The proximate and ultimate bases of regulation of lifespan and reproduction in ants

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Matteo Negroni

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1. Berichterstatterin: Prof. Dr. NA
2. Berichterstatter: Dr. NA

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Summary

Understanding why and how organisms age and die is a major topic in biology, medicine and is of universal interest. The proximate causes of aging are even less well understood than why aging evolved in the first place. One possibility is that aging results from the accumulation of molecular damage due to incomplete somatic repair. Longevity and fecundity are traded-off in most organisms and the negative association between these two traits involves conserved molecular pathways. Social insects are an exception, as fertile individuals live longer, but the mechanisms underlying this positive association are unknown. We are still far from understanding the interactions between environment, genotype, and the molecular pathways determining variation in longevity both within and between species. Social insects are ideal models, not only because they exhibit strong intra-specific variation in lifespan and reproduction with extreme values recorded in queens, but also because this variation can arise from the same genome. I investigated here the ultimate and proximate bases of the regulation of lifespan and reproduction and their reversal association in social insects, using *Temnothorax* ants as a study system.

I first review ultimate factors underlying variation in life-history strategies in female social insects. I highlight in **chapter 1** the importance of colony size, colony founding strategy and social structure (number of queens in the colony) for the evolution of lifespan, reproduction, and body size in queens and workers. Social insects with large colony sizes and independent colony foundation exhibit the longest queen lifespans. In **chapter 2**, I experimentally tested the effect of colony size, body size and social structure, on egg production in ant queens. I show that colony size and social structure, but not body size, determine queen fecundity. While in most solitary insects, female body size is strongly linked to fecundity, this life-history parameter appears to be replaced by colony size in social insects.

Next, I investigated the link between fertility and somatic maintenance within castes, as well as the role of other factors for the regulation of lifespan and reproduction. By connecting phenotypic

results to gene expression data, I shed light on the molecular basis of this regulation as well as the proximate mechanisms underlying the reshaping of the longevity/fecundity trade-off. In **chapter 3** I investigate how longevity and fecundity are linked, and show that both egg removal and nutrient intake have a positive effect on queen fertility, but also activate body maintenance mechanisms. These results contrast with findings on solitary organisms, suggesting an alteration of molecular pathways in our species. **Chapter 4** reveals that queens switch from investment into immunity to the production of antioxidants with age and increasing fertility, suggesting a role of immunity in the reversal of the longevity/fecundity trade-off. Focussing then on workers, I show in **chapter 5** that behavioural castes differ in their rate of intrinsic mortality, where foragers that performed risky tasks outside the nest survive shorter than nurses. The removal of the queen induces worker fertility but also extends their lifespan. I thus investigated in **chapter 6** the molecular basis of this fertility induction and lifespan extension in relation with immunity and the gut microbiome composition. I demonstrate that fertile workers upregulated repair mechanisms, explaining their extended longevity. Fertile workers respond differently to an immune challenge than non-fertile ones. Results suggest that lifespan extension is linked to a better stability of the immune response but unlikely linked to the gut microbiome.

In conclusion, this dissertation sheds light on the molecular bases of aging and the regulation of lifespan in social insects, but also reveals how social life reshapes the association of life-history traits. I bring evidence for an alteration of certain pathways in our focal social species compared to solitary ones and identify a number of candidate genes and pathways for the reshaping of the trade-off between lifespan and reproduction. Finally, I point to an important role of immunity and nutrients in the regulation of lifespan and reproduction in social insects.

General Introduction

Matteo Negroni

“We are survival machines - robot vehicles blindly programmed to preserve the selfish molecules known as genes. This is a truth which still fills me with astonishment.”

— Richard Dawkins

As death is our primal fear, immortality is one of the most ancient dreams of humanity. Humans tried to answer the question *why we age and die* in mythological tales and by religion. Understanding why and how something as detrimental for an organism as senescence and death evolved is also one of the major challenges for evolutionary biology. The concept “survival of the fittest” seems to suggest at first that senescence, is strongly selected against, yet it occurs in almost all multicellular organisms.

The evolution of senescence

Senescence is omnipresent in the animal, fungal and plant kingdom and is characterized by a decline in biological functions, including reproduction and a reduction in survival with age (Box. 1). This deteriorating process has been termed an evolutionary paradox, as it represents a clear cost to individual fitness (Stearn 1992) raising the question: why do organisms not live forever? This intriguing problem has puzzled researchers for a long time leading to the development of evolutionary theories of aging (Medawar 1952, Williams 1957, Kirkwood 1977).

Box. 1

The definition of the terms senescence and aging are used in a quite inconsistent manner throughout the literature. While **senescence** is used only to describe the age-related deterioration of the body (biological aging), **aging** describes the fact of simply growing older (chronological aging; Medaward 1957). Other authors have frequently ignored this distinction using either senescence, or aging, or in some cases both, to define the same concept: the age-related deterioration of the body (Kirkwood and Rose 1991, Lucas and Keller 2014). In this thesis I decided not to make the distinction between the two, and use **senescence and aging** equally as defined by ***a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration*** (Rose 1991).

An early theory, the "**programmed death**" theory advised first by Weismann (1891) proposed that senescence is programmed and adaptive allowing to limit population size or accelerate the turnover of generations, thereby accelerating the speed of adaptation in a changing environment. This theory has been abandoned, as in most species, senescence is rarely observed in the wild, but only in artificially protected environments (Axel and Kirkwood 2016). Indeed, in nature, organisms mostly die due to extrinsic factors such as disease, starvation, accident, and predation, and this occurs long before they grow old enough to die intrinsically from senescence. As a consequence, only a small fraction of the population has the chance to survive long enough to reproduce late in life, while most individuals reproduce at an earlier age and would have already transmited their genes to the next generation. This creates a progressive decline in the power of selection with age (Medawar 1952), which is the principle of the current evolutionary theories of aging.

Among them, the "**mutation accumulation**" theory asserts that senescence results from deleterious mutations that randomly accumulate over generations, because they are expressed late in life (Medawar 1952). Those deleterious mutations are loosely selected against if effective at the so-called shadow of selection (later in life), which leaves space for an accumulation in the genome.

The second theory is known as "**antagonistic pleiotropy**" (Williams 1957), which postulates that a mutation, or an allele that brings a cost to individual fitness late in life, such as senescence and death, can still be selected for, as long this cost is out-weighted by earlier fitness benefits, for example in terms of reproduction. This theory thus introduces the concept of a trade-off, which is also at the core of the "**disposable soma**" theory (Kirkwood 1977, Kirkwood and Rose, 1991), which states that organisms face an allocation compromise. They have to decide which fraction of their limited resources to invest into body maintenance (the soma) or in reproductive functions such as growth, mating or parental care. In other words, if an organism invests into the transmission of its genes to the next generation, it pays a price in term of maintenance of the soma that is thus called "disposable", as opposed to the germ line that survives across generations and is, somehow, immortal.

These three theories are not mutually exclusive and have in common the idea that senescence as such is non-adaptive and that the evolution of lifespan is modulated by the extrinsic mortality rate. It is hence often difficult to quantify the contribution of each model to explain the evolution of senescence. In that regard, looking at the molecular basis of senescence as well as regulatory mechanisms of lifespan can bring crucial insights. Understanding evolution of senescence requires identifying the genes that underlie senescence, how they contribute to this process and how selection shapes their evolution.

Senescence and regulation of lifespan

The complexity of the mechanisms underlying senescence, which are ultimately responsible for death, comes from the fact that they involve many interconnected biological processes, leading to convergent phenotypical changes, such as molecular and cellular damages, or cancer. This makes it difficult to identify the importance of one or several process(es), and to establish causal relationship. For example, an increase in somatic damages can accelerate senescence but also activate various somatic repair mechanisms. On the other hand, an overactivation of somatic repair mechanisms could also delay senescence.

What are the theories about proximate causes of senescence?

Since senescence has important societal and economic consequences, substantial effort has been made in understanding the proximate causes of this process, and many theories have been proposed, namely the mechanistic theories of aging. Currently not all of them have been tested empirically, and studies have shown discrepancy between results, due to the intrinsic problems of a multitude of factors affecting senescence. One of the most popular theories, the "**molecular damage**" theory, notes that senescence is ultimately caused by the accumulation of molecular damage on macromolecules of somatic cell, including protein, lipids, nuclear DNA or mitochondrial DNA, with age due to incomplete somatic repair (Kirkwood et al. 1979, Kirkwood and Austad 2000,

Lombard et al. 2005, Schulz et al. 2007, Sun et al. 2016). The consequences of those damages could result in perturbation of cell functions, cell death, uncontrolled cell proliferation, which ultimately lead to the death of the entire organism. In support for this, molecular damages on the nuclear DNA or mitochondria DNA are indeed increasing with age and are maximal in cancerous cells (Sun et al. 2016). A causal link between molecular damage on the mitochondria and lifespan has been shown in mammals, where transgenic mice that have a higher mutation rate in their mitochondria senesce faster (Lombard et al. 2005, Khrapko et al. 2006, Vermulst et al. 2007). Origins of molecular damage mainly includes replication, transcription and translation mistakes, other spontaneous biochemical errors, and the production of *reactive oxygen species* (ROS) from mitochondrial respiration (Corona et al. 2005). An organism that is long-lived is thus predicted to have a lower production of ROS and/or higher production of antioxidants and/or better repair mechanisms. The "**free radical**" theory of aging specifically considers ROS production as the main source for molecular damage (termed oxidative damage). In the last two decades, many studies found support for this theory. For example, the overproduction of antioxidants including the enzymes *catalase or superoxide dismutase* extends lifespan in the fruit fly and in *Caenorhabditis elegans* (Orr and Sohal 1994, Sampayo et al. 2003, Melov et al. 2000). Going in the same direction, a shortening of lifespan in response to an experimental increase in oxidative stress has been demonstrated repeatedly in insects (Remonila and Hughes 2008, Rzezniczak et al. 2011). However, a study on in *C. elegans* found results that are difficult to reconcile with the free radical theory as glucose restriction increased oxidative damage, but extended lifespan (Schulz et al. 2007). Furthermore, in the same species, the knock-out of all copies of *superoxide-dismutase*, a powerful antioxidant, resulted in more oxidative damage, while lifespan remained unchanged (Van Raamsdonk and Hekimi 2012). One interpretation for those observations could be that oxidative damage is only partly contributing to the accumulation of molecular damage. Another interpretation is that molecular damage might not be the cause of senescence.

The alternate "**hyperfunctioning**" theory, proposed by Blagosklonny (2008), explains molecular damage not as a cause of senescence, but as its consequence. It also suggests that the real cause of senescence is hypertrophy - an excess of protein synthesis. Hypertrophy would allow for growth and reproduction, but at the same time might lead to age-related diseases, molecular damage and increasing mortality. Such a mechanism would be congruent with the ultimate explanation of the antagonistic pleiotropy hypothesis: at young age, high levels of biosynthesis are beneficial, but they are detrimental later in life. Support for this theory are still scarce (Gems and de la Guardia 2013, de Verges and Nehring 2016)

The molecular bases of senescence are therefore not entirely uncovered and are still debated. The proximate causes of senescence remain difficult to identify and might vary between organisms (Gems and de la Guardia 2013). Considering its links to different physiological traits or biological processes might bring crucial insights.

Which factors are influencing the regulation of lifespan?

i) The link with reproduction

When investigating the regulation of lifespan, we have to consider its relationship with reproduction, as both are often traded-off, in line with the disposable soma theory. A negative association between longevity and fecundity is frequently observed both across and within solitary species (Reiznick 1985, Westendorp and Kirkwood 1998). Moreover, in a large range or organisms, experimental reduction of fertility lengthens lifespan (Sgro and Partridge 1999, Partridge et al. 2005). Social insects are a well-known exception of this ubiquitous trade-off as the most long-lived individuals, the queens, are also the most fertile ones (Figure intro.1). This positive association between lifespan and reproduction appears to occur also within castes, as fertile workers live longer than infertile ones (Heinze et al. 2013, Kohlmeier et al. 2017). What are the proximate causes of this positive association is a major question in evolutionary biology, which I will address in this thesis (Figure intro.1).

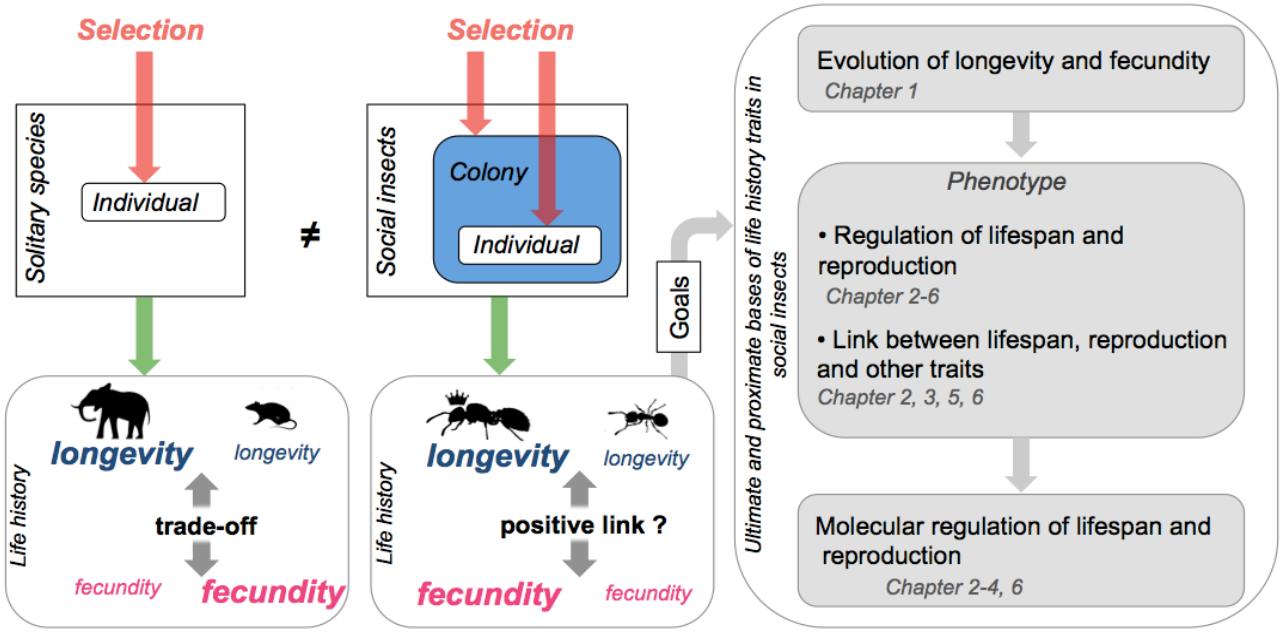

Figure intro.1: Figure illustrating the difference in life history between social insect and solitary organisms, as well as the main goals of this thesis. The red arrows represent the action of selection, the green ones represent the evolutionary consequences and the light grey ones the methodological organization of different research approaches. The double dark grey arrows represent a physiological or an ecological link between longevity and fecundity. The transition from solitary life to social life brought an additional level of selection, which is not only acting on the individual, but also on the colony. Consequently, social life reshaped trade-off commonly observed in solitary species as the one between lifespan and reproduction. In social insect these two traits appear to be positively associated.

ii) The role of nutrients

The idea of a trade-off implies that it occurs between two costly traits. If body maintenance is costly, an increase in resource availability could allow organisms to live longer without paying a price of a reduced fecundity. Actually, the opposite has been observed as a higher food intake often reduces survival, while increasing reproduction in most organisms (Lee et al. 2006, Alder et al. 2013). Conversely, food restriction compared to ad libitum food provisioning, in particular the limiting protein consumption reduces egg production but extends lifespan. Additionally, the relationship between fecundity and longevity is also apparent on the molecular level (Barnes and Patridge 2005, Flatt and

Kawecki, 2007). In insects the highly versatile juvenile hormone (JH) plays an important role in the regulation of life-history traits during development and adult life of insects. In *Drosophila*, a treatment with an analogue of JH increases egg production, but reduces survival (Flatt and Kawecki 2007). The highly conserved Insulin-Insulin-like signalling (IIS) and the target of rapamycin (TOR) signalling pathways are importantly contributing to the negative association between lifespan and reproduction (Kenyon 2005, Narasimhan 2009, Kenyon 2010, Fonseca et al. 2016). In particular, they are activated in response to nutrient intake (in particular methionin) and are responsible of the antagonistic effect of food on the two traits (Lee et al. 2014). It has been proposed that IIS and TOR pathways evolved in response to temporal variation in resource availability by facilitating plasticity in life-history traits, especially in the investment into somatic maintenance (somatic maintenance polyphenism; De Jong and Van Noordwijk 1992, Flatt et al. 2013). The lack of food would induce diapause that may allow organisms to wait for better conditions (Flatt et al. 2013). Conversely, a reduction in lifespan in response to high protein intake could result from a shift in investment into reproduction in line with the **disposable soma** theory, or simply represent the unintended side effect of growth as described by the **hyperfunctioning** theory (Blagosklonny 2008, Gems and Guardia 2013). Although widespread, the hypothesis of a trade-off has sometimes been questioned as there is an increasing number of studies showing that a specific diet or genetic manipulation can lead to an optimisation of growth and reproduction without any cost to lifespan (Flatt et al. 2011). In social insects where longevity and fecundity appear positively associated instead of traded-off, the importance of nutrients intake in the regulation of these traits is only speculative but is a question that I addressed and tested in this thesis.

iii) Importance of the gut microbiome

The important role of diet in the regulation of lifespan should justify examining the role gut symbionts play in senescence as they often provide nutrients, absorbed from the consumed food, to their host. Moreover, gut microbiome composition changes with age, and those changes have been associated with age-related diseases. In *Apis mellifera* and in *C. elegans* gut microbiome composition is causally linked to aging (Kwong and Moran 2016, Ikeda et al. 2007, Komura et al. 2013). Surprisingly the role of the gut microbiome in the regulation of lifespan has long been underestimated and we are just starting to realize its importance (Ottaviani et al. 2011). The mechanisms involved are largely unknown but could include specific nutrients (*i.e.* short-chain fatty acids) provided to the host (Zheng et al. 2017). The link between microbiome and host lifespan can also involve modification in the immune system (Kwong and Moran 2016). In honeybees, and in *C. elegans* gut bacteria improve pathogen resistance and production of antimicrobial substances (Zheng et al. 2017). In my dissertation I investigate how the gut microbiome is linked to lifespan and reproduction in ant workers and furthermore investigate its interaction with immunity.

