When and where to have sex?

Different modes of reproduction and life history traits in the facultative parthenogenetic ant *Platythyrea punctata*

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES (DR. RER. NAT.) DER NATURWISSENSCHAFTLICHEN FAKULTÄT III - BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von

Katrin Kellner aus *Regensburg*

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‘You are all alone with your thoughts for possible hours, and you are fighting temptation to stop. It’s a lonely place to be, but at the same time, beautiful…’

The Loneliness of the Long Distance Runner, Alan Sillitoe, 1959

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I. Chapter 1: Introduction

Conceptual Framework and literature review

Social insects, ants, bees, wasps and termites, but also social thrips and aphids, have been in the focus of research topics that have included ecology, molecular ecology, behavioral ecology, chemical ecology, sociobiology and ethology, networking, bioinformatics, just to name a few. Although a central topic has been the evolution of altruism and aspects of advanced eusocial life, social insects have recently become focal organisms for study on the evolution of sex and parthenogenesis. A small number of species exhibit thelytokous parthenogenesis, which is the emergence of diploid female offspring from unfertilized eggs. Studies on parthenogenetic eusocial insects combine two ongoing and exciting issues: the evolution of sexuality and parthenogenesis on the one hand, and the evolution of social behavior on the other. The aim of this thesis was therefore to investigate both aspects in the facultative thelytokous ant *Platythyrea punctata*. In this introduction, I want to give an overview on thelytokous social insects and discuss the potential that their study has to offer.

The general rule in the Hymenoptera is that females are produced sexually and are diploid; whereas males are produced by arrhenotokous parthenogenesis- *i.e*. unfertilized meiotic eggs develop into haploid individuals. The resulting haplo-diploidy leads to relatedness asymmetry within colonies because females are more closely related to their sisters than to their own offspring. This has been considered to play an important factor in the evolution of altruistic worker castes in social Hymenoptera, and consequently, much research has focused on the impact of varying relatedness on sex allocation, cooperation, conflict and conflict resolution, and the division of labor in insect societies (*e.g.*, Bourke and Franks 1995; Crozier and Pamilo 1996).

Hamilton's theory of kin selection (Hamilton 1964) explains the paradox of individuals helping others by foregoing their own reproduction, and the paradox of evolution of sterile worker casts. By helping close relatives, an individual can increase its indirect fitness. In Hamilton's rule $C<rB$, where C is the cost of the helper (the average number of offspring the helper would have produced instead of helping), B is the benefit of the recipient (the number of offspring produced because of the help), and r is the relatedness between the helper and the recipient. The 'typical' social Hymenoptera colony has been considered to present a simple family with one mother, single mated (monogyny, monandry), resulting in a full-sister colony structure characterized by an average within-colony relatedness of 0.75. Putting this high degree of kinship into Hamilton's rule has been considered as the driving

force of eusocial evolution in the Hymenoptera. It is unambiguous that kinship and relatedness are important factors in the evolution of social behavior in the Hymenoptera (Hölldobler and Wilson 1990; Bourke and Franks 1995; Crozier and Pamilo 1996; Foster et al. 2006; Crozier 2008), and also in other group-living animals (Griffin and West 2003). However, because the factors of costs and benefits also play important roles in the evolution of social behavior, relatedness probably has been overestimated as the sole driving force in social evolution. The case of the termites demonstrates this (*e.g.*, Thorne 1997; Korb 2007; Korb and Schneider 2007), which are all social and diplo-diploid, thus lacking major relatedness asymmetries.

Social insect colonies do not always display simple family structures with high relatedness asymmetries. With the development of variable genetic markers, which provide useful tools to accurately determine nestmate relatedness (Queller et al. 1993; Pamilo et al. 1997), it has become clear that nestmate relatedness can range from zero to one. Multiple queens (polygyny), multiple mated mothers (polyandry) (Crozier and Pamilo 1996), adoption and colony takeover by unrelated queens (*e.g.*, Foitzik and Heinze 2000) and worker reproduction (Bourke 1988; Ratnieks and Wenseleers 2005) can lead to colony structures with relatedness values much smaller than the theoretical 0.75, even going down to zero.

The case of the thelytokous social insects challenges the notion that relatedness is the most important factor. Thelytokous reproduction can result in colony structures, with nestmate relatedness values reaching 1, exhibiting colonies with all members being identical clones. In such true clone colonies, indirect and direct fitness are practically the same, since it has the same genetic outcome if a nestmate reproduces on herself, or her clone mate. These clone colonies exhibit now the ideal society with relatedness among nestmates even above 0.75. Therefore conflicts, as found in other Hymenopteran societies due to the asymmetric relationship structure, are not expected. But conflicts are even found here, and this might demonstrate that relatedness is only one out of three parameters in Hamilton's rule.

Aggression and dominance hierarchies have been reported from thelytokous ants. For example, it was reported that in colonies of *Platythyrea punctata* near-linear dominance orders exist among unmated virgin workers, with only one individual within the colony serving as a reproductive, whereas the other colony members had undeveloped ovaries and forego reproduction (Heinze and Hölldobler 1995). Only in some cases, where colonies were extremely large, several fertile egg layers where found. This is in contrast to the observed pattern within the thelytokous ants *Pristomyrmex punctatus* (former *P. pungens*) and *Cerapachys biroi* where reproduction is shared among all young individuals (Tsuji 1988b; Tsuji and Yamauchi 1995). However, even in such systems selfish cheaters can be found

because some unusual large workers in *Pristomyrmex punctatus* colonies can lay more eggs and work less than other workers (Sasaki and Tsuji 2003), which ultimately can undermine colony performance. Therefore these large workers have been considered cheater genotypes (Dobata et al. 2009) and should be selected against at the colony level (Tsuji 1994). Very surprisingly, 'policing behavior' was found in *P. punctata* (Hartmann et al. 2003). Policing behavior is widespread in colonies of social insects, and serves as a mechanism to prevent workers from laying eggs in the presence of a fertile queen (Ratnieks 1988; Ratnieks et al. 2006). It has been often discussed that policing behavior is influenced by within-colony relatedness (Wenseleers and Ratnieks 2006).

Fig1. Unexpected aggression – workers of *P. punctata* attack a supernumerary reproductive. Photograph by B. Barth

The most prominent example of thelytoky within the social Hymenoptera is the Cape honeybee, *Apis mellifera capensis*, in which the clonality arises from thelytokous reproduction by queenless workers and provides a unique tool for quantitative genetics and studies on the honeybee genome (*e.g.*, Baudry et al. 2004). Thelytoky appears to be determined by a single locus, controlled by the so-called *thelytoky* gene (Lattorff et al. 2005; Lattorff et al. 2007), which influences also other traits, such as egg production and the exhibition of queen pheromones by workers (Moritz et al. 2004; Lattorff et al. 2005; Lattorff et al. 2007). Thelytoky in the Cape honeybee is due to automictic parthenogenesis with central fusion of non-sister nuclei (Tucker 1958; Verma and Ruttner 1983), however, the offspring are often showing a clonal structure, because of a reduced recombination rate during meiosis (Moritz and Haberl 1994; Baudry et al. 2004). Thelytoky has had profound ecological consequences in honeybee populations in southern Africa to the extent that non-thelytokous populations may be extinct within ten years, since Cape honeybee workers invade and lay eggs and thus essentially parasitize colonies of the non-thelytokous sympatric *Apis mellifera*

scutellata (Oldroyd 2002). Thelytoky has also been found not only within Hymenoptera, but also within termites (Howard et al. 1981). Unmated primary queens of *Reticulitermes speratus* can found colonies through thelytokous parthenogenesis, with automictic mechanism with terminal fusion (Matsuura and Nishida 2001; Matsuura et al. 2004). Recently it was shown that thelytoky in that case also serves for queen succession within colonies (Matsuura et al. 2009).

In ants, thelytokous reproduction is known only from ten out of the approximately 12,000 described ant species, which have received less detailed investigation than the Cape honeybee. Older reports considering thelytoky in ant species like *Lasius niger*, *Formica polyctena*, three species of *Aphaenogaster,* two species of *Oecophylla* and four species of *Crematogaster* (listed in Slobodchikoff and Daly 1971; Wilson 1971; Hölldobler and Wilson 1990), could not been confirmed, attributed probably to the long survival rate of queen derived brood in orphaned colonies and the possibility of worker produced trophic eggs. So far, thelytoky has been unambiguously demonstrated in only 10 ant species within different subfamilies, suggesting that thelytoky evolved several times independently as a derived trait within the Formicidae (Brady et al. 2006; Moreau et al. 2006). In addition to the ponerine ant, *Platythyrea punctata* (Heinze and Hölldobler 1995), which is the subject of this study, it has been found in the myrmecines *Pristomyrmex punctatus* (Itow et al. 1984; Tsuji 1988b; Hasegawa et al. 2001), *Messor capitatus* (Grasso et al. 1998; Grasso et al. 2000), in the formicines *Cataglyphis cursor* (Cagniant 1983; Lenoir and Cagniant 1986), recently in *Cataglyphis sabulosa* (Timmermans et al. 2008), and probably in *Anoplolepis gracilipes* (Drescher et al. 2007). It has been well documented in *Cerapachys biroi* (Cerapachynae; Tsuji and Yamauchi 1995; Ravary and Jaisson 2004). Additionally it has been found in a myrmecine fungus farming ant, *Mycocepurus smithii* (Fernández-Marín et al. 2005; Himler et al. 2009), and in queens of the myrmecines *Wasmannia auropunctata* (Fournier et al. 2005) and *Vollenhovia emeryi* (Ohkawara et al. 2006).

In two ant species, *Cerapachys biroi* and *Pristomyrmex punctatus*, thelytoky is obligate, the queen caste is absent and all individuals are fertile (Itow et al. 1984; Tsuji 1988b; Ravary and Jaisson 2004). Moreover, workers do not possess a spermatheca and thus cannot mate. All workers lay thelytokous eggs early in their life cycle and become foragers when they are older. Because of this lack of a sterile caste / lack of a reproductive division of labor (except for the age polyethism), it has been argued if these ants can be really called eusocial anymore, and if they might be the only non-eusocial ants (Tsuji 1990; Furey 1992; Tsuji 1992; Crespi and Yanega 1994). Life history and geographical distribution (widely in Southeast Asia) of *P. punctatus* and *C. biroi* are very similar (Tsuji 1988b; Tsuji 1988a; Tsuji

and Yamauchi 1995), despite the phylogenetic distance between them. Both species nest in open and disturbed habitats, and exhibit a nomadic lifestyle with frequent nest relocations.

In other species, *e.g. Messor capitatus* (Grasso et al. 1998; Grasso et al. 2000), worker thelytoky appears to be facultative and occurs only in orphaned colonies after queen loss. *Vollenhovia emeryi*, *Cataglyphis cursor* and *Wasmannia auropunctata* perform a mixed strategy of reproduction (Pearcy et al. 2004; Ohkawara et al. 2006; Foucaud et al. 2007): mated queens produce female worker offspring sexually, whereas female queens are produced by thelytoky. Moreover, in *Vollenhovia emeryi* and *Wasmannia auropunctata* (Fournier et al. 2005; Kobayashi et al. 2008) it was found that males are produced clonally from males (the maternal genome gets eliminated within the fertilized egg, with the outcome of a haploid male, identical to its father)! This reproduction mode is so far unique, and leads in *W. auropunctata* to a complete separation of female and male gene pools (Fournier et al. 2005). In contrast to the former examples, the ponerine ant *Platythyrea punctata* F. Smith, has been argued to perform facultative thelytoky which might be linked to geographical distribution (Hartmann et al. 2005), with some populations being predominantly thelytokous, while in other populations mated workers (so called 'gamergates') were found.

The proximate mechanisms underlying thelytoky have been so far investigated only in some of these species, although the different mechanisms each have important consequences on the genetic structures within the progeny of an individual, but also within the colony, and therefore also on the genetic structure of a population of species. Whereas in many solitary Hymenoptera thelytoky is caused by the endoparasitic bacterium *Wolbachia* (Bourtzis and O'Neill 1998) and can be 'cured' by treatment with antibiotics (Stouthamer et al. 1990), it has not been detected in any of the known thelytokous ant species (Wenseleers and Billen 2000). Infection with *Wolbachia* causes arrhenotokous species becoming obligate thelytokous through feminization via gamete duplication. In species with single locus sex determination system, frequently observed in social Hymenoptera (reviewed in Normark 2003), gamete duplication would lead to complete homozygous offspring, resulting in diploid males.

Several cytogenetic mechanisms underlay thelytoky, all of which have genetic consequences on progeny. It is a common misbelief that parthenogenesis always leads to monoclonality. Automixis, the mechanism mostly reported in Hymenoptera, involves meiosis with subsequential fusion of egg nuclei to restore diploidy. Depending on crossover events during meiosis and which of the nuclei fuse (sister or non-sister nuclei), offspring can be genetically different than the parent. For example, automixis can lead to an increase in homozygosity (Suomalainen et al. 1987), i.e., it can have consequences similar to inbreeding. Such mechanisms have been demonstrated in *Cataglyphis cursor* (Pearcy et al. 2006) and also

in the Cape honeybee (Baudry et al. 2004). In contrast, apomixis, a mechanism without meiosis, produces offspring genetically identical to the mother. This mode has been recorded in *Wasmannia auropunctata* (Fournier et al. 2005; Foucaud et al. 2006; Foucaud et al. 2007) and probably in *Mycocepurus smithii* (Himler et al. 2009).

Very little is known about the mechanisms of thelytoky in the other ant species. To determine the mechanism underlying thelytoky, cytogenetic analyses have to be performed to screen for meiosis, as has been done for the solitary thelytokous Hymenoptera *Venturia canescens* (Beukeboom and Pijnacker 2000) or in the parasitoid wasp *Lysiphlebus fabarum* (Belshaw and Quicke 2003). In *Pristomyrmex punctatus*, meiosis stadia were found in cytogenetic analyses, suggesting automixis (Itow et al. 1984). Performing such analyses often fails in technical difficulties due to high chromosome numbers, *e.g.* in *Pristomyrmex punctatus* it was possible because this species contains a relatively low number of chromosomes (2n=24). Another possible route is specific mother-offspring comparisons with highly polymorphic genetic markers, which has been done in *Cataglyphis cursor* (Pearcy et al. 2006). This method also gives the possibility to screen for cross-over events and determine recombination rates (Baudry et al. 2004). Which method is best suitable depends on the number of chromosomes a species has (the more chromosomes the more difficulties with cytogenetic analyses) and the number of suitable genetic markers available (the more markers the better the result). However, inferring the mechanism of thelytoky from genotype patterns found within field colonies is not appropriate, since the history of freshly collected colonies is always unknown, and the actually observed pattern of genotypes can be influenced by different unknown circumstances like colony fusion, adoption of stray individuals, brood-raiding, several reproductives and more. It is therefore difficult, if not impossible, to come to accurate conclusions about the thelytoky mechanism by looking at natural colonies alone.

The ultimate causes of thelytoky in ants are even less well understood than the proximate mechanism. Thelytoky evolved several times independently within the different subfamilies. The loss or partial loss of sexuality might be an adaptation to different habitats and live styles. Thelytoky in *Pristomyrmex punctatus* and *Cerapachys biroi,* with their nomadic lifestyle and budding mechanism (splitting) of colony propagation, makes them good colonizers, with thelytokous reproduction of workers compensating for frequent queen loss in disturbed habitats (Tsuji and Ito 1986; Tsuji 1988b; Tsuji 1988a; Tsuji and Yamauchi 1995). A similar argument can be made for *Wasmannia auropunctata*, which has evolved to one of the major pest species around the world (Mikheyev and Mueller 2007). The argument of good colonizers fits to all parthenogenetic organisms, because it assures reproduction without the need to find a mating partner (Sakai et al. 2001). Consistently, parthenogenetic plants and

animals have been shown to be effective colonists (Baker 1955; Samadi et al. 1999). In Hymenoptera, however, a single inseminated queen may have the reproductive potential to establish a new population, as the queen does not have to encounter a male when mated once (Moller 1996). The reproductive system in *Wasmannia auropunctata*, *Cataglyphis cursor* and probably *Vollenhovia emeryi* contains both the advantages of sexual and parthenogenetic reproduction: while queens pass on all of their genes to their female sexual offspring, their sexually produced worker offspring can exhibit genetic diversity, which is thought to be beneficial in terms of work force, task allocation and might help colonies to better deal with environmental stress (*e.g.*, Hughes and Boomsma 2006; Smith et al. 2008).

The study system *Platythyrea punctata* and aims of thesis

Within the thelytokous social Hymenoptera, the ponerine ant *P. punctata* presents an extremely interesting study object, due to the variety of geographic distribution, mechanisms of reproduction and behavioral performance. The distribution range of the species includes the archipelago of the Caribbean Islands, from the Bahamas to the West Indies. On the mainland, the species is found in Southern Florida and Texas, and in Central America. In most populations queen caste is absent, and reproductive workers cannot be distinguished by morphological traits. However, in populations of Florida, winged queens were found regularly, and even intercastes between queens and workers were found here (Schilder et al. 1999a). Males have been reported to occur in some populations, however, the occurrence of matings seems to take place only rarely.

Fig2. *P. punctata* winged male, female and worker caste (from left to right). Photographs by A. Nobile from www.antweb.org

Colonies of these ants are relative small in size, ranging from a few workers to some hundreds, and nest in preformed cavities in rotten wood. Colonies are nesting in branches and twigs on the ground, or in dead branches hanging from trees. Rotten logs and stems are also preferred nest sites. Although some nests were found in soil, even here they used preformed cavities, since these ants are not able to dig or construct nests on their own. Preferred habitats

are wooden and dense vegetation in primary and secondary tropical rainforest, but colonies can also be found in so-called 'Hardwood Hammocks' and disturbed open habitats, like open areas among roadsides.

Fig3. Typical habitats of *P. punctata.* Secondary rainforest at the Trunquillo Experimental Forest on Puerto Rico (on the left), a tropical Hardwood Hammock at The Retreat Garden, New Providence Island, Bahamas (in the middle) and a untypical habitat (but typical in Texas): nests were found at the base of Texas Palms (*Sabal mexicana*) at The Nature Conservancy's Southmost Preserve (on the right). Photographs by K. Kellner and J. Seal

Fig4. Nests of *P. punctata*. Soil nest (on the left) and a typical wood nest in a rotten branch (on the right), both found in Turner's Hall Forest, Barbados. Photographs by B. Barth

In *P. punctata*, the evolution of thelytoky appears to be strongly linked with the geographical distribution of the species. In former studies colonies from Florida, Barbados and Puerto Rico were investigated (Schilder et al. 1999b; Hartmann et al. 2005) and been found to be predominantly thelytokous. There would appear to be rare events of sexual reproduction, since one colony from Puerto Rico contained a mated worker and offspring with variation consistent with sexual reproduction. Sexual reproduction on the mainland could be

obligate, since one colony from Costa Rica was observed to contain mated workers (Hartmann et al. 2005) and unmated workers were not able to reproduce by thelytoky.

One aim of this thesis was to gain a better and more detailed overall picture of the distribution of sexual and thelytokous populations, obtained by collecting numbers of colonies from populations not studied so far. This included several collection trips to Caribbean islands and the North and Central American mainland. Colonies were genotyped with microsatellite markers which were developed especially for *P. punctata* by (Schilder et al. 1999b), and which were partly used by Hartmann et al. (2005). By reanalyzing all of the 10 markers, I found one additional marker to be highly polymorphic, which enabled me to increase the number of suitable markers to five.

In **chapter two** I present results of population genetic analyses for the complete *P. punctata* distribution range on several levels, and test the hypothesis that thelytoky in *P. punctata* evolved in a pattern of 'geographic parthenogenesis' (Vandel 1928). With the additional marker I was also able to reanalyze the Puerto Rico population of *P. punctata* in great detail, which is presented in **chapter three**. This population was studied by Schilder and Hartmann before, but contradicting results existed about the population structure and the occurrence of sex on this island.

Prior studies on *P. punctata* suggested that thelytoky follows an apomictic mechanism (no meiosis, no recombination through crossover), since most colonies collected in the field in former studies exhibit clonal structures. Additionally, colonies containing variation were also reported (Schilder et al. 1999b; Hartmann et al. 2005). Another aim of this thesis was therefore to investigate the mechanism underlying thelytoky in *P. punctata* in more detail by mother-offspring comparisons with a set of highly polymorphic microsatellite markers (see **chapter four**). I chose this method over cytogenetic investigations, since *P. punctata* has been reported to have a chromosome set of 2n=84 (Schilder 1999), which is an extreme high number of chromosomes found in ants (chromosome numbers in ants range from 2n=2 to 2n=94; Imai et al. 1990), and therefore would have made a screening for meiosis stadiums impossible. Additionally, males were analyzed in field and laboratory colonies. Since this is likely difficult under an apomictic mechanism, I address how male production provides clues to the actual mechanism of thelytoky in this species. The results on the mechanism of thelytoky in *P. punctata* are presented in **chapter four**.

Former studies (Schilder et al. 1999b; Hartmann et al. 2005) and this thesis report low intra-colony relatedness in *P. punctata* to the extent that colonies often contain genotypes that could not arise from thelytokous or sexual reproduction. This is the topic of **chapter five.**

Genetic analyses of intra-colonial variation were performed and with a behavioral approach the hypothesis that variation can arise from the fusion of unrelated colonies was tested.

Chapter six is a behavioral study which followed directly on the behavioral set up of chapter five. We tried to answer the question if aggression in colonies of *P. punctata* is due to an aggressive potential or 'personality' or if it is situation-dependent.

Chapters seven and **eight** present the results from studies concerning aggressive behaviors, policing and nepotism. Dominance hierarchies (Heinze and Hölldobler 1995) and aggressive policing behavior (Hartmann et al. 2003) have been reported in this ant. It has been demonstrated that policing behavior in *P. punctata* serves as a mechanism of 'birth control', preventing the colony from uncontrolled brood production (Hartmann et al. 2003). In **chapter seven**, it is showed that additionally selfish behavior of single individuals is expressed: individuals engage in aggression and policing behavior not only for the good of the colony, but also because to climb up in the rank order, giving them the chance to inherit the colony and become reproductives themselves one day. In **chapter eight**, the same experimental set up is used, but with the expansion of comparing aggression and policing behavior in artificial single clone and mixed clone colonies. It is demonstrated, that the selfish component observed before even plays a more important role than relatedness and kinship.

Both facultative and obligate thelytokous Hymenoptera provide interesting systems for the investigation of various phenomena of social life, which are thought to be affected by variation in relatedness. The case of thelytokous social ants challenges previous hypothesis and theories, who considered kinship as the major factor in social evolution. At the same time, thelytokous ants might provide novel model systems to investigate what has been termed as 'the queen of problems in evolutionary biology' (Bell 1982) which is the evolution of sexual and parthenogenetic reproduction. It is the aim of this thesis, to gain new insights in both fields from a thelytokous ant.

II. Chapter 2: Distribution of sexual and thelytokous populations and their genetic structures in the facultative parthenogenetic ant *Platythyrea punctata*

K. Kellner and J. Heinze*

Abstract

The ponerine ant species *Platythyrea punctata* F. Smith is distributed over the Caribbean Islands, from the Bahamas to the West Indies, South Florida, and over the Mesoamerican mainland from south Texas to Costa Rica. *P. punctata* is one of the few ant species which have been demonstrated to produce by thelytokous parthenogenesis, which is the development of diploid female offspring from unfertilized eggs. Among the thelytokous social Hymenoptera *P. punctata* is of special interest because the thelytokous parthenogenesis seemed to be facultative, with populations from Puerto Rico, Barbados and Florida being predominant thelytokous, while a colony from Costa Rica was shown to contain mated individuals. Therefore this ant species provides an interesting model system to test the hypothesis of geographic parthenogenesis, which states different geographic distributions of parthenogenetically and sexually forms within one species. In this study we analyze genetic population and colony structures throughout the whole distribution range of this species and determine the distribution of sexual and thelytokous populations by intensive microsatellite genotyping. The results show that whereas parthenogenetic reproduction is mainly concentrated on the Islands and Florida, colonies from Texas, Belize and Honduras are likely to produce sexually.

Keywords: biogeography, geographic parthenogenesis, Hymenoptera, isolation by distance;

** manuscript*

Introduction

Despite the two-fold cost of sex, the majority of eukaryotic species reproduce sexually (Maynard Smith 1971; Bell 1982). Many models and theories have been proposed to explain the paradoxical evolutionary success of sexual reproduction, whereas, aside from a few examples (Mark Welch and Meselson 2000), asexual organisms have been considered as evolutionary dead ends (Kondrashov 1993; Barton and Charlesworth 1998; West et al. 1999).Theoretical arguments and empirical evidence suggest that asexual organisms accumulate deleterious mutations (Muller 1964; Kondrashov 1988), may be exploited by rapidly evolving enemies and might be more vulnerable in an unstable environment with rapidly evolving parasites (Hamilton et al. 1990). On the other hand, because of the two-fold cost of sex, sexual forms are thought to get out-competed by asexual reproducing forms.

Geographic parthenogenesis (GP) is a model that explains the co-occurrence of both sexual and parthenogenetic forms (Vandel 1928; Bell 1982). Specifically, parthenogenetic forms may escape evolutionary forces by occupying particular environmental settings, such as higher latitudes, higher altitudes, deserts and islands, which could be considered 'marginal' or 'disturbed' environments when compared to their closest sexual relatives. It is predicted that sexual forms have a central or limited distribution, whereas asexual forms are found in the surrounding area and have a wider geographical distribution (Beaton and Hebert 1988; Parker and Niklasson 2000; Schön et al. 2000; Stenberg et al. 2003). Insects, especially, have been in the focus to test predictions of GP and the underlying mechanisms (Bell 1982; Lundmark and Saura 2006). GP has been found in for example in several Coleoptera species (cited in Suomalainen et al. 1987), Lepidoptera (Suomalainen et al. 1987), Blattoptera (Knebelsberger and Bohn 2003), but so far social insects have received less attention as model systems studying the evolution of sexuality and parthenogenesis. Thelytoky (the emergence of diploid female offspring from unfertilized eggs) has been believed to occur in numerous species of social Hymenoptera (Slobodchikoff and Daly 1971; Normark 2003), more detailed investigations has proved it only for a small minority of taxa, including the Cape honey bee (*Apis mellifera capensis*, *e.g.*, Baudry et al. 2004) and several ants, including *Pristomyrmex punctatus* (Tsuji 1988b), *Cerapachys biroi* (Tsuji and Yamauchi 1995), *Cataglyphis cursor* (Cagniant 1983; Pearcy et al. 2004), *Mycocepurus smithii* (Fernández-Marín et al. 2005; Himler et al. 2009) and *Wasmannia auropunctata* (Fournier et al. 2005). In several of these species, thelytoky co-occurs with normal sexual reproduction, resulting in complex genetic colony and population structures (*e.g.*, Pearcy et al. 2004; Fournier et al. 2005; Foucaud et al. 2006; Ohkawara et al. 2006; Pearcy et al. 2006; Foucaud et al. 2007).

However, one ant species which might be of special interest in testing predictions of the GP model is *Platythyrea punctata* F. Smith 1858. *P. punctata* is a neotropical species in the subfamily Ponerinae which occurs from south Florida to the extreme southern West Indies (Barbados) and on the mainland from southern Texas to Costa Rica. *P. punctata* appears absent from South America, Trinidad and Tobago and Panama. Populations from Florida, Barbados and Puerto Rico were found to be predominantly thelytokous (Schilder et al. 1999a; Schilder et al. 1999b; Hartmann et al. 2005). In contrast, a single colony from Costa Rica contained three mated workers and unmated workers appeared to be incapable of producing diploid offspring (Hartmann et al. 2005). Mated workers and mated queens (winged females, which are found regularly only in Florida) have also been reported from Florida and Puerto Rico, but the evidence for female reproductives utilizing sperm to fertilize their eggs in such populations is ambiguous (Hartmann et al. 2005) and a more detailed quantification of population and colony structures is needed.

The aim of this study was to collect and investigate *P. punctata* colonies through the whole distribution range to obtain new insights in populations which have not yet been studied. By using a set of highly polymorphic microsatellite markers, which were developed especially for *P. punctata* but not used intensively in prior studies (Schilder et al. 1999a; Schilder et al. 1999b; Hartmann et al. 2005), we determined the distribution of sexual and thelytokous populations, and the genetic structure of single populations, regions and the total population.

Material and Methods

Sample collection

Entire colonies of *Platythyrea punctata* were collected from several Caribbean islands: Puerto Rico (October 2005), Dominican Republic (November 2006), Barbados and Grenada (June 2007), New Providence Island and Grand Bahama Island, Bahamas (July 2007, November 2008), Commonwealth of Dominica (October 2008), south Florida (July 2007). On the mainland, colonies were collected in Texas, (January 2008), Belize (November 2007), and Honduras (May 2008) (see **Figure 1**). Colonies were found by breaking twigs and branches on the ground or hanging from trees, by opening up rotten logs and stems or baiting and following foragers to their nests. Complete colonies were stored after collection in 100 % ethanol (Colonies from Grenada, New Providence, Dominica and Grand Bahama) or were transferred to the laboratory alive for further investigations. Colony sizes were recorded right after collection when possible or later in the lab. An overview over sample localities, number of colonies and colony sizes is given in **Table 1**.

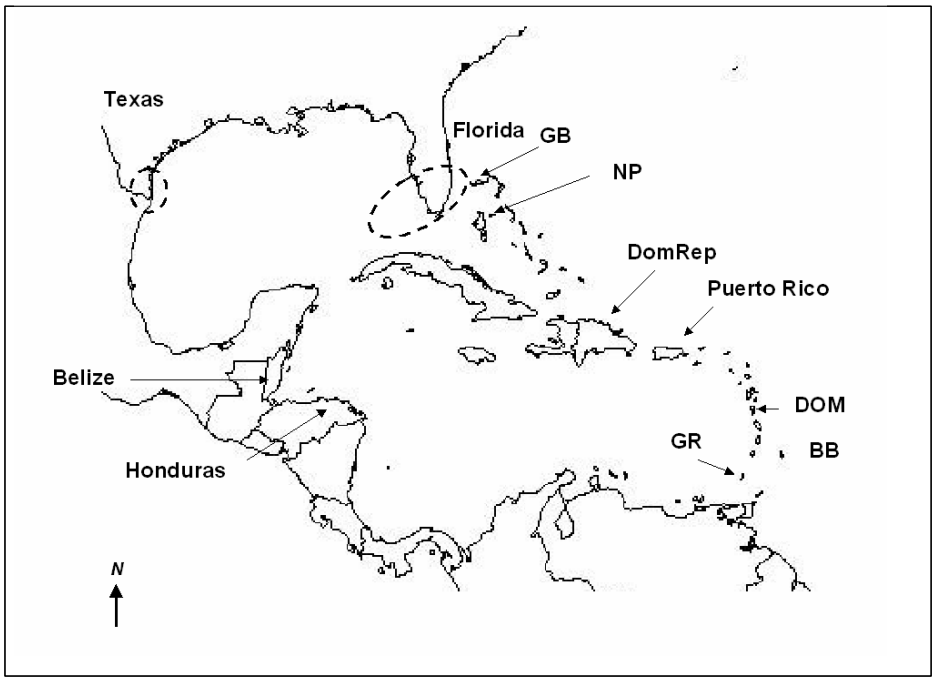

Fig1. Distribution range of the ant *Platythyrea punctata*. Colonies were collected from 2005 to 2009 in the indicated localities. GB: Grand Bahama, NP: New Providence, DomRep: Dominican Republic, DOM: Commonwealth of Dominica, BB: Barbados, GR: Grenada.

