# Aging and Social Homeostasis in Social Insects

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**Erklärung**

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation von mir selbständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde. Diese Dissertation wurde weder in gleicher, noch in ähnlicher Form in einer Prüfungsbehörde bereits vorgelegt noch veröffentlicht.

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# Publications

This thesis is based on the following manuscripts:

Walter, B., Rueppell, O., Leonard, A., Heinze, J. *Insemination and longevity in honey bee (Apis mellifera) queens.*

Heinze, J., Walter, B. *Social insects die lonely – ants altruistically protect their nestmates from infections*.

Walter, B., Brunner, E., Heinze, J. *Policing effectiveness depends on relatedness and group size.*

Walter, B., Heinze, J. *The potential fecundity hypothesis: reduction of potential fecundity leads to precocious foraging*.

# Chapter I

# General Introduction

### *Evolution of eusociality – theories and concepts*

Two major questions of evolutionary biology are how eusociality evolved and how it is maintained (Wilson 1971, Maynard Smith and Szathmáry 1995). Eusociality is defined as a system where individuals display three fundamental traits: cooperative brood rearing, reproductive division of labour (*i.e.* occurrence of reproductives and non-reproducing workers) and overlapping of at least two generations in a colony (Wilson 1971). All ants and termites, some bees and wasps (Wilson 1971), an ambrosia beetle (Kent and Simpson 1992), fifty aphids (Aoki 1987, Benton and Foster 1992), seven thrips (Crespi 1992), six snapping shrimps (Duffy 1996) and two mole rat species (Sherman et al. 1991, Jarvis et al. 1994) fall within the definition of eusociality (reviewed in Korb and Heinze 2008).

The existence of non-reproducing individuals was puzzling evolutionary biologists from Darwin on (Darwin 1859). Because natural selection favours individuals that have the greatest personal reproductive success it was unclear why some individuals forego reproduction but help others to reproduce. It may be, as Darwin suggested, that “with social insects selection has been applied to the family and not to an individual” (Darwin 1859). Two major factors driving natural selection are competition and cooperation, but the importance of the latter was overseen for decades. The natural selection theory of Darwin was considered to be a pitiless struggle for life (Huxley 1887). Therefore, when Kropotkin presented his *Mutual Aid: A Factor in Evolution* (1902) he brought back attention to the role of cooperation in evolution (for more details see Gardner and Foster 2008).

Modern concepts concerning eusocial evolution include life history hypotheses as well as genetic theories (Fletcher and Ross 1985). As to life history hypotheses there are three major ones, namely: subsocial hypothesis, semisocial hypothesis and familial hypothesis. According to the subsocial hypothesis (Wheeler 1923) eusocial insects evolved from species with parental brood-care by increasing cooperation between a mother and her offspring which was possible due to the increasing life span of a mother.

The semisocial hypothesis (Mitchener 1958) implies that high sociality evolved from cooperative breeding with reproductive division of labour among non-related individuals sharing the same nest side. Originally the hypothesis was created for semisocial bees as an alternative to eusocial evolution. The reproductive division of labour between non-related individuals lasting for a single generation serves as an example that a system where individuals are not related can not evolve into eusociality (Mitchener 1958, Wilson1971, Fletcher and Ross 1985). However a recent hypothesis (Wilson and Hölldobler 2005) proposes that eusocial species have evolved from the assemblages of unrelated individuals, likewise explained by the semisocial hypothesis. Similarly, the skew selection model theory (Cassill 2003) assumes that eusocial systems originated from unrelated groups, mainly because they used to share scarce resources. Note that there is only little evidence supporting two last concepts and especially the skew selection model theory is logically severely flawed (e.g. wrong interpretation of Hamilton's rule). The familial hypothesis (West-Eberhard 1978) proposes that eusocial insects evolved from associations of related individuals of which some forewent reproduction. Note that subsocial and familial hypotheses assume relatedness among group members as an important pre-adaptation for evolution of eusociality, while Wilson and Hölldobler suggest that eusocial species evolved from unrelated assemblages and that often observed high relatedness in modern eusocial species is only consequence of eusociality.

Apart from the aforementioned hypotheses, a number of theories have been presented to explain the evolution of eusociality. Three of them are of the special importance, namely: inclusive fitness (known also as kin selection theory), group selection and parental manipulation concept. Inclusive fitness theory (Hamilton 1964) states that a behaviour increasing their inclusive fitness of the actor is favoured, according to the inequality: $br - c > 0$, where $b$ refers to the fitness benefit for the recipient, $c$ denotes direct fitness cost for the actor and $r$ is the genetic relatedness of the recipient to the actor as a result of having common ancestors. Therefore, $-c$ denotes a decrease of direct fitness of the actor and $br$ represents gain in indirect fitness by the actor. Hamilton's rule indicates that even costly behaviour, such as refraining from reproduction and helping to rear alien offspring, can be favoured under conditions of sufficient benefits for the actor and its relatives.

Group selection theory (Allee 1951, Wynne-Edwards 1962, Okasha 2006) implies that eusociality evolved from cooperating individuals, according to the rule that selection acts mainly on a group level (between and within groups). Although the theory used to be considered as useless because the canonical derivation of Hamilton's rule can be applied to all levels of selection (Price 1970, 1972, Hamilton 1975, Wilson 1975, reviewed in Gardner and Foster 2008) sometimes it comes back into discussion (Wilson and Hölldobler 2005). Note that although group selection theory is often presented as the opposite to inclusive fitness theory (Cassill 2003, Wilson and Hölldobler 2005), in fact both theories are 'only' two sides of the same coin (reviewed in Gardner and Foster 2008) *i.e.* Price's theorem (Price 1970, 1972).

According to parental manipulation theory (Alexander 1974) eusociality evolved due to unequal parental investment in offspring leading to reduction of reproductive potential of same progeny. Therefore, a parent manipulates (reduces) fitness of some progeny to maximize it's own fitness through other progeny. The main concern of the theory is that any mutation leading to offspring being resistant to the manipulation breaks down the mechanism is unlikely to be evolutionary stable (Crozier 1979).

Although the Hamilton rule provides theoretical framework for the evolution of eusociality, it does not tell what particular mechanisms are involved. Thus, impressive body of data has documented the importance of several traits for social evolution. Many authors point out the fortress-like nest system as an important adaptation for eusocial life (review in Korb and Heinze 2004), others stress the occurrence of a sting apparatus (Kukuk et al. 1989), bivoltism (Seger 1983, Hunt and Amdam 2005), haplodiploidy (Trivers and Hare 1976) and maternal longevity (Wheeler 1923). There are also several ecological factors studied in a diverse set of taxa that may be of major importance for eusocial evolution, namely: population size and density, birth rate, level of exposure to predators and variation in trophobiosis for aphids (Pike and Foster 2008), food-shelter coincidence, strong predator pressure and defence ability for thrips (Crespi 2005), cost of nest-sides and insurance advantages in hover wasps (Field 2008) and type of resources provided for the progeny in superfamily Apoidea (Strohm and Liebig 2008), and this list is certainly not exhaustive by any means. It shows, however, how many different ecological factors can influence the costs and benefits of helping, thereby shaping the evolution of eusociality in manifold ways.

Chapter V of this thesis presents data on non-reproductive division of labour in ants with discussion on evolution of eusociality in social insects in general.

### *Aging theories*

Eusocial insects are particularly suitable for studying ageing and senescence, as they usually have two distinct female castes: long-living queens and short-living workers (Keller and Genoud 1997, Page and Peng 2001). Furthermore, workers are usually not a homogeneous group, but are subdivided into behavioural castes such as foragers working outside the nest and nurses working inside. Both groups tremendously differ in external mortality and life expectancy (Hölldobler and Wilson 1990). Since differences in life span are caused by environmental factors, it is possible to investigate how identical genotypes differ in aging patterns in response to different nutrition, social conditions, workload, performed tasks and exposure to external mortality.

In order to discuss the evolution of aging in detail it is necessary to define crucial terms: aging and senescence. Aging is defined as changes in individual's trait(s) over the time, while senescence refers to changes decreasing individual's performance (*e.g.* fecundity). In principle aging and senescence refer to an individual, but those terms, under circumstances, can also be applied to groups (*e.g.* demographic data). Note that aging is sometimes used as a synonym of senescence. Aging studies use many terms coming from demography, such as life span (*i.e.* how long an individual lives), life expectancy (*i.e.* how long an individual as a member of certain group is expected to live), external mortality (*i.e.* individual's death caused directly by environmental factors) or internal mortality (*i.e.* individual's death caused by physiology).

As senescence decreases individual's fitness, the questions (1) why organisms age and (2) whether aging is favoured in evolution are of fundamental importance. The argument that all organisms have to eventually die because of the entropy (disorder) does not stand up to scrutiny, as biological systems juxtapose the entropy (Prigogine and Stengers 1984) and can repair themselves. Moreover the organisms reproducing by body splitting (single-cellular organisms, *Hydra*) seem to escape from senescence entirely. There are three theories (mutation accumulation theory, agonistic pleiotropy, disposable soma theory) attempting to explain why aging could have originated and has been stable in the course of evolution. Mutation accumulation theory (Medawar 1952) points out that most individuals in nature die because of parasites or predators before they attain old age, and therefore, there is not much selection pressure to keep viability past the point when most individuals would be dead anyway. Thus, detrimental mutations, which show their negative effect only late in life, are likely to accumulate and decline individual's performance in advanced age. The agonistic pleiotropy theory (Williams 1957) implicates that aging is an effect of genes, which offer benefits in early life (such as head-started reproduction, higher fecundity in early life) but cause costs when individuals are old. The disposable soma theory (Kirkwood 1977) argues that an organism must compromise the investment into reproduction and body maintenance because resources are scarce. As the investment in body maintenance is not sufficient, not all damages are repaired and their accumulation leads to senescence.

Note that some assumptions of evolutionary theories of aging are contested. Mutation accumulation theory was already criticized by Williams (1957), who pointed out that aging itself causes individual's death, *e.g.* by decreasing individual ability to escape predation pressure. Agonistic pleiotropy theory expects trade-off between longevity and reproduction, which is found in some organisms indeed but not found in others. Furthermore, selection should favour mutations which minimize the negative effect of pleiotropic genes.

Besides three aforementioned evolutionary theories of aging, literature abounds with theories explaining the occurrence and evolution of aging patterns based on the inclusive fitness theory (after Hamilton 1964, Burke 2007) or group selection (Mitteldorf 2006) as well as the decreasing evolutionary ability of older individuals (Skulachev 1997, Goldsmith 2008).

The short overview of theories of aging presented here shows clearly that evolution of aging and senescence remains puzzling and that testing these theories is one of the crucial issues in modern biology. Chapter V of this thesis presents data concerning different aging patterns in ant workers of the same age performing different tasks.

### *Aging in social insects*

Two major characteristics of social insects make them particularly important for studying aging and senescence: (1) reproductives (gamergates, queens) outlive non-reproductives (workers) though both groups are of the same genetic background, and (2) social insects escape trade-off between longevity and reproduction, as social insects' reproductives live usually live much longer than non-reproducing workers (Keller and Genoud 1997). The latter contradicts predictions of antagonistic pleiotropy theory (Williams 1957). On the other hand it favours the disposable soma theory (Kirkwood 1977), as protected and well-fed reproductives are likely to have optimal investment in both reproduction and body maintenance. Moreover, the colony usually produces sexual brood after several weeks, months or even years after foundation and therefore reproductives are expected to live long (Medewar 1952). Indeed, the independent-founding species commonly live longer than dependent-founding, in which sexual brood production is head-started (Hölldobler and Wilson 1977, Keller and Genoud 1997).

The workers in a colony resemble functionally somatic cells in a multicultural organism and reproductives resemble germ-line cells (Wheeler 1923). In this context workers are replaced over time by new generations, similar to somatic cells, while reproductives live as long as the colony itself, similar to long-lasting germ-line cells (Rueppell and Kirkman 2005).

Social insects seem to minimize the sexual conflict between a male and a female, which often decreases life expectancy of mated female in solitary insects (Schrempf et al. 2005). Instead, mating has been shown to increase female longevity in one ant species, implying sexual cooperation (Schrempf et al. 2005, reviewed in Heinze and Schrempf 2008).

Mutation accumulation and agonistic pleiotropy theories predict that intrinsic mortality positively correlates with extrinsic mortality (Medawar 1952, Williams 1957). Phenotypic plasticity of social insects (West-Eberhard 2003) provides perfect conditions to test this prediction. Indeed, reproductives living in protected centre of a colony experience relatively low rate of intrinsic mortality (Keller and Genoud 1997). On the contrary, workers exposed to environmental hazards have substantially shorter life expectancy (Page and Peng 2001, Tofilski 2002). Moreover, in most species workers divide into two task-related groups: intra-nidal workers and foragers (Hölldobler and Wilson 1990). In such species foragers experience both higher external and intrinsic mortality (Chapuisat and Keller 2002). Honeybee (*Apis mellifera*) workers divide into foraging summer workers and non-foraging winter workers (Winston 1987). As predicted winter workers have significantly lower rate of intrinsic mortality than summer workers due to lower extrinsic mortality (Maurizio 1950, Page and Peng 2001).

As mentioned before, contrary to the disposable soma theory, eusocial insects seem not to follow the trade-off between reproduction and longevity. Instead, highly fecund individuals outlive those with low fecundity (Hölldobler and Wilson 1990, Tsuji et al. 1996, Keller and Genoud 1997, Andre et al. 2001, Page and Peng 2001, Hartmann and Heinze 2003), suggesting that in eusocial hymenoptera longevity is positively correlated with fecundity or mating itself (Schrempf et al. 2005). The same pattern is found in mole-rats (Damman and Burda 2006) but does not occur in termites (Thorne et al. 2002, Korb 2008). Note also that the molecular mechanisms behind reproductives' longevity are still unclear, since comparative studies of gene expression in reproductives and workers have not provided any clear answer thus far (Parker et al. 2004, Corona et al. 2005, Corona et al. 2007).

In most social insects species a single worker does not perform all tasks necessary for colony's maintenance and reproduction, but specializes in performing some tasks (Hölldobler and Wilson 1990, Gordon 1996). The task allocation in a colony is often age dependent: workers change from low-risk tasks to high-risk tasks as they age (Jeanne 1986, O'Donnell and Jeanne 1995, Tofilski 2002).

Interestingly, social insects exhibit environment-related reverse of senescence. Peculiar increase of longevity was shown in honeybee workers kept without a brood (Amdam et al. 2004) as well as reverse immune senescence in honeybee foragers forced to take care of a brood (Amdam et al. 2005). Another striking pattern of aging in social insects was found in honeybee workers: there was no decline in learning performance with age (Rueppell et al. 2007).

Chapter II of the thesis presents studies on insemination and queen longevity in honeybees (*Apis mellifera*).

### ***Social homeostasis***

Change of life mode from solitary (or group-living without reproductive division of labour) to eusocial is widely recognized as one of the major transitions in evolution (Maynard Smith and Szathmáry 1995). Eusocial insects colonised almost all terrestrial habitats of the Earth and overcompeted other animals of similar size in exploitation of more less stable resources (Wilson 1971, Hölldobler and Wilson 1990, Wilson and Hölldobler 2005). It seems that the transition to eusociality, similar to the transition from a single-cell to a multi-cell organism (Maynard Smith and Szathmáry 1995), ensured their enormous evolutionary success. On the other hand, eusociality caused severe problems concerning compromises between individual's and colony's optimum, labour division or pathogen defence. Therefore, the question how social homeostasis is achieved remains a major theme concerning eusocial species.

As in eusocial insects most of the individuals do not reproduce, it has been crucial for both an individual and a colony to solve the problem of assigning reproductive tasks. Although Hamilton's rule provides general explanation how (functionally) sterile individuals could have been favoured in the evolution, it does not explain mechanisms assuring workers sterility (Gardner and Foster 2008). A recently presented model (Gardner and Grafen 2009) argues that reproductive strategy had to be evolutionary optimised and stabilised already at the individual level and only then it was possible to apply it at the colony level. That does not mean however that refraining from reproduction by a worker is not enforced and that a colony lacks coercion mechanisms. For instance, policing, which is a mechanism assuring workers functional sterility, is enforced (Ratnieks 1988, Frank 1995, Monin and Ratnieks 2001).

Another issue which had to be solved in the course of evolution was the division of non-reproductive labour. The most common mechanism is age polyethism, meaning that workers undertake tasks outside a nest when old (Wilson 1971, Gorgon 1996). However, many deviations from this simple pattern are documented, such as precocious or delayed onset of foraging behaviour (Hunt and Amdam 2004) or specialization of workers to perform a task (Oettler and Johnson 2009). Therefore, a number of alternative hypothesis were tested to explain this deviations, such as the mortality risk hypothesis (Jeanne 1986, O'Donnell and Jeanne 1995, Tofilski 2002), the life expectancy hypothesis (Woyciechowski and Kozłowski 1998, Moroń et al. 2008) or the potential fecundity hypothesis (see chapter V).

Besides age polyethism, in many species the non-reproductive division of labour is influenced by genetic factors in manifold ways. In honeybees (*Apis mellifera*), workers from different patrilines undertake different tasks in a colony (Breed et al. 1990). In addition different strains of honeybee workers differ in their foraging efficiency, point of onset of foraging and type of collected resources (high pollen hoard strain and low pollen horde strain bees; Page and Fondrk 1995, Page et al. 1998, Fewell and Page 2000, Pankiw and Page 2001, Page and Erber 2002). In some ant species workers specialize in performing tasks according to their matriline or patriline (*Gnamptogenys striatula* Blatrix et al. 2000, *Solenopsis invicta* Krieger and Ross 2002, *Acromyrmex versicolor* Julian and Fewell 2004, *Eciton burcellii* Jaffe et al. 2007).

Eusocial groups consisting of highly related individuals living in proximate distance provide ideal conditions for spread of diseases and pathogens. Therefore, mechanisms allowing the control of pathogen pressure are of special importance for such groups. Indeed, social insects evolved many mechanisms to deal with diseases. For example an ant colony seems to develop immunity against a pathogen, if any individual ant ever came into contact with it (Ugelvig and Cremer 2007). Some ants store pieces of resin in the nest, which serve as antifungal agents (Chapuisat et al. 2007) or reduce contact to workers handling garbage, while termites wall-off their infected nestmates and thus minimize the risk of spreading infections (Oi and Pereira 1993, Cremer et al. 2007, Wilson-Rich et al. 2009).

Chapter III of this thesis presents how social homeostasis is achieved in terms of pathogen control. Chapter IV shows data on maintaining workers' sterility in colonies of different size and different relatedness among individuals. Chapter V presents and discusses data concerning the division of non-reproductive labour. It's main focus is the potential fecundity hypothesis, which may substantially contribute to understanding patterns of labour organisation in insect societies.