iv) The role of immunity

A strong immune system is essential to survive infection by pathogens. Yet, it is in itself energetically costly and may negatively affect lifespan, in the absence of pathogens. In bumblebees, an immune challenge in the absence of pathogens reduces survival when resources are limited, owing to an energetic trade-off (Moret and Schmid-Hempel 2000). The immune reaction, and more specifically the inflammatory response, triggers a higher production of ROS, which can cause damage and shorten life. In addition, senescence is characterized by a decline in the efficiency and the homeostasis of the immune system often causing an uncontrolled inflammatory reaction. Fabian *et al.* (2018) have recently shown that artificial selection for longevity and late reproduction in *Drosophila melanogaster* mainly affects immunity genes. The selected alleles conferred to the long-lived flies a better stability of the immune system, pathogen resistance and survival. In conclusion, a

less active immune system can be more efficient and can delay aging. The molecular mechanisms of this link are not elucidated and could result from autoimmunity and/or oxidative damages directly or indirectly caused by uncontrolled immune reactions (Martin et al. 2003, Fabian et al. 2018). Indeed, the immune response not only increases energy expenditure, but also induces an elevation of metabolic rate, which can affect lifespan via a higher ROS production (Finkel and Holbrook 2000, Ots et al. 2001, Martin et al. 2003, Adria et al. 2012). In this thesis, I studied the role of the immune system in the regulation of lifespan and egg production at the molecular level, in ants where these two traits are highly plastic.

v) The link with metabolism

A cross-species a correlation between lifespan and metabolic rate per mass unit has been noticed more than a century ago (Rubner 1908). This finding gave birth to the so-called "**rate of living**" theory first proposed by Pearl (1928) and later reformulated by Harman (1956). This theory proposes a causal link between metabolic rate and lifespan involving the production of ROS from aerobic respiration, which is in line with the **free radical** theory. In contrast to the evolutionary theory of aging, this theory is purely mechanistic and is supported by the fact that larger animals having a lower metabolic rate per gram of tissue, live longer than smaller ones (West et al. 1997, Gillooly et al. 2005, Speakman 2005, Chown et al. 2007). Yet, this theory failed to explain why birds that have a higher metabolic rate than mammals, generally live longer. Moreover, experimental studies sometimes reveal contradictory results depending on the type of organism or manipulation. For example, voluntary exercise increases food intake in female rats and lengthens lifespan (Holloszy 1993). Long-term cold exposure largely increases energy expenditures in mice (Vaanholt et al. 2009), rats (Holloszy and Smith 1986), and voles (Selman et al., 2008), but has no effect on lifespan. Dietary restriction, which extends lifespan in many organisms, does not reduce metabolic rate in *Drosophila* (Hulbert et al. 2004). One difficulty in testing the influence of metabolic activity on lifespan is to control for confounding factors such as food intake, temperature or different physiological

reactions. At any rate if metabolic rate is indeed negatively and causally linked to lifespan, the proximate mechanism remains to be established. I investigated molecular processes underlying variation in metabolic rate, and examined how it is linked with survival.

Social insects' extraordinary life-history traits: evolution and applications

Consequences of social life in life-history evolution

Eusociality represents the latest major evolutionary transition toward a higher complexity (Szathmary and Smith 1995, Rehan and Toth 2015), which brought an additional level of selection that is not only acting on the individual, but also on the colony (Wheeler 1911, Korb and Heinze 2016, Figure intro.1). This has led to extraordinary life-history characteristics and reshaped trait associations of individuals compared to solitary species (Keller and Genoud 1997, Keller 1998, Korb and Thorne 2016, Figure intro.1). Insect societies represent the highest degree of sociality and often display a marked reproductive division of labour. Ant, wasp, bee and termite colonies are headed by a single or few reproductive females, the queen(s) (which in the case of termite can be paired with a male king) and is assisted in every way by their non-reproductive female workers that gain, by so-doing, indirect fitness benefits. As a consequence of reproductive division of labour, evolution of sociality has been accompanied by a divergence in phenotype between the reproductive and the non-reproductive castes, typically queen and workers, in term of longevity, fecundity and body size (Kramer and Schaible 2013, Negroni et al. 2016). The queen caste has experienced a spectacular decrease in extrinsic mortality as protected by the rest of the colony due to its crucial function (Keller 1998). This led to the evolution of extreme lifespan of this caste (Keller 1998). In many species, the queens can live more than two decades and up to ten times longer than their workers that have a lifespan similar to their solitary ancestors (Plateau 1986, Keller 1998, Kramer and Schaible 2013). Task specialisation resulted in the evolution of extreme fecundity of the queen, which can reach in some species a rate of 2000 eggs produced per day (Kaib et al. 2001), and a reduced fertility or a total sterility of the worker caste (Boorke 1999). Hence an evolutionary consequence of social life is

an apparent positive association between lifespan and reproduction instead of a trade-off between the two traits, as observed in solitary species (Heinze et al. 2013, Rodrigues and Flatt 2016). Different colony-level or individual-level traits may modulate life-history evolution in social insects, and sometime their influence can be retroactive (Negroni et al. 2016). For example, colony size influences the workforce of the queen, caste-specific extrinsic mortality, the complexity of division of labour and the per-capita productivity, which may influence divergent evolution in lifespan between queen and worker, queen fecundity and task specialization (Kramer and Schaible 2013, Chapuissat and Keller 2002, Ferguson et al. 2014, Negroni et al. 2016). In turn, worker lifespan and queen fecundity may influence colony size (Giraldo and Traniello 2014, Negroni et al. 2016). Queen number may negatively affect selection for individual queen longevity and fecundity (Keller and Genoud 1997). Furthermore, in social insects' life history traits, and especially lifespan and reproduction are highly plastic, as queen and worker rise from the same genotype, and both traits can be regulated at the adult stage in response to environmental factors (Kohlmeier et al. 2017, Negroni et al. in prep. a, Negroni et al. in prep. b, Negroni et al. in prep. c).

Applying evolutionary theory of aging to social insects requires considering that selection operates at the colony level and within the same genome (Kramer et al. 2016). The **mutation accumulation** theory can thus be true if one part of the genome is only expressed in worker but not in queen and vice versa. Differential senescence between queens and workers can be conciliated with the **antagonistic pleiotropy** theory if a mutation with antagonistic effects is not apparent within all individuals, but only within a specific caste. The **disposable soma** theory should be applied by considering the colony as a super-organism, where colony resources are maybe traded-off between queens and males, representing the germ line, and the workers, representing the soma. Regarding these aspects, the analysis of regulatory gene network related to lifespan in social insects can bring answers to understand the evolutionary mechanisms resulting in their extraordinary life history (Kramer et al. 2016).

Social insects: A good system for understanding the molecular bases of aging

Most of our current knowledge of the molecular bases of senescence results from work that has been done using model organisms, such as yeast, nematodes, fruit flies or mice (Keller and Jemielty 2006). All of these organisms are extremely short-lived raising the question of how representative these studies are for the proximate mechanisms underlying senescence in long-lived organisms. Because of their life-history characteristics, social insects are an excellent system to complement research on aging performed in model organisms (Keller and Jemielty 2006, Kramer et al. 2016, Rodrigues and Flatt 2016, Korb 2016). The first reason for this is the extreme lifespan of queens that count among the longest-lived insects and can reach up to 28 years of age in some species (Keller and Genoud 1997). Second, the remarkable plasticity of longevity and fecundity gives a unique opportunity for identifying the molecular bases of their regulation (Keller and Jemielty 2006, Lucas et al. 2016). Third, the atypical and apparent positive association between lifespan and reproduction in social insects may contain keys for understanding the molecular constraints resulting in a trade-off between these two traits in solitary species (Rodrigues and Flatt 2016, Kramer et al. 2016).

The genetic bases of the regulation of longevity and fecundity in social insects, and the factors underlying the extreme longevity of the queen, remain poorly known. It is possible that species differ in mechanisms involved in this regulation. Termite queens overexpress antioxidants compared to workers, which correlates with a reduction in their oxidative damage (Tasaki et al. 2017, 2018), while in the ant *Lasius niger* the queens overexpress molecular repair related genes compared to workers (Lucas et al. 2016). The way social insect queens circumvent the trade-off between lifespan and reproduction on a proximate level is a mystery. One possible explanation for this may be that a colony level trade-off in resources allocated between the germ line (the sexuals) and the soma (the workers) masks an existing trade-off on the individual level (Kramer and Schaible 2013, Lucas and Keller 2014, Kramer et al. 2016). Indeed, resources might not be limited for the queen, who could invest importantly in both longevity and fecundity, while workers having fewer resources can invest

less in both traits. Body size is a trait discriminating the two castes with queens being much larger than workers. Moreover, body size is commonly connected to the amount of body resources and metabolic rate, which could affect lifespan (Hou et al. 2010). Benefiting from social immunity (Cremer et al. 2007, Stroeymeyt et al. 2018), the queen may invest less into personal immunity, which would possibly allow investing more into both longevity and fecundity (Negroni et al. 2019). Not excluding the latter, another possibility would be that the molecular pathways responsible for the negative association between lifespan and reproduction have been reshaped in social insects compared to solitary organisms, resulting in a modification of the antagonistic effect of nutrients intake on lifespan and reproduction (Rodrigues and Flatt 2016). In my thesis I investigated regulation of lifespan in relation with other features including fertility, immunity, gut microbiome, metabolism or body size using social insects as study system.

Scientific questions of my dissertation on the regulation of lifespan and reproduction in social insects

My research focussed on the ultimate and proximate basis of the reversal of the longevity-fecundity trade-off in social insects, investigating six main questions. My project started with a review of the ultimate factors driving the evolution of life-history traits. Next, I conducted five different projects, in which I experimentally tested the influence of different factors important for the regulation of lifespan and reproduction as well as their molecular bases (Figure intro.1). I used two North American ant species of the genus *Temnothorax: T. longispinosus* (from New York) and *T. rugatulus* (from Arizona).

In **chapter 1** of my thesis I review the ultimate factors underlying variation in life-history strategies in female social insects. I explain how social life reshapes trait associations, in particular the reversal of the trade-off between lifespan and reproduction. I highlight the importance of colony size, founding strategies and social structure on the evolution of longevity, fecundity and body size in queens and workers (Negroni et al. 2016). Life-history divergence between queens and workers

is positively associated with colony size, while queen number that is strongly linked with the founding strategy negatively correlates with queen body size, lifespan and reproduction.

The ant *T. rugatulus* displays two queen morphs differing in body size, where the morph is loosely associated with social structure (Rueppell et al. 1998, Rueppell et al. 2001a). The large queens are mostly found as single queens in their colony while multiple smaller queens mostly coexist within the same nest. In **chapter 2**, I used *T. rugatulus* to test the independent effect of colony size, body size and social structure, on individual queen egg production, as well as the link between queen body size, metabolic rate and survival to oxidative stress. I show that albeit microgynes weigh less than half of macrogynes, they are able to maintain a similar high egg-laying rate apparently by sustaining a high metabolic rate and soliciting more food from workers. These small queens, however, do not seem to pay a price for their high fecundity in form of a lower survival rate, as they were able to withstand oxidative stress to a similar degree, as did the larger macrogynes.

More than displaying an atypical positive association between lifespan and reproduction, in social insects the antagonistic effect of food intake observed in solitary organisms may not occur. Additionally, our previous results suggest that in *T. rugatulus* a higher rate of feeding may allow small queens to reproduce as much as large ones without a necessary lifespan reduction. In **chapter 3**, I experimentally tested in *T. rugatulus* the effect of food restriction and egg removal on fertility and investigated related changes in gene expression in the fat bodies in the large queens. Egg removal had a positive effect on the number of developing eggs in the ovaries, and triggered the expression of genes, important for somatic maintenance but also related to immunity. Food restriction decreased queen fertility and transcriptomic results suggest a decreased investment into cellular homeostasis and an increase in inflammatory and oxidative stress response.

In **chapter 4**, I investigated changes in gene expression with age and fecundity in *T. rugatulus* queens, by comparing tissue-specific gene expression between young founding queens and older highly fecund ones. Results suggest that during their life, queens switch from investment into

immunity and resistance to starvation toward the activation of anti-aging mechanisms with an overproduction of antioxidants. Moreover, reverse age-related expression patterns of certain pathway compared to solitary species suggest that they enclose some modification in our species and are potentially involved in the reversal association between lifespan and reproduction.

In the last two chapters, I focussed on regulation of lifespan and fertility in workers. In **chapter 5** I investigated on the phenotypic level the influence of fertility on lifespan as well as variation in survival in relation with worker task in the ant *T. longispinosus*. As many social insects, *Temnothorax* ants display an age-related division of labour (age polyethism), whereby individuals going outside to forage are commonly older than the ones performing task inside the nest and care for the brood. I therefore expected foragers to survive less than nurses, and the results fit my prediction (Kohlmeier et al. 2017). We also compared worker survival between queenless and queenright nests and demonstrate that workers survive longer in the queens' absence. In this case, *Temnothorax* ant workers fight over reproduction and dominant workers start laying eggs (Kohlmeier et al. 2017).

Comparable to my previous results, recent studies suggest a link between immunity and senescence (Fabia et al. 2018). The gut microbiome is also known to affect aging of its host, though the underlying processes are not established. In **chapter 6** I investigated the molecular bases of fertility induction and lifespan extension in relation with immunity and the gut microbiome composition in worker of the ant *T. rugatulus*. I subjected fertile workers to an immune challenge to investigate responses in fat body gene expression and gut microbiome composition. Fertile workers upregulated repair mechanisms, potentially explaining their extended longevity. Results suggest that lifespan extension is linked to a better stability of the immune response but unlikely linked to the gut microbiome. The immune challenge triggered the expression of immune genes, which is associated with a dramatic loss in bacterial diversity.

Biology of the study systems of *Temnothorax*

Temnothorax ants are easy to collect and maintain in the laboratory and due to their small and variable colony size, facilitate experimental manipulations. As queens can get up to 20 years old, whereas workers live only a few years, they also exhibit quite a strong variation in lifespan (Plateau 1986, Keller 1997).

T. longispinosus is widespread in the North of the United States and southern Canada. Colonies are polygynous, found in cool forests and nest into small cavities such as acorns, stick holes and crevices (Kohlmeier et al. 2017). Colony size ranges from a few dozen, to more than a hundred of workers. Queen body size is variable but always larger than workers and both have a black and shiny cuticule (Figure intro.2).

Figure intro.2: Pictures of *T. longispinosus*, with field colony naturally nested in an acorn (left; picture from Alex Wild) and a queen with its workers and larvae taken from a colony kept artificially in the laboratory (right; picture from Barbara Feldmeyer).

T. rugatulus is widely distributed in the western-south United States and North Mexico. Their colonies are found in the cool forest of pine and oaks and colonies establish into rock crevices (Figure intro.3; Rueppell et al. 1998). Colony size varies between less than a hundred to almost a thousand of workers. This species has alternative reproductive strategies and exhibits two queen morphs, deferring in body size (Rüppell et al. 1998). The morph is associated with social structure and founding strategy. The large queens (macrogynes) can start their colony out of their own body

reserve (independent colony founding) and are mostly found alone in their colony (monogynous). In contrast, the smaller microgynes, slightly bigger than a worker size can only start a new colony via budding (by taking workers from the mother colony) and mostly coexist within the same nest (polygynous; Rueppell et al. 2001 a). This association between morph and social structure is however not strict and colonies, where both morphs coexist, are occasionally found in the field (Rueppell et al. 2001 a, b).

Figure intro.3: Pictures of *T. rugatulus*, with field colony naturally nested in a rock crevice (left; picture from Gary D. Alpert); a queen (macrogyne) with its workers and larvae taken from a colony kept artificially in the laboratory (right; picture from Romain Libbrecht).

CHAPTER 1

Life History Evolution in social insects: a female perspective

Matteo A. Negroni, Evelien Jongepier, Barbara Feldmeyer, Boris H. Kramer and Susanne Foitzik

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Abstract

Social insects are known for their unusual life histories with fecund, long-lived queens and sterile, short-lived workers. We review ultimate factors underlying variation in life history strategies in female social insects, whose social life reshapes common trade-offs, such as the one between fecundity and longevity. Interspecific life history variation is associated with colony size, mediated by changes in division of labour and extrinsic mortality. With increasing colony size, queens and workers' body size, fecundity, and longevity diverge more and more, while colony reproductive output increases. Conversely, individual queen fecundity, queen number and worker turnover influence colony size. In addition to the ratio of juvenile to adult mortality, social factors such as queen number influence life history trajectories. We discuss two hypotheses explaining why queen fecundity and lifespan is higher in single-queen societies and suggest further research directions on the evolution of life history variation in social insects.

Introduction

At a time at which deeper insights into the proximate basis of life history trade-offs are gained, including those in social insects (Rodrigues and Flatt 2016, Flatt and Heyland 2011), we take a fresh look at the evolutionary basis of life history traits in this group. Due to their social lifestyle, ants, termites, social bees and wasps exhibit not only extraordinary life histories (Keller and Genoud 1997, Keller 1998, Korb and Thorne 2016), but also strong variation in these traits both within and between species (Kramer and Schaible 2013). As this is in the social Hymenoptera especially true for queens and workers, we will focus here on female social insects. Eusociality represents the latest of the major evolutionary transitions towards higher complexity (Szathmáry and Smith 1997, Rehan and Toth 2015). In eusocial groups selection not only acts on individuals, though individual selection is an important selective force in many social insects (Korb and Heinze 2016), but on entire colonies as well, which represent the reproductive units here (Wheeler 1911). Therefore, life history traits of social insects include not only individual traits such as queen lifespan, but also colony-level traits, such as colony size or queen number (Shik et al. 2012). Like multicellular organisms, insect societies grow — in worker number instead of body size — and mature when colonies start to produce sexuals. However, in contrast to solitary individuals, they do not necessarily senesce (Heinze et al. 2013). Polygynous colonies that re-adopt daughter queens, are potentially immortal (Sanetra and Crozier 2002), though their genetic composition changes over time in contrast to that of multicellular organisms. Interestingly in lower termites, the replacement of reproductives by neotenics can also lead to immortal colonies without with the link to polygyny (Korb and Thorne 2016). Yet, in most single-queen, that is, monogynous societies, the colony's lifespan hinges on that of the queen and therefore selection on longevity led to extremely long queen lifespans (Keller and Genoud 1997, Keller 1998). Ant and termite queens can live for several decades, and their fertility may remain constant or even increase throughout their life (Heinze and Schrempf 2012, Kramer et al. 2015). A positive association between longevity and fecundity is apparent in queens and defies the predictions of life history theory, as both traits are usually traded-off against each other. The opposite is true for

workers that show reduced lifespan and no or low fertility (Kramer et al. 2015, Von Wyschetzki et al. 2015). Evolutionary theories explain lifespan evolution by the declining force of selection with age with extrinsic mortality as the main driver. Colony life leads to low external mortality for queens, as the security of the nest shields her from predators and parasites and the care of the workers protects her from other environmental hazards such as starvation or desiccation (Keller 1998). In the following, we highlight the impact of colony size, reproductive strategies and social structure on the evolution of life history traits in social insects and emphasize the need to take these factors into account when studying life history evolution in eusocial societies.

