt and Heinze 2003). According to microsatellite analyses, daughters of multiply mated queens are all offspring of a single father, either the first or the last male (Oberstadt and Heinze 2003), which might be caused by some males being incapable of transferring sperm, sperm competition, or cryptic female choice. Unfortunately, no multiply mated queens were available in this study, but further histological studies might certainly help to elucidate the role of the spermatophore and/or the bottleneck in the spermathecal duct. Remainders of the spermatophore appear to be discarded several hours after copulation, as in *A. versicolor* (Reichardt and Wheeler 1996) and *A. mellifera* (Koeniger and Koeniger 1991; Koeniger et al. 2000). To what extent substances from the spermatophore

are absorbed by the female epithelium remains unknown; however, given that male accessory gland secretions often serve multiple functions (Gillott 2003; Frattini-Colonello and Hartfelder 2005), it is likely that not all constituents are expelled.

Previous studies in bees (Bresslau 1905), bumblebees (Schoeters and Billen 2000), and ants (Wheeler and Krutzsch 1994; Gobin et al. 2006) have described muscular tissue along the spermathecal duct, which is thought to be involved in sperm storage and the regulation of sperm release (Dallai 1975; Baer 2005). Such a "sperm pump" appears to be absent in *Atta* (Baer and Boomsma 2006) and *L. gredleri*. As yet, it is unclear whether in *L. gredleri* its function might be taken over in part by the constriction of the spermathecal duct, which presumably does not only impede sperm uptake but might also limit sperm migration during egg fertilization. This suggestion is inspired by the location of the constriction close to the reservoir, exactly in the region where a "sperm pump" would be expected. Our study documents that despite of the meticulous anatomical descriptions of the morphology of ant genitals (e.g., Janet 1902), their function in inter- and intra-sexual selection is still incompletely understood and merits further attention.

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CHAPTER 4

Conserved male accessory gland protein pattern in *Leptothorax gredleri* and related ant species

(Manuscript)

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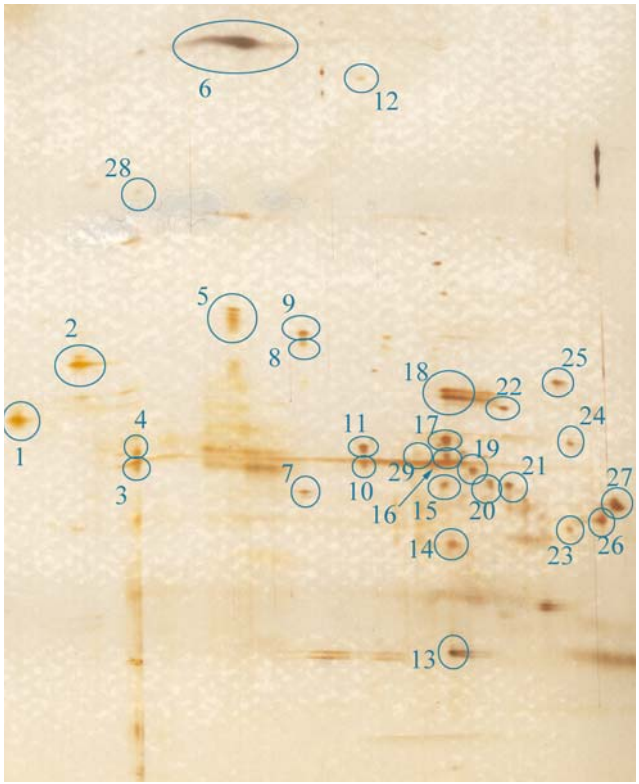

Keywords: 2D gel electrophoresis, SDS page, rapid evolving genes, sexual conflict, sexual cooperation, sexual selection

Abstract

The life history of social insects requires that mated queens first found a prospering colony and produce numerous workers before they invest into sexual offspring. This remarkable difference to solitary insects results in a convergence of male and female interests. Males should therefore by all means avoid harming their mates. Arms races between the sexes with a more and more complex composition of seminal fluids that – like in *Drosophila* – end up being even toxic, should be replaced by sexual cooperation and a conserved pattern of accessory gland proteins.

We tested this hypothesis in the ant species *Leptothorax gredleri* by two-dimensional SDS-polyacrylamid gel electrophoresis of male accessory gland proteins and compared their variation to that of thorax proteins. Accessory gland protein patterns did not vary considerably among males from different colonies and also closely resembled those of the related ants *Leptothorax muscorum*, *Leptothorax acervorum* and *Harpagoxenus sublaevis*. Variation was considerably higher in the thorax proteins. Our results indicate that the protein composition of the seminal fluid from the accessory glands of formicoxenine ant males is highly conserved within and between species.

Introduction

Sexual reproduction requires that male and female sexuals cooperate to propagate their genes into the next generation. Whenever male and female disagree in their evolutionary interests (Parker 1979), a sexual conflict can arise. This, in turn, paves the way for phenomena like sexual antagonistic coevolution (Rice 2000) and elaborate secondary sex traits like the well-known peacock's tail which already caught the interest of Charles Darwin (1871). In more recent years, research on the non-social insect genus *Drosophila* showed that sex- and reproduction-related genes (SRR) are often rapidly evolving (Singh and Kulathinal 2000; Swanson et al. 2001, 2004; Swanson and Vacquier 2002; Panhuis et al. 2006; Haerty et al. 2007). Genes expressed in reproductive tissue showed faster evolution than genes from non-reproductive tissue (Thomas and Singh 1992; Civetta and Singh 1995; Jagadeeshan and Singh 2005). This implies differential selective pressure on reproductive and non-reproductive tissue, with obviously even stronger selective pressure on the male reproductive system (Jagadeeshan and Singh 2005).

Especially male accessory glands and their secreted proteins, which constitute a major part of the male seminal fluid (Gillott 2003), came into the focus of interest. In insects, the accessory gland proteins are transferred during copulation with the sperm to the female, where they induce various changes in female physiology, behaviour and reproduction (Gillott 2003; Wolfner 2002). Furthermore, accessory gland products are known to be responsible for reduced pathogen transmission, mating plug or spermatophore formation and sperm competition (Poiani 2006; Ram and Wolfner 2007b). One- and two-dimensional electrophoretic analysis showed that male *Drosophila* accessory gland proteins are highly species-specific (Chen 1976; Chen et al.1985). Although ovulation can be stimulated in many *Drosophila* females only by the accessory gland products of their own males (Chen et al. 1985), injection experiments showed that viable hybrids could be produced when male extracts of their own species are injected after copulation (Chen 1984). This demonstrates that accessory gland products can efficiently act as isolation factor between species (Chen et al. 1985). Moreover, even within single species the protein patterns of accessory glands show a considerable amount of polymorphic variants. In electrophoretic studies Whalen and Wilson (1986) found polymorphism in almost half of the major protein bands of *D. melanogaster* male accessory glands. This high level of polymorphism could be confirmed also for other *Drosophila* species (Ram and Ramesh 2007) and additionally also by DNA sequence analyses (Aguadé et al. 1992; Tsaur et al. 1998, 2001; Aguadé 1999; Begun et al. 2000). Sperm

competition, sexual conflict, sexual selection as well as host-pathogen interactions are discussed to be the most likely reasons for the observed rapid evolution of seminal fluids (Findlay et al. 2008). These varying opportunities for coevolution might lead to positive Darwinian selection that could explain the high interspecific divergence observed in *Drosophila* accessory gland proteins. Unfortunately, such directional selection does not explain the observed high intraspecific polymorphism (Chapman 2001). Balancing selection (Prout and Clark 1996) might be a better explanation or the idea of reduced selective constraints on genes involved in sex and reproduction (Kulathinal and Singh 2004). However, the high level of polymorphism observed in accessory gland proteins and the traits they influence need further examination to find appropriate explanations.

Interestingly, the special dynamics responsible for the rapid evolution of SRR do not always result in a positive outcome for both sexes. Male accessory gland proteins bear a "cost of mating" for *D. melanogaster* females – their toxic side-effects reduces female lifespan (Chapman et al. 1995; Lung et al. 2002). How life history traits can influence the outcome of such dynamics shows a study of Holland and Rice (1999), where they enforced monogamous mating with random mate assignment to two *D. melanogaster* populations. Males evolved to be less harmful to their mates, while females became less resistant to male-induced harm in these "monogamous" populations. Altogether, monogamous populations showed a higher net reproductive rate when compared to the naturally promiscuous populations. Therefore, the change of the study object might bring a new perspective to the topic and reveal new insights. While in the last decades accessory gland proteins were studied exhaustively in various *Drosophila* species, research on other species are seriously lagging behind (Gillott 2003).

Social insects with their apparently different life history from solitary species bear a special attraction. Sexuals are produced in social insects only after rearing first several generations of workers (Boomsma et al. 2005), which postpones the relevant reproductive success for males. Consequently, males should by all means avoid harming their queens because every damage (either right away or with a time lag) will reduce the total amount of sexuals produced and, thus, also male reproductive success. The discovery that a concentrate of male accessory gland extracts of honey bee (*Apis mellifera*) drones also is toxic to queens was thus very astonishing (Frattini-Colonello and Hartfelder 2005). Again, the explanation for this comes with the life-history traits of the species because contrary to the majority of social Hymenoptera, where queens mate only once (Strassmann 2001), *A. mellifera* is polyandrous. Thus, in honey bees the mating sign of an individual drone is soon removed by the following

one and the toxicity of the single last mating sign is not expected to have a relevant impact on the queen's life expectancy (Frattini-Colonello and Hartfelder 2005).

Recent findings on ants, which are in general monogamous, confirm the idea of reduced sexual conflict. In the ant *Cardiocondyla obscurior*, mating is correlated with a positive effect on lifetime reproductive success. Mated queens lived substantially longer and produced sooner eggs than virgin queens, even when mated to sterilized males (Schrempf et al. 2005a). Here, sexual cooperation might replace sexual conflict, since it is beneficial to both sexes. What influence this observation might have on the variation of male accessory gland proteins is an interesting resulting question. In this study, we therefore analysed the polymorphism of accessory gland proteins of *Leptothorax gredleri* males using two-dimensional gel electrophoresis and compared it to the polymorphism of thorax proteins. Additionally, some single samples of *L. muscorum*, *L. acervorum* and *Harpagoxenus sublaevis*, more or less related ant species, served as between-species comparison for an initial idea about how much divergence exists between these species.

L. gredleri is an ant species that mates by "female calling", i.e. after leaving their maternal nests, queens attract males with a sexual pheromone that is released when females are positioned at an elevated site (Heinze et al. 1992; Oberstadt and Heinze 2003; Oppelt et al. 2008 - chapter one of this thesis). Sperm is transferred with a gelatinous substance that stays inside the female for several hours until the sperm is transferred to the spermatheca (Oppelt and Heinze 2007 - chapter three of this thesis). After the copulation, mated queens change their hydrocarbon pattern immediately so that the difference is detectable already within half an hour. These changes are produced by the queens themselves and are no male anti-aphrodisiacs (Oppelt and Heinze 2009 - chapter two of this thesis). The mechanism and trigger for this reaction is so far unknown, but the participation of male seminal fluids cannot be excluded. We expect both sexes to benefit from this reaction and would therefore anticipate that the mechanism underlying this reaction might be rather conserved than rapidly evolving. Furthermore, queens are mostly monandrous but sometimes additional mating occurs mainly within half an hour after the first copulation (Oberstadt and Heinze 2003; Oppelt and Heinze, 2009 - chapter two of this thesis). Nevertheless, multiply mated queens produced only offspring of a single father (Oberstadt and Heinze 2003). How this exclusive paternity is achieved and whether sperm competition and accessory gland proteins are involved is yet unknown. The analysis of variance in accessory gland proteins of this species therefore further promotes the research in mating biology of ants, which still is so far a rather

unexplored field (Hölldobler and Bartz 1985; Hölldobler and Wilson 1990; Boomsma et al. 2005).

Materials and methods

Collection and laboratory rearing

L. gredleri colonies were collected with their nests in dead branches in small oak and pine stands on an abandoned army drill ground in Erlangen, Germany (49°35'09"N, 11°02'02"E). *L. acervorum*, *L. muscorum* and *H. subleavis* colonies were collected at a road parking site in Abensberg, Germany (48°49'00"N, 11°59'47"E). In the laboratory, individuals and brood were removed from their nests and put into a standard three chamber nest with an internal nest consisting of two object slides separated by a plastic spacer. The internal nests were shaded by a red cling wrap. Colonies were kept in the laboratory under standard conditions (Buschinger 1974; Heinze and Ortius 1991). Sexuals developed in the nests and were not removed until they left the internal nest at their own. After leaving the nest, the males were put in a 1ml Eppendorf tube and stored at -23°C until dissection.

Preparation of accessory glands and thorax tissue with subsequent protein sample treatment

For accessory gland protein preparation, males were dissected in a droplet of distilled water on a chilled object slide. The accessory glands were separated from the rest of the body and seminal vesicles were removed. The dissected pair of accessory glands was put in 5 µl Destreak Rehydration solution (Amersham Biosciences) mixed with 2% IPG buffer (Amersham Biosciences), where they were crushed with forceps and treated with ultrasound for 10 seconds. The samples were put on ice for immediate application on the IEF gel. Before application to the gel, samples were centrifuged for 5 min to separate the solved proteins form unsolved tissue debris. Supernatant was applied to the gel.

Thorax tissue was obtained by separating the thorax of a congealed male with clean forceps from its head and abdomen and subsequently crushing it in liquid nitrogen. The sample was dissolved in 50 µl Destreak Rehydration solution (Amersham Biosciences) mixed with 2% IPG buffer (Amersham Biosciences). Centrifugation for 15 min with 14000 Upm

before the application of the supernatant to the gel avoided contamination of the sample with chitin-debris and other unsolved tissue.

Preparation of the equipment and running of the first dimension

The isoelectric focusing gel of the first dimension was operated on a Multiphor II Electrophoresis Unit (flatbed system) (Amersham Biosciences) connected to an Electrophoresis Power Supply – EPS 3501 (Amersham Pharmacia Biotech). Each time before usage the whole equipment was cleaned with distilled water and dried with cellulose paper. The temperature of the cooling plate through the Colora WK14 thermostatic circulator was set to 3°C and started 30 min before operating the electrophoresis, so that cooling was already established when electrophoresis started.

1-2 ml Dry Strip Cover Fluid (Amersham Biosciences) was applied to the cooling plate. The gel (Servalyt Precotes 3-10, 300 µm, 125 mm x 125 mm) was cut in half and one half was positioned on the cooling plate, so that the Dry Strip Cover Fluid formed a bubble-free film beneath the gel. For a part of our samples, prefabricated Servalyt Precotes 3-10 were not available, so we had to use Blank Precotes^{TM} (Serva) and equilibrate them with a solution of 48% urea (w/v), 10% glycerol (v/v) and 8% SERVALYT^{TM} (v/v) (pH 3-10, Serva) replenished with double distilled water. After 30 min of equilibration the gel was dried for another 30 min. This treatment correlates with the preparation for the prefabricated Servalyt Precotes 3-10, so that there should be no difference between the prefabricated and self-equilibrated gels.

