QUEEN SIZE DIMORPHISM IN ANTS

CAUSATION AND CONSEQUENCES OF BODY SIZE

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KÖNIGINNEN GRÖSSENDIMORPHISMUS BEI AMEISEN

URSACHEN UND KONSEQUENZEN VON KÖRPERGRÖSSE

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TO MY COLONY

1 Introduction

1.1 Social Insects in Evolutionary Research

Charles Darwin, who centered his theory of evolution by means of natural selection around individuals as "unit of self-interest", noted in 1859 that social insects could provide a special difficulty to his theory. Now, 141 years later, it is clear that the contrary is true. Social insects provide some of the best-studied, convincing examples of "evolution by means of natural selection" (Hölldobler & Wilson 1990; Reeve & Sherman 1993; Bourke & Franks 1995; Crozier & Pamilo 1996a). The Evolutionary Synthesis (Mayr & Provine 1980) which combined molecular genetics, population genetics, systematics and palaeontology (Futuyma 1998), set the stage for a gene-centered view of evolution (Dawkins 1986, 1989). This view that genes are the essential replicators that evolve and are passed on in the course of evolution was already anticipated by Hamilton (1964a,b), when he resolved Darwin's special difficulty by the evolutionary principle of inclusive fitness, also termed kin selection (Maynard Smith 1964).

Kin selection theory holds that genes that decrease direct reproduction (fitness) in their bearers can evolve if they cause an increase in the reproduction of relatives of the bearer (indirect fitness). The reason is that relatives would share by descent an identical copy of that gene with a probability that is equal to the degree of relatedness (Grafen 1984). If the disadvantage of helping a relative is less for direct reproduction than the increase of reproduction of this relative devalued by the relatedness coefficient between helper and receiver, the gene will spread through the population. This verbal argument has been formalised by Hamilton (1964a,b) and is now know as Hamilton's rule.

Kin selection is the only principle that explains the evolution of eusociality (Bourke & Franks 1995), with some individuals altruistically forgoing life-time direct reproduction (Hölldobler & Wilson 1990) and evolving caste-specific adaptations (Oster & Wilson 1978) despite their sterility. Certain conditions are believed to have facilitated the evolution of eusociality in different taxonomic groups, but these do not stand in opposition to kin selection. Rather, they alter parameters of Hamilton's rule and consequently change the fitness consequences of altruism. Parental manipulation of offspring, suggested in the subsocial route to eusociality (Alexander 1974), decreases the benefits for independent breeding (Crespi & Ragsdale 2000). The possession of effective defence mechanisms (e.g. stingers in Hymenoptera: Kukuk et al. 1989) and a defendable, valuable nest site (Alexander 1974; Alexander et al. 1991) are factors that increase the benefits of helping, while brood-care simply makes parent-offspring interactions more likely (Choe & Crespi 1997b). Life-history benefits of sociality, such as a "reproductive head start" (Queller 1989) or an "assured fitness return" (Gadagkar 1990), are dependent on the general life-history (e.g. maturation vs. survival times) of the species. The prevalence of evolution to eusociality in the Hymenoptera has made people aware that the genetic consequences of haplo-diploid sex-determination also facilitates eusocial evolution (Bourke & Franks 1995).

Even before Hamilton's (1964a,b) reconciliation of the social insects with evolutionary theory, the social Hymenoptera, in particular ants, have been important study systems in biology (Wray 1670; Wheeler 1910; Donisthorpe 1927). However, over the last 35 years, ants have become important model organisms to test evolutionary theory. As ants are relatively abundant in fossil amber records, their morphological evolution from wasp-like ancestors is now documented (Wilson et al. 1967). Studies on ant mosaics (Leston 1978; Hölldobler 1983) have strongly influenced the understanding of structuring in species communities, and ants are key species in the investigation of biodiversity (MacArthur & Wilson 1967; Bruehl et al. 1999).

Many studies using ants have demonstrated that competition between species can lead to either strategies for successful competition (reviewed by Hölldobler & Wilson 1990) or competition avoidance by character displacement (Klotz 1984). Character displacement seems also important in mating behavior of coexisting species and thus inbreeding avoidance (Baldridge et al. 1980) and speciation. Speciation has mainly been investigated in the context of social parasitism (Emery 1909; Buschinger 1990; Bourke & Franks 1991). Extensive work on the level of species interactions and co-evolution include social parasitism among ants (Buschinger 1986), symbioses with myrmecophiles (Hölldobler & Wilson 1990), and mutualistic symbioses with trophobionts (Hölldobler & Wilson 1990), fungi (Weber 1982) and plants (Beattie 1985). More recently, social insects have become an important model system in the study of classic parasitism (Schmid-Hempel 1998) but as yet little is known about ants.

At the intra-specific level, virtually all important issues of organismic biology have been addressed in ants. In particular, studies on communication (Hölldobler & Wilson 1990) and the optimality approach to colony design (Oster & Wilson 1978; Bonabeau et al. 1997) attained much attention. With the notion that evolution is a hierarchical process, with drift and selection acting on several levels (Maynard Smith & Szathmáry 1995; Keller 1999), the abundant studies of genetic population and colony structure (Crozier & Pamilo 1996a; Pamilo et al. 1997) have gained enormous importance. Allozymes as quasi-neutral markers were used relatively early in social insects (Crozier 1977), and today more genetic markers of different kinds exist across and within species than in any other undomesticated taxon. Accordingly, important patterns of species sub-differentiation and dispersal have been found (e.g. Stille & Stille 1993; Ross et al. 1997).

The level of selection problem, is especially intriguing in social insects because of the additional level of the colony. This level offers unchallenged opportunities to discern the genetic interests of the individual members of the larger unit (colony) and to observe their behavior. Consequently, the most prevalent area of investigation in social insects, and ants in particular, revolves around kin selection and kin conflict (Bourke & Franks 1995; Crozier & Pamilo 1996a; Frank 1997) and this area has also generated the strongest quantitative support for Darwin's theory of evolution by natural selection.

1.2 Kin selection and kin conflict in ants

Given Hamilton's concept of inclusive fitness, it becomes clear that under certain circumstances altruistic restraint from personal reproduction can be adaptive. In the scientific discussion about the origin of eusociality these conditions have been widely debated (Anderson 1984; Choe & Crespi 1997b) and several potential factors have been identified (see above). Many descriptive (Shakarad & Gadagkar 1995; Danforth & Eickwort 1997) or manipulative studies (Kukuk & Crozier 1990; Arathi et al. 1997) have investigated the importance of subsets of these factors and the main consensus reached is that social evolution in separate taxa is diverse and probably driven by different sets of parameters.

With a few derived exceptions, all ants are eusocial and the problem of the evolutionary onset of eusociality dates probably back to the Cretaceous (Hölldobler & Wilson 1990). Most modern ant workers are highly derived (for exceptions see Peeters 1991) and their reproductive options are severely constrained. Consequently, ants do not provide a particularly good study system for the investigation of the evolution and the maintenance of eusociality.

However, Hamilton's concept of inclusive fitness also predicts kin conflicts within colonies of eusocial organisms. Several potential conflicts have been identified and successfully studied in ants: in many ant species, workers are capable of producing unfertilized eggs that can develop into males (arrhenotoky), and workers and queens might disagree over male production (Hamilton 1964b; Trivers & Hare 1976; Bourke 1988; Ratnieks 1988). Although male production by workers is not commonly observed when queens are present (Bourke 1988; Bourke & Franks 1995), it is expected in monogynous (one queen), monandrous (a single mated queen) colonies from kin selection (Hamilton 1964b), as well as more formal models that take the increased male reproductive value and sex ratio effects into account (Ratnieks 1988). Three reasons have been proposed why worker production is not realized in most species: overall colony productivity might suffer disproportionately (Nonacs 1993a), queens might successfully inhibit worker reproduction (Ratnieks 1988), and the sex of queen-laid eggs might be concealed (Nonacs 1993a).

Even without workers producing males, conflict between them and queens is expected on the produced sex ratio due to asymmetries in intra-colonial relatedness (Trivers & Hare 1976; Boomsma & Grafen 1990). While queens are symmetrically related to sons and daughters, and thus favor a 1:1 sex ratio, workers in monogynous, monandrous colonies are three times more closely related to sisters than to the brothers they raise (Trivers & Hare 1976). The general observation, that population sex ratios comply better to the workers 3:1 sex ratio than to the queens' optimum has provided strong quantitative support for kin selection theory (Trivers & Hare 1976; Bourke & Franks 1995; Chapuisat & Keller 1999). On top of that, colony level sex ratio seems to vary with queen mating frequency in polyandrous populations (and hence worker relatedness asymmetry) according to the theory (Boomsma & Grafen 1990; Ratnieks

& Boomsma 1997). However, facultatively polygynous ant species do not conform so well to simple hypotheses (Aron et al. 1999a; Chan et al. 1999), probably because polygyny entails a syndrome of life-history changes (Bourke & Franks 1995).

Furthermore, there is a potential discrepancy between the interests of queens and workers regarding the sexual allocation, i.e. the investment into current sexual output versus colony maintenance (Pamilo 1991; Bourke & Chan 1999). In polygynous species with queen readoption (Bourke & Franks 1995), queens should favor a more worker-biased female brood than their workers in order to avoid being replaced early by readopted daughters (Nonacs 1988; Bourke & Chan 1999). Although Pamilo's (1991) original report of kin conflict over caste determination in monogynous, monandrous colonies was criticized by Bourke and Chan (1999), it still holds that queen and worker interests can diverge under these circumstances because individual colonies are probably not in sex ratio equilibrium even though the population might be. In populations that have a sex allocation ratio between the queen preferred 1:1 and worker preferred 3:1 worker and queen interests diverge if the colony's sex ratio is not identical to the population ratio. In colonies that specialize in females, queens would favor a decreased overall investment in sexuals, in colonies that produced more males, workers should try to decrease overall investment in sexuals (Herbers pers. commun.).

This is closely related to the conflict over caste determination pointed out by Bourke & Ratnieks (1999). These authors note that individual larvae have a lower interest of themselves developing to workers than the rest of the colony. Given sufficient self-determination of the larvae (Wheeler 1986), they should attempt to disguise their caste fate (Nonacs & Tobin 1992; Aron et al. 1999b) under certain conditions and develop into gynes, whereas existing workers and queens should oppose gyne development. Executions of young, virgin queens (gynes) in Linepithema humile (Keller et al. 1989) and apparent worker-larvae conflict in *Myrmica* (Brian 1973) have been presented as evidence for this theory (Bourke & Ratnieks 1999) but conclusive experimental evidence is lacking so far.

Finally, individual queens in polygynous colonies might reproductively compete among each other (Nonacs 1988; Keller 1993b; Keller 1996) and evolve stable levels of reproductive skew (Vehrencamp 1983). Hostility in multiple-queen colonies of ants are rare (Heinze 1993b), but in some species functional monogyny (all reproduction is monopolized by one of several fertile queens) and fighting (Buschinger 1967; Heinze & Buschinger 1989; Heinze 1993b) reveal high potential for conflict. Even in cases without overt aggression, competition can occur by pheromonal inhibition (Vargo 1992) or egg cannibalism (Bourke 1994). Comparative studies have provided some support for models of optimal skew in ants (Bourke & Heinze 1994; Keller & Reeve 1994) but it is unclear whether assumptions hold, and rigorous experimental approaches have proven difficult (Bourke et al. 1997).

In conclusion, there is great scope for evolutionary hypothesis testing in ants, especially focussing on the various forms of kin conflicts. Many excellent studies have built a solid body of empirical tests of evolutionary theory and strongly support a gene-centered view of evolution and its logical consequence, the concept of inclusive fitness. However, the development of theory in some areas far outweighs its experimental support, and care needs to be taken that assumptions of tested models are fulfilled, the experimental scope is broad enough to be transferable to natural patterns, and that data is not over-interpreted. Thus, a careful, integrative approach with sufficient background knowledge of the natural history of the model system is warranted to yield long-standing insights into the laws and forces that govern biological organization.

1.3 Queen size polymorphisms in ants

Body size is a crucial parameter that predetermines physiological properties Schmidt-Nielsen 1984, 1997), life-history (Roff 1992; Stearns 1992) and fitness (Darwin 1871; Brown et al. 1993) of all organisms. In many animal species across the major taxa, males use alternative tactics to increase their reproductive success: some males attempt to monopolize mating with a large number of females by aggressively defending a harem against their rivals, whereas others engage in quick, unnoticed "sneak" copulations with females (Thornhill & Alcock 1983; Andersson 1994; Taborsky 1994; Choe & Crespi 1997a). A famous example is provided by the marine isopod *Paracerceis sculpta* in which three alternative tactics coexist (Shuster 1992): large α-males defend cavities in sponges (spongocoels) as territorial breeding sites to which females are attracted, middle-sized β-males mimic females in morphology and behavior to gain access to these and tiny γ-males are capable of sneaking into the spongocoels just because of their small size. This system exemplifies well that body size might be considered as one of the most prevalent parameters for the adoption of alternative reproductive tactics (Clutton-Brock et al. 1979; Eberhard 1980; Thornhill & Alcock 1983; Gross 1985; Crespi 1988; Danforth 1991): smaller males often follow sneak tactics while larger males are competitive and often territorial.

Alternative reproductive phenotypes are widespread among males, but much less is known about this phenomenon in females (Carroll & Loye 1986; Gross 1996; Cunningham & Birkhead 1997). Although some purely behavioral alternative tactics in female reproduction have been reported (Caro & Bateson 1986), very few alternative female morphotypes have been described. Even though there is considerable variability in female body size, typically the only biological significances that are related to it are fertility effects (Roff 1992; Stearns 1992; e.g. Kim 1997). Recently however, evidence has been accumulating that in social Hymenoptera, particularly in ants, female alternative reproductive tactics are exceptionally abundant (Heinze & Tsuji 1995) and that they often are correlated with differences in

female body size. Queen size polymorphisms are consequently interesting with respect to alternative reproductive tactics.

Therefore, the state of knowledge about queen size polymorphism in ants at the beginning of this thesis will be reviewed (other polymorphisms are reviewed elsewhere: Buschinger & Heinze 1992). After giving a short overview over alternative reproductive tactics in females that are related to morphology, the alternative reproductive tactics in ants will be described and case studies of queen size polymorphisms in ants summarized to provide an overview of the current knowledge.

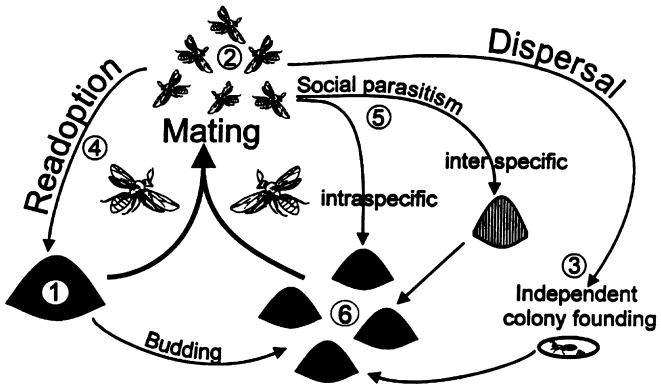
Most cases of female-specific polymorphism are as yet known from insects and they are related to a) the evolution of mimicry, b) dispersal polymorphism, or c) alternating life cycles. In some species of butterflies (Cook et al. 1994) and damselflies (Fincke 1994 and references therein) male-mimicking females coexist with cryptic, ordinary females. This system of female polymorphism is probably stable because male-like females evade sexual harassment at high male concentrations but have lower mating chances when males are scarce (Hinnekint 1987; for an alternative view see Johnson 1975). The evolution of an alternative form of non-dispersing females in insects is often linked to the loss or the reduction of wings (aptery and brachyptery; Braune 1983; Roff 1986) but not always (e.g. Lindquist & Walter 1988). In some cases, such as the cricket *Gryllus firmus* a central trade off between fecundity and dispersion capability appears to be responsible for the reduction of wings (Roff 1984). Finally, when species change their reproductive tactic between generations due to strong environmental fluctuations, this may also be correlated with differences in morphology, as shown in aphids (e.g. Moran 1992), gerbils (Clark et al. 1986), water fleas (Lynch 1980) or rotifers (Gilbert 1980). Still, examples of body size related differences in female reproductive behavior are few, apart from varying number (Parker & Begon 1986) and sex (Clutton-Brock et al. 1984) of the offspring.

1.3.1 Alternative reproductive strategies in ant queens

In eusocial insects, and ants in particular, the size dichotomy between reproductive individuals and workers is related to their different roles in the colony (Wilson 1971; Oster & Wilson 1978). Worker size is selected for efficiency in brood caring, colony maintenance, foraging, etc., while the almost invariably larger queens are specialized for mating, dispersal, colony initiation, and egg production (Hölldobler and Wilson 1990). At least the latter two select for large body size in queens: throughout the insects, egg production is strongly correlated with female body size (Thornhill & Alcock 1983; Fox & Czesak 2000). Colony founding often requires sufficient body reserves (Keller & Passera 1989, 1990; Wheeler & Buck 1995), and thus a comparatively large body size (Stille 1996).

As with other Hymenoptera, the general life cycle of ants leaves males with few options but death shortly after copulation (but see Fortelius et al. 1987; Kinomura & Yamauchi 1987; Heinze et al. 1998b). Females play the major role in reproduction, facing the formidable task of establishing a new reproducing unit, the ant colony. This can be achieved in two fundamentally different ways (Fig.1): the classic life cycle of all Formicidae is presumed to involve independent colony founding: mature colonies release sexuals into mating swarms to find a partner for copulation. After being inseminated, the young queens disperse to suitable spots where they produce their first worker offspring in isolation. These independently and claustrally founding queens rely completely on their body reserves during the initial phase of colony growth and thus are typically well-equipped with fat, muscle tissue to histolyze, storage proteins and carbohydrates (Keller & Passera 1989, 1990; Hölldobler & Wilson 1990; Wheeler & Buck 1995). In some species queens hold less body reserves that forces them to forage occasionally during the colony founding phase (independent, semi- and non-claustral colony founding), despite the high mortality risk. In any case, the success rate of solitarily founding queens typically appears to be low (Hölldobler and Wilson 1990).

Figure 1: Alternative reproductive tactics in ants. Queens have different reproductive options. The mature colony (1) releases sexuals for the mating flight (2). Prevalently, the fertilized queen searches for a suitable spot, builds a nest chamber and rears the first worker offspring in isolation (3) (independent colony founding). After successful growth the colony enters the pool of mature, reproducing colonies (6). In contrast, there are several ways of dependent founding: the newly mated queen may be readopted into her natal colony (4) or seek adoption into an unrelated colony, in some cases coexisting with the original queen(s). The exploitation of an unrelated colony is referred to as inter- or intraspecific parasitism (5).



In contrast, in a broad variety of species alternative modes of reproduction have evolved that bypass the solitary founding step (Wheeler 1933; Buschinger 1974b; Hölldobler & Wilson 1977, 1990; Keller 1991; Heinze & Tsuji 1995). Young queens seek adoption into already established colonies (secondary polygyny), which they either join permanently as additional reproductives or subsequently leave to found a new colony assisted by workers from this colony (colony budding or fission). Although the costs of producing those queens are smaller because they need fewer body reserves and consequently a smaller body size (Buschinger 1974b; Keller & Passera 1989; Stille 1996), the investment in those workers that join young queens in the budding process must at least in part be added to the reproductive

investment (Trivers & Hare 1976; Macevicz 1979; Bulmer 1983), thus considerably augmenting the per propagule cost of dependent reproduction. Socially parasitic species, where young queens invade alien colonies and temporarily or permanently exploit their work force to rear their own young, have the lowest investment cost per propagule because queens neither need large body reserves, (Keller & Passera 1989; Stille 1996) nor assistance by workers of their mother colony. Thus, small queens could be an adaptation to social parasitism or a more pronounced dispersal polymorphism, i.e. wing dimorphism.

To conclude, ant colonies invest their resources either into numerous queens or into a few propagules consisting of queens and workers, depending on environmental conditions and demographic factors. Nowhere in the animal kingdom is this central life history trade-off between number and size of offspring as apparent as in ants: while a single mature fire ant colony consisting of 200000 workers releases thousands of virgin queens during one summer (Hölldobler & Wilson 1990, p.145), an army ant colony of even greater size may split in only two units, each with a single queen (Hölldobler & Wilson 1990, p.583). Ants thus provide a promising study system for the investigation of the factors influencing parental investment and resulting female reproductive tactics. This seems particularly rewarding in species where queens may use both independent and dependent colony founding (e.g. Hölldobler & Carlin 1985), because without genetic separation between the different forms, a dynamic equilibrium has to be stabilized by current selective forces. In general, intraspecific polyphenisms give the opportunity to study phenotypic plasticity or genetic polymorphisms maintained by current selection pressures little affected by evolutionary latency. However, investigations along this line are scarce, especially in the particularly interesting case of queen size polymorphism, where behavioral, morphological and life history traits seem to be linked.

The intraspecific degree to which individual queens are specialized for dependent or independent reproduction varies considerably. In several species, worker-like queens which have secondarily lost adaptations to independent colony founding, such as wings, ocelli and the bulky thorax with well-developed flight muscles, coexist with conventional, winged queens (e.g. *Monomorium* spp., Briese 1983; *Leptothorax* sp. A, Heinze & Buschinger 1987, 1989; Heinze 1993a; for a summary see Buschinger & Heinze 1992). In the fire ant, *Solenopsis invicta*, externally indistinguishable queens may differ only in weight and behavior (Howard & Tschinkel 1978; Tschinkel 1996). In the queens of some species no morphological correlates of differences in reproductive tactic have been reported (e.g. *Leptothorax acervorum*; Douwes et al 1987; Stille & Stille 1993). Intraspecific size dimorphism of ant queens might represent a (probably stable) intermediate stage in the evolution of a stronger morphological divergence between dispersing and non-dispersing forms. However, it may be the morphological manifestation of a facultative, intraspecific social parasitism: small queens might be

efficient searchers of existing unrelated colonies without having the body reserves necessary for independent colony founding.

1.3.2 Case studies

In a small number of species from at least eight different genera in four ant subfamilies queen size is reportedly dimorphic, with small "microgynes" and large "macrogynes", which only differ, almost isometrically, in size (e.g. Fig. 2). Apart from ants, female size polymorphism in social insects is also known from termites (*Nasutitermes princeps* (Isoptera: Termitidae), Roisin & Pasteels 1985) and stingless bees (Imperatriz-Fonseca & Zucchi 1995; Nogueira-Ferreira et al. 1996). In the following, cases of size dimorphism in ants are examined individually.

1.3.2.1 *Myrmica*

The myrmicine genus *Myrmica* provides some of the best studied examples of queen size polymorphism so far (Bourke & Franks 1991). In *Myrmica rubra*, the microgyne form seems to parasitize the work force produced by macrogynes and is now considered to be a separate species, *M. microrubra* (Seifert 1996; but see Buschinger 1997): the gene pools are separated (Pearson & Child 1980), thus the queen morph is presumably genetically determined, despite an effect of the queen/worker ratio in a colony on the size of queens it produces (Elmes 1974). The microgynes generally co-occur with macrogynes and specialize in sexual reproduction (Elmes 1976; Pearson & Child 1980). They produce nearly forty times as many queens as macrogynes (Elmes 1976) probably because microgynes induce gyne suppression while their daughters are immune to this effect (facilitated by their smaller size: Nonacs & Tobin 1992). Complementary data on reduced survival of colonies with only microgynes and a distinct development of microgyne offspring have been collected from a Belgian population (Cammaerts et al. 1987).

By contrast, *Myrmica ruginodis* constitutes a single species in which the dichotomous queen size has been attributed to a dispersal polymorphism (Elmes 1991b). Direct observation of the mating biology (Brian & Brian 1955; Kasugai et al. 1983) and a close link between social structure and queen morph (monogynous macrogynous versus polygynous microgynous colonies) suggest that microgynes preferentially return to their mother colonies after mating in the vicinity (secondary polygyny). However, Elmes (1991b) reports that microgynes are found in mating swarms without indication for assortative mating, and there is no evidence for a genetic differentiation between the two morphs (Seppä 1992, 1994). In European populations the two forms show overlapping size distributions and occur sympatrically probably throughout the range of *M. ruginodis* (Elmes 1991b; Seppä 1992). In Britain, about 25% of nests contain both queen types, however the majority of microgynes live and produce workers independently (Bourke & Franks 1991). *M. kotokai* (considered a subspecies of *M. ruginodis* by

Onoyama 1989) also has macro- and microgynes (Mitzutani 1981), with one population lacking macrogynes altogether (Kasugai et al. 1983).

1.3.2.2 *Solenopsis*

Alternative queen forms have been described in two species of fire ants, *Solenopsis geminata* (McInnes & Tschinkel 1995) and S. *invicta* (Tschinkel 1996), both, however, only from a single population. The two 'morphs' in *S. invicta* differ only in weight (30%), while in *S. geminata* true microgynes are found. As the social structure in both investigated populations is strictly monogynous, this case is fundamentally different from *Myrmica*: the authors suggest that the lighter or smaller queens are not capable of independent colony founding but instead take over orphaned colonies. As these will mostly be unrelated, this strategy amounts to an intraspecific, temporal parasitism (Tschinkel 1996). Microgynes also might accidentally become unfertilized replacement queens in their natal colonies when their mother dies. In both *Solenopsis* species macro- and microgynes swarm in different seasons, however the timing of microgynous swarming flights differ between the two species, as does the extent to which the microgynous tactic is adopted. In *S. invicta* few lighter gynes are produced (Tschinkel 1996) and the frequency of colony usurpation was estimated to be 0.7% per colony per year (DeHeer & Tschinkel 1998). This is frequent enough for the authors to suggest "the origin of polygyny in North-American *Solenopsis invicta* could be explained by the adoption of multiple replacement queens into orphaned monogynous colonies" (DeHeer & Tschinkel 1998). In *Solenopsis geminata* the population allotment of energy to microgynes, as well as the percentage of colonies headed by them are as high as 35%. Thus fitness payoffs for both alternative tactics may be equal (McInnes & Tschinkel 1995) which has also been reported for *S. invicta*.

From observations that queens produce mainly daughters of the same morph as themselves the authors conclude that there may be a genetic basis to the queen size dimorphism in *S. geminata* (McInnes & Tschinkel 1995). However, this needs further testing, as one in seven colonies generated both morphs and social and genetic effects are known to play a role in queen phenotype in *S. invicta* (Keller & Ross 1993; DeHeer et al. 1999).

1.3.2.3 *Leptothorax*

In addition to several cases of pronounced queen polymorphism in the subgenus *Leptothorax* (s. str.) (L. sphagnicolus from Québec, *L.* sp. A from north-eastern North America, *Leptothorax* "*muscorum*" H from Colorado, and probably also *L. oceanicus* from East Siberia, (Francoeur 1986; Heinze & Buschinger 1987, 1989; Heinze 1989; Kupyanskaya 1990), the genus *Leptothorax* provides several examples of queen size polymorphism which have been investigated to various degrees. Microgyny has been claimed for several *Leptothorax* species (Wheeler 1937; Stitz 1939). However, it appears that in *Leptothorax* (s. str.) most, if not all microgynes are in fact separate socially parasitic species, such as *L.*

faberi, *L. goesswaldi*, *L. kutteri*, and *L. pacis* (in part previously considered to belong to its own genus, Doronomyrmex, Heinze 1995b) (Kutter 1945, 1967; Buschinger 1965, 1982).

Some data exist on microgyny in the European species *L.*(M.) *interruptus* and *L.*(M.) *corticalis* (Seifert 1996, unpublished). While the small data set for *L. corticalis* only indicates high variability in queen size, the queen size distribution of *L. interruptus* seems to be bimodal and non-overlapping. As in *Myrmica ruginodis*, microgyny appears to be correlated with polygyny (Seifert, pers. comm.). Herbers (1984) presents some data on the highly variable queen size in *L.*(M.) *longispinosus*, yet the bimodality of the size frequency distribution is not clear.

The best studied examples in this group are *L.* (M.) *spinosior* (Hamaguchi & Kinomura 1996) from Japan and the North-American *L.* (M.) *rugatulus* (Rüppell et al. 1998) In both species the queen size is clearly bimodally distributed and fits two overlapping normal distributions (e.g. Fig. 2). Despite the fact that some colonies with both macro- and microgynes exist, most monogynous colonies are headed by a single macrogyne, while polygynous colonies most often contain several microgynes. In the population studied by Hamaguchi and Kinomura (1996) microgynes constitute about two thirds of all queens collected. In *L. rugatulus*, the frequency distribution of queen size appears to be the opposite because averaged over 14 populations, macrogynes were found twice as often as microgynes. In two sample sites, however, microgynes were found more commonly. While morphological investigations on *L. rugatulus* did not reveal any reductions of flight relevant structures in microgynes, preliminary microsatellite data in *L. spinosior* suggested restricted dispersal because queens within colonies are highly related (Hamaguchi & Kinomura 1996). No genetic separation between the two morphs could be found. In *L. rugatulus* the ovaries of both queen morphs consist of a total of eight ovarioles as opposed to two in workers and neither egg size differs between macro- and microgynes, nor does the size of workers and males they produce (Rüppell et al. 1998). Generally, there was a good correlation between mother and daughter size in both species, although occasionally microgynes matured in colonies with only macrogynes.

1.3.2.4 Other Examples

Well established, further examples of queen size polymorphism are taxonomically dispersed. Satoh (1989) presents a trimodal pattern of queen sizes in the *Camponotus nawai* complex . However, the largest queens, found in monogynous nests only, represent a distinct species, *Camponotus nawai* (s. str.) (Terayama & Satoh 1990). Still, queens from polygynous nests (= *C. yamaokai*) separate into two size groups, which differ over 10% in their mean head width. Indiscriminately, Satoh (1989) suggests budding as the reproductive mode of the polygynous form and high levels of within-colony genetic relatedness (Satoh et al. 1997) support the hypothesis of secondary polygyny by readoption in this species. As the second documented example from the Formicines, the queens of an Australian weaver

ant, *Polyrhachis* cf. *doddi*, have been reported as size-dimorphic (Bellas & Hölldobler 1985). However, a subsequent study, found that thorax structure and wing development varied strongly with size (Heinze & Hölldobler 1993). Furthermore, bimodality of queen size distribution was not well substantiated.

Two other reports of queen size polymorphism exist from two further subfamilies: *Ectatomma ruidum* (Ponerinae) and *Pseudomyrmex veneficus* (Pseudomyrmecinae) are both clearly queen-dimorphic. Despite their worker-like size, the microgynes of *Ectatomma ruidum* seem capable of starting their own colonies independently because they forage during colony founding (semi-claustral colony founding), even though the success of macrogynes is probably higher (Schatz et al. 1996a; Lachaud et al. 1999). This is reflected in fecundity: macrogynes possess more ovarioles. On the other hand, microgynes are reported to have better flight abilities (Schatz et al. 1996b; Lachaud et al. 1999). Smaller colonies seem to produce microgynes while larger specialize on macrogynes, suggesting that the body size of queens is determined by non-genetic factors in this species. Schatz et al. (1996b) report functional monogyny in polygynous nests, however they do not convey any information about whether one of the morphs predominates reproduction in mixed nests.

Pseudomyrmex veneficus might be only one species among several in its genus with size-polymorphic queens (Janzen 1973; Ward, pers. comm.). As in *E. ruidum* morphs do not overlap in size. Microgynes are believed to have advantages when entering the thorns of acacia trees that in this species are used as nest sites, and collection data suggest that microgynes explore the immediate surroundings, while macrogynes potentially specialize on long-range dispersal. This could parallel the reproductive strategy of the acacia trees (Janzen 1973).

Sundström (1995) reports that queens from polygynous and monogynous colonies of *Formica truncorum* differ in mating behavior and in head width. However, the latter difference is so small that a clear bimodality is unlikely. For some further ant species only minimal data exist, if at all. Reports exist of occasional production of microgynes under aggression in *Acromyrmex crassispinus* (Fowler 1977) and laboratory culture in *Atta cephalotes* (Jutsum & Cherret 1977). The queens of the African pseudomyrmecine *Tetraponera tessmani* (formerly *Viticola tessmani*) were reported to be size-dimorphic (Wheeler 1922; Bequaert 1922, in Janzen 1973) and the same was mentioned for the palaearctic formicine *Formica fusca* (Donisthorpe 1927). Additionally, queen size in some *Tetramorium* species seems unusually variable (Sanetra, pers. comm.).

1.3.3 Summary

Queen size polymorphisms in ants clearly relate to alternative reproductive tactics that lead to different life histories of macro- and microgynes. However, not many conclusive studies exist and the only common conclusion that emerges so far is that the microgynes preferentially employ some form of

dependent colony foundation, whereas macrogynes mainly found their colonies independently. The heterogeneity of findings beyond this point is partly explained by the heterogeneity of the underlying studies. Furthermore, the number of cases to be included is debatable, as data on the queen size distribution are not available for all species (e.g. Donisthorpe 1927), or the bimodality of the data has not been tested (e.g. Herbers 1984). Moreover, microgynes might represent a distinct socially parasitic species co-occurring with the macrogyne host queens in some cases (Buschinger 1990; Bourke & Franks 1991). Nevertheless, the presented evidence for the major hypotheses (dispersal polymorphism and social parasitism) will be summarized.

Microgyny presumably has the potential to evolve into both social parasitism and a morphologically more pronounced dispersal polymorphism that might entail speciation into polygynous and monogynous sibling species (Hölldobler & Wilson 1977; Brian 1983; but see Ward 1989). On the other hand, the co-occurrence of macro- and microgynes over a large geographical range in some species (M. *ruginodis* and *L. rugatulus*) suggests that in these cases the dimorphism is rather stable and not a mere transitory stage. A possible scenario is that mating occurs on the wing, but the mode of colony founding has changed, although it is difficult to see why dependent colony founding should not eventually entail a reduction of mating flight and wings in females, given the difficulty to relocate the natal colony and the flexibility of this trait (Sundström 1995). Whether a stable polymorphism or an evolutionary switch point, further investigations should enable us to understand the involved selection pressures in more detail.

1.3.4 Microgynes as "intraspecific parasites"

Both studies in *Solenopsis* explicitly propose small body size of queens to be an adaptation to intraspecific social parasitism. On the other hand, body weight of *S. invicta* is also related to a dispersal polymorphism (Porter et al. 1988; DeHeer et al. 1999) and the overall evidence for social parasitism is not convincing. In contrast to other size-polymorphic species, different queen morphs in the two *Solenopsis* species swarm in different seasons. This mechanism of pre-mating isolation between different size classes might lead to sympatric speciation and to interspecific parasitism (see Buschinger 1990). In South America, *Solenopsis* is indeed parasitized by several workerless species which apparently are closely related to their hosts (Silveira-Guido et al. 1973; Wojcik 1990). From theoretical considerations and the comparison between *S. invicta* and *S. geminata*, Tschinkel (1996) concludes that "a shift from independent to parasitic founding is driven by the degree of habitat saturation to which the species is typically exposed". However, exactly this line of argument has been put forward to explain the transition from monogyny to secondary polygyny (Herbers 1986; Bourke & Franks 1995; DeHeer & Tschinkel 1998, see below).

The conclusion that microgyny is a likely stepping stone in the evolution of inquilinism has also been reached in the genus *Myrmica* (Pearson 1981; Buschinger 1990; Bourke & Franks 1991): the abundance

of small inquilines in this group provides suggestive evidence and the case of intraspecific social parasitism is well supported in M. *rubra* / *microrubra*.

1.3.5 Size polymorphism as morphological correlate of alternative dispersal tactics

In *Myrmica ruginodis* and *Leptothorax spinosior* no genetic differentiation between the two queen morphs could be shown (Bourke & Franks 1991; Hamaguchi et al. 1998). Moreover, some genetic evidence exists in these species, and, additionally in *Camponotus yamaokai* that in polygynous colonies queens are on average highly related. This strengthens the hypothesis that readoption of related queens is much more important than intraspecific parasitism for the establishment of polygynous (mixed) colonies in these species with size-polymorphic queens. However, a high average relatedness coefficient between queens does not exclude rare adoption events of unrelated queens (Stille & Stille 1993). In order to evaluate the facultative tactic of parasitizing macrogynes it needs to be shown how often unrelated microgynes are adopted and whether they bias the colony reproductive output in their favor.