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# Chapter II

## Insemination and longevity in honey bee (*Apis mellifera*) queens*

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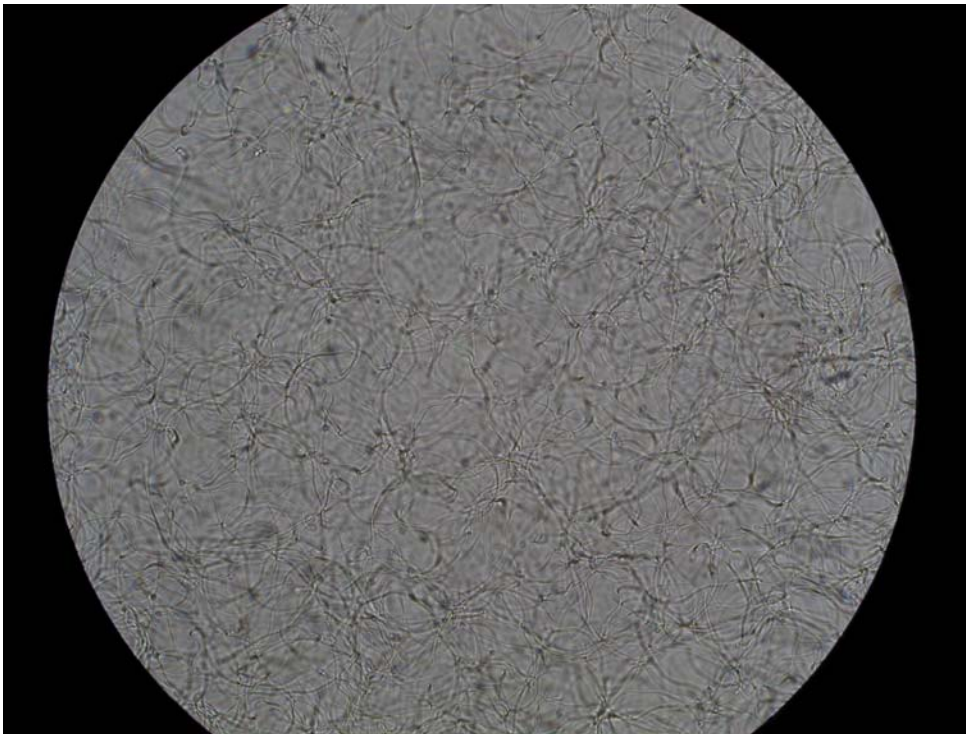

Honeybee (*Apis mellifera*) sperm cells (© Bartosz Walter 2006)

## Abstract

In eusocial Hymenoptera (ants, wasps, bees) there are two types of females that differ strongly in lifespan: long-living reproductives (queens) and short-living non-reproductive workers. As yet it is unknown whether the longevity of queens is a result of their ontogeny, reproductive activity, or insemination. Due to specific life-history features of honey bee (*Apis mellifera*), *i.e.* lifetime pair bond (partners mate only once and the sperm is stored by queen throughout her life) and the fact, that the production of sexuals takes place only after a certain time lag (once the colony reached appropriate size) both queen and drone benefit from prolonged lifespan of the mated queen. This allows to expect that the presence of the seminal fluid in spermatheca has a positive effect on queen's longevity. This study was designed to investigate effects of queen insemination status on longevity in honey bee queens, regardless of their reproductive activity. We instrumentally inseminated queens with viable sperm (NQ), unviable, irradiated sperm (IQ), or saline buffer (SQ) and compared their lifespans with those of virgin queens (VQ). To avoid differential fertility effects, the queens were caged individually in large, queenless hives, which did not allow egg-laying, which is a novel way to study queen's lifespan. Interestingly, inseminated and virgin queens experienced similar lifespans, suggesting that insemination itself does not increase queen longevity. Therefore, we argue that functional fertility, and not insemination status, plays the key role in increasing the lifespan of honey bee queens.

**Keywords:** sexual cooperation, queen longevity, queen lifespan, banked queens, sperm sterilization, *Apis mellifera*

## Introduction

Numerous studies have documented the trade-off between reproduction and lifespan predicted by life history theory in animals and plants (Rose and Charlesworth 1980; Primack and Stacy 1998). The trade-off appears to be reversed in perennial eusocial animals (Keller and Genoud 1997; Dammann and Burda 2006) where reproductive females live significantly longer than non-reproductive individuals. The proximate basis for increased lifespan in reproductives is not well known. First factor that must be taken into consideration is that in many eusocial insects, larvae of reproductives are reared on a more nutritious diet than those of workers and develop into adults that strongly differ from adult workers. Although such nutrition-based differences might explain increased lifespan, reproductives also live longer in those species that lack a pronounced caste dimorphism (Tsuji et al. 1996; Hartmann and Heinze 2003).

Another factor is that in eusocial hymenoptera (ants, wasps, bees) both mating partners have convergent interests. Due to the lifetime pair bond (partners mate only once in their life and the sperm is stored by queen throughout her life) and the fact, that the production of sexuals takes place only after a certain time lag, once the colony reached appropriate size, both partners benefit from prolonged lifespan of the queen (Schrempf et al. 2005). This allows to expect that the presence of the seminal fluid has a positive effect on queen’s longevity.

For example, queens of the ant *Cardiocondyla obscurior* mated to sterilized males lived longer than virgin queen controls though their fecundity did not increase (Schrempf et al. 2005).

In conclusion, it is unclear whether the general observed longevity of female reproductives in social insects is a result of their ontogeny, reproductive activity, or insemination status. Thus, the aim of this study was to investigate the effects of queen insemination status on their longevity regardless of their reproductive activity.

The honey bee *Apis mellifera* provides an excellent model system for studying how insemination influences the longevity of a female. Workers and queens differ tremendously in lifespan (Page and Peng 2001), but it is as yet unknown whether this difference is explained by ovary development (Winston 1987), high hemolymph titres of vitellogenin, which protect from oxidative stress (Seehuus et al. 2006; Corona et al.

2007), or by insemination and sperm storage in the queen's spermatheca, as in *Cardiocondyla* ants (Schrempf et al. 2005).

The long-established method of instrumentally inseminating honey bee queens (Laidlaw 1979) allows testing for the potential effects of insemination on longevity. While the instrumental insemination process is not synonymous with mating, it allows for exact control of the quantity and quality of the insemination fluid. By experimentally inseminating honey bee queens with normal or sterile semen, or saline, we investigated the effect of insemination on their life expectancy. To avoid differential fertility effects, the lifespan was assessed in queen holding cages that do not allow egg-laying. In contrast to *Cardiocondyla* (Schrempf et al. 2005), our results did not reveal a positive influence of insemination on queen lifespan. We discuss the significance of insemination and semen storage and we argue that functional fertility, instead of caste status and insemination, plays the key role in increasing longevity of honey bee.

## Material and Methods

We compared the longevity of virgin queens (VQ, *n* = 15), queens instrumentally inseminated with normal sperm (NQ, *n* = 11), queens inseminated with irradiated sperm (IQ, *n* = 16), and queens inseminated with saline buffer (SQ, *n* = 8). Queens were reared according to standard methods (Laidlaw 1979) in Greensboro (NC), and inseminated at an age of 5 to 10 days.

First, we collected sperm (drones from Raleigh, NC) into ten glass capillary tubes (100 µl sperm each) for a preliminary determination of the correct irradiation dosage to sterilize sperm without affecting its mobility. Each tube was irradiated with a different X-ray dosage (in Gray [1 Gray = 1 J/kg], 4.0 Gy/min: 0.0, 0.5, 1, 5, 25, 50, 100, 250, 500, 1000; $^{137}$Ceasium irradiator, Shepherd Associates). Sperm mobility was observed in sub-samples from each batch within three days after irradiation. Sperm in samples exposed to ≤ 500 Gy was as viable as control sperm, while sperm exposed to 1000 Gy has >50% unviable sperm cells. Hence, we assumed that 500 Gy irradiation was sufficient to sterilize sperm.

In the main experiment, we used normal sperm for NQ and sperm irradiated with 500 Gy for IQ. Both NQ and IQ were injected each with 10 µl of sperm and stored it in their spermathecae, as revealed by dissections of the spermathecae within 12 hours after the queens' death. SQ were injected with 10 µl of saline (1.11% NaCl, buffered with Tris, Harbo and Williams 1987). During insemination, NQ, IQ and SQ were anesthetized by carbon dioxide for 5.0 minutes. Such anaesthesia changes queen behaviour, physiology (Mackensen 1947; Engels and Ramamurty 1976), and possibly also longevity. Therefore, we also anesthetized VQ to insure that experimental treatments were similar.

We conducted first inseminations in June 2006. Because only 3 IQ survived the first week after insemination, we repeated the insemination with irradiated sperm on 13 additional queens in August. The queens in the second run came from the same source colonies and were reared exactly as queens in the first run. However, we used sperm from another source (Falkland, NC) as drones from Raleigh were no longer available. Thus, it should be kept in mind that the different time of queen rearing and sperm sources might have influenced IQ longevity.

After insemination in June, queens (VQ, *n* = 15; NQ, *n* = 11; IQ, *n* = 3 and SQ, *n* = 8) were caged individually in queen banks in a queenless, 3-story hive colony with about

25,000 workers. Worker number was kept constant by adding combs with worker pupae. Queens inseminated in August were caged in a second hive because we did not want to disturb the colony with banked queens from June. We kept the worker number and food resources in the second hive similar to the first hive. However, we cannot exclude the possible effects that any differences between the two hives may have had influence on queen longevity.

The queen banks were checked at least once per week and queen mortality was observed. We found two replacement queens in the first hive. As the mortality rate of stored queens increased when second replacement queen was present, these data were included only as censored data.

We compared the lifespans of queens with different spermatheca content with Cox's F test for two groups. The false discovery rate approach (Benjamini and Hochberg 1995) was used to correct for multiple testing.

## Results

All banked queens died within 6 months after the start of the experiment (before midwinter). Insemination itself did not increase queen longevity. Queens inseminated with normal sperm lived as long as both virgin queens and queens injected with saline buffer (medians: NQ 111 days, n = 11; VQ 83 days, n = 15; SQ 107.5 days, n = 8; survival analysis (log rank test) for multiple samples: $\chi^2 = 0.241$, df = 2, p > 0.89; pair-wise analyses: Cox's F-Test: NQ-VQ: p > 0.22, NQ-SQ: p > 0.44, SQ-VQ: p > 0.25, Figure 1.). However NQ, VQ and SQ lived significantly longer than queens inseminated with irradiated sperm (median: IQ 40.5 days, n = 16; pair-wise analyses Cox's F-Test: NQ-IQ: $p < 0.01$, VQ-IQ: $p < 0.0103$, SQ-IQ: $p < 0.01$).

## Discussion

The median lifespan of banked honey bee queens in our experiment was only three to four months, while normal, fertile honey bee queens usually live much longer. For example, Seeley (1978) reported that 79% of queens survived for one year, 26% for two years, and no queen survived three years. The difference can perhaps be explained by the different reproductive status of queens in the various studies. In previous studies, queens were allowed to move freely through the hive and to lay eggs, but in the current study queens were caged and not able to lay eggs. Hence, any potential influence of oviposition on queen longevity was absent in our experimental design. We therefore conclude that relative queen longevity in the honey bee depends largely on reproductive activity, *i.e.* egg laying.

Our non-laying queens lived only twice as long as sterile workers. In contrast the earlier study (Velthuis et al. 1990) showed, that egg-laying workers of the cape honey bee (*A. mellifera capensis*) live for five months, *i.e.* three times longer than sterile workers do. Furthermore, queens banked only after they had already laid eggs lived as long as normal queens (Engels 1974). We therefore suggest that reproductive history, and not morphology or successful insemination, positively affects the lifespan of honey bee females. Similarly, lifespan depends on an individual's reproductive status in the thelytokous ant *Platythyrea punctata* (Hartmann and Heinze 2003). One possible physiological mechanism of the phenomenon may be increased vitellogenin expression when a queen starts to reproduce. Recent studies (Corona et al. 2007) showed such increase in one-month-old queens which started to lay eggs while one-month-old workers lacked the increase.

We nested the queens in a bank to ensure equal condition for all queens, because if they had been placed into individual colonies, an effect of insemination on queen longevity might have been masked by social interactions. Hence, our design with queen banks had the advantage of a controlled social environment, but at the same time our queens could not lay eggs and presumably suffered from additional, physiological stress.

Our experiment was inspired by recent findings that, in the ant *Cardiocondyla obscurior*, mating itself appears to have a positive affect on queen lifespan (Schrempf et al. 2005). In contrast, the honey bee queens in our experiment had, on average, the same longevity, irrespective of being inseminated or not. Interestingly, honey bee queens that had been inseminated with irradiated sperm (IQ) died significantly earlier than other queens. This might be due to degrading sperm being toxic. While normal bee sperm is resistant to long storage (Collins et al. 2004, 2006), irradiated sperm may have started to decay earlier, which in turn could have poisoned the queens and lead to their premature death.

## Figures

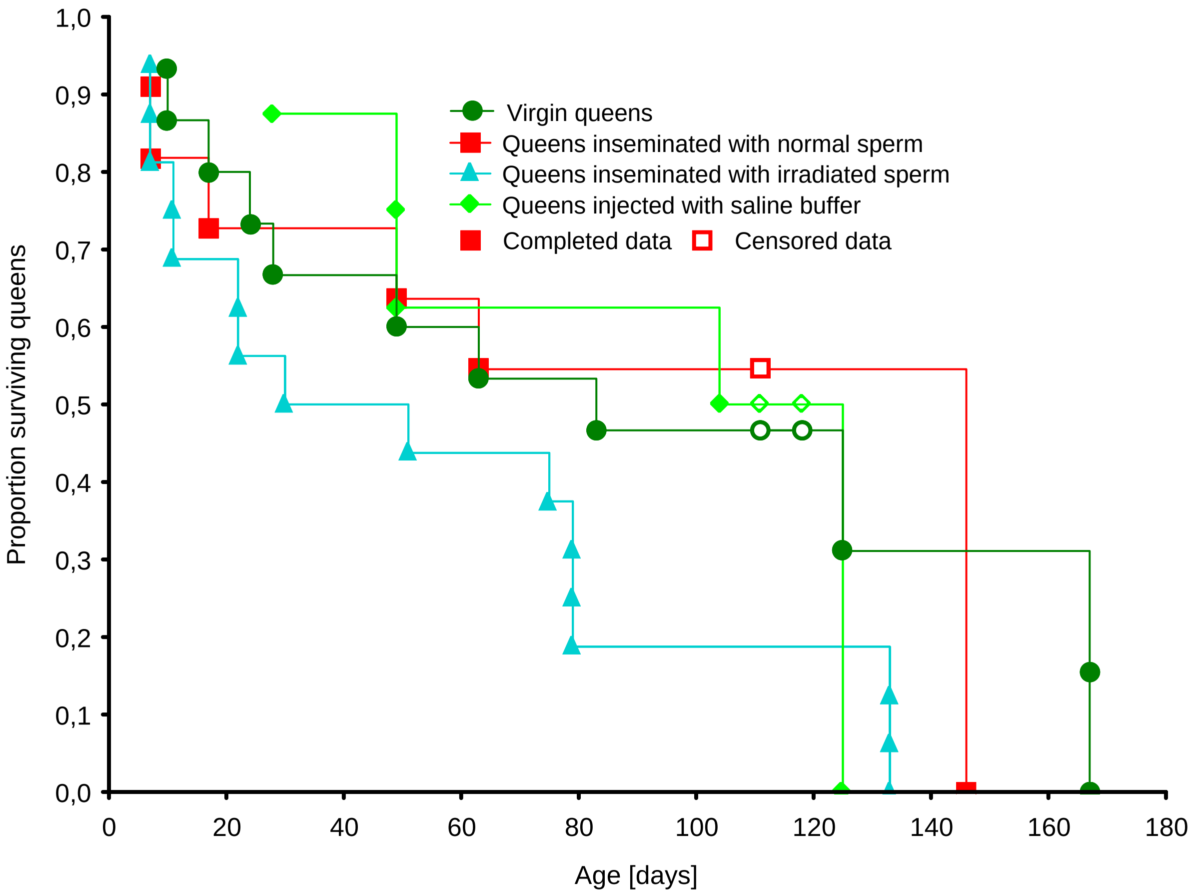

**Fig. 1** Proportion of surviving Honeybee (Apis mellifera) queens according to the insemination status. Positive effect of insemination on longevity was not found.

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# Chapter III

## Social insects die lonely – ants altruistically protect their nestmates from infections*

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Infected worker of *Temnothorax unifasciatus* dying in solitude
(© Bartosz Walter 2008)

## Abstract

Animal societies provide ideal conditions for the spread of infections and are therefore expected to employ mechanisms that reduce the probability of disease transmission to group members. Here, we show that diseased workers of the ant *Temnothorax unifasciatus* stop interacting with their nestmates and actively leave the nest several days before they die. To confirm that dying in solitude has evolved because of its altruistic benefits requires refuting the alternative explanation of pathogen manipulation of host behaviour. We therefore compared the behaviour of workers dying from an experimental infection with the fungus *Metarhizium anisopliae* with two controls: uninfected workers, whose life expectancy was reduced by exposure to $CO_2$, and workers that spontaneously died in observation colonies. All dying workers showed the same behavioural syndrome of isolating themselves from their nestmates before they died. $CO_2$-exposed workers survived longer when we prevented them from leaving the nest by blocking its entrance, *i.e.* leaving the nest accelerates the process of dying in moribund workers. Our data suggest that actively emigrating from the nest and breaking off all social interactions is an altruistic trait that serves the inclusive fitness of workers.

## Introduction

According to models of host-pathogen co-evolution, strains of pathogens that have successfully infected a certain host are more easily transmitted to individuals that are genetically similar, *i.e.*, related, to the already diseased host (*1, 2*). Most social animals live in more or less extended families and frequently engage in intimate interactions (Fig. 1A) (*3*). Both factors greatly facilitate the spread of pathogens from infected individuals to other group members (*4, 5*). Social animals have evolved a number of fascinating group-level mechanisms that prevent infection and the transmission of diseases, including mutual grooming, minimizing the contact to group members that handle garbage or faeces, and walling off of infected individuals (*5-8*). Our experiments reveal an additional, striking adaptation at the individual level in ants that reduces the spread of pathogens or parasites to other group members: moribund workers stop interacting with their nestmates and leave the nest in order to die in isolation (Fig.1B). Death in nature commonly results from infection and parasitism and rarely from old age. Isolating themselves from the group and dying in solitude might therefore be a mechanism to reduce the probability of disease transmission.

Popular science and literature abound with anecdotes of moribund animals, including humans (*9-11*), leaving their families, but few reports stand up to scrutiny. Furthermore, it has been questioned whether this behaviour reflects the selflessness of infected individuals or rather promotes the spread of pathogens to other groups (*6*). For example, social insects infected with pathogens and parasites, such as pathogenic fungi or larvae of liver flukes, disperse from their nests and die on the tips of grass stems. This obviously increases the likelihood that fungal spores are dispersed by wind or that the liverfluke is taken up by a final host. To substantiate earlier reports about the dispersal of moribund ants and to exclude that this behaviour is a parasite-driven manipulation, we studied the behaviour of workers of the ant *Temnothorax unifasciatus* a) that died because of infection with an entomopathogenic fungus, b) of uninfected workers that died of unknown causes in unmanipulated colonies, and c) of uninfected workers whose life expectancy was severely reduced by $CO_2$ exposure (*12*).

## Results and Discussion

Of 44 *T. unifasciatus* workers from seven colonies, which we experimentally infected with conidia of the entomopathogenic fungus *Metarhizium anisopliae*, at least 31 stopped interacting with their nestmates and permanently left the nest several hours before death without ever returning into it (median 6 h, range 1 h - 50 h; Fig. 1, interactions before / after leaving, Wilcoxon matched pairs test, n = 31, Z = 4.68, $P < 0.0001$; four workers died in the nest and nine additional workers died overnight and their behaviour before death could not be observed). In contrast, 65 uninfected control workers continued to interact with their nestmates at the same rate as before (Fig. 1, n = 65, Z = 1.1, $P > 0.27$). Infected workers were neither attacked nor carried out of the nest by other workers but left the nest on their own and also did not receive any special treatment by their nestmates, in contrast to diseased individuals in other species (*13, 14*). This suggests that in *T. unifasciatus* infection and closeness to death is not perceived by other individuals and that the dying workers themselves responded to their own condition by actively retreating from the rest of the society.

Similarly, 12 of 13 workers that died spontaneously in unmanipulated colonies amongst a total of 1609 individually-marked workers stopped interacting, left the nest permanently, and died outside in the foraging arena, away from adult nestmates and brood. In contrast to fungus-infected workers, emigration occurred between one and 15 days (median 3 days) before death, presumably because fungal infection results in a much faster deterioration of the workers' condition than other causes of death. One exceptional worker, which died in the nest, was carried back into it by a nestmate six days before death after staying outside for six days without ever interacting with its nestmates. Moribund workers ceased interacting with their nestmates between 1 and 23 days before their deaths (median 6 days; Fig 1, n = 12, Z = 2.75, $P < 0.01$), while workers, which did not die, did not change their rate of interaction (Fig. 1, n = 499, Z = 0.49, $P > 0.62$). When we returned five additional moribund workers that were no longer capable of walking into their nests, they were first groomed by their nestmates and later left alone in the nest, where they eventually died. They were removed from the nest by other workers only one or two days after their deaths, again showing that closeness to death is not immediately recognized by the nestmates.