According to the gel size, electrode wicks were cut in half, soaked with anode fluid 3 and cathode fluid 10 (Serva Electrophoresis GmbH) and positioned on the surface of the gel. 5µl of sample was applied by dropping it onto the cathode side of the gel. To proof-correct isoelectric focusing, a standard marker (Serva Electrophoresis GmbH) was run along with the samples. The electrophoresis was conducted for 4.5 hours with 2000 V, 2 mA and 5 W for the accessory gland protein samples and for 3.75 hours with 2000 V, 3 mA and 6 W for the thorax samples. In preceding test runs this conditions revealed to be optimal for isoelectric focusing of the corresponding protein samples.

After the electrophoresis the gel was cut, so that the different samples became separated. The resulting gel-stripes of the different samples were enclosed in a test tube, sealed and stored at -70°C until the second dimension could be operated.

Equilibration of the IEF-gel-stripes

The IEF-gel-stripes were defrosted and separately put into a petri dish. In the first step of the equilibration the stripes were left each for 10 min in 50 ml equilibration solution with DTT (see Appendix 1 and 2) and in the second step the gel-stripes were put another 10 min in 50 ml equilibration solution with iodoacetamide and bromophenol blue (see Appendix 1 and 2).

Preparation of the equipment and running of the second dimension

The isoelectric focusing gel of the second dimension was also operated on a Multiphor II Electrophoresis Unit (flatbed system) (Amersham Biosciences) connected to an Electrophoresis Power Supply – EPS 3501 (Amersham Pharmacia Biotech). Each time before usage the whole equipment was cleaned with distilled water and dried with cellulose paper. The temperature of the cooling plate through the Colora WK14 thermostatic circulator was set to 8°C and started 30 min before operating the electrophoresis, so that cooling was already established when electrophoresis started. 3-4 ml Dry Strip Cover Fluid (Amersham Biosciences) was applied to the cooling plate. The gel (ExelGel® XL SDS 12-14) was positioned on the cooling plate, so that the Dry Strip Cover Fluid formed a bubble-free film beneath the gel. Then the cathodic buffer strip and the anodic buffer strip (GE Healthcare Excel Gel™ SDS Buffer Strips) were positioned on the gel.

After the equilibration, moisture was drained from the IEF-gel-strips by placing the strip with one edge at a cellulose paper for a few minutes. Then the IEF-gel-strips were put gel-side down on the ExcelGel, next to the cathodic buffer strip. In this way, two IEF-gel-strips could be placed next to each other on one SDS gel. To ensure a good contact between the IEF-gel-strips and the SDS ExcelGel, a plastic stick (275 mm x 0.7 mm x 0.7 mm) was placed onto the IEF-gel-strips. To absorb water during electrophoresis, sample application pieces (Amersham Biosciences) were placed at the ends of the IEF-gel-strips.

The electrophoresis was conducted for 2 hours with 1000 V, 10 mA and 40 W. Then IEF-gel-strips and sample application pieces were removed. Then electrophoresis was conducted for 10 min with 1000 V, 20 mA and 40 W. After this, the cathodic buffer strip was moved some millimetres towards the anodic buffer strip, so that it covered 1-2 mm of the area where the IEF-gel-strips had been removed. The electrophoresis was finished by running it another 4.5 hours with 1000 V, 20 mA and 40 W.

Silver staining, desiccation, digitalisation and comparison of the SDS gels

The SDS gel was stained according to a standard staining protocol (Appendix 3 and 4). The gel was dried with vacuum on a 50°C heating plate for half an hour. The gels were immediately scanned after desiccation with an hp scanjet 5400c scanner and the scan software hp Precisionscan Pro 3.0 using a resolution of 300dpi and a scaling of 100%.

The gels were compared by overlay of the reference gel or the gel of a sister species with the different *L.gredleri* gels using Adobe Photoshop 5.5 and 7.0. Only well-resolved protein spots were scored.

Statistical analysis

We compared protein variance in the accessory glands to the protein variance in thorax tissue by evaluating for each single spot the proportion of its presence in all samples for the respective tissue. Using these proportions for each spot, we compared the medians with a Median-Test. The median should be high when protein patterns are very similar, while high variance between the samples is expressed by a low median. Calculations were done using Statistica 6.0.

Results

We analysed the accessory gland protein pattern variation of ten *L. gredleri* males originating from ten different colonies. Additionally, two accessory gland samples of *L. muscorum*, one sample of *L. acervorum* and one sample of *H. sublaevis* could be obtained and compared to the accessory gland pattern of *L. gredleri*. To assess the variation in protein pattern of *L. gredleri* accessory glands we later compared thorax proteins of eight *L. gredleri* males from eight different colonies with each other and one *H. sublaevis* thorax protein sample.

First, we chose the accessory gland protein gel of the *L. gredleri* individual from colony 120 (Fig.1a) as reference gel because it showed all relevant protein spots very well. Therefore, a spot absent in this sample but visible in another sample would account for qualitative differences in the protein pattern between the samples. We identified 29 different protein spots. 27 spots were normally expressed in our reference gel, while two, though very faint shown in our reference sample, were included into our analysis only because they were

much stronger expressed in the *L. acervorum* sample (Fig.1c). Another sample, of a male from colony 132, showed also all 29 spots visible in the reference gel and was therefore 100% identical. All the other samples were equally very similar, showing at least 20 of the 29 spots (Table 1). In very rare cases, gel quality was not good enough to decide whether a spot could be observed. We chose to note these spots in the table as not defined (Table 1). For statistical analysis we treated these cases like missing spots, since this would only make gels of the accessory glands dissimilar, which acts contrary to our prediction that accessory gland proteins should be very similar. The thorax gels did not show such fuzzy spots. Interestingly, none of the accessory gland gels of *L. gredleri* males showed an additional spot, not shown in the reference sample. This is already a clear indication for low variance of the proteins in accessory glands of *L .gredleri* males.

When compared to the *L. acervorum* sample (Fig. 1c), almost no difference was distinguishable. In fact, not a single spot could be found which not at least could be observed in one *L. gredleri* gel, although two spots (28 and 29) were very prominent compared to their faint counterparts in the *L. gredleri* samples. However, two spots (10 and 11) which were present in 100% of all *L. gredleri* samples could not be found in the one *L. acervorum* gel. The two samples of *L. muscorum* were not as strongly expressed as the *L. gredleri* reference sample, probably because this species has smaller individuals (Fig. 1d). Apart from this, the pattern is to a large extent identical to the one of *L. gredleri*. The better expressed sample showed 25 of the 29 *L. gredleri* spots (Fig. 1d); the second sample showed 21 spots. In both samples two spots (12 and 20) were missing, but were present in all *L. gredleri* samples. No additional spot could be found, not even stronger expressed ones than in *L. gredleri* males. Finally, the *H. sublaevis* sample (Fig. 1b) showed 5 spots never observed in any of the other accessory gland samples. Nevertheless, it showed also 24 spots of the *L. gredleri* pattern, but also missed 3 spots (10, 12 and13) which were in 100% of all *L. gredleri* samples. The *H. sublaevis* sample was therefore the most divergent compared to *L. gredleri*, which reflects the relatively far relation between *H. sublaevis* and *L. gredleri* compared to *L. muscorum* and *L. acervorum* – which are direct sister species. The accessory gland protein patterns were thus remarkably conserved within the species *L. gredleri* and between its sister species, including a somewhat more distantly related species.

Conservation of proteins, however, can only be evaluated when compared to a reference dataset for proteins that should not be subject to a special form of evolution, neither rapid sexual antagonistic coevolution nor especially conserved proteins. Therefore, we chose as reference to analyse the thorax proteins of eight *L. gredleri* males and also one *H. sublaevis*

male (Fig. 2). Unlike the accessory gland protein samples, the gels of the thorax proteins were so different that a reference gel had to be created by overlaying three gels (of males from colony 272, 109 and 254) using Adope Photoshop 7.0. This artificial reference gel (Fig. 2a) showed almost all 55 spots found in the 8 different gels. Nonetheless, 6 of the 55 identified spots where only visible in one of three other gels, so that 6 of the 8 gels showed at least one spot not found in one of the other *L. gredleri* samples (Table 2). This is a clear contrast to the accessory gland protein gels where not a single gel showed a spot exclusive to a unique sample. The gel (of the male from colony 42f) that showed the most spots observed in a single sample had only 33 spots which corresponded to only 60% of all spots (Table 2). This variance in *L. gredleri* thorax protein samples was also observed when looking at the consistency of single spots. Only six of the 55 spots (11%) were found in all eight samples of *L. gredleri*, whereas eleven spots (20%) could be found in a single sample only. 30 spots (55%) were found in at least 50% of all *L. gredleri* males, while 25 spots (45%) were found in less than 50% of all animals.

The one *H. sublaevis* sample (Fig. 2b) showed 5 additional spots not observed in any *L. gredleri* gel. Nevertheless, also 31 spots observed in *L. gredleri* were found in the *H. sublaevis* gel. These spots consisted mainly of the rather conserved proteins found in more than 50% of the *L. gredleri* samples. Only one spot (18) found in more than 50% of the *L. gredleri* gels was not observed in *H. sublaevis*. However, none of the spots observed in only one *L. gredleri* sample could be found in the *H. sublaevis* gel.

To be able to compare the protein variance of both types of tissue, we calculated for each single spot the proportion of its presence in all samples for the respective tissue. With this data we conducted a Median-Test to compare protein variance. The test revealed that the two datasets are significantly different ($Chi^2 = 26.93$, $DF = 1$, $p<0.001$). In comparison, the 29 spots of the accessory gland tissue showed a median of 1.00, reflecting that more than 50% of the spots were shown in all *L. gredleri* samples (Fig. 3). The thorax tissue had a median of only 0.5, resulting in 50% of the spots that were at least found in 50% of the animals (Fig. 3). This is a clear difference in protein spot consistency which demonstrates the strong conservation of the accessory gland proteins.

Table 1 Spots observed in the ten *L. gredleri*, two *L. muscorum* and respectively one *L. acervorum* and *H. subleavis* 2D SDS gels of male accessory gland proteins.

Accessory glands																
Colony		120	102	106	134	100	107	95	94	105	132	Proportion of *L.gredleri* with spot	*L.acervorum*	*H.sublaevis*	*L.muscorum* 1	*L.muscorum* 2
Spot	1	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	3	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	4	1	1	1	1	1	0	0	1	1	1	0.80	1	1	1	1
	5	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	6	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	7	1	0	0	0	0	0	1	0	0	1	0.30	1	1	1	1
	8	1	1	1	0	1	0	1	0	1	1	0.70	1	1	1	1
	9	1	1	1	0	1	1	1	0	1	1	0.80	1	1	1	1
	10	1	1	1	1	1	1	1	1	1	1	1.00	0	0	1	1
	11	1	1	1	1	1	1	1	1	1	1	1.00	0	1	1	1
	12	1	1	1	1	1	1	1	1	1	1	1.00	1	0	0	0
	13	1	1	1	1	1	1	1	1	1	1	1.00	1	0	1	0
	14	1	1	n.d.	0	1	1	0	0	1	1	0.60	1	1	1	1
	15	1	1	1	1	1	1	1	1	n.d.	1	0.90	1	1	1	0
	16	1	1	1	1	1	1	1	0	n.d.	1	0.80	1	1	1	n.d.
	17	1	1	1	1	1	1	1	0	1	1	0.90	1	1	1	1
	18	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	19	1	1	1	1	1	1	1	0	1	1	0.90	1	1	1	0
	20	1	1	1	1	1	1	1	1	1	1	1.00	1	1	0	0
	21	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	22	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	23	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	24	1	1	1	1	1	0	1	1	1	1	0.90	1	1	1	1
	25	1	1	1	1	1	0	1	0	1	1	0.80	1	1	1	1
	26	1	1	1	1	0	1	1	1	1	1	0.90	1	1	1	1
	27	1	1	1	1	1	1	1	1	1	1	1.00	1	1	1	1
	28	1	0	0	1	0	0	0	1	1	1	0.50	1	0	0	0
	29	1	0	0	1	0	0	0	n.d.	0	1	0.30	1	0	0	0
Total number of spots		29	26	25	25	25	22	25	20	25	29		27	24	25	21
Proportion of all 29 *L.gredleri* spots		1.00	0.90	0.86	0.86	0.86	0.76	0.86	0.69	0.86	1.00		0.93	0.83	0.86	0.72

Table 2 Spots observed in the eight *L. gredleri* and one *H. sublaevis* 2D SDS gels of male thorax proteins.

Thorax Colony		209	109	42f	254	272	M27	M13	M11	Proportion of *L.gredleri* with spot	*H.sublaevis*
Spot	1	0	1	1	0	0	1	1	1	0.63	1
	2	1	1	1	1	1	0	0	0	0.63	1
	3	0	0	1	1	1	0	0	0	0.38	1
	4	0	0	1	0	1	0	0	0	0.25	1
	5	1	1	1	1	0	0	0	0	0.50	0
	6	0	0	0	0	1	1	1	0	0.38	0
	7	0	0	0	0	1	1	0	1	0.38	0
	8	1	1	1	1	0	0	0	0	0.50	0
	9	1	1	1	1	0	1	1	1	0.88	1
	10	1	0	1	1	0	0	0	0	0.38	0
	11	1	1	1	1	0	1	1	0	0.75	1
	12	0	0	0	0	1	0	0	1	0.25	0
	13	1	1	1	1	0	1	1	1	0.88	1
	14	1	1	1	1	1	1	1	1	1.00	1
	15	1	1	1	1	1	1	1	1	1.00	1
	16	1	1	1	0	1	1	1	1	0.88	1
	17	1	0	1	1	1	1	1	1	0.88	1
	18	1	0	1	1	1	1	1	1	0.88	0
	19	1	1	1	1	1	1	1	1	1.00	1
	20	0	1	0	0	0	0	0	0	0.13	0
	21	1	0	1	1	0	1	1	0	0.63	1
	22	1	0	1	1	0	1	1	1	0.75	1
	23	1	1	1	1	0	1	1	1	0.88	1
	24	0	1	1	1	0	1	0	1	0.63	1
	25	0	1	0	0	1	0	0	0	0.25	1
	26	1	0	1	1	0	1	1	1	0.75	1
	27	0	1	1	0	0	0	1	0	0.38	1
	28	0	1	1	1	1	0	1	1	0.75	1
	29	0	0	0	0	1	1	1	1	0.50	0
	30	1	1	1	1	1	1	1	1	1.00	1
	31	1	1	1	1	1	1	1	1	1.00	1
	32	0	0	1	0	0	0	0	1	0.25	1
	33	1	0	1	0	0	0	1	1	0.50	1
	34	0	0	0	0	1	0	0	0	0.13	0
	35	0	0	0	0	1	0	0	0	0.13	0
	36	0	0	0	1	1	0	0	1	0.38	0
	37	0	0	0	1	1	0	0	0	0.25	0
	38	0	0	0	0	1	0	0	0	0.13	0
	39	0	0	0	1	0	0	0	0	0.13	0
	40	1	1	1	1	0	1	1	1	0.88	1
	41	1	1	1	1	1	1	1	1	1.00	1
	42	0	1	1	1	0	1	1	1	0.75	1
	43	1	1	0	1	0	1	1	1	0.75	1
	44	0	1	0	0	0	1	1	1	0.50	1
	45	0	1	0	1	0	0	1	1	0.50	1
	46	0	0	0	0	0	1	1	1	0.38	0
	47	0	0	0	1	0	0	1	1	0.38	0
	48	0	1	1	1	1	1	0	0	0.63	1
	49	0	0	0	0	0	0	1	1	0.25	0
	50	0	0	0	0	0	1	0	0	0.13	0
	51	0	0	0	0	0	0	0	1	0.13	0
	52	0	0	1	0	0	0	0	0	0.13	0
	53	0	0	1	0	0	0	0	0	0.13	0
	54	0	1	0	0	0	0	0	0	0.13	0
	55	0	1	0	0	0	0	0	0	0.13	0
Total number of spots		23	28	33	31	24	28	30	32		31
Proportion of all spots		0.42	0.51	0.60	0.56	0.44	0.51	0.55	0.58		0.56