Readoption of daughter queens was also suggested for *Polyrhachis cf. doddi* (Bellas & Hölldobler 1985) and *Pseudomyrmex veneficus* (Janzen 1973), though genetic data are missing, and hostile, intrusive behavior was observed in the former (Bellas & Hölldobler 1985). Nevertheless, *P. cf. doddi* might classify as an intermediate between species with wing-dimorphic and size-dimorphic queens and could provide, in future studies, evidence for the hypothesis of a causal link between the two.

The data currently available on *Ectatomma ruidum* do not allow any firm conclusion about the underlying life history tactics. The microgynes certainly have the potential for independent (semi-claustral) colony founding, however it remains to be shown whether this is their preferred mode of reproduction. It is important to note that a more favorable wing load (= body mass per unit wing area) of microgynes (which has also been found in *L. rugatulus*: Rüppell et al. 1998) does not necessarily imply better dispersal capability because absolute as well as relative physical parameters account for flight performance (Ellington 1984; Vogel 1994). The relationships between relative queen size and the mode of colony founding across species (Buschinger 1974b; Stille 1996) and between social system and queen size within many species (Brian and Brian 1949; Sundström 1995; Hamaguchi & Kinomura 1996; Rüppell et al. 1998) suggest otherwise. Small queens are typically found where dependent founding is common, and larger queens presumably disperse and attempt independent founding. Some queen size-dimorphic species in which macrogynes may also be found in polygynous nests (e.g. *Myrmica ruginodis*: Wardlaw & Elmes 1996; *Leptothorax rugatulus*: Rüppell et al. 1998) provide a rigorous test system for comparing dispersal behavior of the different morphs, irrespective of social structure.

1.3.6 Proximate factors underlying size polymorphism

Apart from the ultimate factors, the proximate causation of alternative tactics is of general interest (Austad, 1984; Gross, 1996) because queen size and related to it the mode of reproduction could be based on a genetic polymorphism, environmental effects, or a combination of both. As mentioned above, queen weight in *Solenopsis invicta* is mainly due to the social structure of the nest in which they are raised, hence this "cultural transmission" can be viewed as adaptive phenotypic plasticity. This is in sharp contrast with reports on species where wing dimorphism in queens is brought about by the inheritance of a single locus or a closely linked set of loci as demonstrated both by breeding experiments and quantitative genetic analysis (Buschinger, 1975, 1978; Heinze and Buschinger, 1989; Heinze, 1998). Generally, body size is considered a quantitative trait to which environmental factors as well as numerous loci contribute (Stearns, 1992; Roff, 1997).

So far, in queen size-dimorphic ant species the knowledge of proximate determinants of queen size is only based on occasional observations. McInnes and Tschinkel (1995) conclude a genetic basis from the fact that queens produce daughters of the same morph as themselves. While this is also true for *Leptothorax spinosior* and *L. rugatulus*, in these cases the same conclusion is not drawn, because of high variability in offspring size from single colonies in *L. spinosior* and some exceptional small queens produced in large-queened colonies in *L. rugatulus*. The high overall correlation of body size in mothers and offspring in natural colonies (Hamaguchi and Kinomura, 1996; Rüppell et al., 1998) might result from genotype-environment covariance, i.e., relatives sharing not only genes, but also the same micro-environment (Falconer, 1989), or from maternal effects (Bernardo 1996a). The fact that gyne size seems to be related to colony size in *E. ruidum* gives some support for the hypothesis that queen size in this species is a plastic response.

1.4 This study

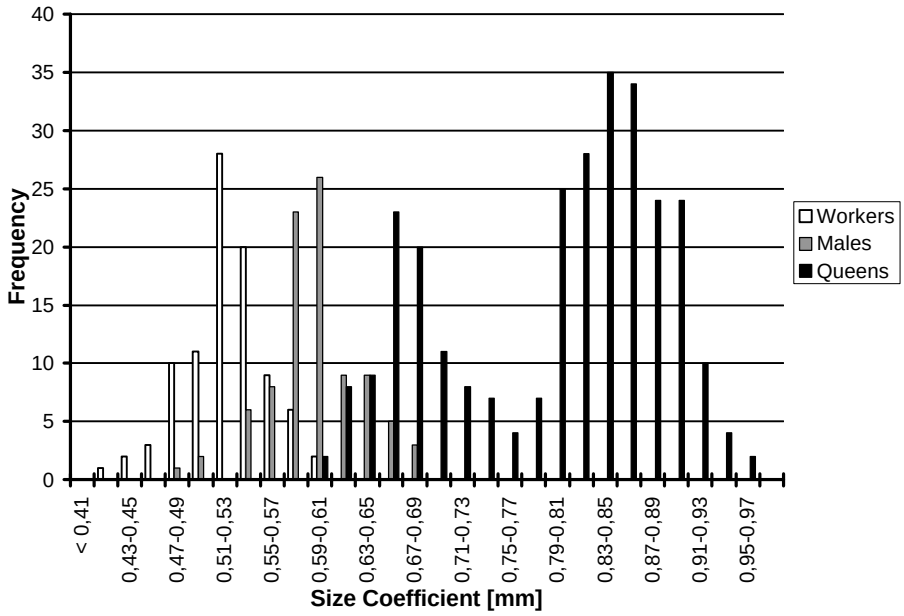
This study aims at a deeper understanding of the phenomenon queen size polymorphism in ants by investigating its proximate and ultimate causation, as well as its consequences. It is based on previous work on the natural history and the morphological description of the queen size dimorphism in *Leptothorax rugatulus* (Fig. 2) and the finding of a contingency between queen body size and colony composition (Rüppell et al. 1998).

Leptothorax rugatulus is widely distributed in the western part of the United States (Creighton 1950) and a number of subspecies have been recognized (Muesbeck & v.Krombein 1951). It belongs to the subgenus "Myrafant" and exhibits a typical morphology. It nests at relatively high densities in mixed forests, predominantly in rock crevices or under stones (Fig.3). The colonies are up to 500 workers in size and contain one or several queens (facultative polygyny). Within the well-defined nest, workers move relatively slowly which facilitates complete colony collection and censusing. Laboratory rearing of

Leptothorax follows well established methods (Buschinger 1974a) which have been successfully employed for *Leptothorax rugatulus* (Rüppell et al. 1998). In sum, this makes *L. rugatulus* an ideal model system to study queen size dimorphism in ants.

The primary goal of this study was to establish that macro- and microgynes employ, and are selected for, alternative reproductive tactics and to determine what ecological correlates exist. Evidence was sought to support either the dispersal polymorphism or the social parasitism hypothesis for microgynes, and the consequences for population genetics were analysed (chapter 2).

Figure 2: In *Leptothorax rugatulus* macro- and microgynous queens coexist and overlap in size. However, the size distribution is clearly bimodal, in contrast to worker and male size distribution. Microgynes are intermediate in size between workers and macrogynes.



In a quantitative genetic framework, the proximate causations of body size differences were investigated (chapter 3), and the differentiation of queen body size between populations compared to other parameters including neutral genetic markers (chapter 4).

The sex ratio pattern of *Leptothorax rugatulus* at population- and colony level was studied (chapter 5) with respect to its queen size dimorphism, and the impact of queen body size on the pattern of intra-colonial sharing of reproduction was examined (chapter 6). In both cases the data was compared to predictions of more general hypotheses.

Finally, a new queen size polymorphism was detected in a Mexican *Leptothorax* species and the morphology, colony social organisation, and the genetic structure of colonies, populations and morphs were described in a comparative perspective to *L. rugatulus* (chapter 7). The general conclusion (chapter 8) summarizes the studies and puts them into perspective with each other. Additionally, it discusses queen size polymorphism in a general context.

Figure 3: *Leptothorax rugatulus* occurs in a wide variety of habitats and nests in variouss structures on the ground. Mixed coniferous forest with rock crevices as nesting sites were typical in the main study area, the Chiricahua Mountains.

2 Alternative reproductive tactics in *Leptothorax rugatulus*

2.1 Introduction

A clear bimodal size distribution specific to queens has been previously established for *Leptothorax* rugatulus (Rüppell et al. 1998). From comparative analyses (Keller & Passera 1989; Stille 1996), a specialization of macrogynes and microgynes in independent and dependent reproduction respectively is expected. Nevertheless, this study seeks for unambiguous evidence, to establish the biological significance of the queen size dimorphism in *L. rugatulus*. As microgynes in queen size dimorphic species might be interpreted as intermediates in the evolution of inquilinism (Buschinger 1990; Bourke & Franks 1991) or a more pronounced dispersal polymorphism with wing reduction (Heinze & Hölldobler 1993), evidence for one or the other hypothesis is required. Furthermore, few studies have addressed the possibility that queen size polymorphism might be a stable phenomenon in itself. In the genus *Leptothorax*, a taxon comprising many social parasites and wing dimorphic species, these questions are of particular interest (Bourke & Franks 1991). Moreover, queen size dimorphism seems to be more common in the genus *Leptothorax* than in other genera (chapter 1).

Queen size dimorphisms are an important case of female-specific alternative phenotypes. However, it is currently unknown how closely behavior is linked to morphology in queen size dimorphic species. The potential consequences for life-histories, mating structure, socio- and population genetics mainly await clarification. Apart from a reduction in queen body size, secondary polygyny entails a syndrome of life history adaptations (Keller 1991; Bourke & Franks 1995), including colony budding for short range dispersal. This commonly leads to clusters of related colonies and thereby increases population viscosity (Stille & Stille 1993; Seppä & Pamilo 1995; Chapuisat et al. 1997).

Queen size dimorphism in the facultatively polygynous ant *Leptothorax rugatulus* (Rüppell et al. 1998) has proven widespread and populations are well differentiated. Thus, it provides a promising study system to address the following five questions: 1. Do large queens (macrogynes) and small queens (microgynes) belong to the same species or are their gene pools separated? 2. What is the evidence for the hypothesis that microgynes found colonies dependently while macrogynes disperse and initiate new colonies independently? 3. If microgynes start reproduction dependently, do they employ social parasitism or secondary polygyny? 4. What environmental factors are ultimately affecting the balance between macro- and microgynes? 5. How is the genetic population structure of this queen dimorphic species affected by the alternative life histories? In this study, answers to these questions were sought by evaluating social, physiological and behavioral data and by genetic analyses.

2.2 Methods

2.2.1 Field methods

For this study 1310 colonies (244 microgynous, 669 macrogynous, 231 mixed and 166 queenless) were collected predominantly in mixed coniferous forests, in Arizona, New Mexico and Colorado in the summers 1996 - 1999. Voucher specimens have been deposited in the collection of P. Ward (University of California, Davies).

In most colonies sexuals, workers and queens were counted immediately. From colonies that showed swarming activity within 2 days after collection, one male and/or one gyne was collected for determination of their fat content according to the method of Keller and Passera (1989). To investigate ecological correlates of queen morphology, the following parameters were determined for collection sites with more than 20 colonies: geographical location, altitude, vegetation type, nesting substrate, sun exposure and an estimate of colony density (Tab.1).

The relationship between the morphology of a colony's queens and its social structure (queen number) and the correlation across sample sites between the frequency of macrogynes in polygynous colonies (i.e. the tendency for dependent founding by macrogynes) and the relative abundance of microgynes was investigated. Furthermore, the relationship between colony size, queen morphology and queen number was analyzed. Despite deviations of the data from normality, parametric statistics were most appropriate for these analyses (Lindman 1974; StatSoft 1994).

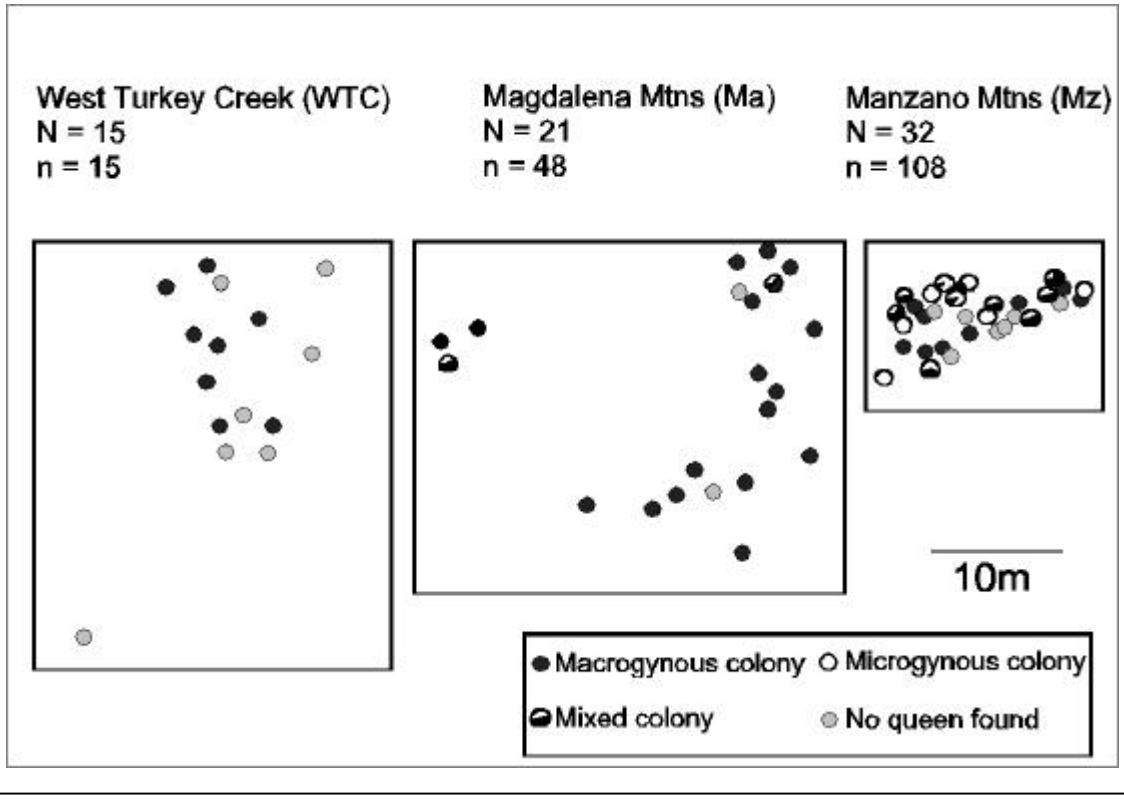
During the swarming period, alates were trapped in the Chiricahua Mountains, Cochise County, AZ., at three sites (subpopulations "OS", "NBL" and "BL") that differed in the frequency of macro- and microgynes (Tab.1). In each location one Malaise trap (2m x 1.4m capturing area), three aerial eclectors (Mühlenberg 1993) and 20 sticky traps (30cm x 20cm foil-covered cardboard with brushed-on insect glue) were set up from the 15 June 1998 to 20 August and checked on average every four days.

A systematic subpopulation comparison was performed in 1998 by sampling every visible potential nesting site and mapping colony positions at three sample sites (Fig.4): West Turkey Creek Valley ("WTC", 800m²; Chiricahua Mts., Cochise County, AZ), Magdalena Mts. ("MA", 800m²; Socorro County, NM) and Tajique Valley ("TA", 200m²; Manzano Mts., Torrance County, NM). Colonies were immediately stored in 96% ethanol for later investigation of colony contents, exact size measurement of the queens (Rüppell et al. 1998) and genetic analysis of all queens or one arbitrary worker in queenless colonies.

Table 1: Collection data and ecological parameters from the major subpopulations (collection sites with n > 20). Location is given as geographic coordinates (latitude north, longitude west). "monog" = monogynous, "polyg" = polygynous.

Subpopulation (Abbrev.)	Location	Altitude [m]	Vegetation	Nest sites	# of macros /micros	# of colonies						Population density	Average queen number	Average worker number
						queenless	macrogynous		micrognes		mixed			
							monog	polyg	monog	polyg				
Barfoot Lookout (BL)	32°00' 109°08'	2630	mixed oak-pine-fir forest	rock crevices	54 / 391	5	3	34	10	5	11	high	6.9	89.3
Huachuca Mtns (HU)	31°36' 110°25'	2400	pine forest	under stones	72 / 30	6	1	5	17	17	5	high	2.3	55.7
Magdalena Mtns (MA)	34°25' 107°30'	2270	coniferous mixed forest	under stones	158 / 18	9	0	1	30	19	5	low	2.7	107.6
Tajique (TA)	34°45' 106°20'	2040	open pine-oak forest	rock crevices	373 / 348	8	16	31	20	31	50	high	4.9	80.7
Manzano Way (Z)	34°45' 106°32'	2060	pine-oak forest	rock crevices	57 / 35	0	3	6	8	2	9	low	3.4	69.3
Mapping Area (Map)	32°03' 109°05'	2300	coniferous mixed forest	under stones	142 / 52	28	4	5	69	26	8	high	1.4	68.1
Monte Vista (MV)	31°47' 109°24'	1850	mixed oak forest	under stones	24 / 0	9	0	0	12	3	0	low	1.0	106.2
Monte Vista Peak (BVP)	31°45' 109°23'	2700	low pine forest	rock crevices	57 / 127	1	1	8	4	7	7	high	6.6	88.6
Mtn Graham (GR)	32°42' 109°45'	2100	low oak-pine forest	under stones	36 / 7	1	0	2	13	3	3	low	2.0	?
Mtn Lemmon (LE)	32°30' 111°00'	2250	mixed forest	under stones	58 / 29	10	0	4	33	6	2	high	1.6	83.1
North Barfoot (NBL)	32°01' 109°08'	2540	mixed bushes	rock crevices	105 / 678	6	8	76	28	9	19	high	5.3	54.1
Onion Saddle (OS)	32°02' 109°05'	2320	mixed forest	under stones	196 / 132	21	9	17	63	31	22	high	2.2	90.1
Pinery Camp (PC)	32°02' 109°08'	2120	mixed pine-fir forest	under stones	65 / 7	4	1	1	24	8	4	low	2.3	140.8
Roosevelt Forest (CO)	40°40' 105°20'	1800	pine forest	?	32 / 2	2	0	1	12	10	0	?	1.4	54.4
West Turkey Creek (WTC)	31°50' 109°25'	1880	coniferous mixed forest	under stones	96 / 0	10	0	0	64	11	0	high	1.1	62.9

Figure 4: In three subpopulations colony density, colony composition and genetic structure of *Leptothorax rugatulus* were compared. Relative colony position and queen content for these sites are shown here.



2.2.2 Laboratory methods

For the allozyme analysis, ten different enzymes were initially screened using polyacrylamide gels and cellulose acetate plates following the protocols given in (Heinze et al. 1995). Glucose-6-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, phosphogluconate dehydrogenase and xanthine dehydrogenase showed no scorable alleles and isocitrate dehydrogenase, malate dehydrogenase and malic enzyme were monomorphic. Only glucose-6-phosphate isomerase (GPI) and phosphoglucomutase-1 (PGM-1) were polymorphic and could be scored consistently on polyacrylamide gels. Subsequently, the genotype of 467 workers at the GPI locus and 473 at the PGM locus was determined from 191 either macro- or microgynous colonies.

For a more detailed genetic analysis, I attempted to adopt 15 primers for microsatellite loci in other *Leptothorax* species (Hamaguchi et al. 1993; Bourke et al. 1997; Foitzik et al. 1997) using various PCR-protocols. Four loci (L18 from *L.* nylanderi and LXGT104, LXGT218, LXGT228 from *L. spinosior*) were

finally used with slightly different PCR conditions for each locus to genotype 471 queens (263 macrogynes, 160 microgynes, 48 unclassified) from 195 colonies.

Queens from alcohol samples were dried for 40 minutes and frozen queens were directly used for DNA extraction, in 50% of the colonies only head and thorax were used and the results did not differ from those using entire queens. DNA was extracted by grinding the ant in a microcentrifuge tube cooled in liquid nitrogen and the addition of 200 µl 5% solution of Chelex® 100 resin (Bio-Rad). This solution was vortexed 10 sec, boiled 8 min and vortexed again before the Chelex® beads were spun down in a microcentrifuge (8000 rpm, 5 min). One microliter of the supernatant was used subsequently per 10µl PCR reaction.

Final concentration of reactants in the PCR mix are given in table 2. The buffer was used according to the manufactures (MBI-Fermentas) instruction. Generally, dATP was used at 1/10th of the concentration of the other dNTPs to increase incorporation of the radioactively labelled P33-α-dATP (ICN Biochemicals) which was added at approximately 0.02 µCi/µl.

Table 2: PCR-conditions for the four microsatellite loci used in this study. Final concentration are given for all reactants and cycling conditions are given in °C (and the time interval in seconds). Initial denaturation was always performed for three minutes at 94°C and the final elongation step of 70°C for five minutes.

Locus	dNTPs	$MgCl_2$	Primer	Taq®	Denaturation	Annealing	Elongation	# of cycles
L18	200 µM	2.5 mM	0.63µM	0.04 u/µl	92 (60)	51 (60)	70 (90)	36
LXGT104	311 µM	2.5 mM	0.63µM	0.03 u/µl	92 (60)	51 (60)	70 (90)	36
LXGT218	200 µM	1.25 mM	0.56µM	0.03 u/µl	92 (60)	57-47 (90)	70 (90)	2x10 +20
LXGT228	200 µM	1.84 mM	0.41µM	0.03 u/µl	92 (60)	57-47 (90)	70 (120)	2x10 +20

Reactions were carried out in a Genius thermocycler (Techne) using 0.2 ml reaction thin-walled tubes (Biozym Diagnostik). The thermoprofiles for the different primers are also given in table 2. Two microliters of 95% formamide solution (containing bromophenol blue and xylene cyanol) were added to the PCR product. Separation was achieved by electrophoresis through 6% denaturing polyacrylamide gels (1xTBE and 8M urea) on a heatable 2010-001 Macrophor Electrophoresis unit (LKB) at 60°C and 2500V for 2:30 to 4:00 hours. For size determination of the alleles SequaMark® sequencing size marker (Research Genetics) was used. Gels were transferred to Whatman paper, dried and exposed on X-ray film for 6 to 72 hours.

Furthermore, 18 similarly sized, monogynous colonies (6 microgynous and 6 macrogynous colonies from the predominantly microgynous sample sites "NBL" and "TA", and 6 macrogynous ones from the macrogynous site "WTC") were selected to study the distribution of queen- and 6 worker genotypes. Their compliance with the hypothesis of independent colony founding (i.e. all workers are daughters of the present queen) was investigated. Colonies were assigned to "Independent founding" when in addition to the queen's alleles only one other allele was present, to "Multiple mating" when worker genotypes were compatible with the present queen assuming multiple mating (which is unlikely but could not be ruled out), or to "Dependent

founding" when at least one of the six workers did not share alleles with the present queen at one or more loci.

2.2.3 Genetic data analysis

The computer program GDA (Lewis and Zaykin 1999) was used to calculate the number of alleles, and observed and expected heterozygosity for each allozyme and microsatellite locus. In order to investigate the genetic separation between macro- and microgynes the allozyme and the microsatellite data were subjected to a hierarchical analysis of molecular variance (AMOVA). One hundred new data sets were generated by drawing at random one individual from each analysed colony (chapter 13.1) because genotypes within a colony are not independent. Subsequently, each resampled data set was subjected to a three-level AMOVA using GDA and values averaged over the 100 resamples. As there was no a priori reason for nesting morphs within populations (Ross, pers. comm.), both possible hierarchies ("individuals within morphs within populations" and "individuals within populations within morphs") were analyzed.

The "morph-groups" (all individuals from either a macro- or a microgynous colony) were clustered with the program TFPGA (Miller 1998) in a UPGMA-tree based on Reynolds coancestry coefficient (Reynolds et al. 1983). Clustering by Wright´s modification of Roger´s distance (Wright 1978) and Nei´s D (Nei 1972), as well as using GDAs UPGMA- and neighbor-joining algorithms, or excluding rare alleles did not change the results.

Average within-colony relatedness estimates for queens from polygynous colonies were obtained from all microsatellite genotypes by the method of Queller and Goodnight (1989), using the computer program RELATEDNESS 5.0.2 (Goodnight and Queller 1998). Subpopulation allele frequencies were calculated from individual genotypes with bias correction by colony. Relatedness values were averaged over individuals, and 95% C.I. and S.E. were obtained by jackknifing over colonies (Pamilo 1990a).

In order to assess potential effects of queen morphology and the correlated life history on the genetic population structure, population viscosity in three subpopulations (Fig.1) was investigated: relatedness coefficients and spatial distance between colonies were correlated using Mantel tests (with TFPGA).

In the two polygynous subpopulations, "TA" and "MA", the hypothesis that queens within the same colony were unrelated to each other was evaluated against two null hypotheses, namely a mother-daughter and a full-sister relationship using Kinship 1.1.2 (Goodnight and Queller 1999). As a conservative estimate only those queens were scored as unrelated to the colony, for which all intranidal comparisons resulted in a significant rejection of the two null hypotheses. To avoid undefined likelihood ratios (Kinship 1.1.2

Manual), the theoretical relatedness coefficient of 1 (for paternal relatedness of full-sisters and maternal relatedness of mother-daughter) was approximated by 0.99.

2.3 Results

2.3.1 Field data

Social colony structure (queen number) and the frequencies of macro- and microgynes varied strongly among sample sites, even within a few kilometer (Tab.1). These two parameters were strongly correlated: only 51 of 2056 microgynes were found in monogynous colonies compared to 472 of 1665 macrogynes (χ^2 = 509.6, df = 1, $p < 0.0001$). 58.6 % (472 of 806) of colonies with macrogynes were monogynous, while this fraction for microgynes was only 12.9 % (51 of 394) (χ^2 = 224.0, df = 1, $p < 0.0001$). These overall contingencies became even stronger when subpopulation effects were accounted for by multiway frequency analyses: (χ^2indiv.(max. lik.) = 3086.6, df = 78, $p < 0.0001$ and ($\chi^2_{\text{colony (max. lik.)}}$ = 302.7, df = 66, $p < 0.0001$).

The size of a colony (number of workers) was strongly dependent on its social structure (monogynous / polygynous) and on the morphology of its queens (2-way ANCOVA: $F_{social}(1,927) = 27.5$, $p < 0.0001$; $F_{morph}(1,927) = 26.1$, $p < 0.0001$, $F_{interaction}(1,927) = 2.4$, $p = 0.12$, "subpopulation" as covariate). Polygynous colonies were larger than monogynous colonies (91 ± 85 S.D. vs. 65 ± 42) and colony size decreased from mixed colonies to macrogynous to microgynous ones (122 ± 125 vs. 79 ± 62 vs. 65 ± 58). Nevertheless, 20 very small colonies (putative founding colonies with one founding queen and less than five workers) headed by a macrogyne were found, and only one such colony with a microgyne. Colony size was more strongly correlated to the number of macrogynes in the colony than to the number of its microgynes (partial correlation: r_{macro}= 0.66, r_{micro}= 0.39, n = 954, $p < 0.0001$). When the total number of queens was accounted for, the partial correlation coefficient of the number of microgynes became slightly negative (r = -0.11, n = 954, $p < 0.001$), while that of macrogynes was insignificant (r = 0.05, n = 954, p = 0.12).

The trapping yielded 5/0, 4/1 and 2/1 macro-/microgynes at "OS", "BL" and "NBL" respectively. A comparison of these data to the values that would be expected under the null-hypothesis that queen morphs did not differ in their reproductive behavior and thus were trapped according to their subpopulation frequencies indicated that macrogynes were significantly more likely to be trapped. The combined probability test of the three binomial tests (Sokal and Rohlf 1995) was highly significant (Cp=22.4, df =6, $p < 0.005$).

When comparing different sample sites, a significant positive correlation between the proportion of macrogynes in polygynous colonies and the relative abundance of microgynes (Spearman's rank correlation:

R_S = 0.69, N = 15, p = 0.004) was found. Angular transformed microgyne frequency was neither correlated to latitude (partial correlation coefficient r_{part} = 0.17, t(15) = 0.23, p = 0.82), nor to altitude (r_{part} = 0.48, t(15) = 2.04, p = 0.06) or sun exposure (north facing slopes vs. south facing slopes; r_{part} = -0.35, t(15) = -1.41, p = 0.19), but there was a significant relationship with nest density (high vs. low; r_{part} = 0.51, t(15) = 2.22, p = 0.04; e.g. Fig.3) and nest site stability (in the soil under a stone = low vs. in rock crevices = high; r_{part} = 0.60, t(15) = 2.82, p = 0.01).

2.3.2 Fat content

Males contained the smallest amount of fat in absolute (10.1 µg ± 9.0 S.D., n = 40) and relative (6.2 % ± 5.0) terms, and their size was not correlated to their relative fat content (Pearsons correlation r_p = -0.10, p = 0.52). In female sexuals however, more fat was stored by macrogynes ($fat_{absolute}$: 466.4 µg ± 146.9, n = 35, $fat_{relative}$: 47.7 % ± 8.1) than by microgynes ($fat_{absolute}$: 51.4 µg ± 12.3,n = 10, $fat_{relative}$: 23.5 % ± 5.4). Differences were significant in both, absolute (Mann-Whitney's $U_{(10,35)}$ = 350.0, p < 0.0001) and relative terms ($U_{(10,35)}$ = 339.0, p < 0.0001). Relative fat content was not correlated to head width in microgynes (r_p = -0.19, p = 0.60), but in macrogynes (r_p = 0.42, p = 0.01).

2.3.3 Allozymes

For both loci, GPI and PGM, over all populations, five alleles could be distinguished electrophoretically, but allele frequencies were highly skewed and consequently both loci were only weakly polymorphic (Tab.3). The genetic differentiation between morphs was only significant when they were nested within populations, whereas populations had significantly different allele distribution in both hierarchical AMOVA orders (Tab.4). There was no indication of inbreeding. The amount of information gained from the two loci was insufficient to derive any significant clustering of morph groups or meaningful intra-colonial relatedness coefficients.

Table 3: Descriptive statistics of genetic data from allozymes and microsatellites over all populations of *Leptothorax rugatulus.*

Locus	Overall sample size	# of alleles	H_{exp} / H_{obs}
Glucose-6-phospate-isomerase	464	5	0.184 / 0.183
Phospo-gluco-mutase	470	5	0.373 / 0.283
L18	410	32	0.894 / 0.824
LXGT104	390	9	0.653 / 0.438
LXGT218	409	9	0.528 / 0.528
LXGT228	370	31	0.942 / 0.900

2.3.4 Microsatellites

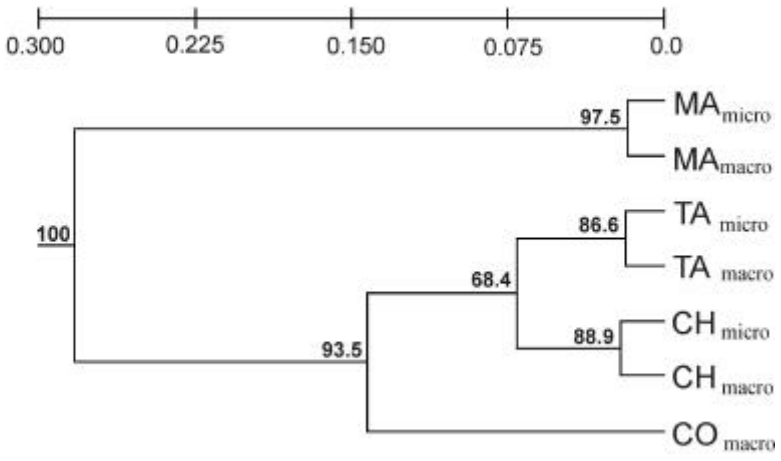
The four microsatellite loci were more polymorphic than the allozymes (Tab.3). In parallel to the allozyme analysis, the three-level AMOVA indicated, for both hierarchies, a significant differentiation at the population level, but differentiation between morphs was only significant when morphs were nested in populations (Tab.4). The mating structure did not deviate significantly from random mating.

Table 4: Results of the 3-level hierarchical analyses of molecular variance for allozyme and microsatellite data. 95% C.I. are given in brackets and significant positive F-values ($p < 0.05$) are printed in bold.

AMOVA model	F_{IS}	F_{IT}	$F_{population}$	F_{morph}
Allozymes 1: morphs nested in populations	0.090 (-0.003 – 0.154)	0.167 (0.100 – 0.220)	0.066 (0.032 – 0.106)	0.085 (0.057 – 0.115)
Allozymes 2: populations nested in morphs	0.090 (-0.003 – 0.154)	0.144 (0.079 – 0.200)	0.059 (0.038 – 0.084)	-0.031 (-0.043 – -0.021)
Microsatellites 1: morphs nested in populations	0.040 (-0.005 – 0.072)	0.146 (0.048 – 0.285)	0.114 (0.051 – 0.234)	0.110 (0.044 – 0.235)
Microsatellites 2: populations nested in morphs	0.040 (-0.005 – 0.072)	0.121 (0.034 – 0.243)	0.084 (0.031 – 0.188)	-0.025 (-0.048 – -0.012)

The cluster analyses further supported the view that the genetic separation was stronger between populations than between morphs because the different "morph-groups" of the same population strictly clustered together (Fig.5).

Figure 5: Cluster analysis (UPGMA) of the different morphs (macro- and microgynes) in different populations based on Reynold's coancestry distance computed from allele frequencies at four microsatellite loci. Numbers over the nodes indicate bootstrapping values in percent.



Significant genetic structuring was found at the colony level. Overall, the average queen-queen relatedness within colonies was r = 0.400 ± 0.036 (S.E.). The relatedness coefficient among macrogynes (r = 0.438 ± 0.038) was slightly higher than the relatedness coefficient among microgynes (r = 0.365 ± 0.089) or between macro- and microgynes (r = 0.351 ± 0.079) but differences were not significant (Tab.5).

Table 5: Queen-queen relatedness in colonies of *Leptothorax rugatulus*. 95% confidence intervals (95% C.I.) were obtained by jackknifing over colonies. "N" refers to the number of individuals; "n" indicates the number of colonies.

Relatedness between	Coefficient of relatedness	95% C.I.	n	N
all queens	0.400	(0.329 – 0.471)	102	379
macrogynes	0.438	(0.363 – 0.514)	59	190
microgynes	0.365	(0.183 – 0.547)	30	134
macro- and microgynes	0.351	(0.188 – 0.514)	25	122

Inter-colonial relatedness was not correlated to spatial distance in any of the three compared subpopulations ("WTC": r_{matrix}= -0.07, n = 15, p > 0.1; "MA": r_{matrix}= -0.08, n = 21, p > 0.1; "TA": r_{matrix}= 0.001, n = 32, p > 0.1) at the investigated scale (Fig.3).

The tests whether queens were more likely to be unrelated than related as mother-daughters or full-sisters to the rest of their colony were significant for four macrogynes (out of 40) and no microgyne (out of 4) in "MA" and one macrogyne (out of 50) and five microgynes (out of 51) in "TA". The ratios of macrogynes to microgynes did not differ significantly from the expected values derived from the subpopulation frequencies (χ^2_{MA} = 0.82, p = 0.36; χ^2_{TA} = 1.68, p = 0.19; combined probability: p > 0.1).

Table 6: The most parsimonious explanations of worker genotypes suggest different modes of colony founding in macrogynous and microgynous colonies.

Worker genotypes compatible with	Microgynous colonies from “TA” and “NBL”	Macrogynous colonies from “TA” and “NBL”	Macrogynous colonies from “WTC”
Independent founding under single mating	0	0	3
Independent founding under multiple mating	0	2	2
Dependent founding (multiple matrilines)	6	4	1

The genetic composition of monogynous colonies revealed strong differences between the three compared groups ($\chi^2_{max.lik.}$ = 13.8, df = 4 p < 0.01, Tab.6): the genotypes of workers could not be explained by the present queen (as single foundress) in any of the six microgynous colonies, whereas this was the case in at

least half of the macrogynous colonies from "WTC". As multiple mating of queens cannot be ruled out (see chapter six), macrogynous colonies from "NBL" and "TA" are intermediate.

2.4 Discussion

2.4.1 Species consistency

Allozyme and microsatellite allele frequencies equally indicate that the separation of the gene pools between macro- and microgynes of *Leptothorax rugatulus* is weaker than that between different populations. This supports the hypothesis that both morphs belong to the same species, which was initially based on the production of similar males and workers by macro- and microgynes and their overall morphological resemblance (Rüppell et al. 1998). Thus, queen size dimorphism in *L. rugatulus* is one of the rare cases of alternative reproductive morphotypes in females in one species (Gross 1996).