Third, we observed colonies consisting of young workers of which some had a reduced life expectancy due to $CO_2$ exposure (*12*). Out of 52 $CO_2$-exposed ants, 27 died within two weeks after the treatment, compared to 11 of 70 unexposed control workers (Cox's Mantel test, I = 7.20, $P < 0.001$). Most dying $CO_2$-exposed ants (22 of 27), and also most dying control ants (6 of 11), were observed leaving the nest 5 to 239 hours before their deaths (median 36 h). All other dying ants were also found dead outside the nest, but their behaviour before death could not be observed. As above, moribund ants stopped interacting with their nestmates (Fig. 1, n = 22, Z = 0.00, $P < 0.0001$), while control workers did not change their behaviour (Fig. 1, n = 65, Z = 0.89, $P > 0.39$). Interestingly, $CO_2$-exposed workers survived significantly longer when they were prevented from leaving the colony by closing the nest entrance (Cox-Mantel test, I = 6.23, U = -12.79, test statistic = -5.12, $P < 0.0001$), *i.e.*, emigration from the nest further decreases the life span of exposed ants and accelerated the process of dying, probably due to desiccation in the foraging arena.

Taken together, our experiments clearly give evidence of moribund workers actively emigrating from the nest and separating themselves from their nestmates to die in solitude. Our observations of $CO_2$-exposed, but otherwise unharmed ants, suggest that their emigration does not result from behavioural manipulation by a parasite or pathogen. Instead, workers appear to react to the closeness of death by breaking off all social interactions and actively emigrating from the nest, instead of seeking the company of their nestmates and attempting to restore their health in the safety of the nest. Emigration behaviour likely reduces the spread of diseases to nestmates, while even further decreasing the life expectancy of dying workers. Thus, dying in solitude constitutes another striking example of altruism of social insects, comparable to the suicidal sting of the honey bee worker (*3*) or the closing of the nest from outside by workers of *Forelius* ants (*15*). Moribund emigration in ants shown here underpins earlier anecdotal reports of dying individuals leaving their families and should be further investigated in other social species, including man.

## Supporting online material

*Ant collecting, nesting in captivity and marking*

We collected complete colonies of the ant *Temnothorax unifasciatus* (Myrmicinae) from their nests in rock crevices in a population at Gargnano, Lago di Garda, Italy, and transferred them into 10cm x 10cm x 3cm plastic boxes with a plaster floor and a 50mm x 12mm x 3mm cavity between two microscope slides serving as a nest site. Ants were provided with water and food (honey and pieces of fresh cockroaches) ad libitum and reared at 12/12 hours day/night cycles with temperatures of 20°C and 25°C, respectively. All workers used in the experiments were individually marked with 30µm thin copper wires knotted around their petioles and postpetioles.

*Observations – general method*

Observations were made at 23°C +/-2°C at day light between 8.00 and 20.00. The duration of ant behaviour was noted with an accuracy of 1 second. We recorded the place (outside and inside of the nest; inside the nest: in nest entrance, near or away from brood pile) and type of behaviour (solitary activities: staying, walking, foraging for water or food, cleaning the nest; social interactions: antennation, food exchange, self and allogrooming, brood tending, carrying dead or live workers or brood, antennal boxing, biting). When an individual interacted with two nestmates at the same time, e.g., when it was offering food to two workers or was groomed by two workers, we counted both interactions.

*Observations of infected workers*

The generalist entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* (S1) (Entomophtorales, strain Ma275, obtained from Dr. Sylvia Cremer and Simon Tragust, Regensburg University, Germany), was cultivated on 6.5% Sabouraud-dextrose-agar plates at 23°C (S2, S3). For the infection of ants, we created a (conidia) spore suspension from a recently sporulating culture plate in a 0.05% solution of a surfactant (Triton X-100 [Sigma]). The suspension was quantified with a haemocytometer (Neubauer-improved counting chamber) and diluted to a concentration of $10^9$ spores/ml. Before infecting the ants we determined the germination capacity of spores by spreading 100µl of the suspension on a SM agar plate (selective medium containing dodine, chloramphenicol, and streptomycine sulphate) and incubation for 18 hours at 23°C (S3). Germination rate was 99% (*i.e.*, 990 of 1000 spores germinated). Quantities of 0.3 µl of the suspension were applied onto the cuticle of individual ants with a pipette (S2, S4). Preliminary tests revealed that >70% ants treated with the suspension died within a week after treatment (three colonies each with 10 infected and 10 control workers). Control ants received the same amount of 0.05% Triton X solution without any spores. Preliminary test showed that the application of 0.3 µl Triton X solution does not affect the survival rate as compared to untreated ants, which is in accordance with results from previous studies (S2).

We set up seven colonies with a queen, brood, and 20 individually marked workers of unknown age. The behaviour of ants was recorded three times for 10 minutes each and seven scans per days over ten days in two weeks. Thereafter, ten randomly chosen workers were infected and ten others served as a control. Two hours after the treatment, infected and control workers were returned into their nests. After allowing the ants to adjust again to the colony for one hour, we observed the ants again for three times of 10 minutes each and seven scans each during the following 10 days. In addition the mortality of workers was monitored until the 21st day after infection. All ants that died did so during the first ten days after infection.

To confirm that the workers had died of the infection we sterilized the surface of dead workers (distilled water, ethanol, sodium benzoate (S3) and transferred it into a sterilized, closed Petri dish with a water-soaked filter paper. First fungal hyphae were observed 12 hours after incubation and first spores (or spore clusters) within the next two to four days. *Metarhizium* spores were found on 44 of 52 corpses, spores of other fungi were found on seven corpses, and no fungus grew on one corpse. The workers that had died without being infected by *Metarhizium* were excluded from the analysis in this experiment.

*Observations of naturally dying workers*

All workers in 28 laboratory colonies (range of worker number: 15 – 100, median 40, total 1609) were observed in ten 1-min observation sessions per day for 5 days per week over four weeks and three times for 10 minutes per day for six days per week for additional two weeks. In addition, dying workers (both dying inside and outside the nest) were observed for seven more scans.

### *Observation of $CO_2$-exposed workers*

We established seven colonies, each consisting of twenty individually marked workers, brood and a queen. We randomly choose 70 workers (ten per colony) and exposed them to 95% $CO_2$ for 80 hours, while 70 additional workers served as a control. Of the exposed workers, 52 recovered after the treatment (4 to 10 per colony, median: 8), and only they were used for the next analyses. After the exposure, workers were put back into their nests and the behaviour of exposed and control workers was observed for ten minutes per day in one minute intervals for two weeks). We analysed the time invested in social interactions by workers before and after leaving the nest and compared the data to control workers.

In a parallel experiment we forced workers to remain in the nest by blocking the nest entrance with a paper plug. In each of seven colonies we exposed 20 randomly collected workers to 95% $CO_2$ for 80 hours and 20 workers remained unchallenged as a control. The cohorts of 20 workers were then kept in closed nests with five larvae. Food was provided ad libitum directly into the nest (cotton soaked with honey and water, peaces of cockroaches). The survival of the ants (140 exposed and 140 control) was checked every day for two weeks and compared with the survival of ants, which were allowed to leave the nest and use foraging arena (52 exposed and 70 control in seven colonies). During the first two weeks after exposure, only eight of 140 exposed ants died in the closed nests compared to 27 of 52 exposed ants allowed to leave the nest (Cox-Mantel test, I = 6.23, U = -12.79, test statistic = -5.12, $P < 0.0001$). None of the 140 control ants in closed nests died compared to 11 of 70 control ants allowed to leave the nest (Cox-Mantel test, I = 2.38, U = -7.5, test statistic = -4.87, $P < 0.0001$).

### *Statistic analysis*

Statistic analyses were performed with Statistica ver. 6. We compared the duration of interactions between moribund workers and other ants during the last 10 minutes of observation before emigration and 10 min observation time after emigration by Wilcoxon matched pairs test. It should be noted that moribund workers usually completely ceased to interact with nestmates, which might in part be due to the lack of opportunity outside of the nest. In principle, interaction frequency would therefore have to be corrected for the likelihood of encountering another ant, which cannot easily be estimated. As moribund workers did neither actively interact when they were encountered by a forager or another ant outside the nest nor when we returned them into the nest (in five colonies in an additional experiment), we feel that we can safely conclude that the low social activity of moribund ants is not caused only by opportunity. In addition, we statistically compared the duration of interactions in control ants between similar time spans.

To compare life spans we used Cox's Mantel test because of the large number of censored life span data from control workers, of which many survived the duration of the experiment.

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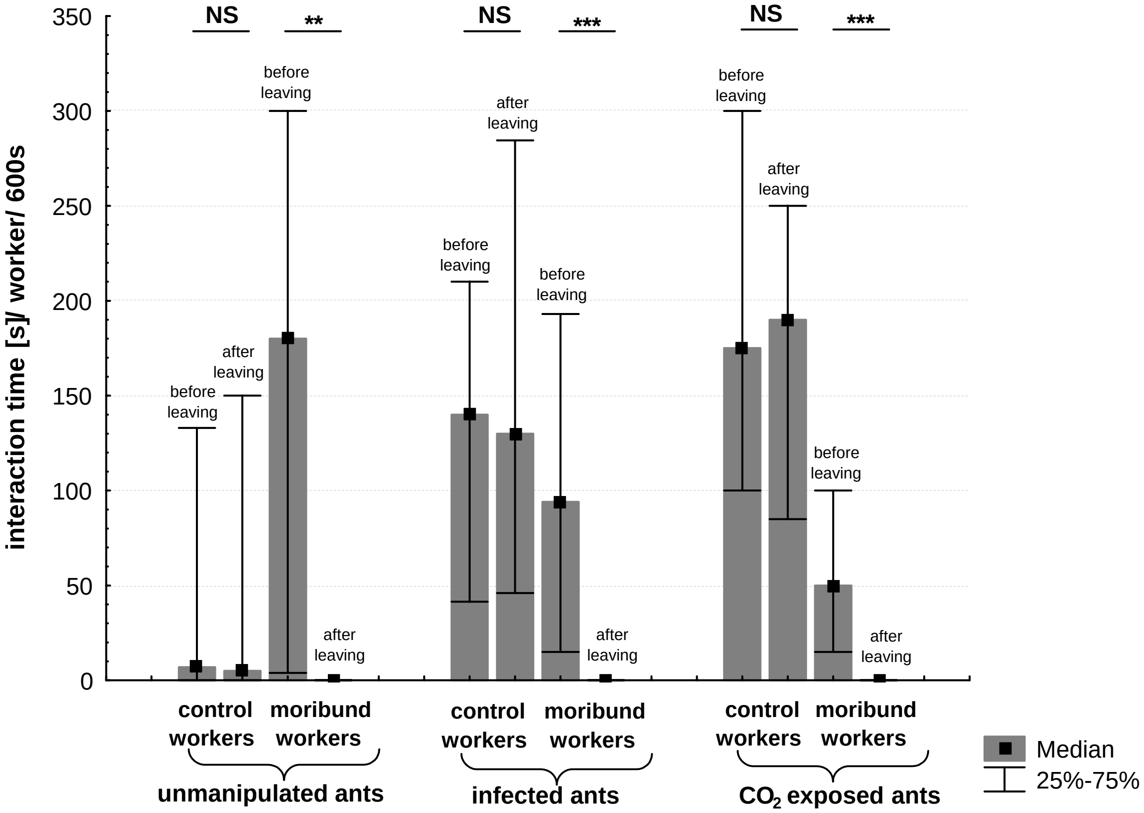
## Figures

**Fig. 1 Behavior of healthy and moribund ants**. While healthy ants frequently engage in intimate interactions (A), moribund individuals cease all social interactions, actively leave their colonies and die in isolation (B).

**Fig 2. Duration of social interactions of control and moribund ants before and after nest leaving.** In all three experiments moribund individuals ceased all social interactions after leaving the nest, while control individuals from the same colony in the same period of time did not significantly change their interaction rate (Wilcoxon matched pairs test, ****** - *P* < 0.01, ******* - *P* < 0.0001, **NS** – difference is non-significant).



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# Chapter IV

## Policing effectiveness depends on relatedness and group size*

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*Manuscript

Policing in queenright colony: immobilization of a reproducing worker of *Temnothorax unifasciatus* (© Bartosz Walter 2008)

## Abstract

The cohesion of social groups requires the suppression of individual selfishness. As predicted by theory, worker egg laying is usually suppressed in insect societies by aggression and egg eating. The effectiveness of “policing” increases with decreasing relatedness, as this strongly affects inclusive fitness of group members [1-3]. As inclusive fitness is also influenced by the costs and benefits of helping[1,4,5], the effectiveness of policing is also expected to decrease with increased colony size, because costs for colony caused by a worker which reproduces instead of helping are proportionally reduced in large groups as we demonstrate here (but compare ref.[6]). Here we show for the first time that policing effectiveness is low in large colonies and high in small colonies when relatedness is high. When we experimentally decreased the relatedness in colonies of the ant *Temnothorax unifasciatus*, the policing effectiveness reached the same level as in small homogeneous colonies, irrespectively to colony size. Therefore, our results indicate that policing effectiveness is simultaneously shaped by relatedness and colony size, *i.e.* an ecological factor. This may have major implications for testing policing across species of animals.

## Introduction

Colonies of social Hymenoptera (wasps, ants, bees) are commonly thought to be harmonious superorganisms without much disagreement about the division of labour. However, insect societies indeed face severe intra-colonial conflicts about reproduction. One of the most significant issues is the conflict over male parentage between the two female castes: mated queens and unmated workers. The latter, in most of the species, retain ovaries and although unmated are able to produce male-destined haploid eggs[7]. The conflict between a worker and her mother queen arises due to the relatedness asymmetry. It means that for a worker her own sons ($r = 0.5$) and sons of other workers' ($r = 0.375$) are closer related to her than sons of the queen ($r = 0.25$), while for the queen, her sons ($r = 0.5$) are closer related to her than workers' sons ($r = 0.25$). However, in nature worker reproduction is rare, because it is prevented by three control mechanisms: policing by the queen, policing by the workers and self-restraint, out of which two latter are most important[1]. By definition, policing inhibits workers direct reproduction[8]: eggs of fertile workers are destroyed and themselves are threatened or even killed. That should enforce functional sterility of workers but those mechanisms are not fully effective, as shown in several empirical studies (revised in[2,3]). Policing effectiveness measures the proportion of worker-laid eggs being killed relative to proportion of queen-laid eggs being killed[2]. That definition fits to wasps, bees and some ant species where policing against worker-laid eggs is common, but does not apply to *Temnothorax* ants where policing against eggs is rare[11]. Instead, in *Temnothorax* ants fertile workers are commonly subject of policing[10,11]. Therefore, the policing effectiveness in *Temnothorax* can be defined as proportion of fertile workers being policed relative to all fertile workers in a colony.

Why workers police each others reproduction? To answer this question three hypotheses were presented: the relatedness hypothesis, the costs hypothesis and the adaptive reproduction schedule hypothesis. The relatedness hypothesis predicts that policing effectiveness should increase when relatedness among workers decreases (due to queen multiple mating or multiple queening), because then a worker becomes less related to an average workers' son than to an average queens' son[1-3,9]. The costs hypothesis proposes that workers police each others' reproduction to improve colony performance, and in that way, gain the maximum inclusive fitness[1,4]. The recently presented adaptive reproduction schedule hypothesis expects policing to be dependent on developmental stage of a colony: (1) policing effectiveness should be high at an ergonomic stage regardless of intra-colonial relatedness, (2) while at a reproductive stage the effectiveness should be low when relatedness is high and high when relatedness is low[5]. The latter notion points out the importance of intra-colonial relatedness, which is consistent with the relatedness hypothesis. The hypothesis stresses also the importance of the colony size, since an ergonomic stage refers to a small colonies and reproductive stage to large colonies. A similar prediction can be concluded from the costs hypothesis, as cost of a reproducing worker is proportionally lower in large colonies.

A reproducing worker imposes direct costs on the colony-level in at least three ways: (1) the workload of the reproducing individual is not performed, (2) it consumes resources and invests them into worker-laid eggs and (3) worker-laid eggs have to be reared into adult individuals. A reproducing worker is expected to consume similar amount of resources and lay similar amount of eggs in large and small colonies. Therefore, the cost of worker's reproduction in large colonies, though the same in absolute terms, is per capita smaller, as (1) it is distributed over a higher number of individuals and (2) workers in large colonies work more effectively and rear per capita more sexual brood than they do in small colonies (see Fig. 2).

Therefore both relatedness and colony size seem to influence policing effectiveness in manifold way. Although influence of relatedness on the effectiveness has been intensively studied[2,3], there are no empirical studies dealing with simultaneous influence of colony size an relatedness on policing effectiveness. Here we demonstrate for the first time that policing effectiveness depends on simultaneous effect of those both factors. As a study system we used *Temnothorax unifasciatus* ants – social species with one single-mated queen[10] (relatedness between workers is high, r = 0.75), relatively small colonies (rarely more than 100 workers) and policing against fertile workers[11]. First, we tested policing in small (n workers = 40) and large (n worker = 100) colonies of a high relatedness (homogeneous colonies: r = 0.75), *i.e.* colonies on ergonomic and reproductive stage. Second, we tested small and large colonies in which relatedness was experimentally reduced (heterogeneous colonies: r = 0.1875). As predicted, policing effectiveness was low (only proportion of 0.28 fertile workers were policed) in large homogeneous colonies, while high (0.78-0.87) in small homogeneous, small heterogeneous and large heterogeneous colonies. Overall the policing effectiveness is affected simultaneously by the colony size and relatedness among individuals.

## Material and Methods

*Types of colonies*

We collected random hibernating colonies at Gargnano, Lago di Garda, Italy 45$^{o}$ 41’ N, 10$^{o}$ 40’ E (n = 40, worker number: 67 ± 30 SD). After three months of being kept under spring conditions (day/night: 12h/12h, +20$^{o}$C/+25$^{o}$C) the colonies produced pupae of workers, males and gynes (unmated queens). The number and type of produced brood served to examine whether workers’ performance depend on colony size (see paragraph below). The colonies served to set up four types of colonies: small homogeneous (SHo), large homogeneous (LHo), small heterogeneous (SHe) and large heterogeneous (LHe). Homogeneous colonies consisted of workers produced by a single colony, and therefore were highly related to each other (r = 0.75). Heterogeneous colonies mimicked colonies with decreased relatedness and consisted of equal proportion of workers from four source colonies (r = 0.1875). Small colonies consisted of 40 workers each and represented colonies on the ergonomic stage of development, while large colonies consisted of 100 workers and represented colonies on reproductive stage (see Fig. 1 thick lines). All types of colonies were headed by a single queen and had 1:1 worker : brood ratio. All workers in a colony were of a single cohort (1-5 days old at the beginning of the experiment) to exclude potential age effect on the policing.

*Brood production and colony size*

Colonies were kept under spring conditions for three months and produced pupae of workers, males and gynes (unmated queens). After that time the number and type of brood was noticed, corrected for difference in body mass between male and female brood and compared with workers’ number. After logarithmic transformation of the data, linear regression between worker number and brood production was tested. If test was significant, the slope coefficient was taken, 1 was subtracted, and the answer was divided by the standard error of the regression. This gave us a t-statistic, the significance of which we determined using a t-distribution. If it was significant, we concluded that the slope coefficient was greater than 1.