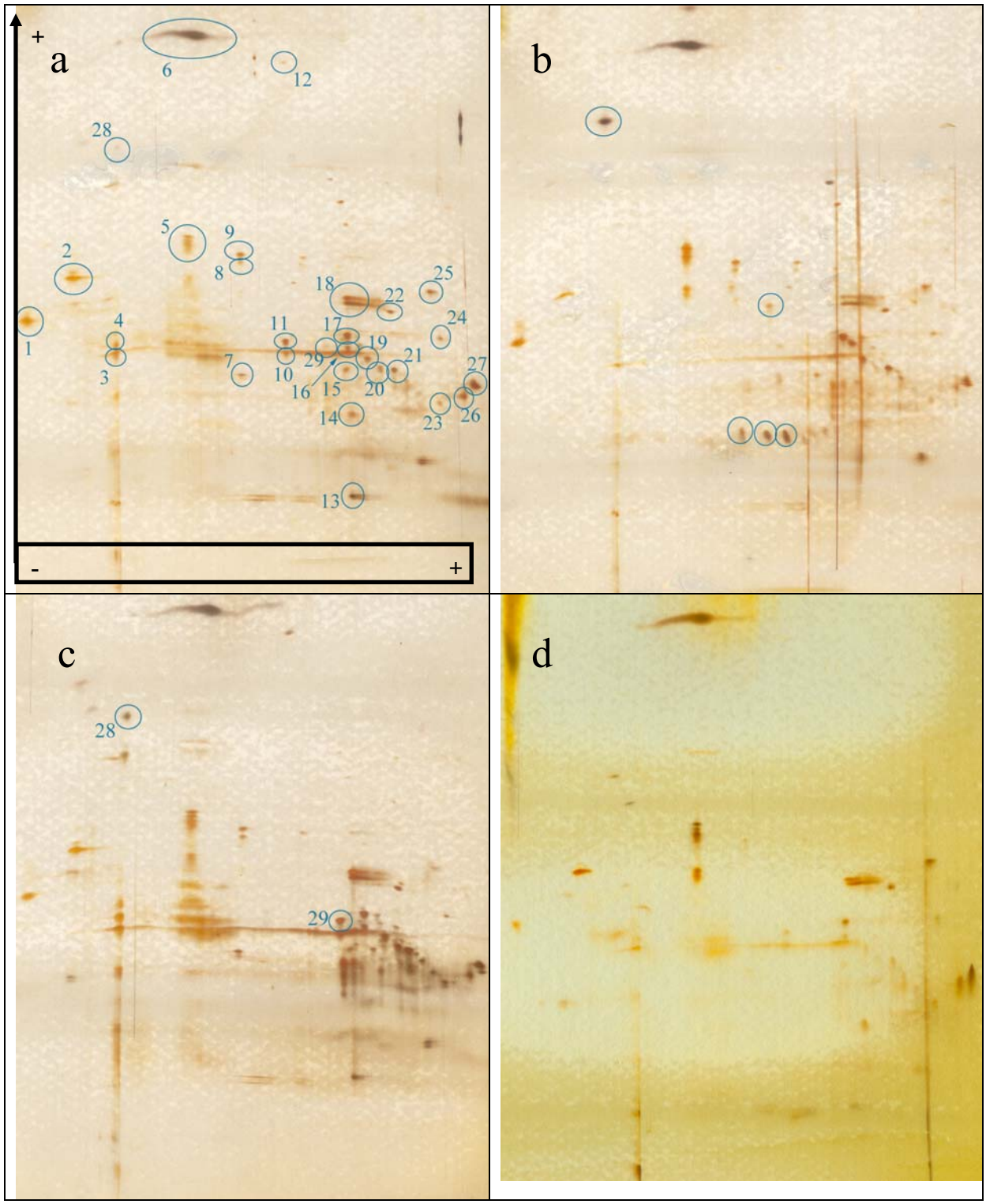

Fig.1 2D SDS gel samples of accessory gland proteins.

a) *Leptothorax gredleri* Master gel (colony 120)

b) *Harpagoxenus sublaevis* gel; additional spots are marked with circles

c) *Leptothorax acervorum* gel

d) *Leptothorax muscorum* gel

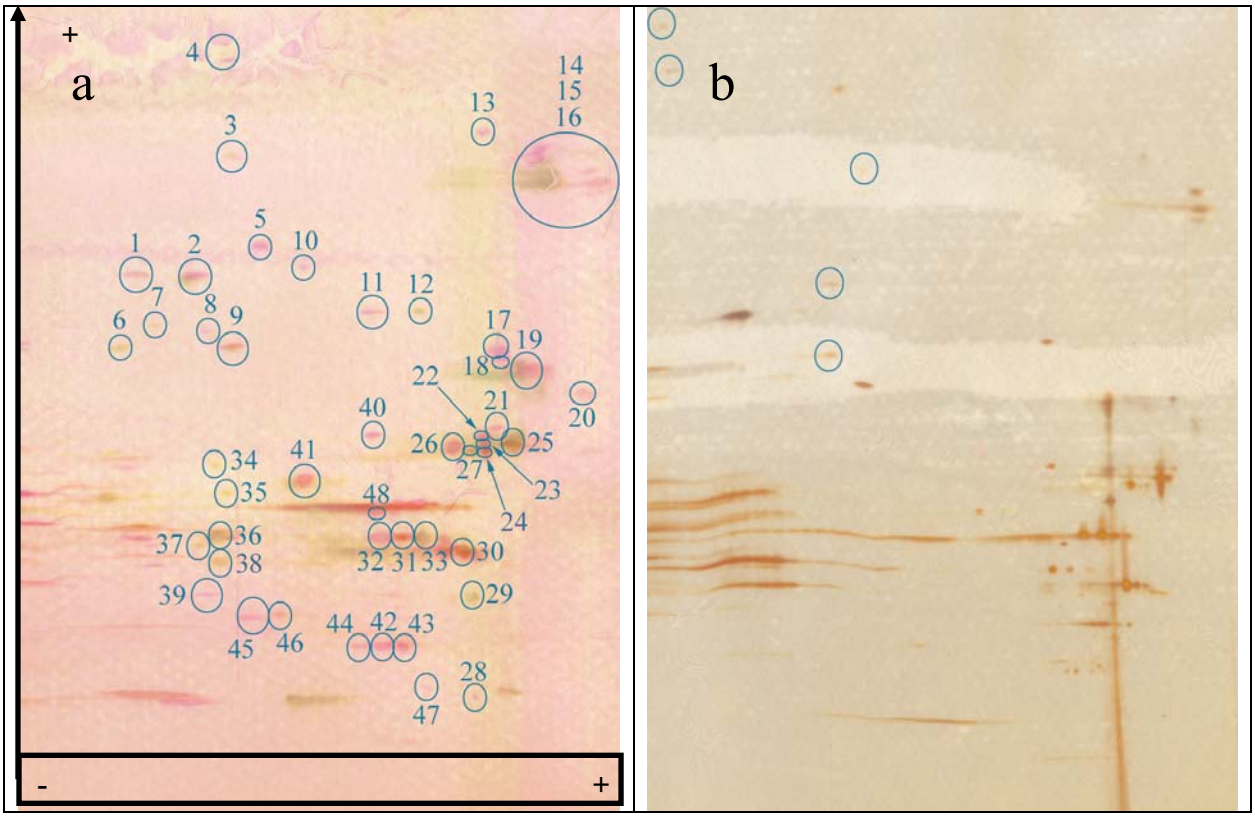

Fig.2 2D SDS gel samples of thorax proteins.

a) *Leptothorax gredleri* Master gel

b) *Harpagoxenus sublaevis* gel; additional spots are marked with circles

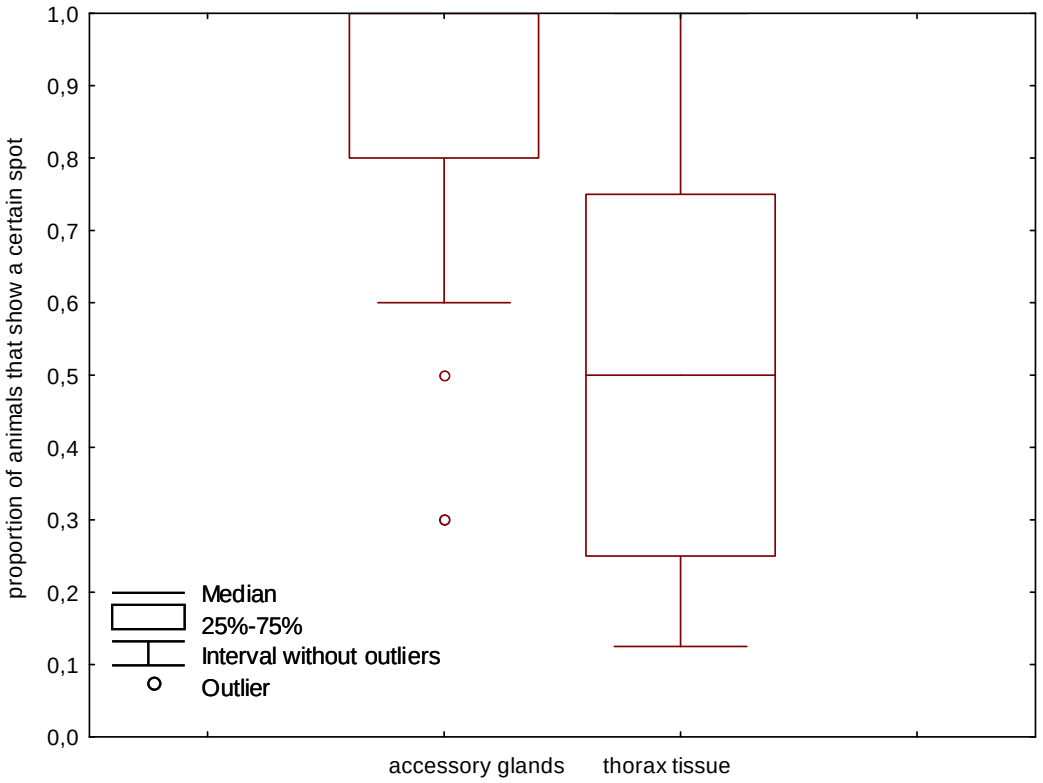

Fig.3 Box plot of proportion of animals that shows a certain spot for accessory glands and thorax tissue of *Leptothorax gredleri*.

Discussion

In *L. gredleri* we observed a lower polymorphism in accessory gland proteins than in thorax proteins. Furthermore, the divergence between the samples of *L. gredleri* and *L. muscorum* and *L. acervorum* were minor. The accessory gland proteins of *H. sublaevis* showed at least five additional spots never observed in the *L. gredleri* sample. In spite of this, the sample was still very similar to the pattern observed in the *Leptothorax* samples.

Our observations are contrary to the notion that accessory gland proteins are rapidly evolving. This has been demonstrated so far mainly in *Drosophila*, where also a high rate of accessory gland protein polymorphism was observed (Coulthart and Singh 1988; Ram and Ramesh 2007; Whalen and Wilson 1986). We explain our results in the light of the already mentioned unusual life history of ants. The interests of both sexes should be rather similar in social insects compared to other animal species and should therefore lead to reduced sexual conflict. The prevalent single mating observed in ant queens should reduce the necessity of ant males to invest into sperm competition. Generally, the sexual conflict hypothesis predicts an escalation of sexual conflict at an increased mating rate, which subsequently generates an even higher evolution rate in multiply mated species (Arnqvist et al. 2000). Interestingly, ant species with multiply mated queens have no or extremely reduced accessory glands (Mikheyev 2004). The implications of this are rather difficult to interpret. It might be possible that although accessory gland proteins regulate mating frequency in monandric species this does not result in antagonistic coevolution with the females, since their objectives might be similar to male intentions. If queens do not gain from additional matings, but rather lose time and energy, it might be favourable for both sexes to avoid additional matings, e.g. by proclaiming the mating status soon after the first copulation analogous to the immediate cuticular hydrocarbon changes in *L. gredleri* (Oppelt and Heinze 2009 - chapter two of this thesis). In this regard and related context, substances beneficial for the receiving queens might be transferred with the seminal fluids and express that sexual selection in ants might be governed rather by sexual cooperation than sexual conflict (Schrempf et al. 2005a).

Since crucial mechanisms for successful reproduction are no subject for gambling rapid evolution, the conservation of molecular pathways for seminal fluid controlled mating responses seems reasonable (Wolfner 1997). It is therefore not surprising, that in spite of rapid evolution of SRR genes, some genes evolved in specific sperm functions remain constrained (Haerty et al. 2007). Even in *Drosophila*, genes for accessory gland proteins were identified that belong to conserved protein classes (reviewed by Ram and Wolfner 2007a). These protein

classes are found in seminal fluid of animals as different as *Drosophila* and mammals (Mueller et al. 2004). Recent results indicate that these conserved proteins classes are essential for important reproductive processes (Ram and Wolfner 2007b). Interestingly, some accessory gland proteins are rapidly evolving at the primary sequence level although they attend to protein classes which show similarities between species (Mueller et al. 2004). A cross-species comparison of *Drosophila* male accessory gland protein genes demonstrated that they represent a combination of divergent and conserved proteins and thus participate to very different patterns of sequence evolution (Mueller et al. 2005). We argue that by reducing sexual conflict the life history of ants might favour the prevalence of conserved proteins in accessory glands.

Sexual selection lives from the interaction of males with females. Various studies on sperm competition and sexual conflict suggest that the interactions with females are important to maintain variation (see Chapman 2001). Females are by no means "silent partners" (Gillott 2003) and interactions can result in profound evolutionary consequences (Clark et al. 1999). In ants, females have some very important opportunities that might give them an advantage in matters of sexual conflict. As social Hymenoptera species, ants are haplodiploid, which means males are produced from unfertilized eggs, while females are diploid. As a result, also unmated queens and sometimes even workers can produce males. In *L. gredleri*, as in many other ant species, it is common that mated but also unmated queens return to their maternal nests, where they hope to inherit the nest as soon as the egg-laying queen dies or is defeated after aggressive interactions that occur often after hibernation (Heinze et al. 1992). In the meantime, they might help the related queen (often their own mother) to raise their own relatives (e.g. sisters and brothers). In this way, the returning queens can promote their own genes without reproducing on their own.