Reproductive polymorphism commonly leads to reproductive isolation and thus sympatric speciation (Coyne and Orr 1998) which has also been proposed for the case of microgyny in ants (Buschinger 1990; Bourke and Franks 1991). Given species identity in *L. rugatulus* (and consequently the nesting of "morph-groups" within populations), a slight but significant genetic differentiation between morphs was apparent. This indicates the potential for sympatric speciation in this case, but gene flow by males may maintain the species' integrity. Comparisons between mitochondrial and nuclear genetic structure of ant populations have been successfully employed to derive male gene flow and separation between alternative social forms (Ross et al. 1997; Ross & Shoemaker 1997), whereas studies confined to the nuclear level have mostly failed to find any differentiation (e.g. Seppä 1994). Thus, to evaluate the prospects for speciation further, investigations of mtDNA population structure combined with a determination of the heritability of queen morphology are needed.

2.4.2 Alternative tactics

Direct observations of colony founding behavior in ants are difficult to obtain in most species. Consequently, only for *Myrmica ruginodis* (Brian & Brian 1955) direct evidence exists that microgyny is linked to dependent colony founding. In all other queen size polymorphic species the conclusions about alternative reproductive tactics are drawn from indirect evidence (e.g. Janzen 1973, McInnes & Tschinkel 1995, DeHeer & Tschinkel 1998). This study provides five separate lines of indirect evidence, which together strongly support the view that microgynes and macrogynes are specialized for and employ predominantly alternative reproductive tactics:

I) Relatively more macrogynes were found in monogynous colonies than microgynes. As monogynous colonies mostly originate by independent colony founding of one self-contained queen, and polygynous colonies arise mainly by adoption of additional queens into existing colonies (Hölldobler & Wilson 1977, 1990; Bourke & Franks 1995), the correlation between queen morphology and social colony type provides evidence that macrogynes establish independent colonies more frequently. This was further supported by the genetic investigations of monogynous colonies which suggested that microgynes become queens in monogynous colonies only by budding or the death of their nest mate queens, whereas at least a part of the macrogynes found independently. However, it is important to stress that the behavior of macrogynes seems to be plastic or at least uncoupled from their morphology to some extent.

II) The ratio of 20 putative founding colonies with a macrogyne to only one with a microgyne cannot be explained by the higher abundance of macrogynes. Thus, the most parsimonious explanation is that macrogynes are more likely to initiate colonies independently, or that they are more successful in doing so.

III) Although sample sizes were small, there is evidence that macrogynes are more likely to be trapped in flight traps during swarming than microgynes. This suggests that macrogynes show a higher flight activity during mating and/or dispersal flights. Both are typically correlated with each other and with independent colony founding (Bourke & Franks 1995), while readoption into the mother nest requires restricted flight activity (e.g. Sundström 1995), as relocating the natal nest over distance may be extremely difficult after swarming.

IV) Generally, polygyny is a good indicator of dependent colony founding (Hölldobler & Wilson 1977; Keller 1991; Bourke & Franks 1995). Thus, the degree of polygyny of macrogynes at a given sample site identifies the tendency of *L. rugatulus* queens at this site to engage in dependent colony founding, irrespective of body size. This behavioural decision may be regarded as an adaptive response to environmental conditions. Its positive correlation with the frequency of microgynes indicates that both, dependent colony founding and microgyny are favored by similar environmental conditions. This corresponds well to the hypothesis that microgynes constitute an adaptation to a dependent mode of colony founding.

V) Macrogynes are physiologically adapted to independent colony founding because they harbor more body reserves prior to mating and colony establishment. In a cross-species comparison (Keller & Passera 1989) macrogynes of *L. rugatulus* classify as independent foundresses, while the relative fat content of microgynes is comparable to species that found dependently. Purely allometric effects can be ruled out on the basis of the fact that neither in males nor within microgynes relative fat content and body size are correlated.

2.4.3 Dependent colony founding

In some arguments above it has already been implied that microgynes are specialized for secondary polygyny by readoption. However, so far it has been only argued for their dependent mode of colony founding which could involve intraspecific social parasitism or secondary polygyny by readoption or both. In *Solenopsis invicta*, microgyny has been viewed as a parasitic tactic (Tschinkel 1996), and also for *L. rugatulus*, it has been argued previously (Rüppell et al. 1998) that microgynes might be efficient "searchers" of unrelated host colonies. Their low flight activity in the field contradicts this hypothesis, and in general *L. rugatulus* resembles more the facultatively polygynous species for which the link between microgyny and readoption has been shown (*Camponotus yamaokai*: Satoh et al. 1997) or suggested (*Myrmica ruginodis*: Elmes 1991b). The high intra-colonial queen-queen relatedness revealed in this study supports the view that most dependently founding macro- and microgynes are readopted into their natal colonies. Although, similar to other facultatively polygynous ant species (e.g. Stille and Stille 1992), occasional adoptions of unrelated queens cannot be excluded, no significant differences in relatedness were found between macro- and microgynes. Thus, microgyny seems not to be a special adaptation to intraspecific social parasitism.

Nevertheless, the slightly negative correlation between a colony's size and its number of microgynes (when total queen number is controlled for) can be interpreted as negative effects of microgynes on the colony fitness. While the term "social parasite" seems too strong in this case, microgynes might be selfishly pursuing their own interests in conflict with the rest of the colony, an idea that has been suggested on theoretical grounds (Bourke and Ratnieks 1999). However, adaptive differences in the colony dynamics (e.g. budding) provide an alternative explanation.

2.4.4 Ecological parameters

This study is the first that systematically investigates social and genetic structure in relation to queen morphology in a queen size dimorphic ant species across several populations. As morph frequencies and social structure of *Leptothorax rugatulus* colonies varied strongly among subpopulations, it is clear from this study that it is most important to study a number of populations that differ in their ecological conditions (Travis 1994) in order to understand a species' evolutionary ecology.

Dispersal polymorphism in female ants is linked to habitat patchiness (Heinze & Buschinger 1989; Heinze 1993a; Heinze & Tsuji 1995). In *L. rugatulus* this patchiness is twofold: at a large scale (> 50km), mixed forests on mountain ranges that constitute suitable habitats are patchily distributed in the semi-deserts of Southwest North America ("sky islands", Heald 1951), and at a finer scale (< 5km), patches with suitable nest sites seem to be separated by largely uninhabitable areas (Rüppell et al. 1998). Presumably, macrogynes

are better colonizers, whereas microgynes may establish new colonies more competitively within high-density patches by budding and profit from stable nesting substrate (rocky outcrops) which makes a potential inheritance of the mother colony (Nonacs 1988) more rewarding.

At the meta-population level the variability of queen size is maintained by the contrasting selection pressures within and between populations (Olivieri et al. 1995) and different ecological conditions in different locations. The sharp clines in morph frequencies without dispersal barriers in *L. rugatulus*, but also in other species (e.g. Hamaguchi et al. 1998), indicate strong advantages of one or the other morph, probably related to environmental parameters.

2.4.5 Population genetic consequences

A clear effect of the alternative reproductive tactics and correlated social colony structure was expected on population viscosity. An increased viscosity in polygynous ants has been demonstrated in other ant species at a larger spatial scale (Seppä and Pamilo 1995; Chapuisat et al. 1997) but in this study no isolation-by-distance effects were detected in any of the three subpopulations. This homogenous population structure might be due to long-range budding or gene flow via males. The latter explanation seems more plausible because *Leptothorax* ants are not very mobile on foot (Herbers 1984; Heinze et al. 1996) and the random breeding structure allows for substantial gene flow via the males if females were philopatric. Similarly, weak nuclear differentiation between social forms, opposed to strong mitochondrial differentiation has also been reported from *Leptothorax acervorum* (Stille et al. 1991; Stille and Stille 1993) and *Solenopsis invicta* (Ross and Shoemaker 1997).

3 Proximate causation of queen size

3.1 Introduction

Quantitative genetics has contributed enormously to the understanding of evolution of behavioural, life-history and morphological traits (Endler 1986; Mousseau & Roff 1987; Roff 1997). Although its theoretical principles have been around for a long time (Fisher 1918), quantitative genetics was long confined to applied questions in animal and plant breeding (Falconer & MacKay 1996). However, with recent emphasis on underlying mechanisms of adaptations (Roff 1997) and the distinction between phenotypic plasticity and genetic differentiation (Via 1993, 1994), quantitative genetics has gained increasing importance in evolutionary studies (Lynch & Walsh 1998).

Surprisingly, this approach remains mainly unexplored in the social insect literature, which otherwise are in many aspects at the forefront of evolutionary research (see chapter one). The honeybee is the only exception to this lack of quantitative genetic work in social insects. The heritability of a number of traits has been evaluated (e.g. Soller & Bar-Cohen 1967; Rothenbuhler et al. 1968; Collins et al. 1984; Collins et al. 1987; Brandes 1988). More recently, *Apis mellifera* has proven to be a model system for research in quantitative trait loci (QTL) identified for behavior (Hunt et al. 1995, 1998), morphology (Hunt et al. 1998) and pheromones (Hunt et al. 1999). Primed by studies in the honeybee (e.g. Robinson & Page 1988), genetic differences among patrilines have been reported in a wasp (O'Donnell 1998), and three ant species (Stuart & Page 1991; Snyder 1993; Fraser et al. 2000).

The lack of formal quantitative genetic studies in social insects is particularly startling because these provide many advantages over non-social organisms for disentangling environmental and genetic variability components. Many species can be reared under defined conditions in the laboratory and experimental brood exchange (cross fostering) provides a particularly powerful approach to separate external from intrinsic factors. In colonies of social Hymenoptera with multiple mating, patrilines of individual fathers coexist that are identical with respect to their paternal genome, but differ completely from their half sisters, while the maternal part of the genome is 50% identical.

On the other hand, many social insects have large colonies, and a number of colonies sufficient for quantitative genetic experiments is difficult to maintain in the laboratory (Hölldobler & Wilson 1990). Additionally, controlled crossing experiments may be difficult or impossible, which reduces the number of experimental designs. Laboratory crossing are particularly difficult in most ant species, though important exceptions exist which have been used to investigate the genetic basis of discrete polymorphisms. In this respect, wing polymorphisms in queens have been found to be under the simple genetic control of a single

locus (e.g. Buschinger 1975; Winter & Buschinger 1986; Heinze & Buschinger 1987, 1989). One study has looked at quantitative traits (body fat reserves and weight) in queens of the fire ant, *Solenopsis invicta*, by means of a cross-fostering study (Keller & Ross 1993). Evidence was reported for phenotypic plasticity in both traits mediated by the social environment, probably the brood care behaviour of workers. However, a genetic polymorphism also is known to play a role in this case (Keller & Ross 1993; DeHeer et al. 1999) and no formal evaluation of the heritability has been attempted (Keller & Ross 1993).

In *Leptothorax rugatulus*, a high correlation of mother and daughter size in nature was found when average queen size of field-collected colonies was correlated to the mean size of the gynes they produced (Rüppell et al. 1998). Body size constitutes one of the most studied traits in quantitative genetics (Mousseau & Roff 1987; Reinhold 1994; Fox 1998) across the animal kingdom because it has important consequences for virtually any other biological trait of the organism (Calder 1984; Schmidt-Nielsen 1984). Generally body size has proven to be a polygenic parameter (Falconer & Mackay 1996).

Body size plays also an important role in the life-history of ant queens: A certain body size is required for independent reproduction (macrogyne), while relatively small queens (microgynes) rely on existing colonies for initiating reproduction (Keller & Passera 1989, 1990; Stille 1996; chapter two). While differences between species are well-established, and it has been shown that queen weight and gyne weight might correlate within species (Herbers 1990; Backus 1993; Herbers & Stuart 1996) no heritability estimates for size exist. The only values again stem from the honeybee (Rothenbuhler et al. 1968; Collins et al. 1984), and the solitary bee, *Osmia lignaria* (Tepedino et al. 1984).

Across populations, the body size of queens in *Leptothorax rugatulus* is bimodally distributed (Rüppell et al. 1998). Body size therefore violates the assumptions of simple quantitative genetics models. However, even discrete characters can be analyzed by a quantitative genetic approach, assuming an underlying, normally distributed parameter that is influenced by several genes (Roff 1994, 1996; Falconer & Mackay 1996; Lynch & Walsh 1998). The individual score in this underlying parameter translates into one or the other discrete phenotype (Fig.6). These threshold traits typically exhibit a large additive genetic component but also vary with environmental variables, such as photoperiod, temperature, or population density (Roff 1996).

For the queen size dimorphism in *Leptothorax rugatulus*, genetic, environmental and social variables are conceivable as proximate causes. This study aimed at determination of the importance of these factors by a combination of several laboratory rearing experiments.

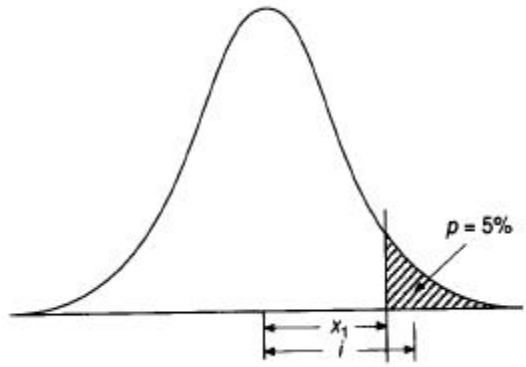


Figure 6: Schematic drawing of the threshold model in quantitative genetics. Discrete character state one develops when the underlying parameter with normal distribution is above a certain threshold (shaded area), the other state develops when the underlying parameter is below the threshold. Thus groups of individuals (populations) are not characterized by an average character value (the underlying parameter is not measurable) but a certain incidence (p = 5%) of the phenotpyic character. Under the assumption of a normal distribution, the distance of the threshold from the population mean (x_1) and the mean difference between the population of individuals of character state one and the overall population (i) can be inferred.

3.2 Methods

Four laboratory fostering experiments were conducted to investigate the proximate causation of the queen size dimorphism in *Leptothorax rugatulus*. Two experiments were used to obtain independent heritability estimates of queen size in *Leptothorax rugatulus*. The third experiment was concerned with food and temperature effects and the fourth investigated the impact of the number of queens present in a colony.

3.2.1 Experimental Design

In experiment one, environmental variance and the effect of worker behaviour were excluded by cross-fostering offspring of single queens. Forty groups of 100 workers were derived from colonies by removal of all queens and brood. Queens were introduced to these groups for one month to lay eggs. For two subsequent months additional eggs were transferred to the worker groups from that queen (returned to its original colony). Microgynes were introduced into groups derived from fourteen macrogynous, three mixed, and three microgynous colonies (Fig.7) Macrogynes were introduced into six microgynous, eight mixed, and six macrogynous worker groups (Fig.7). The design was imbalanced because of a restricted availability of microgynous colonies of sufficient size and further, some of these colonies never produced queens. Worker groups were maintained at standard laboratory conditions (Buschinger 1974a) and all produced gynes were collected in the subsequent two years and size measured (see below). All female brood was presumably derived from the introduced eggs, because five queenless colonies produced, in a separate experiment, only 130 males and no females. Hence female offspring production by *L. rugatulus* workers seems unlikely.

Figure 7: Set-up for the cross-fostering experiment.

Host colony / Mother	Macrogynous	Microgynous	Mixed
Macrogyne	6	6	8
Microgyne	14	3	3

The second experiment involved an independent investigation of the effect of queens and workers present during larval growth. Two experimental groups were used. In the first group, workers from 50 different colonies were randomized and macrogynes, microgynes, or both introduced. The second group consisted of original colonies with macrogynous, microgynous or both queens. In both experimental groups all gynes produced in the subsequent two years were assigned to mothers by microsatellite analysis. Mothers and daughters were genotyped at as many microsatellite loci as necessary to exclude for each daughter all but one mother on the basis of the combined multi-locus genotype.

Apart from the four loci described in chapter two, three additional microsatellite loci were recruited for maternity assignment: L4 (Foitzik et al. 1997), MYRT3 (Evans 1993; Bourke et al. 1997) and LXAGT1 (Bourke et al. 1997). L4 was used at 0.8 µM concentration, with 200µM dNTPs, 2.5mM $MgCl_2$ and 0.008 units/ml of Taq®-polymerase (all from MBI-Fermentas). A primer concentration of 0.36µM was sufficient for MYRT3 and LXAGT1, with 250µM dNTPs, 2.5 mM $MgCl_2$ and 0.01 units/ml of Taq®-polymerase. Annealing temperature for L4 was 10x 56°C and 28x 51°C, for both other loci 10x 60°C and 24x 55°C. Otherwise, temperature profiles were identical to those given in chapter two.

The third experiment involved four different treatment groups (with ten macrogynous, monogynous colonies each) with two temperature regimes in combination with either high or low food treatment (Tab.7). Colonies were set up at the end of the reproductive cycle (August) for two years during which time all produced gynes were collected, frozen and size-measured (see below).

Table 7: Two-by-two factor treatment to investigate food and temperature importance on queen body size. Day/night temperature [°C], day-length in hours and feeding events per week are given for each season treatment, as well as its duration in weeks. Proteinaceous food (cockroaches) was given exclusively in summer.

Food / Temperature	Plus					Minus				
Warm	Winter:	7° / 4°	10	0.5,	12	Winter:	7° / 4°	10	0.5,	12
	Spring/Fall:	15° / 10°	12	2,	5	Spring/Fall:	15° / 10°	12	1,	5
	Summer:	25° / 10°	14	3.5,	30	Summer:	25° / 10°	14	1,	30
Cold	Winter:	7° / 4°	10	0.5,	24	Winter:	7° / 4°	10	0.5,	24
	Spring/Fall:	15° / 5°	12	2,	5	Spring/Fall:	15° / 5°	12	1,	5
	Summer:	20° / 10°	14	3.5,	18	Summer:	20° / 10°	14	1,	18

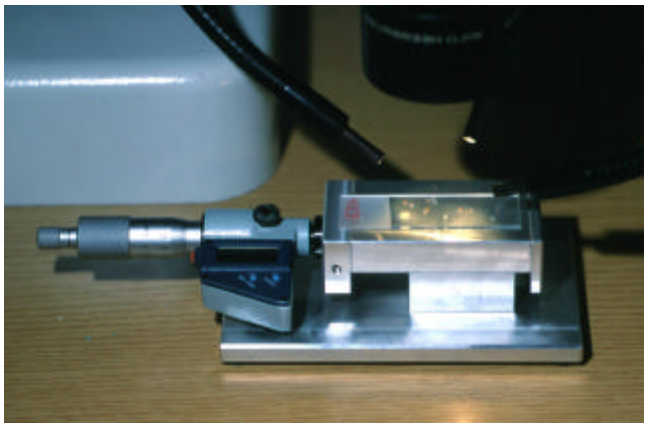
In the fourth experiment, the socio-environmental factor "number of queens per colony" was evaluated. Brood and workers of ten polygynous, macrogynous colonies were evenly split into two halves and one queen was re-introduced into one half. The remaining queens (2-18) were introduced into the other half. As above, any offspring produced was collected after hatching and frozen for subsequent size measurement.

In order to test the possibility that maternal effects could be accounted for by different egg sizes of micro- and macrogynes, eggs were collected in the middle of the reproductive period (July 1997) from ten macrogynous and ten microgynous colonies of the second experiment. The length and width of ten eggs from each colony was determined under a stereomicroscope at 50x magnification. Volume was calculated using a cylindrical approximation (height $\times$ radius ^{2} $\times$ π). Differences between macrogynous and microgynous eggs were tested with an ANOVA nesting eggs within colonies.

3.2.2 Size measurements

All size measurements were carried out with a Wild™ M3Z (Heerbrugg, Switzerland) stereomicroscope at 50× magnification. In the first experiment, maximal head width, thorax width and thorax length were measured with an ocular micrometer and combined to a size coefficient (Rüppell et al. 1998). In the other experiments only head- and thorax-width were determined to the nearest micrometer with a table mounted on a micrometer screw (Fig.8) and then averaged. The size threshold, that was previously established by the minimum of the size distribution (Rüppell et al. 1998), was used to differentiate between macro- and microgynes.

Figure 8: The experimental set-up for size measurements. A table mounted on a micrometer screw minimized measurement error.



3.2.3 Statistical analysis

Since queen size distribution in *Leptothorax rugatulus* across natural populations is bimodal and most data sets of this study were not skewed to the right, no logarithmic transformation of size measurements was performed.

Under the assumption of no colony effect (see results), no inbreeding (chapter two), and no selection on the parents, to derive heritability a formal mother-daughter regression analysis was performed in experiment one. Unequal sample size between families was accounted for by weighting in proportion to the inverse of the residual sampling variance of family means about the unweighted parent-mean offspring regression (Kempthorne & Tandon 1953, in Lynch and Walsh 1998). Despite the bimodality, neither mother size (Kolomogorov-Smirnov's d = 0.18, n = 25, n.s.) nor offspring size distribution (K.-S. d = 0.04, n = 153, n.s.) differed significantly from normality (Fig.9). Nevertheless, simple regression models are not appropriate (Falconer & Mackay 1996; Roff 1996; Lynch & Walsh 1998). Therefore, in addition to the continuous model described above, a threshold model was evaluated.

Macro-and microgyny are regarded as discrete phenotypes which are caused by an underlying, normally distributed parameter ("liability", e.g. juvenile hormone concentration, Fig.6). Individuals in which this character exceeds a particular threshold develop into macrogynes, while those below the threshold develop into microgynes. Under this assumption, heritability is calculated by comparing the mean liability of the parental population to the mean liability of offspring of "affected" parents, where the mean liabilities and the selection differential are calculated from the fraction of "affected" individuals ("incidence": Falconer & Mackay 1996). Mean liability of the parents was calculated from the incidence (the proportion of the morph under consideration) over all natural populations, offspring liability was calculated directly from the experiments according to Falconer & Mackay (1996). Standard errors (S.E.) were calculated from an estimate of the sampling variance as given in Falconer & Mackay (1996): $\sigma^2 = (1-p)/(i^2_r \times i^2_p \times A)$, where “p” is the incidence in the parental population, “i_r“ and “i_p“ are mean deviations of "affected" individuals in offspring and population from the population grand mean, and “A” is the number of "affected" offspring measured.

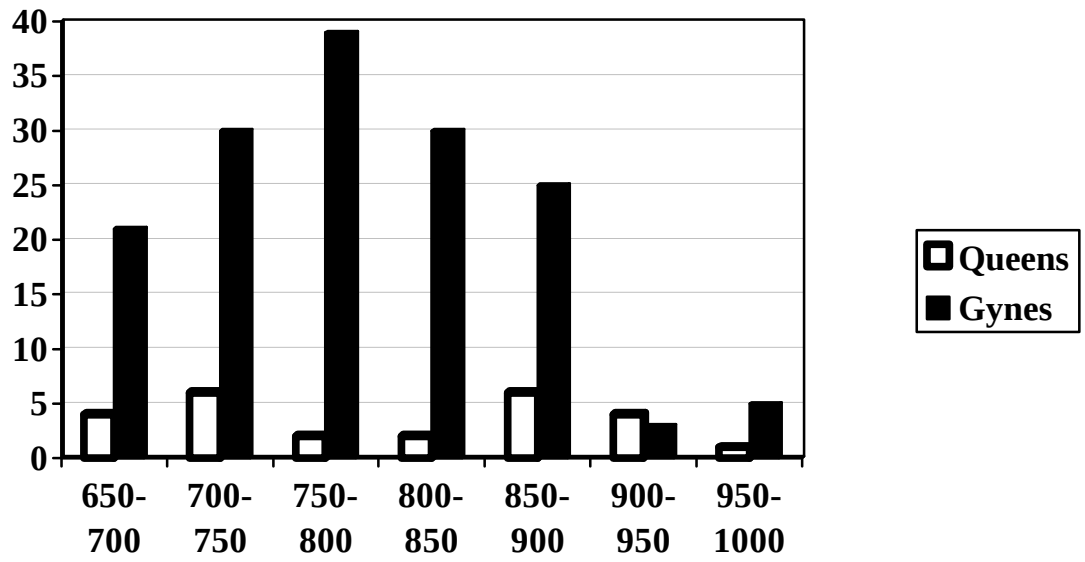
3.3 Results

3.3.1 Experiment one

In the cross-fostering experiment an initial two-way ANOVA demonstrated that mother class (macro- versus microgyne) had a much stronger effect on the mean size of cross-fostered offspring than the class of fostering colony did (F_{colony} (2,19) = 0.62, p = 0.548; F_{mother} (1,19) = 16.62, p = 0.001; $F_{interaction}$ (2,19) = 3.13, p = 0.067). Figure 8 demonstrates that offspring exhibited considerably lower size variance (σ^2 = 5336) than mothers (σ^2 = 8898). The regression of mean daughter size on mother size was significantly positive (b = 0.44 ± 0.10 (S.E.), n = 25, p < 0.001) which translates into a heritability estimate of $h^2 = 0.88 \pm 0.20$ (S.E.).

The threshold model can only be applied to one class of mothers at a time, and consequently two separate estimates were obtained. Both differed significantly from the above value and are outside the biologically realistic range, in opposite directions. The heritability estimate from macrogynes and their offspring yielded $h^2 = 2.48 \pm 0.25$ and the value using microgynous families was $h^2 = -0.52 \pm 0.05$.

Figure 9: Distribution of offspring size from macrogynes in macrogynous colonies ("macros"), microgynes in micrgynous colonies ("micros") and macrogynes and microgynes from mixed colonies ("macro mixed" and "micro mixed" respectively).

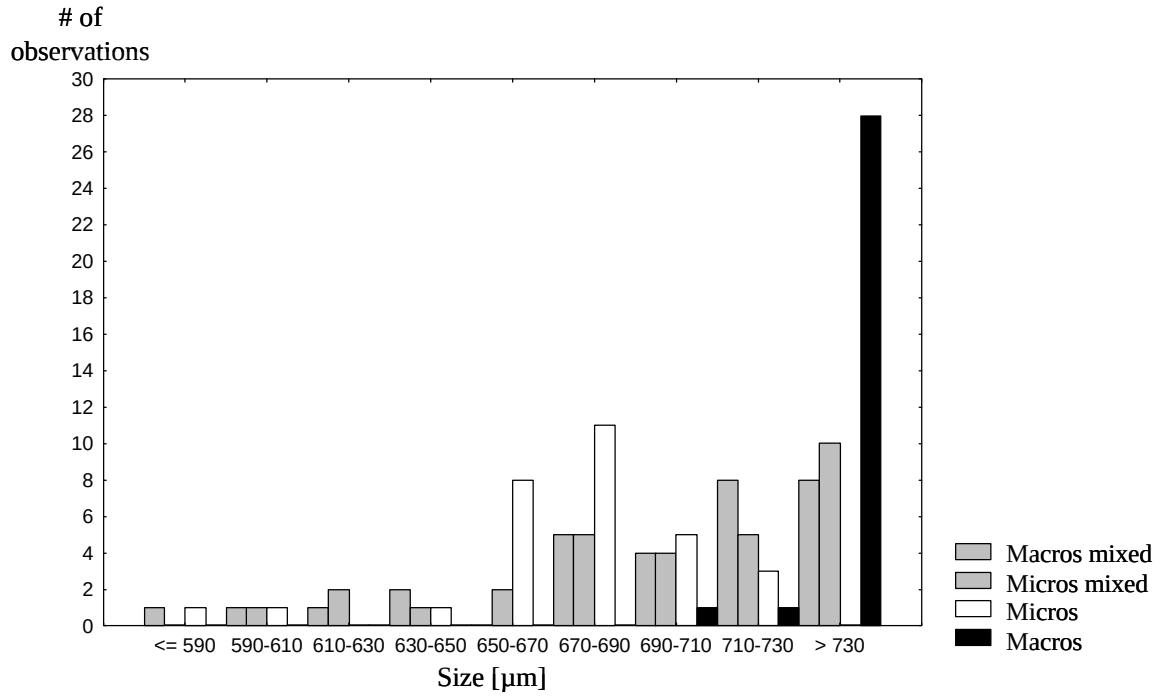


3.3.2 Experiment two

Gyne size was not significantly different between randomized groups and original colonies ($F_{(1,28)} = 1.71$, $p = 0.202$), therefore both groups are pooled in the following analyses. Overall, a two-way ANOVA indicated a significant effect of colony and mother type ($F_{colony\ (2,26)} = 40.29$, $p < 0.001$; $F_{mother\ (1,26)} = 28.64$, $p < 0.001$) on mean daughter size. Overall, daughters of macrogynes (737.8 µm ± 46.9) were larger than daughters of microgynes (685.9 µm ± 37.0). Daughters in macrogynous colonies were larger (776.2 µm ± 28.0) than daughters in mixed (705.6 µm ± 23.6) and microgynous colonies (659.9 µm ± 29.2). In contrast to experiment one, colony type had an effect on daughter size, in the presence of queens. These effects were also true when original colonies and random groups were analyzed separately. The distribution of offspring sizes of the different queen classes did not indicate a single-locus mode of inheritance (Fig.10).

To take the colony effect into account, all mother- and mean daughter size values were divided by the average value of their colony. A regression was performed on these relative values in the three colony types separately, providing within-macrogynous, within-microgynous and across-classes estimates of body size heritability. The regression was not significant in macrogynous ($b = 0.05 \pm 0.44$, $n = 8$, $p = 0.905$) or microgynous colonies ($b = -0.10 \pm 0.13$, $n = 6$, $p = 0.478$). The regression was slightly negative in mixed colonies ($b = -0.12 \pm 0.05$, $n = 10$, $p = 0.047$). These results correspond to zero heritability of body size within or across size classes, and within mixed colonies offspring of macrogynes was not significantly different from microgyne offspring ($F_{(1,12)} = 0.98$, $p = 0.342$). For comparison with experiment one, an overall regression analysis for all groups of experiment two (across mixed, macro- and microgynous colonies) was performed which indicated an overall positive relationship between mother- and daughter size ($b = 0.43 \pm 0.11$, $n = 30$, $p < 0.001$).

Figure 10: Gynes raised in macrogynes colonies were significantly larger than in mixed or microgynous colonies. Within mixed colonies, the offspring of macrogynous and microgynous mothers was not significantly different from each other.



In mixed colonies the threshold approach yielded $h^2 = 1.43 \pm 0.06$ for macrogynes and $h^2 = -1.98 \pm 0.14$ for microgynous. Macrogynes and microgynes analyzed separately in their respective colonies resulted in $h^2 = 3.67 \pm 3.50$ and $h^2 = 2.44 \pm 0.80$, respectively.

Egg volumes between macrogynes (3.301×10^{-2} mm^3 $\pm$ 0.5698×10^{-2}) and microgynes (3.3789×10^{-2} mm^3 $\pm$ 0.4609×10^{-2}) were not significantly different ($F_{(1,180)} = 1.96$, $p = 0.163$).

3.3.3 Experiment three

No gynes were successfully reared under warm conditions, so that the effect of temperature could not be evaluated. Queen production in the colder groups was also low: eleven colonies produced no gynes, seven colonies produced one gyne, and two and three gynes were produced once. The average gyne size in a colony, relative to their mothers' size, was not significantly different between groups (Mann-Whitney $U_{(4,5)} = 13$, $p = 0.462$).

3.3.4 Experiment four

A pair-wise analysis of the split colonies to test for an effect of queen number was precluded by the low overall queen production, especially in the polygynous colony segments. Both colony halves produced gynes only in two cases. In one case the monogynous half produced larger gynes, in the other the polygynous half produced larger gynes. Across the whole data set however, mean gyne size of a given colony half, relative to the mean of potential mothers, was negatively correlated to queen number across all colony halves ($R_S = -0.62$, $n = 12$, $p = 0.030$).

3.4 Discussion

To my knowledge, this study is the first in ants to use the power of quantitative genetics to determine the heritability of a natural trait. Thus far, studies have only demonstrated an unquantified genetic influence (Stuart & Page 1991; Snyder 1993; Fraser et al. 2000), or shown an inheritance pattern that supports predictions from a single-locus despite environmental influence model (Winter & Buschinger 1986; Heinze & Buschinger 1989). The approach of regressing the phenotypes of lab-reared progeny on that of their field-collected parents provides a minimum estimate of h^2 (Riska et al. 1989, see also Coyne & Beecham 1987).

However, queen size determination in *Leptothorax rugatulus* seems to be a complex phenomenon, as is the case with many threshold characters (Roff 1996; Doums et al. 1998), and the results presented here do not allow a coherent estimation of heritability. While it is generally accepted that each experimental estimate of heritability has certain biases (Mitchell-Olds & Rutledge 1986) and independent estimates seldom agree (Roff & Mousseau 1987), the disparity of the estimates presented here demands further exploration.

The only realistic heritability values for body size across the whole parameter range, namely 0.88, was obtained by regressing mean daughter size on mother size in the cross-fostering experiment. This suggests that without the (social) influence of queens, heritability of queen body size in *L. rugatulus* would be high.

On the other hand, queenless colonies are rare in *L. rugatulus*, and hence queen-presence is the biologically more relevant situation. In mixed colonies (experiment two), the inherent developmental bias of macro- and microgynous larvae was rendered insignificant and the regression coefficient of mean offspring- on mother size was even slightly negative. However, statistically these regression values are flawed by violation of the assumption of normality because at least the mother size is bimodal (Fig.8). Thus, the results are not quantitatively valid (Sokal & Rohlf 1995).

The threshold model in quantitative genetics has been devised for discrete traits that nevertheless appear under the influence of several genes and maybe a more statistically valid analysis. Yet, all heritability values calculated in the threshold framework lie outside the biologically reasonable range. Values higher than one are plausible when inbreeding, maternal effects or common environmental influence have not been taken into account (Falconer & Mackay 1996; Lynch & Walsh 1998). Negative values, on the other hand, could arise by inverse maternal effects, or negative environmental associations between relatives, though neither of these have been reported in the literature. While inbreeding at the individual level ($F_{IS} = 0$) could be excluded for *Leptothorax rugatulus* (chapter two), individuals in this study have non-random partners ($F_{IT} > 0$) because they are drawn from different, genetically differentiated populations, and this possibly inflates the heritability estimates. Maternal effects (Bernardo 1996a) can not be completely excluded either, but the parameter "egg size", which is of prevalent importance for maternal effects (Bernardo 1996b), seems not to be responsible. In experiment two, significantly larger gynes were produced in macrogynous than in microgynous colonies, with no differences in egg size.

By laboratory rearing under standardized conditions, most environmental factors could be held constant and when varied, food regime seemed to have no significant effect. Only the social environment varied across experimental treatments and the number of macrogynes in a colony showed a significant negative effect on queen size. Also, the inflated heritability estimates in macrogynous and microgynous colonies of the second experiment suggest that size of queens present in the colony might influence gyne size (see also Backus 1993). Worker brood care could be influenced by physical interference or pheromones by queens (Brian & Hibble 1964; Brian 1973, 1979; Vargo & Fletcher 1986). Alternatively, larvae could be manipulated directly (Keller et al. 1989). A social effect of queen size on gyne size might well explain the high mother-daughter size correlation in field colonies (Rüppell et al. 1998).

However, social environment was identical in the mixed colonies of experiment two and a possible worker influence randomized in experiment one. Furthermore, the confounding effects discussed above influence heritability estimates from continuous and threshold models alike and can not explain the differences between the macro- and microgynous estimates. Consequently, erroneous estimates in the calculation of the

threshold values have to be considered. The fact that in both experiments, the macrogyne estimate always gave h^2 values larger than 1, while that of microgynes resulted in negative h^2, suggests a systematic error because both estimates should be approximately identical.

Two explanations are conceivable: the morph frequency (incidence) of the combined maternal population might have been incorrectly estimated in both experiments and for both morphs independently. Alternatively, and more probably, the difference between estimates from macro- and microgynes is explained by a slightly incorrect size threshold for separating macro- and microgynes. In traits with a bimodal but continuous character distribution, estimation of the threshold is not trivial. In *L. rugatulus* the estimation was originally based on the minimum of the size frequency distribution across populations in the field (Rüppell et al. 1998). A minor shift in the threshold value does not affect maternal assignment to the two classes because mother size is either much larger or much smaller than the threshold, whereas offspring could be frequently misclassified (Fig.8). In fact, a 4.4% increase of the threshold size would yield heritability estimates of $h^2 = 0.63 \pm 0.63$ (macrogynes) and $h^2 = 0.91 \pm 0.04$ (microgynes) in the cross-fostering experiment, and -0.05 ± 0.03 (macrogynes) and 0.05 ± 0.10 (microgynes) in the mixed colonies of experiment two. These values accord much better among themselves and with the respective regression results. This extreme sensitivity of the threshold model to the actual value of the threshold makes it difficult to apply to continuous traits without a priori knowledge of the trait value at the threshold. The regression approach, although statistically problematic, is more robust and seems preferable in cases such as this study.