Table 1. Overview of populations and colonies sampled and used in this study. Colony sizes are given by median and upper and lower quartiles.

population	localities	GPS coordinates	# of colonies collected	colony sizes	# of colonies analyzed
Puerto Rico	El Verde	N 18° 19' 21.0" W 65° 49' 13.08"	14	119 (77.00; 250.00)	9
	Sabana	N 18° 19' 30.96" W 65° 43' 18.0"	9	32 (17.00; 36.00)	6
	Pico	N 18° 6' 5.94" W 67° 2' 23.28"	19	24 (13.00; 38.00)	12
	Rio Grande	N 18° 24' 30.36" W 65° 49' 34.02"	16	32 (17.50; 48.00)	9
	Way to San Lorenzo	N 18° 16' 26.76" W 65° 54' 19.68"	2	31 (25.00; 37.00)	2
	Sabana II	N 18° 19' 13.44" W 65° 42' 58.74"	2	52.5 (48.00; 57.00)	2
	El Tunel	N 18° 29' 3.48" W 66° 58' 3.72"	11	17 (5.00; 26.00)	7
Dominican Republic	El Laurel	N 18° 46' 41.40" W 69° 53' 36.42"	14	20 (15.00; 23.00)	6
	Anton Sanchez	N18° 49' 25.32" W 69° 41' 25.26"	16	15 (4.00; 20.00)	6
	Miches Gallistico	N1 8° 58' 40.62" W 69° 2' 18.06"	10	18 (12.00; 22.50)	6
	Rancho Wendy	N 18° 53' 15.18" W 70° 27' 39.60"	18	23 (12.00; 30.00)	9
Barbados	Harrison's Cave	N 13° 11' 8.28" W 59° 34' 26.70"	1	24	1
	Turner's Hall	N 13° 13' 18.96" W 59° 35' 11.82"	13	20.5 (13.50; 43.50)	6
	Hackleton's Cliff	N 13° 12' 5.22" W 59° 31' 28.56"	5	26 (17.00; 42.00)	4
	no name P8	N 13° 13' 23.70" W 59° 34' 20.50"	2	5.5 (5.00; 6.00)	2
Grenada	Lake Grand Etang	N 12° 5' 37.80" W 61° 41' 41.7"	8	33.5 (22.0; 52.5)	6
	Mirabeau	N 12° 8' 20.22" W 61° 39' 30.36"	6	28.0 (26.0; 31.0)	4
Florida	Crane Point / Marathon	N 24° 43' 0.66" W 81° 4' 36.12"	1	14	1
	Curry Hammock	N 24° 44' 28.98" W 80° 59' 49.50"	1	35	1
	J.Pennekamp / Key Largo	N 25° 7' 34.26" W 80° 24' 28.32"	7	23.0 (12.0; 24.0)	5
	Matheson Hammock / Miami	N 25° 40' 56.64" W 80° 16' 16.38"	2	15.5 (12.0;19.0)	1
	Sebastian Inlet / Ft.Pierce	N 27° 51' 19.32" W 80° 27' 4.86"	1	60	1
New Providence	The Retreat / Nassau	N 25° 3' 49.02" W 77° 18' 43.08"	5	44.0 (33.0; 49.0)	5
Grand Bahama	Lucayan National Park	N 26° 36' 19.56" W 78° 24' 4.86"	2	4.50 (4.0; 5.0)	2
Dominica	Archbold/Springfield	N 15° 18' 43.83" W 61° 22' 24.32"	7	38.0 (18.0; 72.0)	7
Belize	Ian Anderson Lodge	N 17° 10' 3.66" W 88° 41' 2.34"	5	4.0 (4.0; 5.0)	4
	Guanacaste	N 17° 15' 41.52" W 88° 47' 16.14"	3	11.0 (10.0; 32.0)	2
	Cockscomb	N 16° 46' 46.26" W 88° 27' 30.36"	2	14.5 (5.0; 24.0)	2
Texas	Sabal Palm Grow	N 25° 51' 1.86" W 97° 25' 1.8"	6	6.0 (1.0; 11.0)	3
	Southmost Preserve	N 25° 50' 29.52" W 97° 23' 57.66"	1	17	1
Honduras	Lancetilla Botanical Garden	N 15° 44' 20.76" W 87° 27' 23.04"	5	21.0 (20.0; 35.0)	5

Molecular techniques and genotyping

In total 3134 individuals were genotyped from 137 colonies. From each colony, 12 individuals (six adult individuals and six brood items (larvae or pupae) or callows (freshly eclosed workers)) were genotyped. Due to colony sizes and/or lack of sufficient brood, in some colonies it was not possible to obtain six adults and/or six brood items. The sample size was therefore restricted in 11 out of 137 colonies, which ranged from 3 to 9 analyzed individuals. Since colonies of *P. punctata* are normally headed by a single worker which monopolizes reproduction (Heinze and Hölldobler 1995), brood and callows within a colony are expected to originate from one mother. Separating the analyses of individuals into callows/brood and adults gives therefore an insight to the mode of reproduction. If variation within the genetic patterns of callows and/or brood item within a colony is found, it is likely that this variation is caused by sexual or thelytokous recombination events.

Individual ants (adults, larvae or pupae) were pulverized in liquid nitrogen and total genomic DNA was extracted following a modified CTAB extraction protocol (Sambrook and Russell 2001). Isolated DNA was washed with 100% ethanol and twice with 70% ethanol, dried and resuspended in double distilled water (50µl for individual ants and pupae, 40µl for larvae) and stored at -20°C until use.

The original microsatellite primer set developed for *P. punctata* (Schilder et al. 1999b) was tested following the described PCR protocol on a subset of 48 randomly selected adults from different sample sites and colonies from Puerto Rico. Amplified fragments were scored on an ABI Prism 310 Genetic Analyzer. After testing each microsatellite locus for polymorphism, we chose a set of five primers being suitable for analyses (loci 3506, 3302, 2902, 4101 and 2801), including those previously studied by Schilder et al. (1999b) and Hartmann et al. (2005).

DNA was amplified in a total reaction volume of 20µl, containing 1µl template DNA. Each reaction contained 2µl of 10x reaction buffer (for 3506, 2902, 4101 and 2801: Fermentas 10xTaq Buffer + KCl – $MgCl_2$; for 3302: Fermentas 10xTaq Buffer + $(NH_4)_2SO_4$ – $MgCl_2$), 2 µl (5pmol/µl) of each primer (forward primer labeled with different types of fluorescent dye, Applied Biosystems), 4µl dNTPs (1mM of each), 0.5µl Taq polymerase (1U/µl Fermentas), 1.2µl 25mM $MgCl_2$ and 7.3µl PCR-H_2O (Sigma). After an initial 5min denaturation step at 94°C, the reaction mix was incubated at the following temperature cycles: 30 cycles of 1min denaturation at 94°C, 1 min primer annealing at 50-54°C (locus 3506: 50°C; 2801: 53°C; 3302, 4101 and 2902: 54°C), and 1min extension at 72°C. The reaction was terminated by a final 5min extension step before cooling to 4°C. The run time for each of the steps,

denaturation, annealing and extension was extended from 1min to 1.5 min for locus 2801 and 3302. The amplified microsatellite fragments were scored on an ABI Prism 310 Genetic Analyzer. Allele lengths were determined using GeneScan© software.

Analyses of reproduction mode, genetic diversity and population structures

Mode of reproduction and within colony variation patterns

For each colony, the patterns of genotypes were investigated and the occurrence of intra-colonial variation was recorded. Individual genotypes were recorded as different from each other then they differed in at least one allele at one locus. Aberrant genotypes were categorized into three types: i) 'A' the aberrant genotype was caused by two foreign alleles at a locus, ii) 'B' the aberrant genotype was homozygous instead of heterozygous at a locus and iii) 'C' the aberrant genotype was caused by one foreign allele at a locus. For each colony within-colony relatedness was calculated using the algorithms implemented in the Software RELATEDNESS 5.0.8 (Goodnight and Queller 1998). Standard errors were obtained by jackknifing over loci. For each population the average relatedness between colonies was calculated, and standard errors were obtained by jackknifing over colonies.

Population structures

Number of multilocus genotypes (number of clone lineages in thelytokous populations) was inferred for each population. To correct for sample sizes, the number of multilocus genotypes was expressed as number of genotypes / number of analyzed colonies.

Departures from Hardy-Weinberg-Equilibrium (HWE) were calculated using the software GENEPOP 4.0 (Raymond and Rousset 1995), with a global test (Score (U) test) for HWE for each population, testing the hypothesis of heterozygote excess using the Markov chain method with 100 batches and 10000 iterations per batch.

Mean number of alleles, private alleles, observed and expected heterozygosities for each population and for each of the five loci were calculated using the program GDA 1.0 (Lewis and Zaykin 2001). Expected and observed heterozygosities were compared statistically using a Wilcoxon test for matched samples in STATISTICA 6.0 (Statsoft 2003). Because members of social insect colonies are related to each other and colonies represent families, individual genotypes are not independent from each other. For each population, two level analysis of molecular variance was performed, defining colonies as subpopulations. Fixation indices, describing differentiation among individuals within colonies (f or F_{IS}), differentiation among individuals within the population (F or F_{IT}), and differentiation among colonies within the population (θp or F_{ST}) were calculated following the methods of Weir and Cockerham (Weir

and Cockerham 1984; Weir 1996) as implemented in GDA 1.0 (Lewis and Zaykin 2001). 95% Confidence Intervals were obtained by bootstrapping over loci with 1000 replicates.

To investigate population structure of the Caribbean region and the mainland, colonies were defined as subpopulations within populations (the different islands or the different countries on the mainland) within regions (the Caribbean or the North -Central American mainland), and a three level analysis of molecular variance was performed using the AMOVA framework algorithms implemented in the program ARLEQUIN 3.1 (Excoffier et al. 2005). In these analyses, fixation indices were obtained for the differentiation among individuals within colonies (F_{IS}), among individuals within the region (F_{IT}), among individuals within colonies within the populations (F_{SC}, describes the differentiation among colonies within the populations) and among individuals within colonies within populations within the region (F_{CT} describes the differentiation among populations within the regions). Tests for significance were performed with permutation tests (1000 permutations).

A similar approach was used for obtaining the overall population structure. In this three-level analysis, the two regions (the Caribbean and the mainland) were pooled. Fixation indices were obtained for the differentiation among individuals within colonies (F_{IS}), among individuals within the total population (F_{IT}), among individuals within colonies within the populations (F_{SC}, describes the differentiation among colonies with the populations) and among individuals within colonies within populations within the total population (F_{CT} describes the differentiation among populations within the total population). Tests for significance were performed with permutation tests (1000 permutations). Pairwise population differentiation F_{ST} were calculated in ARLEQUIN 3.1 and tested against a null distribution obtained by 10 000 permutations of genotypes between populations.

Isolation by distance

To investigate whether a significant correlation coefficient exists between genetic and geographic distances, a Mantel test was performed using the program FSTAT 2.9.3 (Goudet 1995; Goudet 2001).Genetic distance was defined as $F_{ST}/(1-F_{ST})$ (Rousset 1997). A matrix of geographic distances (measured in km) was constructed using GDMG 1.2.3 (Ersts 2006). A Geographic distance matrix was constructed with GPS coordinates for each population (see **Table 2**). Analyses were run by pooling individuals within populations, for the Caribbean population separately, and for the total population (running a similar analysis on the mainland populations was precluded by the low number of populations (n = 3)). To obtain p-values for significance of isolation by distance, 2000 randomizations were performed.

Table 2. Coordinates of *P. punctata* populations used for testing isolation by distance between the different populations.

Population	GPS coordinates
Puerto Rico	N 18° 27' 17.93" W 66° 7' 10.09"
Dominican Republic	N 18° 30' 34.18" W 69° 52' 29.36"
Barbados	N 13° 7' 33.61" W 59° 33' 37.17"
Grenada	N 12° 2' 28.70" W 61° 42' 55.09"
Florida (Miami)	N 25° 42' 4.82" W 80° 16' 26.30"
New Providence	N 25° 6' 28.25" W 77° 19' 45.01"
Grand Bahama	N 26° 38' 36.93" W 78° 22' 58.27"
Dominica	N 15° 18' 15.59" W 61° 22' 7.63"
Belize	N 17° 15' 41.42" W 88° 47' 18.66"
Texas (Brownsville)	N 25° 56' 8.09" W 97° 28' 50.83"
Honduras	N 15° 30' 22.61" W 87° 59' 59.95"

Results

Relatedness patterns within colonies

Microsatellites appeared to be polymorphic with 9 different alleles at the lowest (Loc3506) and 19 alleles at the highest polymorphic locus (Loc3302).

In the Caribbean region, 36.07% (44 out of 122) of the colonies showed intra-colonial variation. In contrast, in colonies from the mainland, 77.78 % (14 out of 18) of the colonies showed variation. An overview for each population is given in **Table 3**. Values for within colony relatedness ranged from R=1 for true clone colonies down to R=0.20 for colonies containing variation in the Caribbean region. Relatedness values from mainland colonies ranged from R = 0.21 to R = 0.95. Mean Relatedness values for each population are given in **Table 3**.

Table 3. Overview of the number of colonies containing variation and the average within-colony relatedness.

population	# of colonies analyzed	# of colonies containing variation	%	Average R (SE)
Puerto Rico	47	20	42.55	0.94 (0.018)
Dominican Republic	27	10	37.04	0.92 (0.046)
Barbados	13	6	46.15	0.90 (0.036)
Grenada	12	1	8.33	0.99 (0.008)
New Providence	5	0	0.00	1.00 (0.00)
Grand Bahama	2	0	0.00	1.00 (0.00)
Dominica	7	1	14.29	0.79 (0.204)
Florida	9	6	66.67	0.90 (0.077)
Caribbean total	**122**	**44**	**36.07**	**0.93 (0.025)**
Texas	4	4	100.00	0.60 (0.135)
Belize	9	8	88.89	0.76 (0.056)
Honduras	5	3	60.00	0.85 (0.124)
Mainland total	**18**	**15**	**83.33**	**0.74 (0.073)**

Within the colonies from the Caribbean region, 14 colonies contained variation caused by callows or brood items showing aberrant genotypes, whereas in 18 colonies variation was due to adult individuals with aberrant genotypes, and in 12 colonies variation was caused by both callows and adults. In the colonies where variation was due to callows, the variation was caused by one callow out of six, only in one colony two callows differed from their nestmates. Variation pattern showed that these callows were different from the others in only one out of five loci, whereas in one colony, two loci were different, and only in one colony, variation was found at three loci. In the colonies containing variation due to callows and adults, a similar pattern was found. Variation was never caused by more than three out of six callows, and three out of five loci. In cases where variation was caused by adult individuals, one to six adults showed aberrant genotypes with variation at one to four loci. Concerning the categories of the aberrant genotypes, 81 adult individuals were found having aberrant genotypes, with 23 loci belonging in category 'A', 21 in category 'B' and 47 in category 'C'. Within the aberrant callows, 34 callows in total carried loci not fitting to the other colony members, with 7 loci in category 'A', 22 in category 'B' and 18 in category 'C'. In summary, the observed variation patterns are unlikely to emerge from sexual reproduction because we should see consistent variation across all loci and ant types. In contrast, most colonies from the mainland contained variation and did not show a genetically identical colony composition. In each colony showing variation, variation was expressed in more than one individual and in more than one locus. The distribution of alleles makes recombination events due to sexual reproduction likely (an example for a mainland colony is given in **Table 4**).The variation pattern observed is likely due to sexual recombination, which is also reflected in the average relatedness values (see **Table 3**). However, three mainland colonies (one from Guanacaste, Belize and two from Lancetilla, Honduras) appeared to show a clonal colony structure (compare **Table 3**), suggesting that colonies from this localities are maybe not restricted to sexual reproduction.

Table 4: Example of a colony of *P. punctata* with variation pattern showing sexual reproduction (Cockscomb1, Belize). Aberrant genotypes are marked in bold. Individuals 1-6 are callows, 7-10 are adults. The mean relatedness within the colony is 0.741 (SE ± 0.03).

Individual ID	Loc1	Loc2	Loc3	Loc4	Loc5
1	193/193	175/175	**213/213**	390/396	256/262
2	193/193	**175/183**	197/213	390/396	256/262
3	193/193	175/175	197/213	390/396	256/262
4	193/193	175/175	**213/213**	**390/390**	**256/256**
5	193/193	175/175	197/213	390/396	256/262
6	193/193	175/175	**213/213**	**390/390**	**256/256**
7	193/193	175/175	**213/213**	390/396	256/262
8	193/193	**175/183**	197/213	**390/390**	**256/256**
9	193/193	175/175	197/213	390/396	256/262
10	193/193	**175/183**	197/213	390/396	256/262

Population structure

Although populations from the Caribbean region are predominantly thelytokous, significant amount of genetic diversity is present, and single islands do not consist exclusively of one clone lineage (with the exception of New Providence, Bahamas). This is also expressed in the fact, that loci appeared to polymorphic even within thelytokous populations with several alleles per locus. Mean numbers of alleles per locus ranged from 1.40 to 4.20 in the Caribbean region. In contrast, predominantly sexual populations from the mainland showed a higher mean number of alleles per locus (ranging from 4.00 to 8.20). Since this is of course influenced by the number of colonies which were analyzed and in the case of thelytokous populations the number of colonies which originated from the same clone lineage (resulting in repeated multilocus genotypes), the mean number of alleles per locus and the number of genotypes encountered were corrected due to sample size. This correction makes it even more clear, that although thelytokous populations showed a remarkable amount of genetic variation, values obtained from the mainland populations are still higher, concerning the mean number of alleles per locus per number of colonies, and also the number of multilocus genotypes per colony (for an overview see **Table 5**). In the Caribbean region, colonies generally consisted of a single clone lineage, and islands are inhabited by more than one clone lineage. This was supported by the average relatedness between colonies encountered for thelytokous populations. Extreme cases were found for Grand Bahama (R=-1.00, number of genotypes/number of colonies=1.00), where the colonies analyzed represent two different clone lineages, and New Providence (R=1.00, number of genotypes/number of colonies=0.20), where the five colonies analyzed all belong to the same clone lineage. Due to

predominant sexual reproduction, mainland populations exhibited a higher number of genotypes / number of colonies.

Table 5. Overview over the mean number of alleles / locus (A), mean number of alleles / locus (A) per number of colonies, average relatedness between colonies and number of genotypes /number of colonies.

	mean number of alleles / locus (A)	**A / number of colonies**	**mean Relatedness between colonies (± SE)**	**number of genotypes / number of colonies**
Puerto Rico	4.00	0.09	-0.0202 (±0.0009)	0.894
Dominican Republic	4.20	0.16	-0.0268 (±0.0119)	0.815
Barbados	2.60	0.20	-0.0751 (±0.0130)	0.923
Grenada	2.20	0.18	-0.1103 (±0.0174)	0.333
Dominica	2.20	0.37	-0.1826 (±0.0661)	0.833
Grand Bahama	1.60	0.80	-1.0000 (±0.000)	1.000
New Providence	1.40	0.28	1.0000 (±0.000)	0.200
Florida	3.80	0.42	-0.0816 (±0.0744)	1.222
Belize	8.20	2.05	-0.1249 (±0.0201)	5.250
Texas	4.00	1.00	-0.2588 (±0.0973)	4.250
Honduras	5.20	1.04	-0.2910 (±0.1045)	2.200

Populations from the Caribbean region showed significant heterozygote excess. Calculated by loci, observed heterozygosity (Ho) was significant higher than expected heterozygosity (He) (Wilcoxon matched pairs test, n=8, Z=2.2404, p=0.025). The same result was found for calculation by colonies (n=8, Z=2.5205, p=0.012). In contrast, no significant differences between observed and expected heterozygosities were found in the mainland populations (n=3; calculated by locus: Z=1.0690, p=0.285; by colony: Z=1.6035, p=0.109). Results of heterozygosities are shown in Table 6. Tests for HWE revealed significant departure ($p<0.001$) in all analyzed populations.

Table 6. Overview of expected and observed Heterozygosities (Ho, He) calculated over loci and populations, and the fixation indices for each population. The upper and the lower bound of the 95% C.I. are given in parenthesis. Values not significant different from 0 are indicated bold.

	by locus		**by population**		F_{IS}	F_{IT}	F_{ST}
population	**He**	**Ho**	**He**	**Ho**	**f**	**F**	**θp**
Puerto Rico	0.576	0.796	0.428	0.795	-0.946 (-0.911; -0.979)	-0.374 (-0.176; -0.657)	0.293 (0.383; 0.161)
Dominican Republic	0.582	0.622	0.338	0.619	-0.864 (-0.790; -0.952)	**-0.053** (0.156;-0.271)	0.435 (0.539; 0.347)
Barbados	0.390	0.449	0.253	0.440	-0.867 (-0.826; -0.924)	**-0.122** (0.057; -0.037)	0.399 (0.484; 0.289)
Grenada	0.611	0.864	0.464	0.887	-0.994 (-0.972;-1.000)	**-0.376** (0.155; -1.000)	**0.310** (0.571; 0.000)
Dominica	0.372	0.453	0.286	0.476	**-0.732** (1.000; -1.000)	**-0.163** (1.000;-0.275)	**0.328** (0.362; -0.013)
Grand Bahama	0.569	1.000	0.539	1.000	-1.000 (-1.000; -1.000)	-0.600 (-0.333; -1.000)	**0.200** (0.333; 0.000)
New Providence	0.504	1.000	0.523	1.000	-1.000 (-1.000;-1.000)	-1.000 (-1.000;-1.000)	**0.000** (0.000;0.000)
Florida	0.450	0.399	0.268	0.432	-0.668 (-0.112; -0.903)	**0.369** (0.884;-0.197)	0.622 (0.924; 0.365)
Belize	0.687	0.462	0.395	0.511	-0.385 (-0.242; -0.472)	*0.369* (0.432; 0.220)	0.545 (0.611; 0.475)
Texas	0.431	0.464	0.349	0.438	-0.478 (-0.061; -0.699)	**0.027** (0.314; -0.096)	0.342 (0.473; 0.237)
Honduras	0.685	0.582	0.373	0.567	-0.686 (-0.555; -0.847)	**0.266** (0.582; -0.110)	0.565 (0.745; 0.378)

Fixation indices (shown in **Table 6**) obtained in the two level analyses for each population revealed strong negative values for f (differentiation of individuals within colonies) in the case of populations from the Caribbean region, ranging from -0.668 (Florida) to -1.000 (New Providence and Grand Bahama). For the mainland populations, f values ranged from -0.686 (Honduras) to -0.385 (Belize). Values were significantly different from 0 (with the exception of Dominica) as shown by confidence intervals. Since colonies represent families, which always exhibit a certain amount of genetic similarity, these findings are in accordance with the obtained values for within-colony relatedness. The strong negative values reflected clonal within colonies structures. In the case of the mainland populations f values get statistically negative because of the finding of colonies containing variation. F values (overall inbreeding coefficient) were also negative or 0 for the Caribbean populations, and in the populations of Texas and Honduras. Only for the Belize population, F was significantly higher than 0. θp (differentiation among colonies with the populations) was significantly positive in the case of Puerto Rico, Dominican Republic and Barbados, the islands in which a high number of multilocus genotypes were encountered. In the Florida population, a similar pattern was found. In contrast, where a poor number of different genotypes were recorded on the islands

(Grenada, Dominica and the two Bahamas Islands), genetic differentiation among colonies was low. In addition, for the mainland populations, significant genetic differentiation was found among colonies. These patterns are influenced by the fact, that colonies in general represent clone lineages in thelytokous populations, which can be very distinct from each other. On the other hand, since colonies might spread by budding / fission, and various colonies originating from the same clone lineages were found, the overall genetic differentiation among colonies becomes influenced.

In the three-level analysis carried out separately among data from the Caribbean region, mainland and total overall population, it seems that the F_{IT} value for the Caribbean region is not significantly different than 0, whereas significant variation is present in the mainland region. The F_{IT} value of the overall population (taken Caribbean and mainland together) however is not significant different from 0. This result gets influenced by wider and more abundant distribution of thelytokous populations. A same influence can be seen for F_{IS} results. In contrast Fsc results (differentiation among colonies within the regions) show significant amount of differentiation among colonies. A similar result was obtained for the differentiation among the different populations within the regions and the total population. Again the overall value gets influenced by the lower value for the differentiation among the different thelytokous populations with the Caribbean region (Fixation indices are shown in **Table 7**).

Private alleles (alleles which were recorded only in one population and nowhere else) were found at each locus in several cases for the mainland population, but also the populations from Florida and Puerto Rico exhibited exclusive alleles (3506: Honduras, Belize, Texas; 2902: Honduras, Belize, Texas; 4101: Honduras, Belize, Texas, Florida; 2801: Honduras, Belize, Texas; 3302: Honduras, Belize, Florida).

Table 7. Differentiation among individuals within colonies (F_{IS}) and populations (F_{IT}), among colonies within populations (F_{SC}) and among populations within the overall population (F_{CT}) for the Caribbean and mainland region and the overall population. Values significant different from 0 are indicated bold.

region	**F_{IT}**	**F_{IS}**	**Fsc**	**F_{CT}**
Caribbean	-0.017	-0.929	**0.357**	**0.18**
p	1	1	0	0
mainland	**0.424**	-0.545	**0.52**	**0.224**
p	0	1	0	0
total population	0.01	-0.888	**0.334**	**0.213**
p	1	1	0	0

Genetic differentiation for all population pairs was highly significant (all F_{ST} values had $p<0.01$: **Table 8**), with the exception of that for New Providence and Barbados ($p<0.05$). Texas was most differentiated from all other populations, followed by Belize. Within the Caribbean region, Florida was most differentiated from the other populations.

In total, 92 distinct clone lineages were encountered within the Caribbean region. Only five of these were not unique for a population. Two clone lineages were found on Puerto Rico and the Dominican Republic, but nowhere else. One clone lineage was found on the two Bahamian Islands, as well as in Barbados and Dominica. Another clone lineage was found in Barbados and in Dominica. The fifth non-unique clone lineage was found in Dominican and Grenada. An overview is given in **Figure 2**.

Table 8. Genetic differentiation (pairwise F_{ST}) between the eleven *P. punctata* populations. DR: Dominican Republic, PR: Puerto Rico, BB: Barbados, NP: New Providence, GB: Grand Bahama; Significance indicated by asterisks: **p<0.01, *p<0.05

F_{ST}	DR	PR	BB	Dominica	Grenada	NP	GB	Florida	Texas	Honduras
DR										
PR	0.015**									
Barbados	0.181**	0.231**								
Dominica	0.181**	0.232**	0.032**							
Grenada	0.136**	0.199**	0.040**	0.047**						
NP	0.213**	0.264**	0.012*	0.029**	0.094**					
GB	0.157**	0.210**	0.037**	0.090**	0.090**	0.084**				
Florida	0.246**	0.300**	0.298**	0.348**	0.284**	0.348**	0.264**			
Texas	0.442**	0.434**	0.632**	0.648**	0.639**	0.669**	0.644**	0.582**		
Honduras	0.187**	0.226**	0.303**	0.323**	0.280**	0.315**	0.270**	0.209**	0.456**	
Belize	0.309**	0.305**	0.53**	0.53**	0.515**	0.535**	0.519**	0.473**	0.459**	0.289**

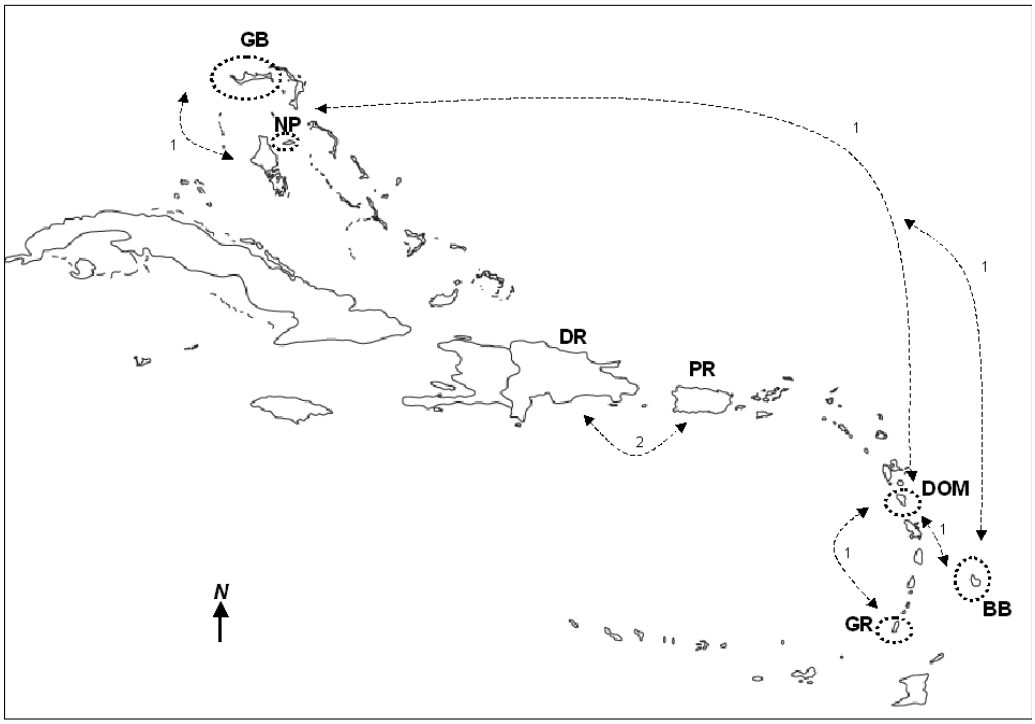

Fig2. Identical clone lineages of *P. punctata* on different Caribbean islands. GB: Grand Bahama, NP: New Providence, DR: Dominican Republic, PR: Puerto Rico, DOM: Dominica, BB: Barbados, GR: Grenada. Numbers indicate number of clone lineages being identical.

Isolation by distance

Genetic distance had a strong positive correlation to geographical distance (**Figure 3**) within the total population, with 27.56% of variation explained by isolation by distance (R^2 =27.56, $p<0.05$). In case of the Caribbean populations, only 0.54% of the variance is explained by the model (R^2 =0.54, $p>0.05$), with no significant correlation.

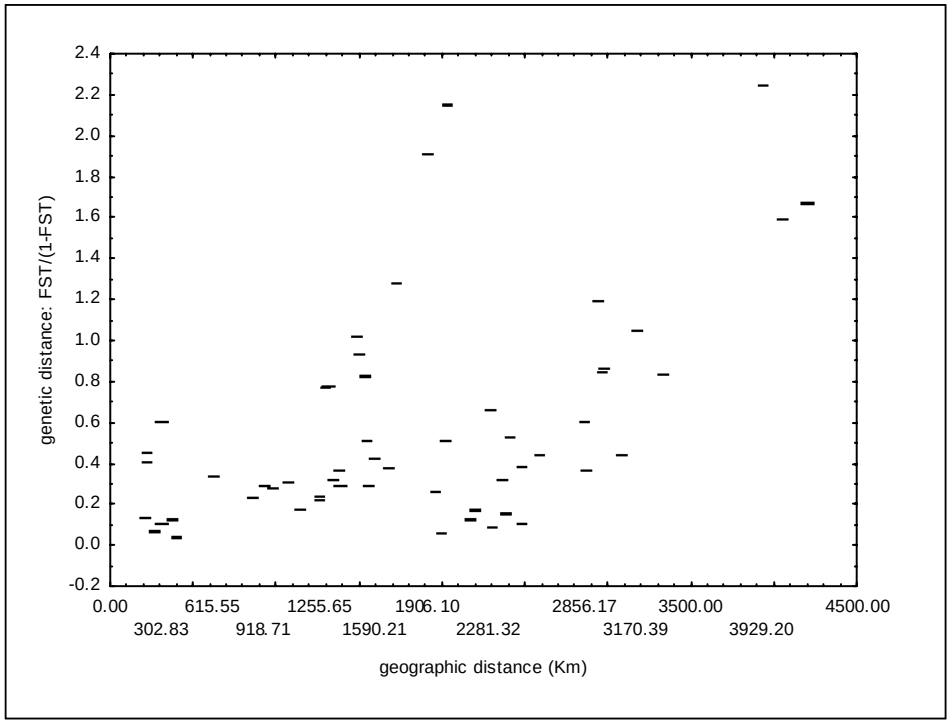

Fig3. Pairwise genetic distances (F_{ST} / (1-F_{ST})) plotted against geographic distance (km) between 11 populations of *P. punctata*.

Discussion

Our data demonstrate that facultative thelytoky in *Platythyrea punctata* is strongly linked to the geographical distribution of the species. We demonstrated that populations from the Caribbean region and Florida are predominantly thelytokous, whereas sexual reproduction is restricted to the mainland. Whereas the population in Texas exhibited sexual reproduction by showing a clear sexual pattern of genotypes within colonies, populations in Belize and Honduras showed mixed patterns, with colonies exhibiting sexual reproduction and other colonies exhibiting thelytoky. However, the low number of colonies collected on the mainland is not sufficient to draw further conclusions, but we can suspect that populations in Honduras and Belize may consist of sexual and thelytokous reproducing colonies. Furthermore, our surveys of the different populations across a large geographic range indicate profound population genetic consequences of the different modes of reproduction, with lower genetic diversity found within the thelytokous populations than the mainland populations. Significant heterozygote excess was also found only in the thelytokous populations, a classic finding for predominantly unisexual reproducing organisms (Balloux et al. 2003; de Meeûs and Balloux 2005). However, genetic variation within thelytokous populations was higher than previously thought (Schilder et al. 1999b; Hartmann et al. 2005) and expected from

unisexual reproducing forms. In contrast to former studies on *P. punctata*, we found several clone lineages on each island and Florida, which might be due to the additional used microsatellite loci and the very high number of colonies obtained.

Although colonies on islands exhibit thelytokous parthenogenesis, the populations do not consist of one clone lineage exclusively (with the exception of New Providence Island, Bahamas). Different clone lineages might arise through mutation events and rare recombination event during the automictic mechanism of parthenogenesis (Kellner and Heinze, *in review*). Isolation by distance pattern revealed also that clone lineages are likely to arise through founding effects and genetic drift, with restricted gene flow between populations, especially between the Caribbean islands and the Central American mainland.

P. punctata is presumably a poor disperser. Winged females have only been found regularly in the Florida population, and because they lack of ocelli and developed wing muscles (Schilder et al. 1999a), they are unlikely to fly and thus represent reliable dispersal forms. *P. punctata* is relatively big in size and not a typical tramp species which can be likely dispersed by human influence (McGlynn 1999; Mikheyev and Mueller 2007). Distribution of the species however might be likely through drifting individuals in seasonal Hurricanes or drift wood between islands. The strong genetic differentiation between the Caribbean region and Central America suggest that the separation of populations and the colonization of islands is not a recent event. Preliminary phylogenetic data reveal that Honduras is likely to be the center of distribution and source population (Seal, Kellner and Heinze *unpublished data*). It has to be tested if populations which show a very low number of different genotypes, with New Providence Island, Bahamas as an extreme with a single clone lineage on the island, are younger in age than thelytokous population exhibiting a large number of different genotypes, like *e.g.* Puerto Rico and Dominican Republic, since the number of different genotypes might have evolved due to mutation events through time.