The effect of colony size on life history evolution in social insects

Just as body size in solitary organisms, colony size in eusocial societies has important consequences, but in social insects, individual as well as colony-level life history traits are affected (Shik et al. 2012, Hou et al. 2010). Species with larger colonies exhibit increased social complexity, communication skills and resource holding potential and intraspecific comparisons show that larger colonies benefit from improved colony survival and reproductive output (Figure 1.1); (Elmes and Wardlaw 1982, Kaspari and Vargo 1995, Changizi 2002, Franks et al. 2006, Shik 2008). Division of labour and task specialization mainly explain these fitness-relevant traits of colonies and leading to caste differentiation and the evolution of divergent ageing phenotypes (Bourke 1999, Ferguson-Gow 2014, Chapuisat and Keller 2002). In species with larger colonies, life history traits of queens and workers, such as body size, fecundity, and longevity increasingly diverge (Figure 1.1); (Kramer and Schaible 2013, Bourke 1999, Ferguson-Gow 2014). According to the evolutionary theory of ageing, differences in lifespan between queen and workers, may be explained by colony size-associated changes in extrinsic mortality (Flatt and Heyland 2011, Medawar 1952, Parker 2010, Kramer and Schaible 2013). In larger colonies, queens are better protected from extrinsic sources of mortality and benefit from higher survival due to improved colony defence, homeostasis and resilience (Figure 1.1); (Kramer and Schaible 2013, Shik 2008, Hölldobler and Wilson 1990, Schmid-Hempel 1998,

Palmer 2004). Yet, how the relationship between worker lifespan and extrinsic mortality shifts with colony size both within and between species is less clear. Increasing colony size causes a rise in resource demand, which in turn leads to increased foraging distances with higher risks of desiccation, predator and parasite encounters.

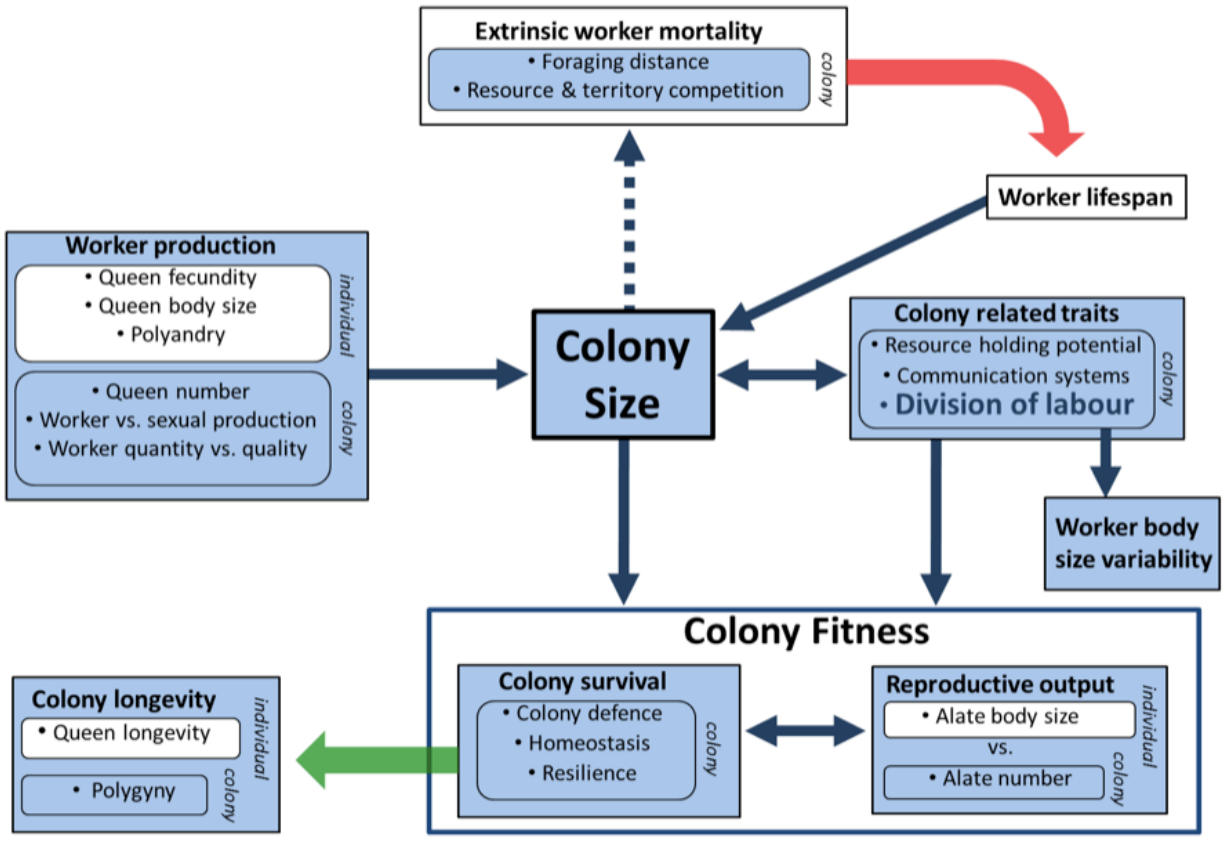

Figure 1.1: This figure illustrates the central role of colony size in life history evolution of social insects. The flowchart depicts the influence of size-related changes on the evolution of individual (in white) and group-level (in blue) life history traits. Dark blue arrows indicate causal relationships, which are condition-dependent when lines are dotted. Colony size affected selective pressures are shown by large arrows, the colour of which indicate the direction of selection (positive: green; negative: red). Colony size largely depends on worker production and worker lifespan, which in turn is influenced by the extrinsic mortality of workers. Intra-specific and interspecific comparisons revealed an increase in division of labour, improved resilience and colony defense, better resource holding potential and communication systems in larger social insect colonies. Although species with stronger division of labour are likely to evolve morphological divergence within the worker caste, increased colony survival selects for colony longevity and in turn for increased queen lifespan or queen replacement, for instance via secondary polygyny.

Species with larger colonies also have a higher potential to control resources and are more likely to engage in intra-specific or interspecific fights (Figure 1.1); (Palmer 2004, Retana et al. 2015). As a consequence, an increase in extrinsic worker mortality could result in relaxed selection for worker longevity (Figure 1.1); (Kramer and Schaible 2013). Alternatively, the overall fitness benefits of increased colony size could select for increased longevity in workers (Figure 1.1); (Giraldo and Traniello 2014). To resolve this issue, more empirical data comparing colony size-related changes in extrinsic mortality and worker lifespan both within and between species are needed. Life history theory predicts a trade-off between offspring quality and quantity (Smith and Fretwell 1974), which appears to shift in insect societies with colony size (Meunier et al. 1999). Within a species, larger colonies produce fewer alates, but the body size of new males and queens increases (Figure 1.1; (Shik et al. 2012, Shik 2008)). Queen body size is associated with better independent founding success and faster colony growth within ant and bee species (Wiernasz and Cole 2003, Fjerdingstad and Keller 2004, Rangel et al. 2013). Hence, smaller colonies focus on producing more queens of lower quality and aim for quantity, whereas larger colonies invest more in offspring quality, which can lead to an increased founding success (Shik 2008, Wiernasz and Cole 2003). Similarly, worker quality and quantity could be traded-off (Figure 1.1); (Kramer and Schaible 2013, Cassill 2003, Holway and Case 2001), albeit it is less clear what characterizes worker quality. As workers do not normally reproduce, the quality of a worker can be regarded as its contribution to the reproductive output of a colony. As in most social insects, per-worker productivity decreases with colony size (Kramer et al. 2014, Michener 1974), is worker quality thus lower in larger colonies? A facet of worker quality could be size, but there is little evidence that worker size decreases with colony size (Kramer and Schaible 2013). A comparison of non-invasive and invasive species revealed that the supercolonial invasive ants have smaller workers (McGlynn 1999). Yet, they differ in so many traits from non-invasive species that it remains unclear whether colony size differences alone explain this pattern. Variation in worker body size clearly increases with colony size both during colony ontogeny and in interspecies comparisons and is associated with higher degree of specialization and task

efficiency in larger colonies (Figure 1.1); (Changizi 2002, Ferguson-Gow 2014). Species with polymorphic workers exhibit diverging worker morphologies as their colonies grow adding the highly specialized larger worker castes (majors) only later in colony development (Hölldobler and Wilson 1977). An exception is fungus-growing termites, in which the early production of soldiers decreases the growth of incipient colonies (Chouvenc et al. 2015). As reduced investment into each worker may help colonies to grow faster, many species with monomorphic workers increase worker size over early colony ontogeny (Espadaler and Rey 2001). Fecundity is another life history trait that can be affected by colony size. Although the likelihood of worker reproduction is reduced in highly eusocial species, queen reproductive output is associated with colony size especially among monogynous species (Figure 1.1); (Shik et al. 2012, Schmid-Hempel 1998, Burchill and Moreau 2016). Furthermore, increased fecundity is associated with physiogastry in army ant and termite queens with colonies of several million of individuals (Hölldobler and Wilson 1977, Sieber and Leuthold 1982, Dornhaus et al. 2012). In monoandrous social Hymenoptera, a queens' total life-time production of diploid offspring may be limited by the amount of sperm she receives from a male during her only mating flight. Evolution of large colony size may thus favour polyandry or several mating events during a queens' social life (Figure 1.1); (Cole 1983, Fjerdingstad and Boomsma 1998, Kraus et al. 2004, Hartke and Baer 2011, Barth et al. 2014). Alternatively, fecundity constraints of body size and sperm availability and the strong fitness dependence on the queens' life-span, are overcome by multiplying the number of reproductives in secondary polygynous species (Figure 1.1); (Keller and Genoud 1997, Boulay et al. 2014).

Social structure and reproductive strategies

Social insects exhibit a diversity of reproductive strategies, often associated with the social organization of their colonies. Whereas ants and termites invariably form long-lived societies, many social bees and wasps are shorter lived and often exhibit an annual semelparous lifestyle (McGlynn 1999, Macevicz and Oster 1976). Life history theory predicts that a species should reproduce only

once in life, if adult mortality exceeds juvenile mortality (Gadgil and Bossert 1970). The semelparity of bees and wasps in temperate climates (McGlynn 1999, Macevicz and Oster 1976), could thus be an evolutionary consequence of high overwintering mortality of established nests. If however, juvenile mortality is higher than adult mortality, several reproductive events are selected for. Albeit a semelparous lifestyle is hard to abandon (Cole's paradox; (Boomsma et al. 2014)), there are several transitions to perennial iteroparous life, for example in the ancestors of the ants, termites, honey and stingless bees (Keller and Genoud 1997, Parker 2010). Reproductives of these taxa benefit from a lower adult mortality due to well-developed nest defences against predators and parasites (Palmer 2004), but suffer from a high juvenile mortality due to a dangerous mating flight and colony foundation phase (Schmid-Hempel 1998, Gordon and Kulig 1996). Obligate eusociality is believed to have evolved under lifetime monogamy (Boomsma 2007, Boomsma 2009, Boomsma and d'Ettorre 2013, Hughes et al. 2008), yet many eusocial lineages have secondarily developed multi-queen breeding (i.e. polygyny; (Hölldobler and Wilson 1990, Schmid-Hempel 1998, Bourke and Franks 1995, Keller 1993)). Monogyny and polygyny are associated with distinct life-history syndromes (Bourke and Franks 1995), as queens of polygynous species are typically shorterlived, less fecund and smaller compared to queens from monogynous ones. According to classical life history theory, these differences in queen lifespan can be explained by the higher juvenile to adult mortality in monogynous queens (Figure 1.2 a), which disperse over longer distances and found new colonies independently (Hölldobler and Wilson 1990, Schmid-Hempel 1998, Peeters and Ito 2001, Cronin et al. 2013). Contrastingly, queens from polygynous colonies suffer lower juvenile to adult mortality because they often mate in or near their natal nests and start to reproduce either in the mother colony or establish new nests with the help of workers. This relaxes selection for longevity thus favouring early reproduction (*i.e.* production of sexuals; Figure 1.2 c). That queen number is less important than the founding mode becomes apparent when looking at the monogynous honeybees, where young queens return to the mother nest and show fast reproduction and relatively short lifespans. Alternatively, differences in queen lifespan between monogynous and polygynous social

Hymenoptera that adopt mated queens may be driven by kin selection (Boomsma et al. 2014, Bourke 2007, Schrempf et al. 2011, Heinze and Schrempf 2008). Central to this kin-selected life history theory, is that worker loyalty erodes over the lifetime of a queen in polygynous colonies, driven by the replacement of workers from older queens by workers from newly adopted ones (Ozan et al. 2013, Bourke and Heinze 1994). A decrease in worker loyalty may result from a switch from worker to sexual production over the reproductive life of a queen. Worker daughters from resident queens are expected to raise eggs of newly adopted queens into workers (Figure 1.2 b). These workers will contribute to the future reproductive success of their mother, corroding the loyalty of the workforce to the older queens with negative consequences on their reproductive success (Figure 1.2 d). Hence, kin selected life history theory predicts a reduction in queen reproductive success over time and thus relaxed selection for longevity once species have evolved obligate polygyny (Figure 1.2 f). This hypothesis thus depends on workers behaving altruistically depending on the degree of relatedness, hence nepotistic. Yet, evidence for true kin recognition and nepotistic behaviour in social insect colonies remains scant (Boomsma and d'Ettorre 2013). Although both classical and kin selected life history theory predict lower queen longevity in polygynous versus monogynous species, the key mechanisms hampering the evolution of queen lifespan in polygynous species markedly differ (Figure 1.2).

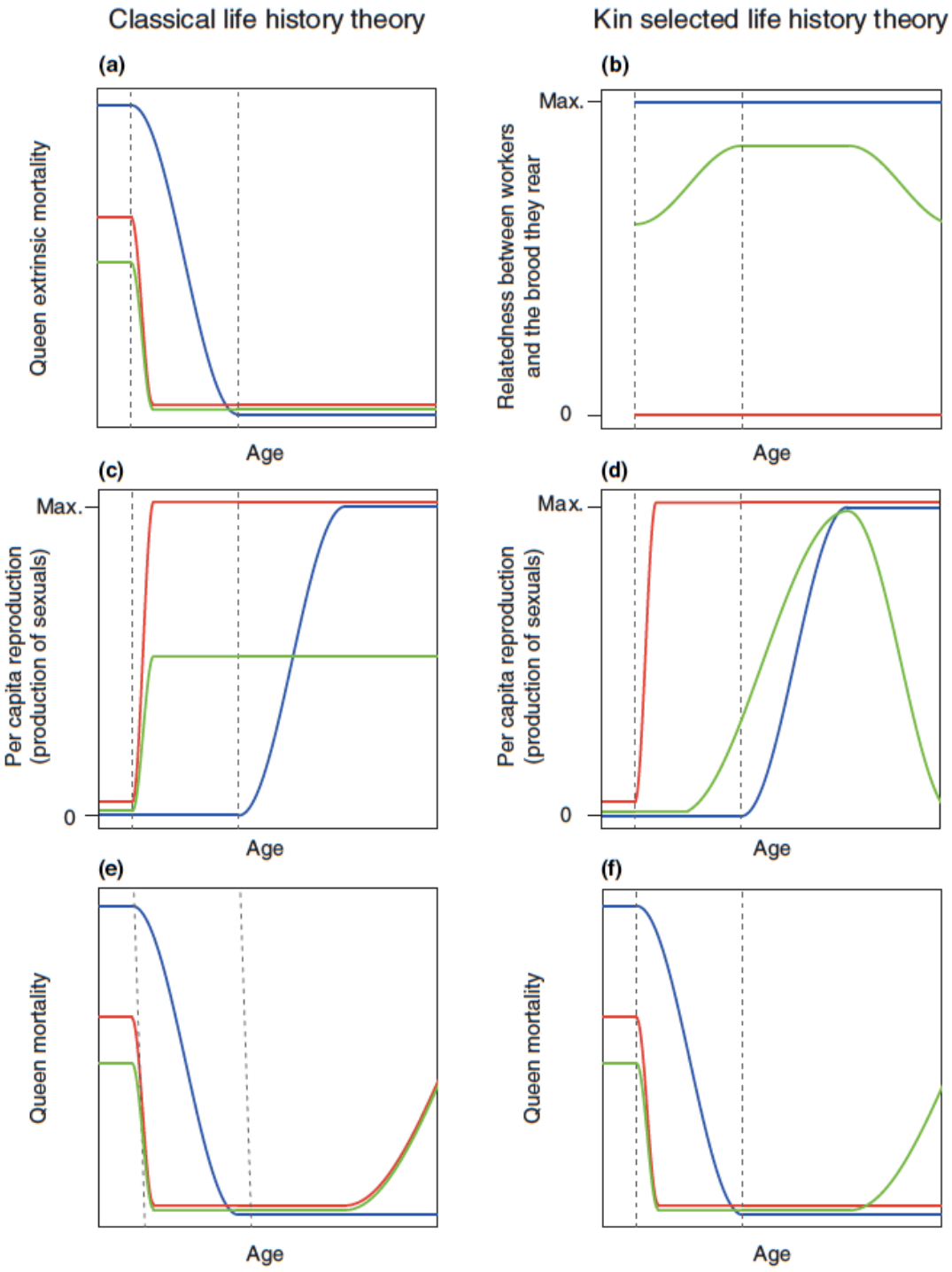

Figure 1.2: Conceptual model of social insect life history traits according to classical (left) and kin-selected life history theory (right). Queen age is represented by three phases, the dispersal phase, the ergonomic phase and the reproductive phase (from left to right), separated by the grey dashed lines. Blue lines represent queens from monogynous species, green lines queens from polygynous species and red lines inquiline social parasites.