The threshold model has been developed for discrete morphs in a fixed environment (Roff 1994). In the experiments and in the populations of origin, the social environment fluctuates widely and queen number and -morphology play a role in gyne size determination. Queen number effects have also been reported for *Leptothorax longispinosus*, but only data on gyne weight is available (Backus 1993). With the presence or absence of queens, a number of colony parameters presumably change (Hölldobler & Wilson 1990; Bourke & Franks 1995; Crozier & Pamilo 1996a) and it is consequently not surprising to find no correspondence of the results of experiment one and two (Roff 1994).

To summarize these complex conclusions, it could be demonstrated that mother size is highly predictive of daughter size when queen effects are removed from the rearing environment. While egg size could be excluded as a possible cause for maternal effects, qualitative maternal effects, such as RNA or protein content of the egg or cuticular hydrocarbons might still play a role (Bernardo 1996b). However, this high mother-daughter correlation can be completely superseded by queen effects in rearing colonies, since in mixed, queenright colonies offspring of macro- and microgynes are mainly microgynous. In colonies of

either queen type, gynes similar to their mother are produced, although within the size classes macrogyne / microgyne no correlations between mother and daughter exist.

The principle that microgyne colonies mainly produce microgynous offspring and macrogyne colonies mainly produce macrogynous offspring, is unambiguous under laboratory conditions but even stronger in the field (Rüppell et al. 1998). This raises the question of whether it can be concluded from a heritability estimate of zero (in the queenright condition of mixed colonies) that no additive genetic variability influences queen size in *Leptothorax rugatulus*, and body size variation is solely due to phenotypic plasticity (Via 1993, 1994). This question has significant implications for the evolutionary interpretation of the queen size polymorphism (Björklund 1996). Given plasticity and sufficient information, colonies could adjust the offspring size and consequently their reproductive options according to the ecological optimum (Johnson & Gaines 1990). Macrogynes could present a "beach-head" strategy to colonize new patches and immediately switch under favourable conditions to microgyne production for exploitation of the surroundings (Denno 1994). If there were constraints on plasticity, the queen size dimorphism is *L. rugatulus* is better interpreted as a polymorphism that is balanced by the conflicting selection pressures for colonization and within patch competition (Hamilton & May 1977; Heinze & Buschinger 1989; Bourke & Heinze 1994).

The strong morphological differentiation of populations (chapter two and chapter four), the high mother - daughter size correlation in natural colonies and the genetic separation between morphs (chapter two) argue against phenotypic plasticity as the sole cause of different queen sizes. If progeny size is considered simultaneously a maternal and offspring character (Fox & Czesak 2000), and larval bias and queen effect are considered as correlated traits (the offspring reaction considered as an extended phenotype of the mother), their "combined heritability" would be considerable. This possible case of "cultural inheritance" is distinct from the case of *Solenopsis invicta* (Keller & Ross 1993) because in *L. rugatulus* the cultural effect is not mediated autonomously by workers, but it is queen-dependent.

To rephrase the biologically relevant question: are queens or their female sexual offspring capable of reacting flexibly to environmental conditions by adjusting gyne size to immediate responses? The negative effect of queen number on gyne size in experiment four suggests that such rules might exist, given that colony queen number is a good indicator of future colony founding success. However, this effect could be also a by-product of the queen effect (Brian 1979), or arise from intra-colonial kin conflicts (Herbers 1990; Backus 1993; Bourke & Ratnieks 1999).

4 Comparison of morphological, genetic and social variability between northern and southern populations

4.1 Introduction

Ever since C. Linné published his "Systema Naturae" in 1735, it has been formally recognized that biological diversity is hierarchically organized. Today, the scientific concepts of taxonomy and phylogeny reside in the notion that organisms can be clustered at various levels. The best-defined clustering level is the species level, with a species being defined as "groups of actually or potentially interbreeding populations which are reproductively isolated from other such groups" (Mayr 1942). Based on this notion, much evolutionary research has focused on the species level (often measured in a single population), and variation between populations and individual variability have been neglected (Endler 1986; Real 1994).

However, the pattern of variability at these lower levels play a prevalent role in evolution (Endler 1986; Bonnin et al. 1996), and with the advent of molecular markers (Avise 1994) and theoretical developments (e.g. Hanski & Gilpin 1991), population structure and sub-species variability have become major areas of evolutionary research (Spitze 1993; Prout & Parker 1993; Olivieri et al. 1995; Bohonak 1999; Fox & Czesak 2000). Today, numerous studies exist on the genetic structure of populations measured with neutral genetic markers, like allozymes, microsatellites or restriction fragment length polymorphisms. This is true across taxa, and in ants many studies on the genetic population structure at various scales exist (e.g. Pamilo 1983; Seppä & Pamilo 1995; Chapuisat et al. 1997; Pamilo et al. 1997; Ross et al. 1997).

Population structure of quantitative traits has been shown to differentiate according to similar rules as molecular markers (Rogers & Harpending 1983; Felsenstein 1986; Rogers 1986; Lande 1992). Population structure of quantitative traits has also proven informative to compare quantitative traits with neutral markers to infer selection on the quantitative traits (Spitze 1993; Prout & Parker 1993; Yang et al. 1996). Two approaches are possible when several populations are studied: the concurrence of divergence patterns in marker and quantitative trait can be investigated, or the degree of overall divergence between them can be compared (Spitze 1993). Both, quantitative and qualitative deviations of the divergence pattern of traits from the null-model (that is derived from the presumed neutral evolution of allozyme or microsatellite markers) suggest that other evolutionary forces have been acting on the trait(s) under investigation. Particularly by demonstrating a significantly higher overall divergence for quantitative traits than expected under the null model of neutral differentiation by drift, selection has been inferred in a variety of organisms (Argyres & Schmitt 1991; Spitze 1993; Prout & Barker 1993; Podolsky & Holtsford 1995; Bonnin et al. 1996; Yang et al. 1996).

For social insects few comprehensive reports on morphological variation across populations exist. The major work has been performed on the differentiation between different races or regional varieties of the honeybee (Lobo et al. 1989; Crewe et al. 1994; Lobo 1995; Hepburn & Radloff 1996, 1997; Radloff et al. 1998). Additionally, few reports for bumble bees (Pekkarinen 1979) and ants (Herbers & Stuart 1996; Heinze et al. 1998a) exist. A co-analysis of quantitative and genetic marker variation has been performed only for the honeybee (Lobo et al. 1989; Lobo 1995) and a general concordance between morphological traits and molecular markers was reported although a formal comparison was not attempted.

Between populations within one species, higher genetic correlations among traits are expected than between species because the period of independent evolution is shorter. Intra-specific analyses have successfully demonstrated genetic correlations that might constrain independent trait adaptation (e.g. Conner & Via 1991). Across ant species an important correlation exists between social and morphological traits: polygyny, the co-occurrence of several queens in one colony, is linked to smaller and less polymorphic workers (Elmes 1974; Ross & Fletcher 1985; Frumhoff & Ward 1992; Keller 1995) and to smaller, relatively short-lived queens (Hölldobler & Wilson 1990; Keller & Passera 1989, 1990; Stille 1996; Keller 1998). While an adaptive explanation for the impact of social structure on queen parameters is provided from life-history theory (Bourke & Franks 1995; Keller 1998), the correlation to worker morphology is hard to explain (Bourke & Franks 1995) and might be caused by adaptation or non-adaptive causes. Thus, a comparison of populations with regard to several morphological and social parameters in a variable species, as *Leptothorax rugatulus*, is an important first step towards a deeper understanding of these associations.

Finally, an investigation of separate populations of a widely distributed species might allow inferences about the ancestral character states in the population of origin, given that a true phylogeny for these populations can be derived. This phylogeny can then be used for a reconstruction of ancestral character states by parsimony analysis (Maddison & Maddison 1999).

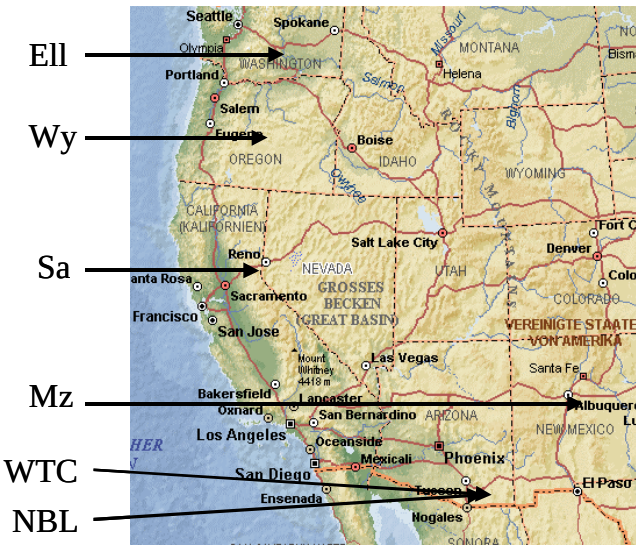
4.2 Methods and materials

4.2.1 Field methods

Morphological, genetic and life-history parameters were compared among six populations (Fig.11) that span almost the entire distribution range: three populations near the southern distribution limit ("WTC" and "NBL" from the Chiricahua Mountains, Arizona, "MZ" near Albuquerque, New Mexico) and three northern populations ("SA" from the Sagahen Field Station, California, "WY" near Bend, Oregon, and "ELL" near Ellensburgh, Washington). Colonies were collected in 1997-1999. Sample sizes were highly variable ("ELL": 13 colonies, "WY": 17, "SA": 20, "WTC": 84, "NBL": 148, "MZ": 157) because of different colony

abundance and sampling effort. Directly after collection, number of queens and number of workers were determined for each colony. Most colonies were taken back to the laboratory alive and some were stored in 96% ethanol for DNA preservation.

Figure 11: The six collection sites (populations) which were sampled for comparative study of worker and queen morphology, colony characteristics and microsatellite frequencies.



4.2.2 Morphological measurements

All size measurements were performed with a micrometer table mounted under a 50x Wild™ stereomicroscope which allowed for an accuracy of one micrometer. Individuals were first killed by freezing, then dissected and measured. In each of ten colonies per population, five workers were randomly selected for measurements of maximal headwidth, headlength, maximal thorax length, petiolus height and the length of both scapi which were averaged to scapus length. Additionally, coloration of the workers' head capsule was assessed by scanning the mounted workers with a commercial scanner and probing the grey score of each head capsule five times using the Adobe Photoshop® eyedropper function. Scores were adjusted for different light intensity of the scanning procedure.

Two morphological queen parameters were determined: maximal head width (as described for workers), and the number of ovarioles (by dissection of the reproductive tract). As queens were not available ad libitum, an unbalanced design was accepted in order to increase overall sample size. The number of queens studied varied for both parameters (queen head width: ELL: 15, WY: 17, SA: 19, WTC: 20, NBL: 28, MZ: 113; ovariole number: WY: 15, WTC: 15, ELL: 19, NBL: 22, SA: 24, MZ: 25).

4.2.3 Genetic analysis

In order to derive the amount and pattern of neutral genetic divergence between the populations, the allele frequency distribution at four microsatellite loci (chapter two) was used. All queens present in a colony were genotyped, and one random worker from queenless colonies was used. PCR-amplification, allele

visualization and -scoring followed standard procedures (for details see chapter two). Sample sizes were "ELL": 18, "WTC": 21, "WY": 26, "NBL": 26, "SA": 36, "MZ": 149.

Overall differentiation of the microsatellites was investigated by resampling 1000 times one individual per colony and subjecting these resamples to a variance analysis with the aid of the computer program GDA (Lewis & Zaykin 1999). GDA was also used to calculate from the resampled data sets Nei's D (Nei 1972) and Reynold's coancestry coefficient (Reynolds et al. 1983) between populations. All statistical parameters of the resamples were averaged to yield an unbiased estimate. Based on average Reynold's coancestry coefficient, UPGMA- and NJ-clustering of the populations were performed and a phylogeny derived, this topology was derived from all other UPGMA analysis, while the NJ algorithms resulted in a number of tied alternatives.

4.2.4 Statistical methods

Morphological worker measurements were normally distributed and queen number per colony and worker number did not differ significantly from normality after square-root transformation. However, both parameters concerning queen morphology showed bimodal distributions which could not be transformed to improve the fit to normality and consequently untransformed data was used in the analyses.

The term "differentiation" is used in this chapter in the sense of an F-statistic, i.e. as the ratio of variance between and within the compared groups. Between-population differentiation of all morphological and life-history traits was first quantified by univariate, nested ANOVAs (colony level nested in population level) and pairwise differences between populations checked post-hoc with Scheffé-tests. In the cases of ovariole number, queen head width, and queen number, the significance of the results were confirmed by a non-parametric Kruskal-Wallis ANOVA.

After high overall correlations between the size parameters of workers had been found (Pearson's r_P ranging from 0.72 to 0.93), the size parameters were combined in a principal component analysis. The first principal component, correlating positively with all single parameters (r_P ranging from 0.88 to 0.96), was taken as general worker size indicator (1.PC) and explained 86.3% of the total morphological variance in workers. With body size removed, the second principal component can be interpreted as a descriptor of body shape (2.PC) (R. Strauss, pers. commun.): it explained almost half of the remaining variance (6.1% of total variance) and correlated best with petiolus height (r_P = -0.46) and scapus length (r_P = 0.26). The between-population differentiation of both multivariate variables was investigated by nested ANOVA and used in further analyses instead of the single worker parameters.

In order to infer selective divergence in quantitative traits among populations, it is necessary to compare the genetic differentiation among populations of neutral marker loci and of quantitative traits (Roger 1986; Lande 1992; Spitze 1993). Measurements of across-population heritabilities of quantitative traits are required (Spitze 1993) because population differentiation of phenotypic characters can be caused by environmental influences or genetic differentiation. This is true, even though colonies of social insects in many ways provide a constant environment (Oster & Wilson 1978; Hölldobler & Wilson 1990) and thus decrease environmental variance on the individual.

However, laboratory maintenance of *Leptothorax rugatulus* colonies from all populations proved impossible under equal conditions, and consequently no heritability estimates across all populations could be obtained. Furthermore, laboratory estimates of the heritability of queen number and colony size of ants are probably too far removed from the natural condition to make any meaningful inferences. Even for morphological characters (see chapter 3), heritability estimates have proven difficult because they are clearly context dependent.

Despite the missing heritability estimates, two approaches were used to make an inferential comparison between quantitative traits and molecular markers possible: First, all differentiation was assumed to be due to a genetic basis or broad-sense heritability to be equal among and within populations. This allows under Hardy-Weinberg equilibrium for a direct comparison between microsatellite F_{ST} and morphological or social differentiation (measured as $F_{ST} = \sigma^2_b / (\sigma^2_b + 2\ \sigma^2_w)$; σ^2_b : phenotypic variance between populations, σ^2_w : phenotypic variance within populations) (Wright 1969; Bonnin et al. 1996; Yang et al. 1996). The sampling distributions of both F_{ST} -values was generated by bootstrapping (chapter 13.2) and the difference between them was considered significant when 95% C.I. were non-overlapping.

The second approach was to determine the maximum relative decrease of the between-population variance (by attributing a proportion of population differentiation to environmental effects) at which the differentiation of the quantitative traits is still significantly larger than the neutral genetic value. As a conservative estimate, the width of the 95% C.I. was maintained in this reduced variance scenario.

For all parameters, the absolute parameter distances between population means were converted to relative population distances by division by the parameters' overall standard deviation. The correlations between the matrices of the various population distances (in geographic, microsatellite and quantitative trait space) were assessed by Mantel tests (Sokal & Rohlf 1995), with Bonferroni corrections for multiple comparisons. Parameters with a high matrix correlation show a similar divergence pattern between populations and thus are either genetically correlated or similarly influenced by the environment.

Based on the UPGMA-clustering, character states for all investigated traits in the presumed ancestral population were derived. Weighted squared change parsimony (Maddison 1991; Schluter et al. 1997) on the unrooted tree was performed, as implemented in the computer program MacClade 3.08 (Maddison & Maddison 1999). Presumably, this ancestral population was continuous at the beginning of the warming phase after the last ice age, roughly ten-thousand years ago when the northern parts of North America were recolonized from southern refugia (Pielou 1991).

4.3 Results

4.3.1 Quantitative traits

The data of the seven morphological traits from the six investigated populations are summarized in table 8. Even though considerable variability resided within populations, all quantitative traits showed significant differentiation between the populations. The colony parameters differed less among populations (only few single comparisons were significant) and morphological queen parameters were most strongly differentiated, with worker morphology intermediate (Fig. 12; Tab.9).

Table 8: Averages (± S.D.) are given for all populations and parameters that entered the final analyses. The ancestor values refer to the phylogenetically reconstructed character range of the ancestral population, given that the population differentiation analyzed was not exclusively due to phenotypic plasticity.

Population:	WTC	NBL	Mz	Sa	Wy	Ell	Ancestor
Colony size (# of workers)	67 ± 37	57 ± 54	82 ± 100	92 ± 39	67 ± 35	72 ± 38	74 – 74
Queen number	1.1 ± 0.8	5.4 ± 7.2	4.9 ± 5.7	3.2 ± 4.5	1.1 ± 0.8	1.5 ± 1.1	2.4 – 3.6
Worker color	97.3 ± 5.0	98.4 ±4.2	99.5 ± 4.5	102.3 ± 4.8	102.0 ± 4.6	100.3 ± 4.2	99.4 – 100.4
Worker size (1. P.C.)	-0.15 ± 0.97	-0.34 ± 0.82	-0.29 ± 0.70	0.72 ± 1.03	-0.01 ± 1.07	0.07 ± 1.02	-0.15 – 0.06
Worker shape (2. P.C.)	0.62 ± 0.70	0.49 ± 0.76	0.22 ± 0.95	-0.40 ± 1.03	-0.44 ± 0.73	-0.48 ± 1.11	-0.34 – 0.13
Queen size [µm]	763 ± 25	664 ± 54	664 ± 52	857 ± 25	841 ± 23	816 ± 68	724 – 790
# of ovarioles in queens	7.8 ± 0.6	7.8 ± 0.6	7.9 ± 0.6	10.1 ± 1.3	9.6 ± 0.6	9.8 ± 1.1	8.4 – 9.3

All parameters concomitantly indicated a north and a south cluster with three populations in each (Tab.9, Tab.10). Workers were darker and larger in the north, and also had a relatively higher petiolus. Queens in the north were much larger on average and possessed more ovarioles. In contrast, there were two predominantly polygynous populations in the south (NBL, Mz) and one in the north (Sa), and colony size did not comply to the north-south separation either.

Figure 12: Colony means of the first and second principal component of worker morphology. Despite some overlap, southern populations (WTC, NBL, Mz) are well separated from northern (Sa, Wy, Ell).

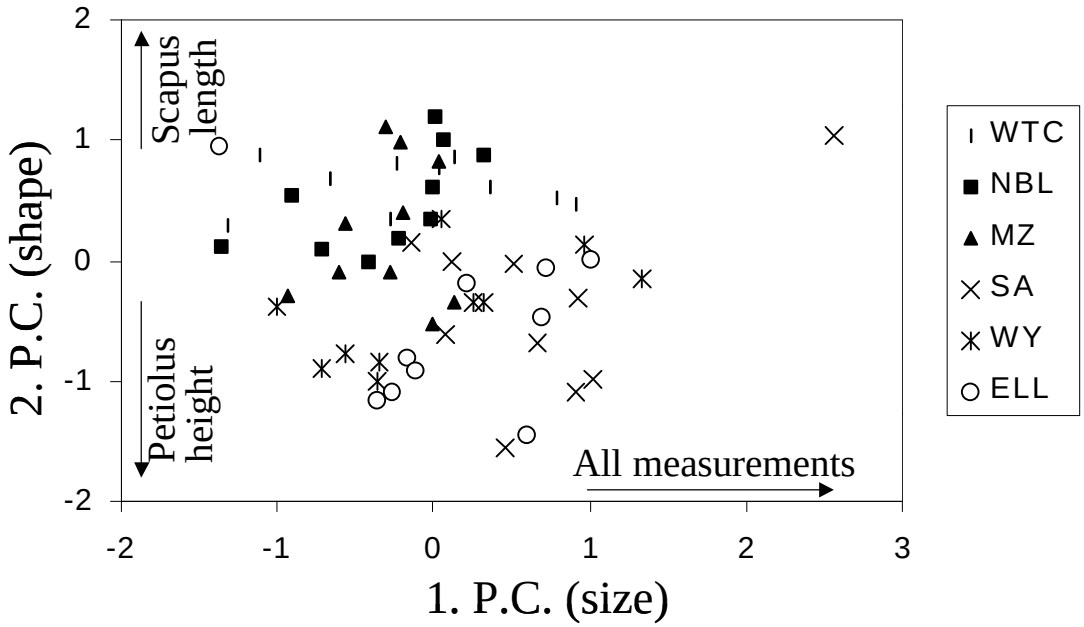


Table 9: Differentiation among the six investigated populations was significant in all investigated parameters. However, the pairwise Scheffé tests indicated that not all populations differed from each other (only differing populations are listed).

Trait	Population effect	Post-hoc Scheffé test results
Colony size (# of workers)	$F_{(5,374)} = 4.14$, $p < 0.001$	Sa > NBL
Queen number	$F_{(5,430)} = 8.19$, $p < 0.001$	(NBL = Mz) > WTC
Worker color	$F_{(5,231)} = 9.83$, $p < 0.001$	(Sa = Wy) > (WTC = NBL)
Worker size (1. P.C.)	$F_{(5,229)} = 11.23$, $p < 0.001$	Sa > (Wy = Ell = Mz = NBL = WTC)
Worker shape (2. P.C.)	$F_{(5,229)} = 19.24$, $p < 0.001$	(WTC = NBL = Mz) > (Wy = Sa = Ell)
Queen size [µm]	$F_{(5,115)} = 144.37$, $p < 0.001$	(Sa = Wy) > Ell > WTC > (Mz = NBL)
# of ovarioles in queens	$F_{(5,43)} = 20.05$, $p < 0.001$	(Sa = Ell = Wy) > (WTC = NBL = Mz)

4.3.2 Genetic data

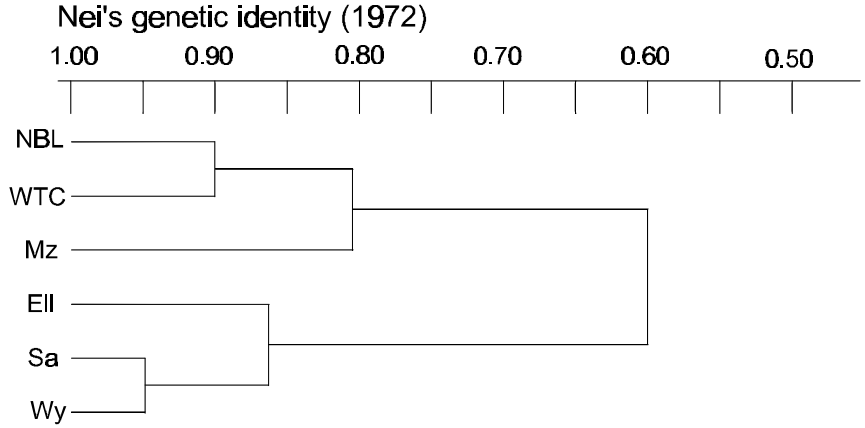
Across all populations all four microsatellite loci proved variable, but to different degrees (Tab.10). There was a significant overall differentiation between populations (F_{IS} = 0.008 (-0.035 - 0.044), F_{ST} = 0.097 (0.045 - 0.191) and F_{IT} = 0.104 (0.055 - 0.172)). The tree topology of genetic distances between populations (Fig.13) was identical to a clustering based on geographic distance and the two matrices were correlated,

although not significantly so (Mantel test: r_M = 0.68, p = 0.077). Genetic variability, measured as heterozygosity (Nei 1978) was evenly distributed among the populations and no hybridization zone was indicated by a significant heterozygote excess (Barton & Hewitt 1985).

Table 10: Descriptive statistics for four microsatellites loci from each population. The total number of individuals genotyped is given with the resample size in brackets.

Popu-lation	Sample size	# of alleles					Heterozygosity: H_{exp} / H_{obs}				
		LXGT 104	LXGT 218	LXGT 228	L18	Mean	LXGT 104	LXGT 218	LXGT 228	L18	Mean
WTC	21 (21)	3	6	17	15	10	0.50/0.55	0.57/0.48	0.91/0.76	0.89/0.80	0.72/0.65
NBL	25 (12)	4	6	15	17	11	0.47/0.50	0.63/0.71	0.84/0.88	0.92/0.92	0.72/0.75
Mz	147 (49)	4	5	25	19	13	0.49/0.43	0.56/0.64	0.91/0.94	0.79/0.80	0.69/0.70
Sa	35 (20)	11	4	12	14	10	0.74/0.86	0.55/0.67	0.86/0.94	0.87/0.88	0.76/0.84
Wy	26 (17)	9	5	13	17	11	0.70/0.65	0.45/0.38	0.88/0.85	0.89/0.85	0.73/0.68
Ell	17 (13)	6	2	9	11	7	0.77/0.71	0.39/0.39	0.86/0.89	0.87/0.69	0.72/0.69

Figure 13: Dendrogram of the six populations constructed from UPGMA clustering analysis on the basis of Reynold's coancestry coefficient. Clustering by geographic distance results in the same topology.



4.3.3 Comparing traits and populations

Assuming complete heritability of all traits (or identical within- and between population heritabilities), morphological traits diverged more strongly than expected under neutral evolution, while differentiation of social (colony) traits (queen number and worker number) was not distinguishable from drift (Tab.11).

Table 11: Summary of the comparison of overall between-population differentiation of quantitative traits and neutral differentiation as measured by microsatellites. F_{ST} for all parameters are given with 95% C.I.

Trait	Colony size (# of workers)	Colony queen number	Worker color	Worker size (1.P.C.)	Worker shape (2.P.C.)	Queen head width	Queen ovariole number	Micro-satellites
F_{ST}	0.072	0.074	0.673	0.601	0.758	0.892	0.861	0.097
95% C.I.	0.034 – 0.121	0.057 – 0.093	0.517 – 0.791	0.431 – 0.730	0.632 – 0.844	0.863 – 0.917	0.810 – 0.904	0.045 – 0.191

Under the conservative estimate of unchanged C.I., significantly stronger differentiation would still be observed for worker color if 74% of the between-population variance in the previous model was caused by environment (within-population heritability was 3.86 times stronger than among populations). The according values for worker size were 62% and 2.67×, for worker shape 85% and 6.76×, for queen size 97% and 29.23×, and for ovariole number 95% and 19.37×. In contrast, a 5.46× lower within- than among-population heritability would be required for colony size (and 4.46× lower for colony queen number) to infer selective differentiation among the populations.

Although none of the mantel tests for correlation of inter-population distance matrices (Tab.12) were significant after Bonferroni correction, these indicated that three classes of traits existed (Tab.13). Colony characteristics were completely unrelated to microsatellite coancestry distance (Reynolds et al. 1983), and concomitant geographic distance (results not shown). Worker morphology followed the pattern of neutral genetic separation reasonably well, whereas queen morphology showed the highest correlation, and colony characteristics did not agree with marker differentiation.

Table 12: Among population distances for all of the seven investigated traits. The absolute distances between population means were divided by the overall standard deviation of the parameter to yield comparable, dimensionless estimates. The difference is indicated as population on the left subtracted from the population on the top.

Population	Colony size \ Queen number						Worker size \ Worker shape					
	WTC	NBL	Mz	Sa	Wy	Ell	WTC	NBL	Mz	Sa	Wy	Ell
WTC	---	0.75	0.66	0.37	0.00	0.07	---	-0.13	-0.40	-1.02	-1.06	-1.10
NBL	0.14	---	-0.09	-0.38	-0.75	-0.68	0.19	---	-0.27	-0.89	-0.93	-0.97
Mz	-0.22	-0.36	---	-0.30	-0.66	-0.59	0.14	0.05	---	-0.62	-0.66	-0.70
Sa	-0.36	-0.50	-0.14	---	-0.37	-0.30	-0.87	-1.06	-1.01	---	-0.04	-0.08
Wy	0.00	-0.14	0.22	0.36	---	0.07	-0.14	-0.33	-0.28	0.73	---	-0.04
Ell	-0.07	-0.22	0.14	0.29	-0.07	---	-0.22	-0.41	-0.36	0.65	-0.08	---
	Queen head width \ Queen ovariole number						Worker color \ Microsatellite distance					
	WTC	NBL	Mz	Sa	Wy	Ell	WTC	NBL	Mz	Sa	Wy	Ell
WTC	---	0.00	0.07	1.69	1.32	1.47	---	0.01	0.04	0.12	0.12	0.09
NBL	1.13	---	0.07	1.69	1.32	1.47	-0.21	---	0.08	0.11	0.12	0.08
Mz	1.13	0.00	---	1.62	1.25	1.40	-0.43	-0.21	---	0.15	0.15	0.14
Sa	-1.07	-2.20	-2.20	---	-0.37	-0.22	-0.97	-0.76	-0.54	---	0.00	0.02
Wy	-0.89	-2.01	-2.01	0.18	---	0.15	-0.91	-0.70	-0.49	0.06	---	0.01
Ell	-0.60	-1.73	-1.73	0.47	0.28	---	-0.58	-0.37	-0.16	0.39	0.33	---

The relative population distances of all traits are summarized in table 6, and yet again the highest values were obtained for the characters of queens, followed by that of workers and then colonies. Although a clear division between the three northern and southern populations exist, within the clusters the corresponding clinal trends (larger queens, more ovarioles, larger and darker workers), could not be found.

Table 13: Results of 6×6 matrix correlations (Mantel test) for population differences of all investigated quantitative traits with neutral population distances, measured as genetic coancestry distance (Reynolds et al. 1983). P-values are given before Bonferroni correction.

Trait	Colony size	Queen number	Worker color	Worker size	Worker shape	Queen head width	Queen ovarioles
Correlation	r_M = -0.08 p_M = 0.420	r_M = 0.07 p_M = 0.257	r_M = 0.55 p_M = 0.054	r_M = 0.24 p_M = 0.208	r_M = 0.76 p_M = 0.069	r_M = 0.72 p_M = 0.049	r_M = 0.86 p_M = 0.101

The phylogenetic reconstruction produced for all parameters values that were intermediate between the north and south clade and generally close to the arithmetic mean (Tab.8). The strongest deviation from these values was obtained by the population "Sa" for colony size, worker size, queen size and ovariole number of queens. Queen number was most different in "NBL", and worker shape and -color differed from the inferred ancestral states strongest in "WTC".

4.4 Discussion

Leptothorax rugatulus displays extraordinary high levels of morphological variability which has been recognized in earlier times by the description of a number of subspecies (Muesbeck & v. Krombein 1951). For all investigated parameters, this variability is hierarchically organized: individuals vary within populations but are more similar to each other than to individuals from other populations. This is accompanied by a similar differentiation of neutral genetic markers. This overall differentiation is not surprising because populations are far apart, and separation between them has probably been complete for several thousand years (Heald 1951). The weaker differentiation in colony characteristics is mainly attributable to larger within-population variability based on the undetermined growth of colonies (Hölldobler & Wilson 1990; Bourke & Franks 1995).

As no methodology exists for age determination of ant colonies independent of size, it cannot be excluded that differences between populations in colony characteristics are due to different age structures of the populations. This is certainly true for colony size, but the degree of polygyny (queen number) seems stable over several years in *Leptothorax rugatulus* and is clearly attributable to ecological correlates (chapter two). The hypothesis that social differences are relatively stable in *L. rugatulus* is also supported by the fact that

its differentiation in social system exceeds the inter-population differences in other *Leptothorax* species (Heinze et al. 1995; Herbers & Stuart 1996; Chan et al. 1999).

Due to this pronounced differentiation, it was expected to find the correlation between social structure and queen- and worker-size that is generally found across species (Bourke & Franks 1995). A link between social system and queen size had been established earlier (chapter two) and this result was reconfirmed in three southern populations. Concomitantly, worker size varied with social system. However, queens and workers in the northern populations were invariably large, demonstrating that there no obligate link exists in ants between polygyny and morphology. In the population "Sa" the largest queen- and worker size was associated with considerable polygyny. This population deviated most from the "ancestral" states in many characters but the causal link between these deviations is not clear. Due to its combination of high latitude and high altitude it was probably the harshest of the investigated environments.

A clear reduction in weight of newly raised queens has been found in the ant *Leptothorax longispinosus*, whereas workers and males were not affected (Backus 1993; Herbers & Stuart 1996). This contrasts with the significant correlation of worker and queen size found in this study. However, a comparison is somewhat speculative because for *L. longispinosus* only weight is available. Weight is a much more flexible character than actual size in ants (Keller 1988). In *L. rugatulus* the correlation between queen and worker size sufficiently explains that smaller workers exist in more polygynous populations, and therefore there is no need for an adaptive explanation.

Clearly, the morphological differentiation between populations exceeds that of neutral genetic markers in this study. Although no heritability estimates were performed, the magnitude of the observed differences makes selective divergence a likely scenario (Fox & Czesak 2000). This is particularly true for the queen parameters, because queen size and ovariole number normally do not vary within species of the Formicoxenini to the extent shown here (Plateaux 1979; Buschinger & Winter 1976; Buschinger & Alloway 1978; Stille 1996;). In fact, the number of observed ovarioles in the northern populations is the highest reported so far (Buschinger 1974b; Heinze, pers. commun.). Interestingly, the northern populations also showed a significantly higher asymmetry in their ovaries (Mann-Whitney U-test: z-approximation for large samples ($n1 = 62$, $n2 = 63$) $z = -3.38$, $p < 0.001$), and asymmetry can generally be taken as an indicator for recent directional selection (Møller & Thornhill 1997).

The number of ovarioles in queens also showed the clearest division between the cluster of northern and southern populations with almost no overlap between them. The consistent differences between northern and southern populations justify the question of whether these populations belong to the same species or not. A

fundamental difference also appeared in the laboratory rearing. While colonies from southern populations reproduced normally, colonies from "Sa", "Wy" and "Ell" did not produce any brood under the same conditions. As *Leptothorax rugatulus* does not mate under laboratory conditions, testing of potential interbreeding (biological species concept) is not possible. However, based on the species' flying capability, and the distance and degree of isolation between the populations, it seems safe to assume that at least the north and south clusters constitute independent evolutionary lines and have evolved different adaptations. This would classify them according to the evolutionary species concept (Wiley 1978) as two separate species, but the general conclusion of a selective differentiation of morphological characters (versus random differentiation) is not affected by this.

The clear differences between southern and northern populations are not exclusively attributable to latitude. Clinal trends depend additionally on altitude and the mixture between altitudinal and latitudinal clines can create complex patterns. Climatic differences not attributable to latitude are probably the reason why the north-south differences between clusters are not repeated within each cluster.

Adaptive explanations for the different trends in parameters are conceivable, but no conclusive inferences can be made from this descriptive study. Body size increases with geographic latitude in many organisms (Bergman's rule: Bergman 1847), and in endothermic organisms the facilitated thermoregulation in larger organisms provides an adaptive significance to this pattern (Schmidt-Nielsen 1984; Graves 1991). However, a similar body size distribution is found in many taxa that reportedly do not regulate their body temperature (Lonsdale & Levinton 1985; Coyne & Beecham 1987; McCabe & Partridge 1997; Heinze et al. 1998a). Although several explanations have been put forward, the evidence is unclear (Cushman et al. 1993; Heinze et al. 1998a). Large queens with a higher reproductive capacity could have advantages at higher latitudes, where relatively little time for reproduction is available, and individuals have to survive long overwintering periods. Furthermore, large queens may have colonized the northern areas only recently after postglacial warming and the transition to microgynes has not yet occurred. The darker coloration is likely to be associated with better heat absorption (Larsen & Nault 1994; McQuillan & Ek 1996) but experimental evidence is lacking in *Leptothorax rugatulus*, as it is for the adaptive value of increased body size and higher ovarioles number.