In general, the distribution of sexual and thelytokous reproducing populations of *P. punctata* fits the distribution patterns predicted by geographical parthenogenesis (Vandel 1928; Bell 1982), since we found that predominantly thelytokous populations are distributed over the Caribbean islands, whereas sexual reproduction occurs on the mainland of Central America and South Texas. Geographic parthenogenesis (GP), which is strongly linked to underlying ecological patterns, may provide insights about the advantages and disadvantages of sexual reproduction (Bell 1982): Four main, non-exclusive classes of hypothesis have been proposed to explain GP. First, ‘Reproductive assurance’ (Baker 1955; Cuellar 1977; Gerritsen 1980) means that unisexual organisms may be better in colonizing new habitats since they do not need to find mating partners; second, sexual organisms in marginal habitats might be

maladapted because of constant gene flow from the distribution center, whereas well adapted parthenogenes are isolated and can maintain well adaptation (Peck et al. 1998); third, sexual reproduction might be advantageous in habitats with many biotic interactions like predators, parasites and competitors favored by co-evolutionary arms race (Hamilton 1980; Hamilton et al. 1990; Lively et al. 1990), whereas parthenogenesis is advantageous in habitats where biotic interactions are rare (Hamilton et al. 1990), and finally, the 'general purpose genotype' hypothesis, stating that parthenogenes might occupy a broader range of habitats because of selection for generalist clones (Parker et al. 1977; Lynch 1984), because of their hybrid origin (Lynch 1984) or because of many differentially well-adapted clonal 'microspecies' (frozen niche model: Vrijenhoek 1987). Recently a fifth hypothesis has been proposed (Haag and Ebert 2004), called the 'metapopulation hypothesis': genetic bottlenecks cause genetic drift in both sexual and asexual organisms, but factors like inbreeding depression are more harmful in sexual than in asexual reproducing populations.

Testing these hypotheses in the field is problematic, but since all of them are non-exclusive, several are likely to explain the pattern we found in *P. punctata*. Climate conditions for the Caribbean region and the Central American mainland are generally subtropical to tropical, except for the 'winter' in Texas and Florida that brings occasional freezing temperatures. Biotically, regional differences are pronounced with lower biodiversity on the islands, including ant species that could be competitors such as army ants and other species of *Platythyrea*. On the Caribbean islands, *P. punctata* is among the most common ants in their preferred habitat (secondary rainforest and tropical Hardwood Hammocks), whereas on the mainland, with a few exceptions, this species is not very abundant (this partly explains the low sample sizes we obtained from the mainland despite intensive searches). Also genetic factors as proposed in the 'metapopulation hypothesis', bottlenecks, founding effects, isolation by distance and restricted gene flow and genetic drift when colonizing islands, might be an explanation for the pattern of geographic parthenogenesis found in this ant species. Further investigations on the phylogenetic relationship between populations are strongly needed here.

However, considering *P. punctata* as a social insect, provides an additional level of selection, the colony level. Evolutionary forces act on the colony level as well as on the individual, however this might be different or even contradictious forces. The high amount of colonies showing intra-colonial variation, possible gained through adoption of foreign workers and fusion of colonies (Kellner, Barth and Heinze, *unpublished data*), might demonstrate that parthenogenetic reproduction might be advantageous in certain habitats for

the individual reproductive, however for the colony as a unit of selection, intra-colonial divergence might be of high advantage in terms of parasite resistance and work force.

Our study is the first evidence for geographic parthenogenesis in social insects. However, further investigations explaining the evolution of thelytoky in this species are needed, and populations with sympatric occurrence of sexual and thelytokous reproducing colonies might become hotspots for further studies.

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III. Chapter 3: Population structure of a parthenogenetic ant: *Platythyrea punctata* (Hymenoptera: Formicidae) on Puerto Rico

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Abstract

Although most female Hymenoptera are capable of producing haploid males from unfertilized eggs by arrhenotokous parthenogenesis, in only a few species they can rear diploid females from unfertilized eggs (thelytokous parthenogenesis). Very little is known on the population structure of such species. Previous, descriptive work indicated that thelytokous parthenogenesis is the dominant mode of reproduction of the ponerine ant *Platythyrea punctata* in several populations from Puerto Rico. Though many colonies consisted of genetically identical workers, some showed genetic heterogeneity. Here, we in more detail quantify the population structure of *P. punctata* and determine the frequency of genetically mixed colonies. We found a surprisingly high degree of genetic variation within colonies, between colonies and between sample sites, which suggests the occasional occurrence of colony fusion, mutations or recombination.

Keywords: clonality, heterozygosity, thelytoky, Ponerinae, Caribbean;

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Introduction

One of the key characteristics of the insect order Hymenoptera (ants, bees, wasps etc.) is arrhenotokous parthenogenesis, i.e., unfertilized eggs develop into haploid males, while fertilized eggs develop into diploid females. In addition, in a few exceptional species unfertilized eggs can develop into diploid females through thelytokous parthenogenesis (Normark 2003). Though thelytoky has been believed to occur in numerous species of social Hymenoptera (Slobodchikoff and Daly 1971), more detailed investigations has proved it only for a small minority of taxa, including the Cape honey bee (*Apis mellifera capensis*, *e.g.*, Baudry et al. 2004) and several ants, including *Pristomyrmex punctatus* (Tsuji 1988b), *Cerapachys biroi* (Tsuji and Yamauchi 1995), *Cataglyphis cursor* (Cagniant 1983; Pearcy et al. 2004), and *Wasmannia auropunctata* (Fournier et al. 2005). In several of these species, thelytoky co-occurs with normal sexual reproduction, resulting in complex and even bizarre genetic colony and population structures (*e.g.*, Pearcy et al. 2004; Fournier et al. 2005; Foucaud et al. 2006; Ohkawara et al. 2006; Pearcy et al. 2006; Foucaud et al. 2007).

Platythyrea punctata F. Smith 1858 is a neotropical species in the subfamily Ponerinae which occurs from Florida and the Florida Keys to Barbados on the West Indies and on the mainland from southern Texas down to Honduras and Costa Rica. Populations from Florida, Barbados, and Puerto Rico have been found to be predominantly thelytokous (Schilder et al. 1999a; Schilder et al. 1999b; Hartmann et al. 2005). In contrast, a single colony from Costa Rica contained three mated workers, and unmated workers appeared to be incapable of producing diploid offspring (Hartmann et al. 2005). Mated workers and mated queens have also been reported from Florida and Puerto Rico, but the evidence for female reproductives utilizing sperm to fertilize their eggs in such populations is ambiguous (Hartmann et al. 2005) and a more detailed quantification of population and colony structures is needed. The aim of this study was to elucidate inter– and intra-population genetic variation of *P. punctata* on Puerto Rico in more detail and to quantify clonal richness and genetic heterogeneity using appropriate population genetic statistics. We show that although parthenogenesis is the predominant mode of reproduction, the populations on Puerto Rico do not exclusively consist of a single clonal lineage but show a fair amount of genetic variation, both between and within colonies.

Material and Methods

Study sites and sample collection

Entire colonies of *Platythyrea punctata* were collected on Puerto Rico in October 2005 in seven sampling sites: El Verde field station in the Luquillo Experimental Forest (N 18° 19.0' W 65° 45.0'), Rio Grande (N 18° 24.5' W 65° 49.6'), Sabana (N 18° 19.5' W 65° 43.3'), Pico (N 18° 6.1' W 67° 2.4'), El Tunel (N 18° 29.1' W 66° 58.1'), WSL (on the way to San Lorenzo, N 18° 16.4' W 65° 54.3'), and SAII (on the way to Sabana, N 18° 19.2 W 65° 43.0'). A map of sampling localities is given in **Figure 1**. Colony sizes ranged from 20.4 ± 15.6 workers (mean worker number ± SD) in El Tunel, where the smallest colony was found (only 4 workers, but contained brood items), to 163.6 ± 127.2 workers in El Verde, where the largest colony was found (475 workers). From El Verde, 9 colonies were used for analyses (180.6 ± 145.1 mean worker number ± SD), Sabana 6 colonies (33.8 ± 21.6), Rio Grande 9 colonies (33.8 ± 14.5), El Tunel 7 colonies (27.9 ± 14.2), Pico 12 colonies (28.0 ± 12.2) and 2 colonies each from Sabana II and WSL (SaII: 52.5 ± 6.4; WSL: 31.0 ± 8.5). From each colony, 12 individuals in total (6 brood items and/or callows - freshly eclosed workers and 6 adults) were stored in 100 % ethanol directly after collection.

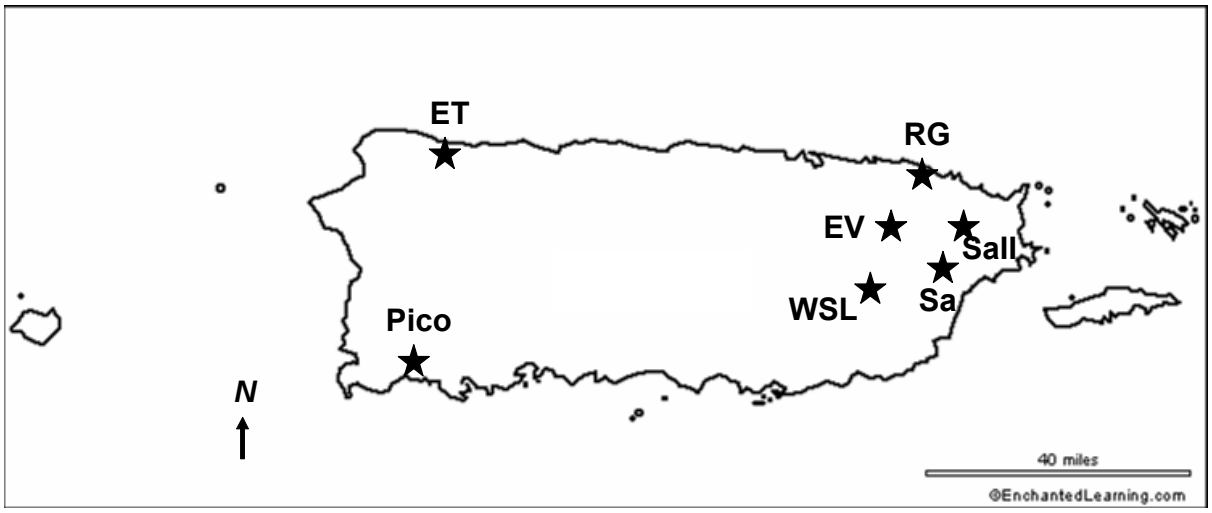

Fig1. Sampling localities of *P. punctata* on Puerto Rico. ET: El Tunel, RG: Rio Grande, EV: El Verde, Sa: Sabana, WSL: Way to San Lorenzo, SaII: Sabana II.

Molecular techniques and genotyping

Whole ants were pulverized in fluid nitrogen and total genomic DNA was extracted following a modified CTAB extraction protocol (Sambrook and Russell 2001). Isolated DNA was washed with 100% ethanol and twice with 70% ethanol, dried and resuspended in double distilled water (50 µl for individual ants and pupae, 40 µl for larvae) and stored at -20°C until

use. The original microsatellite primer set developed for *P. punctata* (Schilder et al. 1999b) was tested following the described PCR protocol on a subset of 48 randomly selected adults from different sample sites and colonies. Amplified fragments were scored on an ABI Prism 310 Genetic Analyzer. After testing each primer for polymorphism, we chose a set of five polymorphic microsatellite loci being suitable for analyses (loci 3506, 3302, 2902, 4101 and 2801), including those previously studied by Schilder et al. (1999b) and Hartmann et al. (2005), loci had between two (3506) and seven alleles (3302).

DNA was amplified in a total reaction volume of 20µl, containing 1µl template DNA. Each reaction contained 2µl of 10x reaction buffer (for 3506, 2902, 4101 and 2801: Fermentas 10xTaq Buffer + KCl – $MgCl_2$; for 3302: Fermentas 10xTaq Buffer + $(NH_4)_2SO_4$ – $MgCl_2$), 2 µl (5pmol/µl) of each primer (forward primer labeled with different types of fluorescent dye, Applied Biosystems), 4µl dNTPs (1mM of each), 0.5µl Taq polymerase (1U/µl Fermentas), 1.2µl 25mM $MgCl_2$ and 7.3µl PCR-H_2O (Sigma). After an initial 5min denaturation step at 94°C, the reaction mix was incubated at the following temperature cycles: 30 cycles of 1min denaturation at 94°C, 1 min primer annealing at 50-54°C (locus 3506: 50°C; 2801: 53°C; 3302, 4101 and 2902: 54°C), and 1min extension at 72°C. The reaction was terminated by a final 5min extension step before cooling to 4°C. The run time for each of the steps, denaturation, annealing and extension was extended from 1min to 1.5 min for locus 2801 and 3302. The amplified microsatellite fragments were scored on an ABI Prism 310 Genetic Analyzer. Allele lengths were determined using GeneScan© software.

Genetic data analyses

For each colony, the patterns of genotypes were investigated and the occurrence of intra-colonial variation was recorded. Individual genotypes were recorded as different from each other then they differed in at least one allele at one locus. Aberrant genotypes were categorized into three types: i) 'A' the aberrant genotype was caused by two foreign alleles at a locus, ii) 'B' the aberrant genotype was homozygous instead of heterozygous at a locus and iii) 'C' the aberrant genotype was caused by one foreign allele at a locus.

Due to the family structure of social insect colonies, individuals within colonies are related to each other and cannot be considered independent. To avoid pseudo-replication, we included only a single individual per colony or, when colonies contained several clones, a single individual per clone lineage in the analyses (n = 71).

Linkage disequilibrium between each pair of loci and deviations from Hardy-Weinberg equilibrium (HWE) at each locus were examined by exact tests using the program GENEPOP

4.0. (Raymond and Rousset 1995) Test for genotypic differentiation were obtained using GENEPOP 4.0 with exact G test. Allelic richness for each sampling locality was calculated using FSTAT (Goudet 1995; Goudet 2001).

Colony and population genetic structure were assessed by performing a hierarchical F statistic analysis, allowing the estimate of the amount of genetic variation found at each hierarchical level using the method of Weir and Cockerham (Weir and Cockerham 1984; Weir 1996) as implemented in the program GDA 1.1 (Lewis and Zaykin 2001). Colonies were treated as subpopulations (since individual workers are dependent samples) in a three level analyses for each sample site separately. In this case, hierarchical variation is partitioned among the individual (I), the colony (C) and the total population (T) (the sample site the colonies were collected). Using this notation, F_{IT} (individual within the sample site, equivalent to the standard inbreeding coefficient), F_{CT} (genetic differentiation among colonies within the sample site, equivalent to F_{ST}) and F_{IC} (differentiation among individuals within colonies, equivalent to F_{IS}) values were obtained with GDA 1.1. The 95% confidence intervals were constructed by bootstrapping over loci with 1000 replicates.

F-statistics were also performed for all colonies and all sample sites together in a three level analyses of molecular variance (AMOVA) considering colonies as subpopulations within the sample sites (populations) within the island of Puerto Rico (total). Here differences among individuals within the total population are expressed by F_{IT}^{total}, colonies within the total population are expressed by F_{CT}^{total} (equivalent to θ_S) and differences among sample sites within Puerto Rico are expressed by F_{ST}^{total} (equivalent to θ_P). 95% CIs were again obtained by bootstrapping over loci with 1000 replicates. To correct for sample size and not overestimating genotype frequencies, from each colony one representative individual was chosen (from colonies containing variation, one individual for each genotype) (n = 71; n = 66 after exclusion of individuals with missing loci). The number of distinct multilocus genotypes (MLGs) were calculated by hand and using the program GenClone 2.0 (Arnoud-Haond and Belkhir 2007). GenClone 2.0 was also used for assessing genotypic vs. clonal membership, the clonal diversity/richness index *R*, the clonal heterogeneity expressed by the Simpson's complement index D', the Shannon-Wiener's index H'' and an index for clonal evenness corresponding to the Shannon-Wiener's index as reviewed in (Arnoud-Haond et al. 2007).

Results

Composition of colonies

In total 47 colonies were analyzed, of which 28 (60 %) did not show any genetic variation, i.e., all analyzed nestmates had identical genotypes at all loci. The remaining 19 colonies

(40%) contained variation among the analyzed individuals. In 11 colonies (23%), this variation was due to 1 to 6 adult individuals, which did not show the same genotype as the analyzed callows, but were identical to each other. In two colonies (4%), variation was due to a single callow each, which was not identical to the rest of the analyzed individuals. In six colonies (13%), variation was caused by both callows and adult ants.

Intra-colony variation was mostly restricted to only one of five analyzed loci. Four individuals were found to differ from their nestmates in two loci, 5 workers were found to differ from their nestmates in three loci, and only one individual was found to differ in four loci from the rest of the colony. Divergent individuals had genotypes differing from those of their nestmates in both alleles (**Table 1**, type A, eight workers from three colonies), being homozygous instead of heterozygous (type B, 24 workers from eight colonies), or differing in only one allele (type C, 20 workers from 10 colonies). The cases where variation was found in more than one locus were four workers from two colonies showing variation at two loci with types AC and BC. Five ants from two colonies showed variation at three loci (types BCA, BCB, ACC, CAA and CCC) and a single ant was found to differ in four loci, showing types B, C, C, C (**Table1**).

Population structure

For the seven sample sites as well as for the overall population, the observed heterozygosities were around twice as high as the expected heterozygosities (see **Table 2**). Consequently, the departure from Hardy-Weinberg equilibrium was highly significant for all loci and all populations (exact G test χ^2: 194.3881, df: 60.0000, $p< 0.0001$). Two tests for linkage disequilibrium were significant (3506 & 2801: $p < 0.05$; 4101 & 2801 $p< 0.05$; all other pairs $p>0.05$), suggesting dependency among these loci. Such non-random associations between loci are common due to clonal mechanisms when no recombination breaks up loci (Halkett et al. 2005).

Table 1. Variation in colonies of the thelytokous ant *Platythyrea punctata* from Puerto Rico:
Type A: variation is due to two foreign alleles at one locus; Type B: the variation is due to homozygosity instead of heterozygosity; Type C: Variation is due to one foreign allele. Aberrant genotypes are printed in bold.

colony	number of individuals	3506	2902	4101	2801	3302	Type of individual	category
Rio Grande 6	11	193/203	183/185	211/211	368/384	238/242		
	1					**236/240**	callow	A
Rio Grande 13	6	203/203	183/185	211/211	384/386	238/246		
	6				**386/386**		adults	B
Rio Grande 15	11	203/203	183/185	211/211	384/384	238/244		
	1				**368/384**		callow	C
El Verde 2	11	203/203	183/185	199/211	368/386	238/242		
	1	**193/203**					adult	C
El Verde 6	11	203/203	183/185	199/211	368/386	238/244		
	1			**211/211**			adult	B
El Verde 17	10	203/203	183/185	199/211	368/386	238/244		
	2			**203/211**			adults	C
Pico 12	6	193/203	183/185	203/211	368/386	238/244		
	6			**211/211**			callows	B
Pico 15	9	193/203	183/185	199/211	382/388	238/242		
	1	**203/203**	183/185	199/211	**382/386**	**240/244**	adult	B,C,A
	1	**203/203**	183/185	**211/211**	**386/386**	238/242	adult	B,C,B
	1	193/203	183/185	199/211	**382/386**	238/242	adult	C
Pico 18	5	203/203	183/185	203/203	382/382	236/242		
	1	**193/203**	183/185	**211/211**	382/382	236/242	callow	C,A
	6	203/203	183/185	203/203	382/382	**240/242**	adults	A
WSL 1	11	203/203	185/185	201/211	384/384	238/244		
	1	**193/203**	185/185	201/211	384/384	238/244	adult	C
SaII 1	11	193/203	183/185	203/211	386/386	238/238		
	1	193/203	183/185	203/211	**368/386**	238/238	adult	C
SaII 2	5	193/203	183/185	203/211	386/388	238/238		
	1	193/203		203/211	**386/386**	238/238	callow	B
	6	193/203	183/185	203/211	**386/386**	238/238	adults	B
Sabana 1	10	193/203	183/185	199/203	368/368	238/244		
	1	193/203	183/185	**199/199**	368/368	238/244	callow	B
	1	193/203		**199/211**		238/244	adult	C
Sabana 4	3	193/203	183/185	199/211	382/386	**238/246**	callows	C
	3	193/203	183/185	**211/211**	382/386	**238/246**	callows	B,C
	6	193/203	183/185	199/211	382/386	238/244	adults	
Sabana 5	5	193/203	183/185	199/211	382/386	238/242	callows	
	1	193/203	183/185	199/211	368/386	**234/238**	callow	A
	1	**203/203**	183/185	**199/203**	368/386	**234/238**	adult	A,C,C
	2	193/203	183/185	199/211	382/386	238/242	adults	
	1	193/203	183/185	**199/203**	**368/368**	**234/238**	adult	C,A,A

	1	**203/203**	183/185	**203/211**	**368/382**	**234/238**	adult	B,C,C,C
	1	193/203	183/185	**203/211**	**368/386**	**234/238**	adult	C,C,C
Sabana 7	11	193/203	183/185	199/211	382/386	238/244		
	1	193/203	183/185	**203/211**	382/386	238/244	adult	C
El Tunel 3	11	193/203	183/185	199/211	382/386	238/244		
	1	193/203	183/185	199/211	**382/382**	238/244	callow	B
El Tunel 6	3	193/203	183/185	211/211	382/386	238/244		
	1	193/203	183/185	211/211	**386/386**	238/244	callow	B
	8	193/203	183/185	**199/211**	382/386	238/244	7 adults / 1 callow	C
El Tunel 10	11	193/203	183/185	199/211	382/386	238/244		
	1	193/203	183/185	199/211	**382/382**	238/244	adult	B

Test for genotypic differentiation revealed that the sample sites were significantly different from each other in their compositions of genotypes (exact G test χ^2 reaching infinity, df =10, $p<0.0001$ at all loci).

Table 2. Sample sizes (number of colonies), expected and observed heterozygosities for the different sample sites of the ant *Platythyrea punctata* on Puerto Rico. From each colony 12 individuals were analyzed (6 callow and 6 adult workers). Results were obtained with GDA 1.1 and FSTAT.

Sample site	n	$H_{expected}$	$H_{observed}$	Allelic richness
Rio Grande	9	0.337	0.635	1.651
El Verde	9	0.410	0.778	1.792
El Tunel	7	0.505	0.952	1.998
Pico	12	0.444	0.821	1.860
Sabana	6	0.510	0.908	2.086
WSL	2	0.376	0.709	1.699
SaII	2	0.354	0.641	1.599
overall	47	0.428	0.795	-

F_{IT} values were significantly different from 0 and ranged from -0.881 (Sabana) to -0.982 (El Verde; see table 3), i.e., they approached -1, as expected for strictly clonal populations (Balloux et al. 2003). In the absence of recombination, ancestral heterozygosity will be maintained and F_{IC} values are expected to approach -1 (Balloux et al. 2003). F_{IC} values ranged from -0.360 (Pico) to -0.887 (SaII) and were significantly different from 0 except in WSL (only two colonies studied; -0.093). Finally, F_{CT} values, representing the amount of differentiation between colonies within a sample site, were ranging from 0.005 to 0.447 and were significantly higher than 0 (see **Table3**), except for the two colonies from SaII (95% CI. 0.022 to 0.000). Analyses of variation calculated for the total population of the island of Puerto Rico revealed considerable differentiation among colonies within the island with a

F_{CT}^{total} value of 0.309 (95% CI 0.405 to 0.175), moderate difference among sample sites within Puerto Rico with a F_{ST}^{total} value of 0.136 (95% CI 0.215 to 0.059) and a F_{IT}^{total} value of -0.344 (95% CI -0.1333 to – 0.631).

Table 3. Three-level hierarchical analyses showing Fixation indices for each sample site of the ant *Platythyrea punctata*: differentiation among individuals within the sample sites (F_{IT}), differentiation among individuals within colonies (F_{IC}) and variation among colonies within the sample sites (F_{CT}). The significance of F statistics estimates were obtained by bootstrapping over loci (1000 replicates), 95% confidence intervals are given in parenthesis. After de Meeûs and Balloux (2005), under strictly clonal reproduction, F_{IS} (F_{IC}) values are expected to get negative, whereas F_{ST} (F_{CT}) values get positive and F_{IT} values are 0.

Sample site	F_{IT}	F_{IC}	# of colonies containing variation	F_{CT}	# of genotypes
Rio Grande	-0.963 (-0.910 to -1.000)	-0.444 (-0.083 to -0.865)	3	0.264 (0.436 to 0.063)	8
El Verde	-0.982 (-0.947 to -1.000)	-0.785 (-0.468 to -0.995)	3	0.099 (0.259 to 0.000017)	6
El Tunel	-0.969 (-0.935 to -1.000)	-0.852 (-0.734 to -0.976)	3	0.060 (0.112 to 0.004)	4
Pico	-0.932 (-0.886 to -0.975)	-0.360 (-0.147 to -0.687)	3	0.296 (0.392 to 0.146)	12
Sabana	-0.881 (-0.793 to -0.958)	-0.587 (-0.340 to -0.886)	4	0.156 (0.264 to 0.029)	12
WSL	-0.976 (-0.909 to -1.000)	-0.093 (0.304 to -0.638)	1	0.447 (0.652 to 0.181)	3
SaII	-0.896 (-0.511 to -1.000)	-0.887 (-0.479 to -1.000)	2	0.005 (0.022 to 0.000)	4

Clone lineage composition

A visual inspection of data suggested the occurrence of 46 different multilocus genotypes (MLGs) on Puerto Rico, while GenClone gave 41 distinct MLGs (n = 66; 4 individuals had to be excluded because of missing data at one locus). Only eight MLGs were present in several colonies (*e.g.* representatives of the colonies RG5, RG7, RG14, RG16 and P12 have the same multilocus genotype), while the majority, 33 MLGs, were found only once. The probability that the eight repeatedly found MLGs were identical by chance, *e.g.*, due to recombination or sexual events, was low (P*gen*(fis) < 0.01 under departure from Hardy-Weinberg equilibrium and P*sex* < 0.05). This suggests that the replicates of the different MLGs are indeed members of the same clone lineages and did not emerge from recombination and/or distinct cryptic sexual events. The occurrence of multiple clones was also reflected in a clonal richness *R* of 0.615, which is more or less halfway between monoclonality (0) and complete heterogeneity of the samples (1). Clonal heterogeneity expressed by Simpson's complement index D' was 0.968. MLGs were rather evenly distributed in the populations (Shannon-Wiener's index H''

= 3.425, maximal possible value H_{max} = 3.715). This means that there is not one major clone lineage on the island with several colonies belonging to it, but that colonies represent several clone lineages with a nearly equal abundance on the island.

Discussion

Our data demonstrate that the ant *Platythyrea punctata* on Puerto Rico reproduces predominantly by thelytokous parthenogenesis, i.e., workers produce genetically identical worker offspring from unfertilized eggs and colonies therefore consist mostly of clone mates. Using quantitative population genetic analyses we show that the organization into colonies and clone lineages has an important influence on the overall population structure. Moreover, we show that despite of predominant clonal reproduction, genetic variation on the population level is not necessarily low.

As expected from presumed clonality (Balloux et al. 2003), observed heterozygosities were approximately twice as high as the expected ones. F_{IS} values (here F_{IC}: individuals within colonies) were negative, which has been considered as 'the ultimate signature of clonal diploid populations' (Halkett et al. 2005).

Nevertheless, our data also reveal a surprisingly high amount of variation both between and within colonies, which is not expected for a clonal organism. Clonal diversity, Simpson's complement index, and Shannon-Wiener's index consistently indicate that the number of clonal lineages is high in relation to the number of analyzed individuals and colonies. Indeed, most colonies represent unique clonal lineages. Cases in which several, neighboring colonies shared a multilocus genotype (*e.g.*, in El Verde and Rio Grande) might result from colony founding by fragmentation of large existing colonies. Several multilocus genotypes were found in colonies from different localities (*e.g.*, Sabana and El Tunel, Pico and Rio Grande), which might reflect migration and suggests that sampling sites at least in disturbed habitats are not completely isolated from each other. In contrast, the population in the relatively undisturbed secondary rainforest at El Verde showed exclusive multilocus genotypes not found anywhere else.

Nineteen of 47 studied colonies had workers with different multilocus genotypes. The error rate of Taq DNA polymerase is 2.2×10^{-5} errors/nucleotide/cycle and the accuracy of PCR is thus 4.5×10^{4}(average number of correct nucleotides incorporated before an error occurs). Since analyses for aberrant individuals (PCR and fragment analyses) were repeated, the variation within colonies is highly unlikely to result from mistakes during PCR. Similarly, genetic heterogeneity does not result from sexual recombination, since genetically different workers differed in most cases from their nestmates in only one or two of the five analyzed

loci and in some cases, their alleles were not shared by other nestmates. Furthermore, in contrast to an earlier study, in which one of 18 studied colonies from Puerto Rico contained a single mated worker (Hartmann et al. 2005), our study did not reveal mated individuals. Nevertheless, even though rare such sexual events will greatly contribute to between-colony variation.

Genetically heterogeneous colonies in our sample likely originate from reproductive turnover or parasitism by unrelated individuals, as in *Pristomyrmex punctatus* (Dobata et al. 2009) and colony fusion (K. Kellner, B. Barth, J. Heinze, *unpublished data*). In addition, mutation or recombination during automictic thelytokous parthenogenesis may contribute to intra-colonial variation and presumably also explains much of the variation between colonies.

A detailed inspection of within-colony variation allows excluding some of the above-mentioned mechanisms for some colonies, but not for others. For example, variation of type A, in which a worker has a genotype with two alleles that are not present in the genotypes of its nestmates, cannot result from recombination but is likely to reflect the adoption of individuals from other colonies. Variation of type B, in which one worker is homozygous while its nestmates are heterozygous, suggests recombination, whereas genotypes of workers of type C, which have one allele not present in other nestmates, may result from adoption of alien workers or mutation. A former study found similar results, with 6 out of 18 colonies showing variation (Hartmann et al. 2005). While one of these six colonies contained a mated worker and showed a variation pattern typical for sexual reproduction, the other five colonies showed a similar variation pattern as the here analyzed colonies, with one to three workers per colony showing aberrant genotypes due to single foreign alleles (type C), homozygosity (type B) and also non common alleles (type A). As the history of field colonies is not known, the different causes of variation cannot be separated by genetic analyses. Genotyping of mothers and daughters in laboratory colonies did not reveal recombination or mutation (Kellner and Heinze, *under review*), suggesting that most variation in field colonies results from adoption.

In part, genetic heterogeneity among colonies might be also explained by ongoing founding events. Since workers of *P. punctata* are totipotent, every single individual can serve as a foundress of new populations. Though *P. punctata* appears to be a poor disperser because of the lack of flying queens (Schilder et al. 1999a), they are widespread in the Caribbean and even occur on the rather isolated island of Barbados, whose ant fauna is relatively depleted. Human activities or driftwood might have repeatedly introduced new clonal lineages to Puerto Rico.

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IV. Chapter 4: Mechanism of facultative parthenogenesis in the ant *Platythyrea punctata*

K. Kellner and J. Heinze*

Abstract

Thelytokous parthenogenesis, the production of diploid female offspring from unfertilized eggs, can be caused by several cytological mechanisms, which have a different impact on the genetic variation within the offspring. The ponerine ant *Platythyrea punctata* is widely distributed throughout the Caribbean Islands and Central America and exhibits facultative parthenogenesis. Workers in many colonies from the Caribbean Islands have identical multilocus genotypes and are thus probably clonal, but the occurrence of males makes an ameiotic mechanism unlikely. To clarify the details of thelytoky in this species we compared the multilocus genotypes of mothers and their offspring and analyzed the genotypes of haploid and diploid males. According to these data automixis with central fusion is the most likely mechanism of thelytoky, as in the Cape honeybee and the ant *Cataglyphis cursor*.

Keywords: apomixis, automixis, diploid males, heterozygosity, sex determination, unisexuality;

** under review in Heredity*

Introduction

A few hundred species scattered throughout the animal kingdom are characterized by thelytokous parthenogenesis, through which mothers can produce diploid, female offspring from unfertilized eggs. Thelytoky appears to be particularly common in the Hymenoptera, i.e., bees, ants, wasps, etc. (Slobodchikoff and Daly 1971; Lamb and Willey 1987; Suomalainen et al. 1987; van Wilgenburg et al. 2006), probably because the typical sex determining mechanism of Hymenoptera, in which haploid males develop from unfertilized eggs and diploid females from fertilized eggs (haplodiploidy: Cook 1993; Cook and Crozier 1995), is rather easily transformed into thelytoky by parasitic bacteria (Bourtzis and O'Neill 1998) or mutations (Lattorff et al. 2005; Lattorff et al. 2007).

Among the social Hymenoptera, thelytoky regularly occurs in the Cape honeybee (*Apis mellifera capensis* Baudry et al. 2004), and several phylogenetically unrelated ant species, including *Pristomyrmex punctatus* (Tsuji 1988b), *Cerapachys biroi* (Tsuji and Yamauchi 1995), *Cataglyphis cursor* (Cagniant 1983; Pearcy et al. 2004), *Wasmannia auropunctata* (Fournier et al. 2005), and *Platythyrea punctata* (Heinze and Hölldobler 1995; Schilder et al. 1999a; Schilder et al. 1999b; Hartmann et al. 2005). Treatment with antibiotics (Schilder 1999) and DNA amplification with *Wolbachia*-specific primers suggest that thelytoky in these species is not induced by bacteria (Wenseleers and Billen 2000), but the cytological details of the mechanisms underlying parthenogenesis are as yet understood only in a few species.