A further consequence of the haplodiploidy is that females experience a relaxed selection pressure on hybridization avoidance (Feldhaar et al. 2008), since they can always produce males – even unmated. If the hybrids are viable, cross-mated queens can additionally produce workers for their colony. Moreover, in some species there is more than one reproducing queen in a nest, which might provide a cross-mated queen with the opportunity to reproduce without a major impact on their reproductive success. This theory is in accordance with the commonness of hybridization in ants (reviewed in Feldhaar et al. 2008). Hybridization might be further promoted by the fact that according to Haldane's rule (Haldane 1922) predominantly the heterogametic sex is sterile or unviable in the offspring of species crosses. As a matter of fact, ants do not have a heterozygotic sex but only haploid and diploid

individuals. The impact of hybridization should be therefore lower in ants than in common diploid species. The few samples included in our study of accessory gland protein pattern in species related to *L. gredleri* demonstrate minor differences between these ant species. This implies that contrary to *Drosophila* (Chen et al. 1985) speciation should be resolved by other means than seminal fluid proteins, e.g., time of mating, habitat preference, etc. It would be interesting to know how much hybridization occurs in *L. gredleri* and related ant species.

Females of *L. gredleri* and also other ant species have several alternatives to normal mating: they can stay unmated, help in their maternal nest or mate with a partner of a related species while still promoting their own genes. Males should therefore be willing to be beneficial to their mates rather than to harm them. The late reproduction and lifetime commitment should decrease sexual conflict and promote cooperation (Schrempf et al. 2005a). Our results, showing low variation in the accessory gland protein pattern of *L. gredleri* compared to their thorax proteins, implicate that the seminal fluid proteins in this species are conserved and crucial for successful reproduction in this species.

Acknowledgements

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Appendix

Appendix 1 Stock equilibration solution: 0.5 mol/l Tris-HCl, pH 6.8 (quantities for a final volume of 1.0 l).

Tris	61g
1.0 mol/l HCl	460-490ml

Appendix 2 Equilibration solution (quantities for a final volume of 100ml).

Tris-HCl stock solution pH 6.8	10ml
Urea	36g
Glycerol (87%w/w)	30ml
SDS	1g
First equilibration step	300mg/50ml DTT
Second equilibration step	1.25g/50ml iodoacetamide and some grains of bromophenol blue

Appendix 3 Solutions for the silver staining.

S1	30% Ethanol / 10% HAc
S2	30% Ethanol / 0,4M NaHAc / 0,15g $Na_2S_2O_3*5H_2O$ per 100ml /2ml 25% Glutardialdehyd per 100ml pH6!
S3	0,1% $AgNO_3$ solution + 30µl formaldehyde (37%!) per 100ml
S4	2,5% Na_2CO_3 solution + 50µl formaldehyde per 100ml
S5	5% HAc solution

Appendix 4 Procedure of the silver staining.

1.	2 times 5 min in S1
2.	15 min in S2
3.	4 times 5 min rinse in distilled water
4.	20 min in S3
5.	Develop in S4 until clear appearance of the spots
6.	Stop the reaction with S5

CHAPTER 5

Differential gene expression in the accessory glands of the ant *Leptothorax gredleri*

(Manuscript)

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Keywords: RDA library, male, sexual selection, cryptic female choice, mating, spermatophore, mating plug

Abstract

As major component of the male seminal fluid in insects, accessory gland proteins regulate various factors relevant for sperm transfer and subsequent reproduction. While their role has been studied in *Drosophila* for more than a decade, similar studies in other insects lag seriously behind. Social insects have a completely different life history and are therefore expected to show a divergent pattern when it comes to reproductive strategies. In *Drosophila*, it has been shown that accessory gland proteins can harm the female and thus reduce its lifetime as a by-product of sexual antagonistic coevolution. In ants, we do not expect such a strategy, since males should avoid harming the queens, because they only produce sexuals after giving rise to several generations of workers. A reduced lifespan of queens would therefore have a direct negative impact also on male fitness. Using representational differential gene expression analysis (RDA), we studied genes preferentially expressed in accessory glands of *Leptothorax gredleri* ants. We obtained 20 unique sequences (US) and subjected them to searches in nr databases. The ortholog of one sequence has been already recorded as differentially expressed in *Drosophila* females after mating. As this gene has also been annotated in the context of immune responses, we assume that it might be a gene crucial for insect reproduction that is conserved across species. For nine US we did not find significant matches in any other species, suggesting that they might represent rapidly evolving genes that could be relevant for speciation processes.

Introduction

Different from solitary insects where females may mate repeatedly during their adult life, females of highly eusocial Hymenoptera only mate at an early age, store the sperm and then spend the remainder of their adult life laying eggs to build up the colony. In ants, a mated queen sheds her wings and completely changes her behavioural repertoire. While she was attracting males prior to mating, she should and does reject them afterwards. In addition, she has to find a nest-site or, depending on the species, may return to the maternal nest, where she has to start laying eggs for reproduction. In insects, this change in female physiology, behaviour and reproduction is known to be triggered by seminal fluids (Gillott 2003; Wolfner 2002), which are secreted by male accessory glands and are transferred during mating. Accessory gland products are known to be responsible for reduced pathogen transmission, mating plug or spermatophore formation and sperm competition (Poiani 2006; Ram and Wolfner 2007b). The major components of biologically active male accessory gland (MAG) products are proteins (Gillott 2003).

In the non-social insect *Drosophila melanogaster*, theses accessory gland proteins (Acps) have been extensively studied in recent years. Gene expression analyses of accessory gland activity identified the sequences of over one hundred Acps (Ram and Wolfner 2007a). These proteins are responsible for reduced female receptivity (Chen et al. 1988; Kalb et al. 1993; Chapman et al. 2003b; Liu and Kubli 2003), antibacterial protein transfer (Lung et al. 2001), stimulation of the female immune system (McGraw et al. 2004; Peng et al. 2005), efficient sperm storage (Neubaum and Wolfner 1999; Tram and Wolfner 1999), as well as stimulation of ovulation and egg maturation (Chen et al. 1988; Chapman et al. 2003b; Liu and Kubli 2003). They are also important for fertilization, as shown in experiments where sperm fertility of infertile mutant males lacking accessory glands could be restored when the females were subsequently mated with males that provided accessory gland proteins (but no sperm) (Xue and Noll 2000).

Besides stimulatory effects on female fertility, there are also clearly negative effects of toxic male accessory gland products on female lifespan (Chapman et al. 1995; Lung et al. 2002) as a by-product arising from sexual conflict (Chapman et al. 2003a). Differing reproductive interests of males and females might result in sexually antagonistic coevolution leading to a rapid evolution of accessory gland proteins (Rice and Holland 1997; Clark et al. 1999; Rice 2000). In fact, positive Darwinian selection could be demonstrated, when

sequences of accessory gland proteins from different *Drosophila* species were compared (Swanson et al. 2001; Mueller et al. 2005; Ram and Wolfner 2007a).

In this regard, research on accessory gland products of species other than *Drosophila* is seriously lagging behind (Gillott 2003). Only in a few other insects have accessory gland proteins been identified (Ram and Wolfner 2007a), including the honey bee *Apis mellifera* (Collins et al. 2006; Frattini-Colonello and Hartfelder 2009) as a member of the eusocial Hymenoptera. Due to the different life history of social insects, as compared to solitary ones, investigating their accessory gland proteins promises further insight into the diversity of sexual selection that acts on both parts, male and female. In social insects, females produce sexuals only after rearing several generations of workers (Boomsma et al. 2005) and thus postpone the only reproductive success relevant for male fitness. As a consequence, males should strongly avoid harming the female in any respect, since every longterm damage as a consequence of mating will inadvertently reduce the total amount of sexuals produced and, consequently, male reproductive success. Finding biological activity in male accessory gland extracts of honey bee drones that was toxic to queens thus was quite surprising (Frattini-Colonello and Hartfelder 2005). Since, however, in this polyandrous species the mating sign left by one drone in the mating chamber of the queen is quickly removed by the next one, the small amount of toxic material contained in the single last mating sign left after 15 or more matings should not negatively impact on the queen's life expectancy.

Such polyandrous mating strategies as seen in *A. mellifera* queens are, however, exceptional, and in the majority of the social Hymenoptera species, queens mate only once (Strassmann 2001). This includes the majority of ant species and should result in an even stronger reduction of sexual conflict. Holland and Rice (1999) showed the impact of monogamy on sexual conflict by enforcing monogamous mating with random mate assignment to two *D. melanogaster* populations. While males evolved to be less harmful to their mates, females became less resistant to male-induced harm in these "monogamous" populations. Moreover, monogamous populations had a greater net reproductive rate than their promiscuous counterparts.

Recent findings on ants confirm this idea of reduced sexual conflict. Rather than reducing female lifespan, as observed in *Drosophila*, mating has a positive effect on lifetime reproductive success in the ant *Cardiocondyla obscurior*. Regardless of being mated to a fertile or sterilized male, mated queens lived considerably longer and started egg laying earlier than virgin queens (Schrempf et al. 2005a). Sexual conflict might thus be replaced by sexual cooperation if favoured by the reproductive circumstances. Though being an important

fraction of social insects, ants are still underrepresented when it comes to any kind of studies on mating biology (Hölldobler and Bartz 1985; Hölldobler and Wilson 1990; Boomsma et al. 2005).

L. gredleri is an ant species that, through research over the last two decades, is now becoming established as a model system for reproductive biology in ants. It exhibits "female calling", i.e., virgin queens climb up grass stems or similar elevated positions near their maternal nests, where they attract males with a sexual pheromone (Heinze et al. 1992; Oberstadt and Heinze 2003; Oppelt et al. 2008 - chapter one of this thesis). During mating, sperm is transferred with a gelatinous substance. It stays inside the female for several hours, while the sperm is transferred to the spermatheca (Oppelt and Heinze 2007 - chapter three of this thesis). Newly mated queens change their hydrocarbon pattern within half an hour after copulation (Oppelt and Heinze 2009 - chapter two of this thesis). The mechanism of this reaction is so far unknown. Only, these changes represent no male anti-aphrodisiacs but are produced by the queens themselves. Again, we do not know what triggers this reaction in freshly mated queens. Possibly, one or a few of the substances which are transferred with the sperm induce this process. More knowledge of the substances transferred with the sperm is therefore necessary. Furthermore, queens immediately stop "calling" after mating but continue to elicit courtship from nearby males for a few minutes. This sometimes results in additional mating, (Oberstadt and Heinze 2003; Oppelt and Heinze 2009 - chapter two of this thesis) which shows that although mainly singly mated, the females of this ant species are able to mate repeatedly. The offspring of multiply mated queens is nevertheless fathered by a single male only (Oberstadt and Heinze 2003). How this exclusive paternity is realized is yet unknown, but sperm competition mediated by accessory gland proteins might be an option. Thus, investigation into the accessory gland proteins is promising to answer so far unsolved questions in this field.

In general, almost nothing is known about the accessory gland secretions of ant males and already for this reason, knowledge of the genes which are expressed in these glands could give new insights into gland and gene functions. To obtain information on gene products specifically produced by the accessory glands, we generated subtractive libraries for transcripts using a Representational Difference Analysis (RDA) strategy. Such a strategy enriches for tissue-specific transcripts by removing from the library sequences that are common in control tissue(s). In the current study we performed an RDA analysis for accessory glands of *L. gredleri* males against the remaining body carcasses from which these accessory glands had been obtained.

Materials and methods

Colony collections and male rearing

Colonies of *Leptothorax gredleri* Mayr 1855 (Hymenoptera: Formicidae) were collected from their nest-sites at the edge of an abandoned army drill ground in Erlangen, Germany (49° 35' 09" N, 11° 02' 02" E). Each single colony was transferred into a three-chamber plastic box with a cavity between two microscope slides serving as a nest. Colonies were kept under standard rearing conditions (Buschinger 1974; Heinze and Ortius 1991) until they produced sexuals.

Representational difference analysis

Male dissection

For the representational difference analysis we used exclusively male sexuals that were completely developed and ready to leave the nest. They were collected before leaving the nest and thus had not yet engaged in mating activities. Males were dissected in chilled Beadle solution (128.3 mM NaCl, 4.7 mM KCl, and 2.3 mM $CaCl_2$; Darrouzet et al. 2002). The accessory glands were separated and the seminal ducts were removed. The intestine was eliminated to avoid contamination of libraries with microorganismal RNA and then the rest of the body served as reference tissue. Glands and body carcasses were stored separately in RNAlater® (Ambion) for subsequent RNA extraction.

RNA extraction and cDNA synthesis

RNA of both the glands and the remaining body carcasses were extracted with TRIzol (Invitrogen) according to a standard manufacturer's protocol. 2µg of each RNA sample was used to synthesize the two cDNA libraries. Reverse transcription and long distance PCR were carried out with the SMART PCR cDNA synthesis kit (Clontech) resulting in double stranded cDNA.

Subtractive hybridization

In a first step, the double stranded cDNA was digested with *MboI* (restriction enzyme Fermentas) and subsequently ligated to R adaptors. A subsequent PCR created the initial cDNA representations used in the RDA protocol of Hubank and Schatz (2000). Adaptor primer sequences and PCR conditions were those used by Judice et al. (2006) and Frattini-Colonello and Hartfelder (2009) in honey bee RDA analyses. Differentially expressed

transcripts were enriched in two rounds of PCR amplification using different adaptors which were followed by a subtractive hybridization of the tester (gland) cDNA to driver (body) cDNA in proportions of 1:100 and 1:800. Purification of the cDNA profiles between these consecutive steps was realized by using the GFX PCR and Gel Band Purification Kit (GE Healthcare).

Cloning and sequencing of the resulting cDNA strands

cDNA from the two tester libraries of the second round of selection was used for ligation into pGEM®-T Easy Vector and T4 DNA Ligase and transformation of *E. coli* DH5α chemocompetent cells. The cells were reared on solid LB containing ampicillin, X-Gal and IPTG. Selected clones were grown in liquid LB medium containing ampicillin. For the sequencing, the cells were lysed and the inserts extracted and sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) with M13 primers on an ABI-PRISM 3100 (Applied Biosystems) gene analyzer.

Bioinformatics analysis

In the E-Gene annotation pipeline (Durham et al. 2005), the sequencing reads were first filtered to detect and remove ribosomal RNAs, mitochondrial DNA, as well as bacterial and fungal DNA contaminants. Subsequently, vector sequences were trimmed using Crossmatch. Read quality was checked and reads were assembled by the Phred-Phrap program module. Quality reads were submitted to a CAP3 analysis for definition of unique sequences (US). All contigs and singlets were dynamically translated and compared by BLASTX to a non-redundant (nr) database (GenBank).