Obviously, this study only constitutes a first step in the description of variability of various parameters in *Leptothorax rugatulus* and their adaptive geographic variation. More detailed studies are needed to determine the heritability of the investigated traits, disentangle genetic correlations between suites of characters, and provide evidence for adaptive scenarios. Nevertheless, important differences between traits concerning worker morphology, queen characteristics, and colony traits were established and some

interesting trends were observed which deserve future investigation. Among all traits, queen size in *L. rugatulus* turned out to be most variable trait, and it might hence be most adaptable to different environmental conditions. Furthermore, the data suggest that the investigated populations of *L. rugatulus* belong two species.

5. Patterns of colony reproductive investments

5.1 Introduction

The combination of sex ratio and kin selection theory provides significant tests of both theories (Trivers & Hare 1976). This has led to a tremendous interest in the patterns of allocation of resources to reproductive males and females (sex ratio) in social Hymenoptera (Bourke & Franks 1995; Crozier & Pamilo 1996a; Chapuisat & Keller 1999). The haplo-diploid sex determination of Hymenoptera has two major consequences. It allows for full maternal control over the sex of produced eggs and results in asymmetric relatedness values among colony members. Outbred queens are symmetrically related to their sons and daughters, but workers are three times more related to their sisters than their brothers (relatedness asymmetry) in the simplest case of a monogynous (one reproductive queen), monandrous (singly mated queen) colony.

If queens were to control the sex ratio, a stable population equilibrium with equal investment in male and female offspring would be expected. In contrast, worker control should lead to a 3:1 investment ratio in favor of females, according to the workers' RA (Trivers & Hare 1976). Overall, empirical findings have suggested worker control (Chapuisat & Keller 1999, but see Helms 1999) and supported the case for kin selection in social evolution. The argument has been considerably strengthened by investigating polyandrous species, in which worker relatedness asymmetry and as a consequence sex ratio are lowered in a straightforward fashion (Ratnieks & Boomsma 1997).

Predictions for polygynous ant species are more complex because multiple effects on genetic colony and population structure, and on colony life history (Bourke & Franks 1995) make a variety of parameters potentially influential. Most polygynous ant species readopt some of their daughter queens. Thus, female dispersal is reduced leading to local resource competition in females and reduced female bias (Frank 1987). On the other hand, an increased population viscosity might cause local mate competition in males and favor female bias (Frank 1987). Colony reproduction occurs often via budding and therefore the accompanying workers have to be partially added to female investment (Pamilo 1991; Nonacs 1993b). Furthermore, worker relatedness asymmetry is lowered by the number of matrilines in a colony, but this effect has to be weighted by the relatedness of the coexisting queens (Trivers & Hare 1976; Boomsma & Grafen 1990). Herbers (1984) argued on proximate grounds that the outcome of the queen-worker conflict was dependent on their ratio. Colonies with relatively more workers per queen pursued worker interests by investing into females (Herbers 1984). Queen turnover makes the present queen number in a colony only a crude predictor of worker relatedness asymmetry (Heinze & Hartmann, unpubl.) and adds complications when male and female sexual brood have different development times.

Overall, polygynous ants have equal or male-biased population sex ratios (Bourke & Franks 1995; Crozier & Pamilo 1996a), probably due to a combination of the above-mentioned factors with the degree of polygyny at center stage (Nonacs 1986). However, as interspecific comparisons suffer from many potential drawbacks (Pamilo 1990b; Chapuisat & Keller 1999), and the number of queens per colony is essentially a variable trait within most species, the strong intraspecific variability at the population- and colony level in sex ratio offers improved prospects to test the importance of the different parameters (Herbers & Stuart 1996; Chan et al. 1999). The majority of studies have found the expected relative decreases in female investment with increased number of queens per colony (Herbers 1984; Evans 1995; Chan et al. 1999). These results are generally regarded as evidence for the relative relatedness asymmetry hypothesis (Boomsma & Grafen 1990; Chapuisat & Keller 1999).

However, there are a number of correlated changes that have not always been taken into account in previous studies, and the effects of colony queen number are generally weak (Herbers 1990; Deslippe & Savolainen 1995). In addition, there are some studies that do not fit the common pattern (Pamilo & Seppä 1994; Pearson et al. 1997; Aron et al. 1999a). Thus, the question to what extent relatedness structure on its own explains within-species variation of sex ratios has not been resolved, at least in facultatively polygynous ants. Comparisons of several differing populations from variable species will provide insight into natural sex ratio patterns (e.g. Herbers & Stuart 1996; Chan et al. 1999), and manipulative studies will yield supplementary information on isolated factors (e.g. Mueller 1991). A combination of both approaches will lead to an enhanced understanding of the intriguing phenomenon of resource allocation in social insects (Crozier & Pamilo 1996a).

The sex ratio produced by a colony also strongly depends on reproductive allocation. That is, the compromise between energy investment into current sexual output and workers (colony growth). The alternative development of female larvae into workers or new queens sometimes is the focus of intra-colonial conflict (Crozier & Pamilo 1996a; Bourke & Chan 1999). Apart from the relative power of workers and queen(s), the outcome of this conflict may be influenced by the degree of self-determination of female larvae. This is because under certain conditions, developing females are expected to favor their development into new queens against the interest of the other colony members (Bourke & Ratnieks 1999). In particular, the occurrence of small queens (microgyny) has been suggested as a selfish strategy of developing females (Bourke & Ratnieks 1999). Given stronger larval self-determination in microgynes, the hypothesis of kin conflict over caste determination predicts a greater (numerical) female bias in sex ratio and a higher reproductive allocation in microgyne-producing than in macrogyne-producing colonies for queen size dimorphic ant species.

In the present study, the influence of queen morphology on sex ratio was investigated, to test the predictions of the hypothesis of selfish self-determination of microgynes (Bourke & Ratnieks 1999). Furthermore, three additional factors in the field and in laboratory experiments were examined, to understand the pattern of resource allocation to male and female offspring in *L. rugatulus*. In addition to queen morphology, the following parameters were studied. 1) The queen number per colony and relatedness estimates, to check for evidence for the relative relatedness asymmetry hypothesis (Boomsma & Grafen 1990). This hypothesis predicts a positive association between worker relatedness asymmetry and proportional investment in females. As direct measurement of relatedness asymmetry by genetic markers was not feasible for all collected colonies, the worker-worker relatedness (which is generally a reasonable predictor of worker relatedness asymmetry) in a small subset of colonies with extreme sex ratio was investigated. Additionally, queen number was taken as indicator of worker relatedness asymmetries because queens in *Leptothorax rugatulus* are on average related by 0.4 (chapter 2) regardless of their morphology. 2) The worker / queen ratio to test Herbers' (1990) conflict hypothesis that predicts that the more workers outnumber queens, the more female biased the colony sex ratio should be. 3) The total sexual productivity, a crucial parameter for the local resource or mate competition hypotheses (Frank 1987), the cost variation hypothesis (Crozier & Pamilo 1996a), and the multifaceted parental investment hypothesis (Rosenheim et al. 1996). With increasing productivity, resource- or mate competition should increase. Consequently Frank (1987) predicts, respectively, a falling or raising relative investment in females. The cost variation hypothesis predicts a positive correlation of female investment with overall output because females are larger quantities of energy that might be difficult to recycle for unproductive colonies. The multifaceted parental investment hypothesis predicts the same correlation because the number of offspring produced might be limited by factors other than energy. Given that, the fitness returns from additional energy investments are larger for females, which should create a positive association between overall investment into sexuals and proportional female investment. A problem with all these predictions is that correlations can arise spuriously because overall sexual productivity depends largely on investment into females (Jasienski & Bazzaz 1999). To measure resource availability the sum of produced worker-, male- and gyne-weight divided by colony size would be a better parameter than overall investment into sexuals.

5.2 Methods and Material

5.2.1 Study species and field data

Whole colonies were collected from six different sites in three mountain ranges in Arizona and New Mexico during the pre-swarming period (June/July) in 1999 and their composition was immediately censused. Colonies were maintained alive and constantly monitored for newly eclosing adults until no further pupae

were found (up to three months, although no more sexuals and few workers initiated pupation after collection). To determine population-specific dry weights of males, workers and micro- and macrogynes 10 to 27 individuals (1-3 per colony) per caste and population were dried until weight constancy and individual dry weights measured to the nearest microgram (Tab.14). For this, young workers were used whose cuticle had darkened and mature sexuals that exhibited 'nest-leaving' behavior (e.g. flight activity). Caste-specific dry weight did not differ from normality and thus a simple, nested one-way ANOVA was used to assess population differentiation. The cost ratio for macro- and microgynes relative to males was calculated according to Boomsma (1989) as (female dry weight / male dry weight)$^{0.7}$. All analyses gave similar results without Boomsma's correction of the dry weight ratio, therefore these results are not presented.

In order to estimate the importance of relative relatedness asymmetry eight colonies with extreme sex ratios were selected (four colonies that produced only males and four with more than 80% investment in females) from one mostly monogynous population (WTC) and from one mainly polygynous population (NBL). Eight workers were genotyped from each colony at the microsatellite loci L18 and LXGT228 (chapter two) and their intra-colonial relatedness calculated with the computer program Relatedness 5.0.2 (Goodnight & Queller 1998). Allele frequencies were estimated from an extended data set with bias correction by group (chapter two).

5.2.2 Laboratory experiments

Colonies for laboratory experiments were collected in August 1996 from the different populations (Tab.1), and the number of workers and queens determined immediately. All colonies were housed under standard conditions in three-chamber nestboxes and kept in incubators (Buschinger 1974a). Caste-specific dry weight was determined as above (Tab.14).

In June 1997 three experiments were conducted in the laboratory to investigate reproductive investment patterns. The effect of queen morphology was studied in two experiments. In the first experiment (I), we removed the brood from 28 colonies. Of these 28 colonies 8 were macrogynous, 8 were microgynous and 12 were colonies containing both queen morphs. These colonies were then monitored for their composition (worker and queen number) and all newly produced offspring were counted and removed. After completion of the experiments, the colonies were frozen, the size of all queens was measured and their insemination verified by dissection. To evaluate the effect of intra-colonial queen-queen relatedness on investment patterns, all queens were genotyped and their relatedness was calculated as in chapter two.

In the second experiment (II), 30 groups of 100 workers were generated randomly from a pool of workers originating from approximately 50 different colonies. We introduced four microgynes, four macrogynes, or

two micro- and two macrogynes into these groups (10 colonies per treatment). Some aggression was observed during the first days after adoption, and killed queens were replaced during the first month of the experiment. These 'artificial' colonies received the same treatment as the colonies in experiment one. The third experiment (III) assessed the effect of multiple queens. Workers and the brood of 14 polygynous, macrogynous colonies were split into two equally-sized halves. One queen was assigned to one half and the remaining queens (2 - 18) were assigned to the other half. Offspring production was compared between the two halves.

5.2.3 Statistical methods and parameter definition

To take into account the specific data structure (many parameters were bimodally distributed and could not be transformed to normality) significance levels and C.I. of all statistical tests were calculated by bootstrapping. This was performed with the software program Matlab 5.3® (The MathWorks, Inc) and the statistics toolbox 2.2 written by R. Strauss (Texas Tech University) and with self-written bootstrap procedures (chapter 13.3). Non-parametric tests were performed in the few cases where data structure was less crucial (paired tests) and sample size was low.

From the census data of individual colonies and the site-specific dry weights (Tab.14), the following parameters were calculated: 'morph ratio' (old microgynes / all old queens), 'worker / queen ratio' (old workers / old queens), 'sexual production' (total sexual dry weight production), numerical sex ratio (ratio of the number of new females to the sum of new males and females), sex allocation ratio (numerical sex ratio * cost ratio), productivity (dry weight of all newly produced individuals) and reproductive allocation (ratio of total sexual dry weight to productivity). In order to reduce chance effects, colonies were disregarded for within-population analyses on sex ratio when they had produced fewer than 5 sexuals, and for analyses on reproductive allocation when they had produced fewer than 5 offspring altogether. One exceptionally large colony in the population MA also was omitted.

5.3 Results

The weight of workers and microgynes was similar among populations, but males and macrogynes differed significantly (Tab.14). The two predominantly monogynous populations (WTC, MA) contained the heaviest macrogynes, workers and males, but overall there was no significant correlation among the average weights of any castes across populations.

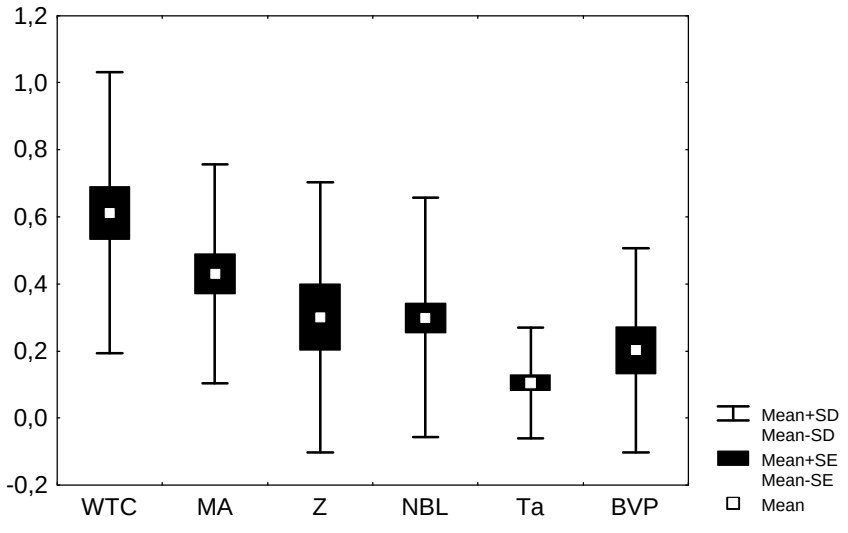
Table 14: The dry weight [µg] of newly produced sexuals and workers from different populations and laboratory colonies (mean ± 1 S.D.). Variation between populations was tested with a nested one-way ANOVA.

Population	NBL	BVP	WTC	TA	Z	MA	ANOVA	Laboratory
Males	153.1 (±26.9) n=18	172.4 (±31.5) n=15	180.8 (±22.5) n=10	141.5 (±10.9) n=15	143.0 (±20.1) n=10	219.1 (±43.2) n=20	$F_{(5,40)}$ = 34.00 p < 0.001	141.8 (±26.9) n=18
Microgynes	239.2 (±55.2) n=17	232.0 (±64.6) n=11	n/a	211.5 (±19.0) n=10	224.3 (±45.6) n=10	n/a	$F_{(3,20)}$ = 2.11 p = 0.132	196.9 (±32.1) n=54
Macrogynes	768.1 (±239.6) n=10	584.7 (±165.3) n=10	924.4 (±196.3) n=27	647.5 (±122.3) n=10	617.0 (±194.4) n=10	1024.7 (±147.4) n=15	$F_{(5,34)}$ = 35.42 p < 0.001	509.4 (±203.4) n=142
Workers	146.4 (±36.0) n=17	153.0 (±32.5) n=17	160.6 (±35.8) n=17	147.2 (±34.4) n=17	146.2 (±33.9) n=17	160.2 (±34.3) n=17	$F_{(5,96)}$ = 0.65 p = 0.659	142.6 (±19.8) n=20
Cost ratio (macro/male)	3.09	2.35	3.13	2.90	2.78	2.94	n/a	2.45
Cost ratio (micro/male)	1.37	1.23	n/a	1.33	1.37	n/a	n/a	1.26

5.3.1 Field data

5.3.1.1 Population comparison

Figure 14: Average queen number per colony and relative investment in females were negatively correlated. The increased investment in new workers (lowering reproductive allocation) was less apparent at the population level.



Colony size, the size of individual queens and the number of queens per nest differed strongly between the six populations. Concomitantly, populations also differed in almost every investigated parameter of offspring production at the colony level (Tab.15).

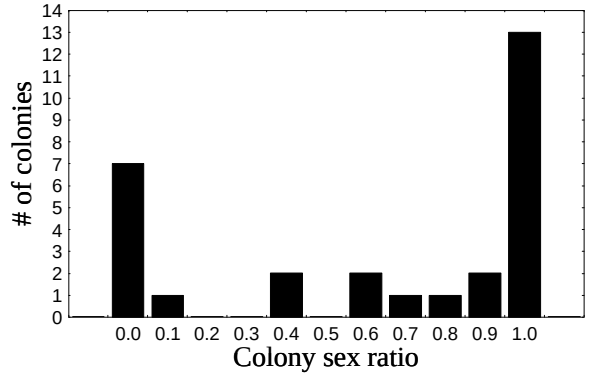
The more polygynous a population was, the fewer new sexual females (relative to males) were produced (Fig.14), and in the most monogynous population (WTC) the sex allocation ratio was significantly female biased (Tab.15). The reproductive allocation showed a similar but weaker trend.

Table 15: Population means (95% CI) of parameters of colony composition and reproductive output and their differentiation between populations (=sites) measured by ANOVA. For reproductive allocation, numerical sex ratio and sex allocation ratio population means and 95% CI were calculated according to (Bourke & Franks 1995).

Population	NBL	BVP	WTC	TA	Z	MA	ANOVA
# of colonies	100	29	70	63	29	37	n/a
Monogyny	27.6%	21.4%	83.3%	29.0%	41.4%	71.4%	n/a
Colony size [# of workers]	53.5 (44.2 – 64.2)	91.2 (67.0 – 117.7)	62.1 (54.6 – 69.8)	92.9 (67.6 – 121.4)	69.5 (42.6 – 105.9)	127.6 (96.1 – 166.2)	$F_{(5,322)} = 6.25$, $p < 0.001$
Macrogynes / microgynes per colony	0.8 (0.4 – 1.4) / 4.5 (3.4 – 5.8)	2.1 (1.1 – 3.3) / 4.8 (1.8 – 9.0)	1.3 (1.1 – 1.5) / 0.0 (0.0 – 0.0)	3.0 (1.9 – 4.4) / 2.7 (1.9 – 3.7)	2.2 (1.2 – 3.3) / 1.3 (0.7 – 1.8)	2.8 (1.5 – 4.8) / 0.0 (0.0 – 0.0)	$F_{(5,322)} = 10.25$, $p < 0.001$ / $F_{(5,322)} = 4.99$, $p < 0.005$
Morph ratio	0.31 (0.22 – 0.40)	0.50 (0.33 – 0.67)	1.00 (1.00 – 1.00)	0.49 (0.39 – 0.60)	0.62 (0.46 – 0.76)	1.00 (1.00 – 1.00)	$F_{(5,312)} = 35.90$, $p < 0.001$
Total # of queens per colony	5.4 (4.1 – 6.8)	6.9 (3.8 – 10.9)	1.3 (1.1 – 1.5)	5.8 (4.1 – 7.5)	3.4 (2.2 – 4.8)	2.8 (1.5 – 4.8)	$F_{(5,322)} = 6.75$, $p < 0.001$
Worker / queen ratio	22.7 (17.2 – 29.4)	28.2 (17.8 – 40.0)	53.7 (46.4 – 60.8)	21.9 (18.1 – 26.4)	26.1 (19.7 – 33.0)	78.7 (60.8 – 95.4)	$F_{(5,312)} = 23.36$, $p < 0.001$
New workers	74.6 (61.1 – 90.6)	120.3 (82.0 – 162.9)	78.8 (64.2 – 94.0)	155.3 (99.3 – 224.3)	77.9 (49.5 – 114.3)	155.2 (66.1 – 238.6)	$F_{(5,322)} = 2.97$, $p < 0.05$
Males	22.4 (13.9 – 33.7)	51.2 (24.1 – 83.3)	5.3 (2.8 – 8.0)	86.9 (52.8 – 127.2)	43.2 (12.6 – 84.1)	70.6 (32.5 – 128.5)	$F_{(5,322)} = 6.64$, $p < 0.005$
New macrogynes / microgynes	1.8 (0.4 – 3.7) / 2.8 (1.9 – 3.8)	1.7 (0.1 – 3.8) / 4.1 (1.1 – 8.5)	4.9 (2.7 – 7.4) / 0.0 (0.0 – 0.0)	1.3 (0.2 – 3.1) / 2.5 (1.4 – 3.9)	3.1 (0.7 – 6.2) / 1.1 (0.1 – 2.5)	21.1 (13.5 – 30.3) / 0.0 (0.0 – 0.0)	$F_{(5,322)} = 17.17$, $p < 0.001$ $F_{(5,322)} = 5.43$, $p < 0.001$
# of eggs per worker	2.59 (2.12 – 3.15)	1.20 (0.98 – 1.46)	0.87 (0.77 – 0.99)	2.14 (1.77 – 2.57)	1.71 (1.47 – 1.99)	1.11 (0.77 – 1.64)	$F_{(5,201)} = 8.01$, $p < 0.001$
Productivity [mg dry mass]	16.5 (12.6 – 21.0)	29.2 (18.3 – 40.7)	18.2 (14.5 – 22.6)	36.6 (23.2 – 51.4)	19.8 (11.2 – 31.5)	62.0 (34.8 – 98.7)	$F_{(5,322)} = 5.95$, $p < 0.005$
Sexual production [mg dry mass]	5.5 (3.6 – 7.9)	10.8 (5.5 – 16.3)	5.5 (3.3 – 8.2)	13.5 (7.8 – 20.3)	8.5 (3.3 – 15.8)	37.7 (22.6 – 56.1)	$F_{(5,322)} = 11.83$, $p < 0.001$
Reproductive allocation	0.30 (0.26 – 0.34)	0.35 (0.28 – 0.41)	0.22 (0.17 – 0.28)	0.37 (0.33 – 0.40)	0.40 (0.32 – 0.49)	0.52 (0.45 – 0.59)	$F_{(5,322)} = 13.91$, $p < 0.001$
Numerical sex ratio	0.17 (0.12 – 0.22)	0.10 (0.04 – 0.16)	0.47 (0.38 – 0.56)	0.05 (0.03 – 0.07)	0.08 (0.02 – 0.15)	0.23 (0.16 – 0.31)	$F_{(5,262)} = 5.76$, $p < 0.001$
Sex allocation ratio	0.29 (0.21 – 0.38)	0.15 (0.06 – 0.24)	0.73 (0.59 – 0.88)	0.09 (0.05 – 0.12)	0.18 (0.04 – 0.33)	0.47 (0.32 – 0.62)	$F_{(5,262)} = 6.78$, $p < 0.001$

5.3.1.2 Colony level analysis

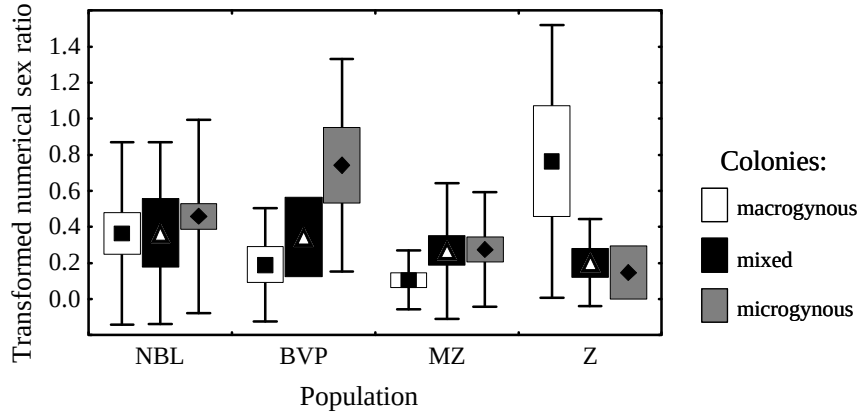
Figure 15: Colony sex ratios in the monogynous population WTC were split in contrast to polygynous populations, in which the colony sex ratios followed rather a negative exponential distribution .



At the colony level (within populations) the full range of sex ratios (0 - 1) was displayed. However, clear split sex ratios could only be observed in the two predominantly monogynous populations (e.g. Fig.15). Correlation analyses revealed that in both predominantly monogynous populations sexual production was correlated to sex allocation ratio (WTC: $r_p = 0.42$, n =29, p = 0.012; MA: $r_p = 0.56$, n = 30, p = 0.003). The polygynous populations showed contrasting patterns: no significant correlations were found in two (NBL, BVP), and in the other two, the worker / queen ratio emerged as the only correlate (TA: $r_p = 0.43$, n = 51, p = 0.006; Z: $r_p = 0.72$, n = 17, p = 0.001).

Morph ratio had no significant influence on sex allocation ratio in either population. In order to test the selfish microgyne hypothesis explicitly, the numerical ratio of new queens to workers (corrected for overall reproductive allocation) was compared between microgyne-producing colonies and macrogyne-producing ones. In the four morphologically mixed populations no significant differences could be detected. Furthermore, I did not find a significant difference in the numerical sex ratio between macrogynous, microgynous and mixed colonies in either population (Fig.16).

Figure 16: In neither population that contained macro- and microgynes produced microgynous or mixed colonies relatively more female sexuals than macrogynous colonies.



Similar to sex ratio, reproductive allocation in the "monogynous" populations was correlated with sexual production ($r_{WTC} = 0.60$, n =68, p < 0.001; $r_{MA} = 0.43$, n = 34, p = 0.010), which could explain the positive

association between sex allocation ratio and reproductive allocation in these cases (r_{WTC} = 0.45, n = 28, p = 0.009; r_{MA} = 0.52, n = 30, p < 0.001). Colonies that produced more absolute sexual biomass allocated more energy to sexuals relative to workers, with biased sex investment ratio toward females. Although in the polygynous populations sexual production was similarly correlated with reproductive allocation (r_{NBL} = 0.44, n = 100, p < 0.001; r_{BVP} = 0.48, n = 29, p = 0.013; r_{TA} = 0.27, n = 63, p = 0.054; r_Z = 0.61, n = 29, p = 0.003), no relationship between sexual production and sex allocation ratio or reproductive allocation and sex allocation ratio existed.

The intra-colonial relatedness coefficient of workers (Tab. 16) was negatively correlated to the number of queens present in a colony (R_S = - 0.51, n = 16, p = 0.044). There was no significant difference between male- and female-specialized colonies in 'NBL' (Mann-Whitney's $U_{(4,4)}$ = 10.0, p = 0.56). In 'WTC', colonies producing only males contained more closely related workers than colonies that produced only females ($U_{(4,4)}$ = 16.0, p < 0.03), This result contradicts the relative relatedness hypothesis.

Table 16 : Queen number and relatedness coefficients among workers in colonies that produced an either extremely male- or female biased sex ratio from the populations 'WTC' and 'NBL'.

	Intra-colonial relatedness coefficient (and queen number) of colonies that specialize in							
	female production				male production			
WTC	0.27 (1)	0.42 (2)	0.61 (1)	0.34 (3)	0.82 (1)	0.78 (1)	0.70 (3)	0.81 (1)
NBL	0.60 (1)	0.60 (4)	0.82 (1)	0.53 (1)	0.38 (7)	0.30 (13)	0.81 (1)	0.76 (2)

5.3.2 Laboratory data

5.3.2.1 Queen morphology

In experiment I, there no significant differences between macrogynous, mixed and microgynous colonies in sex allocation ratio could be detected, neither were there significant sex allocation ratio differences between worker groups in which macrogynes, microgynes or both had been adopted (Tab.17). However, the insignificance in the second experiment could be due to the strong overall female bias, which is in contrast to polygynous colonies in the field.

Table 17: Results of the two laboratory experiments looking at the effects of queen morphology on sex investment ratio (mean is given with 95% CI and sample size).

	Macrogynous colonies	Mixed colonies	Microgynous colonies	ANOVA results
Experiment 1	0.21 (0.00 – 0.52) n = 7	0.73 (0.52 – 0.89) n = 10	0.53 (0.26 – 0.78) n = 8	$F_{(2,22)}$ = 2.44 p = 0.128
Experiment 2	0.98 (0.95 – 1.00) n = 8	0.78 (0.66 – 0.87) n = 8	0.79 (0.48 – 0.98) n = 6	$F_{(2,19)}$ = 1.05 p = 0.391

5.3.2.2 Number and relatedness of queens

The average queen-queen relatedness calculated for the colonies of experiment I ranged from 0 to 1 with a mean of 0.40 (95% CI: 0.28 - 0.53). It did not explain colony sex allocation ratio (R_S = 0.26, n = 22, p = 0.226).

In experiment I, colony queen number and sex allocation ratio were not correlated (r = 0.04, n = 25, p = 0.830) nor was colony queen number correlated to sex allocation ratio relative to the sex allocation of the population of the colony origin (r = 0.13, n = 23, p = 0.556). In contrast, the production pattern of the split polygynous colonies indicated an inhibitory effect of queen number on female production when all other factors were constant. The monogynous colony halves (sex allocation ratio: 0.93 (0.89 - 0.96)) produced significantly more female biased sex ratios than their polygynous counterparts (0.52 (0.30 - 0.72); Wilcoxon-matched-pair test: T=2, n = 7, p = 0.04).

5.4 Discussion

At the population level, sex ratio of the ant species *Leptothorax rugatulus* was correlated with queen number but this was not true for colony sex ratios within populations. Colony sex ratio was related to different parameters within monogynous and polygynous populations. Theoretically, the analyses could be further refined by distinguishing monogynous and polygynous colonies within populations (Chan et al. 1999), but for *L. rugatulus* this would be problematic because colonies can and do change between these social conditions (chapter two). However, the separation in mainly monogynous or polygynous populations seems justified because the differences of the degree of polygyny at the population level are pronounced and relatively constant in *L. rugatulus*.

5.4.1 Population level

The number of studied populations allowed for the tentative conclusion that the evolution of population level sex ratio is strongly influenced by the number of queens per colony. The number of queens per colony has been implicated in a number of studies for among-species sex ratio differences (Trivers & Hare 1976; Nonacs 1986; Bourke & Franks 1995) and colony-level sex ratio variation (Herbers 1984; Chan et al. 1994; Evans 1995), but few studies have identified the average number of queens per nest as an important factor in sex ratio differentiation among populations (Herbers 1996; Chan et al. 1999).

A decreasing relative investment in females with increasing queen number can be related to a number of factors. However, the significantly male-biased sex investment ratios in all four polygynous populations ('TA' ranks among the most male biased sex investment ratios recorded for ants (Crozier & Pamilo 1996a)) cannot be explained by a decreased worker relatedness asymmetry alone (Boomsma & Grafen 1990;

Boomsma 1993) because a 50% investment in females is expected even if relatedness asymmetry is zero. Instead, the male bias suggests an importance of local resource competition in females (Frank 1987) and/or that workers that accompany queens during colony budding have to be added in part as female reproductive investment (Pamilo 1990b; Nonacs 1993b). As Leptothorax ants are generally not highly mobile, and investigations of the genetic structure of monogynous colonies indicate colony budding (chapter two), both factors might occur in *L. rugatulus*.

On the other hand, the observed sex investment ratio in the most monogynous population (WTC) supports worker control of sex investment ratio in *Leptothorax rugatulus* (at least in this population) because the data complied well with the expected 3:1 female investment bias (Trivers & Hare 1976). Although not clear support, the decreased female bias in the other populations is in accordance with this hypothesis. Queen influence on sex ratio in *L. rugatulus* seems difficult, because worker and sexual production are not separated in time as in the *Formica rufa* group (Pamilo & Rosengren 1983) and females develop over two years, probably with caste determination similar to *Myrmica* (Wheeler 1986).

5.4.2 Colony level

The within-population sex allocation ratio pattern depended on the predominant reproductive tactic of queens and the resulting social colony structure in the populations. In the two mainly monogynous populations the sex ratio produced by a colony was associated with its total reproductive output: the higher the overall sexual productivity, the more female-biased the colony sex ratio. This relationship has been frequently found (Nonacs 1986; Hasegawa & Yamauchi 1994; Herbers & Banschbach 1998; Morales & Heithaus 1998; Aron et al. 1999a; but see Deslippe & Savolainen 1995; Chan et al. 1999) and is predicted by the constant male hypothesis (Frank 1987), the cost-variation hypothesis (Crozier & Pamilo 1993, 1996) and the multifaceted parental investment hypothesis (Rosenheim et al. 1996).

The constant male hypothesis may be excluded because its assumptions are not met in monogynous populations of *L. rugatulus*: local mate competition seems unlikely and microsatellite data indicate no inbreeding at the individual level (chapter two). On the basis of the present data, it is not possible to distinguish between the remaining two hypotheses to explain the sex ratio variation within monogynous populations. On the one hand, egg limitation, which is an important assumption of the 'multifaceted parental investment hypothesis', in monogynous colonies of a facultatively polygynous species seems plausible. The average number of eggs in monogynous colonies is reduced by more than twofold compared to polygynous colonies in the investigated populations. Under the 'multifaceted parental investment hypothesis a significant positive correlation between a colony's sex ratio and the offspring provisioning (measured as 'dry weight /

thorax width') is expected. This relationship is found in MA but not in WTC (r_{MA} = 0.62, n = 13, p = 0.017; r_{MA} = 0.60, n = 12, p = 0.018; r_{WTC} : no data; r_{WTC} = 0.32, n = 21, p = 0.206). On the other hand, the data from MA (but not from WTC) also supports the cost-variation hypothesis because colony size is correlated to colony sex investment ratio (r_{MA} = 0.42, n = 30, p = 0.024; but r_{WTC} = -0.01, n = 29, p = 0.580). In conclusion, it is not unambiguously possible to distinguish between these two hypotheses and they are not mutually exclusive but each might explain part of the variation.

Colony sex ratios were predominantly male biased in polygynous populations and many colonies invested exclusively in males. Total sexual output of a colony, was the most prominent factor in the monogynous populations, but was not correlated with colony sex ratio in any of the more polygynous populations. Instead, associations with colony characteristics were generally weak and only in two a significant correlation between sex allocation ratio and worker / queen ratio was detected. Colonies with relatively few workers per queen biased their sex ratio more towards males than colonies with a high worker / queen ratio.

This pattern accords to the prediction of Herbers' (1984) conflict hypothesis, but no evidence for conflict was found in the monogynous populations (in contrast to Herbers 1984) despite a higher potential conflict (higher worker relatedness asymmetry) and strong variability in worker / queen ratio. An additional, adaptive interpretation is possible.

5.4.3 Colony-level sex ratio hypothesis

Given that there is an optimal worker / queen ratio for overall colony productivity (Oster & Wilson 1978) and also an optimal propagule size for colony budding (Stearns 1992; Roff 1992), selection should favor feedback loops to hold these parameters close to the optimum, while at the same time maximizing total reproductive output. This colony-level selection could weaken within-colony conflict and consequently override patterns predicted from relative relatedness asymmetry hypothesis. To adjust worker / queen ratio, colony resources could be differentially directed to the production of new workers, queens and males, and although there might be complex interactions between sex ratio and sexual allocation, the following two effects would be predicted if colony-level selection was important.

First, the worker / queen ratio should be constant, i.e. worker and queen number should be correlated. Second, there should be a positive association between the worker / queen ratio and the fraction of female larvae that develop into new queens. These predictions were met in both populations (TA and Z) in which a significant association between sex ratio and worker / queen ratio were found (Tab.18) that let to the suggestion of the hypothesis that colony sex ratios are explainable by colony-level selection.

Overall, colony-level must not be neglected in the current focus on intra-colonial conflict and might lead to an increased understanding of sex ratio evolution in ants with complex life-histories. This insight gains support from a re-analysis of data on the facultatively polygynous *Leptothorax acervorum* (Chan et al. 1999). In the most polygynous population ("Roydon Wood") the worker / queen ratio could explain more of the sex ratio variation than any other reported parameter ($r^2 = 0.20$, $F_{(1,22)} = 5.59$, $p = 0.027$). As expected, no significant relationship existed in the predominantly monogynous populations.

Table 18: The two relationships that are predicted from our optimal queen-worker ratio hypothesis in the four polygynous populations.

Population	Correlation between colony queen number and worker number	Correlation between the colony's ratio of workers to queens and its proportion of new queens in overall female production
TA	$r = 0.88$, $n = 63$, $p < 0.001$	$r = 0.38$, $n = 61$, $p = 0.015$
Z	$r = 0.68$, $n = 29$, $p < 0.001$	$r = 0.54$, $n = 29$, $p = 0.003$

Nevertheless, it has to be acknowledged that, especially within the polygynous populations, the major part of the variation remained unexplained. This is a common result in facultatively polygynous ants (Herbers 1990; Chan et al. 1999). In part this might be due to within-population genetic heterogeneity of thresholds for sex ratio biasing behavior, as it is expected for traits that evolve under frequency dependent selection. However, this should also be true for monogynous ant species. Therefore, the amount of unexplained variability additionally suggests that a large part of the sex ratio variability at the colony level is random, that there are complex interactions of different factors involved, or that an important internal or external factor has been omitted from the analyses.