One promising avenue to distinguish between these two alternatives is to focus on species where the predictions of classical and kin selected life history theory diverge. In particular, kin selected influences on life history evolution are entirely negated in socially parasitic species (e.g. inquilines), which exploit the workforce of another species to raise their often-exclusive sexual brood (Schmid-Hempel 1998, Wilson 1971, Heinze and Schrempf 2008, Buschinger 2009). Thus, (most) inquiline social parasites do not produce workers and can instead maximally invest in sexual offspring (Figure 1.2 c, d). In inquilines that disperse and usurp host colonies, the extrinsic mortality risk during early life phases may resembles that of independently founding, non-parasitic queens. However, early mortality risk of inquilines that circumvent risky dispersal and colony usurpation by intranidal mating may be comparable to the polygynous strategy. In the latter, theory thus predicts that the age-dependent extrinsic mortality resembles that of polygynous species, in the absence, but not in the presence of kin-selected life history evolution (Figure 1.2 e, f).

Conclusions

The social lifestyle reshapes common life history trade- offs, such as the one between longevity and fecundity, but it is less clear what exactly allows queens to be both highly fecund and long-lived. Although the field currently focusses on revealing how genetic pathways were reset in social insects (Rodrigues and Flatt 2016), it is worth investigating the impact of ultimate factors such as resource availability or extrinsic mortality as well. We have revisited some of the evolutionary drivers of the remarkable life histories of female social insects and highlight four future research areas:

1. Colony size is a colony-level trait tightly connected to intraspecific and interspecific variation in life histories. However, the relative importance of possible underlying factors, such as division of labour, resource holding potential and foraging strategies including communication skills are still unclear. We suggest to study how and why queen and worker mortality, lifespan and body size change with colony size both within and between species. In this context, we consider division of labour of particular importance.

2. As predicted by life history theory in general, the relationship between juvenile and adult mortality should also affect social insect reproductive strategies. However, reliable field data on extrinsic mortalities for social insect queens during mating flight, colony foundation and adult life are scarce. Trapping sexuals, mapping and tracking colonies combined with genetic methods (Ingram 2013, Cole and Wiernasz 2002) allows to obtain these data that will lead to a better understanding of life history evolution.

3. The dependency of colony survival on queen survival is loosened in polygynous social Hymenoptera and in the lower termites, which can replace queens by their daughters. Although this is linked in Hymenoptera to polygyny, this is not necessarily the case in the lower termites, which allows to test whether social structure itself or the possibility to replace the mother queen is associated with relaxed selection and shorter lifespan in social insects.

4. Polygyny-associated shifts in queen life history traits may be imposed by their social rather than their physical environment. The conflict of interest between queens and workers in polygynous species paves the way for intraspecific exploitation, and ultimately inquiline social parasitism (Boomsma et al. 2014, Hughes et al. 2008, Buschinger 2009, Bourke and Franks 1991, Buschinger 1986). Although the role of life history strategies in the evolution of inquilinism has received considerable attention, social parasites are generally excluded from comparative life history studies. Our conceptual model however emphasises that inquiline life history data could be particularly instrumental to test the predictions of kin selected life history theory.

CHAPTER 2

Social organisation and the evolution of life-history traits in two queen morphs of the ant *Temnothorax rugatulus*

Matteo A. Negroni, Marah Stoldt, Marie Oster, Ann-Sophie Rupp, Barbara Feldmeyer, Susanne Foitzik

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Matteo A. Negroni, Marah Stoldt, Marie Oster, Ann-Sophie Rupp, Barbara Feldmeyer, Susanne Foitzik. Social organisation and the evolution of life history traits in two queen morphs of the ant *Temnothorax rugatulus*.

Abstract

Life history theory explains the widespread negative association between longevity and fecundity with a trade-off. Yet, costs of reproduction on lifespan are difficult to quantify as both traits covariate with others. Social insects are ideal to investigate life history trait relationships as these shifted during social evolution. We disentangled the influence of colony size, body size and social structure on ant queen fecundity and investigated links between body size, metabolic rate and survival under oxidative stress. Our model, the ant *Temnothorax rugatulus,* exhibits two queen morphs varying in size and we compared their molecular physiology by analysing fat body transcriptomes. Instead of body size, colony size was positively associated with queen and colony fecundity. Per-queen egg production was lower in polygynous colonies as fecundity was limited by worker care. That colony size was a determinant of fecundity rather than body size or queen number highlights the super-organismal properties of these societies. The smaller microgynes were more frequently fed by workers and exhibited increased metabolic activity, yet they were similar resistant to oxidative stress. Small queens differentially expressed metabolism genes indicating that shifts in molecular physiology and resource availability allow them to compensate their small size without survival costs.

Introduction

Reproductive success and lifespan are important fitness components that are under strong selection. In most solitary organisms these two life history traits are traded-off, as organisms, which invest a lot in reproduction, live shorter (Alonso-Alvares et al. 2004, Barnes and Partridge 2005, Flatt and Kawecki 2007, Flatt et al. 2013). This negative association is also apparent on the molecular level (Flatt et al. 2013), but the proximate mechanisms of this association are poorly understood (Rodrigues and Flatt 2016). Fecundity and longevity are also associated with many other life history traits, such as body size or metabolic rate. For example, a negative association between lifespan and metabolic rate has been reported across and within species, which gave birth to the *rate of living theory* (Harman 1956, Speakman 2005). This theory postulates a causal link between metabolic activity and lifespan involving the generation of reactive oxygen species (ROS) from respiration and causing oxidative damages (Harman 1956, Speakman 2005, Finkel and Holbrook 2000). In line with this, the *free radical theory* of aging, considers oxidative damages as the main cause for aging (Finkel and Holbrook 2000). Yet some results contrast with these theories revealing a more complex link between metabolism, ROS and aging (Hou and Amunugama 2015). Mass-specific metabolic rate is not only associated with lifespan, but also with body size (West et al.1997, Speakman 2005, Gillooly et al. 2005, Chown et al. 2007). Indeed, across-species metabolic activity decreases with increasing body size, so that larger animal species generally live longer (Speakman 2005). Within-species, this negative association between lifespan and body size is often absent or difficult to show given the limited variation in body mass (Speakman 2005, but see Calabi and Porter 1989). However, while across-species, body size is generally negatively associated with fecundity due to a trade-off between growth and reproduction, within-species a larger body mass often indicates higher resource availability, and is thus linked to a higher reproductive success (Stearns 1992). This is especially true for females, which carry in most species the heavier burden of reproduction.

The associations between body size, metabolic rate, longevity and fecundity are mainly documented in solitary organisms. Moreover, the molecular basis of their relationships are as yet unresolved (Da Costa et al. 2016), especially as the proximate causes of aging are not well understood. In social species with reproductive division of labour, i.e. a non-even share of reproduction among group members, selection pressures are allocated differently altering the evolution of life history traits (Kramer et al. 2013, Negroni et al. 2016, Kramer et al. 2016). The degree of reproductive division of labour can be described by the reproductive skew (Keller and Reeve 1994), which is most extreme in the eusocial societies of the ants, termites, bees and wasps (Clutton-Brock 2002). In social Hymenoptera, the colony is generally headed by a single queen (monogynous colony), or several (polygynous colony), who monopolize(s) reproduction, assisted in every way by related, infertile workers, mostly their daughters. Consequently, during social evolution, life history traits of queens and workers such as longevity, fecundity and body size increasingly diverge (Kramer et al. 2013, Negroni et al. 2016). Across the female castes, longevity and fecundity become positively linked (Kramer et al. 2015, Kohlmeier et al. 2017) suggesting a reshaping of the molecular pathways and making social insects a good model for investigating proximate mechanisms involved in the association between these traits (Rodrigues and Flatt 2016).

In many polygynous species, all queens of a colony contribute to reproduction, albeit often not equally ranging from equal share of reproduction to functional monogyny, where a single queen monopolizes reproduction (Heinze et al. 1992). If several queens reside within a colony, they have to share the support of their workers, so that social structure could affect queen fecundity. Although, the overall egg production of a colony increases with queen number (Boulay et al. 2014), the number of reproductive queens negatively affects egg-laying rates of individual queens (Vargo and Fletcher 1989, Negroni et al. 2016). Although queen fecundity is central to colony fitness and individual life history trait evolution, what determines queen fecundity and its consequence on / or link with, other life history traits is not well understood.

Here, we experimentally explore the independent influence of colony size, body size and queen number on queen fecundity and worker-care behaviour. Moreover, we investigate the link between body size, metabolic rate and survival under oxidative stress in queens. We selected as our model the facultatively polygynous ant *Temnothorax rugatulus,* which exhibits a bimodal distribution of body size in queens: the large macrogynes, and the smaller, worker-sized microgynes (Rueppell et al. 1998, Figure 2.1a).

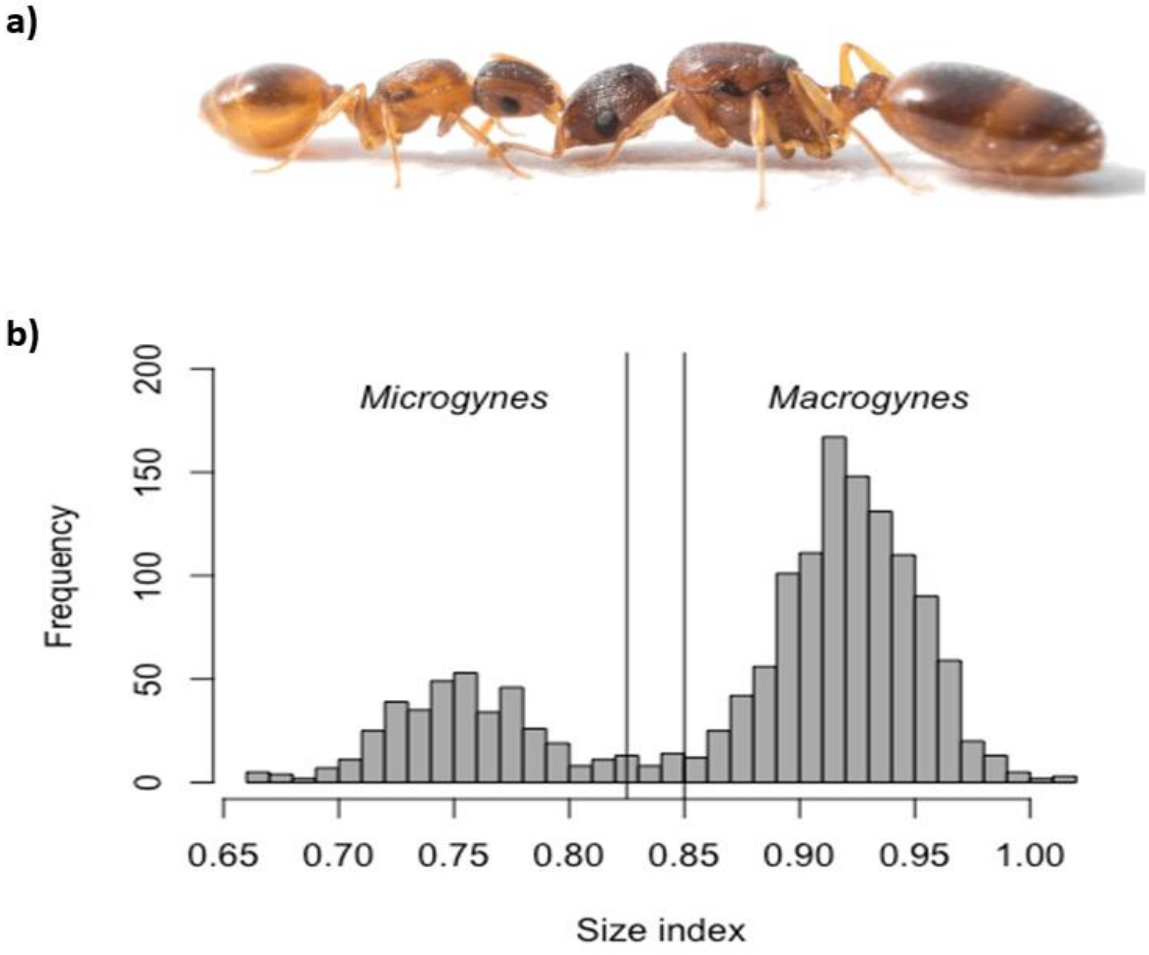

Figure 2.1: Picture of a microgyne (left) and a macrogyne (right) queens a), from Romain Libberecht, with the bimodal distribution of queen body size b), as revealed by the body size index (I = [√ (TL x TW) + HW]/2) based on the meusurement of 1567 queens from 557 colonies.

Queen morph is associated with social structure and founding strategy. Macrogynes can start their colony independently and are residing mostly alone in their colonies, whereas microgynes re-join their mother colony after the nuptial flight and often coexist with other queens (Rueppell et al. 2001a). Determination of queen body size is rather complex in this species, with some heritability being demonstrated (Rueppell et al. 1998, Rueppell et al. 2001b). However, occasionally also colonies with both macro- and microgynes are found, either because macrogynous queens can have microgynous daughters or unrelated microgynes enter macrogynous colonies. Finally, colony size varies strongly in this species and is associated with queen number, ranging between a few dozen to several thousand workers (Rueppell et al. 1998).

In a first experiment, we experimentally varied queen number, queen morph and colony size and recorded egg-laying rates and worker care directed towards the queens. We predicted that queen fecundity should depend on queen body size and/or worker number: larger macrogynes are expected to lay more eggs and queen egg production in general should increase with worker number. Secondly, we contrasted the mass-specific metabolic rate and survival under paraquat-induced oxidative stress, which severely damages macromolecules (Rzezniczak et al. 2011), of the two queen morphs. Thirdly, we compared gene expression in a physiological-important tissue in insects, the fat body (Corona et al. 2007), to gain insights into the molecular regulation of fecundity and longevity in the two queens morphs. Negroni et al. (2019) have shown for this species that macrogynes switch from a strong investment in immunity and starvation resistance at young age to the production of antioxidants such as *catalase* and *superoxide dismutase* later in life. If the two queens morphs would differ in their resistance to oxidative stress, in the rate of aging, or in their metabolic ROS production, we would expect them i) to show a differential expression of such antioxidant genes, and ii) to vary in survival in response to paraquat-induced oxidative stress.

Materials and Methods

Field collection and categorization of queen morph

The ant *Temnothorax rugatulus* occurs throughout the Western United States and Mexico, and lives in rock crevices or under stones in higher elevation oak and pine forests (Rueppell et al. 1998). We collected 557 colonies in Arizona in August 2015 at 15 sites throughout the Chiricahua Mountains. Colonies were kept individually in nest boxes, under artificial conditions, provided with food and water ad libitum and maintained in climate chamber at 22°C 12h light / 12h dark. We followed Rüppell et al. (1998) and calculated a body size index (using the formula $I = [\sqrt{(TL \times TW)} + HW]/2$) for each queen (1567 queens from 557 colonies, Figure 2.1 b) in order to determine their morphs (see bimodal queen body size distribution Figure 2.1b). Queens with an index < 0.82 were grouped into the microgyne category, whereas queens with an index > 0.85 were considered to be macrogynes. 76.8% of the colonies contained macrogyne queens only, 12.6% microgynes only, and 6.5% were mixed colonies containing queens of both morphs. As expected, macrogynes resided more often in monogynous than polygynous colonies (61.7% vs. 38.3%, chi square test, $X^2 = 22.7$, df = 1, $P < 0.001$), while the reverse pattern was found for microgynous colonies (33.8% monogynous vs. 66.2% polygynous, chi square test, $X^2 = 7.2$, df = 1, $P < 0.001$, see Table 2.1).

Table 2.1: Frequency of queen morphs in colonies of different social structure based on 545 colonies collected at 15 different sites in the Chiricahua Mountains, Arizona, August 2015.

	Queen morph			
Social structure	*Macrogynes*	*Microgynes*	*Mixed*	*Total*
Monogynous	48,8 %	4,6 %	-	53,4 %
Polygynous	28,4 %	18,0 %	9,7 %	46,6 %
Total	77,2 %	13,1 %	-	N = 545

Queen fecundity experiment

This experiment was designed to disentangle the influence of body size, colony size, social structure and queen morph on behavior and egg production. Next to standardizing colony composition and maintenance, we used a full-factorial design to investigate the effect social structure and queen morph and their interaction. We split 13 microgyne colonies (with 1-11 queens) and 12 macrogyne colonies (2-5 queens) into smaller experimental units, each receiving either one or two queens, 50 workers and 12 larvae, thereby creating polygynous and monogynous treatments for each queen morph (Table 2.2). A mixed treatment was set-up to study the influence of queen morph on fertility and reproductive skew. These units contained one macrogyne and one microgyne queen from 12 originally mixed colonies. Finally, in order to test for the effect of colony size, we added a treatment consisting of 2 macrogynous queens, 125 workers and 30 larvae derived from independent colonies (Table 2.2).

Table 2.2: Overview of the experimental design showing the six different treatments, characterized by queen morph, social organisation and colony composition. Experimental colonies did not differ in their original worker or queen number between macrogynous, microgynous and mixed colonies (worker number: $F = 2.90$, $df = 2$, $P = 0.07$, queen number: $X^2 = 3.98$, $df = 2$, $P = 0.14$). Independent colonies were used for the large colony treatment and their original worker and queen number did not differ from those used in the polygynous and monogynous macrogyne treatments (worker number: $F = 0.54$, $df = 1$, $P = 0.07$, queen number: $X^2 = 0.53$, $df = 1$, $P = 0.47$). Each experimental colony received a similar proportion of workers from each worker caste (four foragers, two guards, 18 brood careers, 26 intra-nest workers, see Kohlmeier et al. 2017). In a few cases, intra-nest workers replaced workers of rarer worker castes, but this occurred at similar frequencies across treatments (linear mixed-model, $X^2 = 0.62$, $df = 3$, $P = 0.89$).