Thelytoky can be caused by several cytological mechanisms, which have different consequences for the genetic structure of colonies and populations (Suomalainen et al. 1987). It is therefore possible to deduce the mechanism underlying thelytoky by analyzing how commonly heterozygous mothers produce homozygous offspring (Pearcy et al. 2006). In apomictic (or ameiotic) parthenogenesis, there is no meiosis and therefore no recombinations, i.e., all offspring are identical clones of their mothers and heterozygosity is maintained. In automictic parthenogenesis, meiosis takes place and diploidy is restored by the fusion of the second division sister nuclei (terminal fusion) or non-sister nuclei (central fusion). Automictic parthenogenesis therefore may involve an increase in homozygosity: terminal fusion results in immediate homozygosity at all loci, except when recombination occurs, while heterozygosity is maintained in central fusion, except when recombination occurs. In automictic parthenogenesis with random fusion of nuclei, all four chromatides segregate independently, and each heterozygous locus can become homozygous, independent on the position of the locus on the chromosome. The most extreme form, gamete duplication, in which the meiotically produced haploid egg undergoes an extra round of DNA replication without cell division, results in complete homozygosity.

Thelytoky in the Cape Honeybee and the ant *Cataglyphis cursor* follows automictic parthenogenesis with central fusion (Verma and Ruttner 1983; Baudry et al. 2004; Oldroyd et al. 2008; Pearcy et al. 2006; Pearcy et al. 2009), but apomixis has been suggested for the little fire ant, *Wasmannia auropunctata* (Fournier et al. 2005) and the fungus growing ant *Mycocepurus smithii* (Fernández-Marín et al. 2005; Himler 2007). Little is known about the mechanisms in the other ant species.

The ponerine ant *Platythyrea punctata* is widely distributed throughout the Caribbean islands, Florida, and Mesoamerica (Schilder 1999; Schilder et al. 1999a; Hartmann et al. 2005). Colonies from Costa Rica contained mated workers and unmated workers were not capable of producing diploid offspring (Schilder et al. 1999b; Hartmann et al. 2005). In contrast, colonies from Florida and the Caribbean islands almost exclusively produced offspring through thelytoky. Although males do occasionally occur (Wheeler 1905; Schilder et al. 1999a; Hartmann et al. 2005), sexual reproduction appears to be rare in the latter populations and only one reproductive worker and a few non-reproductive workers and queens from Puerto Rico and Florida were found to be inseminated (Hartmann et al. 2005). Most colonies consist of workers with identical multilocus genotypes, which were interpreted as evidence of apomictic parthenogenesis (Schilder et al. 1999b). Some field colonies show significant intra-colonial variation, but since colonies may have adopted alien workers, no definite conclusions on the thelytoky mechanism could be drawn.

To elucidate the mechanism of thelytoky in *P. punctata* in more detail, we compared mother-offspring multilocus genotypes in laboratory colonies and combined this approach with the analysis of diploid males. In the standard system of sex determination in social Hymenoptera, (single-locus complementary sex determination, sl-CSD (Whiting 1939; Cook 1993)), zygotes homozygous at the sex locus develop into diploid males. Under apomictic parthenogenesis diploid males are not expected to occur because the heterozygous state of the sex locus remains fixed. Diploid males are therefore only possible under automictic parthenogenesis (Suomalainen et al. 1987; van Wilgenburg et al. 2006; Oldroyd et al. 2008). Searching for diploids among the morphologically conspicuous males is probably more sensitive to detect rare recombination events than screening workers for homozygosity at microsatellite loci.

Material and Methods

Collecting, housing and set up of experimental colonies

Colonies of *Platythyrea punctata*, which were collected in Puerto Rico in 2005, were genotyped and screened for heterozygosity at five polymorphic microsatellite markers (3506,

2902, 4101, 2801, and 3302; (Schilder et al. 1999b). From each of 22 colonies, which were found to contain no variation and were heterozygous at least at two loci, three callows (freshly eclosed workers, emerged in the lab) and 10-15 foragers were transferred into new nests. The callows were individually marked with dots of Edding marker paint.

Nests consisted of plastic boxes (20cm x 10cm x 6cm) with plaster floors. A preformed cavity in the plaster (7cm x 5cm x 0.5cm) covered with a glass plate and red foil, served as a nest chamber. Experimental colonies were kept under near natural conditions (27°C, 60% humidity, 12 L:12 D cycle) and fed *ad libitum* a mixed diet of honey and pieces of crickets, cockroaches and *Drosophila* flies. The plaster was regularly moistened and a tube with water and cotton served as a permanent water supply.

From each colony, freshly eclosed new callows and pupae were collected and stored at -20°C. Eggs and larvae were not used to insure that the collected offspring consisted only of females and no haploid males. After six to nine months, six of the 22 experimental colonies had produced 23 callows each, i.e. a number sufficient for the comparison of genotypes. These six experimental colonies was observed to determine the egg layer, which is generally the individual most often observed resting on the egg pile (Hartmann et al. 2003). At the end of the experiment, the reproductive individual was also genotyped.

Male production in the field and laboratory

During field work, we collected males in the Dominican Republic (4 males from 4 colonies, number of colonies collected: n=51, November 2005), Barbados (2 males from 2 colonies, n=22, June 2007), and Florida (6 males from 2 colonies, n=12, July 2007). No males were found in Puerto Rico (n=73, October 2005), Grenada (n=14, June 2007), New Providence Island, Bahamas (n=6, July 2007), Grand Bahama Island, Bahamas (n=2, November 2008) and Dominica (n=7, October 2008). In the laboratory, additional males were produced in colonies from Puerto Rico (10 males from 7 colonies during November 2005 to January 2009), the Dominican Republic (6 males from 6 colonies during December 2006 to January 2009), Barbados (2 males from 2 colonies during July 2007 to January 2009), Florida (16 males from 9 colonies during August 2007 to January 2009), Honduras (6 males from 3 colonies during May 2008 to January 2009), Belize (7 males from 3 colonies during December 2007 to January 2009), and Texas (3 males from 3 colonies during February 2008 to January 2009). Colonies from the Bahamas, Grenada and Dominica were brought to the lab as alcohol material and could therefore not be screened for male production. In total, 37 males were obtained for genotyping.

Genotyping of experimental set ups and males

From each of the six experimental colonies, the egg layer and 23 offspring, and in total 37 males were genotyped. DNA was extracted from complete individuals following modified CTAB method (Sambrook and Russell 2001). Isolated DNA was washed with 100% ethanol and twice with 70% ethanol, dried and resuspended in 50 µl double distilled water and stored at -20°C until use. Microsatellite fragments were amplified for the loci the mother colony had been found heterozygous (=informative loci) and in the males, microsatellite fragments were amplified for all loci. PCR conditions were modified after Hartmann et al. (2005). DNA was amplified in a total reaction volume of 20µl, containing 1µl template DNA. Each reaction contained 2 µl of 10x reaction buffer (for 3506, 2902, 4101 and 2801: Fermentas 10xTaq Buffer + KCl – $MgCl_2$; for 3302: Fermentas 10xTaq Buffer + $(NH_4)_2SO_4$ – $MgCl_2$), 2µl (5pmol/µl) of each primer (forward primer labeled with different types of fluorescent dye, Applied Biosystems), 4µl dNTPs (1mM of each), 0.5µl Taq polymerase (1U/µl Fermentas), 1.2µl 25mM $MgCl_2$ and 7.3µl PCR-H_2O (Sigma). After an initial 5min denaturation step at 94°C, the reaction mix was incubated at the following temperature cycles: 30 cycles of 1min denaturation at 94°C, 1 min primer annealing at 50-54°C (locus 3506: 50°C; 2801: 53°C; 3302, 4101 and 2902: 54°C), and 1min extension at 72°C. The reaction was terminated by a final 5min extension step before cooling to 4°C. The run time for each of the steps, denaturation, annealing and extension was extended from 1min to 1.5 min for locus 2801 and 3302. The amplified microsatellite fragments were scored on an ABI Prism 310 Genetic Analyzer. Allele lengths were determined using GeneScan© software.

Determining the mode of thelytoky

For determining the mechanism of thelytoky in *P. punctata*, we adopted an approach similar to that of Pearcy et al. (2006). If thelytoky were apomictic, all offspring would be identical clones of a heterozygous mother and the proportion of homozygous offspring would be 0. If thelytoky followed an automictic mechanism, we would expect the proportion of homozygous offspring to be: a) for central fusion: 0 without recombination (all offspring being heterozygous) and ranging from 0-1/3 in case of recombination; b) for terminal fusion without recombination 1 (all offspring being homozygous) and ranging from 1/3 to 1 in case of recombination. Possibility c) random fusion, would lead to a probability of 1/3 for each locus, independent of the location of the locus. Under gamete duplication, the proportion of homozygote offspring would be 1 in any case, but this mechanism is incompatible with sl-

CSD, as all offspring would be homozygous at the sex determining locus and therefore diploid males.

Determination of haploid and diploid males

Individuals were sexed based on their morphology prior to DNA extraction. Diploid males were identified by heterozygosity at a least at one locus (3506, 2902, 4101, 2802, and 3302). For males which were found to be diploid, PCR and sequencing were repeated to exclude scoring errors. The error rate of the used Taq DNA polymerase is 2.2×10^{-5} errors/nucleotide/cycle and the accuracy of PCR is thus 4.5×10^{4} (average number of correct nucleotides incorporated before an error occurs). Male genotypes were compared to the available genotypes of the colony of origin.

Results

Mode of parthenogenesis

In each of the six experimental colonies, all 23 analyzed offspring were identical to each other and to their mother at all scored microsatellite loci (**Table 1**). No changes from heterozygosity to homozygosity were observed at the informative loci. The proportion of homozygote offspring is therefore 0. Males were never produced in the experimental colonies.

Table 1. Genotypes of reproductives of the thelytokous ant *Platythyrea punctata* and their offspring at the informative, heterozygous loci. Number of offspring is given in parenthesis; all analyzed offspring show identical genotypes to the mother.

colony	analyzed individuals	informative loci			
		3302	**4101**	**2801**	
Tm1	**reproductive**	240/244	201/203	382/386	
	offspring (n=23)	240/244	201/203	382/386	
		3302	**2801**	**4101**	
Tm2	**reproductive**	240/244	368/386	203/211	
	offspring (n=23)	240/244	368/386	203/211	
		3302	**4101**		
Tm3	**reproductive**	238/244	201/211		
	offspring (n=23)	238/244	201/211		
		3302	**4101**	**2801**	
Tm4	**reproductive**	240/242	201/203	382/386	
	offspring (n=23)	240/242	201/203	382/386	
		3302	**3506**	**2801**	
Tm5	**reproductive**	238/244	193/203	382/386	
	offspring (n=23)	238/244	193/203	382/386	
		3302	**4101**	**3506**	**2801**
Tm6	**reproductive**	238/244	199/211	193/203	382/386
	offspring (n=23)	238/244	199/211	193/203	382/386

Male production

Four of the 37 genotyped males were found to be heterozygous at one of five loci (locus 2801) and therefore considered to be diploid. Each of the heterozygous males carried the same heterozygous allele combination as their worker nestmates. The remaining four loci were not heterozygous, which can be explained by homozygosity of the mother colony at these loci. Three of the four males stemmed from thelytokous colonies from Florida and Puerto Rico, and one male came from a sexually reproducing colony from Honduras. All other analyzed males appeared to carry only one allele, even though female individuals in their colonies were heterozygous. We therefore consider these males to be haploid, hemizygous males. An overview over males and colony genotypes for thelytokous colonies is given in **Table 2**.

Table 2. Male and colony genotypes in thelytokous colonies of the ant *Platythyrea punctata*.
Most colonies consisted only of workers with a single multilocus genotype, except colonies JP1, SI, and Mi1. For these, both multilocus genotypes are shown. Males appear to carry only one allele, even when the mother colony is heterozygous. Heterozygous diploid males are indicated in bold.

			loci				
population	**colony**	**Genotypes**	**3506**	**2902**	**4101**	**2801**	**3302**
Florida	JP5	**male**	205	185	201	386	238
		colony	205/205	185/185	201/201	368/386	238/238
	JP4	**m**	205	185	201	386	238
		m	205	185	201	386	238
		m	205	185	201	**368/386**	238
		colony	205/205	185/185	201/201	368/386	238/238
	JP1	**m**	205	185	201	**368/386**	238
		m	205	185	201	368	238
		colony	205/205	185/185	201/201	368/386	238/238
			205/205	185/185	201/201	386/386	238/238
	JP2	**m**	205	185	201	**368/386**	238
		colony	205/205	185/185	201/201	368/386	238/238
	SI	**m**	205	185	201	368	238
		m	205	185	203	386	238
		colony	205/205	185/185	201/203	386/388	238/238
			205/205	185/185	201/203	368/386	238/238
Dominican Republic	Mi1	**m**	193	183	203	386	238
		colony	193/201	183/185	201/203	386/386	238/244
			193/201	183/185	201/203	384/384	238/244
Puerto Rico	WSL1	**m**	203	185	211	384	238
		m	203	185	211	384	238
		colony	203/203	185/185	201/211	384/384	238/244
	EV2	**m**	203	185	211	386	242
		colony	203/203	183/185	199/211	368/386	238/242
	Sa1	**m**	193	183	203	368	238
		colony	193/203	183/185	199/203	368/368	238/244
	Sa5	**m**	203	183	203	368	234
		colony	193/203	183/185	199/203	368/386	234/238
	Sa9	**m**	203	181	211	382	244
		m	203	181	211	382	244
		colony	193/203	181/183	211/211	382/386	238/244
Barbados	HaC4	**m**	203	185	211	386	242
		m	203	185	211	386	242
		colony	203/203	185/185	201/211	386/388	242/244

Discussion

Our results show that in the thelytokous ant *P. punctata*, offspring and their mothers exhibit identical genotypes and that recombination during parthenogenesis is rare. This is in

accordance with the frequent genetic uniformity of field colonies (Schilder et al. 1999b; Hartmann et al. 2005) and quantitative population genetic analyses (Kellner, Barth, Heinze *unpublished data*), which reveal a population structure with a heterozygote excess, as is typical for clonal diploid organisms (Balloux et al. 2003). Proximately, clonality of offspring without an increase of homozygosity can result from apomictic thelytoky or automixis with central fusion and a very low recombination rate. From the absence of transitions to homozygosity we can exclude other mechanisms, such as random fusion, gamete duplication, or automixis with terminal fusion.

Whereas males are commonly produced in colonies from the Central America mainland, which are thought to be sexual (Hartmann et al. 2005), male production is relatively rare in the thelytokous island populations. The majority of the analyzed males appeared to be haploid, which means that meiosis must take place and that males originate from haploid egg cells like in other ant species.

Furthermore, assuming that sex determination in *P. punctata* follows the typical sl-CSD mechanism, the occurrence of diploid males indicates that recombination occasionally occurs, because the mothers of such males must have been heterozygous at the sex locus. We therefore can exclude the hypothesis that thelytoky in *P. punctata* is apomictic.

Instead, both mother-offspring comparisons and the occurrence of diploid males suggest that thelytoky in *P. punctata* involves automixis with central fusion, associated with a very low rate of recombination (**Figure 1**). Under this scenario, male production is possible and compatible with the sex determination model, and changes from heterozygosity to homozygosity can occur if the loci are far from the centromere. Only three of several thousand diploid individuals from thelytokous colonies were diploid males, suggesting a recombination rate at the presumed sex locus close to zero – if the investigated microsatellite loci have a similarly low recombination rate it not surprising that we failed to detect recombination in our mother-offspring comparisons. *P. punctata* has a very large number of small chromosomes (2n=84, Schilder 1999) and crossing over is therefore an unlikely event. Reduced rates of recombination have previously been reported for the Cape honey bee (Baudry et al. 2004; Oldroyd et al. 2008). Evolution is expected to decrease recombination rates under automixis with central fusion to maintain genetic diversity and heterozygosity at the sex locus (Belshaw and Quicke 2003; Baudry et al. 2004).

Haploid nuclei that develop without fusion may develop as a sort of ‘genetic accident’ into the few males in the thelytokous population. The even rarer occurrence of diploid males can be explained by recombination at the sex determination locus. If recombination takes place at the sex determination locus, but not at the microsatellite loci, the result would be a heterozygous

individual, which shows the same genotype as the mother but is male. The recombination rate might perhaps be slightly higher, because from microsatellite data alone it is not possible to distinguish between haploid, hemizygous male and diploid, homozygous males. Some of the male offspring of heterozygous mothers might in principle have been homozygous because of recombination both at the sex locus and a microsatellite locus, though the occurrence of two crossing overs in one individual appears to be quite unlikely.

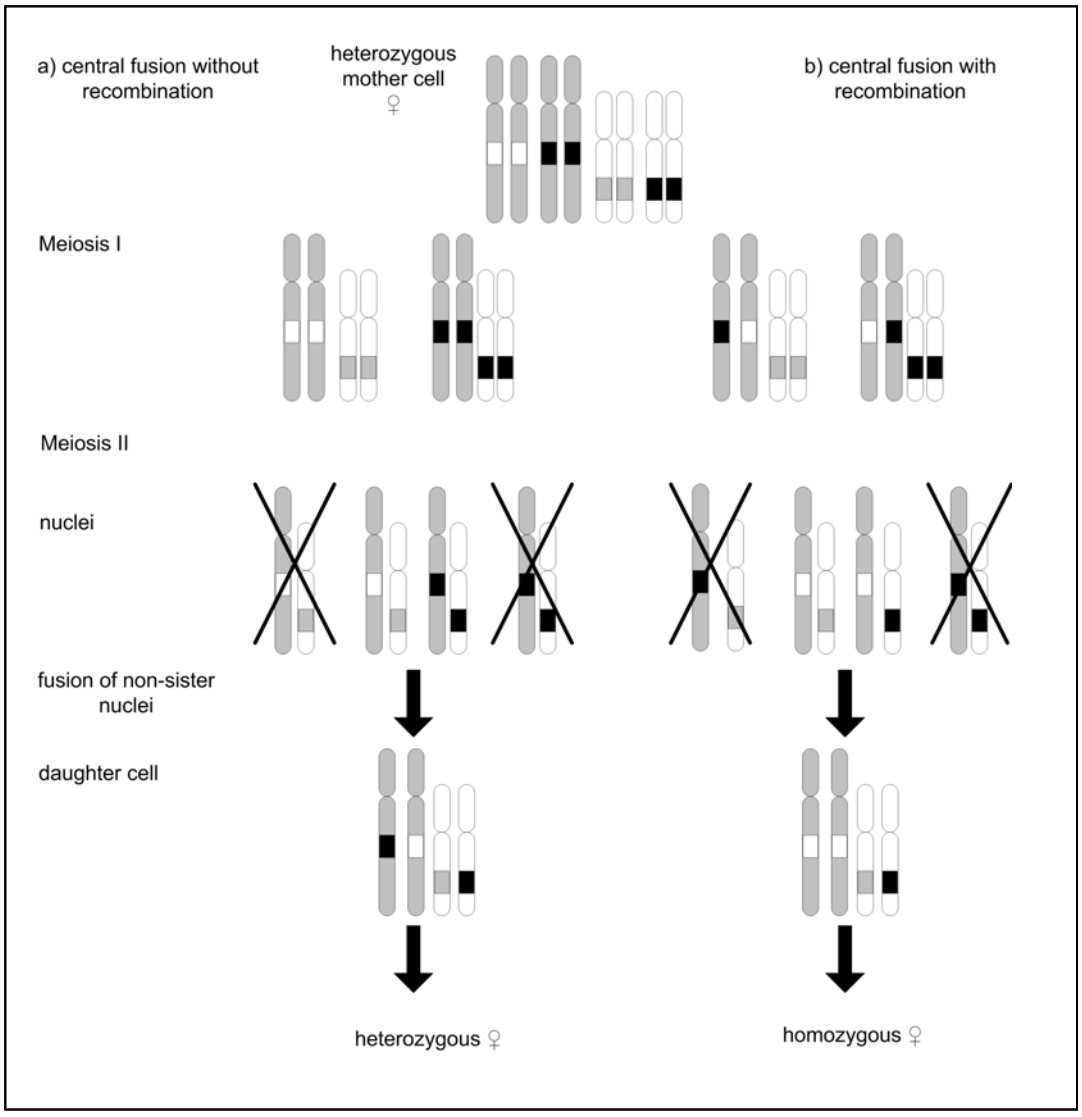

Fig1. Automictic parthenogenesis with central fusion, the presumed mechanism of thelytoky in the ant *Platythyrea punctata*. A heterozygous egg cell can undergo meiosis I without (a) or with recombination (b) at a microsatellite locus. After meiosis II, the two non-sister nuclei fuse to restore diploidy. Under central fusion without recombination, the daughter cell will become a heterozygous diploid, identical to the mother cell, whereas with recombination, the outcome is a homozygous diploid. The sex determination locus (CSD) is not affected under this mechanism, as long as no recombination occurs.
Chromosomes are shown as bars. Grey bars indicate the chromosome carrying the microsatellite locus (alleles as white and black fields), and white chromosomes carry the CSD locus (alleles as grey and black fields).

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V. Chapter 5: Colony fusion causes within-colony variation in a clonal ant

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Abstract

Social insect societies are thought to be closed systems, which prevent non-nestmates from intruding into colonies. *Platythyrea punctata* F. Smith is a thelytokous parthenogenetic ant species, which shows a clonal system of reproduction: female diploid offspring emerge from unfertilized eggs. Microsatellite data suggest that offspring of one mother shows identical genotypes. Nevertheless, field observations indicate that 40% of colonies within the previously studied population from Puerto Rico contain intra-colonial variation. This variation cannot be solely explained by recombination and mutation events. We tested the hypothesis that the variation is a consequence of colony fusion by investigating the occurrence of within-colony variation in *P. punctata* from the Dominican Republic and Barbados and, in a behavioral approach, showing that the fusion of colonies or adoption of foreign workers is a likely source for the observed pattern.

Keywords: Agonistic behavior, colony takeover, replacement, Thelytoky, within colony relatedness, *Platythyrea punctata*

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Introduction

Ants, bees, and wasps are characterized by haplo-diploid sex determination, through which males arise from unfertilized eggs through arrhentokous parthenogenesis and are haploid, while females are produced from fertilized eggs and are diploid (*e.g.*, Cook and Crozier 1995). This rule, however, is not without exceptions. For example, in the ants *Pristomyrmex punctatus* (Tsuji 1988b), *Cerapachys biroi* (Tsuji and Yamauchi 1995), *Cataglyphis cursor* (Cagniant 1983; Pearcy et al. 2004), *Wasmannia auropunctata* (Fournier et al. 2005), and others and in the Cape honey bee (*Apis mellifera capensis*, *e.g.*, Baudry et al. 2004), females may develop from unfertilized eggs by thelytokous parthenogenesis. Depending on the mechanism of thelytoky, offspring may be genetically identical to its mother (Normark 2003), which leads to a clonal colony and population structure (*e.g.*, Schilder et al. 1999a; Schilder et al. 1999b; Hasegawa et al. 2001; Fournier et al. 2005).

Nestmates in colonies of the thelytokous ant *Platythyrea punctata* F. Smith 1858 are often genetically identical at all studied microsatellite loci, which was suggested to be evidence for an apomictic mechanism of thelytoky (Schilder et al. 1999a; Schilder et al. 1999b; Hartmann et al. 2005). Nevertheless, in some colonies genetic heterogeneity was observed, which could be explained neither with the occasional occurrence of sexual reproduction nor recombination during parthenogenesis (Schilder et al. 1999b; Hartmann et al. 2005).

Colony fusion and the adoption of alien individuals into established colonies are potential mechanisms that lead to genetic heterogeneity within colonies. Both go against the common knowledge that the societies of social insects are normally closed, excepting a number of unicolonial invasive ant species (*e.g.*, Hölldobler and Wilson 2009), and that non-nestmates are attacked when they attempt to enter a colony. Colony closure is achieved through the efficient system of nestmate discrimination, which relies on a more or less uniform colony odor shared by all individuals. Nevertheless, the loss of diversity in genetically determined odor cues or a predominance of environment-derived odor cues may eventually lead to recognition errors, as suggested for the ant *Temnothorax nylanderi* (Foitzik and Heinze 1998).

In this study, we investigated the occurrence and frequency of within-colony heterogeneity in populations from the Caribbean islands Hispaniola and Barbados with polymorphic microsatellite markers, and assign the pattern of variation found in the colonies. We then performed laboratory bioassays that simulate a field situation where a colony in search for a new nest invades another colony, which specifically tested the hypothesis that colony fusion could be responsible for the significant within-colony genetic variation we observed.

Material and Methods

Study sites and sample collection

In November 2006 we collected 27 colonies of *Platythyrea punctata* from four different locations in the Dominican Republic (Anton Sanchez AS: 18°49' N; 69°41' W; 100m; *n* = 6, El Laurel EL: 18°46' N; 69°53' W; 90m; *n* = 6, Miches Gallistico Mi: 18°58' N; 69° 2' W; 2m; *n* = 6 and Rancho Wendy/Bonao RW: 18°53' N; 70°27' W; 255m; *n* = 9). In June 2007 we collected 11 colonies from three different locations in Barbados (Hackleton's Cliff HaC: 13°12' N; 59° 31' W; 80m; *n* = 4, Harrison's Cave HC: 13°10' N; 59° 34' W; 54m; *n* = 1 and Turner's Hall Woods TH: 13°13' N; 59° 35' W; 90m; *n* = 6). Colony size ranged from 6 to 50 (mean: 23.07 ± 10.9 SD) and 16 to 71 individuals (mean: 36.55 ± 20.50 SD) in the Dominican Republic and Barbados, respectively.

Molecular methods

We sampled 12 individuals (6 older foragers and 6 young workers (callows), larvae or pupae) from each colony either immediately after collection or, if not possible because of insufficient callow/brood number, later in July 2007 and stored them in 100% ethanol for further genetic analyses. For one colony from the Dominican Republic, in total 24 individuals were analyzed. DNA was extracted from complete individuals and / or brood items (larvae and pupae) using a modified CTAB-Method (after Sambrook and Russell 2001). Individuals were genotyped at four polymorphic microsatellite loci which were used in former studies of *P. punctata* (2801, 2902, 3506 and 4101 (Schilder et al. 1999b; Hartmann et al. 2005)) and additionally at a fifth microsatellite loci (3302), which was developed by Schilder et al. 1999 for *P. punctata*, but so far has not been used. PCR amplification products were denatured for 1min at 90°C and analyzed with an ABI PRISM 310 Genetic Analyzer. Absolute allele lengths were determined using GeneScan® 3.1 Software (Applied Biosystems). Observed and expected heterozygosities were obtained using the Software GDA 1.1 (Lewis and Zaykin 2001). Within colony relatedness was calculated using the software RELATEDNESS 5.0.8 (Goodnight and Queller 1998). Standard errors were obtained by jackknifing over colonies. Genotypes of adults and callow workers / brood items were compared with each other to investigate the genetic patterns within colonies. Genotypes were recorded as aberrant when they differed in at least one allele at on locus.

Behavioral assays

Nine pairs of *P. punctata* colonies (four pairs from Barbados, collected in June2007 and kept in the lab for one month, and five pairs from the Dominican Republic, collected in November

2006 and kept in the lab for eight months, **Table 1**) were transferred into adjacent plastic boxes with plaster floor (20.0cm × 20.0cm × 9.0cm per box) separated by a wall with a plugged tunnel and each with a nest cavity in the plaster covered with a glass plate (8.0cm × 5.5cm × 0.5cm) and red foil. Colonies were fed a mixed diet of honey and pieces of cockroaches *ad libitum*. Ants were marked individually with dots of Edding marker pens. After marking and transferring the colonies in the set up boxes the colonies were left undisturbed for two weeks to recover and get adapted to the new conditions. Some workers were injured during marking and the size of the colonies used in this experiment was therefore lower than that of field colonies (4-20 workers, **Table 1**).

Table 1. Colony sizes of single colonies, fusion pairs and number of reproductives in the used *P. puncata* colonies. Egg laying activity after the fusion is described in yes: active, no: inactive; in three cases, the fusion event resulted in the death of one of the reproductives.

Colony fusion pair	population	single colonies	Colony size before fusion (n)	Colony size after fusion (n)	total size of fused colonies (n)	reproductives before fusion (n)	reproductive activity after fusion
1	Barbados	HaC-01	8	2	11	1	no
		HaC-05	12	9		1	yes
2	Barbados	HC-01	11	11	25	2	yes
		TH-11	16	14		2	dead / no
3	Barbados	TH-07	12	10	20	1	yes
		TH-13	11	10		1	no
4	Barbados	HaC-04	10	4	13	1	no
		TH-01	16	9		1	yes
5	Dom Rep	AS-01	12	9	11	2	no / yes
		AS-14	4	2		1	yes
6	Dom Rep	EL-01	11	4	12	1	dead
		RW-09	20	8		1	yes
7	Dom Rep	Mi-03	15	15	24	1	yes
		AS-15	12	9		2	no
8	Dom Rep	EL-08	15	14	27	1	yes
		EL-09	12	13		1	dead
9	Dom Rep	RW-06	13	12	25	1	no
		RW-07	14	13		1	no
mean			12.44	9.33	18.67		
SD			3.45	4.06	6.84		

Behavioral observations were performed in two phases: In phase 1, colonies were observed in their separate plastic boxes, and in each colony the reproductive individual was determined from its distinct behavioral profile, *e.g.*, its high frequency of sitting on the egg pile (see Hartmann et al. 2003). Reproductive status was confirmed by the appearance of eggs after isolating the individual for 24 to 48 hours in a separate plastic box (10.0cm × 3.0cm × 3.0cm) with a nest cavity, food and water (see Hartmann et al. 2003). After separation the

reproductives were returned to the colonies, and after three days (giving the reproductives time to reintegrate), phase 2 was started. In phase 2, the tunnels in the separation wall between the two boxes were opened, the nest cavity in the box with the smaller colony was destroyed by removing the glass and foil cover, and the colony was thus forced to move out of their nest. We recorded the frequency of sociopositive behavior (antennal contact and allogrooming), and aggressive behavior (antennal boxing, biting, sting smearing, immobilization of opponent, i.e. pulling on legs and antennae (see Hartmann et al. 2003), before (24 – 26 observation sessions of 5 min each per colony over 24 days, total 120 – 130 min per colony) and after this manipulation (one observation session of 30min each beginning with the first contact between workers from the two colonies, five sessions of 10 min each per day over 5 consecutive days after the manipulation, total 270min per colony). Additionally, in each observation session the position of each individual within the colonies was recorded (on eggs, on brood, inside nest cavity / outside nest cavity). Reproductive status of the presumed egg layers was investigated as above after the experiment. All statistical tests were performed with STATISTICA 6.0 (Statsoft 2003).

Results

Colony and population structure

In total, 132 individuals from Barbados and 336 individuals from Dominican Republic were analyzed. In Barbados, one microsatellite locus (3506) did not show variation and was excluded from the analysis. The others had two (2902, 4101, 2801) and four alleles (3302), with expected and observed heterozygosities (calculated over colonies) of 0.277 ± SD 0.129 and 0.509 ± SD 0.246, respectively. In the Dominican Republic, microsatellites had two (3506, 2902), four (4101), six (2801) and 7 alleles (3302), with mean expected and observed heterozygosities of 0.338 ± 0.120 and 0.619 ± 0.199, respectively. The observed heterozygote excess is in accordance with previous analyses in other *P. punctata* populations (Kellner and Heinze, *unpublished data*) and matches expectations for predominantly clonal populations (Balloux et al. 2003; de Meeûs and Balloux 2005). The resulting fixation indices are -0.911 (95% CI: -0.841 to –0.980) for Barbados and -0.864 (95% CI: -0.790 to -0.952) for the Dominican Republic. Fixation indices for the three level hierarchical analyses of variation are shown in **Table 2**. In Barbados we found in total nine different clonal lineages (multilocus genotypes) and 22 in the Dominican Republic.

Table 2: Three-level hierarchical analyses showing Fixation indices for Barbados and Dominican Republic: differentiation among individuals within the total population (F_{IT}), differentiation among colonies within the population (F_{CT}) and variation among sample sites within the total population (F_{ST}). The significance of F statistics estimates were obtained by bootstrapping over loci (1000 replicates), 95% confidence intervals are given in parenthesis. Number of analyzed colonies is given in parenthesis.

	F_{IT} (95% CI)	F_{CT} (95% CI)	F_{ST} (95% CI)
Barbados (n=11)	-0.142 (0.115 to -0.525)	0.403 (0.333 to 0.061)	0.156 (0.519 to 0.221)
Dominican Republic (n=27)	0.019 (0.229 to -0.198)	0.474 (0.574 to 0.379)	0.302 (0.422 to 0.193)

Mean nestmate relatedness was 0.913 ± SE 0.058 in colonies from Barbados and 0.917 ± 0.085 in colonies from the Dominican Republic. The deviation from the relatedness of 1 expected for clonemates is explained by genetic variation among workers in 4 of 11 colonies (36%, 3 colonies with aberrant adults, one with aberrant adult and callow workers) from Barbados and 10 of 27 colonies (37%, 2 colonies with aberrant adults, 5 with aberrant callows, 3 with both aberrant callows and adults) from the Dominican Republic. In these colonies nestmate relatedness ranged from 0.61 to 0.86 in Barbados and from 0.34 to 0.97 in Dominican Republic (see **Table3**), while it was 1 in the remaining colonies.