*Colony splitting and reunion procedure*

Because workers’ reproduction in queenright colonies is very rare, we used the splitting-reunion procedure, commonly used for social insects[4,11,12], in order to uncover workers’ potential fecundity (existing but not expressed in queenright colonies) and in order to detect response of queenright workers to reproducing individuals after colony reunion. Such a procedure seems to be artificial because splitting of colony followed by later reunion its parts in nature is unlikely. However the procedure allows us to easily uncover hidden mechanisms how reproducing workers are treated in a presence of the queen, what otherwise would be difficult to detect. First, the worker pupae ready to enclose were nested together with a queen. After enclosure, all workers were kept with the queen for 30 days. Second, as in queenright conditions worker reproduction is rarely observed[10,13], we split the colonies in queenless and queenright parts in order to induce worker reproduction. Queenless parts in all types of colonies consisted of 20 workers and 20 larvae. Queenright parts consisted of 80 workers and 80 larvae in large colonies, and 20 workers and 20 larvae in small colonies. In queenless parts workers were marked individually with copper wire (courtesy of Elektrisola, Eckenhagen) tied around body segments[10]. In queenright parts, workers were marked as a group that enabled us later to distinguish them from the workers from the queenless parts. Within a month after splitting, workers in queenless parts established hierarchies and worker-laid eggs appeared, indicating that at least some of queenless workers became fertile. Third we reunited the queenless and queenright parts by putting them into a new nest.

### *Ovary dissection*

The experiment was finished one month after reunion, ants frozen and dissected in order to check their ovary status. We distinguished five stages of ovary activation: 0 for non-elongated ovaries, 1 for elongated but without eggs, 2 for elongated with trophic eggs and 3 for elongated with viable male-designed eggs (for more details see ref.[14,15]), 4 for elongated ovaries with degenerated viable eggs. Trophic eggs were round and transparent, while viable eggs were similar to queen-laid eggs: oval and white. The degenerated eggs were oval but only partially white due to well visible transparent vacuole in the centre suggesting that the primarily viable egg was either absorbed by a worker or was turning into a trophic egg. The occurrence of degenerated eggs was not reported previously in literature and its occurrence should be investigated more carefully in very next future. We only considered individuals with ovary stage 3 or 4 as fertile workers.

*Fertility and fecundity of workers in queenless and queenright conditions*

Ants in queenless parts of colonies were observed for five hours in ten-minute intervals over 35 days. We noticed time spent by the ants on following actions: egg lying, worker touching by antennae, brood tending, self- or allo-grooming, trophalaxis, threatening by opening mandibles, antennal boxing and biting. In addition we noticed workers that were foraging outside or were present in a nest but separated from a brood pile. As egg laying by workers was rarely observed, we used dominance interactions as a proxy for fertility[11,15,16]. Individuals showing dominance behaviour (threatening, boxing, biting) usually become fertile workers, since only top-ranked individuals are allowed to reproduce[10,11,15,16]. Indeed, the ovary dissections confirmed that most of aggressive individuals had fully developed ovaries (stage 3 or 4, see results). Viable eggs in queenless parts were counted every second day and the final egg number (*i.e.* fecundity of workers' cohort) was compared between all types of colonies. Behaviour of workers in queenright parts was not observed but their fertility was checked by ovary dissections after the experiment.

*Policing against fertile workers after reunion*

Since we expected reproductive workers to be policed within few days after reunion, each worker from queenless parts was intensively observed for five hours in ten-minute intervals for a week after reunion. Later, each of them was observed for 100 minutes in ten-minute intervals over next three weeks. Indeed if policing was observed, it occurred only in the first week after the reunion. During the observations we noticed time spent on actions as in queenless parts of colonies. In addition we counted time invested in aggressive interactions applied by queenright workers against fertile nestmates, including biting them for several consecutive minutes, immobilization by up to ten workers that were pulling the fertile workers in all directions for a few hours and dragging them through a nest. Those measures were never observed in queenless colonies, and therefore, we counted them as policing acts and the workers that the measures were applied against as policed ones. Furthermore, we counted those workers that were mutilated (they had cut legs and antennae), excluded from a nest or even killed. Much longer duration and seriousness of measures allowed us to distinguish the policing from ordinary aggressive interactions in queenless colony parts, such as antennal boxing and short biting (up to one minute). When we proved the fertility of workers by ovary dissections we compared proportion of fertile workers being policed and a total number of fertile workers. The proportion was counted for each type of colony separately. In the same way we noticed behaviour of queens interacting with queenless workers, both peacefully (antennal contact, grooming, trophalaxis) and aggressively (biting and smearing with gaster tip). The latter was counted as queen policing and after worker ovary dissections, we assessed proportion of queen-policed workers and a number of fertile workers. The proportion was counted for each colony type separately.

*Policing against worker-laid eggs*

To distinguish between worker-laid and queen-laid eggs we fed workers from queenless part and queens with different fat-soluble dyes (Sudan IV and Sudan black B) diluted in tuna oil[11]. As a result, workers laid eggs of different colour than those laid by a queen and therefore we were able to detect whether workers continued reproduction after reunion and whether their eggs were policed. The number of eggs of both types was counted twice a day. We checked whether survival of worker-laid eggs differed between types of colonies, as well as whether the proportion of surviving worker and queen eggs differed between colonies types.

## Results

*Brood production and colony size*

While small colonies (n = 15, worker number: 43 ± 18 SD) produced only workers, medium colonies (n = 12, worker number: 61 ± 21 SD) produced workers and males, large colonies (n = 13, worker number: 96 ± 21 SD) produced workers, males and gynes (see Fig. 1). Furthermore, the production of sexual brood per capita was positively correlated with group size, *i.e.* large colonies used to produce more brood per single worker than small colonies (Fig. 2, regression summary for worker number and sexual brood production: B = 1.66, Standard Error for B = 0.38, t (39) = 4.35, $p < 0.0001$; analysis of slope: value = 1.74, $p < 0.05$, slope > 1). Total brood production per capita was not significantly bigger in large colonies (regression summary for worker number and sexual brood production: B = 1.05, Standard Error for B = 0.20, t (36) = 5.22, $p < 0.00001$; analysis of slope: value = 0.25, $p < 0.40$, slope < 1).

*Fertility and fecundity of workers in queenless and queenright conditions*

Workers in queenless parts of colonies established hierarchy by aggressive boxing and biting within a month after splitting. After the hierarchy was established, out of 20 workers only two to six high-ranking individuals were reproducing (see Fig. 3, differences between colony types non-significant, Kruskal-Wallis test: H (df = 3, n = 29) = 5.1, $p = 0.17$). That was confirmed by dissections of workers' ovaries after the experiment. As well as the number of reproducing workers, the number of eggs laid by queenless workers did not differ significantly between all four types of colonies (Fig. 4, Kruskal-Wallis test H (df = 3, n = 29) = 2.75, $p = 0.43$). This indicates similar fertility and fecundity of workers irrespectively to colony size or relatedness among workers. Additionally, because none of the workers kept in queenright parts were fertile (ovary status 0 to 2), it seems that workers refrained from reproduction in the presence of queen.

*Policing against fertile workers after reunion*

As predicted, most of fertile workers were peacefully integrated in large homogeneous colonies and aggressively policed by nestmates in other types of colonies. In large homogeneous colonies only proportion of 0.28 fertile workers were policed, whereas in small homogeneous 0.78, in small heterogeneous 0.85 and in large heterogeneous 0.87 (see Fig. 5, U Mann-Whitney test: LHo vs. Sho Z = -3.11, $p < 0.01$;

Lho vs. She Z = -3.70, $p < 0.0001$; Lho vs. Lhe Z = -3.65, $p < 0.001$; Sho vs. She Z = -0.51, $p = 0.62$; Sho vs. Lhe Z = -0.41, $p = 0.68$; She vs. Lhe Z = -0.10, $p = 0.92$; data corrected for multiple testing by *False Discovery Rate Approach* FDRA[17]).

The same pattern was observed as far as the duration of policing was concerned. In large homogeneous colonies, fertile workers were policed for a relatively short time (up to 4,800 s/individual; see Fig. 6). In other types of colonies, fertile workers were policed almost three times longer (up to 11,440 s/individual; Mann-Whitney U test: Lho vs. Sho Z = -4.10, $p < 0.0001$; Lho vs. She Z = -3.89, $p < 0.0001$; Lho vs. Lhe Z = -4.01, $p < 0.0001$; Sho vs. She Z = 1.86, $p = 0.07$; Sho vs. Lhe Z = 1.06, $p = 0.29$; She vs. Lhe Z = -1.02, $p = 0.31$; data corrected for multiple testing by FDRA[17]).

Queen policing was not observed in large homogeneous colonies, whereas it was present in small homogeneous (proportion of 0.13 queen-policed workers vs. total number of fertile workers), small heterogeneous (0.07) and large heterogeneous (0.3) colonies (Kruskal-Wallis test: H (df = 3, n = 102) = 12.00, $p < 0.01$; note that in post-hoc testing only the difference between large homogeneous and large heterogeneous was significant, Mann-Whitney test: Z = -3.16, $p < 0.01$). We did not observe policing against non-fertile workers from queenless parts, as well as queenright workers. Only queenright workers performed policing.

*Policing towards worker-laid eggs*

Overall worker-laid eggs had lower survival than queen-laid eggs (data from all colonies pooled, Wilcoxon matched pairs test: n = 29, Z = 3.31, $p < 0.001$, data corrected for multiple testing by FDRA[17]). However, the survival did not differ when we calculated it for four colony types separately (survival of worker-laid eggs vs. survival of queen-laid eggs, Wilcoxon matched pairs test: LHo $p = 0.08$, SHo $p = 0.11$, SHe $p = 0.25$, LHe $p = 0.10$; data corrected for multiple testing by FDRA[17]). Moreover, survival of worker-laid eggs did not differ significantly among all four colony types (Kruskal-Wallis test: H (df = 3, n = 29) = 0.79, $p = 0.86$), as well as proportion of surviving worker-laid eggs and queen-laid eggs (Kruskal-Wallis test: H (df = 3, n = 29) = 0.23, $p = 0.98$).

*Behaviour of non-policed fertile workers*

We observed two types of behaviour of non-policed fertile workers: (1) some kept laying eggs in the presence of the queen until the end of the experiment and retained developed ovaries (stage 3), while others (2), though not policed, ceased reproduction and re-restrained their ovaries before end of the experiment (stage 4). We called the behaviours: rebellion and self re-restraint respectively (see discussion). Interestingly, in small homogeneous colonies all non-policed workers rebelled (five workers non-policed, rebellion proportion = 1), while in large homogeneous colonies all non-policed workers re-restrained themselves (21 workers non-policed, rebellion proportion = 0). In heterogeneous colonies we observed mixtures of both behavioural patterns (small ones: four workers non-policed, rebellion proportion = 0.5, large ones: three workers non-policed, rebellion proportion = 0.77). Overall the data show that behaviour of non-policed workers was influenced by both colony size and relatedness.

## Discussion

Our results show for the first time that policing effectiveness depends on colony size, *i.e.* the proportion of policed workers in small homogeneous colonies (0.78) was three times higher than in large ones (0.28). The data is supported by observations that policing in small colonies lasted three times longer than in large ones. That indicates that workers in small colonies were more determined to police fertile individuals. The difference in policing effectiveness between small and large colonies, however, was not found in heterogeneous colonies: proportion of policed workers (0.85-0.87) and duration of policing did not vary significantly and was similar to that in small homogeneous colonies.

One question that arises is why workers in large homogeneous colonies policed less efficiently. That was not because the fertile workers were more difficult to detect in large colonies: in large heterogeneous colonies policing duration was similar to that in small homogeneous ones. That was also not a result of different fecundity of fertile workers in large homogeneous colonies, as it did not differ among all four colony types. The most likely answer is that the direct costs of worker-reproduction are proportionally lower in large colonies. For example, in case of the first type of costs (reproducing individual's workload is not performed), if an average worker takes care of one larva, then every reproducing worker, that abandons it's work, means one dead larva, irrespectively of the colony size. However, for the colonies consisting of 10 workers one dead larva means 10% of the brood, while for a colony consisting of 100 workers it is only 1%. The same pattern applies to both other types of costs. Therefore, the cost hypothesis can explain why workers in large colonies are less determined to police each other's reproduction.

We observed two types of behaviour of non-policed fertile workers: rebellion and self re-restraint. The adaptive reproduction schedule hypothesis predicts for large homogeneous colonies not only lower policing but also, as a consequence, higher worker reproduction. The latter was not confirmed in our experiment. Instead, (1) in large homogeneous colonies, we observed total cession of worker reproduction, due to peaceful self re-restraint of the fertile workers, and (2) total or partial rebellion (*i.e.* worker reproduction) in other types of colonies. Self re-restraint has been observed in workers of honey bee[18]. One can argue that fertile workers in large homogeneous colonies voluntarily followed honest signals of the queen[19]: queens might be less fecund in small colonies than in large due to different nutrition or age. There is no adequate evidence for this however, therefore this explanation should be rejected as too speculative. On the other hand, lack of self re-restraint in small homogeneous colonies might indicate that the behaviour in large homogeneous colonies was enforced by large workers forces[3]. The rebellion of workers occurred only in small or heterogeneous colonies, indicating that it is possible only when workers forces are small or weakened by internal conflicts. Rebelled workers dominated other nestmates and queen, reaching top-rank position in hierarchy. This supports the selfish policing hypothesis[11], which predicts that worker policing is a form of dominance behaviour by which police-workers ensure their high-rank status. In fact, our rebelled workers were too strong to be policed and instead they dominated other workers and even a queen. From selfish-policing hypothesis solely we should also expect lower effectiveness of policing in large colonies, as hierarchy playing the key role in hypothesis, is more difficult to maintain in large groups. At the same time the selfish-policing hypothesis does not explain why we found high policing effectiveness in large heterogeneous colonies.

However all theories mentioned before do not provide explanation why non-policed workers in large homogeneous colonies stopped, while in other colony types they continued reproduction. As we showed *T. unifasciatus* produces more sexuals per capita in large colonies (Fig. 2). Moreover only large colonies produce virgin queens which in homogenous colonies are the most related type of brood to a worker (Fig. 1). Therefore, from a worker's selfish perspective it pays-off more to refrain from reproduction when colony is large and to rear gynes ($r = 0.75$) rather than produce it's own sons ($r = 0.5$). This condition is not met in small colonies which do not produce sexuals, as well as in heterogeneous colonies where a worker is more related to it's own son than a gyne. That explains why we observed self re-restrain in large homogenous colonies and rebellion in other colony types.

In addition, our results indicate that workers are able to discriminate between small and large, as well as homogeneous and heterogeneous colonies. Although the direct mechanism is not known, the insects' ability to count objects has already been demonstrated[20]. Here it appears that the social insects are also able to assess colony size. Although social insects are thought to not be able to discriminate kin from non-kin (for example see ref.[21]) they still have some discriminating abilities based on genetic factors[22,23]. Since our ants discriminated between homogeneous and heterogeneous colonies, we suggest that workers could detect the decreased relatedness among colony members by cues coming from hydrocarbon profile of colonies. If decreased relatedness would cause higher variation of hydrocarbons in a profile and workers would detect it, then more effective policing could result.

It should be pointed out that there is no evidence that *T. unifasciatus* workers produce males in nature when a queen is present in a colony[10,13]. Therefore, our rebelled workers unveil a hidden pattern which is extremely rare in nature, due to unlikely reunion of split colony. Nevertheless, it shows that under certain circumstances workers can selfishly reproduce in the presence of a queen.

Although there are many behavioural patterns possible, workers always follow simple zero-one response schedule. For the police-workers schedule is: police if a colony is small or heterogeneous (1); not police if colony is large and homogeneous (0); if 1: police till a fertile worker re-restrains, if fertile worker is weaker than you (1 1) or stop policing if fertile worker is stronger (1 0). Response schedule for fertile workers is: if policed (1) re-restrain if weaker than police-workers (1 1) or rebel if stronger (1 0); if not policed (0) self re-restraint (0 0) if colony is large and homogeneous or rebel (0 1) if colony small or heterogeneous.

Overall our results give new insights into how conflicts among lower-level units (workers and queen) are resolved in order to achieve efficient higher-level unit (colony). Simultaneous effect of relatedness and an ecological factor, *i.e.* colony size on conflict resolution mechanism was demonstrated. The lower-level units were able to assess complicated parameters and adjust their behavioural response to them. Despite its complexity, conflict resolution strategy, is based on simple zero-one schema.

## Figures

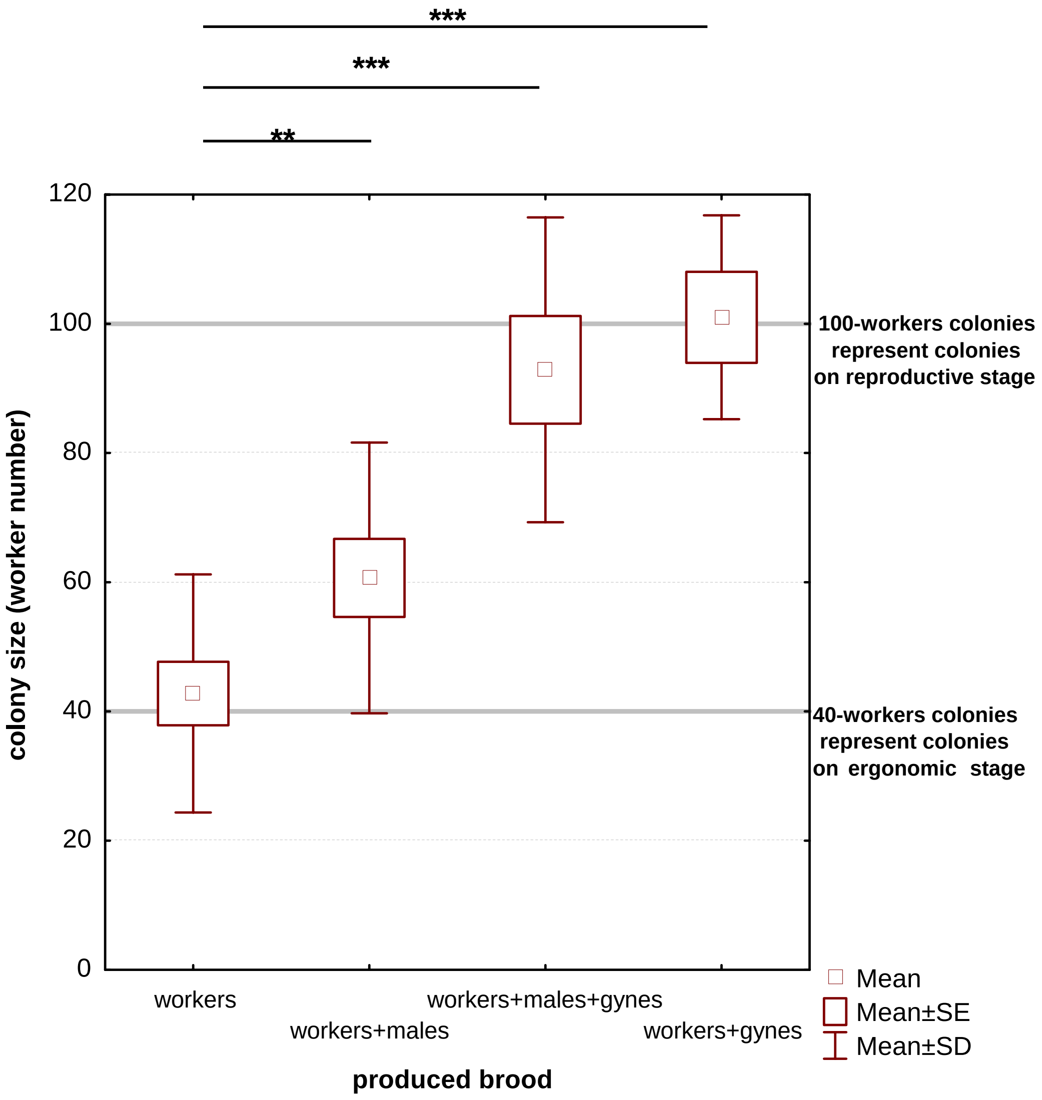

**Fig 1.** Sexual reproduction depends on the size of a colony. Small colonies (mean ± SD: 43 ± 18) were on ergonomic stage of development and produced only workers, whereas large colonies (96 ± 21) were on reproductive stage and produced both workers and sexuals (gynes or males). To test whether policing effectiveness depends on size and development stage of colony we tested policing in colonies consisting of 40 and 100 workers, representing ergonomic and reproductive stage respectively (compare thick horizontal lines on the graph). ** $p < 0.01$, *** $p < 0.001$. Kruskal-Wallis test H (n = 40, df = 3) = 21.6, $p < 0.0001$; Mann-Whitney U post-hoc tests: workers vs. males Z = -1.93, $p < 0.06$; workers vs. gynes + males Z = -3.51, $p < 0.001$; workers vs. gynes Z = -3.19, $p < 0.01$; gynes + males vs. gynes Z = -0.37, $p = 0.71$; data corrected for multiple testing[17].