The data presented on colony-level sex ratios does not comply with predictions of the relative relatedness asymmetry hypothesis (Boomsma & Grafen 1990). However, the hypothesis cannot be dismissed because only indirect measures of worker relatedness asymmetry to male and female offspring were used (queen number and within-colony worker relatedness). Generally, the relative relatedness asymmetry hypothesis seems to have less explanatory power in facultatively polygynous ants (Herbers, 1990; Pearson et al. 1997; Aron et al. 1999a; Chan et al. 1999) than in monogynous species (Ratnieks & Boomsma 1997). However, none of these studies has measured worker relatedness asymmetry directly and thus the detailed internal relatedness structure of colonies might constitute one of the potentially neglected factors. Additional internal factors whose influence could not be ruled out in this study are the colony's past and endoparasites (Chapuisat & Keller 1999), although *Wolbachia* seems to be absent from this species (Wenseleers, pers. commun.).

In order to investigate single factors in the absence of externally variable factors, such as microclimate and food availability, we engaged in laboratory experiments. In contrast to most polygynous colonies from the field, strong female bias was observed in all laboratory set-ups. This observation suggests laboratory artefacts. Of several conceivable factors, the constant and abundant food supply seems most likely to be responsible for this effect. However, this effect does not preclude the laboratory studies from supporting the main conclusions from the field: colony structure (worker / queen ratio), but no within-colony relatedness or queen morphology, affects the colony sex ratio.

5.4.4 Conclusions

Within any species most traits display considerable variability at the lower organizational levels and this is particularly true for sex ratios in ants. Different populations are probably under different selective pressures and thus, it is not surprising that different patterns were found for the sex ratio of *Leptothorax rugatulus*, calling for separate hypotheses. While the recent focus on intra-colonial conflicts has proven powerful in explaining many aspects of social insect biology (Bourke & Franks 1995; Crozier & Pamilo 1996a), it is also clear that all conflicting parties are willing to compromise their personal optima if escalating the conflict is too costly (Bourke & Franks 1995; Maynard-Smith & Szathmáry 1995; Keller 1999). Another reason why intra-colonial conflicts might not be realized is that the available information is insufficient or unreliable. In facultatively polygynous ants the genetic heterogeneity of a colony may be too complex for a reliable cue of the workers' relative relatedness asymmetry.

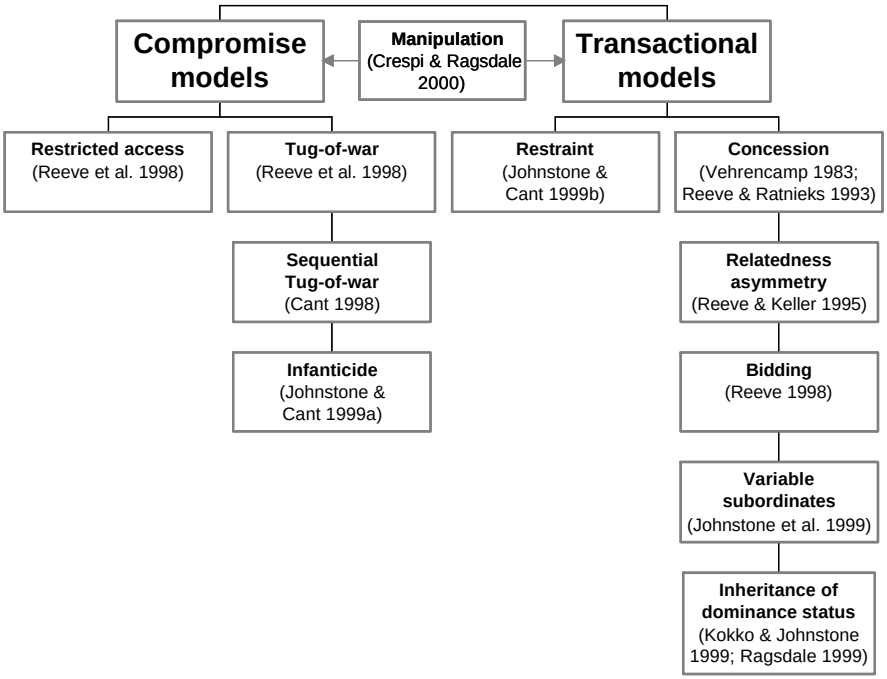
Ultimately, within- and between colony level selection have to be combined in a way that allows empiricists to validate holistic fitness functions and quantitative predictions (Pamilo 1990b; Frank 1998). However, the large proportion of unexplained variation in this and other studies cautions our optimism to find a universally predictive model since nothing is known about the genetic basis of sex ratio evolution in ants. Moreover, some randomness of “individual” colony sex ratio is to be expected because deviations from optimal sex ratio are not selected against when the population is in sex ratio equilibrium (Fisher 1930).

6 Within-colony distribution of reproduction

6.1 Introduction

Animal societies differ markedly in how reproduction is shared among potential breeders at both, the inter- and intraspecific level. The theory that tries to explain patterns of division of reproduction in animal societies is called reproductive skew theory (Vehrencamp 1983), which has received much scientific attention (for a review see Johnstone 2000). While theoretical elaborations abound and create an ever-increasing array of models (Fig.17; Johnstone 2000, Reeve & Keller 2001), independent, empirical tests are scarce and do not provide unified support for any of the models. In our study, we seek to understand how two of the principal factors of almost all models, namely body size and relatedness, influence the division of reproduction among *Leptothorax rugatulus* queens under controlled laboratory conditions.

Figure 17: Overview of hypotheses of reproductive skew. Many factors are not mutually exclusive and elaborations of one common type of model are written below each other (for details see text).



Although skew models differ in several aspects, they can be categorized into two groups: transactional and compromise models (Johnstone 2000). Transactional models comprise the classic optimal skew models and focus on group stability. Under full control of the dominant, subordinates will be apportioned only the minimum fraction of reproduction that they require to remain in the group and cooperate (concession model:

Vehrencamp 1983; Reeve & Ratnieks 1993). If subordinates are not fully controllable by the dominant, they are expected to claim the maximal share of reproduction, which does not induce the dominant to evict them from the group (restraint model: Johnstone & Cant 1999b). Selection will only favor cooperative groups when the inclusive fitness payoff for the dominant and subordinates exceeds eviction of subordinates, and departure or fighting for the dominant position, respectively.

Thus, a minimal or maximal monopolization of reproduction (reproductive skew) is predicted by the different types of transactional models. Both types are based on the principle of group stability and depend therefore on the same parameters: independent breeding opportunities for subordinates, subordinates' helping efficiency, intra-group relatedness and the difference in competitive ability between dominant and subordinate individuals. However, their predictions contrast strongly in the simplest case of a two-member association. The concession model predicts that dominants can safely claim reproductive monopoly when independent breeding is difficult, the dominant is physically dominant over the subordinate, and indirect fitness returns for subordinates are high because helping rises group reproductive output efficiently and intra-group relatedness is high. Except for competitive ability, the restraint model predicts exactly opposite trends (Johnstone & Cant 1999b).

The concession model has been extended in a number of directions with varying degrees of changes in the predictions. Most importantly from a practical perspective, Johnstone et al. (1999) found that skew in multi-member groups with variability among subordinates (as is the case in all groups with linear hierarchies, e.g. Higashi et al. 1994; Clarke & Faulkes 1997) is not comparable to skew models involving dyadic interactions. The model becomes increasingly complex in multi-member groups and the number of parameters to be determined (eight, in the "simple" case of two unequal subordinates) prohibits any accurate testing of quantitative predictions. From a theoretical standpoint, skew depends on relatedness values, fitness returns of subordinates leaving the group, and on how group productivity grows with group size. The incorporation of competition among dominants for subdominant helpers or vice versa also changes theoretical outcomes in non-intuitive ways (Reeve 1998).

Life-history decisions, such as dispersal or onset of reproduction, have current and future consequences for the inclusive fitness of an individual. The incorporation of future benefits, such as inheritance of dominance status and resources augments the probability of subordinates to stay, and consequently a higher skew under these conditions is expected (Kokko & Johnstone 1999; Ragsdale 1999). The concession model has been extended to groups in which dominant and subordinate are asymmetrically related to each others offspring (Reeve & Keller 1995). The skew in these groups (mainly mother-daughter associations) is expected to be higher than in groups with symmetric relatedness values (Reeve & Keller 1995). In additon, all of the above

modifications of the concession model are conceivable for extending the restraint model, although nothing has been published thus far.

The other group of skew models does neglect the issue of group stability and focuses on the compromise between selfish attempts of individuals to maximize their personal share of reproduction and the decrease in overall group productivity that is caused by this act. Dependent on the relatedness of individuals, kin selection results in an optimal degree of conflict and consequently the general prediction arises that group productivity increases with relatedness (Reeve et al. 1998). Dominants do not have full control over reproduction but are defined as those that have access to more resources or are more efficient in their use. This latter difference constitutes the distinction between the two subclasses of compromise models. The restricted access model assumes no efficiency differences but reduced access of the subordinate to group resources. It predicts that the correlation of relatedness and skew is strongly negative, while relatedness does not influence skew significantly in the simple subordinate inefficiency model (Reeve et al. 1998).

Cant (1998) explored how predictions of the subordinate inefficiency model change when the subordinate reproduces after the dominant. In contrast to the other compromise models, skew is expected to increase with relatedness in this case because the subordinate is more likely to refrain from increasing the offspring production to sub optimal levels when it is related to the dominant (Cant 1998). In a further extension of this model, Johnstone & Cant (1999a) incorporated the possibility of indiscriminate infanticide after summarizing empirical evidence for it. Group productivity does not suffer from additional young because any excess is simply eaten (e.g. Bourke 1991) with a probably high recycling efficiency. Consequently, the subordinate is predicted to produce at her highest possible rate even when highly related to the dominant and compared to the model of Cant (1998) skew is significantly decreased under these circumstances. It follows that skew and intra-group relatedness are less correlated.

Crespi & Ragsdale (2000) add the concept of (parental) manipulation (Alexander 1974) to skew theory. Dominants that are able to decrease the benefits of independent reproduction of their subordinates (or their competitive ability) without decreasing their efficiency as helpers by the same amount are expected to gain under all the above models because manipulated subordinates will accept higher skew but remain nonetheless efficient helpers. Thus, parental manipulation could play an auxiliary role in all of the above models.

The classic skew models involving concessions and complete control have so far received most attention with mixed empirical results. There has been support in vertebrates, they found support from early studies (Keller & Reeve 1994; Emlen 1997; Jamieson 1997), but possible alternative explanations were not ruled

out (Clutton-Brock 1998), and have since been shown to be important (e.g. Cooney & Bennett 2000). In some systems the assumptions of classic models do not hold. For example, in banded mongooses dominant females benefit from subordinate reproduction (Cant 2000).

In invertebrates, evidence in favor of concession models comes mainly from inter-specific comparisons in social insects (Keller & Reeve 1994; Heinze 1995a; Hogendoorn & Velthuis 1999; Reeve & Keller 2001). Tests at the intra-specific level are less common and provide conflicting evidence. For example in the wasp genus *Polistes* conflicting reports about reproductive transactions exist (Field et al. 1998; Reeve et al. 2000).

One study in the ant tribe Formicoxenini has generated some support for reproductive skew among nest mate queens at the intra-specific level (Bourke et al. 1997). Additionally, the patterns of reproductive partitioning between species corresponds to the classic transactional skew models (Bourke & Heinze 1994; Heinze 1995a). This correspondence seems surprising because benefits for the dominant from additional queens are not obvious (Bourke 1993). In contrast, in many ant species a decrease in individual reproduction is reported from polygyny (Bourke & Franks 1995). However, it has been shown by Ragsdale (1999) that this assumption of "cooperative benefit" is not required in cases of resource inheritance between related dominant and subordinate. This scenario seems to be fulfilled in Formicoxenines because relatedness of queens in polygynous colonies is generally above zero (Heinze 1995a) and nest sites and/or workers are inherited due to the phenomenon of secondary polygyny (see chapter one). Likewise, incomplete control models (Reeve et al. 1998; Johnstone & Cant 1999b) do not rely on the assumption of cooperative benefit and it is conceivable that dominants gain from additional queens by insured fitness returns (Gadagkar 1990).

From the above descriptions of the various models, it is apparent that relatedness among queens and their body size variability should be influential regardless of what model of reproductive skew in employed. *Leptothorax rugatulus* with its strongly size divergent queens offers an ideal study system to investigate these two factors. Based on the different preferential founding tactics of the two queen morphs the prediction that reproductive skew should be stronger in colonies with microgynes can be made with even more confidence. Furthermore, *L. rugatulus* exhibits variable nest mate relatedness in natural colonies (chapter two). Populations are strongly differentiated with respect to reproductive tactic (c.f. chapter two) due to colony density (cost of independent reproduction increases) and nest site stability (resource inheritance and thus reward for staying increases). Hence, a population effect on reproductive skew is predicted.

In *Myrmica rubra* it was concluded that microgynes constitute a social parasite because they preferentially produce sexuals relative to workers (Bourke & Franks 1991). Microgyny in the social insects has been suggested to be a selfish tactic to disguise caste fate (Brian 1975), either in the context of social parasitism

(Nonacs & Tobin 1992; Aron et al. 1999b) or of intra-colonial conflict for caste determination (Bourke & Ratnieks 1999). In both situations larvae might develop into gynes against the workers' interest by remaining similar in size to worker-destined larvae because larval size might be the only information for workers on larval caste fate (Brian 1975; Bourke & Ratnieks 1999). Although, no support for this hypothesis could be found for *Leptothorax rugatulus* at the colony level under natural conditions (chapter five), it is still conceivable that microgynes bias sexual output within colonies at the individual level. This was concomitantly investigated with overall skew.

Two complementary experiments in the laboratory were performed to investigate the influence of relatedness and body size on the patterns of reproduction in *Leptothorax rugatulus*. This controlled set-up was chosen because preliminary experiments had shown that colonies react to disturbances with nest relocation (Möglich 1978) and ant-tight enclosures are extremely difficult to maintain in the field (Fig. 18). In other social insects, reliable estimates from the field without individual marking and monitoring manipulations have proven extremely difficult (Bourke et al. 1997) and rely on extensive likelihood estimates (Field et al. 1998; Reeve et al. 2000; Sumner et al., unpublished).

Figure 18: Constant colony monitoring of *Leptothorax rugatulus* in observation nests proved impracticable in the field. Ant-tight enclosures suffered heavily from physical wear during winter, and various types of observation colonies provided only inadequate substitutes for original rock crevices.

6.2 Methods

In order to investigate the pattern of reproduction among *Leptothorax rugatulus* queens, two experimental groups of colonies were established in the laboratory: native colonies and arbitrarily mixed groups. Both groups comprised colonies with only macrogynes, only microgynes, or both.

After collection of colonies in August 1996 from various populations, colonies were maintained under standard conditions in three-chamber nest boxes and kept in incubators (Buschinger, 1974). In June 1997 the following experiments were set up. From 28 colonies (8 macrogynous, 8 microgynous and 12 mixed) with 2 - 7 queens, all brood was removed and queens randomly paint marked (Edding™ 780 paint marker). Similarly, 30 artificial groups (10 macro-, 10 microgynous and 10 mixed) with four queens each and without brood were composed from a random pool of workers and queens from approximately 50 original colonies.

In both experiments the primary egg laying rates of all queens were determined two weeks later, by isolating single queens for 16 hours with some workers. The isolation procedure was repeated five times and queens were place back into the original colonies for 32 hours of recovery between trials. For the following two years, until the end of the reproductive phase in August 1999, colonies were monitored at irregular intervals for their queen and worker number. All newly emerging offspring were collected (weekly in summer, the main reproductive season, and less frequently during the rest of the year) and kept frozen at -20°C for DNA-extraction. In order to estimate group productivity, dry mass production was estimated as in chapter five, and standardized to one year and worker. At the end of the experiments, all queens were killed by freezing, their insemination status was checked by ovarian dissection, and head and thorax were size-measured (as in chapter four) and used for DNA-extraction.

DNA was extracted from individuals by a modification of the Chelex®-protocol (as in chapter two, Altschmied et al. 1997) and several PCR-amplification protocols were used with the four different primer pairs (c.f. chapter two). The amplificates' separation by gel electrophoresis and their visualization and allele scoring follows the procedure described in chapter two. From the gained genotypic information, queen-queen relatedness was calculated in all experimental groups using Relatedness 5.0.2 (Goodnight & Queller 1999) based on the principles of regression relatedness (Queller & Goodnight 1989). In order to calculate population-specific allele frequencies, the available genotypes from chapter two were combined with this sample and parameter settings for the computer algorithm were used as in chapter two. For calculation of the relatedness in the artificially assembled groups, allele frequencies of the total sample were used. For maternity assignment, three additional microsatellite loci were used (see chapter three).

From the initial genotyping of queens, the discrimination power between queens as potential mothers was known for individual microsatellite loci. On this basis, colonies were chosen that provided, additionally to a relatively high reproductive output, good prospects for unambiguously assigning offspring to mothers. Furthermore, colonies with queens dying during the course of the experiment were excluded. Starting with the most informative locus, mothers and offspring were genotyped at as many microsatellite loci as necessary (chapter three). Offspring that could not be assigned unambiguously (some males in four of 23 colonies investigated) were omitted from the subsequent analysis. On average 27 (range 16 - 38) offspring per colony could be assigned (Tab.19).

Table 19: Total offspring production, sample sizes and queen-queen relatedness from the 23 investigated colonies. The first 12 data rows refer to artificial groups and the following 11 to original colonies of *Leptothorax rugatulus*. Colonies contained only macrogynes ("macro"), microgynes ("micro"), or both ("mixed").

Colony	Colony type	Queen number	Genetic queen relatedness	# of produced			# of successfully assigned		
				males	gynes	workers	males	gynes	workers
sk1	macro	4	0.00	4	5	30	0	4	20
sk2	macro	4	0.00	0	11	36	0	7	18
sk7	macro	4	0.00	0	21	17	0	18	17
sk10	macro	4	0.15	0	23	56	0	19	19
sk12	mixed	4	0.06	1	7	24	0	7	14
sk13	mixed	2	0.00	0	2	20	0	1	15
sk16	mixed	4	0.05	7	12	13	7	12	12
sk18	mixed	2	0.00	0	21	24	0	17	8
sk20	mixed	2	0.00	4	10	11	4	10	10
sk25	micro	3	0.00	3	6	10	2	6	8
sk26	micro	3	0.00	2	25	17	0	22	15
sk29	micro	4	0.00	6	13	18	6	11	12
ors4	macro	3	0.42	15	0	77	6	0	23
ors7	macro	2	0.74	114	0	143	5	0	20
ors8	macro	3	0.00	5	0	64	5	0	17
ors28	macro	2	0.50	15	31	114	0	15	10
ors34	macro	2	0.29	29	2	53	5	2	13
ors13	mixed	3	0.00	2	19	16	0	17	15
ors16	mixed	2	0.76	20	12	60	13	7	12
ors18	mixed	3	0.28	5	26	42	5	22	10
ors27	mixed	3	0.54	0	34	42	0	20	9
ors22	micro	5	0.35	48	1	25	8	0	18
ors26	micro	3	0.10	11	6	14	11	6	14

Combining the assigned worker, male and female offspring, skew indices were calculated for each group. Two indices of skew were calculated and used in subsequent analyses: "PCskew" (Pamilo & Crozier 1996) and "KKskew" [corrected skew = (observed skew – random skew) / (maximum skew – random skew): Keller & Krieger 1996] because they seemed most appropriate for our study system (Kokko et al. 1999). Both gave similar results and only those for "KKskew" are reported. At the group level, skew was related to variability in queen body size, group productivity and queen-queen relatedness. Original colonies were

grouped based on whether they were collected from a population with high or low ecological constraint (estimated from colony density and average queen number per colony), and skew in the two groups was compared. Similarly, the skew in sexual offspring (the offspring fraction that is relevant for selection: Bourke 1988; Bourke & Franks 1995 p.290) was calculated and analyzed. The hypothesis that queens within groups contribute differentially to worker, male and female offspring was tested by χ^2-tests with subsequent Bonferroni correction.

At the individual level, relative offspring production (reproductive share) was correlated with relative queen body size and relative egg-laying rate. The influence of relative body size on reproductive specialization in males, young queens or both sexuals relative to workers was investigated to test the selfish microgyne hypothesis (Bourke & Ratnieks 1999).

6.3 Results

6.3.1 Colony level

Across all colonies skew in overall and sexual offspring production were correlated (R_S = 0.65, n = 22, p = 0.001). The two skew indices and productivity of this study are summarized in Tab.20. Overall skew was significantly higher in artificial groups than in original colonies. For sexual skew this difference was not significant. Productivity was significantly lower in artificial groups whose queens also produced fewer eggs than queens from original colonies, although this difference was not significant ($U_{(39,31)}$ = 762, p = 0.063).

Within the sample of original colonies regression relatedness was correlated to neither overall skew (R_S = -0.20, n = 11, p = 0.555), nor productivity (R_S = 0.08, n = 11, p = 0.821). However, relatedness was correlated to sexual skew (R_S = -0.69, n = 11, p = 0.019). The population origin of colonies in this sample had no significant effect ($U_{(6,5)}$ = 19, p = 0.460).

Table 20: Skew and productivity [µg/worker] of the investigated groups. "Test" refers to the results of a Mann-Whitney test for group differences between the original colonies (ors4 - ors34) and the artificial groups (sk1-sk29). No skew was calculated when less than four offspring were produced in a group.

Colony	sk1	sk2	sk7	sk10	sk12	sk13	sk16	sk18	sk20	sk25	sk26	sk29
KKskew	0.79	0.09	0.21	0.07	0.01	0.75	0.44	0.10	0.68	-0.06	0.04	0.40
Sexual skew	0.30	-0.01	0.47	0.05	1.00		0.31	0.24	1.00	-0.03	0.11	0.29
Productivity	0.16	0.23	0.32	0.43	0.11	0.17	0.15	0.21	0.13	0.07	0.14	0.13
Colony (ors)	4	7	8	28	34	13	16	18	27	22	26	**Test**
KKskew	0.18	-0.03	0.10	0.00	0.04	-0.01	0.03	0.01	0.04	0.11	0.11	**$U_{(12,11)}$=98 p=0.049**
Sexual skew	0.18	-0.20	0.45	-0.07	-0.13	0.29	-0.02	-0.01	0.05	0.24	0.84	**$U_{(11,11)}$=84 p = 0.123**
Productivity	0.21	0.54	0.23	0.64	0.64	0.56	0.36	0.30	0.62	0.13	0.56	**$U_{(12,11)}$=112 p = 0.005**

No differences between macrogynous, mixed and microgynous colonies were found with respect to colony productivity (Kruskal-Wallis ANOVA: $H_{(2,23)} = 5.37$, $p = 0.068$), overall skew ($H_{(2,23)} = 0.02$, $p = 0.992$), or sexual skew ($H_{(2,22)} = 0.48$, $p = 0.785$). Thus, body size variability of cohabiting queens had no significant influence.

6.3.2 Individual level

The size of queens relative to other queens in the colony did not affect the overall reproductive share of a queen in the original colonies ($R_S = 0.14$, $n = 31$, $p = 0.439$) and artificial groups ($R_S = 0.07$, $n = 37$, $p = 0.697$). The same result was obtained when only micro- and macrogynes in mixed colonies were compared ($U_{(11,11)} = 72$, $p = 0.450$). The insignificance of body size is corroborated by the fact that relative body size and egg laying rate were not correlated in original colonies ($R_S = 0.03$, $n = 31$, $p = 0.890$) or artificial groups ($R_S = 0.11$, $n = 36$, $p = 0.53$) and macro- and microgynes did not differ in mixed colonies in egg laying rates ($U_{(11,11)} = 74.5$, $p = 0.358$). Independent of body size, a positive correlation between primary egg laying rate and reproductive share was found in original colonies ($R_S = 0.42$, $n = 31$, $p = 0.019$) and artificial groups ($R_S = 0.53$, $n = 38$, $p = 0.001$).

Queens in colonies did not contribute evenly to male, female, and worker offspring. The degree of differentiation in offspring production among queens was significant in eight of the 23 colonies. Chi²-values did not differ between original colonies and manipulated groups ($U_{(12,11)} = 72$, $p = 0.712$), but were significantly higher in groups with microgynes (microgynous and mixed colonies) than purely macrogynous groups ($H_{(2,23)} = 6.75$, $p = 0.034$). This result did not change when different numbers of degree of freedom in the χ^2-statistics were accounted for ($H_{(2,23)} = 6.09$, $p = 0.048$). All groups that were significantly differentiated were either microgynous (three) or mixed (five).

Inspection of the reproduction patterns of these differentiated colonies revealed that male specialization caused the differentiation between queens in three cases. In contrast, unequal gyne / worker ratios were responsible for different offspring production in three other colonies, and in the remaining two a mixture of both processes.

Over all mixed colonies, microgynes produced relatively more sexuals than their macrogynous nest mates ($U_{(13,11)} = 124.5$, $p = 0.002$), which resulted primarily from increased relative female production ($U_{(13,9)} = 92.5$, $p = 0.023$) and to a lesser extent from male production ($U_{(5,5)} = 16$, $p = 0.465$).

6.4 Discussion

6.4.1 Reproductive skew

The distinction between sexual skew and overall skew is crucial because skew in worker production does not translate into fitness effects in the absence of worker reproduction and within-colony kin discrimination (Carlin et al. 1993; DeHeer & Ross 1997; Keller 1997). In contrast, differential production of sexuals directly translates into fitness differences, and it could be shown in this study that queens of *Leptothorax rugatulus* differ in their produced caste ratios. However, the high correlation between the two parameters was expected because preferential male production by laying only unfertilized eggs is reported to be rare in ants (Bourke & Franks 1995) and queen determination in *Leptothorax* takes probably place long after individual discrimination (and thus nepotism) is possible, as in *Myrmica* (Wheeler 1986).

Although the results of a dependency of sexual and overall skew on relatedness differed in significance, the directional trends were identical: correlation of relatedness and skew and comparison between artificial groups and native colonies supports a negative relationship between skew and relatedness. However, the low overall skew in original colonies also indicates that under the given circumstances, the potential conflict is not realized and there is little biological variability to explain. In conclusion, there is some evidence that relatedness, or at least familiarity, influences the skew negatively and that queen body size has no effect.

Both these findings do not fit the predictions of the classic concession model of optimal skew (Vehrencamp 1983; Reeve & Ratnieks 1993). Relatedness showed a trend opposite to the expectation. Body size, which affects constraints on independent colony founding (chapter two) and also probably physical competitiveness (Field et al. 1998), had no effect. Thus, the classic concession model is not applicable in *Leptothorax rugatulus*. This result is not surprising because the system may violate some of the assumptions of this model and its variants (Fig.17). In only three of six natural populations queen number had a significant positive effect on colony productivity (as measured by a multiple regression of colony productivity on worker and queen number). In general, additional ant queens do not promote colony productivity (Keller & Vargo 1993; Evans & Pierce 1995). Furthermore, departure for an established, single queen from a nest is not likely because normal budding occurs accompanied by workers who might have differing interests (Reeve & Keller 2001). Workers also play a crucial role in gyne acceptance and partly control the energy flow in the colony, thus it seems generally in ants important to incorporate their interests. While it is likely that *L. rugatulus* queens have asymmetric relatedness to each other's offspring (Reeve & Keller 1995), and conceivable that inheritance of resources or dominant status plays a role (Kokko & Johnstone 1999; Ragsdale 1999), a social market for queens as helpers (Reeve 1998) seems extremely unlikely.

Restraint and compromise models of skew presuppose that the dominant individual has only incomplete control over reproduction of subordinates. In functionally monogynous *Leptothorax* species (Heinze 1995a; Ortius & Heinze 1999) the dominant queen seems to be able to control subordinate reproduction completely. Although, functionally monogynous *Leptothorax* species do not differ in their fundamental biology (colony size, habitat) from *L. rugatulus*, this does not prove the case for the possibility of complete control in *L. rugatulus*. On the contrary, dissection data suggest that almost all queens exhibit at least some egg laying. Both incomplete control models relax the crucial assumption of cooperative benefits and the presence of subordinates might even be detrimental to group output. Occasional findings of single, mutilated queens outside of the colony in the laboratory (pers. observ.) suggest that eviction of subordinates is a realistic threat, which is a crucial assumption of the restraint model. However, the additional assumptions of the compromise models (higher efficiency in claiming reproduction by the dominant or its advantage in accessing resources) might also be met.

The predictions of the restricted access model (Reeve et al. 1998) and the restraint model (Johnstone & Cant 1999b) concerning the impact of relatedness are both supported by the data in this study. However, body size should play a crucial role in eviction (because of different constraints and physical power) and resource access and usage (nutrient storage and physical power) and hence an influence would be predicted by both, restricted access and restraint models. They are only different variants of the same underlying concept, as already pointed out by Johnstone (2000), and both models describe two different outcomes of the same conflict. Dominant and subordinate struggle for reproduction and if the subordinate manages to utilize too many resources, it is exposed to the threat of eviction. Testing whether subordinates deliberately refrain from full reproduction is empirically difficult. However, the higher egg production in polygynous colonies of *L. rugatulus* (chapter five), probably linked to indiscriminate cannibalism (Bourke 1994), suggests otherwise.

A more complex model of reproductive skew is probably conceivable to integrate the results of this study and the general biology of *Leptothorax rugatulus*. Incomplete control models are compatible with the data from this study. On the other hand, relatedness asymmetries over several generations of queens (Reeve & Keller 1995), inheritance of nest structure and workers (Kokko & Johnstone 1999; Ragsdale 1999), infanticide by egg cannibalism (Bourke 1994; Johnstone & Cant 1999a), and sequential offspring production (Cant 1998) are all likely to play a role. Moreover, colonies contain frequently more than two queens, and several, variable subordinates (Johnstone et al. 1999) pose a problem that cannot be overcome simply by restricting experimental testing to two queens per group. The reason for this is that multi-member groups would still characterize the evolutionary history of the species that generated the investigated behavioral

rules. Finally, in many social insects functionally sterile workers are a predominating party for virtually all colony parameters, and their interests must not be neglected (Reeve & Keller 2001). To distinguish worker from queen effects, in this study a third experimental group with related queens in arbitrarily mixed worker groups would have been revealing.

The above considerations are not specific to *Leptothorax rugatulus* (e.g. Field et al. 1998) and thus reports of positive, as well as negative evidence for basic skew models should be carefully scrutinized for correspondence to the assumptions and secondary predictions (e.g. Johnstone 2000). While the growing number of models and variations, with a multitude of factors involved, makes a falsification of general skew theory impracticable, skew theory has also lost its general predictive power. The different skew models might be only variants of the same underlying principle (Johnstone 2000) but then the question arises, what generality beyond Hamilton's rule (Hamilton 1964a,b) remains. In fact, skew theory seems as diverse as social evolution itself.

6.4.2 Sexual bias

In 30% of all colonies, individual queens hold significantly different reproductive shares in male, worker, and gyne offspring. This was mainly explainable by microgynes producing relatively fewer workers and more gynes. Microgynes also overproduced males in two colonies but a general difference in male production between macro- and microgynes is not obvious from the data. The exclusive male production by inseminated queens could be explained by unviable sperm or incompatibility (Godfray 1990), or by queens refraining from fertilizing their eggs to increase their chances of contributing to the future gene-pool. Although this seems rare in ants (Bourke & Franks 1995), it cannot be excluded.

The main result of queen body size influence on the worker/gyne ratio produced is strongest in colonies with microgynes. This result supports the selfish microgyne hypothesis in kin conflict over caste determination (Bourke & Ratnieks 1999). The underlying model suggests that larvae should bias their caste fate towards queens against the interest of simultaneously developing brood, existing workers, and queens. This conflict is predicted particularly in species that reproduce by dependent colony founding and when a certain degree of larval self-determination exists. The intensive brood care by workers reduces the potential for self-determination in most ants, but Bourke & Franks (1999) point out that microgyny might be an effective strategy to evade caste control (see also Nonacs & Tobin 1992).

The long queen life span in social insects poses a threat to conclusions about reproductive skew and differential reproduction because it is unclear how short term measurements of these parameters translate into life-time estimates (Keller 1993a). Especially relevant for this study is the shorter developmental time

of smaller gynes that could have favored microgyne over macrogyne reproduction in the experimental period. Given a certain mother-daughter transmission of body size, this would cause only the smallest daughters of macrogynes to complete their development. A consequence would be a decreased share of gyne production of macrogynes and a reduced mother-daughter size correlation (chapter three). Thus, independent confirmation of the presented bias from colonies under equilibrium conditions (in the third year) would be valuable. Currently, the macrogyne production in macrogynous colonies (Fig.8, Tab.19) has to be taken as evidence that two years are sufficient for macrogyne development in the laboratory. Keeping this provision in mind, the following conclusion seems justifiable.

In contrast to chapter five that investigated reproduction at the colony level, the result of this study is the first to provide independent support for the selfish microgyne hypothesis at the within-colony level. Within the same social and physical environment, microgynes produced relatively more sexual female offspring (microgynes). This demonstrates that microgynous female larvae have a higher tendency to develop into gynes. At the colony level, microgyny had no effect on the caste ratio produced (c.f. chapter five), which could be interpreted as evidence for non-equilibrium in the experiments presented in this chapter. However, worker interest and influence must not be neglected. It is likely that they can effectively control the produced colony caste ratio so that no difference between macro- and microgynous colonies exists. Probably the microgyne bias in mixed colonies arises not due to microgynes manipulating workers to raise their offspring preferentially to gynes or invariant larval factors (e.g. blastogenic caste control: Brian & Hibble 1964; Petersen-Braun 1977) but because microgynous larvae have relatively low (size) thresholds to develop into queens. Clearly, these issues deserve further investigation. Only given similar worker treatment, more microgynous than macrogynous larvae develop into queens, otherwise altered worker brood care might counteract the lowered threshold. As mixed colonies in natural populations are rare (Rüppell et al. 1998; chapter two), developmental threshold and the corresponding worker behavior are not to be interpreted as the outcome of an evolutionary arms race (Dawkins & Krebs 1979) but rather as a co-adopted gene complex (Dobzhansky 1948).

In general, there are only two other studies that provide evidence for intra-specific variability in gyne-versus-worker production. In *Solenopsis invicta*, queens also contribute differentially to worker and gyne pools in artificial groups (Ross 1988), and in natural colonies of *Formica sanguinea* the relatedness among worker and gyne offspring are different (Pamilo & Seppä 1994) which indicates higher sexual than overall skew (Queller 1993). However, neither study can convincingly exclude differential development times as underlying mechanism of differentiation and an independent study found no genetic effect on gyne-versus-worker production (Keller et al. 1997). Genetic influence on different worker caste has recently been

reported (Fraser et al. 2000) but sexual caste bias seems to be rare (Hölldobler & Wilson 1990). Nevertheless, different environmental conditions select for different caste ratios (and sex ratios) and thus differentiation between populations is expected. If queens contribute to the adjustment of the colony reproduction to selective pressures, they are likely to differ genetically between populations. Consequently, groups of queens that are combined from several populations are expected to exhibit different bias in worker and gyne production among these queens (Bourke & Ratnieks 1999). Alternatively, phenotypic plasticity without genetic differentiation of queens could be invoked, or worker behavior could be exclusively responsible for the colony adjustments. These hypotheses await further testing.

7 Queen size dimorphism in *Leptothorax* cf. *andrei*

7.1 Introduction

The genus *Leptothorax* is rich in social parasites, wing-dimorphic species, and cases of queen size dimorphism (Bourke & Franks 1991; chapter one). Consequently, case studies in the genus *Leptothorax* are particularly suitable for studying the evolution of queen size dimorphism in ants (Buschinger 1990; Bourke & Franks, 1991) and in taxonomically independent lineages microgynes might pursue different life-history tactics. They might either specialize in social parasitism, or readoption into their natal colonies. In the first case, a low queen-queen relatedness within colonies and a close association of microgynes with macrogynes were expected. On the other hand, readoption might lead to the loss of mating flights (Fortelius et al. 1987; Bourke & Franks 1995; Sundström 1995) and to a wing polymorphism (Buschinger & Heinze 1992). Also, species might differ in the degree of evolutionary divergence between macro- and microgynes, and thus studies might allow comparative evolutionary "snap-shots" to understand the evolutionary dynamics.