Three different types of aberrant genotypes were encountered. The first case was caused by two foreign alleles at a locus, which were not present in other individuals within the colony. Second, the aberrant genotype was homozygous instead of heterozygous or in the third case, the genotype was caused by a foreign allele at a single locus. Although homozygosity at a locus could be explained by rare recombination events due to the mechanism of thelytoky (Kellner and Heinze *in review*), foreign alleles found inside otherwise clonal colonies could only come from external sources. One theoretical possible explanation for one foreign allele at a locus would be sexual events. However, this was not supported by the data because sexual recombination would result in more evenly distributed variation within colonies. However, two foreign alleles are impossible to originate from sexual events. Instead, the genotypes of workers in one of four heterogeneous colonies from Barbados and in five of ten heterogeneous colonies from the Dominican Republic suggested the mixture of different clonal lineages through colony fusion or adoption, which becomes very obvious for example in colony Mi3 from the Dominican Republic (see **Table 3**), where at least four different clone lineages were present.

Table3. Within colony relatedness and variation patterns in non clonal colonies of *P. punctata*. Aberrant genotypes are shown in bold. Individuals causing variation can be callow workers or brood (c) and / or adults (a).The type of variation is explained by A: two foreign alleles, B: homozygosity instead of heterozygosity, C: one foreign allele; BB: Barbados, DR: Dominican Republic;

			Locus							
	colony	**# ind**	**3506**	**2902**	**4101**	**2801**	**3302**	**individuals causing variation**	**category**	**R value**
BB	TH5	6	203/203	185/185	201/201	386/386	240/242			0.68
		6			**201/211**			2c, 4a	C	
	TH7	11	203/203	185/185	201/201	386/386	238/240			0.86
		1					**240/242**	1a	C	
	HC	11	203/203	183/185	201/211	386/388	240/242			0.78
		1		**185/185**				1a	B	
	HaC2	10	203/203	185/185	201/211	386/388	238/238			0.61
		2					**242/244**	2a	A	
DR	AS6	10	193/203	183/185	199/211	382/382	238/244			0.56
		1			**211/211**	**382/386**	**238/238**	1c	B, C, B	
		1			**201/201**	**382/386**	**236/244**	1a	A, C, C	
	AS11	9	193/193	183/185	199/211	382/382	238/244			0.56
		3	**193/203**				*238/246*	1c, 2a	C, C	
	Mi1	10	193/203	183/185	201/203	384/384	238/244			0.79
		2				**386/386**		2c	A	
	Mi2	11	193/203	183/185	201/203	386/386	238/242			0.88
		1					**240/244**	1a	A	
	Mi3	13	193/203	183/185	203/211	382/390	238/238	5c, 8a		0.34
		9			**201/201**	**382/382**	**236/244**	7c,2a	A,B,A	
		1			**211/211**	382/390	238/238	1c	B	
		1			**203/203**	382/390	238/238	1a	B	
	Mi9	11	193/203	183/185	201/201	382/386	236/244			0.80
		1			**199/211**	**382/382**	**238/244**	1a	A, B, C	
	EL1	11	203/203	183/185	199/211	382/386	238/244			0.96
		1				**386/386**		1c	B	
	EL8	11	203/203	183/185	199/211	386/386	238/244			0.97
		1			**211/211**			1c	B	
	EL11	11	203/203	185/185	199/211	386/386	238/244			0.97
		1			**211/211**			1c	B	
	RW12	11	203/203	185/185	211/211	386/386	242/244			0.93
		1				**368/386**		1c	C	

Colony fusion

All nine pairs of colonies in our experiments eventually fused and finally inhabited the one remaining nest site. Workers from the destroyed nest started moving into the intact nest within 30 minutes after discovering it. Workers did apparently not discriminate between nestmates and non-nestmates, and workers from the resident colony never attempted to prevent non-nestmates from moving into their nest. Instead, workers from both colonies carried brood items and young workers from the destroyed nest cavity into the intact nest.

Nevertheless, the frequency of aggressive behavior (all aggressive acts) increased significantly after the manipulation from 0 (quartiles 0, 0) to a median of 0.011 (quartiles 0.008, 0.020) interactions per individual per minute (Wilcoxon matched-pairs test, $Z = 2.67$; p

< 0.01; **Figure1**). In contrast, peaceful interactions did not change significantly (medians, quartiles 0.013, 0.011, 0.014 and 0.014, 0.012, 0.035) per individual per minute before and after fusion, respectively (Wilcoxon matched-pairs test, $Z = 1.24$; $p > 0.05$; **Figure 1**).

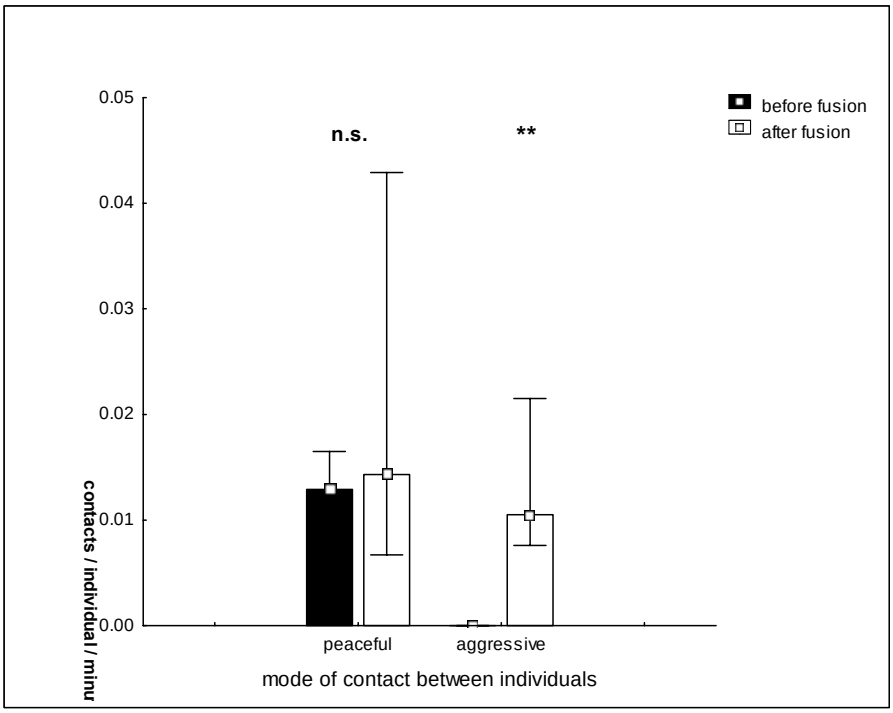

Fig1. Peaceful and aggressive behavior within *P. punctata* colony pairs before and after colony fusion ($n = 9$ fusion pairs). Spots represent medians of interaction per individual per minute over all colonies; boxes show percentile and whiskers the range without outliers. Aggressive behavior increased significantly after fusion, whereas peaceful interaction was constant (Wilcoxon matched-pairs test; ** $p < 0.01$; n.s. = not significant)

Reproductive individuals received proportionally more attacks than other workers in seven of nine colonies over the total observation time (χ^2-test, 1: $\chi^2 = 15.49$; 2: $\chi^2 = 19.77$; 3: $\chi^2 = 25.98$; 4: $\chi^2 = 6.07$; 6: $\chi^2 = 37.52$; 7: $\chi^2 = 7.81$ and 9: $\chi^2 = 35.14$; $p < 0.05$ and for colonies 5: $\chi^2 = 0.62$ and 8: $\chi^2 = 2.71$; $p > 0.05$; **Figure 2**). Over all colonies ($n = 146$ individuals), reproductives ($n = 22$) received 57.7% of all recorded aggressive acts (χ^2-test, $\chi^2 = 115.21$; $p < 0.001$).

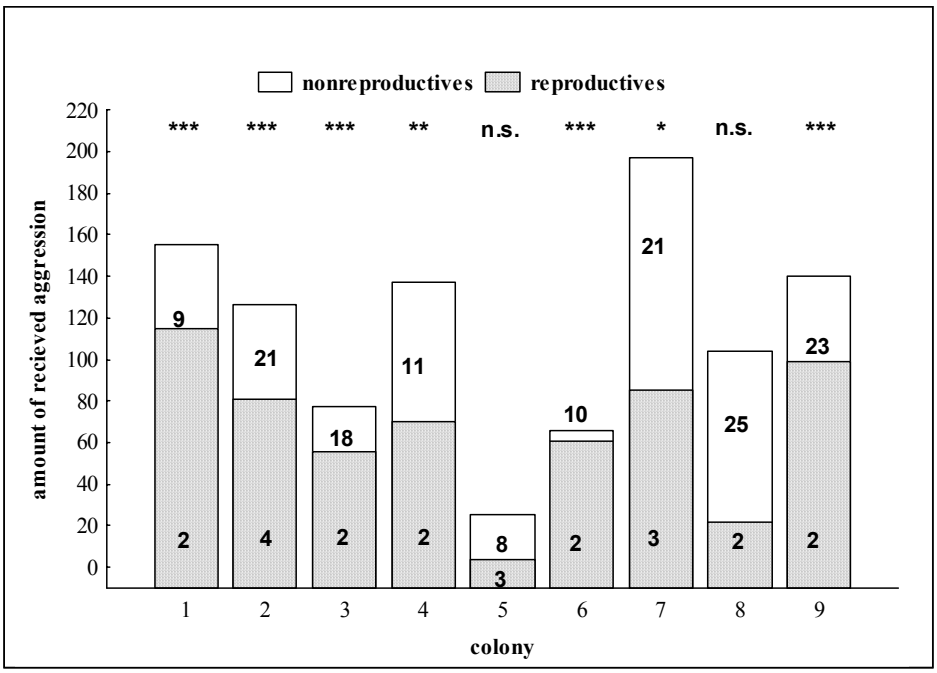

Fig2. Totally received aggressive acts (antennal boxing, biting, smearing and immobilization) by reproductives and non-reproductive workers in nine fused *P. punctata* colonies over total observation time. In all colonies except of colony 5 and 8 aggression ratio was significantly skewed towards reproductives (χ^2-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant). The numbers in the bars represent the number of contributing individuals

Worker aggression was not preferentially directed towards the unfamiliar egg-layer (χ^2 = 72.96; $p < 0.01$ over all colonies). Nonetheless, in at least five of nine colonies one reproductive was more frequently attacked (Wilcoxon matched-pairs test for colonies 3: Z = 3.3; 8: Z = 2.37 and 9: Z = 2.97; $p < 0.05$ and Friedmann test for paired samples for colonies 2: χ^2 = 37.03 and 7: χ^2 = 17.19; $p < 0.05$). In three colonies, aggression started with a delay of one day, during which the reproductives were disproportionately aggressive by themselves (χ^2-test for colonies 1: χ^2 = 7.82; 4: χ^2 = 6.18 and 9: χ^2 = 4.43; $p < 0.05$) towards each other.

Aggression against reproductives resulted in the re-establishment of 'monogyny' in the fused colonies. Before fusion, colonies had one, less commonly two egg-layers, and after fusion two to four reproductives (two in colonies 1, 3, 4, 6, 8, 9; three in 5, 7 and four in 2, see **Table 1**). At the end of the experiment at least one egg-layer per colony had ceased to lay eggs or died.

Colony fusion did not cause any bias in task allocation among the former colonies, i.e., workers that had engaged in nursing or foraging continued to do so after fusion. The proportion of indoor and outdoor workers did not differ between the former colonies (Wilcoxon matched-pairs test for indoor workers of fused colonies 2: Z = 1.11, 3: Z = 1.36, 4: Z = 1.83, 6: Z = 0.37, 7: Z = 0.28, 9: Z = 0.12; outdoor workers of colonies 2: Z = 0.66, 3: Z = 1.27, 4: Z = 1.6, 6: Z = 0.37, 7: Z = 0.00, 9: Z = 0.12; p > 0.05 in all comparisons). Only in colony pair 8 workers of the intruder colony performed more indoor than outdoor work (Z =

2.39 for indoor and Z = 2.63 for outdoor; $p < 0.05$). For fused colony pairs 1 and 5 no comparisons could be done due to insufficient sample size.

Discussion

Populations of the thelytokous ant *Platythyrea punctata* on Caribbean islands usually consist of clonal colonies but some fraction of colonies contains workers of genetically different lineages (40% in Puerto Rico, Kellner and Heinze, *unpublished data*, 33 to 36% in the Dominican Republic and Barbados, this study). The genotypes of some aberrant workers may reflect mutation or recombination events during parthenogenesis. However, in cases where workers have genotypes that are completely incompatible with those of their nestmates, genetic heterogeneity can only be explained by the fusion of different colonies or the adoption of alien workers. While brood-raiding could also result in similar patterns in variation, brood raiding probably does not occur in *P. punctata*. Brood raiding is most commonly found in derived ants, *e.g.*, *Solenopsis*, *Myrmecocystus* and *Acromyrmex* (Bartz and Hölldobler 1982; Pollock and Rissing 1989; Fowler 1992; Bourke and Franks 1995; Tschinkel 2006), which typically reproduce by independent founding, possess large colonies and exhibit territoriality, traits unlike those found in *P. punctata*.

The observation that genetically different colonies easily merge in the laboratory suggests that genetically heterogeneous colonies in the field may indeed result from fusion. When their nest was experimentally destroyed, ants readily moved in with another colony and after some initial aggression permanently lived together. In the field, *P. punctata* nests in preformed cavities in rotten wood on the ground, dead branches in trees or soil. Workers are apparently not capable of enlarging their nests when the colonies grow (in colonies nesting in soil cavities no digging behavior could be observed and no loose soil was found around nest entrances). Nests in dead wood appear to be fragile and when they are destroyed by strong winds or the seasonal hurricanes, colonies have to seek for new nests.

Nest site fragility and habitat saturation might lead to limitation of nest sites. Previous studies have shown that the availability of nest sites strongly shapes the communities of wood-dwelling ants in temperate forests. Nest site limitation can lead to intraspecific competition and replacement of an established nest by another species (Yamaguchi 1992). Nest site limitation also can result in fusion of mature colonies, temporal polygyny and even usurpation and take over, which might be a first step to intraspecific parasitism (Herbers 1986; Herbers and Banschbach 1999; Foitzik and Heinze 2000; Strätz et al. 2002).

We suggest that similar phenomena explain the structure of *P. punctata* populations in disturbed woodland on the Caribbean islands. As yet, common colony fusion in response to

nest site limitation has only been observed in *Temnothorax nylanderi*, a myrmicine ant from deciduous forests in Central Europe, which also lives in ephemeral nests sites, such as hollow acorns or hazelnuts. After fusion, one of the two reproductives from the two originally monogynous colonies is eliminated (Foitzik and Heinze 1998; Strätz et al. 2002). Our observations reveal a strikingly similar pattern in *P. punctata*. Resident ants accepted the adoption of foreign workers into their colonies after some initial aggression, and in most cases at least one original reproductive individual ceased laying eggs or died. Like in *T. nylanderi* (Strätz et al. 2002), reproductives engaged in fighting and were attacked both by nestmates and non-nestmates.

In contrast to expectations from kin and nestmate recognition models and previous observations in other ants (Kikuchi et al. 2007), workers did not preferentially attack the alien reproductive but directed their aggression randomly to both clone mate and alien reproductive. In *T. nylanderi* it was shown that workers have a tendency to attack the older of two queens, even when it was their own, and showed a preference for the younger and probably more fertile queen (Strätz et al. 2002). In *P. punctata* nestmate discrimination and nepotism might be weak and attacks perhaps are directed to the less fertile reproductives as shown in monogynous colonies of *Solenopsis invicta* (Fletcher and Blum 1983), i.e., fertility signals might in this case override colony odor. Weak nestmate discrimination may result from low genetic variability as suggested for unicolonial, invasive ants (Tsutsui et al. 2000; Vásquez et al. 2008) and also for *T. nylanderi*, which apparently has lost genetic variability after postglacial expansion of its range into Central Europe (Pusch et al. 2006). Though the populations of *P. punctata* consist of different clonal lineages, mtDNA indicates that the overall genetic similarity is very high (Hartmann et al. 2005). We suggest that the high genetic similarity impairs the nestmate discrimination ability in these ants and facilitates the fusion of colonies. To mention another aspect, the adoption of foreign workers into an already established colony might even be beneficial, since after solving the problem with the supernumerary reproductives, the resident colony obtains an increased genetic heterogeneity, which might be beneficial in terms of parasite resistance (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Hughes and Boomsma 2006), productivity and fitness (Jones et al. 2004; Mattila and Seeley 2007), and / or task efficiency and division of labor (reviewed in Oldroyd and Fewell 2007; Smith et al. 2008).

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VI: Chapter 6: The police are not the army: context-dependent aggressiveness in a clonal ant

B. Barth, K. Kellner and J. Heinze*

Abstract

Animals often exhibit particular 'personalities', i.e., their behavior is correlated across different situations. Recent studies suggest that this limitation of behavioral plasticity may be adaptive, since continuous adjustment of one's behavior may be time-consuming and costly. In social insects, particularly aggressive workers might efficiently take over fighting in the contexts of both nest defense and 'policing', *e.g.*, the regulation of kin conflict in the society. Here, we examine whether indeed those workers that engage in aggressive policing in the ant *Platythyrea punctata* play a similarly prominent role also in nest defense against alien ants. The participation of individuals in policing and nest defense duties was highly skewed, i.e., in each colony only a minority of workers behaved aggressively. Workers that attacked supernumerary reproductives after experimental colony fusion refrained from defending the nest and vice versa. This suggests that workers show situation-dependent behavioral plasticity rather than consistently aggressive personalities and support the interpretation of policing behavior as being selfish at least in part.

Keywords: *Platythyrea punctata*, colony fusion, worker policing, animal personalities, behavioral syndromes

** under review in Biology Letters*

Introduction

Animals often differ consistently in their behavior across a wide range of situations. Certain individuals may be always more or less aggressive than others, regardless of whether confronted by a conspecific rival, a predator or a prey. Such 'behavioral syndromes' or 'animal personalities' are well known from mammals, birds, and other vertebrates, but have recently also been reported from arthropods, such as spiders and honey bees (Burns 2005; Johnson and Sih 2005; Rueppell et al. 2006). Though 'behavioral syndromes' severely limit behavioral plasticity, recent studies suggest them to be adaptive rather than constraints (*e.g.*, Sih et al. 2004; Réale et al. 2007; Wolf et al. 2007).

Group-living animals might benefit particularly from a division of labor among individuals with different 'personalities', as exemplified in workers from a certain honey bee strain, which invest more in pollen collection and recruitment to pollen sources than other bees (Rueppell et al. 2006). Likewise, particularly 'bold' or aggressive individuals might efficiently suppress within-group conflict about the partitioning of reproduction ('policing', Ratnieks et al. 2006) and also defend the group and its territory against rivals and competitors from outside (Clutton-Brock and Parker 1995; Frank 1996; Nunn and Deaner 2004; Kitchen and Beehner 2007).

We tested the prediction that group members with an especially aggressive 'personality' take over both nest defense and policing in the ant *Platythyrea punctata* Smith 1858. All workers of this species are potentially capable of producing female offspring from unfertilized eggs by thelytokous parthenogenesis (Heinze and Hölldobler 1995). Many colonies are clones with a nestmate relatedness of one (Schilder et al. 1999b; Hartmann et al. 2005). Though kin conflict is not expected from relatedness, reproductive division of labor with one or a few laying workers is maintained by dominance and policing (Heinze and Hölldobler 1995; Schilder et al. 1999a), presumably because egg laying by too many reproductives lowers colony efficiency and the average inclusive fitness of nestmates (Hartmann et al. 2003). All individuals benefit from policing, but theoretical and empirical studies suggest that it is often taken over by a 'policing elite' (Frank 1996; Stroeymeyt et al. 2007; van Zweden et al. 2007; Brunner, Kellner and Heinze, *under review*). We hypothesized that individual workers show similar levels of aggression across different situations, i.e., that police workers engage more in colony defense against intruders, competitors or potential predators.

Material and Methods

In laboratory experiments *P. punctata* colonies readily fuse without much aggression, but a subset of workers quickly begin to fiercely attack at least one of the reproductives (Kellner, Barth and Heinze, *unpublished data*). Though colony fusion may result in genetically heterogeneous colonies, these attacks are not preferentially directed against unrelated reproductives and also not against non-reproductives. Instead, they resemble aggression patterns in policing experiments in *P. punctata* (Hartmann et al. 2003) and can therefore be considered as policing. Thus, we use fused *P. punctata* colonies and the subsequent introduction of alien ants, i.e. enemies, to test our hypothesis.

Colonies were collected in Barbados and the Dominican Republic and housed in artificial nests in the laboratory. Water and food were provided *ad libitum*. Fusion experiments were carried out with nine colony pairs with individually paint-marked workers (for details see Kellner, Barth and Heinze, *unpublished data*). Between ten and 67 days after fusion single workers of four enemy ant species were successively (one day intervals) introduced into the nests (colony sizes: 5 to 21, mean: 11.3 ± 5.7 SD): *Odontomachus* sp., *Pachycondyla stigma*, *Solenopsis invicta* (majors only) and *Camponotus* sp. (in this order). All these species co-occur with *P. punctata* and may inhabit similar nesting sites (*e.g.*, rotten wood). Alien workers were gently placed directly into the nest chambers to ensure contact with all *P. punctata* workers. Nest entrances were closed to avoid escape.

In both situations, beginning with the first contact between the respective parties, we recorded the frequency of aggressive acts (antennal boxing, biting, stinging, gaster smearing and immobilization, i.e. pulling on legs and antennae) per colony (fusion: 270 min over five consecutive days; enemy: 30 min per enemy). To determine whether some individuals were more aggressive than others frequency distributions of aggressive acts of each colony and over all colonies in both experiments were fitted to a Poisson-distribution by χ^2-tests, assuming random sharing of aggression (one colony had to be excluded from the 'enemy'-assay because of too small colony size).

In each colony we identified the four most aggressive individuals during fusion and against alien ants, respectively. Since colony size and composition changed between the two experiments due to the death and the eclosion of some workers, we compared the aggressiveness of individuals relative to the level of aggression expected from equal contribution of all workers. Only in four colonies (9 to 21 workers) all the four individuals that had been most aggressive after fusion were still alive during the introduction of alien ants. For each of these workers ratios between observed and expected number of attacks were compared using a Wilcoxon matched-pairs test. Furthermore, if workers were equally

aggressive across the situations, aggressiveness during fusion and against alien ants would be positively correlated. We therefore analyzed the data also with non-parametric Spearman R statistics. All statistical analyses were performed with STATISTICA 6.0 (Statsoft 2003).

Results

Like the fusion of two colonies (Kellner, Barth and Heinze, *unpublished data*), the introduction of alien ants into the nest of a *P. punctata* colony resulted in immediate aggression. In both cases, a minority of workers fiercely attacked the intruder, whereas others avoided fights. In eight of nine colonies during fusion (Kellner, Barth and Heinze, *unpublished data*) and in all eight colonies during the introduction of alien workers, the frequency distribution of attacks per worker showed a significant departure from random (χ^2 -tests, $p < 0.05$ in each colony; summed over all colonies, **Figure 1**). In contrast to colony fusion (Kellner, Barth and Heinze, *unpublished data*), reproductive individuals were never observed to be aggressive. Attacks usually resulted in the death of alien ants within 30 minutes, except of *Pachycondyla stigma* workers, which even killed some *P. punctata* workers in seven of eight colonies.

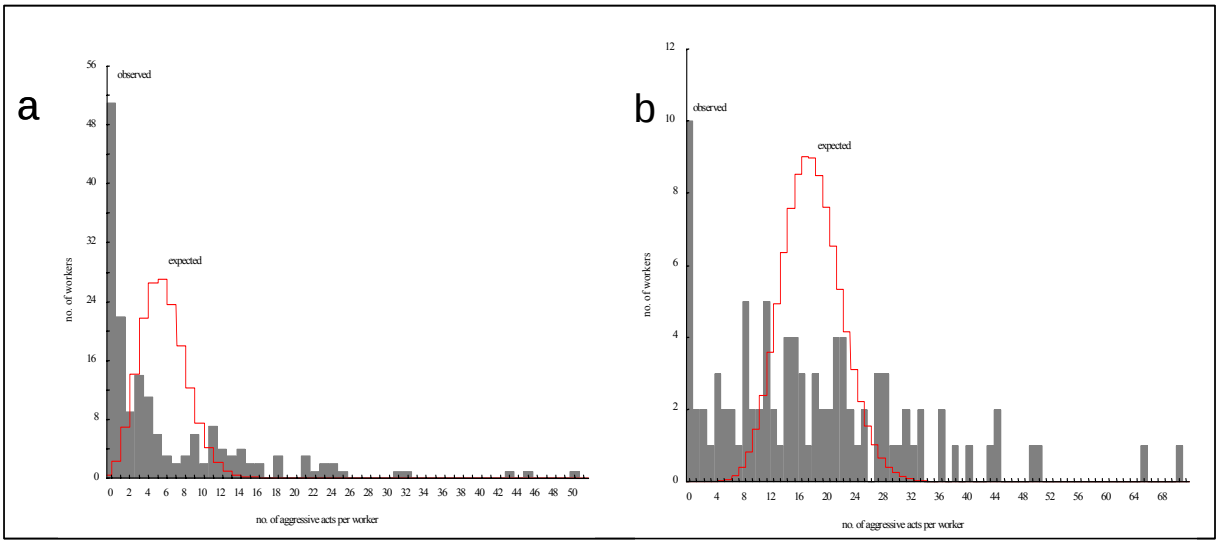

Fig1. Frequency distribution of aggressive behavior among *P. punctata* workers within colonies during **a)** colony fusion (n = 9) and **b)** 'enemy'-introduction (n = 8). Observed distributions were significantly more skewed than expected from random sharing of aggressive tasks (χ^2-test, assuming a Poisson-distribution: **a)** χ^2 = 203.34; **b)** χ^2 = 44.64; $p < 0.001$ in both cases)

The 16 workers that were most aggressive during the fusion experiments engaged 2.64 (median, quartiles 1.87, 3.86) times more frequently in attacks than expected under equal contributions, but were significantly less aggressive against enemies (median 0.75, quartiles 0.38, 1.04, Wilcoxon matched-pairs test, n = 16, Z = 3.26; $p < 0.01$). Similarly, those 16

workers that most frequently attacked alien ants (ratio observed / expected, median 1.8, quartiles 1.55, 2.02) were usually significantly less aggressive during the fusion experiment (median 0.38, quartiles 0.15, 0.85, $Z = 3.46$; $p < 0.001$). Only two individuals were among the four most aggressive workers in both situations. The ratios between observed and expected aggressiveness of individual workers in both experiments were negatively correlated ($r_S = -0.43$; $p < 0.05$, **Figure 2**).

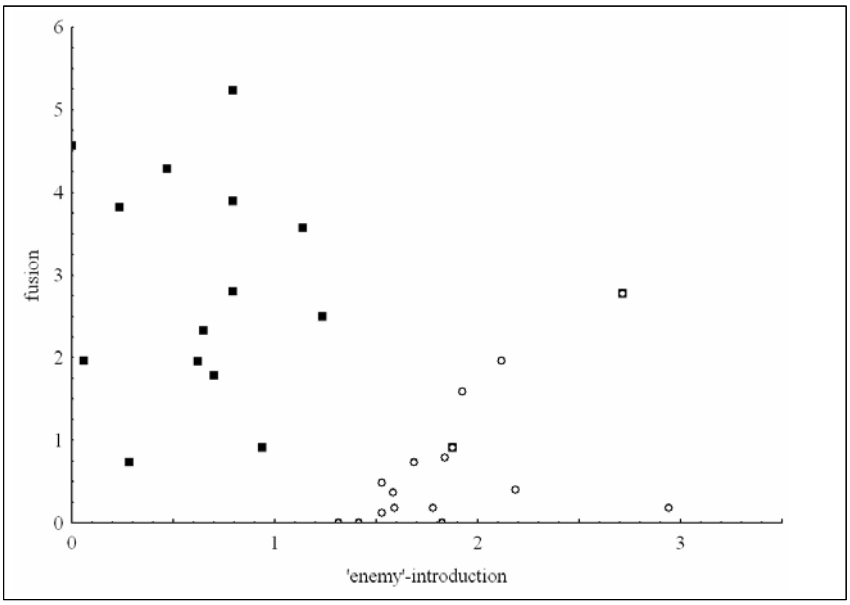

Fig2. Correlation between the ratios of observed to expected aggressiveness in the colony fusion experiment and against an introduced alien ant. Black squares and circles indicate the four most aggressive workers of *Platythyrea punctata* from each of four colonies ($n = 16$) during fusion and in the 'enemy'-introduction experiment, respectively. Aggressiveness is situation-dependent, as indicated by the negative association between the two ratios. Two workers (circles in squares) were among the four most aggressive individuals in both situations.

Discussion

In colonies of *Platythyrea punctata*, a minority of individuals engage in worker policing in colony fusion experiments (Kellner, Barth and Heinze, *unpublished data*), and likewise only few workers attack alien ants introduced into the nest cavity. Our study shows that different workers take over aggression in the different situations. Police workers less frequently attack alien intruders, while the nest defenders are less active in policing. This suggests that the level of aggressiveness is context-specific. Aggressiveness is often governed by a single physiological and endocrine pathway (*e.g.*, Kravitz and Huber 2003) and therefore correlated across situations (Sih et al. 2004; Johnson and Sih 2005; Rueppell et al. 2006; Réale et al.

2007). In contrast, the physiological mechanisms underlying aggression in *P. punctata* appear to be more flexible.

The observed behavioral plasticity rejects the idea of an 'aggression syndrome' but nicely corroborates the hypothesis that policing in social insects has a selfish component. In several species of wasps and ants, police workers appear to be high-ranking 'hopeful reproductives', which themselves lay eggs when the old reproductives are removed (*e.g.*, Wenseleers et al. 2005; Stroeymeyt et al. 2007). Such 'hopeful reproductives' often engage less in helping than low-ranking individuals and in this way appear to save their resources for future reproduction (*e.g.*, Field et al. 2006). In analogy, *P. punctata* workers with a high expected future fitness refrain from bearing the risks of attacking alien ants and cede nest defense to lower ranking, presumably older workers with low future direct fitness. Though the age of individual *P. punctata* workers was not determined in our study, previous research suggested a temporal polyethism, i.e., workers move from relatively save indoor tasks to more risky outdoor tasks with age (Hartmann and Heinze 2003). This matches the prediction that individuals should invest less in helping when their expected future fitness is greater and that helping effort increases with decreasing social status (Cant and Field 2005). In contrast, dominant, younger workers of *P. punctata* obviously benefit from aggressively excluding rivals from egg laying.

Division of aggression between policing and nest defense therefore differs strikingly between highly eusocial animals, such as honeybees and naked mole-rats (*e.g.*, O'Riain and Jarvis 1997), and social animals with totipotent individuals, such as primates or 'primitively' eusocial paper wasps. In the latter taxa, nest defense both against predators and potential usurpers are usually taken over by high-ranking individuals. They have the most to lose (Judd 2000; Kitchen and Beehner 2007), while low rankers may have a reasonable chance of reproducing away from the nest or would suffer higher costs from aggression (Heinsohn and Legge 1999; Cant and Field 2005). This reflects the distinction between indirect fitness driven sociality with sterile helpers and cooperative breeding, in which helpers may gain both indirect and direct fitness (Korb and Heinze 2008). Although colonies of *P. punctata* consist of totipotent individuals, the species has evolved from highly eusocial ancestors (Hartmann et al. 2005) and thus belongs to the first class of social animals. Selfish fighting for dominance and policing may have been retained despite of regular clonality because of occasional colony fusion in the field and sexual reproduction. Furthermore, aggression may be necessary to maintain a division of reproductive labor (Hartmann et al. 2003).

Acknowledgements

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VII. Chapter seven: Selfish policing in the parthenogenetic ant *Platythyrea punctata*

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Abstract

In the parthenogenetic ant *Platythyrea punctata* policing behavior is not expected on relatedness grounds as workers are normally clone mates and thus equally related to all offspring in the colony. Nevertheless, colonies usually contain only a single reproductive and other workers that begin to lay eggs are policed, i.e., attacked by their nestmates. In this study we show that policing individuals not only eliminate surplus reproductives and thus serve for maintaining an efficient reproductive division of labor, but simultaneously increase their own chances of later becoming reproductives themselves. Individuals that were most aggressive towards new reproductives later developed their ovaries after reproductive individuals had been removed from the colonies. This suggests that direct fitness gains may be of considerable importance even in this predominantly clonal species, presumably because infrequent sex, recombination, or the adoption of alien workers may eventually introduce genetic heterogeneity.

Keywords: Selfishness, dominance hierarchy, worker policing, Thelytoky

** under review in Animal Behaviour*

Introduction

The societies of eusocial Hymenoptera (ants, bees, and wasps) give the appearance of harmoniously cooperating groups, in which most individuals refrain from producing their own offspring and instead help rear the progeny of one or a few reproductives in the colony. Nevertheless, conflicts of interest occur in such societies, and several mechanisms, such as dominance, punishment, and policing, have evolved to resolve such conflicts and to prevent the break down of the society (Ratnieks 1988). For example, in the presence of a fertile queen workers may prevent other workers from reproducing by policing, i.e., they attack egg laying workers or eat their eggs. Policing is expected to evolve when workers are less closely related to the male offspring of other workers than to the sons of the queen(s), *e.g.*, when colonies have multiple related queens or when queens mate multiply (Woyciechowski and Lomnicki 1987; Pamilo 1991; Ratnieks et al. 2006). Furthermore, even when workers are more closely related to worker-produced males than to queen-produced males as in colonies with a single, singly-mated queen, policing may be beneficial if the costs associated with worker reproduction or the benefits of policing to colony efficiency are high (Frank 1996).