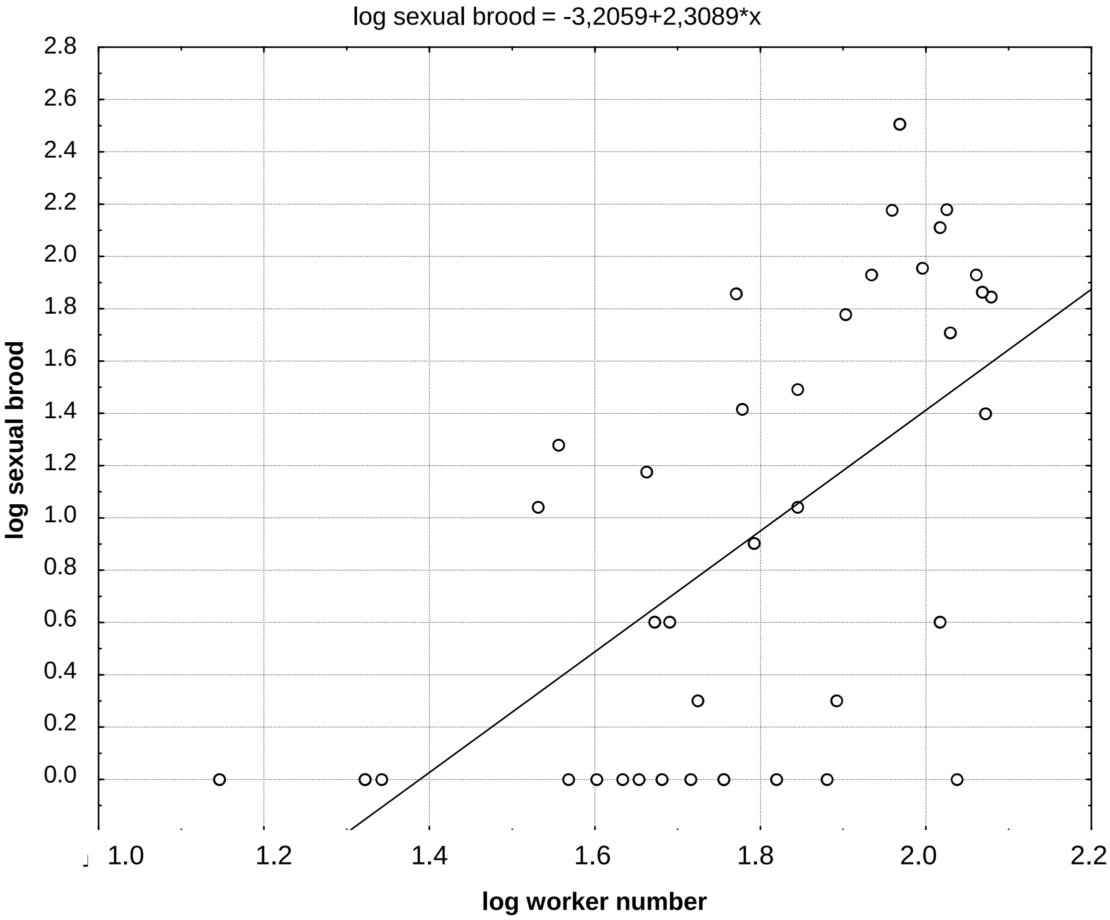

**Fig 2.** Larger groups produce more sexual brood (males and queens) per single worker (data corrected for different weight of males and queens; regression summary for worker number and sexual brood production: B = 1.66, Standard Error for B = 0.38, t (39) = 4.35, $p < 0.0001$; analysis of slope: value = 1.74, $p < 0.05$, slope > 1). A reproducing worker imposes direct costs on the colony-level in at least three ways: 1) the workload of the reproducing individual is not performed, 2) it consumes resources and invests them into worker-laid eggs and 3) worker-laid eggs have to be reared into adult individuals. Those costs are proportionally easier to bear in large colonies because a single worker rears per capita more brood than it does in small colonies.

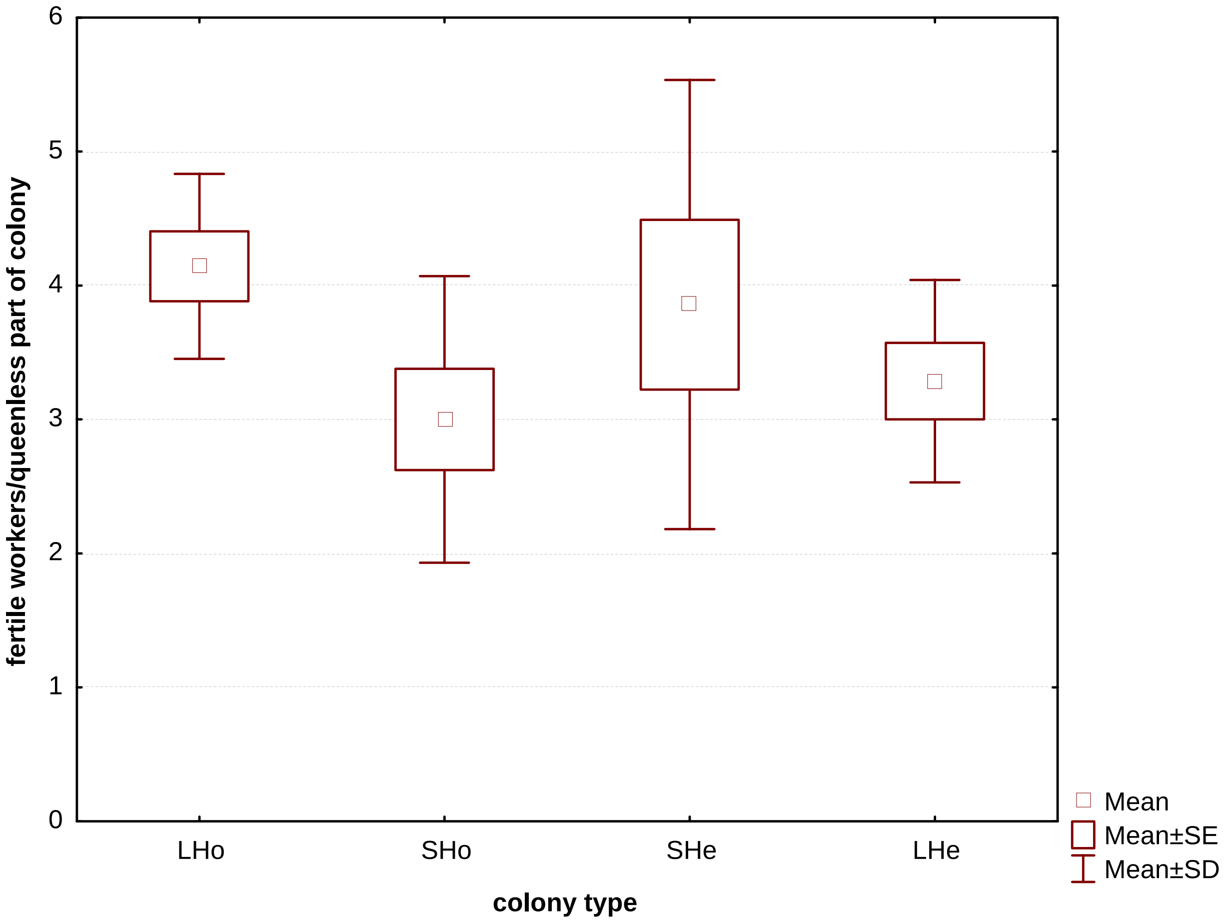

**Fig 3.** Number of fertile workers per queenless part of a colony in different types of colonies. The number did not differ significantly among colony types: Kruskal-Wallis test H (df = 3, n = 29) = 5.1, $p = 0.17$). LHo – large homogeneous colonies, SHo – small homogeneous colonies, SHe – small heterogeneous colonies, LHe – large heterogeneous colonies; large colonies = 100 workers, small colonies = 40 workers; homogeneous colonies r = 0.75, heterogeneous colonies r = 0.185.

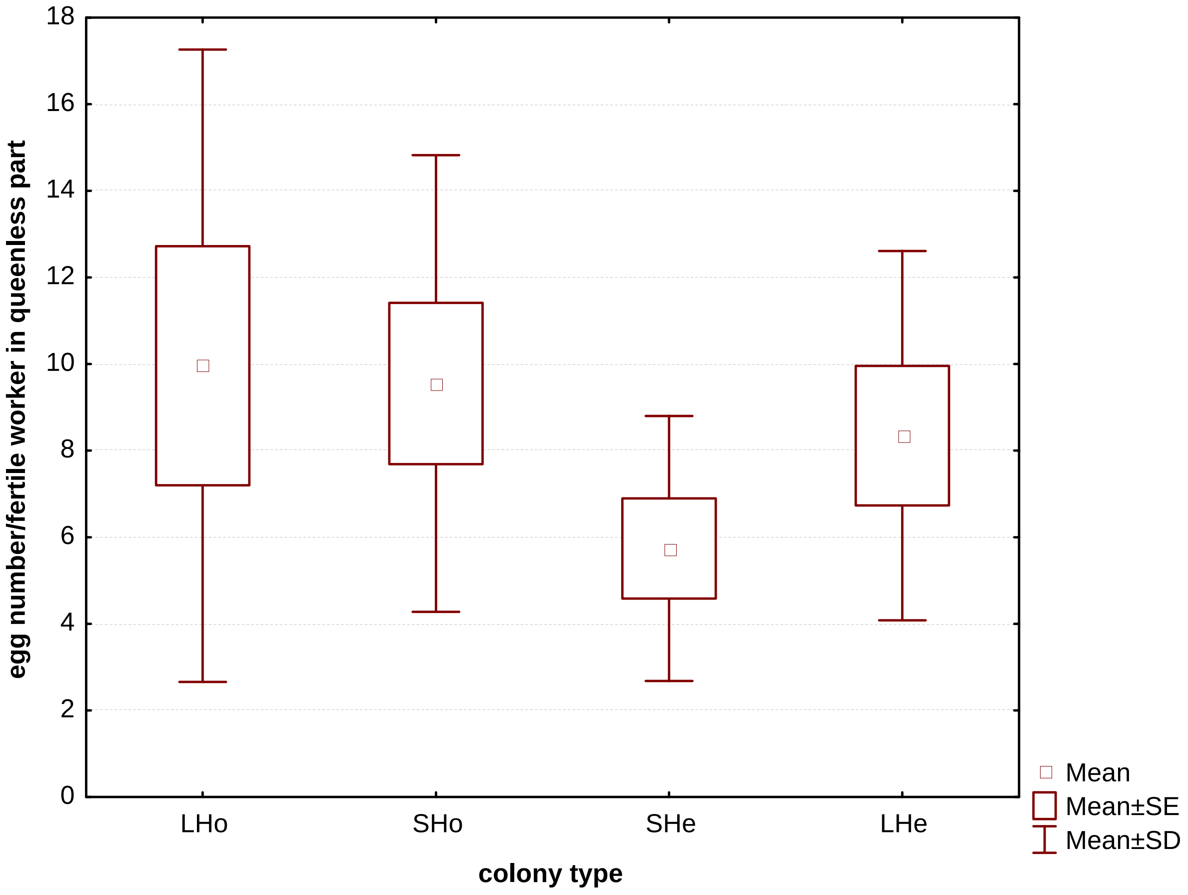

**Fig 4.** Egg number per fertile worker per queenless part of a colony in different types of colonies. The number did not differ significantly among colony types: Kruskal-Wallis test H (df = 3, n = 29) = 2.75, *p* = 0.43). The abbreviations as in Fig. 3.

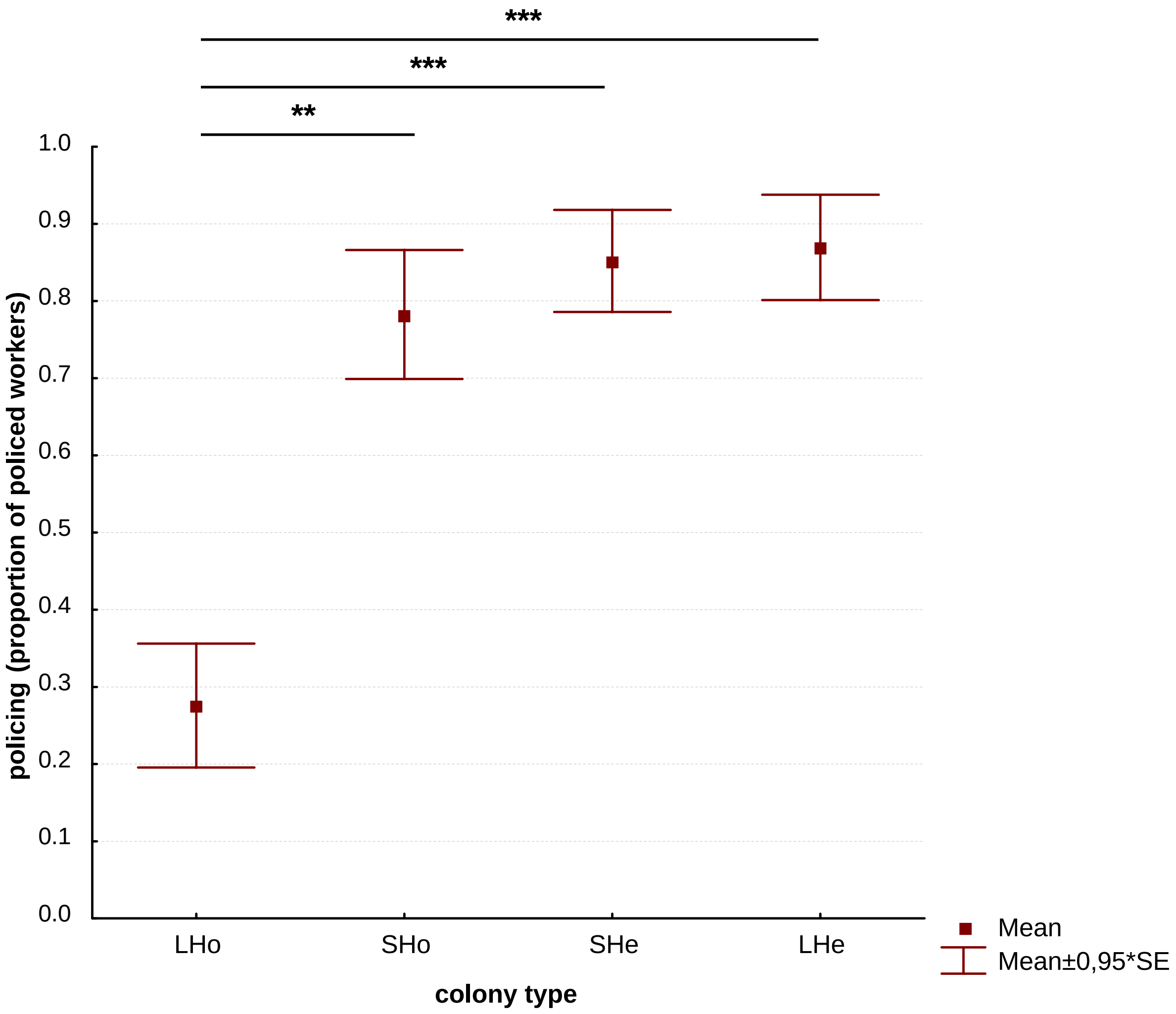

**Fig 5.** Proportion of policed workers depending on colony size and relatedness. LHo – large homogeneous colonies, SHo – small homogeneous colonies, SHe – small heterogeneous colonies, LHe – large heterogeneous colonies; large colonies = 100 workers, small colonies = 40 workers; homogeneous colonies r = 0.75, heterogeneous colonies r = 0.185; Mann-Whitney U tests: ** $p < 0.01$, *** $p < 0.001$, data corrected for multiple testing[17].

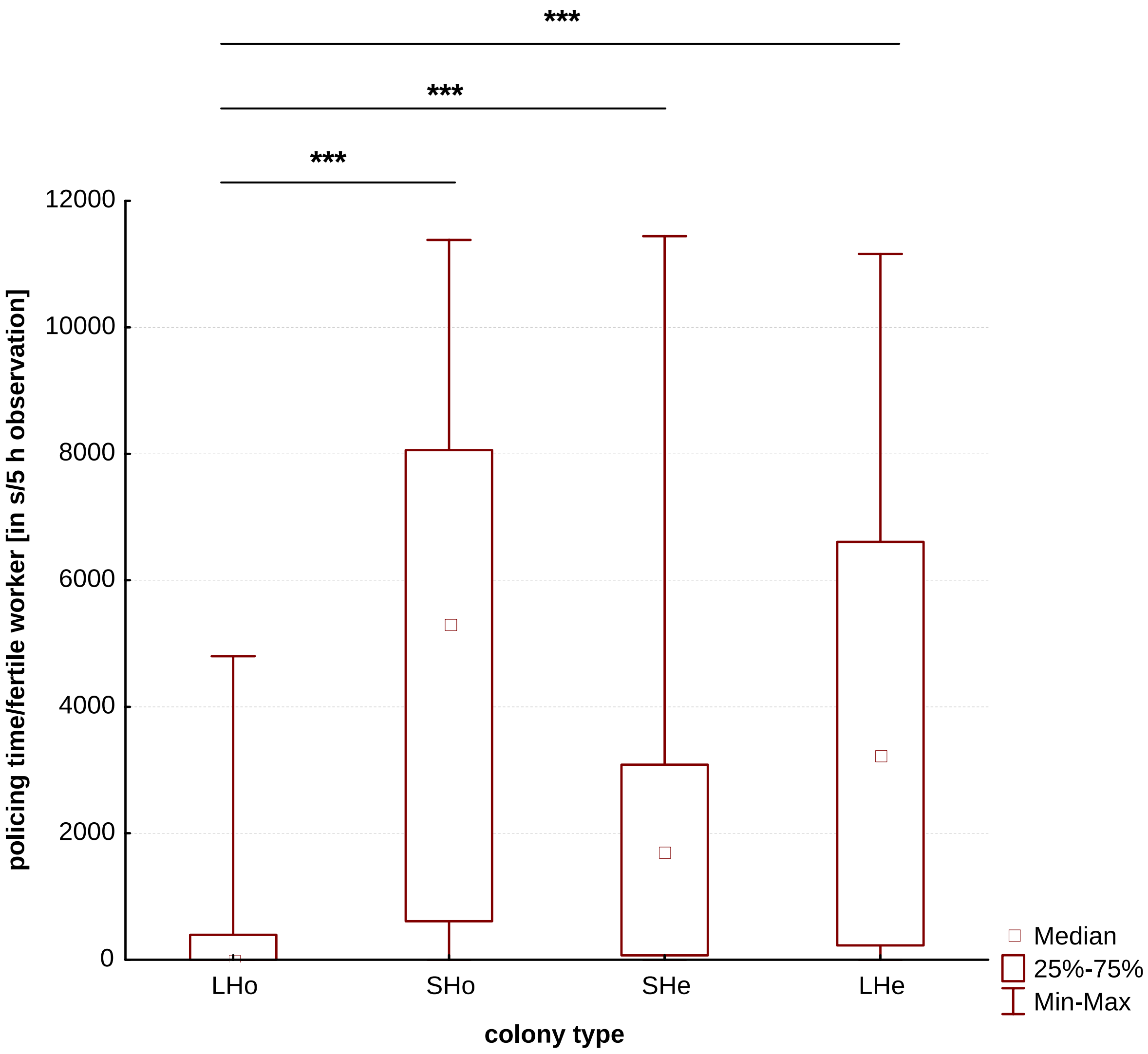

**Fig 6.** Policing time per fertile worker depending on colony size and relatedness. Legend as in Fig. 5.

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# Chapter V

## The potential fecundity hypothesis: reduction of potential fecundity leads to precocious foraging*

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*Manuscript

Two reproducing intra-nidal workers of *Temnothorax unifasciatus*
(© Bartosz Walter 2007)

## Abstract

Most of eusocial insects are characterised by precise labour division among reproductives, nurses and forages. Although intensively studied from colony-level to genes mechanisms underlying the division of labour between nurses and foragers are not yet fully understood. Here, I show that in a myrmicine ant *Temnothorax unifasciatus* (1) nurses possess high potential fecundity, while foragers do not; (2) decrease in potential fecundity is not dependent to chronical age, but instead some workers decrease their fecundity early in life and others later, (3) reduction of potential fecundity is primary to onset of foraging. The results suggest that decision to start foraging or not is self-decision of a worker and that workers stay in relatively safety nest as long as possess high potential fecundity, possible as hopeful reproductives. Similarly, laying eggs and foraging are mutually exclusive among colony founding queens of the ant *Pogonomyrmex rugosus* (Dolenzal et al. 2009). The findings may indicate general mechanism of division on nurses and foragers in insect societies, as well as demonstrate how reproductive division of labour was achieved in early stages of eusocial evolution.