Verification of differential gene expression by quantitative PCR

For real time quantitative RT-PCR (qPCR) analysis, gene-specific primers were designed for a set of selected US using the Gene Runner Version 3.05 and Primer3 softwares. As reference genes we chose the elongation factor 1-alpha (*elf1-α*) and ribosomal protein 49 (*rp49*), both of which are recommended control genes for honey bee qPCR studies (Lourenço et al. 2008). A partial sequence of the *L. gredleri rp49* ortholog sequence was obtained by a standard RT-PCR using *A. mellifera rp49* primers. The obtained fragments were cloned and sequenced. Based on the *L. gredleri* specific sequence (Table 1), we designed primers suitable for qPCR (Table 4). To obtain an *L. gredleri* sequence for *elf1-α*, we aligned the corresponding sequences of *Leptothorax muscorum* (a direct sister species to *L. gredleri*) and *Temnothorax rugatulus* (a more distantly related ant species) (GenBank accession numbers

ABK54792.1 and ABK54711.1, respectively) and designed primers to a conserved region. An *L.gredleri elf1-α* fragment was cloned and sequenced, and qPCR suitable primers were designed (Table 2). For the qPCR analyses, we collected mature males that were still inside the nest. These males were dissected in Beadle solution, as described above, separating glands and intestine-free body carcasses into RNAlater solution. Each sample consisted of 4 male bodys or 4 glands. RNA of the glands was extracted using the extraction kit Gen Elute Mammalian (Sigma Aldrich). RNA of the body carcasses was extracted using TRIzol. The RNA samples were treated with 0.1 U DNaseI (Promega) for 40 min to eliminate possible DNA contaminants. First strand cDNA was produced using a SuperScript II (Invitrogen) protocol at 42°C for 50 min and 70°C for 15 min.

Quality and annealing temperatures of the gene-specific qPCR primers were tested in a temperature-gradient PCR protocol run in a PTC200 thermal cycler (MJ Research). Product length varied from 305bp for *elf1-α* and 98bp for the shortest of the *L. gredleri* singlets. Subsequently, for each primer pair a dilution series was run using first strand cDNA from glands of mature males collected inside the nest. These and subsequent qPCR amplifications were conducted using a SYBR Green (Applied Biosystems) protocol in an ABI Prism 7500 detection system (Applied Biosystems). The protocol for amplification was: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15s and 60°C or 57°C (according to the optimal annealing temperature) for 1 min. Subsequently, (in an additional dissociation step 95°C for 15s, 60°C for 1 min and 95°C for 15 min) the melting curve was acquired. The serial dilution curves were checked for contaminants by verifying that a single melting peak was obtained in all replicas for each triplicate sample.

Results

RDA library characteristics and bioinformatics analysis of genes

In the suppression subtractive hybridization approach to detect differential expressed genes in male accessory glands of *L. gredleri* we obtained a total of 158 reads (Phred quality ≥ 20), representing 58.09% of all sequences. After passing these sequences through the EGene pipeline, and the CAP3 assembly of the valid reads, this resulted in 12 contigs and 8 singlets as unique sequences (US) (Table 1).

The BLASTX (Table 3) analyses of the US revealed significant matches (E-scores < e^{-5}, a cut-off level frequently used in EST projects; Nunes et al. 2004) for 7 of the 12 contigs and for 4 of the 8 singlets. Thus, 9 (45%) of the 20 identified sequences showed no significant match to any other species.

Most of the ESTs (47, 29.7% of all valid reads) clustered to form Contig 12. This US matches a predicted *A. mellifera* gene. The fly ortholog of this gene is annotated in Flybase as being involved in carboxylic acid metabolism. The second most frequently sequenced transcript is represented by Contig 10 with 36 reads (22.8% of all valid ones). This sequence could not be assigned to any known sequence. Contig 8 is composed of 15 reads and is also similar to a predicted *A. mellifera* gene, and there is no Gene Ontology function or process associated with in Flybase. Contig 9 was formed by 12 reads and was without significant similarity to any known gene sequence. A protein domain analysis revealed conserved elements characteristic of transmembrane proteins.

In functional terms, the searches for orthologs revealed that, apart from Contig 12, only four of the *L. gredleri* contigs actually correspond to functionally defined genes (Table 3). Two of these four sequences are similar to genes involved in energy-dependent transport processes. In one case this is a putative sodium/potassium ATPase (Contig 7, 9 reads) and in the other a mitochondrial ATPase gamma chain (Contig 5, 6 reads). The third US (Contig 1, 2 reads) was similar to a gene for which a transmembrane receptor protein tyrosine phosphatase activity is predicted in *Drosophila*. And the last US (Contig 4, 4 reads) corresponded to a gene that is functionally defined in *Drosophila melanogaster* as having a cyclin-dependent protein kinase regulator activity. Furthermore, it is known to be involved in male germ-line stem cell division and in the transforming growth factor beta receptor signaling pathway.

Of the eight singlets, only two could be assigned to functionally defined genes. The functions of the ortholog of Singlet 5 in *Drosophila* are described as inositol oxygenase activity and iron ion binding. More important, however, may be that it is one of the genes up-regulated in *Drosophila melanogaster* females after mating (Lawniczak and Begun 2004) and is mentioned in this context with immunity related genes. Finally, Singlet 8 is similar to a gene which in *Drosophila* shows hormone activity but cannot be assigned to any known biological process.

For all of these functionally defined genes an *Apis mellifera* gene was the one with the best similarity score, and for two US (Contig 2 and Singlet 2) the closest matches were with proteins predicted for the parasitic wasp *Nasonia vitripennis.*

Validation of RDA results

Primers for the obtained sequences were designed to validate the RDA results (Table 4). In a standard RT-PCR the optimal annealing temperature was determined for each primer pair (Table 5). Seven of the contig primer pairs and two of the singlet primer pairs produced a PCR product with a single band when electrophoresed in an agarose gel. When a dilution series of these PCR products were tested in quantitative PCR protocols, five contig primer pairs behaved satisfactorily producing a single prominent peak in the melting curve. These five pairs of primer are therefore usable for further validation of the RDA results.

Two of the seven singlet primer pairs produced a good PCR product and subsequent testing with quantitative PCR showed that they are also suitable for quantitative analysis. Of the two reference gene primer pairs only one, for elongation factor 1-alpha, amplified at a reasonable temperature to ensure annealing specificity. This primer also passed the quantitative PCR tests.

Altogether seven pairs of primers for RDA sequences and one pair for a reference gene showed to be suitable for further analysis. They can be applied to quantitative PCR to test whether the genes identified in the RDA analysis are in fact specifically expressed in the accessory glands of *L. gredleri* males.

Table 1 Resulting sequences (contigs and singlets) after passing the pipeline and conjunction with Crossmatch and Cap3. Number of reads and percentage of the in total 158 good quality reads.

Name	Sequence	Reads (% of 158 good reads)
Contig1'	GATCGCCACGATATTTGGGATATCGAGTAGCCGACCGCCTTTCGGCCTCGCGACAATCGCGTATCAGCAT GCGTCTGGCACTCCTCGCTGCATGAACTCATTCGATTTCGTATATGCATTCGTGCGCGTTCTGCTTACCGT ACTGTTTGGTGCGAACCGGATACGGTATCGTCGTGGCACCCTCTCCCCTTTGACTCCTCGCGGCCAGCCA GACGAAGTACAGGGTGTTCGGATAAAGTTGGCTCAAAGTGTAGTTCTCGGTGACCGGTATTCTGCGATG ATGCTTTTCCTTCGCGTAAGTATCGTTCCAGTACAGCTCATAGCTGAGAATATTCTCAGCGCTATGAGCG GGCTTGCTCCATTGGAGCGT	2 (1.3%)
Contig2'	GATCCAGATTCGTCAGCTTTCTGACGCCAGCAAAACCGGCACCGTTTGGTTGCGGATTCAGCAGCGTGAA GAGGACCGCTCTCTGGAAGAGCTGGCTTGTTTGCAGCGTTAAGACAGCCTTGGATTTTCCCACTGTTCTC ACAGTGTCGGACACCTCCCGCGATC	3 (1.9%)
Contig3'	GATCATCAATAGCAAAAAGTGATACTTCCCCAGAAAGAGCAAACGTACGACAGTTGTTATCAGTAAGGA TAAGAAACGATATTTTTTGCATCATCTGATTGGAATAATATGATACCAAATAGACATTCAATTCATTTTG ATTCAGTTGTCCCGCGATC	3 (1.9%)
Contig4'	GATCATGAGGATGCTCAAGTTTCAAGTTACGCCTGTGCATCCGCATAAGTACATGCTTCATTATCTGCGT TCTTTGCAAGCCTGGTTCGGCGAAGAAGAATGGTCCAAGTATCCGGTCGCCAAAACTAGCATGGCACTG TTGCAAGACTTTCATCATTCCCCAGCTATTCTTGACTACCCACCGAACTTAATAGCGATAGCTTGTATCAA CTTGTCATTGCAAATTTACGGCGTGGTCGTACCTCTGATGGACGAGTGTGATC	4 (2.5%)
Contig5'	GATCGCTGGTCACGGCGATGACGAGCTTCTTCGGCTTCTCCGGCGGTGCTTCAATCTCAGCCTGCTCGTA GAACACCTTTGTACCGACACCGAGAGGACGAGCCTGCCTCAAATCGCGCTCAGCTCGATTGTACTTGGCC GCCGACACCATCTTCATGGACTGAGTGATC	6 (3.8%)
Contig6'	GATCCACGTCCATCGCATCGCCCGCGTCCGTGGTGTCGGTGATGTCCGAGTCCGAATGATTGGTATTCGA GCTGGAGCTGCTCTCATCGTTGCTGAAGTTCGCGTCATCCACTATGCCGCTGTCCGTTTTTTCGCGCCCCG CGATC	7 (4.4%)
Contig7'	GATCATTCGTCGACCATCAGTTCAAAGTGTACCGCACCCTTCTTTTCGGATTTAATCGAACTGTATTGAAT ATTCTTCGCCCATGCCCGGCATTCCACGTTTATGAGCTGGTTTCTTCTTGGTCGAAGAAAATGAACGGCG ACGACGGGACTTAAGTATCCGGGAATATTTTCATATGGATAATAAAATCCTGGAAAGCCGTGTCCCCCGC GATC	9 (5.7%)
Contig8'	GATCGCGGGGACATCAAGCAGGACCACAAGGGCGAGGACGTCGACAGCTACGGGATGGGCAACAGCGC CGACCCGAAGAACATGCAGGAGCTGACGCAATACGTGCAAACGCTGCTGCAGAACATGCAGGACAAGT TTCAAACCATGTCCGACCAGATC	15 (9.5%)
Contig9'	GATCGCTGTCGACCGCTCATCCCTGGGACTTGCACGAGTGTCCCGACTCTATCGCGCTCGCATCTTTAAA CGGTGCGTCGCGACTCGCGACGCACGCGGTTCCTCCCTCCCCGTGCGCGCAGCTTAGCTCGAAAATAAAT TCTATTTCTCGGGGTCCACGGAGCGTGGGGATC	12 (7.6%)
Contig10'	GATCGAACGATAAACTTCTGCTATCGGTTCCTCAGCAATCAGTATAACGAGCGGCACATGTATTGCGGCA TATTGCAAAAAACATACCTATAAAGAAGGCTAAACTGATTGATTCAAATTAACTTGAGAATACACGATG TTGATGTTCAGTCGAAACAAATCGCTCGCGTATTGTGATC	36 (22.8%)
Contig11'	GATCGCGTGAGCGTCTCAATTATGGCGAGTCTCGAAACGACGAGGAGTGCGTGCATCGAGCTTCAGTCTT AGCGTTTAAGCGCGTGGGCAATAGTGATATACAAAAAAAATAGCCGTGACAAAACCGTGTTATCAACTT TTTGTAGCAGAAAGGCGGTACCCCGTCGAGCTGCATCGGGGATC	6 (3.8%)
Contig12'	GATCGCGGGTATCTTCGATGTAGGGCGAGTCGCGTCACCCTGACCAACCCCTCGAGGTGGTCCTCGTGGA AGGAGGAAGCGCAGTCGACGAAGCGGAAGATGTGGCTGAGCCATCTCGTCGTGTCGGCCGCGAGACGA CCCGCGAGTTTCTGCGCGTGTGACTTTGGTAGCGCTACTGCGTAAGCCGAGATAGTGTGCGTGACTAAGG CCAATTTTGCCGTTTCGGTTAGAGCTGGCAGAGCGGTGATC	47 (29.7%)
Singlet1'	ATCCATTGTGACCCTTAGGCTTAATCGAACAATCCAAAGCCCATATCATCATCTGATTCTGGTTCAGGTTC TTCTTTCTTCTTTTCTTCTTTAGCTGGTGCACTTTCTGTACTGGCAGGTGCAGCTGCTGCTGGTGCTGCTGC TGGCGCTTTTCCAACTCCTGATC	1 (0.6%)
Singlet2'	AGACTATCATTATTTTCCCCACACGAAGCACACCATGCTAGCTGTGTTTCTACATTTGGACGACACAACA CCGGAGAATGGCGGCTTGGCGGTATATCCTG	1 (0.6%)
Singlet 3'	CCCTTAAATATCCTAATGATATCGTCACCGCACGCGGTCATCAGTAAACCCGTGGTCTTACACCAGTCGA TGTCGTAAATAGTTCTCGTATGGTAGCCCGACAACGTGCAGACACACTTCCAAACAGACTCGTTATTCGG CGTAGCGACGCCCATATCGTTCCCAGGTTTATATTCCCGCGATC	1 (0.6%)
Singlet 4'	GGTGTATCTCAATCCATTTTAATAATAATCACACGCCCGATACGATTGCGTATTAAAAATGTAACATAAA TATAACTATGTTTAGCGCTTTGTCGGAATTAATGAGCGCCAAATCGGATTATTCGCAAGCGTATTATTCC AAAGTATATACGTCTTTGTGTGTGATC	1 (0.6%)
Singlet 5'	CCTTCGAGGTGCAAAAGTGCATGTAGTCGCCGCCAGCGTGCCACGGATAAAAGGAATGGTAACGAATCA TCGCCAGAGCCTCCTCGGGCAGCTTGCAGTTGTTGTGCACAAGCACGCGGTACAAATACTCGTCGTGGCC CCAGGACATGAGCAAGTTTTCTATCCCGCATTTCGGCTCGTACATGCCGTATTTCGTG	1 (0.6%)
Singlet 6'	ACGCGCGGTCCAGCCTTTCAATCTTTCTCTCGAAGTTACGCTTTACGCAACGAGATAATTAATTACGCAG CCTCATTTTTTTCACAGATACGCACGACCACGTTTATCGCTGTATACGCCGCTCTTCGACCGATGAGCCGT GGAATGAAAGACACCGAGTATATCGGTCCTGGCCCGATC	1 (0.6%)
Singlet 7'	AGGGGTGGCGGAAGGAGAAGACGGAGCGCAAAATGCGAAACAGCGAGGGTAGAACGAGAAAGAGAAA GAGAGAGAGAGAGAGAGGGAGAGAAAGGAG	1 (0.6%)
Singlet 8'	GATCGATTCGAGCAGAGACAATCGCGCTGAATGGGCGGCGCATTTGGCCATGGTAAATCGAGCACCTGT AAACAGACAGCAGAGAAATGCCTCTGACCGTCTTAATCCTCGAAATAGTACTTTTAATTTATCGTTTTTTC ATCAATATATGTTTTGCGGACGAAGAAACGATTCCCGATAAACATCTCGTGATTATTTCTGGAAAATGCT TC	1 (0.6%)

Table 2 Reference Genes

Name	Sequence	Primer
Ribosomal protein 49	TCGTCATATGTTGCCAACTGGTTTTAGAAAA GTTTTGGTANTTANTGTCAAGGAACTGGAAG TTTTAATGATGCAAAACAGAAAATTTTGTGC TGAAATTGCTCATGGTGTGAGCAGTAAAAAA CGTAAATCCATTGTTGAACGTGCTCAA	Primer–F 5'TCGTCATATGTTGCCAACTG 3' Primer–R 5'GAGCACGTTCAACAATGGAT 3' (ATCCATTGTTGAACGTGCTC)
Elongation factor 1-alpha	CATGATCACCGGTACCTCGCAGGCCGATTGC GCGGTGCTGATCGTAGCCGCTGGTACTGGTG AATTCGAGGCTGGTATTTCAAAGAATGGACA GACCCGCGAGCACGCTCTACTCGCGTTCACC CTCGGCGTCAAACAGCTGATTGTCGGAGTTA ACAAAATGGATTCCACCGAGCCCCCGTACTC CGAGACCCGATTCGAGGAAATCAAGAAGGA AGTGTCGTCCTACATCAAGAAGATTGGTTAT AACCCGGCCGCTGTTGCGTTTGTGCCGATCT CCGGCTGGCACGGAGACAACATGCTGG	Primer-F 5' CATGATCACCGGTACCTCG 3' Primer-R 5' CCAGCATGTTGTCTCCGTG 3' (CACGGAGACAACATGCTGG)

Table 3 Best matches for BlastX searches in the NCBI Database, followed by searches in Flybase whenever a gene was assigned a CG number.