Assuming policing behavior to be costly and the availability of resources to vary among individuals, models predict that policing is performed particularly by the strongest group members (Frank 1996). Indeed, not all individuals engage randomly in policing, but a subset of workers within the colony performs aggressive behavior towards uncooperative individuals (van Zweden et al. 2007). Recent studies suggest such policing workers are the most dominant workers (Saigo and Tsuchida 2003; Wenseleers et al. 2005; Wenseleers and Ratnieks 2006; Stroeymeyt et al. 2007), which lay eggs themselves when the colony's queen is removed (Stroeymeyt et al. 2007). Policing may therefore serve for increasing not only the average inclusive fitness of all group members but also the potential direct fitness of the police worker. In this case of 'selfish policing' (Saigo and Tsuchida 2003; Wenseleers et al. 2005; Wenseleers and Ratnieks 2006; Stroeymeyt et al. 2007), the concept of policing (individuals attack egg-laying workers or eat their eggs in order to increase the indirect fitness gains of themselves and others (Ratnieks 1988; Monnin and Ratnieks 2001), partly overlaps with the concept of dominance (high-ranking individuals attack others in order to increase their own direct fitness (Clutton-Brock and Parker 1995; Monnin and Ratnieks 2001).

Workers of a few species of social Hymenoptera are able of producing female offspring from unfertilized eggs by thelytokous parthenogenesis. As recombination is extremely rare (Schilder et al. 1999b), thelytokous mothers produce genetically identical offspring, and colonies are essentially clones (Onions 1912; Heinze and Hölldobler 1995). Conflicts and mechanisms of conflict resolution based on genetic grounds are not expected to

occur in such clonal societies, as workers then are equally related to offspring produced by themselves or any other nestmate (Greef 1996; Hartmann et al. 2003; Pirk et al. 2003). Similarly, in the thelytokous ant *Platythyrea punctata*, workers, which begin to lay eggs in the presence of an established reproductive, are attacked and their eggs are selectively removed (Hartmann et al. 2003). Decreased colony-level performance due to a mismatch between egg layers and nurses might be the driving force of policing in such species (Ratnieks 1988; Hartmann et al. 2003).

Here, we demonstrate that even in the predominantly clonal societies of *P. punctata* policing as a selfish component is retained. Those workers that had been most aggressive towards new reproductive individuals developed their ovaries themselves after the established reproductive had been removed from the colony. We conclude that direct fitness gains may therefore be important even when societies normally consist of clonally identical nestmates.

Material and Methods

Set Up and Housing of Colonies

This experiment employed six colonies of *Platythyrea punctata* (K1, K2, K3, K6, K7, K8; containing 45 to 59 individuals; $X \pm SD = 50.5 \pm 5.8$) that were collected in Puerto Rico in 2003 and 2005. Colonies from the Puerto Rico population are queenless and headed by a single worker, in rare cases by two workers, which monopolize reproduction (Schilder et al. 1999a; Schilder et al. 1999b). Since these workers are not mated, and due to the mechanism of thelytoky, colonies show a clonal structure, with all colony members being identical (Schilder et al. 1999b). Colonies used in this experiment were genotyped prior to this experiment and found to show no intra colonial variation (K1, K2, K3, K6, K8 analyzed in Hartmann et al. 2005; K7 analyzed by Kellner *unpublished data*).

During the experiment, colonies were housed in plastic boxes (20cm x 10cm x 6cm) with plaster floors. A preformed cavity in the plaster (7cm x 5cm x 0.5cm) covered with a glass plate and red foil served as a nest. Colonies were kept under near natural conditions (27°C, 60% humidity, 12 L:12 D cycle) and fed *ad libitum* a mixed diet of honey and pieces of crickets, cockroaches and *Drosophila* flies. The plaster was regularly moistened and a tube with water and cotton served as a permanent water supply.

Behavioral Observations and Experimental Design

Behavioral observations were carried out under a stereomicroscope. All ants were marked individually with dots of enamel paint. Colonies were observed by opportunistic sampling in each session of 10 minutes each, 3-7 sessions per day. The location of individuals (inside /

outside the nest, sitting on brood) and the occurrence of brood care, foraging, allogrooming behavior, and aggression (ritualized aggression: antennal boxing; overt aggression: biting, biting and dragging, gaster flexing, stinging attempts) were recorded individually.

Since reproductive individuals do not have a distinct morphology, we identified them before the experiment by directly observing egg laying or other traits typical for reproductives (see Hartmann et al. 2003). To corroborate our predictions, individuals suspected to be the reproductive were then separated from the colony overnight (ca.12 hours) and checked for freshly laid eggs the next morning.

To induce policing behavior, we applied a standard experimental design following Hartmann et al. (2003). By splitting the colonies first into two fragments (phase 1) we induced the establishment of a second reproductive individual, by reuniting the two parts (phase 2), we caused aggression to arise because of the presence of a supernumerary reproductive. By expanding the experiment with a third phase (the two reproductives were removed, and the establishment of a further reproductive was induced), we could determine whether individuals engaged in aggression in phase 2 became reproductive in phase 3. This association might clarify if aggression has a selfish component. An overview of the different phases of the experiment is given in **Figure 1**.

Phase 1: splitting of colonies induces the establishment of an additional reproductive

Colonies were split into two fragments with one fragment containing the established reproductive individual (fragment 'A') and one fragment without reproductive individual (fragment 'B'). Non-reproductive workers were distributed randomly between the two colony halves. Both colony fragments were observed, whereby colony fragments without the reproductive individual were observed until new reproductive individuals had been established and new eggs appeared. In consistence with former observations (Hartmann et al. 2003) this generally occurred after a period of 5-15 days with 30-53 observation sessions of 10min each (300min – 530min total observation time; X = 441.7min). To confirm reproductive status, individuals were separated for one night (ca.12 hours) if they were not directly observed laying eggs.

Phase 2: reunion of colonies induces aggression

Thereafter, both fragments were reunited. After reuniting the colonies in Phase 2, policing behavior by aggression against the old and new reproductive individuals (OR and NR respectively) was observed, as shown before by Hartmann et al. (2003). The presence of a supernumerary reproductive caused aggression between the two reproductives and other colony members. The colonies were observed over several days until aggression had ceased

(10-35 days, 35-52 sessions of 10min each; 350min - 520min total observation time; X = 436.6min).

Phase 3: orphaning the colonies induces establishment of next reproductive

We removed all old and the new reproductives plus all eggs and kept them frozen for further analyses. Colonies were than observed until new eggs were discovered (which indicates the establishment of a new reproductive individual) in the colonies and aggression had ceased (observation time: four to five days, 16-17 sessions of 10min each; 160min – 170 min total observation time; X = 165min; first eggs appeared already two to three days after removal of the former reproductives, which suggests that policing workers had already begun to develop their ovaries during phase 3). To test whether aggressive behavior has a selfish component, we investigated whether this new reproductive had been engaged in aggression in phase 3. If aggression serves selfishness, the individual, which becomes reproductive in phase 3, should have been among aggressive individuals in phase 2.

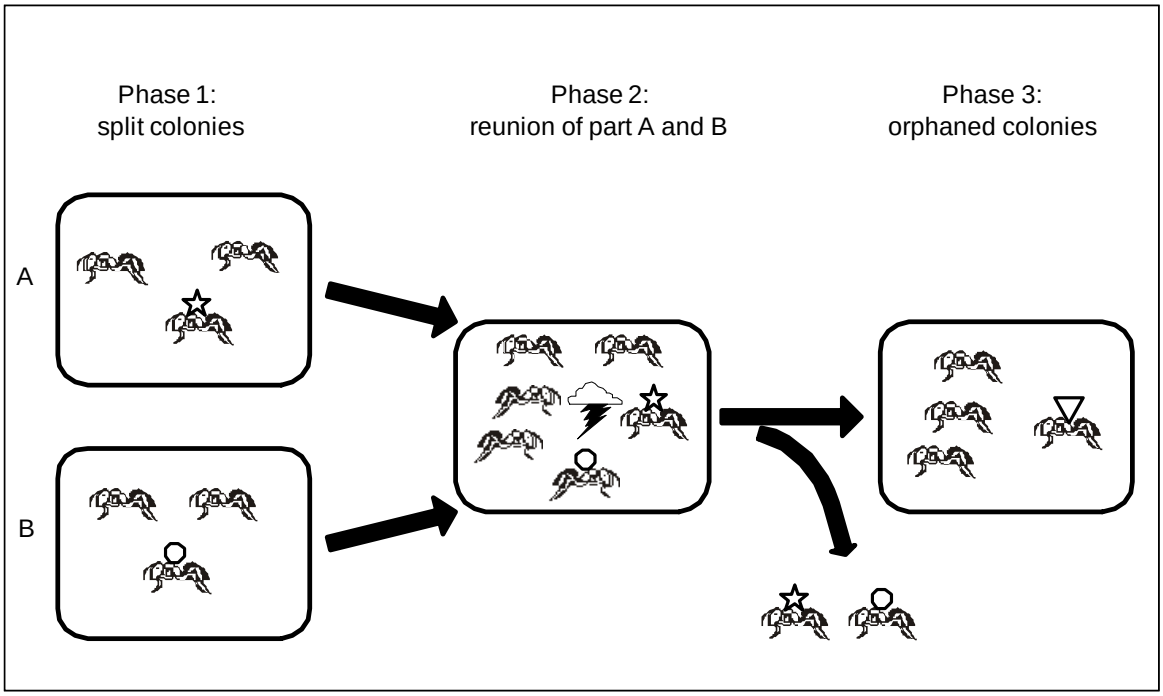

Fig1. Experimental design. Prior to the experiment, the reproductive individual (marked with a star) was determined by directly observing egg-laying. In phase 1, colonies were split into two parts 'A' and 'B', with part 'A' containing the reproductive. In part 'B', a new reproductive individual (marked with a circle) quickly established. In phase 2, both parts were reunited and aggression between the two reproductives and other individuals was recorded. The two reproductives were then removed from the nest, and in phase 3, the orphaned colonies were observed until a new reproductive individual (marked with a triangle) was established. After the observations, all individuals were dissected to record the state of ovary activation.

Ovarian Activation

After observations, all individuals were frozen and dissected under a stereomicroscope to assess the activation of their ovaries. As described in Hartmann et al. (2003), individuals were

categorized by their state of ovarian activity: Status I: undeveloped ovaries (ovary length: $X \pm$ SD = 1.40 ± 0.88 mm), Status II: developed ovaries, but no yellow bodies (ovary length: $X \pm$ SD = 4.76 ± 1.45 mm) and Status III: fully developed, mature ovaries with oocytes and yellow bodies suggesting former egg laying activity (ovary length: $X \pm$ SD = 8.44 ± 2.43 mm). We determined quantitatively that our assignment of categories indeed corresponded to ovary length, since ovary length differed significantly among all three categories (ANOVA: $F_{2,134} = 121.9$, $P < 0.0001$). Specifically, we found that ants with ovary status III had the longest ovaries and ants with status I, the shortest (post hoc Scheffé's test: Status I-II: $P < 0.0001$, Status I-III: $P < 0.0001$, Status II-III: $P < 0.01$).

All analyses were conducted with STATISTICA version 6.1 (Statsoft 2003). For comparing the number of aggressive attacks received by reproductives and non reproductives, two-sample permutation tests were performed with PAST version 1.82b (Hammer et al. 2001).

Results

Whereas in natural, un-manipulated colonies only infrequent ritualized aggression (antennal boxing) was observed (observations before colony splitting), in reunited colonies workers acted much more violently by stinging, biting, dragging, and immobilizing the opponent. ORs and NRs were observed fighting against each other, showing sting smearing behavior, again as previously observed (Hartmann et al. 2003). Sting smearing appears to cause non-reproductives to join the fight and to attack the ORs and NRs. Generally, non-reproductives did not show any preference for either of the reproductives (see **Table 1**). An overview over the number of attacks reproductive individuals received from other reproductives and non reproductives is given in **Table 1**.

Table 1. Number of attacks old and new reproductives (OR, NR) received from non-reproductives and other reproductive individuals in colonies of the parthenogenetic ant *Platythyrea punctata* after the re-unification of colony fragments (for details see text, Chi square tests, χ^2 and *P* values are given in the table).

colony	reproductives	number of received attacks by non reproductives	number of received attacks by reproductives	χ^2	*P*
K1	OR NR	0 7	0 8	15	p<0.001
K2	OR NR	3 1	1 1		-
K3	OR NR	15 0	6 0	21	p<0.001
K6	OR NR1 NR2	15 11 10	36 22 6	0.04	p=0.84
K7	OR NR	0 1	0 1		-
K8	OR NR	1 1	2 1		-

In four of six colonies, reproductives (OR or NR) were significantly more frequently attacked than non-reproductive individuals (two-sample permutation test, see **Figure 2**).

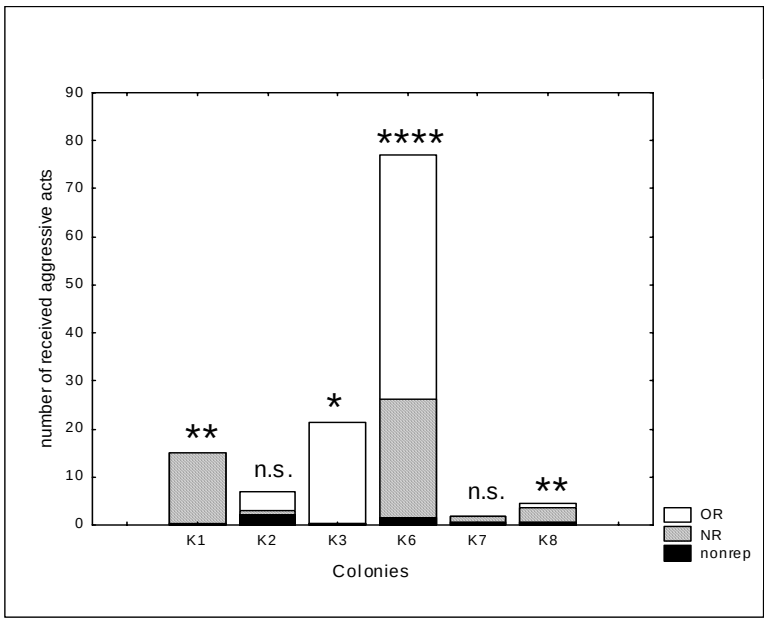

Fig2. Total number of attacks received by old reproductives (OR), new reproductives (NR) and non-reproductive workers (nonrep) during the policing phase (Phase 2) in colonies of the clonal ant *Platythyrea punctata*. Number of individuals and total time of observation in each colony: K1: 42 individuals, 350min observation; K2: 35ind, 520min; K3: 31ind, 440min; K6: 41ind, 430min; K7: 22ind, 450min; K8: 14ind, 430min. *P*-values denote the significant differences between the number of received attacks by ORs and NRs together against nonreproductive individuals (two-sample permutation test; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$).

In the policing phase (Phase 2), individuals were not equally likely to initiate aggressive acts. In all six colonies, only a minority of workers exhibited at least one aggressive act, and not all workers attacked one or both reproductives (see **Table 2**).

Table 2. Distribution of aggression and origin of non-reproductive individuals attacking reproductives after the re-unification of two colony fragments of *Platythyrea punctata*. Not all non-reproductive individuals were aggressive, and not all of the aggressive individuals attacked the reproductives. In the splitting phase (phase1), old and new reproductives (OR and NR) had been separated with non-reproductives in two colony fragments 'A' and 'B'. After reuniting both fragments, ORs and NRs were attacked similarly by non-reproductives from both fragments. Freshly eclosed workers (callows), which were born after reunion, were engaged in aggression in five colonies.

colony	total number of non reproductives	total number non reproductives being aggressive	total number of non reproductives attacking reproductives	reproductives	origin of non reproductive attackers
K1	40	9	7	OR	-
				NR	1 'A'; 3 'B'; 3 callows
K2	33	24	4	OR	2 'A'; 1 'B'
				NR	1 'B'
K3	29	19	15	OR	6 'A'; 6 'B'; 3 callows
				NR	-
K6	38	23	20	OR	6 'A'; 6 'B'; 3 callows
				NR1	7 'A'; 1 'B'; 3 callows
				NR2	7 'A'; 1 'B'; 2 callows
K7	20	9	1	OR	-
				NR	1 'A'
K8	12	5	2	OR	1 'A'
				NR	1 'B'

The expected frequency of number of attacks differed significantly from the observed number of attacks (Chi square test, $P<0.00001$; see **Figure 3**).

Some of these attackers had been in the colony fragment with the OR, some in the fragment with the NR, and in three colonies the attackers were freshly born individuals, which eclosed during phase 2 and therefore had not been present during the splitting process. An overview over the origin of aggressive individuals is shown in **Table 2**. This shows that aggressive behavior against reproductive individuals was not influenced by a change of colony odors during phase 1.

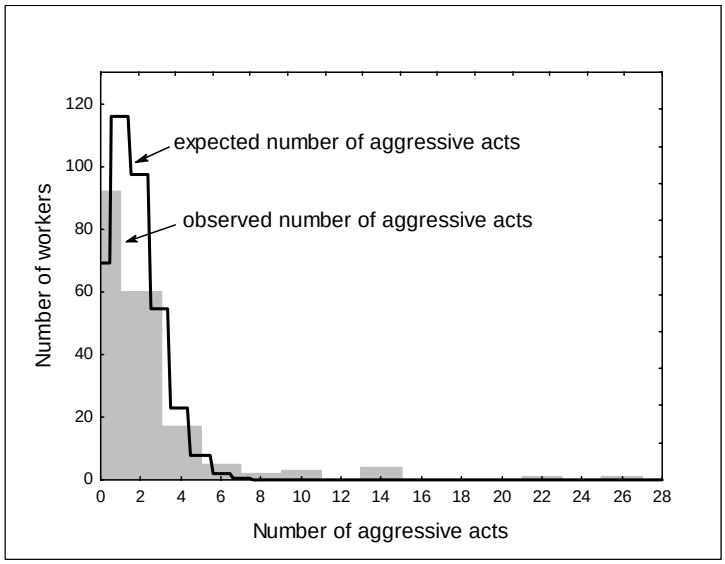

Fig3. Frequency distribution of worker aggression during the policing phase (Phase 2) in colonies of *Platythyrea punctata*. The number of aggressive acts per individual workers is highly skewed, with only few workers initiating most attacks (Chi-Square test: (expected versus observed) of the number of aggressive acts from a Poisson distribution, $\lambda = 1.676$, $X^2 = 149.94$, $df = 2$ (adjusted), $P < 0.00001$).

How often individuals behaved aggressively during the policing phase (Phase 2) was clearly connected with the reproductive status they later obtained in the orphaned colonies (in Phase 3). When dissected after completion of the experiment, individuals being most aggressive during phase 2 had ovaries with the highest status of activation (status III) after orphaning the colonies again in Phase 3 (**Figure 4**; ANOVA: $F_{2, 144} = 3.92$, significant at the 5% probability level after Bonferroni's correction for 3 comparisons ($P' < 0.05$)).

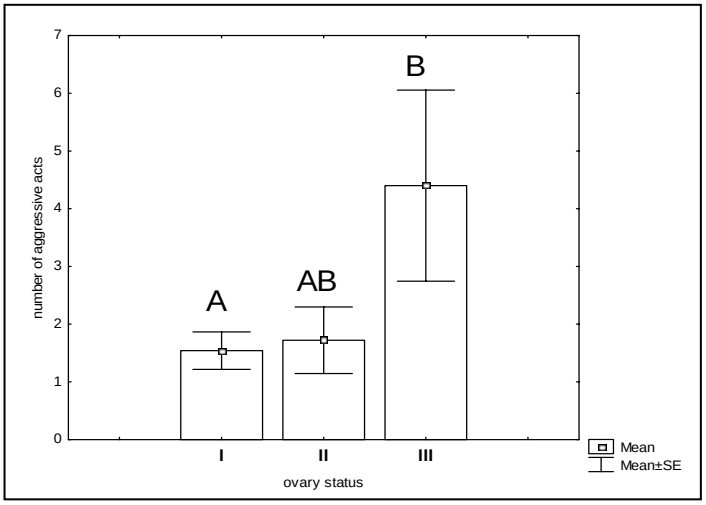

Fig4. Aggressive acts initiated during phase 2 of the experiment by workers of *Platythyrea punctata* with different ovarian status at the end of the experiment. Workers with developed ovaries in orphaned colonies (Phase 3) were most aggressive during the policing phase (Phase 2) (Scheffé's test, $df = 144$). Significant differences are indicated by different letters. Data were square-root transformed prior to statistical analysis but are here depicted untransformed.

In four of six colonies, aggression (number of aggressive acts/individual/10minutes) decreased significantly after orphaning the colonies in Phase 3 (Wilcoxon matched pairs test; K1: $Z = 0.228$, $N = 49$, $P = 0.820$; K2: $Z = 4.432$, $N = 35$, $P < 0.0001$; K3: $Z = 3.823$, $N = 36$, $P < 0.0001$; K6: $Z = 4.457$, $N = 41$, $P < 0.0001$; K7: $Z = 2.366$, $N = 17$, $P = 0.018$; K8: $Z = 0.731$, $N = 14$, $P = 0.465$). Aggression decreased rapidly within a few days, and the appearance of new eggs after 2-3 days suggests that a new egg layer had very quickly become established.

Discussion

Our study demonstrates that policing in *P. punctata* is associated with an increase in the likelihood of an individual becoming reproductive itself in the future. As predicted by policing models (Frank 1996), only the most dominant individuals of a society attacked new reproductives. They were also most likely to become the future reproductives when all established reproductives were removed from the nest. Both freshly eclosed workers, which soon after eclosion establish their position in the hierarchy of un-manipulated colonies (Heinze and Hölldobler 1995; Kellner *unpublished data*), and also a few older workers behaved aggressively. By our manipulation of splitting and later reuniting the colonies, we presumably disturbed the natural rank orders, and individuals that attacked the new reproductives tried to re-establish or improve their social status. When we removed all reproductives and eggs from the colonies, it took only two to three days until new eggs were found within the colonies, and only four to five days until aggression calmed down. This suggests that police workers did not only behave aggressively during the policing phase, but that they at least prepared for egg laying at the same time, probably in response to the changed social structure of the nest.

Our study thus adds to previous reports that policing at least in some species of social Hymenoptera serves not only to increase the average inclusive fitness of all nestmates by minimizing the costs of reduced group productivity (Hartmann et al. 2003) but has a substantial selfish component. Potential direct fitness benefits therefore appear to be important even for workers in societies, which are regularly clonal, such as those of *P. punctata*. Despite of relatedness values close to 1, policing and dominance might be necessary to maintain an efficient reproductive division of labor. By attacking surplus reproductives or destroying their eggs, workers keep up a fine-tuned numerical balance between reproductives and non-reproductives, which maximize the total output of the colony and thus maximize inclusive fitness (Hartmann et al. 2003). But why should individuals bother about who reproduces? Selfish behavior in completely clonal colonies might constitute an atavistic relic

of an ancestral sexual population (Hartmann et al. 2005), maintained in response to the frequent fissions and fusions of colonies that may involve individuals from different clones (Kellner, Barth and Heinze *unpublished*) and also the occasional occurrence of mating and bisexual reproduction (Hartmann et al. 2005). Furthermore, a recent study documents the existence of selfish clones that exploit other clones by monopolizing reproduction in the thelytokous ant *Pristomyrmex punctatus* (Dobata et al. 2009). Policing may minimize the costs of being invaded by such parasitic lineages. Finally, even if sex, recombination, or fusion were extremely infrequent, the resulting small drop in average nestmate relatedness would be sufficient to explain antagonism among potential reproductives, as competition is completely local (West et al. 2001).

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VIII. Chapter eight: It's every ant for herself – selfish and non-nepotistic behavior among and within clone lineages in a thelytokous ant

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Abstract

Kin selection theory predicts not only cooperation, but also conflict within social insect societies. Under kin selection theory, individuals should favor close relatives other non relatives, a behavior known as nepotism. Such nepotistic behavior is expected to occur in policing, where workers prevent each other from laying eggs dependent on the relatedness towards the offspring. The ponerine ant *Platythyrea punctata* reproduces by thelytokous parthenogenesis, and offspring of one reproductive are genetic identical clones. Nevertheless, policing behavior has been documented in this species. When colonies not only exist of one clone lineage, but several, question arises if policing behavior is any different between single clone and mixed colonies, and if individuals show nepotistic behavior towards members of their own clone lineage. To test predictions from relatedness theory, we performed a behavioral assay using artificial colonies of different clone lineage composition. We found that although this species follows parthenogenetic reproduction and individuals are clones, selfishness of single individuals seems to be of more importance than relatedness grounds.

Keywords: dominance hierarchy, nepotism, *Platythyrea punctata*, policing, selfishness, thelytoky;

** manuscript*

Introduction

Kin selection theory, also known as inclusive fitness theory, explains the evolution of altruistic behaviors in the social Hymenoptera (ants, bees, wasps): an individual helping a relative indirectly promotes the transmission of copies of its own genes to the next generation (Hamilton 1964; Hamilton 1972; Trivers and Hare 1976). How many of the genes will be transmitted, depends on the relatedness between the actors, the benefit the act brings to the recipient and the cost the act brings to the actor. Since members of social insect colonies in general are related to each other, but are not genetically identical clones, potential conflicts are expected to occur within colonies over partitioning of reproduction, male parentage, caste determination and colony sex ratio (reviewed in Keller and Chapuisat 1999; Heinze 2004). Kin selection theory predicts a dynamic equilibrium between cooperation and conflict, where the evolution and maintenance of cooperation at the colony level depends on the resolution of conflicts among selfish individuals. Several mechanisms like queen and worker policing, punishment and dominance hierarchies are thought to have evolved to resolve these conflicts (reviewed in Ratnieks et al. 2006). In most social insect colonies workers are not completely sterile, but retain the ability to lay haploid male eggs.

Policing behavior (Ratnieks 1988; Ratnieks and Visscher 1989; Frank 1995; Frank 2003) is an action whereby workers prevent other workers from reproducing such male eggs, and this behavior is considered to be selectively favored then workers are more related to their mother queen's sons than they are to other workers sons, considering genetic relatedness as a key component in Hamilton's rule (Ratnieks et al. 2006; Wenseleers and Ratnieks 2006). However, theory has been shown that if worker policing has colony level benefits, it can be selected for other reasons than relatedness, *e.g.* if it enhances colony productivity (Ratnieks 1988). By studying queenless ponerine ants, where workers can mate and become 'gamergates' (Peeters 1993), the original definition of policing got broadened, so that it can also include worker reproduction of any kind, not only males (Monnin and Ratnieks 2001). One aspect to take into account here is the difference between policing behavior and dominance behavior: whereas in policing the policers seek to increase their indirect fitness (by supporting relatives to reproduce), in dominance interactions competing individuals seek to increase their direct fitness (by monopolizing reproduction) (reviewed in Monnin and Ratnieks 2001). In some cases it has been shown, that the borders between policing and dominance behavior can disappear, resulting in a behavior known as 'selfish policing' or 'corrupt policing' (Wenseleers et al. 2005). For example in *Temnothorax* ants, workers try to prevent each other from laying male eggs, but at the same time establish a dominance

hierarchy, with top ranking individuals reproducing under queenless conditions (dominance hierarchies in Themnothorax: Heinze et al. 1997; selfish policing: Stroeymeyt et al. 2007).

If genetic relatedness is the key component in the evolution of conflict resolution behavior, kin selection theory predicts that individuals should be able to discriminate and favor close relatives over non-relatives, a behavior known as nepotism. Only in a few cases, direct evidence for nepotism has been found (*Formica fusca,* Hannonen and Sundström 2003), and drywood termites (Korb 2006), whereas more evidence exists for nepotism to be absent (*e.g.*, DeHeer and Ross 1997; Goodisman et al. 2007; Atkinson et al. 2008). The conclusion has been drawn, that worker policing based on relatedness grounds also serves as an evidence for the occurrence of nepotism (Foster and Ratnieks 2001; Wenseleers 2007).

One exceptional case, where in contrast to most other ant species colony members are indeed genetic identical clones, and direct and indirect fitness are the same, occurs in the ponerine ant species *Platythyrea punctata.* The predominant mode of reproduction is thelytoky (parthenogenetic reproduction of diploid females from unfertilized eggs, Heinze and Hölldobler 1995), and queens are absent in most populations (Schilder et al. 1999a). Genetic studies show that offspring of a laying worker are genetically identical clones to the mother. Since workers are totipotent in this species, they have particularly strong interest to become reproductively dominant, and therefore conflict is expected to arise about partitioning of reproduction and mothering offspring. On the other hand, since colonies consist in this case of clones, conflicts based on genetic grounds are not expected. However, colonies have been observed to be headed by a single reproductive, which is stabilized within a dominance hierarchy (Heinze and Hölldobler 1995), and even more surprising, policing behavior has been documented in this species (Hartmann et al. 2003). It was suggested that in such thelytokous reproducing species (see also Cape honeybee: Moritz et al. 1999; Beekman et al. 2002; Pirk et al. 2003), worker policing should be selectively neutral on genetic grounds (Wenseleers and Ratnieks 2006), but might have been selected for other reasons, like colony – level costs of worker reproduction, and / or if it gives policing workers a greater opportunity to reproduce directly. Uncontrolled thelytokous reproduction has been referred as 'social cancer' and should not be adaptive in the long run (Oldroyd 2002). Indeed it was shown in *P. punctata* that colonies were not able to rear additional brood, and that the prevention of uncontrolled worker reproduction might be a mechanism of 'birth control' (Hartmann et al. 2003). Lately it has shown that also selfishness might play a role (Brunner, Kellner and Heinze *in review*). However, genetic analyses of field colonies revealed that about 30 - 40% of colonies do not consist of identical clones, but more than one clone lineage. Such

heterogeneous colonies can be the result of fusion of different colonies, something which can be performed easily in the lab (Kellner, Barth and Heinze *unpublished data*).

The aim of this study is to determine whether policing definitely occurs in the absence of genetic conflicts, since natural colonies, used in former studies, cannot always be assumed to be clones. If where are policing and conflicts about partitioning of reproduction for genetic reasons, according to relatedness theory we would expect individuals nepotistically favoring their own clone lineage over foreign clone lineages in mixed colonies. We specifically sought to determine which individual became the new reproductive in orphaned colonies. We discovered that most within colony aggression was caused by dominance interactions between reproductives and high ranking individuals. The individuals, that engaged in aggression and occupied a high rank within the colony hierarchy, were most likely to become reproductives themselves, and this behavior was independent on nepotism towards own clone lineages.

Material and Methods

Setting up experimental colonies

Colonies of *Platythyrea punctata* were collected in Puerto Rico (October 2005), Dominican Republic (November 2006) and Barbados (June 2007). Prior to this experiment, colonies were genotyped with five polymorphic microsatellite markers developed for this species (Schilder et al. 1999b). As source colonies for this experiment, we chose colonies which contained no intra-colonial variation and which stem from different thelytokous populations.

For setting up artificial clone colonies, only freshly eclosed workers and brood items were used. Four single-clone colonies (SC1, SC2, SC4 and SC8) were established by setting up ten to twelve freshly eclosed callows and five to ten cocoons originating from the same source colony in a fresh nest. Four double-clone colonies (DC1, DC2, DC7, and DC8) were established by setting up two to five callows and four to five cocoons from one source colony together with four to seven callows and four to five larvae from another source colony. Earlier eclosing new workers could therefore be assigned to the clone lineage, the cocoons were taken out. For these source colonies, colonies from different populations were chosen. Freshly eclosed callows show no non-nestmate aggression, and with this method we could obtain colonies containing each two different clone lineages (in the following referred to as 'A' and 'B'). All ants in both single and double clone colonies were marked individually with dots of Edding marker. Freshly eclosing new workers were marked right after eclosion. Experimental colonies were reared in the lab over a period of one month under near natural conditions (27°C, 60% humidity, and 12:12 light dark photoperiod) and fed three times per week a mixed diet of honey, *Drosophila* flies and pieces of crickets and cockroaches. The behavioral

assay was started after confirming freshly laid eggs in each colony, confirming the existence of an established egg layer. An overview over colony composition after the rearing period, at the time of starting the experiment is given in **Table 1**.

Table 1. Colony composition of experimental colonies at the start of the experiment.
SC: single clone colonies; DC: double clone colonies; Source colonies stem from different *P. punctata* populations. Whereas single clone colonies represent true clone colonies with R=1, double clone colonies contain two different clone lineages 'A' and 'B', with R=1 within and R=0 between lineages.

colony	source colony	clone lineage	colony composition	
			workers	brood
SC1	Barbados-Turner's Hall 8		13	10 cocoons
SC2	Barbados-Turner's Hall 1		21	1 cocoon
SC4	Dominican Republic-Rancho Wendy 6		12	1cocoon
SC8	Puerto Rico-Sabana 4		8	1 cocoon
DC1	Puerto Rico-Sabana 4	**A**	6	4 cocoons
	Dominican Republic-Monte Plata	**B**	5	5 larvae
DC2	Dominican Republic-Miches 9	**A**	6	2 cocoons
	Puerto Rico-Sabana 9	**B**	6	5 larvae
DC7	Dominican Republic-Rancho Wendy 7	**A**	5	2 cocoons
	Barbados-Turner's Hall 12	**B**	7	4 larvae
DC8	Dominican Republic-Rancho Wendy 12	**A**	7	3 cocoons
	Barbados-Turner's Hall 2	**B**	5	5 larvae

Behavioral assay

Colonies were transferred in plastic boxes (20cm x 10cm x 6cm) with plastered floor. A preformed cavity in the plaster (7cm x 5cm x 0.5cm) was covered with a glass plate and red foil, which served as a nest. The plaster was regularly wetted and a tube with water and cotton served as a permanent water supply. To avoid any aggression by food limitation, the colonies were fed *ad libitum* a mixed diet of honey, pieces of crickets and cockroaches, and *Drosophila* flies.