## Introduction

Enormous evolutionary success of social insects (termites, ants, wasps and bees) was possible due to precise division of labour among colony members into reproductives, intra-nidal nurses and foragers working outside the nest (Wilson 1971). Although intensively studied for several decades mechanisms underlying the division of labour are still not completely understood (review in Gadau and Fewell 2008). The aim of this study was to examine whether natural variation in workers' fecundity makes ants undertake different tasks in the colony. As social insect workers commonly forego reproduction in a presence of queen, workers' fecundity is not usually expressed (Fletcher and Ross 1985). This potential fecundity, however, can be uncover when a queen dies and workers start to reproduce (Burke 1988a, 1988b, Choe 1988, Heinze et al. 1997). Therefore even in queenright colonies, I expected more fecund workers to perform tasks inside the nest, while less or non-fecund workers to forage outside. Testing this hypothesis required refuting two alternative explanations that 1) expected lower fecundity of foragers is a result of foraging; and 2) correlation between foraging and decreasing fecundity is strictly age-dependent, *i.e.* only old workers, expected to have decreased fecundity due to aging, became foragers.

Potential fecundity hypothesis proposed here assumes that 1) workers at the day of enclosure posses some potential fecundity, which can be expressed when reproductive individual or queen dies, 2) already at the time of enclosure workers vary in the reproductive potential, and 3) potential fecundity decreases unequally among workers, *i.e.* some of the workers lose fecundity early in their life and serve as precocious foragers, some in advanced age and forage only when old, and some never lose the potential fecundity and live inside a nest for all their life. First assumption narrows application of the hypothesis down to species where workers have functional ovaries and do not deal with species with sterile worker caste. However, fertility of workers is believed to be ancestral in social insects and sterility of workers has evolved only in minority of lineages (few ants genera and upper termites families; reviewed in Wilson 1971, Fletcher and Ross 1985, Choe 1988). In that few lineages the fecundity pattern could have been remodelled or replaced by other physiological pattern, e.g. hormone titre, dependent on workers quality or age. Second and third assumptions imply that even morphologically and genetically identical workers are not homogeneous group:

they vary in their physiology already at the point of enclosure and this variation can increase as workers age, *i.e.* their potential fecundity decreases at a different pace.

In several species the differences in workers' fecundity have been investigated in context of hierarchy and dominance, as well as in context of labour division among foragers and intra-nidal workers (e.g. Franks and Scovell 1983, Bourke 1988a, 1988b, Heinze 1996, Heinze and al. 1997). The initial variation can result from subtle differences in fate during larval stage, such as slightly better and worse feeding. Therefore it is assumed that there are more and less fitter workers which also lose their potential fecundity more or less quickly . It is important to note here that the hypothesis does not exclude the influence of aging but predicts that some workers age quicker than others as the moment of crossing the foraging threshold in potential fecundity is not normally distributed. Potential fecundity could also correlate with the amount of fat body (BW unpublished data). The fat body tissue serves social insects not only as an energy reservoir when the resources are scarce or colony lacks foragers, but also produces many substances, including a protein vitellogenin, crucial for reproduction. Vitellogenin influences an individual's fecundity both directly by forming yolk in an egg, as well as indirectly as a factor in main physiological cascades, including insulin-like-factor-cascade and juvenile-hormone-cascade, both crucial for fecundity, nutrition, longevity, immunity and behaviour e.g. onset of foraging (Amdam et al. 2003, Guidugli et al. 2005, Seehuus et al. 2006, Nelson et al. 2007). Therefore, intra-nidal workers using reserves from their fat body severely decrease their potential fecundity. Weaker workers, possessing smaller fat body, exhaust it more quickly than fitter workers and once their reserves decline to a certain level, they become foragers. As soon as they provide food to the colony, the process of fat body consumption in intra-nidal workers is stopped and their fat body might be even rebuilt. As to the foragers rebuilding the fat body is unlikely due to proximate factors: wear and tear, exposure to pathogens and desiccation, expensive foraging runs, as well as ultimate factors: high risk of predation and infection with pathogens outside.

The variation in the initial workers' potential fecundity and especially the variation in aging pace (decrease of potential fecundity over time) is expected to be influenced by genetic factors and to be the lowest in clonal social insects, medium in monoandrous and monogynous species with one morphological worker case and highest in polyandrous and polygynous species. In fact clonal system is known only in twelve species (10 in ants, 1 in termites and 1 in bees; note that clonal system is common in aphids which of c.a. 50 species are sometimes consider as eusocial). Monogyny and monoandry is believed to be ancestral in social insects and is predominant in existing species (Hughes et al. 2008).

Most of social ants, wasps and bees have no morphologically distinct worker sub-castes, and worker polymorphism is know in minority of ants genera and is common in termites (Wilson 1971). In many species with worker polymorphism workers' sub-castes are specialized into performing a special tasks, e.g. in *Oecophylla* ants major workers work outside a nest, while minor worker in a nest. In such labour division sub-castes working in a nest are expected to have higher potential fecundity than sub-castes working outside a nest. Similar solder castes in termites are typically sterile (compare Korb 2009). In other cases larger body size may correlate with higher fecundity, as in thelytokous ants *Pristomyrmex punctatus* (former *P. pungens*, Sasaki and Tsuji 2003; note that higher body mass and higher fecundity in major workers *P. punctatus* may results from different genotype, see Dobata et al. 2009).

We examined the potential fecundity hypothesis using an ant *Temnothorax unifasciatus*, a system providing ideal conditions to study labour division. This species live in relatively small colonies (from few up to a few hundreds workers, usually smaller than 100) that allows to mark and observe all ants individually. Workers are produced only a few times per year in cohorts. Freshly enclosed workers are easy to detect due to distinct light pigmentation. That allows to observe many individuals on the same age in a single colony. The colonies of *T. unifasciatus* are headed by one single mated queen (monogyny and monoandry; compare Heinze et al. 1995, Heinze et al. 1997) what fundamentally deceases influence of genetic factors on workers variability. Workers are potentially fertile and reproduce when queen is not present in a colony (Heinze et al. 1995, Heinze et al. 1997), what is crucial because workers' fertility is assumed to be ancestral in social insects evolution (Wilson 1971). There are no morphologically different worker sub-casts, as it is the case in most social species. In nature the ants inhabit rocky crevices or hollowed oak sticks – of which both can be easily mimicked in laboratory conditions. Furthermore the natural nests are fragile and therefore ants are forced to change the nest a few times during a colony life. During migrations colony is likely to split into queenright and queenless parts. All those factors along with relatively long lifespan of workers (up to two years, BW unpublished data) significantly increase chances for intra-nidal workers to become reproductive when queen is lost or dead. Therefore, a worker is expected to remain  an intra-nidal worker as long as its potential fecundity stays above a certain level. When the threshold is crossed a worker is expected to become a forager as it will not be able to reproduce even if the queen dies or is lost. All together *T. unifasciatus* represents an ancestral mode of social organisation (small colonies, monogyny and monoandry, workers with functional ovaries, no morphological castes). Studying this system may allow us to uncover proximate and ultimate mechanisms employed in evolution of social insects.

First, I tested natural variation in fecundity between foragers and intra-nidal workers of unknown age. To unveil their potential fecundity, workers were put into new closed nests (*Closed Nest Approach*, see Methods) with larvae but without a queen. This way foragers were forced to stay inside the nest and take care of the brood and prevented from staying outside, what they normally prefer. Second, I compared fecundity of foragers and intra-nidal workers of the same age, to examine whether foraging and decrease of fecundity are strictly age dependent. I expected to find low fecundity in foragers and high fecundity in intra-nidal workers, independently of age, *i.e.* even young foragers were expected to be less fecund and even old intra-nidal workers were expected to be more fecund than other ants of the same age. Third, as young workers were kept in queenless conditions I tested whether foragers or intra-nidal workers are reproductive workers. I expected to find strict division between non-fertile foragers and fertile intra-nidal workers. Fourth, the fecundity of young workers was experimentally reduced by exposing them to carbon dioxide ($CO_2$) and their foraging activity was compared with the same-age but naturally fecund workers. Workers with reduced fecundity were expected to start foraging earlier than non-manipulated individuals. Fifth, new foragers , *i.e.* ants foraging for one day, were collected and their fecundity checked. I expected them to have low fecundity, as the potential fecundity hypothesis predicts that decreased fecundity stimulates ants to start foraging, rather than the opposite, that decreased fecundity is the effect of foraging.

Here I show that workers in a colony exhibit natural variation in fecundity, which is independent from age: low-fecund workers serve as foragers while highly fecund workers stay in safe nest. The low fecundity is primary to the onset of foraging behaviour, *i.e.* individuals with decreased fecundity become foragers and foraging activity may only advance this process, but not induce it. Those findings not only demonstrate a proximate factor underling labour division but also shed new light on the evolution of altruism in social insects. Though worker forego direct reproduction in a presence of a queen, nevertheless they stay in the safety of the nest as long as they are potentially fertile and wait for chance to reproduce should the queen die or colony split. Only when fecundity of a worker decreases and it has definitely no chance to reproduce directly it starts to perform risky duties, *i.e.* foraging. The colonies consisting of morphologically identical individuals differing only in their individual fecundity is assumed to be ancestral in the evolution of eusocial insects (Wilson 1971). Therefore, the division into low-fecund foragers and intra-nidal reproductives might play key role in evolution of cooperation and altruism in social insects.

## Material and Methods

### *Ants collection, nesting and marking*

Complete colonies of the ant *Temnothorax unifasciatus* (*Myrmicinae*) were collected from their nests in rock crevices and hollowed oak's sticks in a population at Gargnano, Lago di Garda, Italy and transferred into 10cm x 10cm x 3cm plastic boxes with a plaster floor (foraging arena) and a 50mm x 12mm x 3mm cavity between two microscope slides serving as a nest site with one entrance (see Photo 1 in Photo Gallery). Ants were provided with water and food (honey and pieces of fresh cockroaches) ad libitum and reared at 12/12 hours day/night cycle with temperatures of 20°C and 25°C, respectively. If not stated otherwise all workers used in the experiments were individually marked with various colours of 30µm thin copper wires (courtesy of Elektrisola, Eckenhagen) knotted around their petioles and postpetioles. If not stated otherwise all colonies were queenright, headed by a single queen, having worker-larvae ratio close to 1:1.

### *Closed nest approach*

To check workers' fecundity cohorts of workers showing the same behaviour (foragers or intra-nidal workers) or being after the same treatment (exposed or non-exposed) were kept without queen (queenless conditions) using *Closed Nest Approach*. A worker cohort was placed in a 50mm x 12mm x 3mm cavity between two microscope slides with entrance closed by paper plug. Thus ants were not able to forage and potential effect of foraging was excluded. Instead, water and honey was provided directly to the nest ad libitum (cotton soaked with honey and water). If not stated otherwise the cohorts were kept with five larvae from their own nests to force ants to take care of the brood, *i.e.* mimicking nest conditions.

### *Foragers and intra-nidal workers: definitions*

An ant was assumed to be a forager when it was, at least once, collecting food or water outside a nest and bringing it back. All workers which never collected food or water outside were assumed to be intra-nidal workers. Foraging turn refers to an act when a worker left a nest, walked/run in the foraging arena, collected food/water and brought it to a nest. When worker come back to nest without food/water such act was not counted as foraging.

*Fecundity of foragers vs. intra-nidal workers*

First, workers of unknown age were observed in 30 one-minutes intervals per day for five consecutive days in 18 colonies. Than ten random foragers and ten random intra-nidal workers per colony were collected and kept as separate foragers' and insiders' cohorts in queenless conditions for six weeks. Egg number was counted in cohorts every three days. After six weeks all eggs were removed from the nest, counted again and frozen. The number of eggs laid by foragers cohort was compared with the egg number laid by intra-nidal workers cohort from the same colony using Wilcoxon matched pairs test.

Second, to exclude potential effect of age difference between foragers an intra-nidal workers, ants of the same age were tested. In five colonies all workers were removed except from ten per colony which were marked with a cupper wire. Within next five days new cohorts of workers enclosed from pupae (new workers/colony: range 36-80, median 80). All new workers were marked as a group, while ten old workers were removed. In that way a single colony consisted of same-age workers of which some became foragers and others remained intra-nidal workers within two weeks after marking. Than, after the colonies were not manipulated for a few months (2-12 months, median 5), ten random foragers and ten random intra-nidal workers per colony, all of the same age, were collected and kept separately as foragers' and intra-nidal-workers' cohorts using *Closed Nest Approach*. Egg number was counted every three days. After six weeks all eggs were removed from the nests, counted again and frozen. The number of eggs laid by foragers cohort was compared with the egg number laid by intra-nidal workers cohort from the same colony using Wilcoxon matched pairs test.

Third, 19 colonies consisting of a queen, ten old marked workers, 40-100 young (one to five days old) unmarked workers and brood. After one month twenty of young, non-foraging workers were removed, marked individually and placed into queenless nest with open entrance and 20 larvae. The ants were observed for five hours over six weeks (in ten minutes intervals, five intervals per week). Position of each ant (outside the nest, inside the nest: on brood pile, out of brood pile) and their behaviour (staying, walking, brood tending, self and allogrooming, food exchange, antennal contact or boxing, biting, nest cleaning, egg lying) was noticed. Time spend on particular behaviour was noticed with accuracy to one second. Egg number was counted every third day. After six weeks eggs were removed from the nest, counted again and frozen. Ants were also removed, frozen and their ovaries were dissected to confirm their fertility. Four stages of ovary development were recognized: 0 for non-elongated ovaries, 1 for elongated but without eggs, 2 for elongated with trophic eggs and 3 for elongated with viable male-designed eggs (Buschinger and Alloway 1978). Only workers with ovary status 3 were counted as fertile. Number of fertile foragers and fertile intra-nidal workers was compared using $\chi^2$ test.

*Experimental reduction of workers fecundity*

If reducing workers fecundity leads to foraging than its should be possible to induce foragers from intra-nidal workers by exposing the latter to a stressor which reduces fecundity. To test this notion cohorts of ten young (5 to 10 days old) workers were exposed to carbon dioxide ($CO_2$) and their fecundity was compared with fecundity of cohorts consisting of 10 non-treated control workers (queenless cohorts in *Closed Nest Approach*, eggs counted and collected after six weeks, workers from exposed and control cohorts were of the same age and from the same ten source colonies). The treated workers were exposed three times to 95% $CO_2$ for 20 hours with 24-hour break between exposure sessions.

*Foraging of workers with reduced fecundity*

Ten colonies consisting of queen, 20 workers and 20 larvae were set up. All workers in a colony were of the same age (five to 10 days old) and inexperienced in foraging (a colony was monitored for five hours per day after the first worker enclosed; nest entrance was closed by paper plug for the rest of the time). Ten random workers per colony were collected and exposed three times to 95% $CO_2$ for 20 hours with a 24-hour break between exposure sessions. Between exposures workers were kept in closed, queenless nest with water-soaked paper for 24 hours in order to recover. In that time the nest with queenright part of a colony was closed, not allowing workers to start foraging. After exposure both exposed and control ants were marked individually and put back together into nests. Every colony consisted therefore of both workers with natural variation of fecundity (controls) and workers with reduced fecundity (exposed). The colonies where the workers were placed were hungry, since we prevented them from foraging for 5 days by blocking the nest entrance. Foraging arena in each colony was observed for 150 consecutive minutes a day five times per week. Food and water were placed on the arena only during observations, therefore workers were not able to forage when they were not observed. Foraging time and sort of collected food (honey, meat, water) was noticed per every foraging turn. In addition to observation of foraging arena, behaviour of each worker in the nest was observed for five minutes in one-minute intervals per day. After the first worker started to forage observation was continued for the next two weeks. Week after the first worker started to forage, it was collected and placed in a closed nest. All workers which started to forage within a week after the first worker (first cohort of foragers) were nested together. Workers which started to forage later were nested in a second closed nest as a second cohort of foragers. After the observation was finished five random intra-nidal workers per colony were collected and put into closed nests. After six weeks eggs laid by intra-nidal workers' cohorts were counted and compared with a sum of eggs laid by the first and second cohort of foragers.

*Statistical analysis*

Statistical analysis was performed using StatSoft STATISTICA6.

## Results

### *Fecundity of foragers vs. intra-nidal workers*

In all 18 colonies foragers were much less fecund than intra-nidal workers (Fig 1., Wilcoxon matched pairs test: Z = 3.72, $p < 0.0002$). In 11 cohorts foragers did not lay a single egg and maximum fecundity was eight eggs per a cohort (median = 0), while all intra-nidal workers cohorts laid between 12 and 86 eggs (median = 31). The same pattern was observed when foragers were of the same age as intra-nidal workers (Fig 2., Wilcoxon matched pairs test: n = 5, Z = 2.02, $p < 0.05$). While foragers cohorts laid up to 12 eggs (2 of them laid no eggs, median = 3), intra-nidal workers cohorts laid between 33 and 71 eggs (median = 52).

In queenless cohorts, where ants were allowed to forage, notable division into non-fertile foragers and fertile intra-nidal workers occurred. Total of 142 foragers and 258 intra-nidal workers were observed. Dissections revealed that 69 ants were fertile (ovary status 3). Fertile individuals almost exclusively recruited from intra-nidal workers (64 intra-nidal fertile workers vs. 5 fertile foragers, $\chi^2$ test: $p < 0.00001$).

### *Experimental reduction of workers fecundity*

Prolonged $CO_2$ exposure reduced an average workers' fecundity five times. Young workers exposed to $CO_2$ three times for 20 hours were on average six times less fecund as control workers of the same age and from the same colony (Fig 3). As cohorts of exposed workers laid between 0 to 56 eggs (two cohorts laid no eggs; median = 13 eggs), cohorts of non-treated ants laid between 19 to 90 eggs (median = 65.5 eggs; Wilcoxon matched pairs test: n = 10, Z = 2.80, $p < 0.005$). In all cases exposed workers laid less eggs than controls.

### *Foraging of workers with reduced fecundity*

Workers with reduced fecundity became foragers more frequently than workers from unmanipulated groups (Fig 4., Cox's F test: F (80, 130) = 2.19, $p < 0.0001$). Within 3 weeks 70% of $CO_2$-treated ants changed tasks from working inside the nest to foraging outside, while only 40% of controls did alike. Total number of foragers per colony differed between 5 to 13, that was between 3 and 9 for exposed ants and between 2 and 7 for control ants. Before becoming a forager an ant was coming close to nest entrance, making short (few seconds or minutes) walks in the foraging arena and coming back to the nest. After 1 to 5 days of such behaviour ants started to forage: collecting food or water and bringing it to the nest. In total exposed foragers spent more time foraging than control workers (see Fig. 5., Wilcoxon matched pairs test: n = 10, Z = 2.19, $p < 0.03$) and performed more foraging turns (see Fig. 6, Wilcoxon matched pairs test: n = 10, Z = 2.19, $p < 0.03$). However, per capita single control forager foraged for a similar time as an average exposed forager (Fig. 5., Wilcoxon matched pairs test: n = 10, Z = 1.7, $p = 0.24$), as well as performed almost the same number of turns (Fig. 6., Wilcoxon matched pairs test: n = 10, Z = 1.89, $p = 0.06$).