Name	Best matches	E value
Contig1'	>ref|XP_397010.2| PREDICTED: similar to Leukocyte-antigen-related-like CG10443-PA [Apis mellifera] 157 The gene Leukocyte-antigen-related-like is referred to in FlyBase by the symbol Dmel\Lar (CG10443, FBgn0000464). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is described as: protein tyrosine phosphatase activity; transmembrane receptor protein tyrosine phosphatase activity. It is involved in the biological processes described with 11 unique terms, many of which group under: anatomical structure development; cell motion; cell projection organization and biogenesis; cell morphogenesis; neuron differentiation; biopolymer modification; embryonic development via the syncytial blastoderm; cell adhesion; regulation of developmental process; gamete generation. 46 alleles are reported. The phenotypes of these alleles are annotated with 21 unique terms, many of which group under: germarium; organ system; embryonic neuron; embryonic nervous system; female germline cyst; nervous system; multi-cell-component structure; egg; anatomical structure; peripheral nervous system.	2e-37
Contig2'	>ref|XP_001603752.1| PREDICTED: similar to SJCHGC05576 protein [Nasonia vitripennis] 72.4	1e-11
Contig3'	No match!	
Contig4'	>ref|XP_395803.3| PREDICTED: similar to CG31232-PA, isoform A [Apis mellifera] 185 name: kokopelli protein_coding_gene The gene kokopelli is referred to in FlyBase by the symbol Dmel\koko (CG31232, FBgn0051232). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is described as cyclin-dependent protein kinase regulator activity. It is involved in the biological processes: male germ-line stem cell division; transforming growth factor beta receptor signaling pathway.	8e-46
Contig5'	>ref|XP_625078.1| PREDICTED: similar to ATP synthase gamma chain, mitochondrial precursor [Apis mellifera] 102 The gene ATP synthase-γ chain is referred to in FlyBase by the symbol Dmel\ATPsyn-γ (CG7610, FBgn0020235). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is described as: hydrogen-exporting ATPase activity, phosphorylative mechanism; hydrogen ion transporting ATPase activity, rotational mechanism; hydrogen ion transporting ATP synthase activity, rotational mechanism. It is involved in the biological processes: proton transport; phagocytosis, engulfment; ATP synthesis coupled proton transport. 2 alleles are reported. No phenotypic data is available.	9e-21
Contig6'	No match!	
Contig7'	>ref|XP_394384.2| PREDICTED: similar to Sodium/potassium-transporting ATPase subunit beta-2 (Sodium/potassium-dependent ATPase beta-2 subunit) (Protein nervana 2) [Apis mellifera] 97.4 The gene nervana 2 is referred to in FlyBase by the symbol Dmel\nrv2 (CG9261, FBgn0015777). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is described as: sodium:potassium-exchanging ATPase activity; cation transmembrane transporter activity. It is involved in the biological processes: regulation of tube size, open tracheal system; open tracheal system development; regulation of tube diameter, open tracheal system; regulation of tube length, open tracheal system; septate junction assembly; cation transport; establishment of blood-brain barrier; potassium ion transport; sodium ion transport. 23 alleles are reported. The phenotypes of these alleles are annotated with: septate junction; embryonic/larval dorsal trunk; embryonic/larval transverse connective; embryonic/larval tracheal system; embryonic salivary gland; embryonic somatic muscle; embryonic trachea; embryonic dorsal trunk; embryonic ganglionic branch.	3e-19
Contig8'	>ref|XP_001121436.1| PREDICTED: similar to CG5446-PA [Apis mellifera] 94.7 The gene CG5446 is referred to in FlyBase by the symbol Dmel\CG5446 (CG5446, FBgn0032429). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is unknown. The biological processes in which it is involved are not known..	2e-18
Contig9'	>ref|XP_001364637.1| PREDICTED: similar to two transmembrane domain family member A [Monodelphis domestica] 35.4	1.4
Contig10'	No match!	
Contig11'	No match!	
Contig12'	>ref|XP_393418.2| PREDICTED: similar to CG1486-PA, isoform A [Apis mellifera] 154 The gene CG1486 is referred to in FlyBase by the symbol Dmel\CG1486 (CG1486, FBgn0031174). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is described as: carboxy-lyase activity; pyridoxal phosphate binding. It is involved in the biological process carboxylic acid metabolic process.	3e-36
Singlet1'	>gb|AAD40232.1| unknown [Apis mellifera] 42.0	0.015
Singlet2'	>ref|XP_001606773.1| PREDICTED: hypothetical protein [Nasonia vitripennis] 64.3	3e-09
Singlet 3'	>ref|XP_395314.3| PREDICTED: similar to CG12797-PA [Apis mellifera] 116 5e-25 The gene Ciao1 is referred to in FlyBase by the symbol Dmel\Ciao1 (CG12797, FBgn0033972). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is unknown. The biological processes in which it is involved are not known.	
Singlet 4'	No match!	
Singlet 5'	>ref|XP_392190.2| PREDICTED: similar to CG6910-PA [Apis mellifera] 149 The gene CG6910 is referred to in FlyBase by the symbol Dmel\CG6910 (CG6910, FBgn0036262). It is a protein_coding_gene from Drosophila melanogaster. Its molecular function is described as: inositol oxygenase activity; iron ion binding. It is involved in the biological process inositol catabolic process.	5e-35
Singlet 6'	No match!	
Singlet 7'	No match!	
Singlet 8'	>ref|XP_391870.3| PREDICTED: similar to CG32432-PA [Apis mellifera] 52.8 The gene CG32432 is referred to in FlyBase by the symbol Dmel\CG32432 (CG32432, FBgn0052432). It is a protein_coding_gene from Drosophila melanogaster..20878288. Its molecular function is described as hormone activity. The biological processes in which it is involved are not known.	8e-06

Table 4 Primers of contigs, singlets and control genes with the expected resulting fragment length.

Name	Pair of primers	Expected fragment length
LgCon1F LgCon1R	Primer-F 5' TTCGTGCGCGTTCTGCTTACC 3' Primer-R 5' GCATCATCGCAGAATACCGGTC 3'	166
LgCon2F LgCon2R	Primer-F 5' GATTCGTCAGCTTTCTGACG 3' Primer-R 5' ATCGCGGGAGGTGTCC 3'	158
LgCon3F LgCon3R	Primer-F 5' CCAGAAAGAGCAAACGTACG 3' Primer-R 5' GGGACAACTGAATCAAAATG 3'	123
LgCon4F LgCon4R	Primer-F 5' TCAAGTTACGCCTGTGCAT 3' Primer-R 5' CTGGGGAATGATGAAAGTCT 3'	143
LgCon5F LgCon5R	Primer-F 5' CACGGCGATGACGAGC 3' Primer-R 5' GCCAAGTACAATCGAGCTG 3'	129
LgCon6F LgCon6R	Primer-F 5' TCCACGTCCATCGCATC 3' Primer-R 5' AACGGACAGCGGCATAG 3'	126
LgCon7F LgCon7R	Primer-F 5' GCACCCTTCTTTTCGGAT 3' Primer-R 5' GACACGGCTTTCCAGGAT 3'	172
LgCon8F LgCon8R	Primer-F 5' CATCAAGCAGGACCACAAG 3' Primer-R 5' CTGGTCGGACATGGTTTG 3'	145
LgCon9F LgCon9R	Primer-F 5' CTCATCCCTGGGACTTGCAC 3' Primer-R 5' GTGGACCCCGAGAAATAGA 3'	144
LgCon10F LgCon10R	Primer-F 5' CGGTTCCTCAGCAATCAG 3' Primer-R 5' ACGCGAGCGATTTGTTT 3'	146
LgCon11F LgCon11R	Primer-F 5' ATTATGGCGAGTCTCGAAAC 3' Primer-R 5' GTACCGCCTTTCTGCTACA 3'	142
LgCon12F LgCon12R	Primer-F 5' GAAGCGGAAGATGTGGC 3' Primer-R 5' GCCAGCTCTAACCGAAAC 3'	148
S1C01-F S1C01-R	Primer-F 5' CGAACAATCCAAAGCCCATA 3' Primer-R 5' TGCACCTGCCAGTACAGAAA 3'	98
S2BO2-F S2BO2-R	Primer-F 5' AGACTATCATTATTTTCCCCAC 3' Primer-R 5' CAGGATATACCGCCAAGCC 3'	101
S3BO6-F S3BO6-R	Primer-F 5' CCTAATGATATCGTCACCGC 3' Primer-R 5' GGAATATAAACCTGGGAACG 3'	166
S4CO7-F S4CO7-R	Primer-F 5' TAATAATCACACGCCCGATAC 3' Primer-R 5' ATAATACGCTTGCGAATAATCC 3'	115
S5E02-F S5E02-R	Primer-F 5' GGAATGGTAACGAATCATCG 3' Primer-R 5' CACGAAATACGGCATGTACG 3'	145
	For Singlet 7 no primers could be designed.	
S6FO5-F S6FO5-R	Primer-F 5' GTTACGCTTTACGCAACGAG 3' Primer-R 5' CTTTCATTCCACGGCTCATC 3'	117
S8H10-F S8H10-R	Primer-F 5' GATCGATTCGAGCAGAGACA 3' Primer-R 5' TGTTTATCGGGAATCGTTTC 3'	184
LG-ELF1aF LG-ELF1aR	Primer-F 5' CATGATCACCGGTACCTCG 3' Primer-R 5' CCAGCATGTTGTCTCCGTG 3'	305
LeptRP49F LeptRP49R	Primer–F 5'TCGTCATATGTTGCCAACTG 3' Primer–R 5' GAGCACGTTCAACAATGGAT 3'	149

Table 5 Results of standard PCRs and melting curve of serial dilution curves in quantitative PCRs of the different sequences.

Fragment name	Standard PCR	Annealing temperature	Melting curve
LgCon1	OK	60°C	OK
LgCon2	No product		
LgCon3	Amplification problem	57°C	Amplification problem
LgCon4	Multiple products	57°C	Amplification problem
LgCon5	OK	60°C	Multiple peaks
LgCon6	OK	60°C	OK
LgCon7	OK	57°C	Multiple peaks
LgCon8	Multiple products		
LgCon9	Amplification problem	60°C	Multiple peaks
LgCon10	OK	60°C	OK
LgCon11	OK	57°C	OK
LgCon12	OK	60°C	OK
S1C01	No product		
S2BO2	OK	57°C	OK
S3BO6	To be repeated (multiple product)	(57°C)	
S4CO7	OK	57°C	OK
S5E02	No product		
S6FO5	To be repeated (multiple product)	(60°C)	
S8H10	To be repeated (multiple product)	(57°C)	
LG-ELF1a	OK	60°C	OK
LeptRP49	Amplification at very low annealing temperature only	48°C	

Discussion

Using the Representational Difference Analysis (RDA) method, we successfully identified a set of genes being differentially expressed in male accessory glands of the ant *L. gredleri*. Unfortunately, an "ant" genome is not yet available, so that annotation against a genome, as is possible in *Drosophila* and *Apis*, is not yet conductable. A genome comes in handy when to decide which genes might be secreted proteins, since their RNAs should have hydrophobic amino-terminal signal sequences (Harwood 1980). Swanson et al. (2001) found that 24% of the 212 identified genes in their study of *Drosophila* male reproductive proteins had such a putative signal sequence. Due to the digestion steps in the RDA procedure, no entire genes but only expression tags are obtained, even though different pieces can be combined into contigs. In our sequences we are, therefore, missing the termini of the RNA strands and, consequently, we cannot decide whether the respective genes may encode secreted proteins.

The strength of the RDA method is that it is applicable also in systems where genomic information is either completely lacking or still in an incipient stage. RDAs are able to detect both, known and so far unknown genes, being thus more comprehensive. As an "open system" they are in contrast to "closed systems" for expression analysis, such as microarrays, which are limited to the detection of whatever genes that are spotted on a slide.

An important finding with respect to the genes identified for *L. gredleri* is that all significant matches were against *A. mellifera* and *N. vitripennis* genes, emphasizing the importance of these hymenopteran reference genomes in future gene expression studies in social insects. Nevertheless, most of these genes were functionally undefined genes and, together with the "no matches", this finding is an indication of rapid evolutionary processes and species specificity in the secretion products of male accessory glands. There is already evidence for such rapid evolution in the male accessory glands of *Drosophila* (Swanson et al. 2001). About 50% of the *Drosophila* accessory gland proteins had no homologs in non-*Drosophila* species (Swanson et al. 2001; Ram and Wolfner 2007a), which corresponds very well to the 45% of sequences with no significant match in our study. The accelerated divergence of these genes directly involved in reproduction is most likely caused by positive selection that also may drive speciation processes. In fact, sexual antagonistic coevolution is seen as an "engine of speciation" (Rice 1996) with tandem gene duplication providing the genetic redundancy for the rapid evolution of seminal fluid between species (Mueller et al. 2005; Findlay et al. 2008).

However, not all seminal fluid genes can be subject to rapid evolution, since successful reproduction must be assured. Mueller et al. (2005) found in a cross-species comparison of *Drosophila* male accessory gland protein genes that there are not only rapidly evolving but also conserved genes. These conserved genes fall to a much higher extent into predicted protein functional classes than their rapidly evolving counterparts. Therefore, the authors suggested that these proteins might mediate reproductive strategies that are conserved across *Drosophila* species. Furthermore, similar protein classes are also relevant in the seminal fluid of other animals including mammals, crickets and honeybees (reviewed by Ram and Wolfner 2007b). Thus, members of conserved protein classes in the seminal fluids might be important for reproduction across organisms (Ram and Wolfner 2007b). Accessory gland proteins must consequently be considered as a combination of divergent and conserved proteins that undergo distinctly dissimilar evolutionary dynamics (Mueller et al. 2005). This culminates in genes where both, rapid evolution and conservation, can be observed, e.g. the *Drosophila* accessory gland protein ovulin (reviewed by Ram and Wolfner 2007a).