Behavioral observations were carried out in a climate chamber with near natural conditions (27°C, 60% humidity and 12:12 light dark photoperiod), under a stereomicroscope.

Colonies were observed by opportunistic sampling in sessions (each scan 5 minutes, several scans per day). Whereabouts of individuals (inside / outside the nest) and brood care, foraging, allogrooming behavior and aggression events (ritualized aggression: antennal boxing, violent aggression: biting, biting and dragging the opponent, gaster flexing, stinging attempts) were recorded individually. For statistical tests, frequencies of behaviors were calculated by: behavior / number of individuals / 10minutes. All statistical tests were conducted in STATISTICA 6.0 (Statsoft 2003).

Behavioral observations were carried out in four different phases, according to classical policing experiments:

Phase 1:

Colonies were observed over a period of five days (three sessions per day, 5 min per session) until the egg laying individual was identified. If the reproductive was not identified by observing egg laying, the suspicious individuals were separated from the colonies for one night (12 hours). Finding freshly laid eggs the next morning was confirming reproductive status.

Phase 2:

Each colony was split into two parts, one containing the reproductive ('queenright'), and one without reproductive ('queenless'). Individuals were separated haphazardly in the two parts, and in the double clone colonies we made sure to have a 50% distribution of the two clone lineages in each half. The part without reproductive was observed until the first eggs appeared in the colonies (nine days observation period with three sessions per day, 5 min per session, except colony dc7: six days of observation), indicating the establishment of a new reproductive. Again, to confirm reproductive status, individuals were separated for twelve hours over night, and screened for fresh eggs the next morning.

Phase3:

The two split parts were reunited and observed ongoing for 20 minutes each, then following observation sessions with 5 min per session over several days until aggression calmed down (SC2: 3 days, SC1: 4 days, all others: 5 days).

Phase4:

The two reproductives and all eggs were removed from each colony. The now orphaned colonies were observed over the following 3 days (3 sessions per day, 5 min per session) until new eggs were discovered in the colonies (after 3 days!).

State of ovaries

After the experiment, all individuals (including reproductive individuals) were frozen and dissected to measure ovary length and record state of ovaries. Individuals were categorized by their reproductive status: Status I: not developed ovaries, Status II: developed ovaries, Status III: fully developed mature ovaries with yellow bodies suggesting former egg laying activity. Length of ovaries in Status I (n=66 individuals; median ovary length=0.87mm; lower and upper quartile: 0.00; 2.52) is significantly shorter than Status II (n=5; median=6.96; 4.52; 7.83) and Status III (n=22; median=10.35; 8.70; 10.70), but there is no significant difference between Status II and Status III (Kruskal Wallis, $H=55$, $p<0.0001$).

Results

Policing behavior and aggression profiles

Policing behavior was observed in both single and double clone colonies. While aggression rates were low during Phase I and Phase II, aggression rate was significantly increased in Phase III, and went down again in Phase IV (comparison of the total aggression: Friedmann ANOVA, χ^2 =13.5000, d.f. 3, p<0.004; see **Figure 1**). The same pattern was observed both for double and single clone colonies, but remarkable, aggression profile of double clone colonies were overlaying the one of single clone colonies. Violent aggression such as biting, biting and dragging the opponents antennae and legs, gaster flexing with smearing the opponent and stinging attempts were observed in phase III and IV, whereas these behaviors were absent in phase I and II.

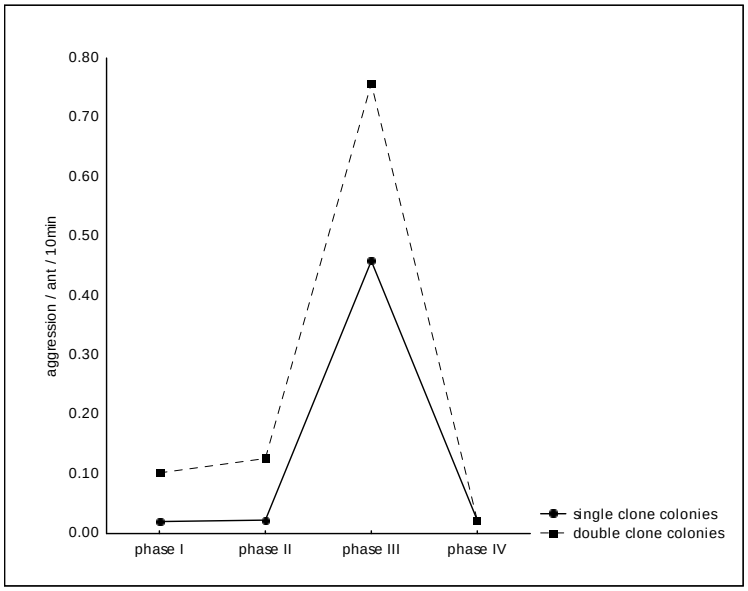

Fig1. Aggression profile during the different phases of the experiment.
Phase I: non manipulated colonies; Phase II: after splitting the colonies, observation of the parts without reproductives; Phase III: reunion of both parts, now each containing a reproductive; Phase IV: orphaned colonies after removing both reproductives; Solid line: single clone colonies; interrupted line: double clone colonies. Aggression rate is significantly higher in phase III than in the other phases (Friedmann ANOVA, χ^2 =13.5000, d.f. 3, p<0.004).

In phase III violent aggressions were significantly higher than ritualized aggression (Wilcoxon matched Pairs test T = 0.00 p=0.0117; see **Figure 2**).

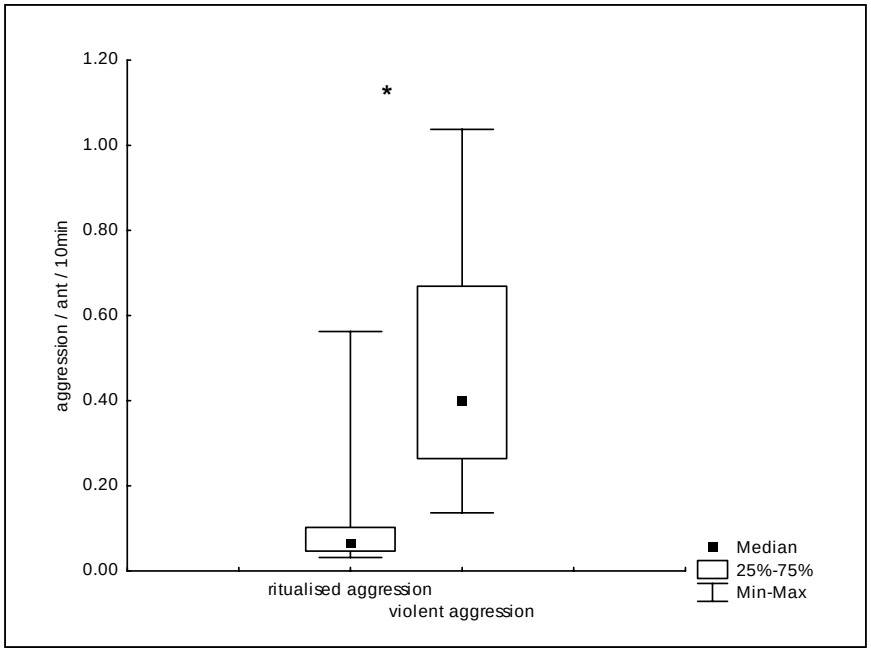

Fig2. Observed aggression in Phase III: Frequency of violent aggressive acts (stinging, biting, gaster flexing) is significantly higher than ritualized aggression (antennal boxing) (Wilcoxon matched Pairs test T = 0.00 p=0.0117).

In 6 out of 8 colonies, in more than 50% of the observed total aggression the two reproductives were involved (In colonies dc7 and dc8 the reproductives were involved in 33% and 40% of the observed total aggression). In total, reproductive individuals were more often attacked than non reproductive individuals (Wilcoxon matched Pairs test T 2.000 p=0.025), suggesting policing behaviors, and no influence of separation during the splitting phase II.

Old reproductives and new reproductives were attacked similar (Wilcoxon matched Pairs Test T=16.000, p=0.779).

Egg laying was observed in SC2, SC8, DC7 and DC8 by the new reproductive. In DC2 the old reproductive was observed egg laying. In all colonies new reproductives were thrown out of the nest by old reproductives and vice versa, but only in one colony the old reproductive died (SC2). In all other colonies, after three days of observation aggression calmed down.

These results show that there is indeed policing behavior, in both single and double clone colonies. Most of the observed aggression took place between the two reproductives. Other individuals joined the fight between the reproductives (probably because of smearing behavior), but did not start a fight with the reproductives.

Differences between single clone and double clone colonies

Comparison of single clone colonies and double clone colonies revealed indeed differences in some of the phases. In phase I, the non manipulated state, double clone colonies showed

significant more aggressive and allogrooming behavior when single clone colonies (MW U test, U=0.00, p=0.021 for aggressive behavior; U=0.50, p=0.03 for allogrooming). In phase II, the separation phase, significant more aggression was observed in the double clone colonies than in the single clone colonies (MW U test, U=1.00, p=0.043), whereas no differences were found in the frequency of allogrooming. In phase III, the policing phase, no differences were observed between single clone and double clone colonies. Concerning aggression between the two reproductives in phase III, were was no difference between single and double clone colonies (MW U= 4.000 p=0.248), suggesting that in any case the two reproductives fight with each other, regardless of relatedness. In phase IV, the orphaned state, double clone colonies showed significant more allogrooming than single clone colonies (MW U test, U=0.00, p=0.021), whereas no differences were observed for aggressive behavior. An overview for the comparison of single clone and double clone colonies is given in **Table2**.

Table 2. Aggressive and allogrooming behavior in single clone (SC) and double clone (DC) colonies. Frequencies of aggression and allogrooming were calculated by behaviour/ant/10minutes. Medians and lower and upper quartiles are given. Significant p values are given in bold (MW U test).

phase	behavior	SC	DC	U	p value	
phase I	aggression	0.0218 (0.0050-0.0357)	0.0879 (0.0610-0.1434)	0.00	**0.021**	**DC > SC**
	grooming	0.1865 (0.1587-0.1944)	0.2444 (0.2056-0.2783)	0.50	**0.03**	**DC > SC**
phase II	aggression	0.0134 (0.0070-0.0389)	0.1173 (0.0617-0.1914)	1.00	**0.043**	**DC > SC**
	grooming	0.1717 (0.1574-0.1920)	0.1914 (0.1451-0.2407)	7.00	0.0773	DC = SC
phase III	aggression	0.4364 (0.3301-0.5872)	0.6291 (0.2893-1.2250)	6.00	0.564	DC = SC
	grooming	0.5988 (0.4138-0.6983)	0.4149 (0.3655-0.4688)	4.00	0.248	DC = SC
phase IV	aggression	0.0093 (0.0056-0.0188)	0.0111 (0.0054-0.0236)	6.50	0.665	DC = SC
	grooming	0.0250 (0.0198-0.0299)	0.0516 (0.0522-0.0145)	0.00	**0.021**	**DC > SC**

Nepotism and clone lineage preferences

Within the four double clone colonies, to test for nepotism and preferences of individuals for their own clone lineage, behaviors within and between clone lineages were compared within the four phases of the experiment.

In phase I, no differences were found for aggression and allogrooming behavior (grooming: MW U=3.00, p=0.144; aggression: MW U=5.00 p= 0.386). In phase II, there was no significant difference in grooming and aggression between compared within lineages (grooming MW U=4.500 p=0.31; aggression MW U=2.000 p=0.081). In phase III, no significant differences in the behavior within and between clone lineages was found (MW U

test; allogrooming: U=3.500, p=0.19; total aggression: U=8.000, p=1.0; ritualized aggression: U=7.000, p=0.77; violent aggression: U=6.000, p=0.56). Phase IV was the only phase of the experiment, where we found differences in the behavior within than between clone lineages. Whereas no differences concerning aggression (total aggression U=5.000 p=0.39; ritualized aggression: U=6.000 p=0.56; violent aggression U=5.000 p=0.39) were found, the allogrooming rate was higher within clone lineages than between clone lineages (MW U=1.000 p=0.043).

But similarly, reproductives of different clone lineages did not fight more against each other than reproductives of the same clone lineage (MW U=2.00 p=1.00). In one of the four double clone colonies, a consistent clone lineage was observed, whereas in the other three colonies turnovers between the two clone lineages were observed (**Table 3**).

Table 3. Clone lineage membership of reproductives in the double clone colonies (DC).
In colony DC1, all three reproductives stem from the same clone lineage, whereas in the other three colonies frequent turnovers of clone lineages were observed. OR = old reproductive in Phase I; NR = new reproductive, established in the part without OR in Phase II; FR= future reproductive, established in the orphaned colony after taking out OR and NR in Phase IV.

colonies	OR in Phase I	NR in Phase II	FR in Phase IV
DC1	A	A	A
DC2	A	B	A
DC7	B	A	A
DC8	A	A	B

Selfishness as the driving factor

Aggression towards the two reproductives by non-reproductives can theoretically be influenced by two factors: the whereabouts of workers during the splitting phase (non-reproductives might favor their own over the foreign reproductive) and the membership to clone lineages (non-reproductives might favor a reproductive from their own clone lineage over the foreign clone lineage). To test which of these two factors has a higher influence on the amount of aggression, we compared aggression towards both reproductives performed by non-reproductives a) foreign reproductive vs. own reproductive and b) foreign clone lineage vs. own clone lineage. No significant differences were found for the comparison of aggression towards the foreign and the own reproductive (Wilcoxon matched pairs test: T=7.000, p=0.46), and no differences were found in the amount of aggression directed towards

reproductive from the own and the other clone lineage (Wilcoxon matched pairs test: T=14.000, p=1.000). Distribution of aggression is shown in **Figure 3**.

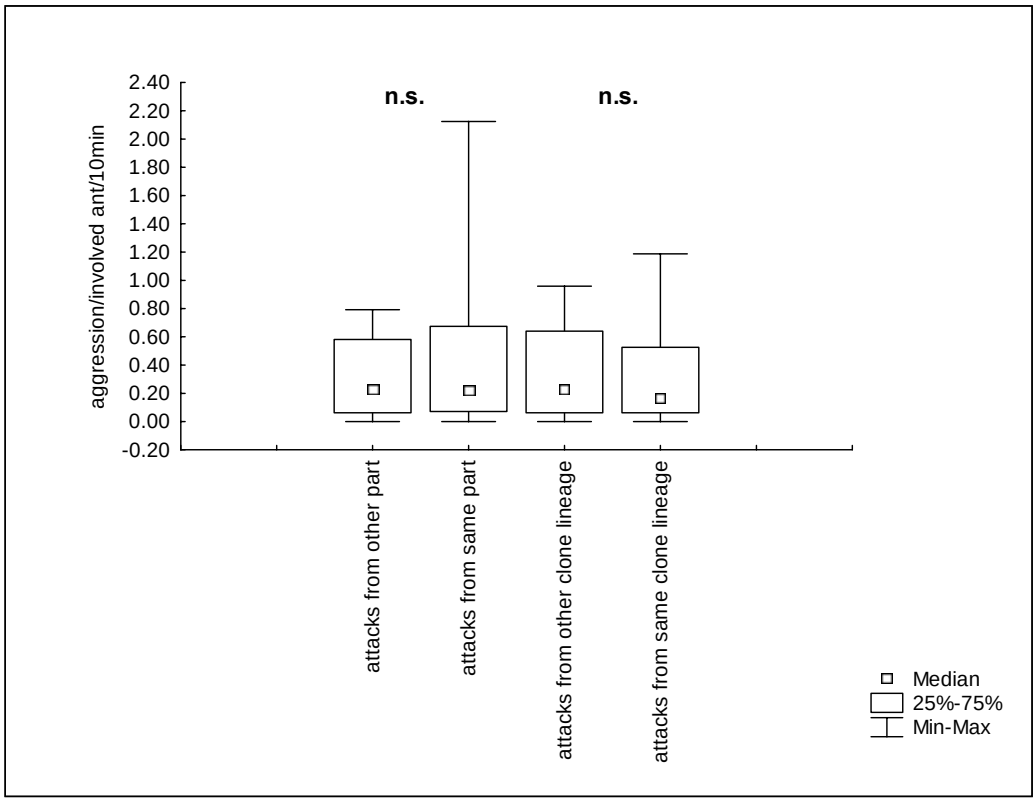

Fig3. Aggression towards the two reproductive during policing phase. Reproductives were similarly attacked by workers which were separated from them during phase II and together with them (Wilcoxon matched pairs test: T=7.000, p=0.463). Concerning clone lineage membership, reproductives were similarly attacked by workers from their own and from the other clone lineage (Wilcoxon matched pairs test: T=14.000, p=1.000).

This suggests that neither the separation phase (interpreted as social affiliation) nor the membership to clone lineages (relatedness) has an influence on aggressive behavior towards reproductives, but we observed a clear trend concerning the question who is becoming the next reproductive. Non reproductive individuals, who were most aggressive in phase III and IV, were likely to develop their ovaries and become the next reproductive after taking out the two former reproductives (phase IV) (MW U test: aggression in phase III: U=77.000, p=0.0031; aggression phase IV: U=68.000, p=0.0018; aggression in phase III and IV: U= 53.500, p=0.0007; see **Figure 4**).

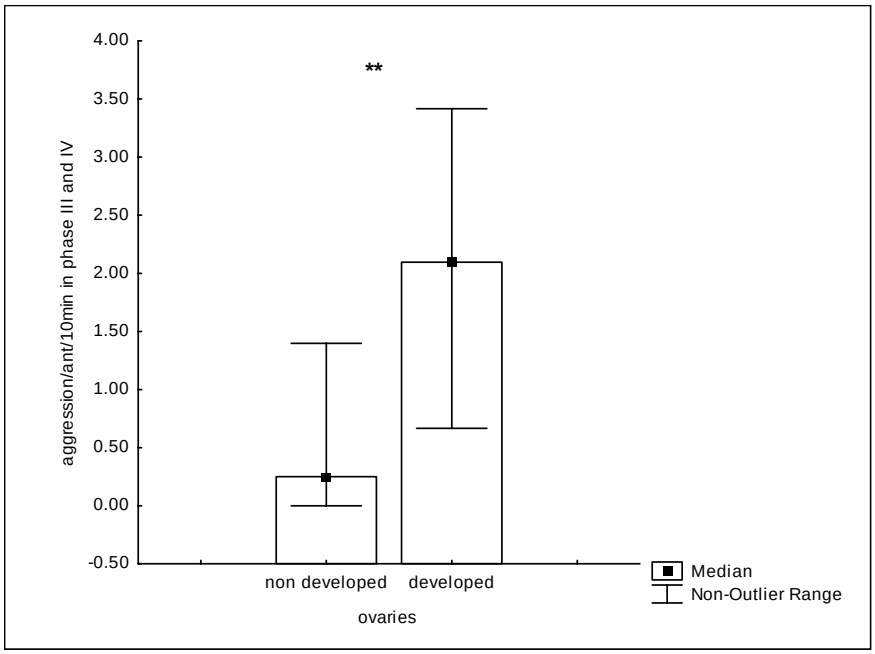

Fig4. Selfishness as the factor influencing aggressive behavior. Non reproductive individuals, which were most aggressive in phase III (attacking the two reproductives) and phase IV (attacking their nestmates) are most likely to developed their ovaries and become reproductives themselves after the colony gets orphaned (comparison of aggression/ant/10min of workers with non developed ovaries and developed ovaries: MW U test: U=53.000, p=0.0007). ** indicate p<0.001.

Discussion

Our data clearly demonstrates that dominance interactions and aggressive policing behavior towards reproductive individuals takes place in both true clone colonies and colonies containing mixed clone lineages. This confirms that policing behavior in this ant indeed occurs without genetic conflicts as suggested before (Hartmann et al. 2003), and that this behavior is not based on relatedness grounds. Moreover, we show that although differences in the behavior of individuals within single and double clone colonies exist, individuals do not show discrimination in the behavior towards their own and the foreign clone lineage. We would therefore state that nepotism in its most extreme form is absent in clone lineages of *P. punctata*. This becomes most clear by comparing the aggressive behavior of individuals during the policing phase. In most cases we observed strong aggression between the old and new reproductive then they encountered, which always resulted in a violent fight between them. Probably stimulated by this, other individuals joined the fight. Old and new reproductives fight with each other in both single and double clone colonies. On relatedness grounds we would have expected that reproductives should fight harder in double clone colonies, when they emerge from two different clone lineages, than they would do in single clone colonies, but no differences were found in the amount of aggression. Also interesting,

individuals joining the fight did not support their own reproductive and chased away the foreign reproductive, nor did they favor the reproductives from their own clone lineage over reproductives from the other clone lineage.

Question arises what is influencing the aggressive behavior then, i.e. what causes individuals to decide whether to join the fight or not? Our data shows, that the driving factor is selfishness: individuals which there most engaged in aggressive behavior, were likely to develop their ovaries and become reproductive themselves then they get the chance.

This selfish behavior was shown before in *P. punctata* (Brunner, Kellner and Heinze *in review*), but with this study we show that selfishness influences aggression even stronger than nepotistic kin discrimination.

Selfishness might be likely to occur in colonies of *P. punctata,* where individuals, unlike in most other ant species, retain reproductive totipotency. Theoretically each group member can become the future reproductive, and we would consider high ranking workers as hopeful reproductives, similar to the case in queenless ponerine ants (Monnin and Ratnieks 2001). Whereas non reproductive workers might voluntary refraining from reproduction (self-policing (Ratnieks 1988), the aggressive behavior of high ranking workers might be a mixture of punishment, social policing and dominance. Selfish punishment as been considered as a second-order altruism and can be an evolutionary stable strategy (Eldakar et al. 2007; Eldakar and Wilson 2008), where altruism can be maintained by selfish individuals punishing cheaters, regardless of kin selection.

In a clonal ant, direct and indirect fitness is only the same within clone lineages, but not between lineages if colonies consist of more than one clone. In single clone colonies, we would have not expected selfish behavior. In double clone colonies, selfishness can be understood at two levels: selfish individuals and selfish clone lineages (which show nepotism towards their own lineage). Even in the double clone colonies we found selfish individuals, not behaving nepotistically. We also did not find evidence for selfish clone lineages, since frequent turnover of reproductives stemming from different lineages took place. This shows clearly, that worker policing in *P. punctata* not only occurs as improvement of colony efficiency (Hartmann et al. 2003), but it might preliminary reflect reproductive competition among selfish individuals.

Absence of nepotistic behavior has been shown in another thelytokous ant, *Pristomyrmex punctatus* (Tsuji and Ito 1986; Tsuji 1988a; Tsuji 1990). In these two studies it was shown that these thelytokous ants show nestmate discrimination and territoriality, and that colony odor labels are transferable and therefore maybe caused by environment rather than genetic conditions. A similar explanation might be the case in *P. punctata*. We showed that the

potential for aggression is higher in double than in single clone lineages, although the ants did not show preferences. Heterogeneous colonies have been found in the field, and colony fusion events have been demonstrated in the lab without aggression towards non colony members but aggression towards reproductive active individuals (Kellner, Barth and Heinze *unpublished data*). The ability to discriminate between nestmates and non nestmates, kin and non kin seems to be very poorly developed in *P. punctata*. It has been suggested that recognition errors in discriminating kin and non kin are too costly, and therefore nepotism might be selected against (Keller 1997). It will also be selected against if helping unrelated recipients is associated with a gain of indirect benefits (*e.g.* larger colonies, higher work force, more genetic diversity within colonies).

When it comes to fight over whom is becoming the next reproductive, it seems better to fight against everybody else than, maybe due to an imperfect mechanism of recognition, favoring and supporting the wrong ones. Therefore it is very likely that individuals behave selfish, because in case they become reproductive they win it all: direct and indirect fitness benefits.

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IX: Chapter 9: Conclusions and Perspectives

The aim of this thesis was to work out genetic and behavioral topics of the facultative thelytokous ant *Platythyrea punctata*. I used previous work as a basis for my own. Specifically, former ambiguous results were clarified, but also new insights in the genetic and behavioral organization of this ant species were gained. Aspects of this thesis range from population genetic analyses, mother-offspring comparisons to behavioral studies investigating policing behavior, selfishness and nepotism. In this general discussion I want to summarize the results, describe some aspects about how thelytoky might have evolved in *P. punctata* and give an outlook on what can be done in the future.

A major contribution of this study was the unraveling of the cytogenetic mechanism underlying thelytoky in *P. punctata*. By mother-progeny comparisons it was showed that offspring of one mother are likely to be genetically identical clones, however, the conclusion to be made is not an apomictic mechanism as previous studies suggested (Schilder et al. 1999b). Rather the appearance of haploid and diploid males suggests that meiosis is occurring during the production of egg cells. Thus an automictic mechanism appears to be operating. The subsequent fusion of non-sister nuclei (central fusion) leads to the observed patterns, if there is no crossing-over. A comparable finding was found in other thelytokously reproducing species, such as the Cape honeybee (Baudry et al. 2004) and the ant *Cataglyphis cursor* (Pearcy et al. 2006). Similar to the findings in the Cape honeybee, recombination rates seem to be reduced in *P. punctata*, at least in the screened microsatellite loci. This might be an adaptation to the parthenogenetic live style, because it prevents well adapted genotypes from breaking up by recombination events.

Another surprisingly finding was that colonies can exhibit intra-colonial variation, which can be partly explained by rare recombination events. However, some genotypes found within colonies were impossible to explain by recombination or sexual reproduction. Adopting an experimental approach, it was showed that the fusion of different colonies belonging to different clone lineages is a likely explanation for the observed pattern of within colony variation. It is unknown yet, if the acceptance of foreign workers into a closed system like a clonal ant colony is due to an inability of nestmate recognition, and /or if it is also a strategy to obtain additional work force. The high intra-colonial genetic diversity is thought to increase work force (Smith et al. 2008) and prevents the spread of diseases (*e.g.* Hughes and Boomsma 2006). Logically, it is possible that colony fusion may be a mechanism that fosters adaptive variation that compensates for the inability to create a high genetic diversity through

conventional sexual means. This question was not completely solved within this thesis, but is certainly an interesting topic for future research.

It was also showed that fusion does not occur without conflicts: while foreign non-reproductive workers are accepted in the colonies, additional reproductives were attacked. This is likely to be a response to supernumerary reproductives within colonies, which is in accordance with a former study (Hartmann et al. 2003), which demonstrated that such 'policing' serves as a mechanism to prevent the colony from uncontrolled brood production. However, the question of who is performing this policing behavior was not fully answered by Hartmann et al. (2003).

In a side project we showed, that aggression in this ant is not due to an aggressive individual 'personality', which might have been a consequence from a higher level of aggressive potential steered by hormone pathway, but that it is situation-dependent in terms of inter-and intraspecific aggression. Different individuals performed aggressive behavior towards reproductives than defended the colony against intruders from foreign species.

The last two chapters demonstrated that such aggressive individuals attacking additional reproductives not only serve as a police to prevent the colony from uncontrolled reproduction, but that they also act in their own selfish interest. In *P. punctata*, colonies exhibit dominance rank orders even in the presence of a fertile reproductive (Heinze and Hölldobler 1995). There seem to be a clear order on who is inheriting the colony after the death of the reproductive. With artificial creation of supernumerary reproductives, this rank order gets disturbed, and high ranking individuals engage in aggression to defend their status. That these individuals indeed act in selfish interest was confirmed by showing that aggressive individuals are likely to become reproductives themselves after removing the old reproductives. Such selfish behavior is not expected to occur within social insect colonies, and finding such a behavior in colonies with clonal relatedness structures is even more surprising.

The last chapter took things one step further, and showed that selfish behavior is even stronger than nepotistic behavior. Selfish individuals showed aggression against reproductives regardless if they were from their own or a foreign clone lineage. Why individuals act selfish is unknown so far. In true clone colonies, indirect and direct fitness gains are the same. For a clone, it plays no role if she herself reproduces or her own clone mate. In mixed clone colonies however, one clone lineage could gain advantage by suppressing the reproduction of the other. But since selfish behavior was also observed within clone lineages, the question remains open. Individuals seemed to notice, if they were in a true or a mixed clone colony (the overall aggression level was higher in mixed colonies), but probably they are not able to discriminate between clone lineages. However, nepotistic behaviors might be also

disadvantaged because they are prone to recognition errors (Reeve 1989; Keller 1997). On the other hand, even if ants are able to discriminate between clone lineages based on relatedness grounds, they still might act selfish: dominance fights and rank orders might be a behavioral leftover from the ancestral state, when reproduction was predominantly sexual. Further studies are needed here, and gas chromatographic analyses might reveal a promising tool for future investigation.

The thesis demonstrates that the life of a parthenogenetic organism is not that simple. True clone colonies with relatedness values of one, do not represent the ideal social insect colony without conflicts, and the organization of clone lineages into colonies reveals much more genetic differentiation .

By reanalyzing the microsatellite primer set originally developed by Schilder et al. (1999b) for *P. punctata*, one more polymorphic microsatellite locus was achieved, which was discovered to be highly suitable for further analyses. These five polymorphic primers and the collection of colonies from more sampling sites, allowed performing accurate population genetic analyses of the whole distribution range of this species on various levels.

The results in the first two chapters show that genotypic variation within as well as between-population variation is much higher than previously observed. It was showed that the distribution pattern of sexual and thelytokous reproducing populations fit the hypothesis of 'geographic parthenogenesis' (Vandel 1928): while thelytoky appears to be indeed the predominant reproduction mode on the Caribbean Islands and Florida, colonies from the mainland were found to reproduce sexually. Populations from Honduras and Belize are likely to show mixed strategies, with populations exhibiting thelytokous and sexual reproducing colonies sympatrically.

These results should probably be considered tentative since the sample size was low (n=20) and sacrificing colonies by killing and dissecting individuals was not conducted. Genotyping individuals within colonies with highly polymorphic microsatellite markers provides a powerful tool to estimate the mode of reproduction underlying the found patterns; however, only dissections screening for mated workers with sperm-filled spermathecea will give unambiguously results. Strong genetic differentiation exists between the island populations and the Mesoamerican mainland, which is probably explained by the poor dispersal ability of this ant. Surprisingly genetic differentiation was not only found between populations, but also within populations. I showed that the number of clone lineages present in different populations can range from one to several, and that most islands contain exclusive clone lineages, which were not found anywhere else. This once more confirms restricted gene flow between populations, and gives a hint that the genetic structure of the different

populations might have evolved through founder effects, gene drift and mutations. Not very much is known about mutation speed of microsatellite markers so far in *P. punctata* (average mutation rates of microsatellite loci range approximately from 10^{-6} to 10^{-2} per generation; reviewed in Jarne and Lagoda 1996; Schlötterer 2000), but the strong differentiation of genotypes suggest that the separation of the island populations and the mainland might have been a long time ago.

The genus *Platythyrea* is a relatively old genus, which evolved probably in the middle Cretaceous (100 million years ago) as a sister group of the other genera within the Ponerinae (Moreau et al. 2006). In the Caribbean region, the genus *Platythyrea* has been found to be at least 20 million years old (late Oligocene or early Miocene), which is shown by specimens found in Dominican amber (Wilson 1985a; Wilson 1985b; Lattke 2003). *Platythyrea* has a pantropical distribution with 37 known species in total, from which eight species are found in the neotropics (Bolton 1995). From this eight *Platythyrea* species, the only living species presently known from the Caribbean region are *P. strenua*, endemic to Hispaniola, and *P. punctata* (Brown 1975). The other neotropic species were never found on the Caribbean Islands sampled in this study, and their distribution seems to be limited to the Central - and South American mainland.

The West Indies have captured the attention of biogeographers for over a century (Wallace 1881). Reasons for this include a large but taxonomically peculiar fauna, high levels of endemism and an intriguing physiography. The Greater Antilles (Cuba, Hispaniola, Puerto Rico and Jamaica) are known to be quite old (middle to late Eocene, ca. 40-30 million years). A large group of smaller, relatively flat islands are on limestone plateaus that are only recently above sea-level, for example, the Bahamas, Barbados and Florida. Most of the Lesser Antilles are volcanically derived. When plate tectonics, sea level fluctuations, water current patterns and hurricane movements are added, the Caribbean region presents a complex challenge for biogeographers. Reviews on Caribbean biogeography can be found in Hedges 2001; Graham 2003; Santiago-Valentin and Olmstead 2004.

The invertebrate fauna of the West Indies have been given attention, with some taxonomic groups studied are ants (Wilson 1985b; Wilson 1988), termites (Scheffrahn et al. 2003), butterflies (Smith et al. 1994), and mites (Niedbala 2004) beside others. Fossil records from Dominican amber have added to the knowledge of the invertebrate diversity (Poinar and Poinar 1999).

The geographical and evolutionary history of *P. punctata* within the Caribbean region remains a mystery yet. Winged females only have been found in Florida and in one specimen in the Dominican Republic. None of the laboratory colonies ever produced winged queens. It

is unknown how the development of a winged female caste is regulated, and if, for example, the island populations lost the ability to produce winged female castes. However, winged females are unlikely to fly due to the lack of ocelli and developed flight muscles (Schilder et al. 1999a). Therefore it is unclear yet how this ant species colonized the islands. If the *P. punctata* population in the Caribbean region evolved through vicariance, i.e. that it is a remnant of a much larger distribution, evolved with land bridges connecting the Greater Antilles with the Central American mainland and a later separation through rising sea levels, or if Caribbean population evolved through dispersal in driftwood, hurricanes and human influence, is the study topic of ongoing phylogenetic reconstruction which is performed at the moment.