After six weeks of being kept in closed nests the foragers' cohorts laid significantly less eggs than cohorts of five random intra-nidal workers (Fig. 7.; egg number/foragers cohort: median = 1, range 0 to 3, 3 cohorts laid no eggs; egg number/intra-nidal workers cohort: median = 14, range 1 to 48; Wilcoxon matched pairs test: n = 10, Z = 2.67, $p < 0.008$). The difference was even bigger when the number of eggs was divided through the number of individuals (Fig. 7.; egg number/forager/cohort: median = 0.1, range 0 to 0.3; egg number/intra-nidal worker/cohort: median = 2.8, range 0.2 to 9.6; Wilcoxon matched pairs test: n = 10, Z = 2.81, $p < 0.006$). Foragers from the first cohort showed the same fecundity as foragers from the second cohort (Wilcoxon matched pairs test: n = 10, Z = 0.0, $p = 1.0$).

## Discussion

The results clearly show that reduction of potential fecundity leads workers to the onset of foraging. Experiment with workers of the same age but performing different tasks clarified that onset of foraging behaviour is not strictly age dependent, *i.e.* there are young workers that forage and old individuals still being intra-nidal workers. Instead, what distinguishes foragers from intra-nidal workers is the difference in their potential fecundity. When it declines to a certain level intra-nidal workers become precocious foragers. Furthermore, fresh foragers (collected on the first or second day of foraging) revealed fecundity level similar to experienced foragers. This two findings indicate that the decrease of potential fecundity is primary to onset of foraging and is not an effect of wear and tear resulting from foraging.

Furthermore workers with experimentally reduced fecundity forage for the similar time and with similar efficiency (number of foraging turns in time) as unmanipulated foragers. It shows that an experimental reduction of fecundity only accelerates the onset of foraging, but does not decrease foragers' efficiency. Moreover, observations of queenless colonies and later dissections confirmed prediction that reproducing workers generally do not forage. By keeping foragers and intra-nidal workers in queenless closed nests their potential fecundity was unveiled and proved to be similar in the-same-task cohorts, irrespectively from treatment and age. All together the results confirm the potential fecundity hypothesis and show that potential fecundity plays key role in labour division among foragers and intra-nidal workers.

As mentioned before the results revealed that decrease in potential fecundity and onset of foraging are not strictly age dependent. I show that even few-days old individuals can be low-fecund and forage, as well as old ants can still work in the nest if they possess high potential fecundity. That indicates that workers in a colony are not the homogeneous group and may experience different fate. The proximate mechanisms underlying that variousness are not yet known. One possibility is different feeding during larval stage, resulting in different fat body size. One can argue that larvae should all be fed the same, and the different treatment is expected only between worker- and queen-destined larvae. It is particularly likely in species which rear larvae in separated wax or paper cells. For example honey bees *Apis mellifera* rear workers' larvae individually in identical wax cells and each larva gets the same amount of food (Tautz 2008). As there is no difference in nutrition, the difference in fecundity between workers is based on genetic factors (for example compare low- and high-pollen-hoard-strain bees: Page and Fondrk 1995, Pankiw and Page 2001, reviewed in Page et al. 2009). The onset of foraging behaviour might be accelerated by suboptimal conditions or infestation by mites (Amdam et al. 2004). In stingless bees *Melipona* nutrition is believed to have no influence on fecundity of imagines (Wenseleers and Ratnieks 2004, Wenseleers et al. 2004). In that species all larvae are fed the same and later development into worker or queen is thought to be dependent on a decision of an individual itself. In ants and termites, where larvae are kept on piles and not in separate cells, equal feeding is less likely. Therefore, differences in worker-larvae nutrition may result in more and less fecund workers, a pattern that resembles the fact that the best nurtured individuals develop into queens that outrange fecundity of workers (Wilson 1971). Moreover many of polistine wasps and ponerine ants have no morphologically distinct queen cast (Gamboa et al. 1978, Dropkin and Gamboa 1981, Ito and Higashi 1991, Peeters 1997, Cuvillerier-Hot et al. 2001). Therefore, slight differences in potential fecundity may decide which individual becomes new reproductive when the old reproductive dies.

Huge variability in fecundity and age of fertility decline in *T. unifasciatus* workers is different from common human experience, as the age of becoming mature or age menopause is normally distributed in a population (Cameron 2002). However social insects are known to not follow aging patterns exhibit by solitary species, *e.g.* queens do not show trade-off between longevity and reproduction, the trade-off which is common for non-eusocial species (Keller and Genoud 1997, Page and Peng 2001). It has to be clarified that aging also affects transition from intra-nidal workers to foragers: workers are expected to senescence (decrease potential fecundity over time) and more age advanced individuals are more likely to serve in a colony as foragers, *i.e.* follow age polyethism pattern. Therefore, the potential fecundity hypothesis does not reject age polyethism itself, but explains the occurrence of precocious or delayed foragers.

Apparently workers of the ant *Temnothorax unifasciatus* stay in the relatively safe nest as long as they have high potential fecundity and start to reproduce when queen is dead or lost. Such pattern was observed in several other *Formicoxenini* ants such as *T. crassispinus, T. lichtensteinii* and *T. albipennis* (BW unpublished data). In ponerine ants *Diacamma* a colony is inherited by strongest young individual after an old reproductive dies (Alle et al. 2001). Similar mechanism occurs in other ponerine ants without distinct queen caste (Sommer et al. 1993, Schiler et al. 1999, Cuvillerier-Hot et al. 2001). In lower wood-dwelling termites *Cryptotermes* a colony consists of a reproductive pair, few sterile solders and hundreds of non-reproducing individuals – "workers" (Korb and Lenz 2004). Intensive studies revealed that the "workers" of *Cryptotermes* in fact are hopeful reproductives waiting for current reproductives to die, or leave a maternal colony in order to establish their own (Korb and Katrantzis 2004, reviewed in: Korb 2008 and Korb 2009). This shows that reproductive potential of social insect workers was not lost with the transition from solitarity or semisocialty to eusociality. Instead, its natural variability may play a key role in evolution of non-reproductive labour division in eusocial species. Most likely eusociality evolved from species with collective brood rearing (revised in Wilson 1971). Under condition that individuals sharing one nest site were related, it would paid-off for some of them to forego their own reproduction and help rising offspring of their kin instead. This way, although indirectly, they produced more copies of their genes (Hamilton 1964). However the question what proximate factors may led some of the individuals to forego reproduction at that early stage remains open. Results concerning workers' fecundity and division of labour shown here suggest that simple variation in potential fecundity might have led to division into reproductive and non-reproductive individuals, as costs of refraining from reproduction are lower for less fecund individuals. Identical situation might still occur in nature in species of pleomentrotic colony foundation (new colony is founded by two or more young queens). In some ants (e.g. *Pachycondyla, Leptothorax gledlerii* and *Lasius meridionalis*) co-founding queens establish hierarchy and only the top ranking queen(s) is/are reproducing while others do not (Kolmer and Heinze 2000, Seifert and Buschinger 2001, Heinze 2004). In *Pachycondyla inversa* the division of labour is even more spectacular and after the hierarchy is established subordinate queens start foraging outside a nest (Kolmer and Heinze). It has to be studied in the future if subordinate queens serving as foragers have decreased potential fecundity in comparison to intra-nidal queens, as it is the case with *T. unifasciatus* workers.

It appears that young *T. unifasciatus* workers posses high potential fecundity which decreases at a different pace when they age. Therefore older workers are more likely to be foragers. Such pattern is expected to be widely spread in social insects. However, opposite situation can also occur. In particular in tropical wasps (hairy-faced hover wasp *Liostenogaster flavolineata*) younger individuals are known to be foragers while oldest inherits a nest when the reproductive female dies (Samuel 1987, Bridge and Field 2007, revised in Field 2008). Should that happen due to increasing potential fecundity with age, it would support the potential fecundity hypothesis.

As mentioned before the potential fecundity hypothesis respects age polyethism theory. The potential fecundity hypothesis is also in favour of life expectancy hypothesis as foragers are expected to have decreased life expectancy both due to costs of foraging and as less fit individuals (Woyciechowski and Kozłowski 1998, Moroń et al. 2008). No conflict between the hypotheses is therefore expected. Instead, the potential fecundity hypothesis may underpin the physiological causes that lead to decreased life expectancy and onset of foraging. For example decreased amount of fat body and decreased vitellogenin production may not only reduce potential fecundity but also affect other physiological mechanisms resulting in accelerated ageing or decreased life expectancy.

The potential fecundity hypothesis is in a favour of threshold theory. The onset of foraging is expected to be linked with crossing a certain threshold by an individual. Although the exact threshold is not yet uncovered the data suggest some relation between fat body amount, changes in vitellogenin titre and it's pleiotrophic effect.

All together the potential fecundity hypothesis is in agreement with behavioural, physiological and molecular data on labour division in social insects. It's novelty consists in pointing out that transition from intra-nidal workers into forages is affected by decrease in workers' fecundity, something what was overseen for a long time (possibly because workers are commonly considered to be altruistic non-reproductive forces, gaining their inclusive fitness indirectly). Because a decrease in fecundity is not strictly age dependent, it explains occurrence of precocious as well as delayed foragers. Remaining questions which have to be studied in near future are (1) if the hypothesis applies to other social insects with workers having functional ovaries or in species lacking morphologically distinct queen caste (queenless ponerine ants, *Polistes*), (2) whether the decrease of potential fecundity with age is reversed in *Liostenogaster* hover wasps, (3) what are the factors underlying variance in potential fecundity. It calls and re-studying of labour division data-body in context of potential fecundity variation. Therefore, as integrative studies on several distinct lineages are necessary the story is just about to begin.

## Figures

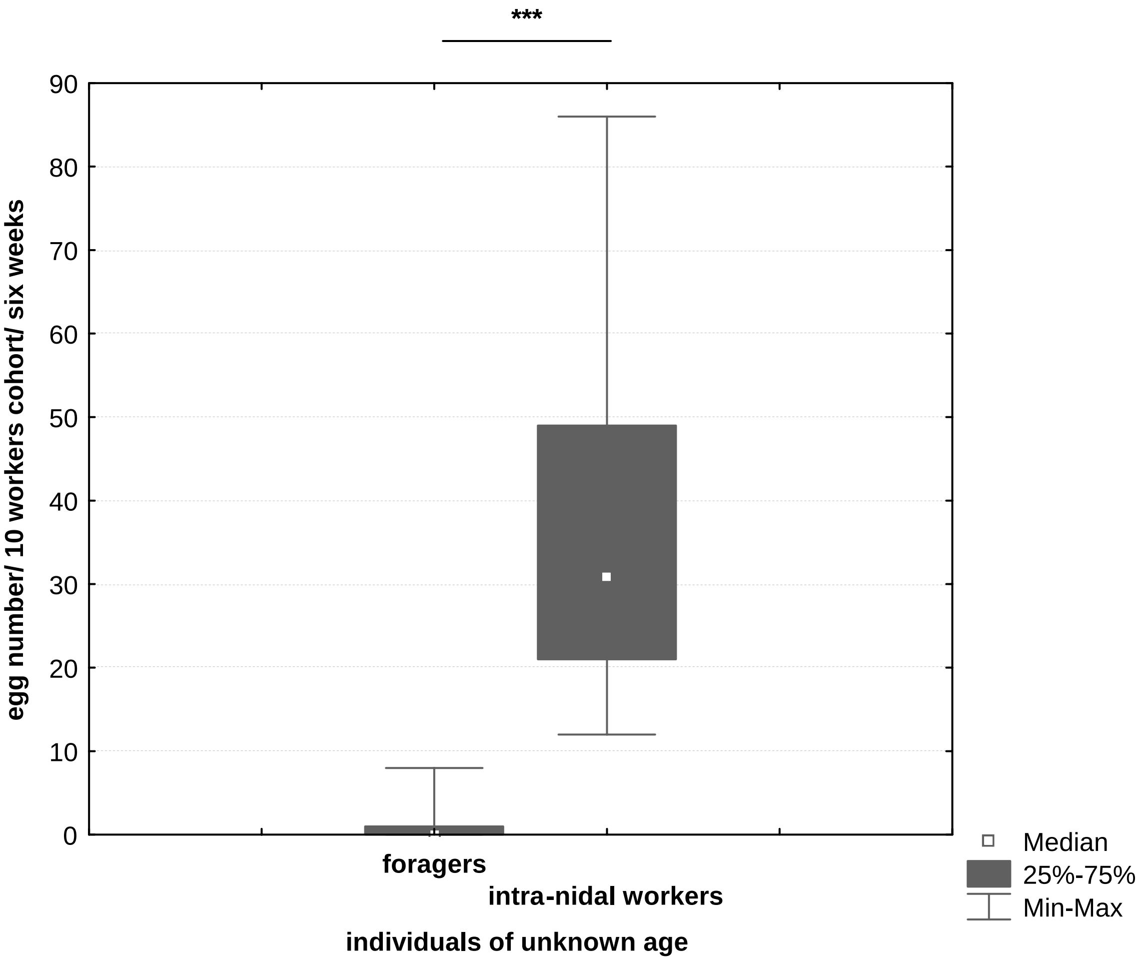

**Fig 1.** Fecundity of two worker subcastes: foragers and intra-nidal workers. Cohorts of foragers were drastically less fecund than cohorts of intra-nidal workers, although both types of ants came from the same source colonies and were kept in identical conditions (Wilcoxon matched pairs test: n = 18, Z = 3.72, $p < 0.0002$).

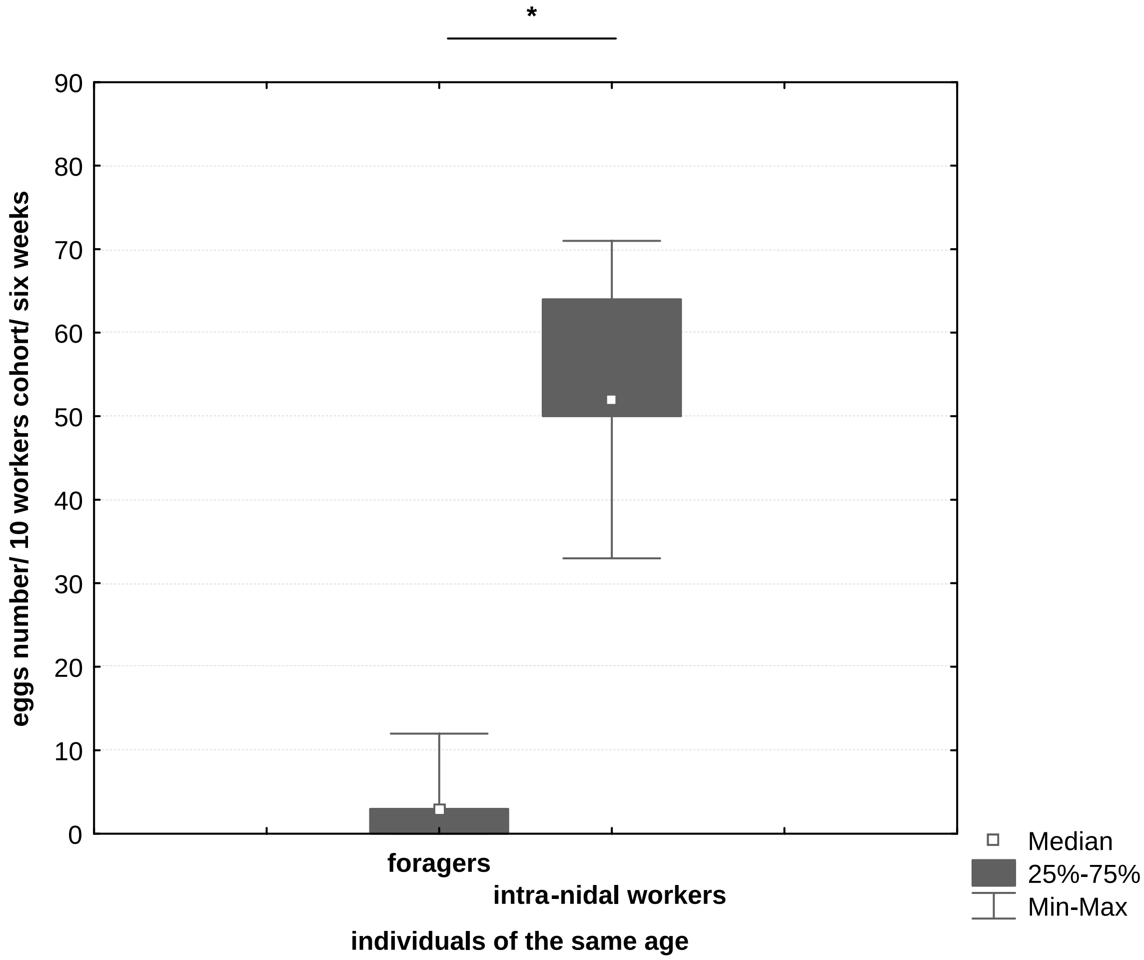

**Fig 2.** Fecundity of foragers and intra-nidal workers of the same age. The difference in fecundity between foragers and intra-nidal workers does not result from advanced age of foragers. When both types of workers were of the same age they still differed significantly in their fecundity (Wilcoxon matched pairs test: $n = 5$, $Z = 2.02$, $p < 0.05$).

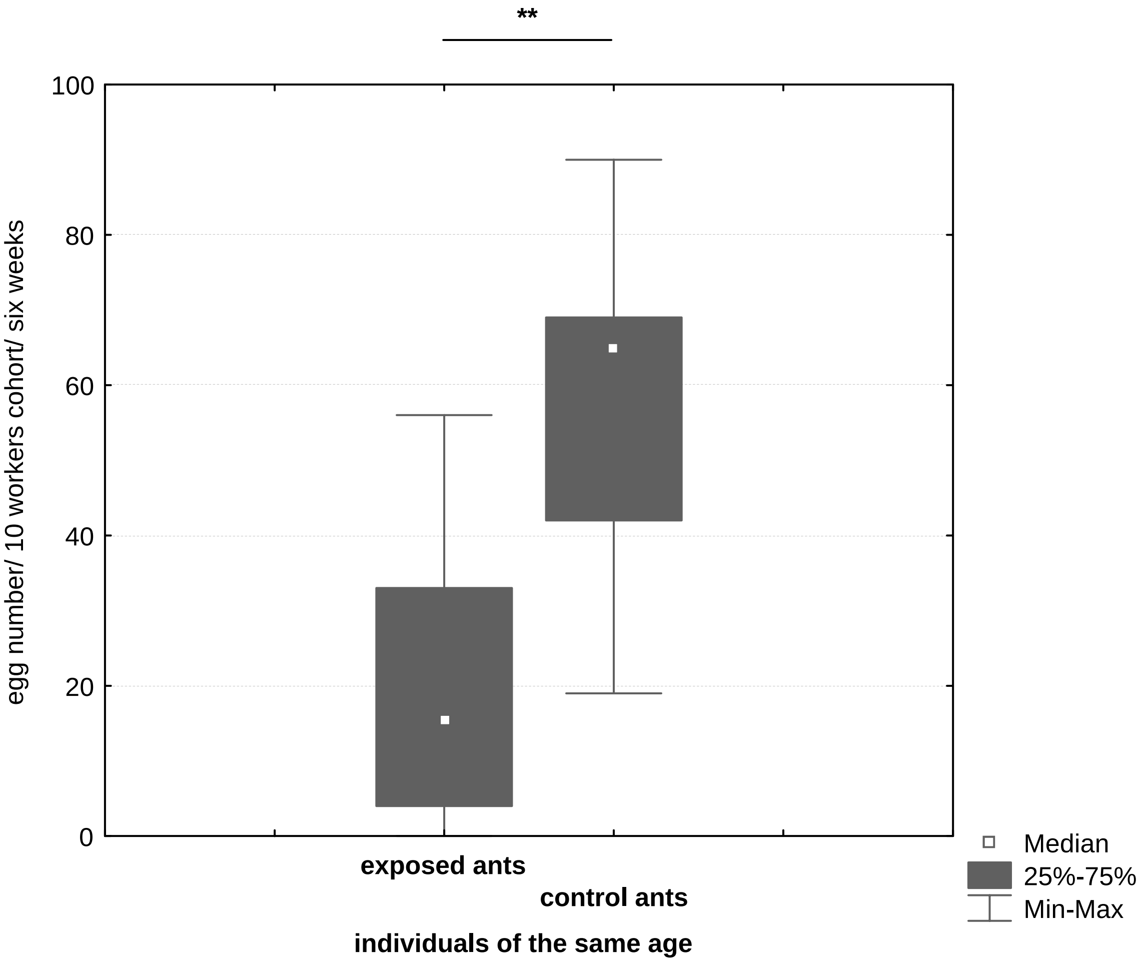

**Fig 3.** Exposure to $CO_2$ reduces fecundity of workers. Fecundity of cohorts of 10 workers exposed to $CO_2$ (3 x 20h) was compared with fecundity of cohorts of 10 non-exposed workers (cohorts from the same source colonies and workers of the same age, Wilcoxon matched pairs test: n = 10, Z = 2.80, $p < 0.006$).