Treatment	Macrogyne	Microgyne	Mixed
Polygynous	2 Queens 50 workers 12 Larvae	2 Queens 50 workers 12 Larvae	1 Macrogyne 1 Microgyne 50 workers 12 Larvae
Monogynous	1 Queen 50 workers 12 Larvae	1 Queen 50 workers 12 Larvae	
Large colony	2 Queens 125 workers 30 Larvae		

To be able to record egg production of individual queens in polygynous colonies, we administrated one of two lipophilic dyes (blue: Sudan Black, 33 gL^{-1}, red: Sudan IV, 40 gL^{-1}) onto the queens 48h before experimental colony establishment. Each queen was isolated from her colony and the administration of the dye consist in applying a mixture of sun-flower oil and dye on the head of the queen which will be ingested afterward via grooming. Ingestion of the dye resulted in a colour change of the queens' cuticle (red or blackish), making them identifiable, and all newly queen-laid eggs (either light red or blue, Figure 2.2 a, b). We used two different colours for queens of polygynous colonies, and randomized queen colour over monogynous treatments and between morphs of mixed colonies.

Starting 24h after experimental set-up, each coloured queen was individually observed for 5 min every other day, for 46 days (total observation time = 115 min / queen). For each 5 min scan, we recorded her walking time, the number and type of interactions and how often she was fed (N of trophallaxis). Additionally, we recorded the number of coloured eggs and deduced their origin (*i.e.* red eggs were produced by red queen). The distance between polygynous queens was measured at under a stereomicroscope.

Statistical analyses were conducted in R v. 3.3.2 using the packages *car* and *lme4 (R Dev. Core Team 2008).* All models were implemented with original colony identity (ID) as random factor (linear mixed-model: LMM, generalized linear mixed-model: GLMM,). For models differentiating individual queen traits, we added fragment ID (experimental colony ID) as 2nd random factor.

In order to investigate the individual contribution of polygynous queens on colony egg production, we calculated the reproductive skew (see Pamilo and Croisier 1996: Skew = $N_b \times V_b$, where N_b is N of queens and V_b is the variance in reproductive output among queens) and analysed differences between polygynous treatments excluding the colony size one. We moreover tested if macrogynes have a higher egg-laying rate in mixed colonies compared to microgynes. We finally

analysed the influence the influence of colony size on egg production and reproductive skew. The post-hoc pairwise comparisons were performed using the package *multcomp* with Bonferroni correction.

Metabolic rate and survival under oxidative stress

In this experiment, we compared mass-specific metabolic rate, egg production and survival under paraquat-induced oxidative stress between the two queen morphs. We used queens from 53 colonies (31 macrogynous, 15 microgynes, 7 mixed colonies) and created a total of 45 macrogynous and 23 microgynous experimental units by colony splitting, each containing one queen, 14 workers and 10 larvae.

Twenty days after splitting, we measured oxygen consumption for each queen separately using the MicroRespiration system from UNISENSE (Denmark). Measurement of O_2 consumption was recorded for 10 min in glass micro chamber containing the queen, using the oxygen microsensor and the software SensorTraceBasic v 3.0.200 (UNISENSE). All queens were weighed directly after the metabolic rate measurement (PESCALE Wägetechnik, accuracy 1µg). We calculated the mass-specific respiration rate by taking the slope of O_2 (O_2 concentration x chamber volume) over time, with a fixed time range of 9 min, corrected for the queens' body mass. This mass-specific respiration rate was used as a proxy of the metabolic activity per mass unit and thereafter referred to a metabolic rate.

To test if the two queen morphs, which differ in body weight and metabolic rate (see below) also differ in survival to oxidative stress, we artificially induced oxidative stress in queens using paraquat. Experimental colonies were divided in three treatments: a) Paraquat-microgyne treatment including all 23 microgynous colonies, b) Paraquat-macrogyne treatment including 29 macrogynous colonies, and c) control without paraquat with 19 macrogynous colonies. The Paraquat-treatments consisted of administrating a single dose of Paraquat (pure from *Sigma-Aldrich*) solution mixed with

sunflower oil, every 2nd day to each queen until the end of the experiment. The macrogyne control only received oil only.

To administrate the Paraquat-oil solution, each queen was isolated in a petri dish and the head of the queen was covered with a Paraquat-oil solution or oil only by using a thin needle. The queen ingested the solution via grooming (45-60 min) before she was placed back into her colony. As the amount of oil applied relative to body weight was lower in macrogynes (LMM: $X_2 = 6.38$, df = 1, P = 0.01) we used a higher concentration of 0.8 M of Paraquat solution for macrogynes and a lower for microgynes (0.46 M). This resulted in the same final dosage of paraquat per body mass of 1.58 $molg^{-1}$ for macro- and microgynes. To measure queen fecundity, we counted the number of eggs in each experimental colony the day before the Paraquat treatment (day 0). From day 1 (24h h after 1st administration of paraquat) we recorded queen survival every day, and the number of eggs produced every 2nd day. The experiment ended on day 44, when all queens from the Paraquat treatments had died.

We compare fecundity and metabolic rate between queen morphs (GLMM and LMM respectively). We analyzed the effect of the treatment on queen survival (survival model with the R package *coxme*). To analyze the influence of paraquat in interaction with queen morph on queen egg production, we created a categorical variable *Paraquat treatment*, which included two levels (*yes* or *no*) differing in time: day zero (one day before starting the treatments: *no*) and day ten (after ten days at least 72 % of each queen morph were still alive: *yes*). The choice of day 10 as second factor level was determined to optimize statistical power (compromise between a long enough period of paraquat treatment and a large enough number of individuals still alive). We tested the influence of queen morph (Paraquat-macrog. / Paraquat-microg.) in interaction with *Paraquat treatment* on queen eggproduction.

Gene expression comparison between queen morphs

To investigate differences in gene expression between queen morphs we used 14 polygynous colonies, 8 with macrogynous queens and 6 with microgynous queens. We standardized queen number to two, worker number to 50 and larvae number to 12. Experimental colonies were maintained in standard artificial condition (maintained in a climate chamber at 22°C, 12h light / 12h dark) for sixteen weeks up to which we randomly chose eight macrogynes and six microgynes, each queen from a different colony. The ants were killed by decapitation, and fat body was dissected on ice. The RNA was extracted from fat body samples separately using the RNeasy mini kit (Qiagen), resulting in 14 RNA samples. RNA sample were sequenced in BGI Hongkong (100bp paired reads, Illumina HiSeq 2000/2500).

The raw reads from RNAseq were trimmed with *Trimmomatic-v0.36* (Okada et al. 2017), checked for quality using *FastQC-v0.11.5 (*Yang et al. 2013). All paired reads were *de novo* assembled using *Trinity* (trinityrnaseq-Trinity-v2.4.8), resulting in an assembly with 166,120 contigs, of which 55.86 % were annotated with *BlastX* (Altschul 1990) against the non-redundant insect protein database (state April 2019) and a cut-off of E-05. The read count estimates per contigs and sample was obtained using *RSEM*-v1.3.0 with the implemented *Bowtie2* aligner. To eliminate spurious reads, all contigs with less than 10 reads in at least four samples were removed (Alleman et al. 2019). The filtering steps reduced the database to 67735 contigs used in differential gene expression analysis, which was performed with pairwise contrasts with the R package of *Deseq2-v1.2.10* (*contrast* function). Nucleotide sequences were translated into amino-acid sequences with *Transdecoder-v5.5.0* (Okada et al. 2017), before conducting a gene ontology (GO) term annotation using *InterProScan-v5.34-73.0* (Quevillon et al. 2005), assuming across-species conservatism of gene functions. A gene ontology (GO) term annotation was conducted using *InterProScan-v5.34-73.0* (Quevillon et al. 2005). We performed a GO term enrichment analyses based on the subsets of differentially expressed contigs using the R package *TopGo -v-3.6* (Alexa and Rahnenfuhrer 2016),

with the “weight01” algorithm. This was done separately for contigs upregulated in macrogynes and in microgynes. We manually annotated the top ten differentially expressed genes (FDR-p < 0.05), with the higher absolute Log2FoldChange using the *Uniprot* database (*www.uniprot.ong*), with *Drosophila* annotations when available, or other organisms if not, looking for candidate biological function either associated with metabolism, longevity or fertility.

Results

Queen fecundity experiment

The number of eggs laid was neither affected by social structure, queen morph nor their interaction (LMM, *structure*: $X^2 = 2.49$, df = 1, P = 0.11, *morph*: $X^2 = 0.12$, df = 1, P = 0.72, *interaction*: $X^2 = 1.36$, df = 1, P = 0.24). The reproductive skew among nestmate queens was moderate with an average of 0.31, that is 71% of all eggs were produced by the most reproductive queen. However, the skew differed from zero for all treatments (polygynous macrogyne: t = 4.63, P < 0.001, polygynous-microgynes: t = 2.36, P = 0.024, mixed: t = 5.25, P < 0.001), but did not vary between polygynous treatments (LM: F = 2.09, df = 1, P = 0.14). Moreover, macrogynes did not lay more or less eggs than microgynes in mixed colonies (macrogynes: mean 0.58 +/- SE 0.32 %, Student test: t = 0.96, df = 11, P = 0.18). The spatial distance between queens in polygynous colonies (without mixed treatment) did not affect the reproductive skew (LMM: $X^2 = 0.68$, df = 1, P = 0.40), nor the number of eggs produced, though this association was marginally negative (LM: F = 4.12, Df = 1, P = 0.051). Moreover, the skew was unaffected by the number of workers in the nest when comparing the polygynous-macrogynous treatments (LM: F = 0.37, df = 1, P = 0.37). Yet queens in larger colonies produced more eggs (LM: F = 10.06, df = 1, P < 0.01).

Individual queen egg production was reduced in polygynous colonies, but was unaffected by morph (Table 2.3, Figure 2.2b). Behavioural observations revealed that microgynous queens were more often fed by workers than macrogynes independent of social structure (Table 2.3, Figure 2.2e). Larger macrogynous queens were more often groomed than the smaller microgynes (Table 2.3, Figure 2.2f). Grooming and feeding rates were not influenced by social structure, nor their interaction with queen morph (Table 2.3, Figure 2.2e, f). Colony size did not affect the rate with which queens were fed or groomed by workers (respectively: df = 1, F = 0.94, P = 0.34, df = 1, F = 0.08, P = 0.77). Finally, queen movement was unaffected by social structure, queen morph or their interaction (Table 2.3).

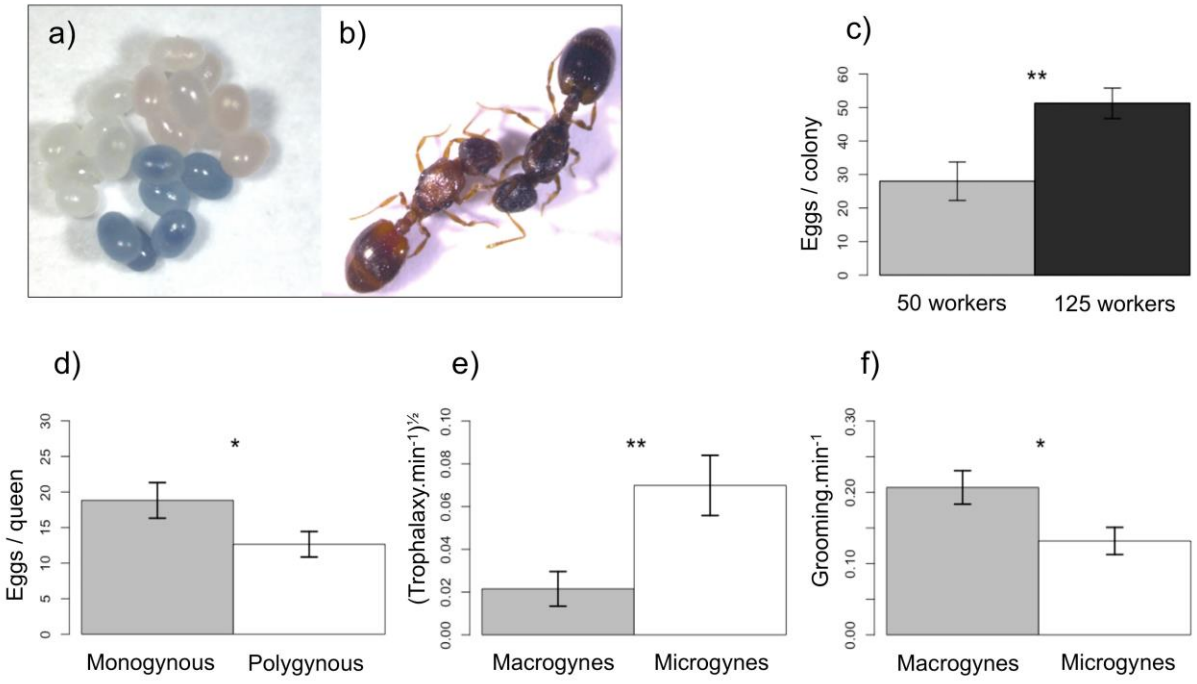

Figure 2.2: Colorations with lipophilic dye of a) queens (Sudan black (left) Sudan IV (red, right), and b) eggs (uncoloured (right), Sudan IV (red, top left), Sudan black (blue, below). c) Number of eggs produced in a colony depended on worker number. d) Queens lay more eggs, when they reside alone in a colony. Macrogyne queens are e) less often fed, but f) more often groomed than microgynes. Results of the full model are presented in Table 2.3.

Table 2.3: Influence of social structure, queen morph and their interaction on individual egg production, grooming, feeding rate and mobility. Significant p values were corrected for multiple testing using the Bonferroni method.

Fixed variables	**Egg number**			**Grooming rate**			**Trophallaxis rate**			**Movement rate**		
	χ^2	**df**	**P**	**χ^2**	**df**	**P**	**χ^2**	**df**	**P**	**χ^2**	**df**	**P**
Morph	0.96	1	0.75	4.97	1	**0.03**	9.71	1	**<0.01**	0.70	1	0.40
Struct.	5.98	1	**0.04**	1.36	1	0.24	0.01	-	0.98	0.76	1	0.38
Morph x Struct.	1.68	1	0.19	0.02	1	0.96	0.08	-	0.77	0.25	1	0.61

Metabolic rate and survival under oxidative stress

Macrogyne queens had about twice the body mass of microgynes (Figure 2.3a), but the two morphs did not differ egg production (GLMM with poisson distribution: $X^2 = 0.22$, df = 1, P = 0.64, Figure 2.3b). Microgynes had a twice-higher metabolic rate than macrogynes (LMM: $X^2 = 21.39$, df = 1, P < 0.001, Figure 2.3c). Though both queen morphs survived less well under paraquat-induced oxidative stress, there was no difference in survival between macrogynes and microgynes (survival mixed-model: $X^2 = 33.11$, df = 2, P < 0.001, Paraquat-macrogyne vs. oil-control: Z = -5.36, P < 0.001, Paraquat-macrogyne vs. Paraquat-microgyne: Z = 1.70, P = 0.19, Figure 2.3 d). Queen survival was unlinked to metabolic rate ($X^2 = 0.70$, df = 1, P = 0.40) nor its interaction with the treatment ($X^2 = 2.24$, df = 2, P = 0.29). Finally, egg production was influenced by an interaction between treatment and morph, with microgynes showing a decrease in egg-laying rate after ten days of daily paraquat administration (Z = 3.09, P = 0.007), whereas macrogynes kept their egg-laying rate constant (Figure 2.3e).

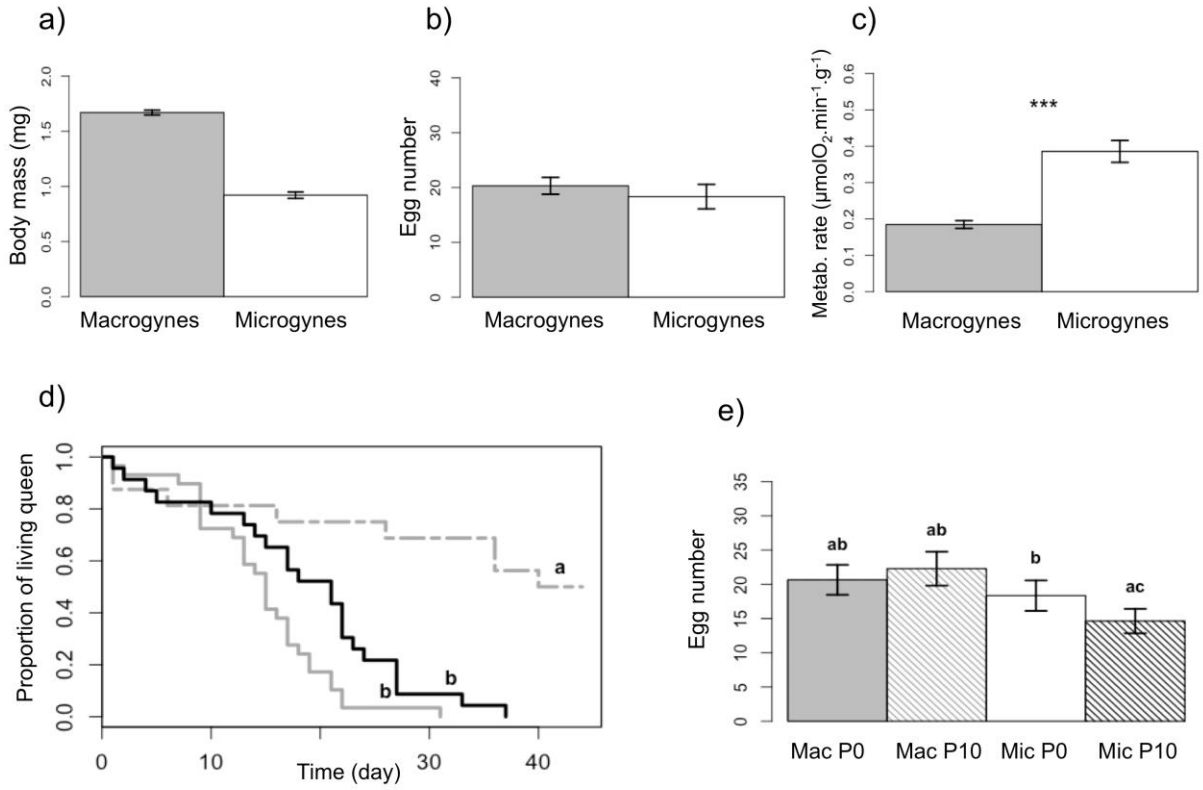

Figure 2.3: Differences between macrogyne (grey) and microgyne queens (white) in a) body weight, b) egg number c) metabolic rate before and c) survival and e) egg-laying rate under paraquat-induced oxidative stress. Letters summarize the results from pairwise post-hoc pair comparisons with Bonferroni correction.