Being important for successful reproduction, conserved proteins should be mainly beneficial for males and females. One kind of proteins is quite interesting in this regard – immune defence proteins. On the one hand, accessory gland proteins include antimicrobially-active proteins (Lung et al. 2001), while on the other hand, some accessory gland proteins regulate the expression of genes with antimicrobial activity in females after sperm transfer (Lawniczak and Begun 2004; McGraw et al. 2004; Peng et al. 2005). Thus, different seminal fluid proteins might successively protect the sperm, the reproductive tracts (of both sexes) and then the eggs from microbial attack. An interesting overlap was found between known and predicted classes of seminal fluid proteins and several gene classes regulated in females by mating (McGraw et al. 2004). This observation fits to the fact that we found a differentially expressed sequence (Singlet 5) in the accessory glands of *L. gredleri*, which, as ortholog CG6910, is already known to be up-regulated in *D. melanogaster* females after mating (Lawniczak and Begun 2004). While its exact role is not yet known, it is described as involved in oxidoreductase activity and immune related gene, since it shows down-regulation after bacterial infection. For the further elucidation of its function, it might be of special interest to investigate whether the same gene is also differentially expressed in virgin and mated *L. gredleri* queens. In this regard, we agree with Poiani (2006), who states in his review about the complexity of seminal fluids that focusing on exclusively one sex, regardless of which one, will always be the wrong path. Paying tribute to the fact that females are no “silent partners” (Gillott 2003), when it comes to seminal fluids, we have to keep in mind that it is

always “an interplay of male and female factors [that] coordinates the gametes for fertilization” (Qazi et al. 2003).

Our study is a first step to elucidate at a molecular level the reproductive interplay between *L. gredleri* queens and their mates. Questions arising from this study might stimulate further investigation, which could be resolved by gene function analyses, like RNAi-mediated gene silencing, similar to the ones already realized in *Drosophila* (e.g. Chapman et al. 2003b). Independent of the method used, the first step must be to confirm our RDA results by quantitative PCR amplification. Furthermore, it would be interesting to see how expression levels change during male maturation. We recommend for the qPCRs the use of more than one reference gene, according to the results of Lourenço et al. (2008). For this end, new primers for the *rp49* gene will have to be designed and tested. This applies also to all the sequences, where no good product could be obtained so far, or where the melting curve revealed the amplification of more than one product. The occurrence of tandem gene duplicates, which are common among accessory gland proteins (Mueller et al. 2005; Findley et al. 2008), can make this effort a complicated business, which demonstrates again the value of a fully sequenced genome.

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GENERAL DISCUSSION

Sexual selection in social Hymenoptera is supposed to be very hard to study. Conspicuous traits as male ornaments and sperm displacement devices are absent and male fighting is rare (Boomsma et al. 2008). Nevertheless, this thesis will help to elucidate the interactions of males and females of the species *L. gredleri* in terms of reproductive success. Chapter one shows that males possess a male specific cuticular hydrocarbon pattern and simultaneously a colony specific pattern. Females should therefore be able to distinguish between males from their own nest – presumably brothers – and foreign males. Behavioural observations in a choice situation revealed a significant inbreeding avoidance. However, a considerable number of queens still chose to mate with nestmate males. This demonstrates that mate choice is certainly not only affected by a single attribute, but rather might be influenced by a variety of factors. Thus, sexual selection may work rather sophisticatedly and one effect might be superimposed by another. Identifying individual parameters in this "equation of mate choice" is for the present the only way to get started. The avoidance of mating with nestmates is obviously such a parameter but probably not the only one. Moreover, *L. gredleri* is an ant species that lives in a patchy environment (Heinze et al. 1992; Oberstadt and Heinze 2003). Being exposed to a restricted gene flow, the populations should suffer under the fragmentation into relatively small subpopulations. Diploid male load should be elevated compared to more panmicticly living ant species. Due to the life in a patchy environment, a species like *L. gredleri* should be under increased selective pressure to evolve an alternative system to the single-locus complementary sex determination system. So far, nothing is known about the diploid male load in *L. gredleri*. Investigation in this field might reveal whether this species in fact applies the single-locus or multi-locus complementary sex determination system or another unknown alternative. In *Cardiocondyla obscurior*, an ant species with mating inside the nest, an inbreeding experiment showed that single-locus and also multi-locus complementary sex determination with only few loci can be excluded (Schrempf et al. 2006). If sex is determined by another mechanism and diploid male load is not a problem in *L. gredleri*, a moderate discrimination against mating with brothers would be more suitable for their lives in a patchy environment than an absolute rejection of sib-mating.

This would secure that at least some queens get mated. The avoidance of inbreeding depression should, however, always favour a moderate discrimination against sib-mating.

In the second chapter, queens of *L. gredleri* showed a change in their hydrocarbon pattern after mating. A similar mechanism was already suggested by Tsuji (1996) as male strategy to inhibit queens from mating multiply. He predicted the use of a marking pheromone in ants and subsequent choosiness. His hypothesis has been proven mostly correct, only that it is not a marking pheromone but a change in the cuticular hydrocarbon pattern that signals to males that a queen has already mated. The fact that the signal is presumably linked to a physiological change of the queen after mating guarantees that it is an honest signal for males. Furthermore, this sort of signal is much more likely to evolve. Since after mating the queen has to change anyway a lot in its physiology – wings must be shed and egg production must be induced, a change in the cuticular hydrocarbon pattern might evolve as side effect that can be used by attentive males to discriminate for mated and virgin queens. Whether this creates now a subject of sexual conflict is hard to say. In the house fly (*Musca domestica*), Arnqvist and Andrés (2006) induced polyandry in females that generally mate only once. They showed that females would have a potential benefit of multiple mating through an increased lifespan that is caused by accessory seminal substances. Meanwhile, other seminal components are apparently responsible for the induction of monandry in females. To prove sexual conflict in our system imposes to demonstrate an advantage of polyandry in queens and additionally that males induce the change of cuticular hydrocarbon pattern. I assume also that the change might be induced by male seminal fluids but I rather doubt that females gain much from mating multiply. In ants there is more and more evidence for the fact that lifetime monogamy is the default breeding system (Boomsma 2007) and that multiply mating of queens is a derived trait (Boomsma et al. 2008). In *L. gredleri*, queens mate generally once but double and triple matings have been observed once in a while during mating experiments (Oberstadt and Heinze 2003; Oppelt et al. 2008 – chapter one of this thesis). The reason for the multiple mating might be that in mating experiments females encounter males in a higher density and thus in a more rapid sequence than in nature. Since the change of cuticular hydrocarbon pattern needs approximately 30 min to manifest, additional matings in the meantime might be more probable in these mating experiments. This, however, is no proof for any advantage of polyandry in *L. gredleri* queens. The change of the cuticular hydrocarbon pattern could just as well be beneficial for them, since it might reduce further harassment by males. The fact that multiply mated queens apparently only produced offspring of a single father (Oberstadt et al. 2003) indicates at least that females do not gain from obtaining diverse sperm that they can

use for fertilization. The observed pattern of the hydrocarbon change, a decrease in branched hydrocarbons and an increase in linear ones, creates a further but not exclusive explanation for the change. Females might become less conspicuous to males but also to workers, e.g. when they search re-adoption in their maternal nest. Whether this mechanism of cuticular hydrocarbon change is a sign of sexual conflict or sexual cooperation can therefore not yet be determined and further research on this topic is needed.

The third chapter elucidated the mechanisms of sperm transfer in *L. gredleri* ants. Sperm is transferred in a gelatinous substance which serves as spermatophore. The male places the ejaculate into the *bursa copulatrix* with the sperm cells in the tip of the spermatophore next to the spermathecal duct. From there, the sperm cells have to migrate into the spermatheca. It takes approximately 3 hours to fill the spermatheca because the migration process is slowed down by an extremely narrow constriction at the end of the spermathecal duct. Here, the sperm cells have to pass virtually one by one. The finding of such a structure that makes it difficult for sperm cells to reach the spermatheca is a common observation in insects and it is directly related to sexual selection processes. Eberhard (1997) suggests that for females it often might have been advantageous to complicate the entry to the spermatheca, since this could limit the sperm that is entering. Limited access of sperm could lead to discrimination between sperm, which might result in cryptic female choice. Species, whose males directly transfer the sperm into the spermatheca, like e.g. *Atta colombica* (Baer and Boomsma 2006) do not have this possibility. Whether the constriction at the end of the spermathecal duct in *L. gredleri* actually serves in this regard can only be answered by additional investigation into this topic. However, an additional indication is given by the fact that multiply mated queens only produce offspring of a single father. In this, the father could be the first mating partner as well as the second one or even the third, if the queen was triply-mated (Oberstadt and Heinze 2003).

While sperm cells are migrating to the spermatheca, the spermatophore might prevent sperm loss until the storage process is concluded. Additionally, the spermatophore could function also as a mating plug, since the entrance of the spermathecal duct should simultaneously be sealed also for subsequent ejaculates of potential second and third matings. Boomsma et al. (2005) suggested that mating plugs may be much more widespread than so far noticed, since they have been probably systematically overlooked. Whether the spermatophore in *L. gredleri* is efficient in this regard cannot be determined so far. The investigation of multiply mated queens is therefore indispensable. In *A. mellifera*, males leave a part of their abdomen together with several gland secretions as a mating sign in the females'

genital tract (Koeniger et al. 1979; Koeniger and Koeniger 2000). However, this mating sign does not prevent queens from multiple mating. *A. mellifera* is highly polyandrous. Queens mate with 15 or more males (Adams et al. 1977; Estoup et al. 1994). Actually the mating sign in *A. mellifera* might be the result of an arms race between the sexes, with males that try to secure sperm transfer and queens that evolve counter adaptations aiming for cryptic female choice (Baer 2005). In contrast to honey bees (*A. mellifera*), the mating plug observed in bumble bees (*Bombus terrestris*) is highly effective. The reason is not that the reproductive tract is thoroughly sealed off against subsequent ejaculates, but that the mating plug reduces the female's willingness to remate (Baer 2003). Only one substance (linoleic acid) is mainly responsible for this reaction (Baer et al. 2001). This mechanism seems to be highly effective, since *B. terrestris* females are monandrous although they could benefit from genetic heterogeneity of their workers (Baer and Schmid-Hempel 1999, 2001, 2003). In *L. gredleri*, the cuticular hydrocarbon pattern changes after mating (see chapter two), which might be responsible for a failure of additional matings. In my opinion, there is good reason to suppose that in *L. gredleri* this reaction is induced by a substance that is included in the gelatinous mass that accompanies the sperm cells. This, at least, would give males the opportunity to manipulate queens to be monandrous, which should be in the interest of male reproductive success.

It can be assumed that the substances responsible for the spermatophore etc. are produced mainly in the male accessory glands that are well developed in *L. gredleri*. In ant species whose queens mate multiply, male accessory glands are lost or extremely reduced (Mikheyev 2004). In the dwarf honey bees *Apis florea* and *Apis andreniformis*, sperm is transferred directly into the spermatheca (Koeniger et al. 1989) and we probably, not by chance, find reduced accessory glands in both species (Koeniger and Koeniger 1991). Accessory glands are therefore of critical importance concerning sexual selection in social insects including *L. gredleri*. Therefore, chapter four and five of this thesis addressed this topic.

The variability of accessory gland proteins was the central issue of chapter four. Two-dimensional SDS pages revealed that accessory gland proteins of *L. gredleri* are less variable than its thorax proteins. Furthermore, some samples of *L. muscorum* and *L. acervorum* as well as *Harpagoxenus subleavis* revealed only minor differences between the accessory gland protein patterns of these species. This conserved pattern in accessory gland proteins is what can be expected from the fact that males cannot invest into substances that harm the queens in any regard, since this would equally reduce their own reproductive success. Male options in

social insects are therefore restricted in this context. Boomsma et al. (2008) stated however that, apart from these imposed constraints in social insects, the system bears opportunities for innovative studies and I want to add that it might facilitate also a somehow innovative view of sexual selection. The explanation therefore comes with the following question: Why have accessory glands not become redundant then in social insects in general, especially with monandry being the prevalent mating system (Strassmann 2001)? One explanation might be that fighting for the own reproductive success can be mediated also by not harming the female. Primarily, this can be realized by avoiding mating errors. If a male secures that his sperm is reliably reaching the queens spermatheca, he already promotes his own reproduction. A mating device, produced by the male accessory glands, as the observed spermatophore (see chapter three) can avoid sperm loss. Proteins therefore should be rather conserved. Furthermore, even in the case that single mating of queens might be optimal for both sexes, this behaviour has to be induced by a certain mechanism. The mechanism of changed cuticular hydrocarbon pattern, which I have proposed already above, would still require some substances to induce the process in mated queens. These substances could be produced by accessory glands and transferred with the sperm. Again, it is rather likely that these substances are highly conserved.

Interestingly, the notion that recent sexual selection theory overestimates the probability that sexual conflict will generate sexual arms races was already proposed by others (Hosken and Snook 2005). In agreement with this idea is the observation that Parker (1979), in his early theory of sexual conflict, predicted the evolution of an antagonistic arms race only for a small proportion of cases. Moreover, conflict is totally avoided, when both sexes, in spite of having different optima, can reach their optima simultaneously, e.g. by sex limitation (Parker 2006). Consequently, sexual selection is much more than sexual conflict and could stimulate also coevolution for beneficial traits. The idea of sexual cooperation has initially been confirmed by the result that mating is beneficial for the lifespan of ant queens (Schrempf et al. 2005a). Now, the observation that seminal fluid enhances sperm viability (den Boer et al. 2008) further supports the idea that ant males pursue various beneficial strategies to enhance their own and likewise the queens' reproductive success. In this regard, it seems to be advantageous for ant males to be a "good guy".

Another reason for the benefit of being a "good guy" is that the evolutionary "power" of the sexes (see Arnqvist and Rowe 2005) seems to be biased in social Hymenoptera in favour of the queens. In contrast to the males, they have some alternatives to mating which enables them to promote their reproductive success anyway. If they stay unmated, they can be

helpful in their maternal nest and/or can try to lay unfertilized eggs that would develop into male sexuals. Moreover, they should lose far less than males by mating with a male of another related species (see chapter four and Feldhaar et al. 2008). The options of queens seem to be far more diverse than the possibilities of males. This again highlights the importance of looking on both sides, because focusing on just one sex is clearly the wrong path (Poiani 2006) to study sexual selection. Females are no "silent partners" (Gillott 2003), especially not in the female dominated group of social Hymenoptera.