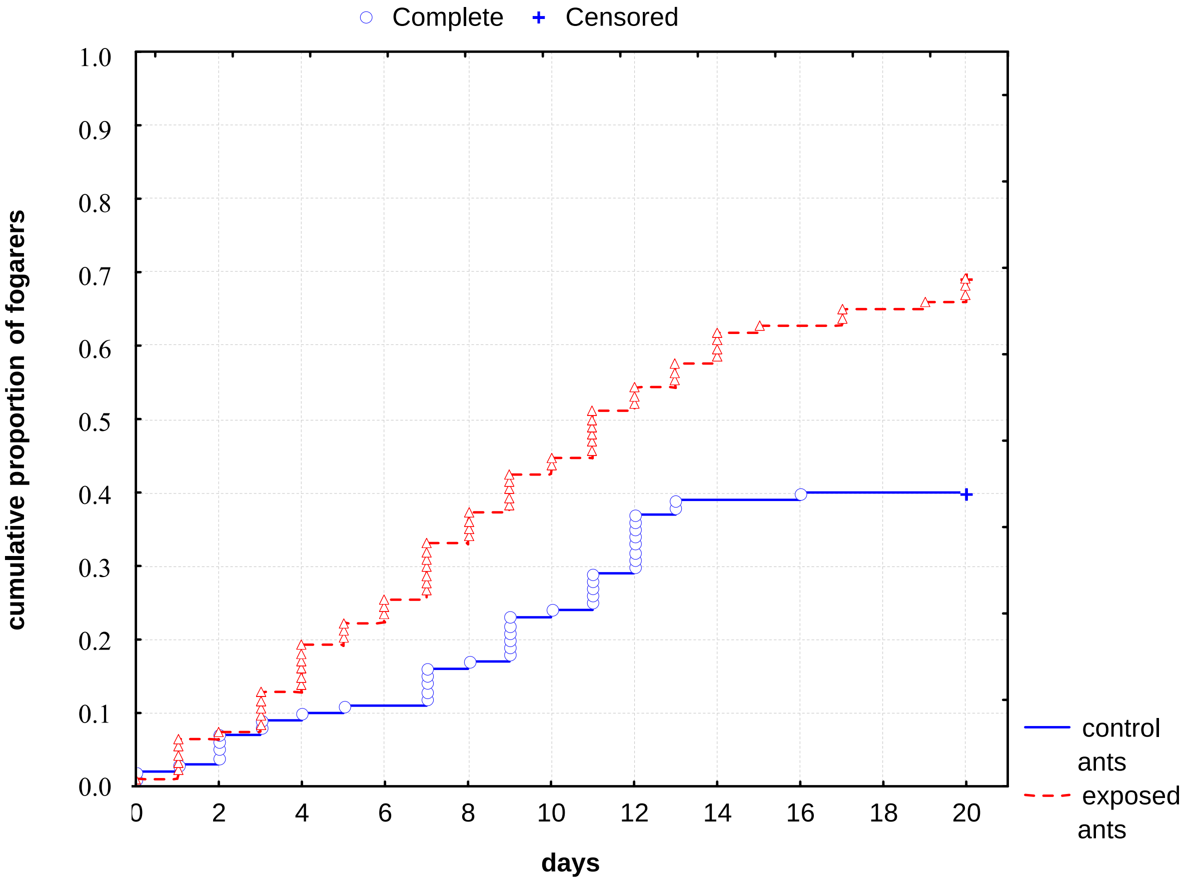

**Fig 4.** Cumulative proportion of ants which become foragers depending on group. Ants with reduced fecundity ($CO_2$ exposed) became foragers more frequently that those of unmanipulated fecundity (control) although both types of workers came from the same source colonies and were of the same age (Cox's F test: F (80, 130) = 2.19, $p < 0.0001$).

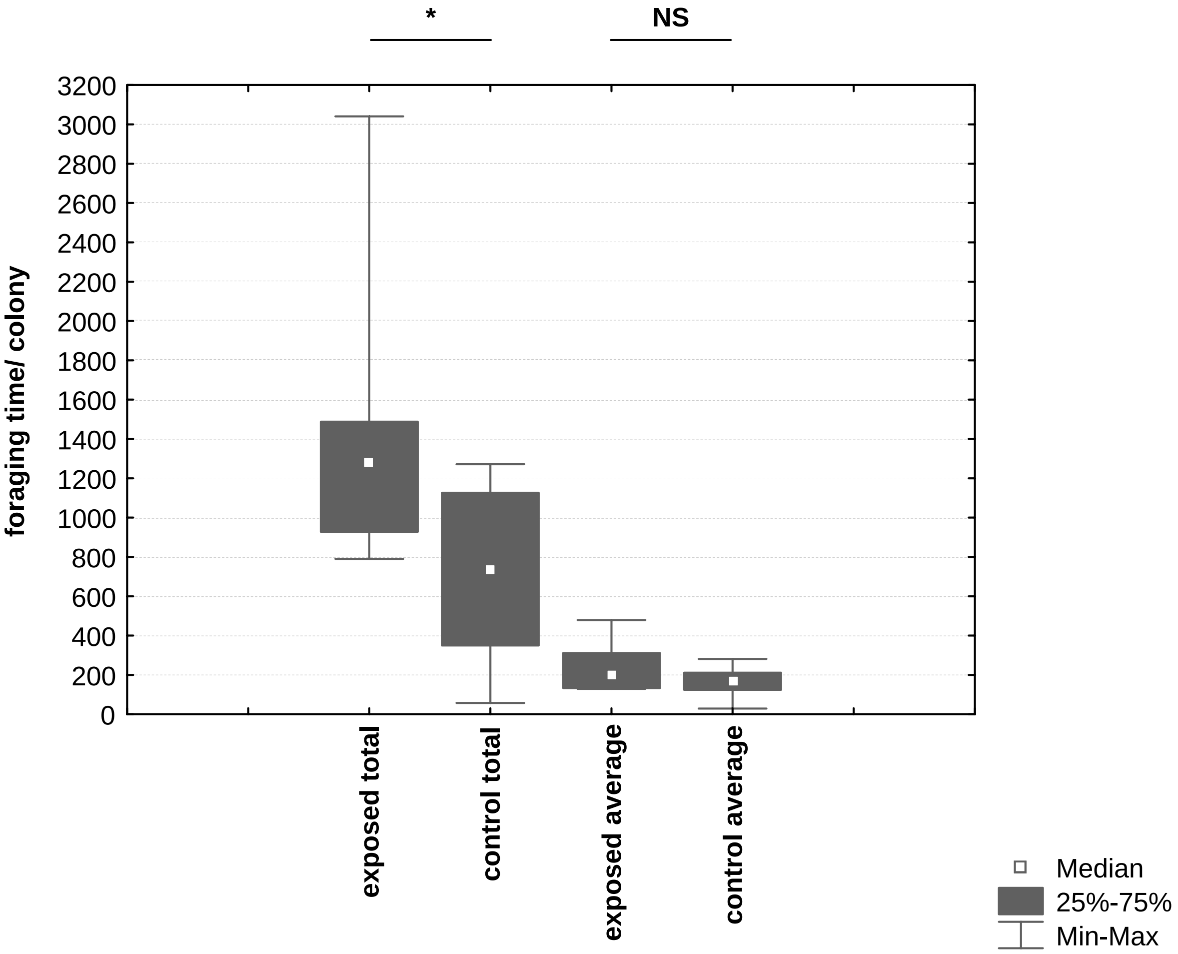

**Fig 5.** Time spent on foraging by workers with reduced fecundity ($CO_2$ exposed) and unmanipulated (control) per colony (n = 10). In total exposed ants foraged longer than controls (Wilcoxon matched pairs test: n = 10, Z = 2.19, $p < 0.03$). However, the single exposed foragers foraged on average for the similar time than single control forager, showing that foragers from both groups exhibit the same behaviour (Wilcoxon matched pairs test: n = 10, Z = 1.7, $p = 0.24$).

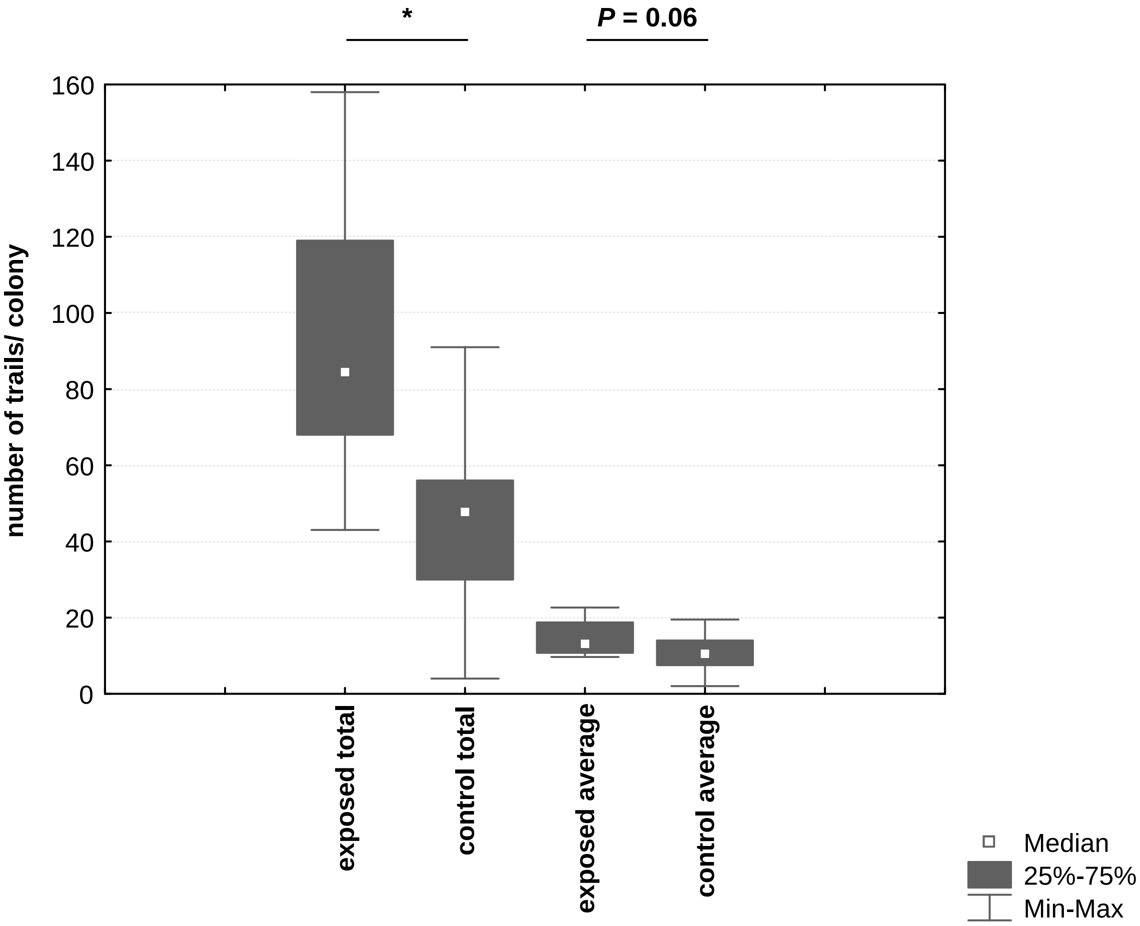

**Fig. 6.** Number of foraging turns performed by workers of reduced fecundity ($CO_2$ exposed) and unmanipulated ones (control) per colony (n = 10). In total exposed ants performed more turns than controls (Wilcoxon matched pairs test: n = 10, Z = 2.19, $p <$ 0.03). However, an average exposed forager performed only slightly more turns than an average control, showing that foragers from both groups exhibit the similar behaviour (Wilcoxon matched pairs test: n = 10, Z = 1.89, $p = 0.06$).

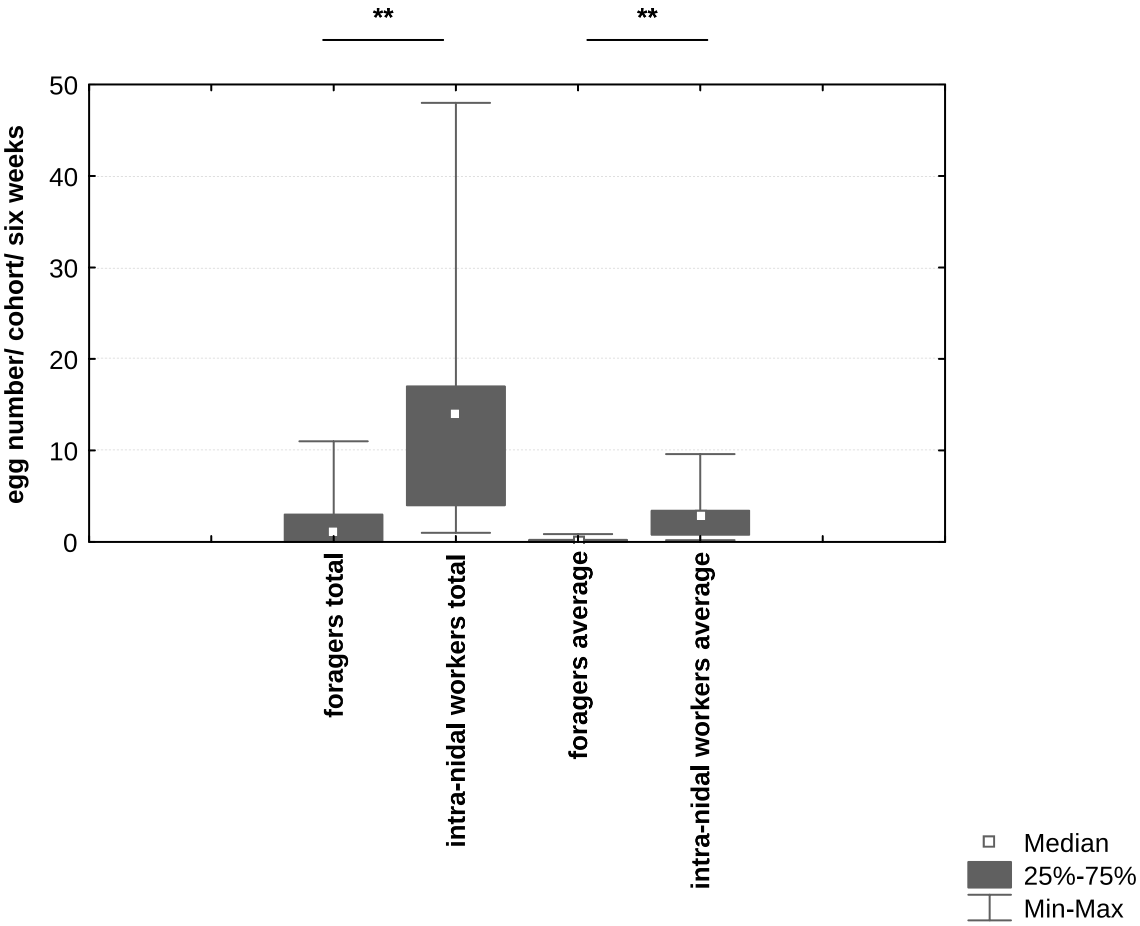

**Fig. 7.** Fecundity of foragers' and intra-nidal workers' cohorts (mixed $CO_2$ exposed and non-exposed) after six weeks of keeping in closed nests. Foragers' cohorts laid less eggs than intra-nidal workers' cohort, though both groups consisted of exposed and unexposed individuals of the same age. It shows that foragers always recruit from workers with decreased fecundity (total egg number per cohort, Wilcoxon matched pairs test: $n = 10$, $Z = 2.67$, $p < 0.008$; egg number per individual per cohort $n = 10$, $Z = 2.81$, $p < 0.006$).

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# Chapter VI

# Summary

Main topics of this thesis are aging and social homeostasis in eusocial insects in light of the evolutionary biology. The keystone of studies presented here are questions how eusociality evolved and is maintained.

Chapter 1 comprises a review of theories concerning evolution of eusociality, evolutionary theories of aging, as well as major issues regarding aging of eusocial insects and social homeostasis.

Chapter 2 presents studies on sexual cooperation and longevity in honeybee (Apis mellifera). Contrary to solitary insects, in eusocial species insemination itself is expected to increase lifespan of a female. However testing this prediction on honeybee turned out to be impossible as (1) queens inseminated with non-viable sperm are recognized and killed by workers, and (2) they experience increased mortality when kept in semi-isolation (queen bank approach).

Studies presented in chapter 3 exemplify mechanism protecting social insect colonies from the spread of pathogen. Moribund workers of the ant *Temnothorax unifasciatus* (*Myrmicinae*) show striking adaptation on the individual level: they cease all social contacts and leave nest to die in isolation.

Chapter 4 presents how functional sterility of workers is achieved in colonies of different size and relatedness among individuals. In the ant *T. unifasciatus* the sterility is enforced by policing: reproducing workers are attacked by their nestmates, that induce workers to refrain from reproduction. I show that policing effectiveness (proportion of reproducing workers which are policed) is influenced by the group size and relatedness among colony members in manifold way.

Chapter 5 discusses proximate and ultimate factors influencing evolution of labour division. I present data indicating that workers of the ant *T. unifasciatus* divide into low fecund foragers and high fecund intra-nidal workers. The reproductive aging of workers is not dependent on chronical age, but is an effect of phenotypic plasticity. The findings shed light on the evolution of eusociality suggesting that reproductive labour division could have evolved from differences in fecundity among related cooperative breeders.

# Photo Gallery

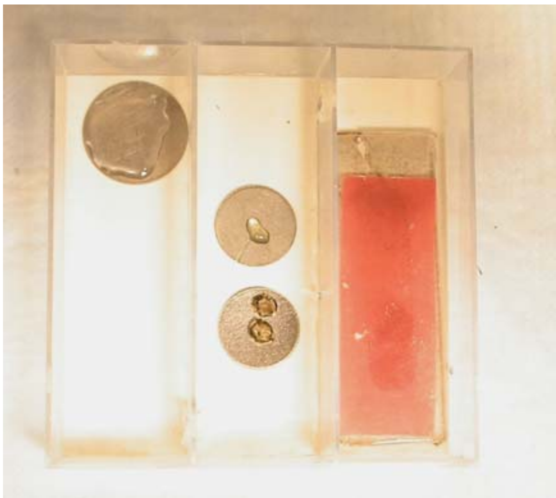

**Photo 1.** Foraging arena and a nest of experimental colonies of *Themnothorax unifasciatus* (© Bartosz Walter 2008)

**Photo 2.** Nest for a colony of *T. unifasciatus* used in experiments requiring the *Closed Nest Approach* (© Bartosz Walter 2008)

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