However, the present-day surface water current flow is predominantly from the southeast to the northwest, and would have been similar in the past. Present hurricanes are similar in direction, and both are important for Caribbean biogeography (King 1962). One of the best documented cases of flotsam dispersal (drifting plant and wood material) involves a West Indian vertebrate, the green iguanas (*Iguana Iguana* Linnaeus), which is consistent with this pattern. The Iguanas from Guadeloupe were transported during Hurricane Luis 1994 by flotsam to Anguilla, 250km to the northwest (Censky et al. 1998). The closest relatives of most West Indians terrestrial vertebrates (excluding birds, bats and freshwater fish) are found in South America, a fact that is compatible with the hypothesis that current flow and hurricanes are responsible for over water dispersal. A further aspect which should be mentioned is accidental dispersal of invertebrates by human influence. *P. punctata* is not a typical tramp species, however, several sampling localities we visited on our collecting trips were botanical gardens exhibiting collections of neotropical plants, which were probably brought from localities on other islands and the mainland up to one hundred years ago (*e.g.* The Retreat Garden, New Providence, Bahamas, includes a section of Cuban vegetation). It remains a possibility that the species has colonized the islands in this manner. Preliminary phylogenetic data reveal, that the Honduras population is likely to be the center of distribution of this species (Seal, Kellner and Heinze *unpublished data*), and probably the origin of island populations. The phylogenetic relationship of *P. punctata* to the other neotropical *Platythyrea* species is a further topic of ongoing research at the moment.

It is unclear yet, how thelytoky evolved in *P. punctata.* Clear however, *Wolbachia* infection can be excluded (Wenseleers and Billen 2000). Further investigation is needed her, especially testing the hypothesis on parthenogenesis evolving through hybridization and polyploidy (Avise et al. 1992; Vrijenhoek 1998), as shown in unisexual vertebrates (Avise et

al. 1992) and other parthenogenetic insects, such as weevils (Tomiuk and Loeschke 1992; Tomiuk et al. 1994), Orthoptera (Honeycutt and Wilkinson 1989) and stick insects (Tinti and Scali 1996; Scali et al. 2003). *P. punctata* has a rather high number of chromosomes (2n=84; Schilder 1999), which is much higher than *Platythyrea quadridenta* (2n=18; Imai et al. 1983), which reproduces sexually by queens and mated workers (Ito 1990). On the other hand, *Platythyrea tricuspidata*, another species from the genus *Platythyrea*, reproduces sexually too, but contains a similarly high number of chromosomes (2n=92-94; Imai et al. 1983). This high diversity in karyotype might reflect the instability of chromosome numbers also known from other ant genera (Imai et al. 1990). However, it would be interesting to perform further investigations on polyploidy through hybridization in *P. punctata*.

Another hypothesis of the origin of parthenogenesis is the so-called 'contagious origin', which means that parthenogenetic lineages arise secondarily from pre-existing parthenogenetic lineages as a result of incomplete reproductive isolation between sexuals and parthenogens, a mechanism which has been observed in another parthenogenetic Hymenoptera the parasitoid wasp *Venturia canescens* (Schneider et al. 2002; Schneider et al. 2003). The actual incidence of this mechanism in nature is largely unknown, and theoretically could be limited by several factors, that i) parthenogentically produced males must be functional, ii) must be able to successfully mate with sexual females and iii) must transmit their parthenogenetic genes to their offspring, which should result in the emergence of new parthenogenetic lineages (reviewed in Simon et al. 2003). Growing evidence has indeed been found for gene flow between sexual and asexual lineages in aphids and *Venturia canescens* (Schneider et al. 2003).

In predominantly thelytokous populations of *P. punctata*, males are produced, even though rarely, and most of them appear to be normal haploid males. However, performing mating experiments in the lab is always problematic in ants. It is unknown for example under which conditions (seasonality, temperature, humidity, age of males and females) most ants mate, and where the mating takes place (inside or outside the nest, on the ground or mating flights). The Honduras population of *P. punctata*, where sexual and parthenogenetic reproducing colonies have been found, is subject of ongoing research to investigate the possibility of gene flow between the two forms.

A further hypothesis is parthenogenesis through mutation, which is a spontaneous loss of sex (reviewed in Normark 2003)). Mutations in genes involved in production sexual forms and successful meiosis might lead to a permanent loss of sexual reproduction. Such origin of parthenogenesis is for example known from the aphid genus *Sitobion* (Wilson et al. 1997; Wilson et al. 1999). In the Cape honeybee it has been demonstrated that thelytoky is

determined by a single locus (Lattorff et al. 2005). We would exclude this hypothesis in *P. punctata*, since mated individuals have been found, even though rarely on Puerto Rico and Florida (Schilder et al. 1999a; Schilder et al. 1999b; Hartmann et al. 2005), and colonies are able to produce males, although in general no mating takes place. Whereas it is possible to rear new colonies from a subset of freshly eclosed workers in the cases of thelytoky populations, unmated individuals from Texas where not able to reproduce by thelyoky (Seal, Kellner and Heinze *unpublished data*), a similar result when Hartmann et al. 2005 found for the Costa Rican population. In contrast, unmated individuals from Belize and Honduras seemed able to start new colonies by thelytokous reproduction (ongoing research at the moment by Seal, Fiesl and Heinze). I would therefore conclude that the appearance of thelytokous reproduction is likely to be caused by ecological factors and constraints, rather than due to a single gene arisen by mutation or an accidental 'loss of sex', at least within populations where both forms seem to be possible.

The evolution of parthenogenesis is one of the best examples demonstrating 'Dollo's Law' (Dobzhansky 1970; Gould 1970): the inability of a population to reacquire a (recent) ancestral state. It is an extreme type of evolutionary restriction. Two important changes accompany the evolution of thelytoky: the loss of males in the population and a mechanism that maintains the same chromosome number in the egg as in somatic cells. To revert to sexual reproduction within a population, two events are therefore required: fertile males must be introduced, and they must mate with females who produce eggs which can be fertilized by the male. Therefore facultative thelytoky is more likely to be reversible than obligate parthenogenesis (reviewed in Bull and Charnov 1985). Of course this excludes the above mentioned cases of thelytoky induced by microorganisms and curable by antibiotics. In ants thelytoky evolved several times within different subfamilies from the ancestral state of sexual reproduction. However, in all thelytokous species (except *Mycocepurus smithii*) males are still produced, even in the obligate thelytokous ants *Pristomyrmex punctatus* and *Cerapachys biroi*, even though they lack a spermatheca and thus cannot mate. In *P. punctata* it is unknown if workers from thelytokous population could be mated with males from sexual populations, and if thelytoky is reversible in such cases.

Unanswered questions include whether thelytoky is genetically determined or ecologically influenced; depending on the factors, individuals may switch back and forth between the reproductive modes. Possibly, mating, storing sperm and fertilization of eggs is situation dependent. Finally, a promising and exciting area of future work, which would yield new insights in the evolution of sex and parthenogenesis, would be to compare differential gene expression in sexually and thelytokously producing individuals.

X. Summary

Hymenopterans generally exhibit a haplo-diploid sex determination mechanism, in which females are produced by sexual reproduction and are diploid and males are produced parthenogenetically and are haploid. In a small number of ant species, workers are able to produce diploid female offspring parthenogenetically from unfertilized eggs, which is termed thelytoky. One of these species, the ponerine ant *Platythyrea punctata*, is of peculiar interest, because it has been found to have both thelytokous and sexual colonies, which makes it an interesting model system to investigate population structures, colony relatedness patterns and behavioral colony performance that may give new insights on the evolution of thelytoky in social Hymenoptera. The aim of this thesis was to investigate consequences of thelytoky on the genetic population and colony structures and behavioral performance within colonies. By combining genetic and ethological studies, I analyzed the geographic range of thelytokous and sexual populations, the mechanisms underlying thelytoky and male production, as well as other aspects of behavioral performance in social insect colonies with clonal structures.

In **chapter two**, 'Distribution of sexual and thelytokous populations and their genetic structures in the facultative parthenogenetic ant *Platythyrea punctata*', I presented the results from analyses of population structure throughout the entire geographic distribution of this species. Reproductive mode of the sampled population was determined by microsatellite analyses. Specifically I tested the hypothesis of geographic parthenogenesis.

Chapter three 'Population structure of a parthenogenetic ant: *Platythyrea punctata* (Hymenoptera: Formicidae) on Puerto Rico' focused on the predominately thelytokous population on the island of Puerto Rico. This chapter detailed the population structure and shows that, although thelytokous is the primary reproductive mode, there is significant genetic differentiation. Moreover, I showed that colonies may not consist exclusively of one clone lineage. Where this intra-colonial variation might come from, is investigated in the following two chapters.

In **chapter four** 'Mechanism of facultative parthenogenesis in the ant *Platythyrea punctata*' I investigated the mechanism of parthenogenesis underlying thelytoky in *P. punctata* by a microsatellite approach, comparing mother-offspring genotypes. Different mechanisms of thelytoky are discussed, each with different consequences on within colony relatedness patterns. The results showed that thelytoky in *P. punctata* is likely to follow an automictic mechanism. Additionally, we discuss and analyze the appearance of haploid and diploid male production in this ant species.

In **chapter five** 'Colony fusion causes within-colony variation in a clonal ant', within-colony relatedness in two different populations of *P. punctata* was investigated. I showed that the fusion of different colonies of different clone lineages is a possible explanation for the observed within-colony relatedness patterns.

In **chapter six** 'The police are not the army: context-dependent aggressiveness in a clonal ant' I tested the hypothesis whether aggressiveness in *P. punctata* workers is due to an aggressive 'ant personality' or if it is situation-dependent is answered with a behavioral approach.

Chapter seven 'Selfish policing in the clonal ant *Platythyrea punctata*' presents the results of a behavioral study, which showed that although colonies exhibit thelytokous parthenogenesis resulting in clonal colony structures, single workers in this ants follow their own selfish interest in competing for future reproduction.

In **chapter eight** 'It's every ant for herself – selfish and non-nepotistic behavior among and within clone lineages in a thelytokous ant' I investigated selfish worker behavior in colonies with artificial clone compositions and demonstrated that nepotistic behavior, i.e., when one clone lineage favors itself over a foreign clone lineage, is absent in *P. punctata*.

XI. Zusammenfassung

Hymenopteren zeigen für gewöhnlich einen haplo-diploiden Sexdeterminationsmechanismus, oder genauer gesagt, „arrhenotoky“, wodurch weibliche Tiere durch sexuelle Fortpflanzung entstehen und diploid sind, während männliche Tiere parthenogenetisch entstehen und haploid sind. In einer kleinen Anzahl von Ameisenarten sind Arbeiterinnen in der Lage diploiden weiblichen Nachwuchs parthenogenetisch aus unbefruchteten Eiern zu produzieren, ein Fortpflanzungsmechanismus der Thelytoky genannt wird. Eine dieser Arten, die ponerine Ameise *Platythyrea punctata*, ist von besonderen Interesse: Es hat sich gezeigt, dass diese Art zugleich sexuelle und thelytoke Kolonien umfasst, was sie zu einem interessanten Modelsystem macht um Populationsstrukturen, Verwandtschaftsgrade innerhalb von Kolonien und Verhaltensweisen zu studieren. Dies ermöglicht neue Einblicke in die Evolution der Thelytoky innerhalb der sozialen Hymenopteren. Das Ziel dieser Doktorarbeit war es die Folgen der Thelytoky auf die genetischen Populations- und Koloniestrukturen und Verhaltensmuster innerhalb Kolonien zu untersuchen. Mit der Kombination von genetischen und ethologischen Methoden, habe ich die geographische Verbreitung sexueller und thelytoker Populationen, den Mechanismus der Thelytoky und Männchenproduktion, sowie verschiedene Aspekte von Verhaltensmustern in Kolonien mit thelytoker Struktur untersucht.

In Kapitel zwei, ‘**Distribution of sexual and thelytokous populations and their genetic structures in the facultative parthenogenetic ant *Platythyrea punctata*’**, präsentiere ich die Ergebnisse populationsgenetischer Analysen für die gesamte geographische Ausbreitung dieser Art. Der Fortpflanzungsmechanismus wurde mit Hilfe von Mikrosatelliten-Markern bestimmt. Getestet wurde dabei die Hypothese der Geographischen Parthenogenese.

Kapitel drei, **‘Population structure of a parthenogenetic ant: *Platythyrea punctata* (Hymenoptera: Formicidae) on Puerto Rico’**, handelt von der hauptsächlich thelytoken Population auf Puerto Rico. In diesem Kapitel wird die Populationsstruktur als Folge der Thelytoky detailliert behandelt. Es wird gezeigt, dass obwohl Thelytoky der vorherrschende Reproduktionsmechanismus ist, dennoch signifikante genetische Differenzierung innerhalb der Population auftritt. Darüber hinaus zeige ich, dass Kolonien nicht nur aus einer Klonlinie bestehen müssen. Woher die beobachtete intra-koloniale Variation stammen könnte, ist das Thema der zwei folgenden Kapitel.

In Kapitel vier, ‘**Mechanism of facultative parthenogenesis in the ant *Platythyrea punctata*’**, untersuche ich den Mechanismus der der Thelytoky in *P. punctata* zugrunde liegt. Dazu wird ein Ansatz mit Mikrosatelliten Markern verwendet, um Vergleiche zwischen

Mutter und Nachkommen zu vollführen. Verschiedene Thelytoky Mechanismen werden diskutiert, von welchen jeder zu unterschiedlichen genetischen Verwandtschaftsverhältnissen innerhalb der Kolonien führen kann. Die Ergebnisse zeigen, dass Thelytoky in *P. punctata* wahrscheinlich auf einen automiktischen Mechanismus zurückzuführen ist. Zusätzlich wird in diesem Kapitel das Auftreten haploider und diploider männlicher Tiere in dieser Ameisenart analysiert und diskutiert.

In Kapitel fünf, **'Colony fusion causes within-colony variation in a clonal ant',** werden die Verwandtschaftsverhältnisse innerhalb von Kolonien in zwei unterschiedlichen *P. punctata* Populationen untersucht. Mit Hilfe von Verhaltensbeobachtungen wird gezeigt, dass die Fusionierung verschiedener Kolonien mit verschiedenen Klonlinien eine Erklärung für die gefundenen Verwandtschaftsverhältnisse innerhalb der Kolonien ist.

In Kapitel sechs, '**The police are not the army: context-dependent aggressiveness in a clonal ant',** wird die Hypothese geprüft, ob Aggression von *P. punctata* Arbeiterinnen auf eine aggressive "Personalität" zurückzuführen ist, oder ob aggressives Verhalten situationsabhängig ist. Diese Fragestellung wird mit Hilfe von Verhaltensversuchen untersucht.

In Kapitel sieben, '**Selfish policing in the clonal ant *Platythyrea punctata***', werden die Ergebnisse einer Verhaltensstudie präsentiert, die zeigt, dass obwohl Kolonien durch thelytoke Parthenogenese bedingt klonale Strukturen aufweisen, einzelne Arbeiterinnen in dieser Art dennoch eigene selbstsüchtige Ziele verfolgen, indem sie um zukünftigen Reproduktionserfolg konkurrieren.

In Kapitel acht, **'It's every ant for herself – selfish and non-nepotistic behavior among and within clone lineages in a thelytokous ant',** untersuche ich egoistisches Verhalten von Arbeiterinnen in künstlichen Klonkolonien. Hier demonstriere ich, dass nepotistisches Verhalten, im Sinne von der eigenen Klonlinie gegenüber der Fremden zu bevorzugen, nicht vorhanden ist in *P. punctata*.

XII. Appendix: Methods of microsatellite analyses in *P. punctata*

A1: DNA-Extraction

DNA Extraction with CTAB-Method (modified after Sambrook and Russell 2001)

In alcohol stored ants have to be equilibrated with 70% alcohol and distillate water, frozen samples can be used directly. For the use of ant pupae with cocoons, cocoons were removed. Frozen adult ants have to be checked for eggs or larvae between mandibles before use. For handling samples, forceps washed in ethanol were used.

1. Place each individual in a 1.5ml Eppendorf Cup, put label (colony number, individual ID) on the Cup (in aluminum rack on ice!)

2. Crush frozen or equilibrated ants, legs of ants, larvae or eggs in fluid nitrogen and add **500µl** of 1% (2%) CTAB-solution (**65°C**). Make sure plastic pestle are clean before starting. Plastic pestle can be autoclaved or stored in HCl. If stored in HCl: make sure to neutralize and remove HCl before you start! Use a fresh pestle for each sample, or wash them with ethanol between each sample.

Incubate for **1h at 65°C**, cool down to **55°C**, add **2µl** Proteinase K (MBI Fermentas), Incubate **3-4h at 55°C** (old alcohol animals: incubate over night)

3. Add **500µl** Chloroform / Isoamylalcohol (24:1), vortex, centrifuge 5min/14.000rpm, room temperature (RT); prepare fresh 1.5ml Eppendorf Cups with the same labels; transfer upper phase in the fresh Cups (if individuals are very old or dirty or very clean DNA is needed, do this step twice). Step 3. and 4. have to be performed under the fume hood!

4. Add **40µl** NaAc (1/10 Vol., 3M, ph: 4.8) and **350µl** cold Isopropanol (stored at -20°C); Incubate 30min at –20°C (old animals: 1h); this step can also be performed over night.

5. Centrifuge for **30min at 4°C**, 14.000rpm (cool down centrifuge before you start!)
Wash pellet with **300µl 100% ethanol (-20°C),** centrifuge **10min at 4°C**, 14.000rpm
Wash pellet with **300µl 70% ethanol (-20°C),** centrifuge **5min at RT**, 14.000rpm
Wash pellet with **150µl 70% ethanol (RT),** centrifuge **5 min at RT**, 14.000rpm

6. Dry DNA-Pellet for 2min at 50°C or for **10min at RT**; use fume hood!; no ethanol has to be left!!

Resolve the pellet in PCR water (Sigma Aldrich Chemie GmbH): depending on size of specimen, use 50µl for an ant (*Platythyrea* size) or larvae and 25µl for eggs or legs; to avoid contaminations of DNA, use for this step fresh water and sealed pipette tips.

The DNA can be stored in the fridge (4°C) while PCR steps are performed. For longer storage, freeze DNA at -20°C. After being done with all PCRs, for long term storage transfer DNA to -70°C.

Ingredients

CTAB-Solution: 2% (1%) Hexadcyltrimethylammoniumbromid (add after autoclave!)
0.75M NaCl
50mM Tris/HCl ph 8.0
10mM EDTA

Chloroform/Isoamylalcohol 24:1

NatriumAcetat 1/10 Vol., 3M, ph: 4.8

Isopropanol, ETOH 70%, ETOH 100%

Quality control of DNA

Check 5µl of DNA together with loading dye on a 0.8 % Agarose gel (add 5µl of Ethidiumbromid to 50ml Agarosegel). Use 5µl of 1kb size marker as reference (Gene Ruler^{TM}, MBI Fermentas). From gel documentation, quality (and quantity) of the extracted DNA can be estimated. Very thick bands are a sign for a high DNA yield; however it also means lots of contaminations from unclean extractions and high RNA content. Too much DNA can also block PCR. The sensitivity of primers towards too high RNA content is primer specific. In some cases, it might be useful to dilute the DNA sample (*e.g.* 1:50). The quality and quantity of the DNA yield is determining the application volume of DNA in PCR reaction.

A2: Primers and PCR conditions

Table 1. Primer sequences of the *P. punctata* primers

Locus	up	down
2801	5' – CGC TTC CCA TCC CTG TGT – 3'	5' – CGG TTT CCT CTC CTT CCC – 3'
3506	5' – GGA TAA GAT TGG CGG TCG – 3'	5' – TCT GCC GAT GAA AAC CTC – 3'
2902	5' – GAC ATC GGG CGT CTC GTA – 3'	5' – TCA GAA GCG AGT CGA TGA – 3'
4101	5' – CTT TGT ACG CCT TGG ACG G – 3'	5' – GCG GGT GAG AAA AGG GAA T – 3'
3302	5' – GAA GAG CGA GGA AGG CAG – 3'	5' – GCG TCT TGG GAC CAT CTC – 3'

Standard PCR protocol

For each of the primers, the following protocol was used.

Master Mix per sample

10x PCR buffer (-$MgCl_2$)	2.0µl
$MgCl_2$ (25mM)	1.2µl
dNTPs (1mM of each)	4.0µl
Primer forward (label / 5µM)	2.0µl
Primer reverse (5µM)	2.0µl
Taq Polymerase (1U / µl)	0.5µl
PCR water (Sigma Aldrich Chemie GmbH)	7.7µl
DNA	1.0µl
Total reaction volume	20.0µl

PCR conditions for 3506, 4101 and 2902

95°C	5 min
94°C	1min
Annealing temperature	1min
72°C	1min
72°C	5min
4°C	end

Number of cycles: 32

PCR conditions for 2801 and 3302

Temperature program:

95°C	5 min
94°C	1.15min
Annealing temperature	1.15min
72°C	1min
72°C	5min
4°C	end

Number of cycles: 32

Table 2. Annealing temperatures and PCR buffers

Primer	Label on forward primer	Annealing temperature	Buffer
3302	FAM, blue	54°C	$+(NH_4)_2SO_4$-$MgCl_2$
3506	TET, green	50°C	+KCL-$MgCl_2$
2902	FAM, blue	54°C	+KCL-$MgCl_2$
4101	HEX, black	54°C	+KCL-$MgCl_2$
2801	TET, green	53°C	+KCL-$MgCl_2$

Quality control of PCR products

PCR products were checked for quality on 1.5 % Agarosegel, with 10µl sample and loading dye, compared with 10µl of 50 bp standard marker (Gene Ruler^{TM}, MBI Fermentas).

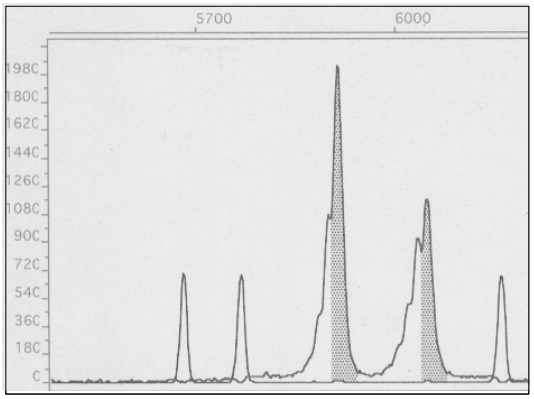
A3: Fragment analyses of microsatellite loci

Depended on strength of PCR product, 0.06 – 1.0µl or dilutions (*e.g.* 1:50, 1:100) per sample were mixed with 18.0 µl PCR-H_2O (Sigma Aldrich Chemie GmbH) and 0.3µl Tamra size standard (Applied Biosystems) for fragment analyses reaction. Samples were incubated 1 min at 90°C for denaturation and analyzed with an ABI PRISM 310 Genetic Analyzer. Running conditions of the sequencer were Inj sec. 5, Inj kY 15.0, Run °C 60.0, Run kY 15.0, runtime 25 min per sample.

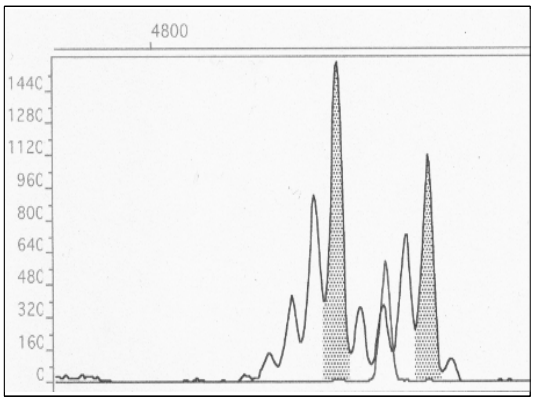
A4: Fragment analyses raw data

Absolute allele lengths of microsatellite fragments were determined using GeneScan® 3.1 Software (Applied Biosystems). Typical shapes of microsatellite peaks are given below. Each locus is shown in the heterozygote state.

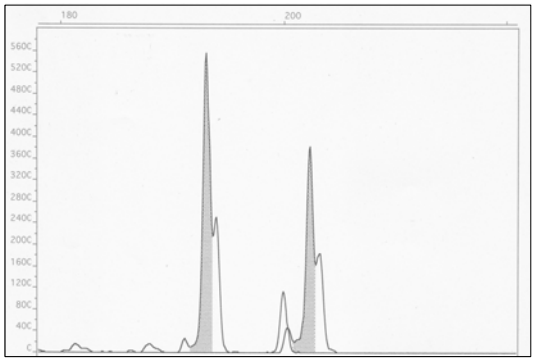
Locus 2801: size range 350 – 400 bp



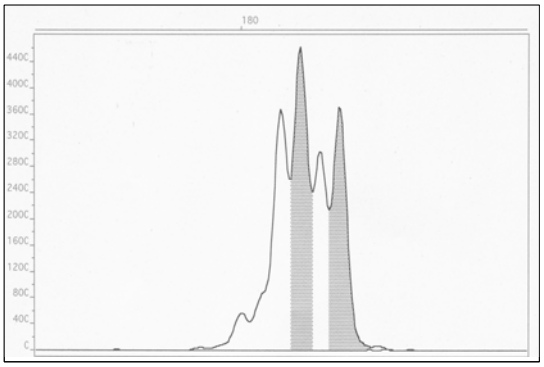
Locus 3302: size range 200 – 270 bp



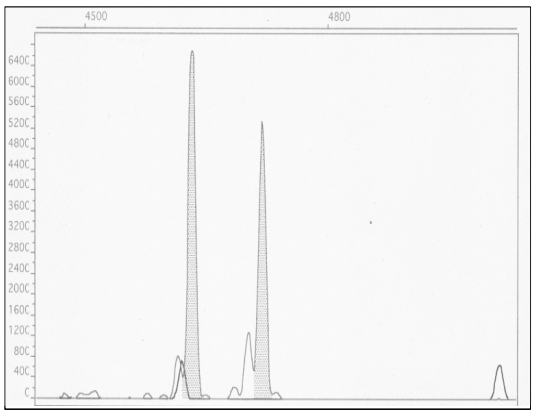
Locus 3506: size range 190 – 270 bp



Locus 2902: size range 180 – 210 bp



Locus 4101: size range 180 – 220 bp



A5: Microsatellite alleles

Table3. Number of alleles for each loci range from 9 to 19. Occurrence of alleles is shown by population: DR: Dominican Republic, PR: Puerto Rico, BB: Barbados, GR: Grenada, GB: Grand Bahama, NP: New Providence, DO: Dominica, FL: Florida, HO: Honduras, BEL: Belize, TEX: Texas.

3506		2902		4101		2801		3302	
measured without +A effect		measured with +A effect							
191	BEL	**171**	HO	**189**	TEX,BEL	**350**	BEL	**200**	TEX,HO
193	DR,PR, BEL,HO	**175**	BEL	**191**	BEL	**362**	TEX,HO	**204**	HO
197	TEX	**181**	PR	**193**	FL	**364**	TEX	**216**	HO
199	HO	**183**	DR, PR, BB, BEL, HO	**197**	BEL,HO	**366**	TEX,HO	**226**	TEX
203	DR,PR, BB,FL, GR,NP, GB,DO, HO	**185**	DR,PR, BB,FL, DO,NP, GB,GR, HO	**199**	DR,PR	**368**	DR,PR, FL,	**230**	TEX
205	FL,BEL, HO	**187**	HO	**201**	DR,PR,BB, FL,GR,NP, GB,DO,HO	**372**	HO	**232**	FL,BEL
255	BEL	**189**	HO	**203**	DR,PR,BB, FL,HO	**382**	DR,PR, BEL	**234**	DR,PR, BEL,HO
257	BEL	**191**	BEL	**205**	HO	**386**	DR,PR, BB,FL, GR,NP, GB,DO, BEL,HO	**236**	DR,PR, FL,BEL
261	BEL	**193**	BEL	**209**	FL	**384**	DR,PR, FL,GR	**238**	DR,PR, BB,FL, GR,GB, TEX,HO
		195	BEL	**211**	DR,PR,BB, FL,GR,NP, GB,DO	**388**	DR,PR, BB,FL, GR,HO	**240**	DR,PR, BB,FL, GR,NP, GB,DO, TEX
		197	TEX,BEL	**213**	BEL	**390**	DR,BB, BEL	**242**	DR,PR, BB,FL, GR,NP, GB,DO, TEX,BEL
		199	TEX,BEL			**392**	BEL	**244**	DR,PR, BB,GR, DO,TEX, BEL
		201	TEX			**394**	BEL	**246**	DR,PR, DO,TEX, BEL
		205	BEL			**396**	BEL,HO	**250**	FL
		211	TEX			**398**	BEL,HO	**252**	TEX,HO
								254	TEX,BEL
								256	BEL
								258	FL,BEL
								262	TEX

Microsatellite loci 3506, 2902, 4101 and 2801 were used in Hartmann et al (2005) to analyze populations from Puerto Rico and Costa Rica. Due to the age of the capillary in the sequencer, running conditions were different, and therefore alleles differ slightly at loci 3506 and 4101 between the former study and this study. An overview over correspondence of alleles for these two loci is given in **Table 4**.

Table 4. Correspondence of alleles. In parenthesis the studied populations are given. PR: Puerto Rico, CR: Costa Rica, TEX: Texas, HO: Honduras, BEL: Belize. The same alleles were found in both studies in the Puerto Rico population. Alleles which were found in Costa Rica in Hartmann et al. 2005 correspond to alleles found in Texas, Belize and Honduras (this study).

3506		4101	
Hartmann et al. 2005	this study	Hartmann et al. 2005	this study
190 (PR)	193 (PR)	198 (PR)	199 (PR)
200 (PR)	203 (PR)	200 (PR)	201(PR)
196 (CR)	197 (TEX)	202 (PR)	203 (PR)
198 (CR)	199 (HO)	210 (PR)	211 (PR)
		204 (CR)	205 (HO)
		196 (CR)	197 (BEL, HO)

A6: Software tools

The number of software packages for the analyses of population genetic data has increased tremendously over the past years. A very good review on software tools for population genetic analyses can be found in Excoffier and Heckel (2006). All software packages used in this study are available for free download in the Internet. Links to download pages are given in Excoffier and Heckel (2006) and below.

CONVERT is a software package which can convert genotypic diploid data files written in standard .txt format into several input file formats for different software programs (Glaubitz 2004). Runs under Windows; this program is extremely useful for the creation of input files for *e.g.* GDA, Arlequin and Genepop.

GDA is a standard program which computes indices of genetic diversity, linkage disequilibrium and F-statistics. It can be used for the performance of hierarchical analysis of genetic variance with up to 4 levels. Private alleles can be detected. Runs under Windows (Lewis and Zaykin 2001);

ARLEQUIN computes various indices of genetic diversity, F-statistics and genetic distances. Exact tests of Hardy-Weinberg-Equilibrium (HWG) and linkage disequilibrium (LD) can be performed. Hierarchical analysis of genetic structures based on the AMOVA framework with

up to 3 levels can be performed. It also includes the feature of Mantel test performance. Runs under Windows (Excoffier et al. 2005);

GENEPOP is an older program running under DOS. It can compute basic indices of F-statistics and genetic diversity, exact test for HWG and LD, and Mantel test. Global test of HWE and LD can be performed using different hypothesis. Additionally it has the function to convert GENEPOP input files into FSTAT input files (Raymond and Rousset 1995).

FSTAT performs calculations for basic indices of genetic diversity, allelic richness and F-statistics. It is possible to compare various statistics among groups of samples. It can also perform multiple regression analysis and Mantel test. A special feature is that it computes column format from matrix format, which is extremely useful for performing Mantel test. Runs under Windows; It also includes the possibility for relatedness calculations (Goudet 1995; Goudet 2001).

RELATEDNESS is a program which has been written especially for the analyses of relatedness relations, *e.g.* in social insects. It can calculate genetic relatedness among and between demographically-defined groups of individuals. This software only runs on Mac-OS systems, and is not easy to use. However, so far no real alternative exists for this program. It is freely available at http://gsoft.smu.edu/Gsoft.html (Goodnight and Queller 1998).

GENECLONE is a software package which provides several tools for the analyses of genetic data of diploid or haploid clonal organisms. It can perform clonal assignment with resampling procedures to sort individuals into identical multilocus genotypes (MLGs), and calculate various indices of genotypic richness and diversity. The program runs under Windows and is available for free at http://si-wagner.ualg.pt/ccmar/maree/software.php?soft=genclon (Arnoud-Haond and Belkhir 2007; Arnoud-Haond et al. 2007).

GDMG (Geographic distance matrix generator) is tool with which GPS data can be transformed into geographic distance matrices, which can serve as input for Mantel tests. GDMG is a Java application, which can be downloaded for free at http://biodiversityinformatics.amnh.org/open_source/gdmg (Ersts 2006).

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XV. Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Dissertation selbständig und ausschließlich unter der Verwendung der angegeben Quellen und Hilfsmittel angefertigt zu haben.

Diese Arbeit wurde bisher weder einer Prüfungsbehörde vorgelegt noch veröffentlicht.

Regensburg, im Mai 2009

Katrin Kellner