Gene expression analyses

The PCA revealed a strong clustering of samples according to queen morph (Figure 2.4a). The two queen morphs differed in the expression of 699 genes, 295 being up-regulated in macrogynes and 404 in the microgynes. *GRB10-interacting GYF protein 2* and *syntaxin-binding protein 5* were found among the top 10 differentially expressed genes and were upregulated in macrogynes and microgynes, respectively. Both genes are involved in the insulin-signalling pathway according *Uniprot* functional annotation (Table 2.4). A gene annotated as *Proteasome complex subunit B4* was upregulated in macrogynes and is potentially involved in cellular response to DNA damage. Generally, our results indicated that strongest differentially expressed genes exhibit metabolic processes or regulation of cellular processes functionalities. The functional enrichment (Figure 2.4b)

revealed that the two morphs differ in various functions mostly related to metabolism including *malate* or *fatty* acid *metabolic processes*. The enrichment of *proteasome assembly* in macrogynes may indicate that macrogynes do more protein degradation (Figure 2.4b), whereas *translation*- the most enriched function in microgynes – points to more protein synthesis. Moreover, the enrichment of *glycerol-3-phosphate catabolism* in microgynes indicated a higher consumption of energy (Nguyen et al. 2003, Figure 2.4 b).

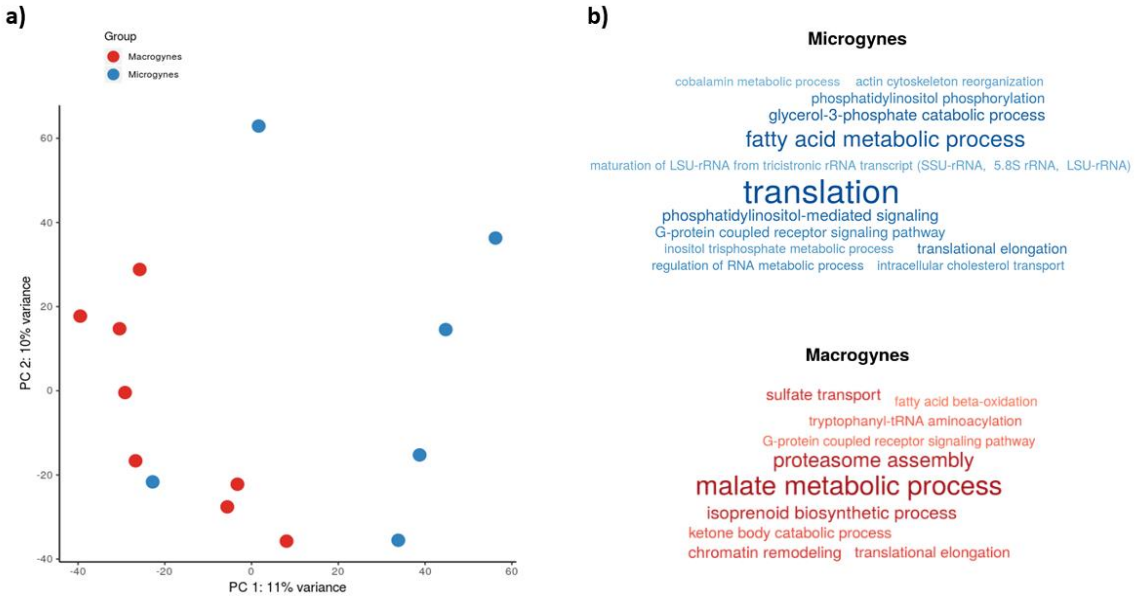

Figure 2.4: a) PCA of all samples colour by queen morph, b) Functional enrichment with all enriched functions represented in word clouds for each morph.

Table 2.4: Upregulated contigs (p value FDR-adjusted) in each queen morph among the top 10 with the highest Log2FoldChange (LFC) and blast annotated, with their blast annotation and species as well as their Uniprot annotation and species.

	Blast annotation	Species	LFC	P	UniProt annotation	Species
Macrogyne	importin subunit alpha-7	*Temnothorax curvispinosus*	25.9	8.3^{E-18}	cell redox homeostasy	*Camponotus floridanus*
	ADP-ribosylation factor GTPase-activating protein 1	*Cyphomyrmex costatus*	25.9	1.1^{E-17}	regulation of GTPase activity	*Drosophila melanogaster*
	flavin-containing monooxygenase FMO GS-OX-like 6	*Temnothorax curvispinosus*	25.6	5.5^{E-18}	glucosinolate biosynthetic process from homomethionine	*Arabidopsis Thaliana*
	patatin-like phospholipase domain-containing protein 3	*Temnothorax curvispinosus*	25.3	9.4^{E-22}	lipid catabolic process	*Drosophila ficusphila*
	60S ribosomal protein L8	*Cyphomyrmex costatus*	25.1	4.5^{E-17}	cytoplasmic translation	*Drosophila melanogaster*
	high mobility group protein HMGI-C	*Temnothorax curvispinosus*	24.8	5.0^{E-17}	cell division	*Mus musculus*
	GRB10-interacting GYF protein 2	*Temnothorax curvispinosus*	24.6	5.4^{E-17}	insulin-like growth factor receptor signaling pathway	*Homo sapiens*
	proteasome activator complex subunit 4B	*Atta cephalotes*	24.4	6.0^{E-17}	cellular response to DNA damage stimulus	*Ictalurus punctatus*
Microgyne	glycerol-3-phosphate dehydrogenase NAD+	*Monomorium pharaonis*	28.4	$4.4E^{-26}$	carbohydrate metabolic process	*Drosophila melanogaster*
	cytochrome b5-related protein	*Temnothorax curvispinosus*	25.3	$4.9E^{-20}$	lipid metabolic process	*Drosophila melanogaster*
	long-chain-fatty-acid-CoA ligase 4	*Temnothorax curvispinosus*	14.8	$1.7E^{-45}$	positive regulation of cell growth	*Homo sapiens*
	syntaxin-binding protein 5	*Temnothorax curvispinosus*	11.6	$2.8E^{-04}$	negative regulation of insulin secretion	*Mus musculus*
	lambda-crystallin homolog	*Temnothorax curvispinosus*	11.2	$4.8E^{-17}$	fatty acid metabolic process	*Drosophila melanogaster*
	restin homolog	*Temnothorax curvispinosus*	11.0	$7.0E^{-04}$	cellularization	*Drosophila melanogaster*

Discussion

Our experiment revealed that in the ant *T. rugatulus* queen egg-laying rate is unlinked to body size, but depends in this social species on the support of workers. Queen egg-laying rate was higher in larger colonies and lower when queens had to share the workforce with other queens, i.e. in polygynous colonies. Albeit microgynes weigh less than half of macrogynes, they are able to maintain a similar high egg-laying rate apparently by sustaining a high metabolic rate and soliciting more food from workers. Interestingly, these small queens do not seem to pay a price for their high fecundity in form of a lower survival rate, as they were able to withstand oxidative stress to a similar degree, as did the larger macrogynes. Our transcriptomic results revealed that a divergent activation of metabolic pathways may allow microgynes to do this.

On the colony level, we found an increase in egg production in larger colonies compared to smaller ones. Queens in polygynous colonies shared reproduction to a certain extent, but not entirely fairly indicating a functional polygyny with a moderate reproductive skew (Medeiros et al. 1992). However, when standardized to worker number, polygynous colonies produced as many eggs as monogynous ones, indicating that worker rather than queen number is the limiting factor for colony egg production. Our findings differ from what has been observed in the ant *Cardiocondyla obscurior* where colony egg production increased almost linearly with queen number, while worker number had no effect on queen egg production (Schrempf et al. 2011). In our study the experimental colonies were rather small compared to natural conditions, which could thus have masked any potential effect of queen number on egg production that maybe visible with a larger colony size (Negroni et al. 2016).

With a functional polygyny and a clear effect of worker number on egg production, it is not surprising that queen number had a negative effect on the individual egg-laying rate queens, corroborating earlier field and experimental studies on other ants (*S. invicta*: Vargo and Fletcher 1989, Vargo 1992, *Temnothorax acervorum*: Bourke 1993, *C. obscurior:* Schrempf et al. 2011). The

lower egg-laying rate of queens in polygynous colonies can be the result of i) direct aggressive competition between queens (i.e. *Cardiocondyla sp.*: Yamochi et al. 2007, *Odontomachus chelifer:* Medeiros et al. 1992), ii) mutual pheromonal inhibition (i.e. *S. invicta*: Vargo 1992), or iii) indirect competition for a limited workforce (food intake, grooming, queen-larvae care, Negroni et al. 2016). We observed no aggression between polygynous queens so that the first hypothesis can be rejected. If mutual pheromone inhibition would play a role, we would expect the reciprocal negative effect on queen reproduction to decrease when the physical distance between them increases (Keller and Nonac 1993, Boulay et al. 2007). Instead, we found a marginal negative effect of the distance between polygynous queens on egg production, making this hypothesis unlikely. This leaves the hypothesis of competition for the workforce. We found that queens in polygynous colonies were as often groomed or fed by workers than those in monogynous colonies. Hence, it is likely that either i) queens adapt their egg production according to or in response to the workforce, ii) that workers adapt the number of eggs in the colony by cannibalizing the surplus of eggs, or iii) that queens can cannibalize each other's eggs (Bourke 1991).

Unexpectedly, as reproduction is commonly strongly linked to female body size in insects (Honěk 1993) including ants (Vargo and Fletcher 1989, Kronauer 2009), we did not find an association with individual queen fecundity or colony egg production, as macrogynes produced as many eggs as microgynes. Moreover, even in mixed colonies the reproductive skew was not higher than in other polygynous colonies, with microgynes producing within those nests, as many eggs as their larger nestmate macrogynes. In contrast to species where queens are physiogastric, that is their gaster extends as a response to ovarian activity and development (Vargo and Fletcher 1989, Kronauer 2009), body size differences between *T. rugatulus* queen morphs concerns the entire body including thorax and head (Rueppell et al. 1998), which are not morphologically plastic during the adult stage. Our results indicate that in *T. rugatulus* queen morph is important during the founding phase (Rueppell et al. 2001a), while it plays a much lesser role later in life when the colony is established. As neither queen number nor body size had an effect on colony egg production,

whereas colony size does, our findings fit to the concept of the super organism where colony size, as body size in solitary organisms, is a major life history trait strongly linked to reproduction (Shik et al. 2012, Negroni et al. 2016). Our experimental colonies were rather small so that queens might not have been limited by worker care, which might have masked any effect of queen morph on egg production (Rueppell et al. 2001a). In the field, colony size is on average about 100 workers – twice the number of our experimental colonies - yet large colonies can contain up to 1900 workers in *T. rugatulus* (unpublished data). In these large colonies, queens might be closer to their reproductive limits, so that more queens in a colony might increase the overall egg production (Rueppell et al. 2001a, Negroni et al. 2016).

Egg production is energetically costly and represents a metabolically active process (Williams 2005). Supporting this, we found that microgynes were more often fed by workers than macrogynes. In addition to their smaller size all of these observations suggest a higher mass-specific metabolic rate in microgynes compared to macrogynes. In fact, the respiration measurements confirmed this prediction as it revealed an almost twice-higher mass-specific metabolic rate in the small queens while both morphs did not differ in their mobility. This observation is one of the few examples confirming the negative association between body size and basal mass-specific metabolic rate within species (but see Calabi and Porter 1989, and West et al. 1997).

According to the *rate of living theory,* higher metabolic activity is predicted to result in higher production of ROS and consequently, in line with the *free-radical theory* of aging, in a shorter lifespan (Sohal 2002, Speakman et al. 2005). As we base our study on ant queens collected in the field and of unknown age, we cannot measure queen lifespan as such. Moreover, as *Temnothorax* queens are with up to 20 years very long-lived (Plateau 1986), monitoring the survival to gain insights into morph-specific lifespan would require long-term observation (only 8.4% of queen death over more than three months of monitoring, unpublished data). We used an alternative approach and induced oxidative stress by a forced ingestion of paraquat (Rzezniczak et al. 2011). However, contradicting our predictions and the *rate of living theory,* we did not find differences in survival between the two

morphs, albeit the paraquat treatment strongly decreased queen survival in general (Rzezniczak et al. 2011). There are several explanations for this finding. First, given their higher metabolic rate, it is possible that microgynes eliminated the poison paraquat faster than the larger queens (Hall et al. 2012). Second, microgynes may produce less reactive oxidative species relative to their metabolic activity, for example due to better mitochondrial efficiency. This is in line with the called *uncoupling to survive theory,* which predicts a positive association between metabolic rate and lifespan (Brand 2000, Speakman et al. 2004). A higher metabolism would result in a lower production of ROS from the complex III of the respiratory chain, and a longer lifespan relative to body mass as shown before in mice (Speakman et al. 2004). Third, it is possible that the effect of paraquat treatment depends more on the paraquat concentration of the administrated solution rather than on the final concentration in the ant body. Indeed, while we standardized paraquat quantity per body mass for the two morphs, the larger queens received a more concentrated solution. This could have induced more severe tissue damages (for instance in the pharynx) in large queens compared to smaller ones, resulting in a similar survival. A higher metabolism without survival reduction under oxidative stress could indicate that small queens invested more into somatic maintenance, for example via the production of antioxidants (Negroni et al. 2019) or in molecular repair compared to large queens. We did not find any evidence for that when looking at our fat body gene expression data, however it is possible that a differential investment into somatic maintenance between the two morphs would only appear in response to the paraquat treatment. Large queens kept their egg production constant under oxidative stress, whereas the microgynes reduced it, potentially revealing a trade-off between somatic maintenance and egg production and providing an example for terminal investment in macrogynes (Heinze and Schrempf 2012). From an evolutionary point of view, there should be a stronger selection for maintaining a high egg production in macrogynes as they mostly reside in monogynous colonies and are thus the only reproductive, whereas microgynes commonly share the reproduction with other queens (Negroni et al. 2016). Finally, the absence of differences in resistance

to paraquat between the two queen morphs despite a higher metabolic rate in microgynes can result from morph-specific metabolism, or physiological responses to paraquat.

Our transcriptomic analyses revealed clear and symmetrical differences in gene expression between the two morphs. Macrogynes and microgynes differed for example in the expression of candidate genes involved in the insulin growth-factor signalling pathway, which is well known as a major regulator of lifespan and reproduction. Indeed, this pathway is involved in the longevity-fecundity trade-off in solitary species (Flatt et al. 2013). However, we did not find any differences in fertility at the phenotypic level and no evidence for differences in lifespan between queen morphs. Hence, those genes are good candidates potentially involved in the apparent absence of trade-off between longevity and reproduction in social insect queens. Consistent with our phenotypic findings, in particular the metabolic rate measurements, the enrichment analysis shows overall that macrogynes and microgynes vary in the activation of various metabolic pathways. The most significant among them is the malate metabolism as malate synthesis was suggested to prevent senescence and cancerous cell proliferation (Willey and Campisi 2016). Though the directionality of the molecular physiological shifts remain unresolved, the differential activation of the malate pathway could potentially contribute to the absence of differences in paraquat resistance between the morph, despite a higher metabolic rate in microgynes. Finally, we found that while microgynes seem to synthetize more proteins, as indicated by an upregulation of translation genes, macrogynes may degrade more damage proteins as they overexpress genes with a proteasome production functionality. Also proteasome activity has been reported to have a positive effect on lifespan (Chondrogianni and Gonos 2008). The gene expression and functional enrichment analyses therefore indicate that the queen morphs have altered their molecular regulation of longevity and fecundity.

Conclusions

Our experimental approach allowed us to disentangle the influence of various factors important for the evolution of life history traits in relation with social life. Contrary to most solitary species, body size had no effect on female fecundity in our ant model. Instead, the major determinant of queen and colony egg production was the number of workers present in a colony, which is in line with the superorganism concept where colony size replaces body size as an important colony-level life history trait. The number of reproductives in a colony led to a reduced egg-laying rate of each queen, but despite a moderate reproductive skew, all queens in polygynous colonies contributed to egg production. But how can the smaller microgynes keep up with the larger macrogynes in egg production? Our data indicate that smaller queens receive more food from workers and upregulate their metabolism, which was also apparent on the molecular level. Surprisingly this rise in metabolic activitiy did not increase their susceptibility to oxidative stress challenging the *rate of living theory* and pointing to a less direct relationship between metabolic activity and lifespan as commonly presumed. Our transcriptome analysis reveals that queen morph specific differences in gene expression are associated with longevity pathways such as malate metabolism, proteasome activity or insulin growth-factor signalling indicating shifts in the molecular regulation of lifespan and potentially fecundity in queens of the same species.

CHAPTER 3

Positive effect of food intake on *sequestosome-1* expression and a positive link between fertility and DNA repair in *Temnothorax rugatulus* ant queens

Matteo A. Negroni, Barbara Feldmeyer, Susanne Foitzik

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Matteo A. Negroni, Barbara Feldmeyer, Susanne Foitzik. Positive effect of food intake on sequestosome-1 expression and a positive link between fertility and DNA repair in *Temnothorax rugatulus* ant queens

Abstract

Nutrient intake often has opposing effects on lifespan and reproduction in solitary organisms. While more food increases fecundity, it often leads to a shorter lifespan. The molecular mechanisms underlying this opposing consequences have been suggested to also be at the basis of the widespread longevity-fecundity trade-off. Social insects display an atypical positive association between lifespan and reproduction: the queen is by far the most fecund and longest-lived individual in the colony and she is provided with more food than the short-lived, sterile workers. Which factors or processes allow social insect queens to circumvent the trade-off between lifespan and reproduction is an open question. Here, we experimentally tested the effect of food intake and egg removal on fertility and investigated related changes in the phenotype and in fat body gene expression in queens of the ant *Temnothorax rugatulus*. Egg removal triggered an increase in queen fecundity, whereas food restriction lead to a decrease in egg-laying rate. The two treatments altered the expression of candidate genes related to longevity, but also some involved in other biological processes. Food restriction had a negative effect on cellular homeostasis and autophagy, the last being required for the diet-restriction-related lifespan extension in solitary species. Egg removal not only stimulated queen fertility, but also triggered the expression of genes important for somatic maintenance, especially DNA repair. Overall, our study identifies candidate genes linked to fecundity and longevity in social insect queens, which might help to uncover how the molecular regulation of these two life history traits shifted during social evolution.