Data were available for only two queen size-dimorphic *Leptothorax* species, *L. spinosior* (Hamaguchi & Kinomura, 1996; Hamaguchi et al. 1998) and *L. rugatulus* (Rüppell et al., 1998). This study presents data on the newly discovered queen size dimorphism in *Leptothorax* cf. *andrei* from Mexico, whose origin is presumably phylogenetically independent of those in the other two species. The morphological size difference between queen morphs is described, the consequences of queen size dimorphism for social and genetic colony structure investigated, and its prospects for speciation discussed.

7.2 Material and methods

7.2.1 Material

Colonies of *Leptothorax* cf. *andrei* were collected from 19 June to 8 July 1998 at three sites in the Sierra Madre Occidental in western Mexico: 14 colonies 25 km west of San Juanito, Chihuahua province (28°05´ N, 107°45´ W), 33 colonies 75 km south of Creel, Chihuahua province (27°15´ N, 107°30´ W), and 16 colonies 65 km south of Durango, Durango province (23°45´ N, 104°30´ W). Nests were found in montane forests under stones or in rock crevices. The small size of colonies made their complete collection and immediate census of their queens and workers possible. Colonies were either taken back to the laboratory alive or stored in 96% ethanol for DNA preservation.

Although this yellowish species with 12-segmented antennae was identified as *Leptothorax andrei* (Creighton, 1950), it might be a different, closely related species (A. Francoeur, pers. comm.). Voucher specimens have been sent to P. Ward (University of California, Davies), A. Francoeur (Centre de données sur la biodiversité du Quebec) and S. Cover (Harvard University).

7.2.2 Laboratory methods

Queen head width and thorax width and worker head width were determined to the nearest µm using a micrometer screw table mounted under a Wild™ stereomicroscope (Heerbrugg, Switzerland). After killing the queens by freezing, their reproductive states were assessed by dissection (Buschinger & Alloway, 1978), and their heads and thoraces were used for DNA extraction (modified from Altschmied et al., 1997: chapter two). Four of 15 microsatellite primer pairs from different *Leptothorax* species (Hamaguchi et al., 1993; Bourke et al., 1997; Foitzik et al., 1997) were adopted to genotype macro- and microgynes. The detailed amplification conditions are given in table 21.

Table 21: Final concentration of reactants and the cycling conditions used in the PCR-amplification of microsatellites in *Leptothorax* cf. *andrei*. All PCR-steps are given in °C with the duration [sec] in brackets.

Locus	dNTPs	$MgCl_2$	Primer	Taq®	Denaturation	Annealing	Elongation	# of cycles
L5	200µM	2.5mM	0.5µM	0.04u / µl	92° (60)	50° (60)	70° (120)	35
L18	200µM	2.5mM	0.5µM	0.04u / µl	92° (60)	50° (60)	70° (120)	35
LXGT218	200µM	2.5mM	0.5µM	0.04u / µl	92° (60)	54° (60)	70° (120)	33
LXGT223	200µM	2.5mM	0.5µM	0.04u / µl	92° (60)	57°-47° (60)	70° (120)	10x2 + 18

The computer program Relatedness 5.0.2 (Goodnight & Queller, 1998) was used to calculate average relatedness of nest mate queens (Queller & Goodnight, 1989) from the genotypic information. Allele frequencies were calculated from individuals using a bias correction for related individuals in the same group as the focal animal. The average relatedness coefficients were obtained by weighting colonies equally.

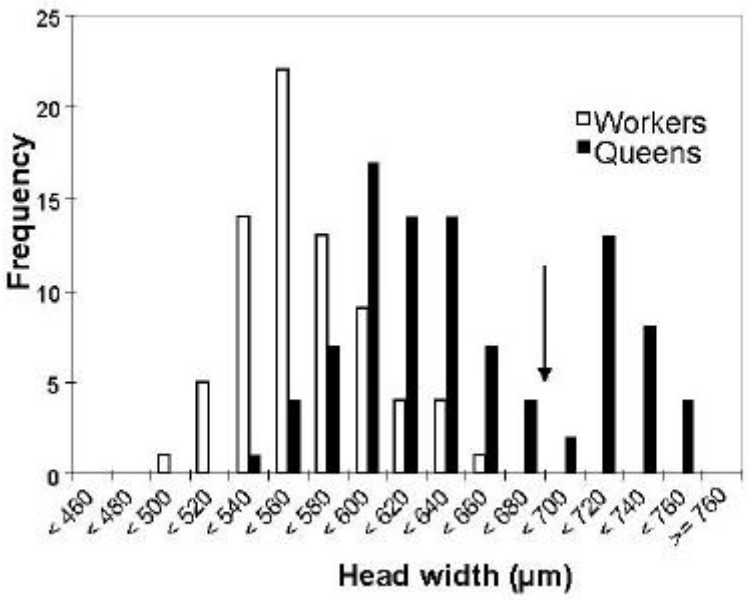
Genetic differentiation between the morphs was studied by two four-level hierarchical analyses of molecular variance (AMOVA) using the computer program GDA (Lewis & Zaykin, 1999). Two nesting orders were used: individuals in colonies in morphs in populations, and individuals in colonies in populations in morphs. Both hierarchies were used, because, in the absence of a priori information on structuring, the correct hierarchy depends on the amount of differentiation at the different levels. The AMOVA analyses were combined with an UPGMA (unweighted pair-group method using arithmetic averages) cluster analysis using the computer program TFPGA (Miller, 1998). Clustering was based on Reynolds' coancestry coefficient (Reynolds et al., 1983) but the tree topology remained robust when Nei's D (Nei, 1972) or Wright's modification of Roger's distance (Wright 1978), or the computer program GDA were used.

7.3 Results

7.3.1 Morphology

The distribution of worker size in *Leptothorax* cf. *andrei* was not significantly different from a normal distribution (Shapiro-Wilk's test: W = 0.96, n = 62, p = 0.06; Fig. 16). In contrast, overall queen size (mean of head width and thorax width) distribution showed a significant deviation from normality (W = 0.91, n = 95, p < 0.001) and conformed better with two overlapping normal distributions (W_{small} = 0.98, n = 68, p = NS and W_{large} = 0.94, n = 27, p = NS). From the observed size distribution of queens (Fig. 19), a head width of 680µm was selected as the threshold value to classify queens as microgynes (< 680 µm) or macrogynes (> 680 µm). Thorax and head width in queens were highly correlated (Spearman's r_s = 0.91, n = 95, p < 0.001) despite a relative increase in thorax width with overall size (thorax : head width ratio correlated positively with the sum of thorax and head width: r_s = 0.84, n = 95, p < 0.001). Worker size did not differ between macrogynous and microgynous colonies (t = 1.31, d.f. = 61, p = NS).

Figure 19. Frequency distribution of head width in workers and queens of *Leptothorax* cf. *andrei*. The critical value to distinguish macro- from micro-gynes is indicated by an arrow.



Twenty of 21 (95.2%) dissected queens were fertilised and their ovaries were developed to some extent. The number of ovarioles ranged from five to nine, with a median of eight. Despite the same median for macro- and micro-gynes (eight), macrogynes contained signify-cantly more ovarioles than microgynes (Mann-Whitney $U_{(13,7)}$ = 28.0, $p_{(adj.)}$ = 0.02). Workers had two undeveloped ovarioles and showed no sign of reproduction in colonies with queen presence.

7.3.2 Colony structure

Overall, 69 microgynes occurred in 24 polygynous colonies, and 11 were found in monogynous colonies (mean queen number of all colonies with microgynes = 2.3). Twenty-six macrogynes were found in ten polygynous colonies and 9 in monogynous colonies (mean queen number of all colonies with macrogynes = 1.8). There was no significant relationship between queen morphology and social colony type ($\chi^2 = 2.43$, d.f. = 1, p = NS). Six mixed colonies with macro- and micro-gynes were included in this analysis However, from the detailed results in the different populations (Tab.22) it is apparent that microgynes occur slightly more often in polygynous colonies than do macrogynes.

The frequency of microgynes varied among the three populations, from 53.7% at Durango and 56.0% at San Juanito to 89.8% at Creel. Creel also differed in terms of its nest sites (rock crevices vs. nests under loose stones) and its richer vegetation. Further, colony size differed significantly among populations (Kruskal-Wallis test: $H_{(2,61)} = 9.79$, $p < 0.01$), with the smallest size at Creel (average worker number = 25 ± 26 S.D.), intermediate size at San Juanito (36 ± 19), and largest size at Durango (48 ± 32). The size of a colony was related to the morphology of its queens ($H_{(3,61)} = 10.07$, $p < 0.05$): microgynous (30 ± 27) and queenless (30 ± 23) colonies were smallest, macrogynous colonies were intermediate (36 ± 27) and mixed colonies (with macro- and micro-gynes) were largest (65 ± 21). Colony size was correlated significantly with the number of small queens (multiple regression: $r_{partial} = 0.28$, $t_{(60)} = 2.25$, $p < 0.05$), but not with the number of its large queens ($r_{partial} = 0.13$, $t_{(60)} = 1.06$, p = NS).

Table 22: Colony composition of *Leptothorax* cf. *andrei* from three populations. The numbers of respective colonies and, in brackets, the numbers of queens (macrogynes/microgynes where applicable) are given.

Population	Queenless colonies	Colonies with macrogynes		Colonies with microgynes		Mixed colonies	Sample size
		Polygynous	Monogynous	Polygynous	Monogynous		
San Juanito	3	1 (3)	4	1 (2)	3	2 (4/9)	14 (11/14)
Creel	9	0	1	11 (25)	8	3 (4/11)	32 (5/44)
Durango	2	3 (14)	4	6 (21)	0	1 (1/1)	16 (19/22)
Total	14	4 (17)	9	18 (48)	11	6 (9/21)	62 (35/80)

7.3.3 Genetic data

Descriptive statistics for the four microsatellite loci (Table 23) showed that all loci were polymorphic in each population. The genetic differentiation was significant at the population level in both hierarchical orders (morphs nested in populations, populations nested in morphs), but between-morph differentiation was only found when morphs were nested within populations (Table 24). Thus, the nesting of morphs in populations is the more appropriate model. Within populations, there is a differentiation of the gene pools

between morphs, but differentiation at the population level is stronger. The latter result was also obtained by the UPGMA-cluster analysis (Fig.20). In both populations where macro- and micro-gynes were investigated, the two morph-groups had similar allele distributions and thus clustered together. The clustering distance to queen groupings of the same morph from different populations were consistently larger.

Table 23: Summary statistics of the four microsatellite loci that were used to investigate genetic structure across the five "subpopulations" in three populations of *Leptothorax* cf. *andrei*.

Population	Sample size	# of alleles at locus				Average H_{exp} / H_{obs}
		L5	L18	LXGT218	LXGT223	
Durango (macro)	16	4	11	4	5	0.65 / 0.78
Durango (micro)	17	5	13	4	4	0.71 / 0.75
Creel (micro)	22	2	23	9	7	0.75 / 0.56
San Juanito (macro)	3	2	4	4	5	0.78 / 0.75
San Juanito (micro)	7	3	8	4	7	0.67 / 0.64

Table 24: Results of the two four-level AMOVAs that were performed to investigate the genetic differentiation between populations and morphs of *L.* cf. *andrei*. Means are given with 95% CI (obtained by bootstrapping over loci). F-values are variance components (structuring) at each hierarchical level (F_{IT} : overall inbreeding coefficient, F_{IS} : individual inbreeding within populations). However, the significantly negative F_{IS} values are an artefact of the structure of the data set (often only one queen per colony).

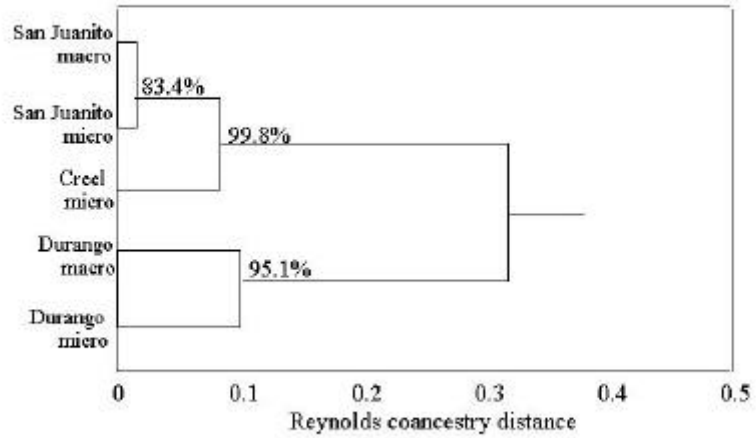
Hierarchy	F_{IS}	F_{IT}	F_{colony}	F_{Morph}	$F_{Population}$
Individuals in colonies in morphs in populations	-0.28 (-0.35 – -0.19)	0.27 (0.05 – 0.45)	0.43 (0.29 – 0.57)	0.21 (0.05 – 0.36)	0.20 (0.02 – 0.39)
Individuals in colonies in populations in morphs	-0.28 (-0.35 – -0.19)	0.21 (0.03 – 0.38)	0.39 (0.28 – 0.51)	-0.09 (-0.15 – -0.02)	0.15 (0.03 – 0.27)

Further, the genetic data suggest strong genetic population structuring at the colony level (Table 24). Most queens within colonies are highly related (average intra-colonial relatedness = 0.44) and the values for microgynous and mixed colonies are not significantly different from this overall estimate, whereas macrogynous colonies have an intra-colonial relatedness coefficient that is higher than the overall value (Table 25).

Table 25. Relatedness coefficients within colonies of *L.* cf. *andrei*. 95% CI and SE were obtained by jackknifing over loci. N is the number of colonies.

	All colonies N = 20	Macro colonies N = 4	Micro colonies N = 13	Mixed colonies N = 4
R (± S.E.)	0.43 ± 0.03	0.64 ± 0.02	0.42 ± 0.05	0.35 ± 0.06
95% C.I.	(0.33 – 0.53)	(0.56 – 0.71)	(0.28 – 0.56)	(0.17 – 0.54)

Figure 20. UPGMA-dendrogram of two macro- and three micro-gyne subpopulations on the basis of microsatellite allele frequency distribution. Bootstrap support is given above each node.



7.4 Discussion

The work reported here describes a new case of queen size polymorphism in ants. Despite some overlap, the bimodal size distribution of queens warrants categorizing the queens of *Leptothorax* cf. *andrei* into macro- and micro-gynes. The small thorax width relative to head width in microgynes could be explained in three different ways: (1) The head width might be conserved because of neurological constraints. (2) Microgynes do not need a large pterothorax because of a smaller wing load (Rüppell et al., 1998). (3) Microgynes do not need a large pterothorax because of lower flight activity, which would be evidence for alternative reproductive tactics.

Alternative reproductive morphotypes in females are rare (Gross, 1996), but ants are exceptional in this respect (Heinze & Tsuji 1995; chapter one). This is further supported by the results on *Leptothorax* cf. *andrei* presented here. A size reduction in this particular case may be possible because selection to maximize individual egg productivity is reduced by the possibility of re-adopting additional queens. Thus, colony productivity, rather than queen productivity, may be optimized and queen number might fine-tune overall egg-laying capacity.

Microgynes are functional queens with slightly less reproductive potential (in terms of number of ovarioles) than macrogynes. The difference in fertility between queen morphs contrasts with findings in other queen dimorphic *Leptothorax* species (Heinze & Buschinger, 1987; Rüppell et al., 1998) but has been suggested for the queen size-dimorphic *Ectatomma ruidum* (Schatz et al., 1996) and *Polyrhachis* cf. *doddi* (Heinze & Hölldobler, 1993). Overall, female body size is a strong predictor of fertility in insects (Thornhill & Alcock, 1983). The lower ovariole number in microgynes, which presumably reduces their fertility, might explain why microgynous colonies in this study were smaller than macrogynous colonies. It may also explain the relationship between colony size and the number of microgynes: the growth of microgynous colonies might be limited by egg laying capacity, whereas total egg production in macrogynous colonies is not affected significantly by the adoption of additional queens but by the number of workers. An alternative explanation is that (micro-) gyne adoption depends on colony size and not vice versa; experimental evidence for both hypotheses is lacking.

Microgyny is generally viewed as an adaptation to dependent colony founding in ants (Bourke & Franks, 1995; chapter one), and good evidence for this exists in some species (McInnes & Tschinkel, 1995; chapter two). For *Leptothorax* cf. *andrei* however, a close link between queen body size and colony structure could not be demonstrated, which would have provided strong support for this hypothesis.

Approximately 14% of the microgynes were found in monogynous colonies, which may be explained either by some independent colony founding by microgynes or by frequent colony budding. The latter hypothesis seems more plausible because no newly initiated colonies could be found, and with regard to nesting substrate, colonies were patchily distributed in high quality locations surrounded by unsuitable habitat. Furthermore, budding is a common reproductive tactic in *Leptothorax* ants (Bourke & Franks, 1995).

The high relatedness coefficients indicate clearly that most polygynous colonies arose by re-adoption of related queens, irrespective of morphology. Colonies comprise a complex mixture of queen-queen relatedness values. The significantly higher relatedness coefficient in macrogynous colonies indicates that most relationships in these colonies are between mothers and daughters and between full sisters. However, it has to be acknowledged that microgynes and even macro- and microgynes in mixed colonies are also related to some degree.

Overall, the general conclusion of secondary polygyny by re-adoption in *Leptothorax* cf. *andrei* is well supported. As in other studies of queen size-dimorphic *Leptothorax* species (Hamaguchi et al., 1998; chapter two), it cannot be excluded that occasional adoption of unrelated queens occur, but social parasitism seems not to be the prevalent reproductive tactic in microgynes and thus not a factor responsible for their evolution.

These results parallel previous findings in *Leptothorax rugatulus* (chapter two) that macro- and microgynes should be considered the same species, in spite of a genetic separation of their gene pools. The genetic separation, also manifested in the lower relatedness within mixed colonies, indicates different mating tactics by macro- and micro-gynes and the potential for sympatric speciation (Buschinger, 1990; Bourke & Franks, 1991). Microgynes might mate locally, while macrogynes join mating swarms (leks), an observation already made in *Myrmica* (Elmes, 1991). Nevertheless, gene flow via males constitutes a potent mechanism in ants with philopatric females to prevent inbreeding and consequently speciation (Bourke & Franks, 1995).

Like macrogynes of *L. rugatulus* (chapter two) or queens of conventional facultatively polygynous ant species, *Leptothorax* cf. *andrei* macrogynes probably switch between independent colony initiation and returning to their natal colonies, depending on environmental conditions. Thus, two different reproductive tactics, independent colony founding and re-adoption, coexist, and some adaptive size modifications have evolved. However, behavioural responses to environmental cues are more flexible and consequently are only correlated loosely with morphotype.

8 General discussion and conclusions

8.1 This study

The study of *Leptothorax rugatulus* represents the most comprehensive, coherent investigation of a queen-size dimorphic ant species, so far reported. This study extends from a description of macro-geographical patterns and inferences about underlying ecological factors to the detailed analysis of the intra-colonial distribution of reproduction and examination of the proximate causation of body size under laboratory conditions.

The main conclusion to be drawn from the various results is that queen size dimorphism in *L. rugatulus* constitutes a complex phenomenon. Despite its plastic response to queen influence, queen morphology generally persists from mother to daughter. Queen body size seems not be adapted rapidly to the environment and is only loosely associated with dependent or independent reproduction. These alternative life-histories, but not body size per se, shape the overall pattern of colony resource investment to worker, male or gyne production. Within mixed colonies, queen body size plays a role for individual reproductive output. However, the high intra-colonial relatedness of queens and the relative infrequency of mixed colonies suggest that queen size polymorphism has evolved as an adaptation to different dispersal tactics, not to social parasitism, though the potential certainly exists. The basic data gathered on *Leptothorax* cf. *andrei* show that it is similar to *L. rugatulus* and thus queen size dimorphism is comparable across species, at least within the genus *Leptothorax*.

8.2 Queen size polymorphism

The cases of queen size polymorphisms in the genus *Leptothorax* seem all to be related secondary polygyny by readoption, which also has been demonstrated for *Myrmica ruginodis* (Brian & Brian 1955; Elmes 1991b) and *Camponotus yamaokai* (Satoh 1989; Satoh et al. 1997) and speculated for *Polyrhachis* cf. *doddi* (Heinze & Hölldobler 1993) and *Pseudomyrmex veneficus* (Janzen 1973). Queen size polymorphism in these facultatively polygynous species is therefore distinctly different from the case of *Solenopsis*, which has been clearly attributed to intra-specific social parasitism. In both *S. invicta* and *S. geminata* the two queen forms mate in different seasons (McInnes & Tschinkel 1995; Tschinkel 1996), which might lead to sympatric speciation and interspecific social parasitism (Buschinger 1990; Bourke & Franks 1991) if gene flow via males is weak. The genetic separation within populations between macro- and microgynes of *Leptothorax rugatulus* demonstrates the potential for speciation also in secondary polygynous species (c.f. Crozier & Pamilo 1996b). In its natal range in South America, *Solenopsis* is indeed parasitized by several workerless inquilines which apparently are closely related to their hosts (Silveira-Guido et al. 1973; Wojcik 1990). The

status of inter-specific parasitism has been reached by *Myrmica microrubra* (Seifert 1996) and probably a number of *Myrmica* inquilines via this route (Bourke & Franks 1991).

However, no conclusive data are available for intra-specific queen size polymorphisms to decide whether microgyny is an adaptive evolution to reduce the costs of female propagules, or represents a selfish strategy of the developing females themselves in the context of caste conflict (Bourke & Ratnieks 1999). For *Leptothorax rugatulus* it could be established that, in contrast to macrogynes, microgynes do not found colonies independently and are presumably not even capable of doing so. Instead, they mainly return to their mother colonies. However, this does not prove that microgyny evolved by colony level selection to reduced dispersal because microgynes do not need many body reserves for independent claustral founding. Readoption of daughter queens also increases the kin conflict over caste determination (Bourke & Ratnieks 1999).

There were conflicting results from the reproduction of field colonies and laboratory experiments. The fact that microgynous colonies did not produce a higher gyne to worker ratio in nature suggests that these colonies are well functioning and microgynes do not preferentially develop into gynes against the interest of the rest of the colony. In contrast, microgynes produced relatively more sexuals, especially new gynes at the individual level in the laboratory. To what extent this is explainable by a longer development of macrogynous larvae is unclear, and therefore evidence for selfish microgynes can not be excluded. The release on queen body size constraint in polygynous populations coincides with increased conflict over larval caste determination and thus might have led to miniaturization. However, brood rearing behavior by workers might have adapted to the lowered threshold of microgynous larvae to develop into gynes which would explains why overall microgynous colonies do not produce more brood, and as a side effect that workers in polygynous populations (with microgynes) are smaller.

The problem with the selfish microgyne hypothesis based on Bourke & Ratnieks' model for kin conflict over caste determination (1999) is that selection differentials (fitness differences) in the parameter range of potential conflict are extremely weak. Under conditions that favor selfish gyne development in larvae and worker opposition to gyne development, the fitness advantage of selfish gyne development for the focal larva is minimal. In contrast, the cost reduction for the colony for raising instead of a macrogyne a microgyne with two to three times less dry weight is probably considerable. Thus, the hypothesis of microgyny as a colony-level selected adaptation to alternative dispersal tactics is based on stronger selection differentials and seems more likely. Nevertheless, at least some colonies (mainly in the population “NBL”) exist with a clearly non-adaptive ratio of coexisting microgynes and workers, and it would be interesting to investigate the causes and consequences of this phenomenon.

The high relatedness coefficients between queens of the same colony, even in mixed colonies, and the relative rarity of these mixed colonies demonstrate that *Leptothorax* microgynes do not systematically exploit the workforce produced by unrelated macrogynes. Consequently microgynes do not classify as social parasites, although high average relatedness coefficients do not exclude rare adoption events of unrelated queens (e.g. Stille & Stille 1993).

Secondary polygyny and intraspecific parasitism might not be as distinct as commonly perceived and one may evolve from the other (Bourke and Franks, 1991; Bourke and Heinze, 1994; Ross and Keller, 1995; DeHeer and Tschinkel, 1998). Furthermore both processes, colony usurpation and queen readoption, might co-occur in one species. In fact, the latter largely facilitates the former because secondary polygyny involves by definition readoption of young queens. The difference to social parasitism is simply the degree of relatedness, and an alien queen might overcome the colony recognition more easily in species, in which the behavioral repertoire of acceptance of newly mated queens exists at all. Likewise, the adoption of unrelated queens in polygynous colonies of *Solenopsis invicta* might be explicable in proximate terms only, i.e. by their genetic homogeneity in North America (c.f. Tsutsuji et al. 2000). From a population perspective, pure social parasitism and pure readoption tactics might represent only ends of a continuum. If so, the distinction between readoption and intraspecific social parasitism would only be quantitative. The similarity of both phenomena is emphasized by the discussion of kin conflict over caste determination (Bourke & Ratnieks 1999) discussed above. However, it would be insufficient to investigate the intra-colonial relatedness among queens, but (at least in theory) the fitness impact for all members of the adopting colony and for the adopted gyne with respect to her body size have to be determined. These fitness pay-offs will differ individually and depend on environmental circumstances, therefore the task seems difficult, if not impossible.

Although the proximate causation of alternative tactics is of considerable interest (Austad 1984; Gross 1996), in general, little was known about queen size polymorphisms in ants. Queen size and concomitantly the mode of reproduction could be based on a genetic polymorphism, environmental effects, or a combination of both. Body size is generally considered a quantitative trait to which environmental factors, as well as numerous loci contribute (Stearns 1992; Mousseau & Roff 1987; Roff 1997). This might also be true for bimodally distributed queen size in some ant species (Roff 1996) when a threshold response in phenotype to an underlying continuous variable or a major environmental effect is assumed. Body size transmission in *Leptothorax rugatulus* resembled the pattern observed in *Leptothorax spinosior* (Hamaguchi & Kinomura 1996) and *Solenopsis geminata* (McInnes & Tschinkel 1995). Queens and daughters in natural colonies are of similar size. For *L. rugatulus* this mother-daughter resemblance is even true under laboratory conditions and in the absence of any queen effects, but the results from mixed colonies prohibit the

conclusion of an unconditionally high heritability. Nevertheless, large differences in colony-external factors, such as food, temperature, or colony density need not to be present for macro- and microgynes to develop. The experiments shown here demonstrate no autonomous effect of the brood raising workers but a probably worker-mediated queen effect (Keller & Ross 1993).

This possibility of a phenotypically plastic response would suggest an adaptive response given that environmental stimuli provide reliable information for the relevant future (Padilla & Adolph 1996). It takes maximally three years for *L. rugatulus* larvae to mature and repeated collections from populations demonstrate that within this time span the degree of polygyny in a population remains stable. The degree of polygyny reflects the ratio of successful readoption to successful independent colony founding or budding. Two correlated factors, nest site stability and population density, provide reliable information about the relative benefits of either tactic. Given phenotypic plasticity, it would seem likely that colonies adjust queen size adaptively to the environment. However, the accuracy of this adjustment may not be high and the social system of a colony and the size of its queen(s) are only loosely linked. This might be interpreted as further evidence that queen size plasticity is (genetically) constrained, or that microgyny is not an adaptive phenomenon at the colony level, and larvae rather react when a certain colony level of polygyny imposes strong constraints on gyne development.

8.3 General evolutionary hypotheses

Apart from studying the phenomenon of queen size dimorphism in the ant genus *Leptothorax*, this study makes important contributions to more general issues in evolutionary biology. The data on *L. rugatulus* demonstrates how versatile social structure in ants is, and that this increases the complexity of life-history options and makes simple sex ratio models not applicable. *Leptothorax rugatulus* has provided interesting information of reproductive sharing among several potential reproductives (queens) and it complies with biogeographic rules and models of dispersal. Furthermore it is the first species that has been independently analysed with respect to the kin conflict over caste determination hypothesis.

The dichotomy between monogyny and polygyny has received much attention in the social insect literature (e.g. Hölldobler & Wilson 1977, 1990; Bennett 1987; Keller 1993b; Bourke & Franks 1995; Lindstrom et al. 1996; Shoemaker & Ross 1996; Tsuji & Tsuji 1996), mainly because of its link to alternative reproductive tactics (e.g. Heinze & Tsuji 1995; Tsuji & Tsuji 1996) and its prospects for speciation (Crozier & Pamilo 1996b). In some groups of ants the social systems seem rather stable (e.g. *Formica*: Rosengren & Pamilo 1983), but more often it varies intraspecifically due to season (Elmes 1991a; Heinze et al. 1995), local ecological conditions (Herbers 1986, 1993) or genetic factors (Shoemaker & Ross 1996). Especially, in many species of the genus *Leptothorax* the social system is extremely flexible and *L. rugatulus* certainly

belongs to this group. Different populations are strongly differentiated with respect to social organization. However, populations seem to be rather stable in their average social structure across years, a fact that might be explained by its, for *Leptothorax* exceptionally, stable nesting sites. In some areas nest sites and "their" ant colonies may be practically immortal, which results in a constantly high population density. Budding is probably conditional on appearing vacancies and thus rare and erratic.

The high quality patches are surrounded by less densely occupied habitat with less stable nesting sites. At a larger scale, the forests lie patchily distributed in uninhabitable deserts. This situation might be also intermediate between completely patchily distributed species as *Leptothorax* sp. A (Heinze & Buschinger 1989) and uniformly distributed species as *Leptothorax acervorum* (Heinze et al. 1995). Habitat patchiness is held responsible for dispersal polymorphisms (Heinze & Buschinger 1989, Heinze 1993a; Heinze & Tsuji 1995) and might also explain why long-range dispersing macrogynes coexist with microgynes. This intraspecific variability is probably balanced by contrasting selection at the intra- and the interspecific level (Olivieri et al. 1995) and also conforms with simpler models of dispersal (Hamilton & May 1977).

In the northern populations, where microgynes are virtually absent, the habitat is more uniform. *L. rugatulus* nests under small stones or in dead wood on the floor of boreal forests and high density patches on rocky outcrops were not found. These northern populations were different from the southern populations, according to Bergman’s and Gloger's rule and the consistent, probably adaptive, differences may justify a separation of *L. rugatulus* into a northern and a southern species.

The partitioning of energy to offspring (Clutton-Brock 1991; Fox & Czesak 2000) has received much scientific attention because it relates closely to the fitness of organisms, it should be well adapted to the environments and quantitative predictions can be made from basic life-history theory (Charnov 1982; Roff 1992). The extremely variable size of gynes in *Leptothorax rugatulus* and the correlated life-history changes were expected to profoundly change the pattern of energy investment into males versus females (sex ratio: Fischer 1930; Trivers & Hare 1976; Charnov 1982; Chapuisat & Keller 1999) and the reproductive allocation ratio (Stearns 1992; Roff 1992; Crozier & Pamilo 1996a; Bourke & Chan 1999). Particularly sex ratio studies in social Hymenoptera have yielded invaluable tests of kin selection and sex ratio theory (Bourke & Franks 1995; Crozier & Pamilo 1996a; Queller & Strassmann 1998; Chapuisat & Keller 1999). The sex ratio variation in *L. rugatulus* supports the view that the transition from monogyny to polygyny is correlated with a reduction of the investment into gynes at the population level (Crozier & Pamilo 1996a). However, queen size and concomitantly gyne size, had no significant effect on sex ratio or reproductive allocation produced by a colony.

The study also shows that simple models, such as the relative relatedness hypothesis (Boomsma & Grafen 1990), cannot account for the sex ratio variation within populations of facultatively polygynous ants (see also Pearson et al. 1997; Aron et al. 1999a; Chan et al. 1999). The existence of alternative reproductive tactics (independent colony founding vs. readoption of daughter queens) makes sex ratio predictions more complex and generally weak or no support is found for model predictions. In the dense polygynous populations of *L. rugatulus* colony competition is probably intense (Rosengren & Pamilo 1983; Banschbach & Herbers 1996). On the other hand, within-colony conflicts are mitigated by the complexity of odour cues (Hölldobler & Michener 1980) that are probably uninformative for intra-colonial relatedness discrimination (Stuart 1991; Sundström 1997). These arguments have led to the suggestion of a novel, life-history based hypothesis for sex ratio evolution in ants with daughter readoption, based on colony level selection (Oster & Wilson 1978).

Reproductive skew theory is the second research area where advances in theory have substantially outpaced empirical data. The number of hypotheses and parameters involved make critical tests of the general theory almost impossible. All assumptions of the tested model have to be fulfilled (Hogendoorn & Velthuis 1999; Johnstone 2000) and this is presumably not the case in *L. rugatulus* for concession-based models. Accordingly, the results do not agree with predictions from these models. Compromise models (Reeve et al. 1998) have less stringent assumptions and the experimental data basically fit predictions of the "restricted access" model. However, in native colonies the overall reproductive skew is low and the potential conflict seems not to be realized. Furthermore, size is presumed to influence fighting ability in other social insects (Reeve 1991), yet it had no influence on overall reproductive skew in *L. rugatulus*. Generally, field studies of reproductive skew face practical problems because environmental conditions are hard to control and difficult to monitor, and unobserved changes in group composition (Bourke et al. 1997; Field et al. 1998) might change the interpretation of data completely. On the other hand, it is unclear how much natural behavioral patterns are observable in laboratory experiments (Hogendoorn & Velthuis 1999). Thus the optimal choice between the two approaches depends on the species under study, and the small colonies of the Formicoxenini certainly have the advantage of little need for simulating natural with laboratory conditions.

At the individual level, important differences between queens were found with respect of the kind of offspring produced. In contrast to the colony-level analysis, the higher ratio of gynes to workers produced by individual microgynes supports the kin conflict over caste determination hypothesis (Bourke & Ratnieks 1999), although it could not be fully excluded that the affect was simply caused by different development times of macrogyne and microgyne offspring. If confirmed, the gyne bias of microgynes would be an

important piece of evidence because support of the hypothesis comes so far only from stingless bees and invading ant species which have suites of characteristics not found in their native ranges (e.g. Tsutsui et al. 2000). Equally interesting and not to be explained by different development time, are colonies in which one queen specialized in male production, despite all queens having been fertilized. Certainly, traits such as produced caste or sex ratio have an underlying genetic basis and evolve according to selection pressures. Even though in social insects it might be extremely difficult to isolate the heritable basis of these behaviors from plastic variability in response to environmental conditions and intra-colonial conflict resolution, it seems a worthwhile enterprise. With the pronounced differences found in this study, *Leptothorax rugatulus* seems a valuable model system, and studies along these lines in combination with an elaboration of the understanding of proximate mechanisms governing body size development in queens, are warranted in the future.

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10 Summary

Female alternative tactics in reproduction are relatively rare in the animal kingdom but common in ants (Formicidae). In some species, alternative reproductive tactics are held responsible for queen polymorphisms that are comparable to the queen-worker dichotomy, but alternative behavior also exist in species with uniform queens. Queen size polymorphic ant species are an interesting intermediate between species with uniform queens and more pronounced dispersal polymorphisms. A transition to social parasitism has also been suggested. However, little was known about the few established cases of coexistence of large (macrogynes) and small queens (microgynes). Hence this study aimed at contributing to the understanding of causation and consequences of queen size polymorphisms in ants, focussing on the model species *Leptothorax rugatulus* (Emery).

Employing allozymes and microsatellites as neutral genetic markers, no evidence for a taxonomically relevant separation of the gene pools of macrogynes and microgynes was found in *Leptothorax rugatulus*. Queens in polygynous colonies were highly related to each other, supporting the hypothesis that colonies with more than one queen commonly arise by secondary polygyny, i.e. by the adoption of daughter queens into their natal colonies. These results and conclusions are also true for the newly discovered queen size polymorphism in *Leptothorax* cf. *andrei*.

The higher fat content of *L. rugatulus* macrogynes, their predominance in colonies with only one queen and especially in small founding colonies, and their higher flight activity, favor the view that macrogynes predominantly found their colonies independently, while microgynes are specialized for dependent colony founding by readoption. Furthermore, genetic analysis showed that colonies with only one microgyne could not have been founded by her independently. These results corroborate well with the ecological correlates nest site stability and population density. Microgynes occur predominantly at sites where high population density makes successful independent colony founding unlikely, and stable nest sites make returning to the natal colony rewarding. However, no significant effect on population viscosity at the investigated scale could be detected when a macrogynous, monogynous population was compared to a macrogynous polygynous and a mixed, polygynous population.