The restricted potential for sexual conflict in our study object may explain also the observed minor differences between the accessory gland proteins of *L. gredleri* and its related ant species *L. muscorum*, *L. acervorum* and *H. sublaevis*. Sexual conflict, especially perpetual antagonistic coevolution between the sexes is known to produce rapid evolutionary divergence of reproductive traits (Arnqvist et al. 2000). This "engine of speciation" (Rice 1996; Rice and Holland 1997) is expected to operate in a significant minor extent for species of social Hymenoptera. To what extent seminal fluids still play a role in species separation is therefore a justified question. The degree of hybridization between *L. gredleri* and its related ant species might give an indication for the importance of sexual conflict and seminal fluids for reproductive isolation.

In the study of sexual interaction, the most successful researchers employed a diverse set of empirical methods (Arnqvist and Rowe 2005). Thus, I investigated the male accessory gland proteins of *L. gredleri* at the level of their gene expression in the fifth and last chapter of my thesis. By applying a representational differential gene expression analysis (RDA), I identified 20 unique sequences (US). Searches in nr databases revealed that nine US could not be significantly matched with any other known sequence. This finding may indicate rapid evolutionary processes, which does not necessarily mean that sexual antagonistic coevolution must be involved. However, I also found sequences that matched significantly to already recorded gene sequences. Apart from energetic relevant genes, especially one sequence delivered a very interesting match to a sequence already observed in the context of mating biology. The corresponding gene was observed to be up-regulated in females of *D. melanogaster* and its function is indicated as immune-related (Lawniczak and Begun 2004). Supposedly, this gene helps both sexes to deal with the risk of microbial attack that is always relevant when mating is realized by internal fertilization. Therefore, it is not surprising that this gene is conserved between species as different as *Drosophila* and *Leptothorax*. Conserved protein classes were already discovered for genes of the accessory glands in *Drosophila* (reviewed by Ram and Wolfner 2007a) and could be found in animals as different as

Drosophila and mammals (Mueller et al. 2004). Assumably, these conserved genes are rather genes that promote the success of mating and should be therefore beneficial for both sexes. Topics as important as immune defence, analogous to the gene I found, should be addressed within these conserved genes. Social cooperation is expected to work on these conserved genes for the benefit of the reproductive success of mating partners.

Conclusion

For this thesis I applied various methods to study sexual selection in the ant species *L. gredleri*. Behavioural observations, gas-chromatographic analyses, histological studies, protein separation and gene expression analyses helped to elucidate the sexual life of this ant. Sexual selection is working sophisticatedly in this species. Sexual conflict is not obviously observable, but a final proof for sexual cooperation also needs further investigation. I found some good arguments for the claim that males might benefit from being the "good guy". However, I am not the first referring to the fact that male and female interactions are more multi-faceted than purely "conflicting" or "cooperative" (Ram and Wolfner 2007). Sexual conflict and sexual cooperation can operate simultaneously at different aspects of an interacting couple. Only in species of social Hymenoptera, e.g. *L. gredleri*, sexual cooperation should be more striking than in other species.

SUMMARY

L. gredleri is an ant species that is mating by female calling. Virgin queens attract males with a droplet of a sexual pheromone in the vicinity of their maternal nest. Both sexes are expected to be choosy because females store the semen in their spermatheca throughout their whole lives and do not remate after this initial mating period. Males are short-lived and survive only as sperm in the spermathecae of queens. Furthermore, males possess only a fixed amount of sperm, since their testes degenerate by the time they reach sexual maturity. Mating is therefore an important event that results in a lifetime investment for both sexes. Haplodiploidy and complementary single-locus sex allocation bears the risk of producing sterile diploid males, especially with sib-mating. After the mating, queens have to found a new colony or they may return into their maternal nest, where they might have the possibility to inherit the colony. In any case, queens generally have to invest first into the production of workers and produce sexual offspring only later. This constellation restricts the possibilities for the evolution of sexual conflict and antagonistic coevolution. Males should avoid harming their mates, because this has a direct negative impact on their own reproductive success. Thus, rapidly evolving accessory gland proteins, as observed in *Drosophila*, are not expected for *L. gredleri*.

The studies showed that males, workers and virgin queens have a distinct cuticular hydrocarbon pattern. Like workers, males show additionally a colony specific pattern which could be used by queens to avoid mating with nestmates. Although mating experiments showed that mating occurred significantly more often between foreign individuals than between nestmates, discrimination was only weak and mating between nestmates was not a rare event.

After the mating, queens change their cuticular hydrocarbon pattern within only 30 min. This might explain why additional mating of queens was mainly observed only few minutes after their first copulation. The change of the hydrocarbon pattern was not caused by males marking females during copulation, since it was not detectable directly after the mating but is produced by the queens afterwards. The pattern shifted towards a lower relative amount of alkenes and branched alkanes and a higher relative amount of linear alkanes. This could help queens to be less conspicuous for males and also for nestmates when re-adoption into

their maternal nests occurs. Males could discriminate in this way against already mated queens to invest more efficiently their sperm in virgins.

During copulation, sperm is transferred with a gelatinous substance that might serve as spermatophore. The ejaculate is placed into the *bursa copulatrix* with the sperm cells in the tip of the spermatophore at the entrance of the spermathecal duct. Sperm cells have to migrate into the spermatheca which takes approximately 3 hours, because at the end of the spermathecal duct a very narrow constriction restricts the amount of sperm that passes. The sperm cells therefore have to pass this section virtually one by one. Whether this enables the queens to exert cryptic female choice and whether the spermatophore also serves as mating plug for males are questions for subsequent studies.

Two-dimensional SDS page investigations of the variability of accessory gland protein patterns in *L. gredleri* revealed that accessory gland proteins are rather conserved compared to thorax protein pattern variability. Also when compared to some few samples of closely related ant species, only marginal differences could be observed between the accessory gland protein patterns. Accessory gland proteins seem to be rather conserved, contrary to observations of *Drosophila*, where antagonistic coevolution might be the reason for rapid evolutionary change and a resulting high variability.

A representational difference analysis (RDA) of the gene expression in male accessory glands of *L. gredleri* resulted in 20 unique sequences (US). Searches in nr databases revealed that nine of these sequences did not significantly match to any known sequences of another species. Whether these represent rapidly evolving genes has to be checked. However, one sequence was already recorded to be differentially expressed in *Drosophila* females after mating. This sequence was annotated also in the context of immune defence and is therefore expected to be a conserved gene and crucial for insect reproduction.

Altogether, the results of this thesis provide further support for the notion that sexual selection in *L. gredleri* ants might be dominated rather by sexual cooperation than sexual conflict. However, research on sexual selection in ants is still in its infancy. Although sexual conflict was not discovered in this work on *L. gredleri*, a final proof for sexual cooperation requires also further verification.

ZUSAMMENFASSUNG

In *L. gredleri*, einer Ameisenart, die sich durch „Locksterzeln“ verpaart, locken Jungköniginnen in der Nachbarschaft zu ihren Herkunftsnestern die Männchen durch ein Sexualpheromon an. Von beiden Geschlechtern wird erwartet, wählerisch zu sein. Weibchen speichern die Spermien in ihrer Spermathek bis zu ihrem Lebensende und verpaaren sich kein weiteres Mal nach dieser Paarungsperiode, die bereits zu Beginn ihres Lebens stattfindet. Männchen sind kurzlebig und überdauern die wenigen Tage ihrer Paarungszeit nur als gespeichertes Sperma in der Spermathek der Königinnen. Des Weiteren besitzen Männchen nur eine begrenzte Anzahl Spermien, da ihre Hoden degenerieren, sobald sie ihre sexuelle Reife erlangen. Die Paarung ist deshalb ein wichtiges Ereignis für beide Geschlechter, welches einer Lebensinvestition gleichkommt. Haplodiplodie und die Geschlechtsbestimmung durch komplementäre Allele eines einzigen Genortes bergen die Gefahr, sterile diploide Männchen zu produzieren, v.a. bei Geschwisterpaarung. Nach der Paarung müssen die Königinnen eine neue Kolonie gründen oder in ihr mütterliches Nest zurückkehren, wo sie ggf. die Möglichkeit bekommen, die Kolonie zu erben. Auf jeden Fall müssen Königinnen normalerweise zunächst in die Produktion von Arbeiterinnen investieren und können erst später Geschlechtstiere produzieren. Diese Konstellation beschränkt die Möglichkeiten für die Entstehung von sexuellem Konflikt und antagonistischer Koevolution. Männchen sollten vermeiden ihre Partnerinnen zu schädigen, da dies auch einen direkten negativen Einfluss auf ihren eigenen reproduktiven Erfolg hätte. Deshalb wird nicht erwartet bei *L. gredleri*, entsprechend *Drosophila,* schnell evolvierende akzessorische Drüsenproteine vorzufinden.

Die Untersuchungen dieser Arbeit zeigen, dass Männchen, Arbeiterinnen und Jungköniginnen unterscheidbare kutikuläre Kohlenwasserstoffmuster besitzen. Wie bei Arbeiterinnen findet man bei Männchen zusätzlich einen Koloniegeruch, den Königinnen nutzen könnten, um zu vermeiden, sich mit Nestgenossen zu paaren. Obwohl Verpaarungsexperimente eine signifikant höhere Anzahl an Fremdpaarungen nachwiesen, war die Diskriminierung sehr gering und die Verpaarung unter Nestgenossen kein seltenes Ereignis.

Nach der Verpaarung ändern Königinnen ihr kutikuläres Kohlenwasserstoffmuster innerhalb von nur 30 Minuten. Dies könnte erklären, warum eine erneute Verpaarung der Königinnen hauptsächlich in den ersten Minuten nach der ersten Kopulation stattfand. Die Veränderung des Kohlenwasserstoffmusters wurde nicht durch eine Markierung durch die Männchen während der Verpaarung hervorgerufen, da direkt nach der Paarung noch nichts nachweisbar war. Die Königinnen selbst verursachten daher die Veränderung erst später. Das Muster veränderte sich hin zu einem geringeren relativen Anteil an Alkenen und verzweigten Alkanen und einem höheren relativen Anteil an linearen Alkanen. Königinnen könnten dadurch weniger auffällig für Männchen werden und auch bei der Wiederaufnahme in ihr mütterliches Nest besser getarnt sein. Männchen könnten die verpaarten Königinnen meiden und besser in jungfräuliche Partnerinnen investieren.

Während der Kopulation wird das Sperma mit einer schleimartigen Substanz übertragen, die als Spermatophore dienen könnte. Das Ejakulat wird in die *Bursa copulatrix* plaziert, wobei die Spermien in der Spitze der Spermatophore am Eingang des Spermathekengangs zum liegen kommen. Die Spermazellen müssen nun in die Spermathek einwandern, was in etwa drei Stunden dauert, da das Ende des Spermathekenganges eine sehr schmale Verengung aufweist und dies den Spermiendurchlauf begrenzt. Die Spermazellen müssen sprichwörtlich eines nach dem anderen einwandern. Ob dies die Königinnen befähigt, kyptische Weibchenwahl auszuüben und ob die Spermatophore auch als Paarungspfropfen dient, sind Fragen, die weitere Untersuchungen bedürfen.

Zwei-dimensionale SDS Gel-Untersuchungen der Variabilität der akzessorischen Drüsenproteinmuster von *L. gredleri* ergaben, dass akzessorische Drüsenproteine stärker konserviert sind als Thoraxproteinmuster. Auch im Vergleich mit einigen wenigen Proben nah verwandter Arten konnten nur geringfügige Unterschiede festgestellt werden. Die akzessorischen Drüsenproteine sind eher konserviert als bei der Gattung *Drosophila*, bei welcher antagonistische Koevolution der Grund für schnelle evolutive Veränderungen und daraus resultierende hohe Variabilität sein könnte.

Eine Untersuchung repräsentativer Unterschiede in der Genexpression in männlichen akzessorischen Drüsen von *L. gredleri* produzierte 20 einzigartige Sequenzen. Die Suche in nr Datenbanken ergab, dass neun dieser Sequenzen keine signifikante Analogie zu bekannten Sequenzen irgendeiner anderen Art aufwiesen. Ob dies schnell evolvierende Gene sind, muss

noch geklärt werden. Bei einer anderen Sequenz wurde dagegen bereits zuvor beobachtet, dass sie stärker in frisch verpaarten *Drosophila* Weibchen exprimiert wurde. Diese Sequenz wurde auch im Zusammenhang mit der Immunabwehr festgestellt und ist daher wahrscheinlich ein konserviertes Gen, welches für die Vermehrung bei Insekten wesentlich ist.

Zusammenfassend liefern die Ergebnisse dieser Arbeit weitere Unterstützung für die Annahme, dass sexuelle Selektion in *L. gredleri* Ameisen durch sexuelle Kooperation und weniger durch sexuellen Konflikt dominiert sein könnte. Jedoch steckt die Untersuchung von sexueller Selektion bei Ameisen noch in ihren Kinderschuhen. Obwohl sexueller Konflikt in dieser Arbeit über *L. gredleri* nicht nachgewiesen werden konnte, müssen für einen zwingenden Nachweis für sexuelle Kooperation auch noch weitere Bestätigungen erbracht werden.

Regensburg,

PUBLICATIONS, CONFERENCES AND WORKSHOPS

Peer-reviewed papers

Oppelt A, Heinze J (2007) Dynamics of sperm transfer in the ant *Leptothorax gredleri*. Naturwissenschaften 94: 781-786

Oppelt A, Spitzenpfeil N, Kroiss J, Heinze J (2008) The significance of intercolonial variation of cuticular hydrocarbons for inbreeding avoidance in ant sexuals. Animal Behaviour 76: 1029-1034

Oppelt A, Heinze J (2009) Mating is associated with immediate changes of the hydrocarbon profile of *Leptothorax gredleri* ant queens. Journal of Insect Physiology 55:624-628

Posters, talks and papers at conferences

Oppelt A, Heinze J (2006) Reproductive physiology of *Leptothorax* ants. Proceedings of XV Congress IUSSI, Washington, p 78

Oppelt A, Spitzenpfeil N, Heinze J (2006) Mate choice in the ant *Leptothorax gredleri*. 99th Annual Meeting of the German Zoological Society, Münster

Oppelt A, Heinze J (2007) Sperm transfer in *Leptothorax gredleri*, Annual Meeting of the Study Group Evolutionary Biology of the German Zoological Society, Bayreuth

Oppelt A, Heinze J (2007) Sperm transfer in the ant *Leptothorax gredleri.* 2nd Central European Myrmecology Meeting, Szeged, Hungary

Oppelt A, Heinze J (2007) Male accessory gland protein patterns of *Leptothorax gredleri* and related ants. Meeting of German section of IUSSI, Schwerte

Oppelt A, Heinze J (2008) Rapid hydrocarbon profile change in *Leptothorax gredleri* queens after mating. Meeting of Ethologische Gesellschaft, Regensburg

Oppelt A, Heinze J (2008) Male accessory gland protein patterns of *Leptothorax gredleri* (Hymenoptera: Formicidae) and related ant species. Annual Meeting of the Study Group Evolutionary Biology of the German Zoological Society, Hamburg

Oppelt A, Hartfelder K, Heinze J (2008) Mating biology of *Leptothorax gredleri* (Hymenoptera, Formicidae). International Congress of Zoology, Paris, France

Presentation at a workshop

Oppelt A (2007) Sexual selection in ants - The males' part. PhD course: Insect Chemical Ecology. Alnarp, Sweden

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