Introduction

Why lifespan is regulated on an ultimate level and how on a proximate one are long-standing questions in evolutionary biology that have been the focus of intensive research (Medawar et al. 1952, Williams 1957, Kirkwood, 1977, Partridge et al. 1993, Kirkwood et al. 1991, Flatt et al. 2013). Yet we are still far from understanding the interaction between environment, genotype and molecular pathways determining variation in longevity within and between species. Senescence is occurring in almost all pluricellular organisms and is characterized by a decline in biological function including reproduction, immunity and homeostasis, reflected in an increasing mortality rate (Partidge et al. 1993, Finkel and Holbrook 2000). On a proximate level it is considered as the result of the progressive accumulation of molecular damage due to insufficient somatic repair (Kirkwood and Holliday 1979, Kirkwood and Austad 2000, Lombard et al. 2005, Schulz et al. 2007, Sun et al. 2016). Endogenous causes of molecular damage include mainly metabolically generated radical oxygen species (ROS) and spontaneous biochemical errors (Kirkwood and Austad 2000, Corona et al. 2005). Higher production of antioxidants and improved molecular repair mechanisms, are two important facets of body maintenance as they should reduce the rate of accumulation of molecular damage (Kirkwood and Austad 2000, Lombard et al. 2005, Khrapko et al. 2006, Vermulst et al. 2007). Although intensively studied the importance of these two parameters for the rate of molecular damage as well as the role of different factors in the regulation of lifespan remain poorly understood.

The negative association between lifespan and reproduction, observed in most solitary organisms, both across and within species, has been described as resulting in i) antagonistic pleiotropic effects or ii), a trade-off in allocation of resources (Williams 1957, Kirkwood 1977, Flatt et al. 2013). The highly conserved target of rapamycin (TOR) signaling and, insulin/insulin-like signaling (IIS) pathways are important regulators of lifespan and substantially contribute to the negative link between longevity and fecundity (Kenyon 2005; Narasimhan 2009, Kenyon 2010, Flatt et al. 2013, Fonseca et al. 2016). Instead of being traded-off, in social insects, lifespan and reproduction appear

to be positively linked: the most fertile individuals in insect societies, the queens, live the longest (Heinze and Schrempf 2008; Kohlmeier et al. 2017, Rodrigues and Flatt 2016, Negroni et al. in prep. a). Moreover, longevity and fecundity are highly plastic traits in this group, as the phenotypic differences between the queen and worker castes can arise from the same genetic background (Keller and Jemielity 2006). These characteristics make social insects a good system for investigating the molecular bases of the regulation of lifespan and its link to fertility. Yet how queens circumvent the trade-off between these two traits on a proximate level is not well understood (Rodrigues and Flatt 2016), but several hypotheses have been proposed.

The immune response is energetically and physiologically costly (Moret and Schmid-Hempel 2000). Moreover, recent studies have shown a link between aging and the inflammatory reaction reflected by an overactivation of the immune system (Xia et al. 2016, Fabian et al. 2018). Benefiting from social immunity (Cremer et al. 2007, Stroeymeyt et al. 2018), the queen may invest less into personal immunity, which would possibly allow investing more into both longevity and fecundity (Negroni et al. 2019). Second, resources might not be limited for the queen, who could invest more both into longevity and fecundity, which would thus mask an existing trade-off (Kramer et al. 2016). Nutrition is one of the best-studied factors in the regulation of lifespan and reproduction. In solitary species, food intake has a positive effect on reproduction, but often shortens lifespan through the activation of the TOR signalling and IIS pathways (Lee et al. 2006, Flatt et al. 2013, Fonseca et al. 2016). In social insects, this antagonistic effect of food intake seems to not occur, which may also suggest a reshaping of related molecular pathways compared to solitary organisms (Rodrigues and Flatt 2016), allowing for a positive link between these two traits.

Here we investigated the link between lifespan and reproduction in queens of the ant *Temnothorax rugatulus* as well as the importance of food intake for the regulation of these two traits, at the phenotypic and molecular level. We conducted two experiments, in which we separately manipulated food availability and queen egg production, through egg removal, and observed the consequences on the phenotype and on fat body gene expression. We have previously shown in

this species that a higher rate of feeding may allow smaller queens to reproduce as much as larger ones without a necessary lifespan reduction (Negroni et al. in prep.). Thus, we expected here, food availability to positively affect fecundity and lifespan. We have also shown in this species that experimental manipulation leading to fertility induction in workers is accompanied by a lifespan extension (Negroni et al. in prep. a). Moreover, this lifespan extension appears to be linked to changes in immunity (Negroni et al. in prep. a). Similarly, we expected here a positive link between experimental increase in egg-laying rate and investment in somatic maintenance in relation with changes in the immunity.

Materials and Methods

Colony collections

We collected 105 colonies in Arizona in August 2015 at a total of 10 sites throughout the Chiricahua Mountains. Colonies were kept in artificial conditions, provided with food and water ad libitum and maintained in climate chamber at 22°C, 12h light / 12h dark.

Food restriction experiment

In this experiment we aimed to manipulate food availability in queens and were interested in the consequences on survival, fertility as well as on gene expression in the fat body. As workers may buffer food restriction of the queen, the queen was isolated from the rest of the colony together with five of her workers to ensure food provisioning to the queen. To do this we used a total of 62 colonies monogynous colonies (average colony size 103.2, SE = 31.5). We created artificial experimental nest sites made of a wire grid sandwished between two Plexiglas perimeters (3 mm high each), the all sandwiched between two microscope slides (7.5 x 2.5 x 0.5 cm). Each Plexiglas perimeters had an entrance located at the opposite side from the other, and each opening at an individual foraging area separated with a central Plexiglas wall. For each colony the queen was isolated with five

workers (two foragers and three nurses as determined by Kohlmeier *et al.* 2017) from the rest of the colony. The queen and her five workers were placed in the upper part of the artificial experimental nest forming thus the queenright (QR) part, while the rest of the colony was placed in the lower part forming the queenless (QL) part (Figure 3.1). The experiment consisted of two treatments the *food restriction* and the *control* (no food restriction). Ant colonies in the *food restriction* treatment were provided with two cricket legs and honey once every second week in the foraging area of the QR part. The *control* treatment received the same amount of food twice per week, so that over a two week period the control treatment was provided with 4 times as much food. For both treatments, the QL part was fed with cricket and honey twice per week and both QR and QL parts were provided with water ad libitum. This organization allows for a control of food availability to the queen in the QR part, while limiting any potential buffering effect by workers for the lack of food in the *food restriction* treatment. The grid stitch does not allow for trophallaxis between individuals from the QR and the QL part, while allowing for keeping integrity of the colony in term of chemical communication of volatile compounds. After the setting of the treatment colonies were kept at 22°C until the end of the experiment, we counted the number of eggs and checked for survival every week until the end of the experiment. As eggs produced at the beginning of the experiment turned into larvae, queens reduced their egg production in both treatments after a few weeks. Thus in order to increase the likelihood of finding an effect of the treatment on fertility, the brood including eggs and young larvae were removed from the QR part in every nest at week 8 after the set-up of the experiments. After 13 weeks of treatment, the experiment ended, and all queens were dissected.

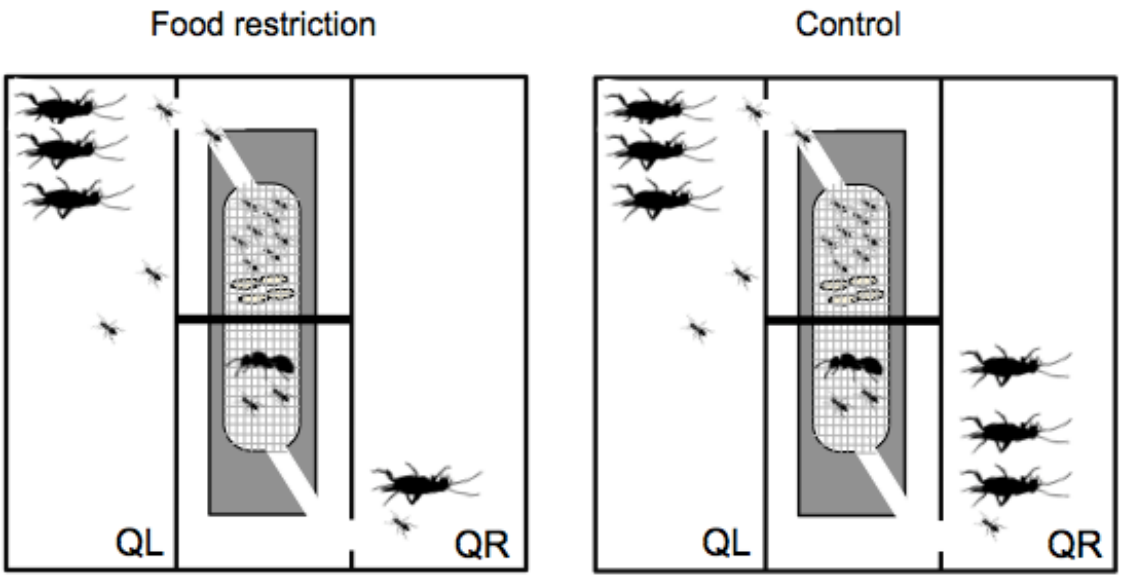

Figure 3.1: Illustration of the experimental design of the food restriction experiment. The queen and its five workers are placed in the upper part of the artificial experimental nest forming thus the queen right (QR) part, while the rest of the colony was placed in the lower part forming the queen less (QL) part. QR and QL opened on individual foraging areas, where food availability can be independently controlled. The food restriction treatment consists in feeding the QR part with cricket legs once every second weeks, while in the control the QR was fed twice per week. The number of cricket silhouettes illustrates the frequencies of feeding. This organisation allows for a control of food availability to the queen in the queen right (QR) part while limiting any potential buffering effect by worker for the lack of food in the food restriction treatment without the possibility for trophallaxis between individuals from the QR and the QL part, and keeping colony integrity for chemical communication of volatile compounds.

Egg removal experiment

In this experiment we aimed to manipulate queen fertility and were interested in the consequences on ovary development, and activity as well as on fat body gene expression. To do so we weekly removed all eggs from the nest aiming to increase thereby the egg production of the queen. We used a total of 43 polygynous colonies and created via colony splitting a total of 60 experimental colony fragments. In order to avoid any confounding effects, we standardized queen number, worker number, as well as larvae number respectively to two, 50 and 12. All eggs were removed during

colony splitting. Seven to five weeks after this set-up, colonies were gradually transferred into an artificial winter (5°C) within a week, and stayed at this temperature for 12 weeks. Then the temperature was gradually increased back to 22°C within two weeks and remained unchanged until the end of the experiment. Starting eight weeks after, all colonies were weekly anesthetized with CO2 to remove the eggs. Nest sites were shortly opened and for the 29 colonies in the *egg-removal* treatment all eggs were removed from the nest with floppy forceps. For the other 31 colonies in the *control* treatment, we opened the nests similarly, but just touched and moved a bit the eggs in the nest with the forceps as a control for manipulation. The forceps was systematically cleaned with hexane after each manipulation in order to prevent from any transfer of hydrocarbons compounds. Some experimental colonies were not independent as they originated from splitting the same original colony in two fragments. However in this case the two colony fragments from the same source colonies were distributed to the two different treatments. Five queens from five different colonies, died before the start of the treatment and the corresponding colonies were then removed from the rest of the analysis. From the beginning of the treatment until the end of the experiment, queen survival was recorded weekly and after the death of one of the two queens the entire corresponding colony was removed from the rest of the analysis. The experiment was performed for six weeks, and two weeks after the experiment ended and all queens were dissected.

Ovary dissection and fat body RNA extraction

At the end of both experiments, for each treatment and for each experiment, eight queens from different experimental colonies were randomly chosen, killed by decapitation, and fat body and ovaries were immediately dissected on ice. Tissue dissection took less than 10 min for each queen. For each experiment the order of dissection was randomized over their two treatments to control for potential confounding effects. For each queen the fat body was individually homogenized in 50µL of TRIZOL for storage at -20°C before RNA extraction. RNA was extracted from fat body samples separately using the RNeasy mini kit (Qiagen), resulting in total in 32 RNA samples (16 queen fat

bodies from the food restriction experiment and 16 queen fat bodies from the egg removal experiment). Library preparation was conducted according to standard protocols at BGI Hongkong, which also sequenced 100bp paired reads on an Illumina HiSeq 2000/2500. The remaining queens (N = 50 and N = 41 respectively for macro- and microgynes) were further dissected only for their ovaries. A photo was taken for fertility measurements including all queens (magnification x20; camera *Leica DFC425*; measurements: Leica software *LAS version 4.5*), resulting in a sample size of N = 29 and N = 27 respectively for experimental and control treatment of the food restriction experiment and N = 43 and N = 57 respectively for experimental and control treatment of the egg removal experiment.

Transcriptomic analysis

For the transcriptomic analysis all reads from all samples were used to build a single *de novo* assembly transcriptome and gene annotation. For the differential gene expression analysis the two experiments were treated separately. All the raw reads were first trimmed with *Trimmomatic-v0.36* (Okada *et al.* 2017, Negroni *et al.* 2019), checked for quality using *FastQC-v0.11.5* Yang et al. 2013), and all paired reads were *de novo* assembled using *Trinity* (trinityrnaseq-Trinity-v2.4.8, Negroni *et al.* 2019), which resulted in a total of 328.731 contigs. To annotate the contigs we conducted a *BlastX* homology search (Altschul 1990) against the non-redundant invertebrate protein database (state June 2018) and an E-value cut-off of E-05.

Separately for each experiment, the read count estimates per contigs and per sample was obtained using RSEM-v1.3.0 with the implemented Bowtie2 aligner. To remove low read counts that likely represent noise, we filtered all contigs with had less than 10 reads in less than four samples out (Alleman *et al.* 2019). The differential gene expression analysis performed with pairwise contrasts with the R package *Deseq2-v1.2.10* (*contrast* function) by comparing the *food restriction* treatment versus the *control* one for the food restriction experiment, and the *egg removal* treatment versus the *control* one for the egg removal experiment. For the egg removal experiment as some of the

samples were dependent from their original field colony, we added colony identity as controlled factor for increasing the power of our focal comparison. Nucleotide sequences were translated into amino-acid sequences with *Transdecoder-v5.5.0* (Okada et al. 2017), before conducting a gene ontology (GO) term annotation using *InterProScan-v5.34-73.0* (Quevillon et al. 2005). We performed a GO term enrichment analyses based on the subsets of differentially expressed contigs using the R package *TopGo -v-3.6* (Alexa and Rahnenfuhrer 2016), with the "weight01" algorithm. This was done separately for each list of upregulated contigs of one group compared to another.

We furthermore manually annotated the top ten differentially expressed genes (FDR-p < 0.05), the *Uniprot* database (*www.uniprot.ong*), with *Drosophila* annotations when available, or other organisms if not, looking for candidate biological function either associated with immunity, longevity or fertility.

Statistical analysis of fertility and survival

The statistical analysis was conducted in R v. 3.0.2 (R Development Core Team 2008) and separately for each experiment but for every model treatment was implemented as fixed factor. For the food restriction experiment, to analyse differences in fecundity between treatment we used a non-parametric Wilcoxon test for ovary length, and we used two generalized linear model with a *poisson* distribution (link function = log) one with the number of maturing (white) eggs (count data) in the ovary as a dependent variable, the other with the final number of eggs observed at the end of the experiment as a dependent variable. For the egg removal experiment differences in fecundity between treatment were tested by using a linear mixed model with ovary length (in mm) as a dependant variable, and a generalized linear mixed model with a *poisson* distribution (link function = log) with the number of maturing (white) eggs (count data) as a dependent variable, and experimental fragment ID as well as colony ID as random effects. For both experiments we separately analyzed queen survival by running two survival model, both with treatment as explanatory variable and time in weeks until death (yes or no). As all queens were independent for

the food restriction experiment we used the R package *survival*, while we used the package *coxme()* for the analysis of the egg removal data by adding colony ID in the model implemented as random factor.

Results

Food restriction experiment

Food restriction had a negative effect on fecundity as it reduced the number of eggs produced (X^2 = 240.2, df = 1, P <0.001), the ovary length (W = 508, P = 0.038; Figure 3.2 a), as well as number of eggs in development in the ovaries (X^2 = 44.6, df = 1, P = <0.001; Figure 3.2 b). Only two queens died during the experiment in the control colonies, compared to eight queens in the food restriction experiments, but statistical analyses revealed no effect of food restriction on queen survival (X^2 = 2.05, df = 1, P = 0.152).

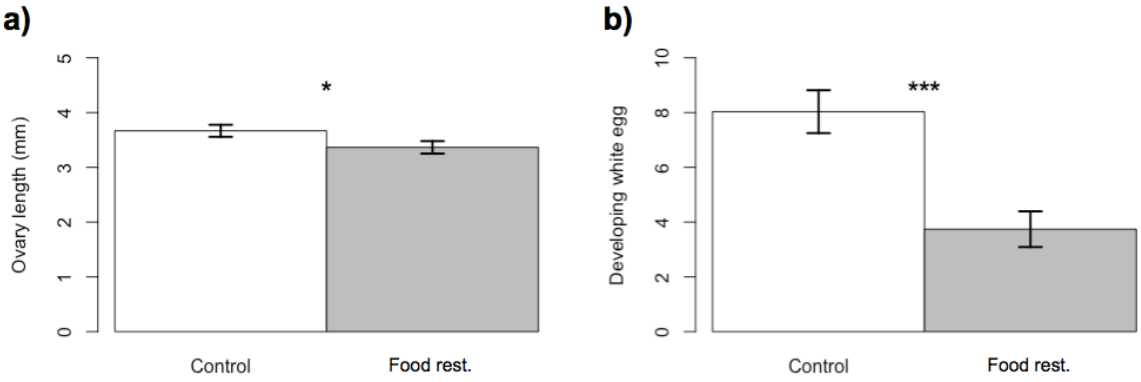

Figure 3.2: Food restriction treatment had a negative effect on queen fertility. Differences in ovary development include (a) length of ovarioles and (b) the number of eggs in development.

After filtering 72.996 contigs were retained for the differential gene expression analysis, which revealed a total of 72 differentially expressed genes, 29 and 43 being respectively up-and down-

regulated in response to the food restriction treatment (heatmap of the differentially expressed genes Figure 3.3a).