Under natural conditions, mother and daughter size are highly correlated and this is also true for laboratory colonies. The results of cross-fostering larvae in worker groups could exclude autonomous worker influence. Most larvae inherently developed their mothers' phenotype. However, the clear bimodality of the size frequency distribution was lost in the offspring. The size of gynes raised in colonies with macro- and microgynous queens did not correlate with mother size. Thus, queen body size in *L. rugatulus* is not

heritable, but certainly transmissible, although a slightly negative effect of queen number on the size of macrogyne offspring was observed.

Comparing populations across the distribution range, queen morphology (head width and ovariole number) is more differentiated among populations than worker morphology (coloration, multivariate size and shape) or colony characteristics (queen and worker number per colony). Neutral genetic variation between populations is closely linked to their geographic distance and a comparison to differentiation of queen morphology suggests an adaptive differentiation of queen morphology. Northern and southern populations differed consistently, which indicates the possibility of two different species.

The queen size dimorphism in *L. rugatulus* did neither influence the sex ratio produced by a colony, nor its ratio of workers to gynes. However, the population sex ratio varied strongly with the average number of queens per colony across populations. Within monogynous populations, more productive colonies invested relatively more in female sexual offspring. In contrast, within facultatively polygynous populations, the proportional female investment increased with an increased ratio of existing workers to queens. No support for the relative relatedness asymmetry hypothesis to explain colony level sex ratio variation was found, and the hypothesis was put forward, that colony level selection accounts better for the sex ratio patterns in facultatively polygynous populations of *L. rugatulus* and other ants than within-colony kin conflict hypotheses.

Queen body size had no significant influence on the amount of reproductive skew among queens. Generally, the skew in native colonies of *L. rugatulus* was low, but in groups of unrelated, unfamiliar individuals it reached high levels. Concomitantly, the relative productivity in these groups was dramatically decreased. These results lend some support to the compromise models of reproductive sharing in animal societies and do not fulfill predictions of optimal concession-based skew models.

In eight of fourteen mixed or microgynous colonies, the relative contributions of individual queens to workers, gynes and males were significantly different. This was mainly due to the fact that relative body size was negatively correlated with the ratio of gynes to workers produced. This supports the hypothesis that microgynes might develop selfishly to gynes.

A further, presumably independent case of queen size dimorphism in *Leptothorax* cf. *andrei* was discovered and described. The genetic structure, and the morphological and social data agree with those in *L. rugatulus* and it is likely that these similarities have arisen by convergent evolution in a comparable habitat.

A fair amount of information has been gained about the queen size dimorphism in *Leptothorax* ants but no unambiguous scenario for its evolution can be inferred. While many findings argue for a dispersal-linked

polymorphism, microgyny as a selfish tactic cannot be excluded. Probably, new patches are colonized by macrogynes, and with rising habitat-saturation the level of polygyny increases until at least some colonies convert to microgyne production. Subsequently, microgynes take over because they have a relatively higher sexual output within mixed colonies, and their dependent colony foundation is favored under high-density conditions.

11 Zusammenfassung

Alternative Taktiken bei der Reproduktion sind relativ selten im Tierreich, jedoch häufig in der Gruppe der Ameisen (Formicidae). Bei einigen Ameisenarten werden alternative Reproduktionstaktiken für die Entstehung von Königinnenpolymorphismen verantwortlich gemacht, die in ihrem Ausmaß der Divergenz von Königinnen und Arbeiterinnen ähneln. Alternatives Reproduktionsverhalten existiert jedoch auch bei Arten mit einer einheitlicher Königinnenkaste. Königinnen-Größenpolymorphismen bei Ameisen stellen eine interessante Zwischenform dar, und es wurden Übergangsmöglichkeiten zu Sozialparasitismus oder einem deutlicheren Ausbreitungs-polymorphismus (mit einer geflügelten und einer ungeflügelten Morphe) diskutiert. Dennoch war bisher über die wenigen wissenschaftlich beschriebenen Fälle von Ameisenarten mit großen (Makrogynen) und kleinen Königinnen (Mikrogynen) relativ wenig bekannt. Daher versuchte diese Studie, dazu beizutragen, die Verursachung und Konsequenzen von Königinnen-Größenpolymorphismen bei Ameisen zu verstehen. Als Modell diente hierzu primär die Art *Leptothorax rugatulus* (Emery).

Die Verwendung von Allozymen und Mikrosatelliten als neutrale genetische Marker deutet eine signifikante aber geringe genetische Aufspaltung zwischen Makro- und Mikrogynen innerhalb von gemischen Populationen an, die allerdings keine Aufspaltung in zwei Arten rechtfertigt. In Kolonien mit mehreren Königinnen erwiesen sich letztere als durchschnittlich hoch verwandt, was die Hypothese unterstützt, daß solche Kolonien durch sekundäre Polygynie (d.h. durch Wiederaufnahme von Töchtern in die Herkunfts-Kolonie) enstehen. Diese Ergebnisse und Schlußfolgerungen können von *Leptothorax rugatulus* gleichermaßen auf *L.*cf *andrei* übertragen werden, bei der ein Königinnen-Größenpolymorphismus neu entdeckt und beschrieben werden konnte, der vermutlich unabhängig in einem ähnlichen Habitat durch konvergente Evolution entstanden ist.

Mehrere Argumente sprechen dafür, daß Makrogyne ihre Kolonien überwiegend unabhängig gründen, während Mikrogyne für eine abhängige Reproduktion durch Readoption spezialisiert sind: Makrogyne besitzen nicht nur absolut, sondern auch relativ mehr Fettreserven im Körper. Sie überwiegen in Kolonien mit einer Königin und vor allem in kleinen Gründungskolonien und zeigen im Freiland eine höhere Flugaktivität. Dahingegen zeigt die genetische Analyse von Kolonien mit nur einer Mikrogynen, daß diese nicht von ihr unabhängig gegründet worden ist. Diese Ergebnisse sind auch gut mit den ökologischen Korrelaten Neststabilität und Populationsdichte vereinbar. Mikrogyne kommen vor allem in Populationen vor, in denen aufgrund von hoher Koloniedichte wenig Chancen zur unabhängigen Koloniegründung vorhanden sind und es gleichzeitig sehr stabile Nestgelegenheiten gibt, die eine Rückkehr ins Mutternest

lohnend machen. Ein Vergleich dreier Populationen mit unterschiedlichem Sozialsystem erbrachte jedoch keinen signifikanten Einfluß dieser Unterschiede auf die Viskosität der genetischen Populationsstruktur.

Unter natürlichen Bedingungen, aber auch im Labor, sind die Größen der in der Kolonie vorhandenen Königinnen hochkorreliert mit der Größe der produzierten Jungköniginnen (Gynen). In einem Kreuzaufzuchtsexperiment konnte ein autarker Effekt durch die Arbeiterinnen ausgeschlossen werden, da sich die Larven nach dem mütterlichen Einfluß entwickelten. Allerdings ging in der Nachkommenschaft dieses Versuches die klare Bimodalität der Größenverteilung der Elterngeneration verloren. In gemischten Kolonien mit Makro- und Mikrogynen war die Größe von Gynen nicht mit der ihrer Mutter korreliert. Daher kann die Größe von *L. rugatulus* Königinnen nicht als erblich, wohl aber als übertragbar ("kulturelle Vererbung"), angesehen werden, obwohl auch die Anzahl der Königinnen einen leicht negativen Effekt auf die Körpergröße von Makrogynen hat.

In einem Populationsvergleich über das gesamte Verbreitungsgebiet zeigte sich, daß die Morphologie der Königinnen (Kopfbreite und Ovariolenanzahl) stärker zwischen den Populationen divergiert ist, als Arbeiterinnen-Morphologie (Färbung, sowie multivariate Größe und Form) und Kolonieparameter (Anzahl von Königinnen oder Arbeiterinnen pro Kolonie). Ein Vergleich der morphologischen Divergenz der Königinnen mit neutraler genetischer Populationsdivergenz (die wiederum gut mit dem geographischen Abstand zwischen den Populationen korreliert) läßt eine adaptive Differenzierung der Königinnen-Morphologie vermuten. Die konsistenten Unterschiede zwischen nördlichen und südlichen Populationen in allen morphologischen Merkmalen, legen die Vermutung nahe, daß es sich um zwei Arten handelt.

Der Königinnen-Dimorphismus von *L. rugatulus* beeinflußt weder das Geschlechterverhältnis, noch das Verhältnis von Gynen zu Arbeiterinnen, das von einer Kolonie produziert wird. Dahingegen variiert das Geschlechterverhältnis auf Populationsebene stark mit der mittleren Anzahl von Königinnen pro Kolonie. In monogynen Populationen investieren produktivere Kolonien stärker in weibliche Geschlechtstiere, während in fakultativ polygynen Populationen das Investitionsverhältnis in Weibchen und Männchen von dem Verhältnis von adulten Arbeiterinnen zu Königinnen abhängt. Es konnte keine Evidenz für die Hypothese der relativen Verwandtschafts-Asymmetrie zur Erklärung des Geschlechtsverhältnisses auf Kolonie-Ebene gefunden werden. Daraufhin wurde eine neue Hypothese vorgestellt, die auf Optimierung der Kolonieproduktivität beruht und die Geschlechterverhältnisse von *Leptothorax rugatulus* Kolonien und anderer fakultativ polygyner Ameisen besser erklärt als Hypothesen, die auf Kolonie-internen Verwandschaftskonflikten beruhen.

Weiterhin wurde kein signifikanter Einfluß der Verteilung der Königinnen-Körpergröße auf die Aufteilung der gesamten Reproduktion zwischen Königinnen gefunden. Generell war das reproduktive

Ungleichgewicht in natürlichen Kolonien von *L. rugatulus* niedrig, erreichte aber in Gruppen von sich fremden Tieren hohe Werte. Gleichzeitig lag die relative Produktivität dieser Gruppen deutlich niedriger, als die der natürlichen Kolonien. Diese Ergebnisse unterstützen Kompromiß-Modelle zur Reproduktionsaufteilung in Tiergesellschaften und erfüllen nicht die Vorhersagen von optimalen "Skew"-Modellen, die auf Zugeständnissen beruhen.

In acht von vierzehn gemischten oder mikrogynen Kolonien waren die relativen Anteile individueller Königinnen an der Produktion von Arbeiterinnen, Gynen und Männchen signifikant unterschiedlich. Hauptsächlich wurde dieses Ergebnis dadurch verursacht, daß relative Körpergröße negativ mit dem Verhältnis an produzierten Gynen zu Arbeiterinnen korrelierte. Das unterstützt die Hypothese, daß Mikrogynie eine egoistische Strategie darstellt.

Es konnten einige Informationen über den Königinnen-Größendimorphismus bei Ameisen der Gattung *Leptothorax* gesammelt werden, aber ein eindeutiges Szenario ist schwer zu entwerfen. Viele Resultate sprechen für einen Polymorphismus, der mit unterschiedlichen Reproduktions- und Ausbreitungstaktiken zusammenhängt, jedoch kann die Möglichkeit einer egoistischen Taktik der Mikrogynen nicht ausgeschlossen werden. Wahrscheinlich werden neue Gebiete durch Makrogyne besiedelt und mit steigender Habitat-Sättigung steigt auch der Grad an Polygynie bis zumindest einige Kolonien auf die Produktion von Mikrogynen überwechseln. Diese ersetzen dann die Makrogynen durch eine relativ höhere Geschlechtstierproduktion in gemischten Kolonien und einen größeren Erfolg der abhängige Reproduktion unter hohen Populationsdichten.

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13 Appendix: bootstrapping and resampling procedures

13.1 Resampling routine in chapter 2.2.3

```
Sub Resampling ()
   Sheets("rawdata").Select
   header = Cells(1, 2)
   Max = 141
   samples = 100
   locus = 4
   oldcol = "???"
   level = 1
   For subsampling = 1 To samples
      For Zeile = 1 To Max
         Sheets("rawdata").Select
         colony = Cells(Zeile + 1, 1)
         Cells(Zeile + 1, 3) = Round(Rnd() * (Cells(Zeile + 1, 2) - 1), 0)
         Number = Cells(Zeile + 1, 3)
         Range(Cells(Zeile + 1, Number * locus + 4), Cells(Zeile + 1, (Number + 1) * locus + 3)).Select
         Selection.Copy
         Sheets("Resample").Select
         While Len(colony) > level
           colony = Left(colony, Len(colony) - 1)
         Wend
         If colony = oldcol Then
            Cells(Zeile + subsampling * (Max + 2) - Max, 1) = ""
         Else: Cells(Zeile + subsampling * (Max + 2) - Max, 1) = colony + "dd"
         End If
         oldcol = colony
         Range(Cells(Zeile + subsampling * (Max + 2) - Max, 2), Cells(Zeile + subsampling * (Max +   2) - Max, locus
         + 1)).Select
         ActiveSheet.Paste
      Next Zeile
      Cells(1 + subsampling * (Max + 2) - Max - 1, 1) = header
      Cells(1 + subsampling * (Max + 2), 1) = "end;"
   Next subsampling
   Sheets("Resample").Select
   For Zeile = 4 To (Max + 2) * samples
      If (Cells(Zeile, 1) = "" Or Cells(Zeile, 1) = "#nexus") Then
         Cells(Zeile - 1, locus + 2) = ""
      ElseIf Cells(Zeile, 1) = "end;" Then
         Cells(Zeile - 1, locus + 2) = ";"
      Else: Cells(Zeile - 1, locus + 2) = ","
      End If
   Next Zeile
   For Zeile = 4 To (Max + 2) * samples
       If Cells(Zeile, 1) = "#nexus" Then Cells(Zeile, locus + 2) = ""
   Next Zeile
   Cells((Max + 2) * samples, locus + 2) = ";"
   Cells((Max + 2) * samples + 1, 1) = "end;"
End Sub
```

13.2 Bootstrapping ANOVAs of quantitative traits in chapter 4.2.4

```
Sub twolevelbootstrap()
   Lange = 435
   Gruppe1 = 84
   Gruppe2 = 148
   Gruppe3 = 157
   Gruppe4 = 19
   Gruppe5 = 14
   Gruppe6 = 13
   For i = 1 To Gruppe1
      Do
         zufall = Round(Rnd() * 100, 0)
      Loop Until ((zufall > 1) And (zufall < Gruppe1 + 2))
      Cells(i + 1, 6) = Cells(zufall, 1)
      Cells(i + 1, 7) = Cells(zufall, 2)
      Cells(i + 1, 8) = Cells(zufall, 3)
   Next i
   For i = Gruppe1 + 1 To Gruppe2 + Gruppe1
      Do
         zufall = Round(Rnd() * 250, 0)
      Loop Until ((zufall > Gruppe1 + 1) And (zufall < Gruppe1 + Gruppe2 + 2))
      Cells(i + 1, 6) = Cells(zufall, 1)
      Cells(i + 1, 7) = Cells(zufall, 2)
      Cells(i + 1, 8) = Cells(zufall, 3)
   Next i
   For i = Gruppe1 + Gruppe2 + 1 To Gruppe3 + Gruppe2 + Gruppe1
      Do
         zufall = Round(Rnd() * 440, 0)
      Loop Until ((zufall > Gruppe2 + Gruppe1 + 1) And (zufall < Gruppe1 + Gruppe2 + Gruppe3 + 2))
      Cells(i + 1, 6) = Cells(zufall, 1)
      Cells(i + 1, 7) = Cells(zufall, 2)
      Cells(i + 1, 8) = Cells(zufall, 3)
   Next i
   For i = Gruppe1 + Gruppe2 + Gruppe3 + 1 To Gruppe4 + Gruppe3 + Gruppe2 + Gruppe1
      Do
         zufall = Round(Rnd() * 440, 0)
      Loop Until ((zufall > Gruppe3 + Gruppe2 + Gruppe1 + 1) And (zufall < Gruppe1 + Gruppe2 + Gruppe3 +
Gruppe4 + 2))
      Cells(i + 1, 6) = Cells(zufall, 1)
      Cells(i + 1, 7) = Cells(zufall, 2)
      Cells(i + 1, 8) = Cells(zufall, 3)
   Next i
    For i = Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + 1 To Gruppe5 + Gruppe4 + Gruppe3 + Gruppe2 + Gruppe1
      Do
         zufall = Round(Rnd() * 440, 0)
      Loop Until ((zufall > Gruppe4 + Gruppe3 + Gruppe2 + Gruppe1 + 1) And (zufall < Gruppe1 + Gruppe2 +
Gruppe3 + Gruppe4 + Gruppe5 + 2))
      Cells(i + 1, 6) = Cells(zufall, 1)
      Cells(i + 1, 7) = Cells(zufall, 2)
      Cells(i + 1, 8) = Cells(zufall, 3)
   Next i
   For i = Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + 1 To Gruppe6 + Gruppe5 + Gruppe4 + Gruppe3 +
Gruppe2 + Gruppe1
      Do
```

```
        zufall = Round(Rnd() * 450, 0)
    Loop Until ((zufall > Gruppe5 + Gruppe4 + Gruppe3 + Gruppe2 + Gruppe1 + 1) And (zufall < Gruppe1 +
Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + Gruppe6 + 2))
    Cells(i + 1, 6) = Cells(zufall, 1)
    Cells(i + 1, 7) = Cells(zufall, 2)
    Cells(i + 1, 8) = Cells(zufall, 3)
  Next i
End Sub

Sub Annova()
Dim submitte(300), nsub(300) As Single
'muss die Matrix uebernehmen
  Gruppe1 = 29
  Gruppe2 = 28
  Gruppe3 = 113
  Gruppe4 = 19
  Gruppe5 = 17
  Gruppe6 = 12
  Gesamtmitte = 0
  mitte = 0      'Anfangsinitialisierung
  SS = 0
  For i = 1 To 300
    submitte(i) = 0
    nsub(i) = 0
  Next i
  For i = 2 To Gruppe1 + 1
    mitte = mitte + Cells(i, 8)
    Gesamtmitte = Gesamtmitte + Cells(i, 8)
    submitte(Cells(i, 7)) = submitte(Cells(i, 7)) + Cells(i, 8)
    nsub(Cells(i, 7)) = nsub(Cells(i, 7)) + 1
  Next i
  Cells(2, 10) = mitte / Gruppe1
  Cells(2, 11) = Gruppe1
 '**************GRUPPE 2*******************************
  mitte = 0
  For i = Gruppe1 + 2 To Gruppe1 + Gruppe2 + 1
    mitte = mitte + Cells(i, 8)
    Gesamtmitte = Gesamtmitte + Cells(i, 8)
    submitte(Cells(i, 7)) = submitte(Cells(i, 7)) + Cells(i, 8)
    nsub(Cells(i, 7)) = nsub(Cells(i, 7)) + 1
  Next i
  Cells(3, 10) = mitte / Gruppe2
  Cells(3, 11) = Gruppe2
  '****************Gruppe3*********************
  mitte = 0
  For i = Gruppe1 + Gruppe2 + 2 To Gruppe1 + Gruppe2 + Gruppe3 + 1
    mitte = mitte + Cells(i, 8)
    Gesamtmitte = Gesamtmitte + Cells(i, 8)
    submitte(Cells(i, 7)) = submitte(Cells(i, 7)) + Cells(i, 8)
    nsub(Cells(i, 7)) = nsub(Cells(i, 7)) + 1
  Next i
  Cells(4, 10) = mitte / Gruppe3
  Cells(4, 11) = Gruppe3
  '****************Gruppe4*********************
```

```
   mitte = 0
   For i = Gruppe1 + Gruppe2 + Gruppe3 + 2 To Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + 1
      mitte = mitte + Cells(i, 8)
      Gesamtmitte = Gesamtmitte + Cells(i, 8)
      submitte(Cells(i, 7)) = submitte(Cells(i, 7)) + Cells(i, 8)
      nsub(Cells(i, 7)) = nsub(Cells(i, 7)) + 1
   Next i
   Cells(5, 10) = mitte / Gruppe4
   Cells(5, 11) = Gruppe4
   '****************Gruppe5*********************
   mitte = 0
   For i = Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + 2 To Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + 1
      mitte = mitte + Cells(i, 8)
      Gesamtmitte = Gesamtmitte + Cells(i, 8)
      submitte(Cells(i, 7)) = submitte(Cells(i, 7)) + Cells(i, 8)
      nsub(Cells(i, 7)) = nsub(Cells(i, 7)) + 1
   Next i
   Cells(6, 10) = mitte / Gruppe5
   Cells(6, 11) = Gruppe5
   '****************Gruppe6*********************
   mitte = 0
   For i = Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + 2 To Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 +
Gruppe5 + Gruppe6 + 1
      mitte = mitte + Cells(i, 8)
      Gesamtmitte = Gesamtmitte + Cells(i, 8)
      submitte(Cells(i, 7)) = submitte(Cells(i, 7)) + Cells(i, 8)
      nsub(Cells(i, 7)) = nsub(Cells(i, 7)) + 1
   Next i
   Cells(7, 10) = mitte / Gruppe6
   Cells(7, 11) = Gruppe6
   '***************ENDAUSWERTUNG********************
   For i = 1 To 300
      If nsub(i) > 0 Then
         Cells(i + 1, 12) = submitte(i) / nsub(i)
         Cells(i + 1, 13) = nsub(i)
      End If
   Next i
   Cells(1, 12) = Gesamtmitte / (Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + Gruppe6)
   Cells(1, 13) = (Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + Gruppe6)
   For i = 1 To 6 'SS among populations
      SS = SS + ((Cells(i + 1, 10) - Cells(1, 12)) * (Cells(i + 1, 10) - Cells(1, 12)) * Cells(i + 1, 11))
   Next i
   Cells(2, 14) = SS
   SS = 0
   ii = 2
   For i = 1 To 6   ' Within population among colonies SS
      zahler = 1
      Do
         SS = SS + (Cells(ii, 13) * (Cells(ii, 12) - Cells(i + 1, 10)) * (Cells(ii, 12) - Cells(i + 1, 10)))
         zahler = zahler + Cells(ii, 13)
         ii = ii + 1
      Loop Until zahler > Cells(i + 1, 11)

   Next i
```

```
   Cells(2, 15) = SS
   SS = 0   ' Error term SS
   For i = 2 To (Gruppe1 + Gruppe2 + Gruppe3 + Gruppe4 + Gruppe5 + Gruppe6)
      SS = SS + ((Cells(i, 8) - Cells(Cells(i, 7) + 1, 12)) * (Cells(i, 8) - Cells(Cells(i, 7) + 1, 12)))
   Next i
   Cells(2, 16) = SS
End Sub

Sub Ubertragung()
   For i = 1 To 1000
      twolevelbootstrap
      Annova
      Cells(i + 1, 18) = Cells(10, 14)
   Next i

End Sub

Sub sorter()
   For i = 3 To 1000
   Cells(i, 9) = ""
   Cells(i, 10) = ""
   Cells(i, 11) = ""
   Cells(i, 12) = ""
   Cells(i, 13) = ""
   Cells(i, 14) = ""
   Next i
   For i = 3 To 1000
      Cells(i, Cells(i, 6) + 8) = Cells(i, 7)
   Next i
End Sub

Sub singleanovas()
   For boot = 1 To 1000
      twolevelbootstrap
      sorter
    SS1 = 0
    SS2 = 0
    SS3 = 0
    For i = 1 To 6
      For ii = 3 To 450
         If Not Cells(ii, i + 8) = "" Then
         SS1 = SS1 + (Cells(ii, i + 8) - Cells(2, i + 8)) * (Cells(ii, i + 8) - Cells(2, i + 8))
         SS2 = SS2 + (Cells(ii, i + 8) - Cells(2, 15)) * (Cells(ii, i + 8) - Cells(2, 15))
         End If
      Next ii
    Next i
   Cells(boot + 1, 16) = SS1
   Cells(boot + 1, 17) = SS2
   For i = 1 To 6
      SS3 = SS3 + ((Cells(2, i + 8) - Cells(2, 15)) * (Cells(2, i + 8) - Cells(2, 15)))
   Next i
   Cells(boot + 1, 18) = SS3
   Next boot
End Sub
```

13.3 Bootstrapping ANOVA and correlation analyses in chapter 5.2.3

```
Sub anovae()
   Worksheets("ANOVA").Activate
   xrow = Cells(1, 9).Value
   yrow = 7
   anzahl = Cells(1, 11).Value
   bootstrapzahl = Cells(1, 13).Value
   groupes = 3
   Range("H2:P5022").Select
   Selection.ClearContents
   Range("R3:S9").Select
   Selection.ClearContents
   Range("R10:S5022").Select
   Selection.ClearContents
   Range("U2:V5005").Select
   Selection.ClearContents
   bootstrap = 0
   While bootstrap < bootstrapzahl
      c1 = 21 c2 = 21 c3 = 21 c4 = 21 c5 = 21 c6 = 21
      warn = 0
      oldcell = 9999
      oldcell2 = 9999
      For zeile = 1 To anzahl
         Zufallszahl = Round(Rnd() * (anzahl - 1), 0)
         If Cells(Zufallszahl + 2, yrow).Value = 1 Then
            Cells(c1, 8).Value = Cells(Zufallszahl + 2, xrow).Value
            newcell = Cells(c1, 8).Value
            c1 = c1 + 1
         End If
         If Cells(Zufallszahl + 2, yrow).Value = 2 Then
            Cells(c2, 9).Value = Cells(Zufallszahl + 2, xrow).Value
            newcell = Cells(c2, 9).Value
            c2 = c2 + 1
         End If
         If Cells(Zufallszahl + 2, yrow).Value = 3 Then
            Cells(c3, 10).Value = Cells(Zufallszahl + 2, xrow).Value
            newcell = Cells(c3, 10).Value
            c3 = c3 + 1
         End If
         If Cells(Zufallszahl + 2, yrow).Value = 4 Then
            Cells(c4, 11).Value = Cells(Zufallszahl + 2, xrow).Value
            newcell = Cells(c4, 11).Value
            c4 = c4 + 1
         End If
         If Cells(Zufallszahl + 2, yrow).Value = 5 Then
            Cells(c5, 12).Value = Cells(Zufallszahl + 2, xrow).Value
            newcell = Cells(c5, 12).Value
            c5 = c5 + 1
         End If
         If Cells(Zufallszahl + 2, yrow).Value = 6 Then
            Cells(c6, 13).Value = Cells(Zufallszahl + 2, xrow).Value
            newcell = Cells(c6, 13).Value
            c6 = c6 + 1
         End If
```

```
        If Not (newcell = oldcell) Then warn = 1 + warn
        oldcell = newcell
        Zufallszahl = Round(Rnd() * (anzahl - 1), 0)
        Zufallszahl2 = Round(Rnd() * (groupes - 1), 0)
        Cells(zeile + 20, 14 + Zufallszahl2).Value = Cells(Zufallszahl + 2, xrow).Value
        newcell = Cells(Zufallszahl + 2, xrow).Value
        If Not (newcell = oldcell) Then warn = 1 + warn
        oldcell = newcell
      Next zeile
      If warn > 2 Then
        schrittzurueck = 0
        Range("H2:N19").Select
        Selection.ClearContents
        Application.Run "ATPVBAEN.XLA!Anova1", ActiveSheet.Range(Cells(21, 8), Cells(1021, 7 + groupes)), _
        ActiveSheet.Range("$H$2"), "C", False, 0.05
        Cells(bootstrap + 2, 21).Value = Cells(10 + groupes, 12).Value
        If Cells(10 + groupes, 12).Value > 10000 Then schrittzurueck = 1
        Range("H2:N19").Select
        Selection.ClearContents
        Application.Run "ATPVBAEN.XLA!Anova1", ActiveSheet.Range(Cells(21, 14), Cells(1021, 13 + groupes)), _
        ActiveSheet.Range("$H$2"), "C", False, 0.05
        Cells(bootstrap + 2, 22).Value = Cells(10 + groupes, 12).Value
        If Cells(10 + groupes, 12).Value > 10000 Then schrittzurueck = 1
        If schrittzurueck = 0 Then bootstrap = bootstrap + 1
      End If
      Range("N21:S1021").Select
      Selection.ClearContents
    Wend
    Columns("U:U").Select
    Selection.Sort Key1:=Range("U2"), Order1:=xlAscending, Header:=xlYes, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    Columns("V:V").Select
    Selection.Sort Key1:=Range("V2"), Order1:=xlDescending, Header:=xlYes, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    grenze = Round((bootstrapzahl + 1) / 1000 * 25, 0) 'truncation process
    helfer = (bootstrapzahl + 1) / 1000 * 25
    If grenze < helfer Then grenze = grenze + 1
    grenze1 = Round((bootstrapzahl + 1) / 1000 * 5, 0) 'truncation process
    helfer = (bootstrapzahl + 1) / 1000 * 5
    If grenze1 < helfer Then grenze1 = grenze1 + 1
    grenze2 = Round((bootstrapzahl + 1) / 10000 * 5, 0) 'truncation process
    helfer = (bootstrapzahl + 1) / 1000 * 25
    If grenze2 < helfer Then grenze2 = grenze2 + 1
    Cells(9, 19) = grenze
    Cells(9, 18) = bootstrap
    Cells(3, 18).Value = Cells(1 + grenze, 21).Value
    Cells(4, 19).Value = Cells(1 + grenze, 22).Value
    Cells(5, 18).Value = Cells(1 + grenze1, 21).Value
    Cells(6, 19).Value = Cells(1 + grenze1, 22).Value
    Cells(7, 18).Value = Cells(1 + grenze2, 21).Value
    Cells(8, 19).Value = Cells(1 + grenze2, 22).Value
    Columns("U:U").Select
    Selection.Sort Key1:=Range("U2"), Order1:=xlDescending, Header:=xlGuess, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
```

```
    Columns("V:V").Select
    Selection.Sort Key1:=Range("V2"), Order1:=xlAscending, Header:=xlGuess, _
        OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    Cells(4, 18).Value = Cells(1 + grenze, 21).Value
    Cells(3, 19).Value = Cells(1 + grenze, 22).Value
    Cells(6, 18).Value = Cells(1 + grenze1, 21).Value
    Cells(5, 19).Value = Cells(1 + grenze1, 22).Value
    Cells(8, 18).Value = Cells(1 + grenze2, 21).Value
    Cells(7, 19).Value = Cells(1 + grenze2, 22).Value
    Range("R2:S8").Select
End Sub

Sub Korrel_Bootstrap()
    Worksheets("correlation").Activate
    Calculate
    xrow = Cells(12, 15).Value
    yrow = Cells(13, 15).Value
    bootstrapzahl = Cells(11, 15).Value
    anzahl = Cells(2, yrow).Value   'get sample size for correlations
    If Cells(2, xrow).Value < Cells(2, yrow).Value Then anzahl = Cells(2, xrow).Value
    anzahl2 = Cells(2, yrow).Value   'get larger number for random draws
    If Cells(2, xrow).Value > Cells(2, yrow).Value Then anzahl2 = Cells(2, xrow).Value
    Application.CutCopyMode = False    'clear data sheet
    Range("G3:J5002").Select
    Selection.ClearContents
    Range("K2:L5002").Select
    Selection.ClearContents
    Range("N3:O10").Select
    Selection.ClearContents
    bootstrap = 0
    While bootstrap < bootstrapzahl 'main loop
        warn1 = 0   'no variability warner
        warn2 = 0
        warn3 = 0
        warn4 = 0
        Do
            Zufallszahl = Round(Rnd() * (anzahl2 - 1), 0) 'first data set without warning control for no variability
            Cells(1 + 2, 7).Value = Cells(Zufallszahl + 3, xrow).Value
            Cells(1 + 2, 8).Value = Cells(Zufallszahl + 3, yrow).Value
        Loop Until Not ((Cells(1 + 2, 7) = "") Or (Cells(1 + 2, 8) = ""))
        Do
            Zufallszahl = Round(Rnd() * (anzahl2 - 1), 0)
            Zufallszahl2 = Round(Rnd() * (anzahl2 - 1), 0)
            Cells(1 + 2, 9).Value = Cells(Zufallszahl + 3, xrow).Value
            Cells(1 + 2, 10).Value = Cells(Zufallszahl2 + 3, yrow).Value
        Loop Until Not ((Cells(1 + 2, 9) = "") Or (Cells(1 + 2, 10) = ""))
        For zeile = 2 To anzahl
            Do
                Zufallszahl = Round(Rnd() * (anzahl2 - 1), 0)
                Cells(zeile + 2, 7).Value = Cells(Zufallszahl + 3, xrow).Value
                Cells(zeile + 2, 8).Value = Cells(Zufallszahl + 3, yrow).Value
            Loop Until Not ((Cells(zeile + 2, 7) = "") Or (Cells(zeile + 2, 8) = ""))
            Do
                Zufallszahl = Round(Rnd() * (anzahl2 - 1), 0)
```

```
            Zufallszahl2 = Round(Rnd() * (anzahl2 - 1), 0)
            Cells(zeile + 2, 9).Value = Cells(Zufallszahl + 3, xrow).Value
            Cells(zeile + 2, 10).Value = Cells(Zufallszahl2 + 3, yrow).Value
        Loop Until Not ((Cells(zeile + 2, 9) = "") Or (Cells(zeile + 2, 10) = ""))
        If Not (Cells(zeile + 2, 7) = Cells(zeile + 1, 7)) Then warn1 = 1
        If Not (Cells(zeile + 2, 8) = Cells(zeile + 1, 8)) Then warn2 = 1
        If Not (Cells(zeile + 2, 9) = Cells(zeile + 1, 9)) Then warn3 = 1
        If Not (Cells(zeile + 2, 10) = Cells(zeile + 1, 10)) Then warn4 = 1
      Next zeile
      If (warn1 * warn2 * warn3 * warn4 > 0) Then
        Worksheets("correlation").Rows("2").Calculate
        Cells(bootstrap + 1, 11).Value = Cells(2, 8).Value
        Cells(bootstrap + 1, 12).Value = Cells(2, 10).Value
        bootstrap = bootstrap + 1
      End If
      Cells(9, 14) = bootstrap
    Wend
    Columns("K:K").Select
    Selection.Sort Key1:=Range("K2"), Order1:=xlAscending, Header:=xlYes, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    Columns("L:L").Select
    Selection.Sort Key1:=Range("L2"), Order1:=xlDescending, Header:=xlYes, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    grenze = Round((bootstrapzahl + 1) / 1000 * 25, 0) 'truncation process
    helfer = (bootstrapzahl + 1) / 1000 * 25
    If grenze < helfer Then grenze = grenze + 1
    grenze1 = Round((bootstrapzahl + 1) / 1000 * 5, 0) 'truncation process
    helfer = (bootstrapzahl + 1) / 1000 * 5
    If grenze1 < helfer Then grenze1 = grenze1 + 1
    grenze2 = Round((bootstrapzahl + 1) / 10000 * 5, 0) 'truncation process
    helfer = (bootstrapzahl + 1) / 1000 * 25
    If grenze2 < helfer Then grenze2 = grenze2 + 1
    Cells(9, 15) = grenze
    Cells(3, 14).Value = Cells(1 + grenze, 11).Value
    Cells(4, 15).Value = Cells(1 + grenze, 12).Value
    Cells(5, 14).Value = Cells(1 + grenze1, 11).Value
    Cells(6, 15).Value = Cells(1 + grenze1, 12).Value
    Cells(7, 14).Value = Cells(1 + grenze2, 11).Value
    Cells(8, 15).Value = Cells(1 + grenze2, 12).Value
    Columns("K:K").Select
    Selection.Sort Key1:=Range("K2"), Order1:=xlDescending, Header:=xlGuess, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    Columns("L:L").Select
    Selection.Sort Key1:=Range("L2"), Order1:=xlAscending, Header:=xlGuess, _
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
    Cells(4, 14).Value = Cells(1 + grenze, 11).Value
    Cells(3, 15).Value = Cells(1 + grenze, 12).Value
    Cells(6, 14).Value = Cells(1 + grenze1, 11).Value
    Cells(5, 15).Value = Cells(1 + grenze1, 12).Value
    Cells(8, 14).Value = Cells(1 + grenze2, 11).Value
    Cells(7, 15).Value = Cells(1 + grenze2, 12).Value
    Calculate
    Range("N2:O8").Select
End Sub
```