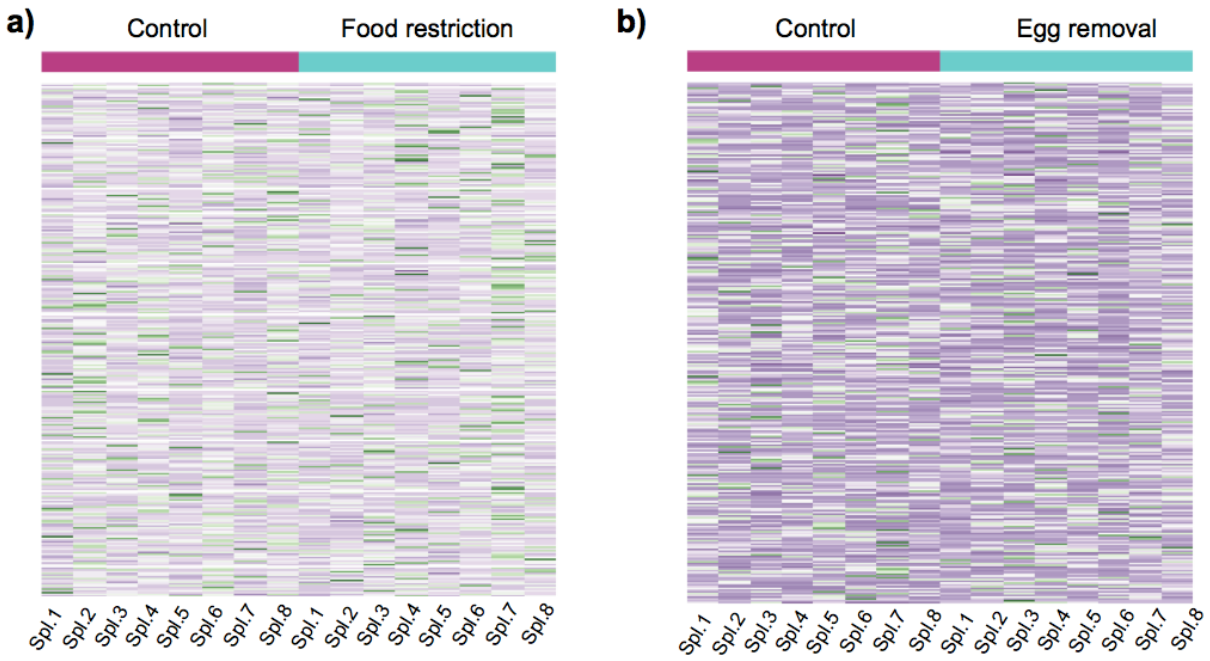

Figure 3.3: Heat maps of differentially expressed genes between control and treatment, per samples for a) the food restriction experiment and b) the egg removal experiment.

The enrichment analysis revealed the function *protein dephosphorylation* (GO:0006470, P = 0.021) enriched in the food restriction and *phenylalanyl-tRNA aminoacylation* (GO:0006432, P = 0.011) enriched in the control. The search of candidate genes (Table 3.1) revealed a negative effect of food restriction on the expression of genes related to autophagy, cellular homeostasis, and DNA integrity. Interestingly also a downregulation of genes related to immunity such as *proteasome subunit alpha type-7-1*, and *tetraspanin-1-like*, where found in queens suffering from food restriction. No obvious fertility candidate genes were found among the differentially expressed ones.

Table 3.1. Candidate genes up-regulated group and for each experiment (exp.: experiment; treat.: treatment; food rest.: food restriction treatment; egg rem.: egg removal treatment). We list only the genes likely involved in the regulation of longevity, immunity and/or fecundity. Shown are the blast annotation with corresponding species, the logFoldChange (LFC) from the comparison between old and young within tissue, the corresponding corrected P-value, the functional annotation made on UniProt as well as the corresponding species (*D. melanogaster* for *Drosophila melanogaster*)

		Blast				Uniprot	
Exp.	Treat.	Annotation	Species	LFC	P-val	Function	Species
Food restriction	Control	sequestosome-1	*Monomorium pharaonis*	10.7	<0.001	autophagy	*Homo sapiens*
		mitochondrial Rho GTPase	*Wasmannia auropunctata*	9.1	0.013	cellular homeostasis	*Drosophila melanogaster*
		proteasome subunit alpha type-7-1	*Monomorium pharaonis*	9.0	<0.001	Toll signalling pathway	*Drosophila melanogaster*
		tetraspanin-1	*Vollenhovia emeryi*	3.6	<0.001	innate immune response	*Caenorhabditis elegans*
	Food rest.	-	-	-	-	-	-
Egg removal	Control	protein toll-like	*Wasmannia auropunctata*	1.6	<0.001	innate immune response	*Drosophila melanogaster*
	Egg rem.	DNA ligase 1	*Vollenhovia emeryi*	11.6	0.006	DNA repair	*Drosophila melanogaster*
		protein toll-like	*Wasmannia auropunctata*	11.6	0.045	innate immune response	*Drosophila melanogaster*
		mitochondrial Rho GTPase	*Wasmannia auropunctata*	9.2	0.032	cellular homeostasis	*Drosophila melanogaster*
		breast cancer type 2 susceptibility protein	*Solenopsis invicta*	5.2	0.029	DNA repair	*Drosophila melanogaster*

Egg removal experiment

Egg removal had no effect ovary length (X^2 = 1.45, df = 1, P = 0.231; Figure 3.4a), but caused an increase in the number of eggs present in the ovaries (X^2 = 8.91, df = 1, P <0.001; Figure 3.4b). Only two queens died during the experiment, so that survival was unaffected by treatment (X^2 = 1.49, df = 1, P-value = 0.223).

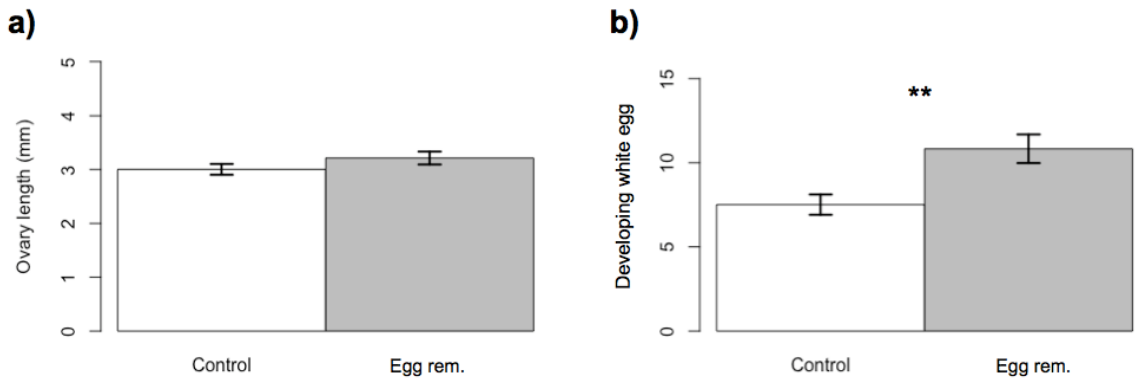

Figure 3.4: The food restriction treatment had a negative effect on queen fertility. Differences in ovary development include (a) length of ovarioles and (b) the number of eggs in development.

After filtering 75,220 contigs were retained for the differential gene expression analysis, which revealed a total of 372 differentially expressed contigs, 194 and 178 being respectively up-and down-regulated in response to the egg removal treatment (heatmap of the differentially expressed genes Figure 3.3b). We found no function to be enriched in neither of the groups, egg removal nor the control. The search of candidate genes (Table 3.1) revealed an upregulation of genes important for DNA repair in response to egg removal. Also two candidate genes of the Toll signaling pathway playing a major role in insect immunity altered their expression with our treatment; interestingly one was up, the other downregulated. Again, we found not obvious fertility candidate genes among the differentially expressed genes. Only one gene, *mitochondrial Rho GTPase* involved in cellular homeostasis, was found differentially expressed across both experiment, and upregulated in response to egg removal as well as food availability (Table 3.1).

Discussion

Nutrient intake in solitary species generally increases fertility at the price of lifespan reduction. Molecular mechanisms involved are thought to contribute to the general trade-off between lifespan and reproduction observed in most organisms. As social insect queens seem to be an exception to this trade-off, they might not experience these opposing effects of nutrients on lifespan and

reproduction. We investigated the underlying molecular mechanisms of these reverse patterns in queens of the ant species *Temnothorax rugatulus*. We show a positive effect of nutrients on fertility and somatic maintenance via autophagy. Moreover, our data reveal a causal positive link between fertility and investment into DNA repair, suggesting that on a mechanistic level longevity and fertility are positively linked. We can thus identify a number of candidate genes potentially involved in the reversal effect of nutrients on lifespan and on longevity/fecundity trade-off reversal in social insects.

Food restriction experiment

Our results confirmed a positive effect of food intake on queen fertility as food restriction reduced ovary development and the egg-laying rate of ant queens. Interestingly, this phenotypic effect was not reflected in the transcriptome of the fat body, as we did not find candidate genes likely involved in fertility regulation. This is consistent with other studies made on the same species (Negroni et al. 2019, Negroni et al. in prep. a) indicating that the fat body might not be the right tissue to look for differential expression of fertility genes. While some fertility genes are known to be expressed outside of the ovaries, they might be nevertheless the tissue reflecting most strongly fecundity (Cong et al. 2015). Ant queens live often for decades and indeed, in *Temnothorax* ants they can reach up to 20 years (Plateaux 1986). This long lifespan might explain, why only few queens died during our relatively short experiment and the unexpected absence of an influence of food restriction on queen survival. However, gene expression analysis revealed that food restriction indeed had a negative effect on the expression of *mitochondrial Rho GTPase* and *sequestosome-1* respectively involved in cell homeostasis and autophagy, both associated with longevity (Madeo et al. 2010, Sun et al. 2016). Especially autophagy has been shown to increase lifespan. The autophagy downregulation via the activation of TOR signalling could indicate a negative impact of food restriction in lifespan in ants (Bergamini et al. 2007, Minina et al. 2013). Or result can thus suggest a reshaping of pathway related to autophagy in our species compared to solitary ones, making of the gene coding for *sequestosome-1* a good candidate potentially involved in the positive association between lifespan and reproduction

in social insects. Experimental dietary restriction has been shown to extend lifespan via enhancing of autophagy (Minina et al. 2013). It is thus possible that the observed positive effect of food availability on autophagy involve modification of food composition in the food ingested by the queen. Indeed, the queen receives its food via the intermediate of workers that process it (Leboeuf et al. 2016). The analysis of the composition trophallaxis fluid given to the queen could allow for testing this hypothesis (Leboeuf et al. 2016). Interestingly the food restriction treatment induced a change in the expression of genes related to immunity (*proteasome subunit alpha type-7-1* and *tetraspanin-1)*. Unexpectedly *tetraspanin-1* involved in the inflammatory response was upregulated in the control. Being a facet of the immune response, inflammatory reaction has been positively linked to aging, but a better immune response allows for better defences against pathogens (Capuron et al. 2014, Schneider et al. 2008, Fabian et al. 2018). The upregulation of *tetraspanin-1* in response to food intake maybe explain by a reduced social immunity due too the low number of workers (Cremer et al. 2007, Stroeymeyt et al. 2018, Negroni et al. 2019) able to take care of the queen and the down regulation of immunity related genes in response to food restriction suggest a cost of immunity in term of resource (Moret and Schmidt-Hempel 2000). In the bumblebee workers, the negative effect of an immune challenge on survival is more pronounced when resources are limited (Moret and Schmidt-Hempel 2000).

Egg removal experiment

At the phenotypic level our results show a stimulation of the queen's ovary activity in response to the removal of eggs. This result indicates that in the control treatment queen egg production is not maximal but probably adapted to the workforce as previously shown in this species where colony size but not queen body size or queen number determine queen and colony productivity (Negroni et al. in prep.). Again, this phenotypical effect on fertility was not reflected in gene expression in the fat body tissue. Similar to the food restriction experiment, the egg removal treatment had no detectable effect on survival, which may be explained by the overall very low death rate over the recorded time

frame. However, results show that the egg removal treatment not only increased queen ovarian activity, but also triggered the expression of genes involved in DNA repair such as *DNA ligase 1* and *breast cancer type 2 susceptibility protein*, or in cellular homeostasis, reflecting a higher investment into body maintenance associated with fertility (Lombard et al. 2005, Schulz et al. 2007, Sun et al. 2016). This is consistent with another study made on *T. rugatulus* workers where fertility induction and lifespan extension correlate with the expression of gene related to molecular repair (Negroni et al. in prep.). This observation at the transcriptomic level could thus result in a lifespan extension in queen caused by the experimental increase in fertility. However, the influence of DNA repair on lifespan depends on the rate of accumulation of DNA damages, and does not necessarily leads to an increase in lifespan. In the ant *Lasius niger*, queens that live ten times more than their workers up regulate molecular repair related genes, but show a similar rate of accumulation of molecular damages (Lucas et al. 2016, Lucas et al. 2017a). Long-term experiments are needed in this long-lived species to detect a potential effect of egg removal on queen survival. Interestingly we found that the treatment changed the expression of toll-like protein involved in the Toll signaling pathway playing a major role in insect immune defense (Valanne et al. 2011). However, we found that two contigs were annotated to the same gene and each of them were upregulated in both treatments. Knowing that this result should be interpreted with caution, it can reflect an opposite effect of the egg removal treatment on the expression of two isoform of the same genes annotated as *toll-protein*.

Our study reveals a clear role of nutrition on fertility and suggests a contradictory positive effect of food intake on autophagy known as essential for experimental extension of lifespan in solitary species. These results make of genes coding for *sequestosome-1*, good candidates potentially involved the absence of antagonistic effect of nutrition on longevity/fecundity on ant queen and on the trade-off reversal in social insects. Our experimental manipulation of fertility revealed a causal positive influence of fertility on the investment into somatic maintenance mostly on molecular repair. Both food intake and fertility stimulation triggered the expression of longevity candidate genes involved in different biological processes including autophagy and DNA repair, reflecting a diversity

in mechanisms underlying queen longevity. Interestingly we only found a single gene to be differentially expressed across both experiment. The expression of this gene, involved in cellular homeostasis correlated with the expression of other somatic maintenance related genes making of it a good candidate for the regulation of this trait in our study species. We furthermore bring additional support for a role of immunity in the regulation of lifespan and reproduction and potentially in their atypical positive association in social insects.

CHAPTER 4

Long-lived *Temnothorax* ant queens switch from investment in immunity to antioxidant production with age

Matteo Negroni, Susanne Foitzik* and Barbara Feldmeyer*

*Authors contributed equally to the study

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Abstract

Senescence is manifested by an increase in molecular damage and a deterioration of biological functions with age. In most organisms, body maintenance is traded-off with reproduction. This negative relationship between longevity and fecundity is also evident on the molecular level. Exempt from this negative trait association, social insect queens are both extremely long-lived and highly fecund. Here, we study changes in gene expression with age and fecundity in ant queens to understand the molecular basis of their long lifespan. We analyse tissue-specific gene expression in young founding queens and old fecund queens of the ant *Temnothorax rugatulus*. More genes altered their expression with age in the fat body than in the brain. Despite strong differences in ovary development, few fecundity genes were differentially expressed. Young founding queens invested in immunity (*i.e.* activation of *TOLL* signalling pathway) and resistance against environmental and physiological stress (*i.e.* down-regulation of TOR pathway). Conversely, established older queens invested into anti-aging mechanisms through an overproduction of antioxidants (*i.e. upregulation of catalase, superoxide dismutase*). Finally, we identified candidate genes and pathways, potentially involved in the association between fertility and longevity in social insects and its proximate basis.

Introduction

Senescence occurs in almost all organisms, despite the fact that it increases intrinsic mortality (Partridge et al. 1993). Understanding the origin of this evolutionary puzzle is a fundamental, but challenging question in biology. Evolution of senescence is considered to result from a decline in strength of selection with age, due to extrinsic mortality (e.g. predation, starvation, diseases, accident), and/or the costs of body maintenance (Medawar et al. 1952, Kirkwood et al. 1991). Evolutionary theories of aging regard senescence as a consequence of deleterious mutations expressed late in life due to antagonistic pleiotropy or mutation accumulation (Kirkwood et al. 1991). Physiologically, senescence is characterised by a general decline in biological functions including immunity and homeostasis (Finkel et al. 2000). At the molecular level, aging is commonly considered as the result of the progressive accumulation of molecular damage due to inadequate somatic repair (Kirkwood et al. 1991, Finkel et al. 2000, Lucas et al. 2017a). Causes of molecular damage include metabolically generated radical oxygen species (ROS), spontaneous biochemical errors in replication, transcription, translation or maturation, and environmental factors such as toxic component or extreme temperatures (Finkel et al. 2000, Rattan et al. 2006). The oxidative stress theory points to the accumulation of oxidative damage as the main proximate cause of aging (Finkel et al. 2000), which implies that long-lived organisms should be characterized by: i) better molecular repair abilities, ii) lower rates of molecular damage (i.e. lower production of ROS or reduced replication mistakes); iii) higher production of anti-oxidants. Although being intensively studied, the highly complex biological process of aging is still poorly understood and many lifespan determining factors remain unidentified (Lucas et al. 2017 a, Bengtson et al. 2016, Da Costa et al. 2016, Korb et al. 2016).

Underlying the cost of body maintenance, lifespan appears to be commonly traded-off with reproduction (Reznick et al. 1985). Although this trade-off is considered widespread across the animal kingdom, there is an increasing number of examples of increased growth and reproduction

through manipulation of diet composition, without a cost on lifespan challenging the trade-off hypothesis (Piper et al. 2017). In particular, social insect queens, are apparently bypassing the common negative association between fecundity and longevity, as they are both, extremely long-lived and highly fecund compared to their workers (Keller et al. 1997, Von Wyschetzki et al. 2015). In most eusocial species, phenotypic plasticity rather than genetic differences cause those tremendous life history differences between female castes, which develop from the same genomic background (Wilson et al. 1971). Hence, social insects offer the unique opportunity to study how differential gene expression regulates different rates of aging (Keller et al. 2006).

Insect queens are well cared for by their workers, and the availability of ample resources could explain why queens can invest in both lifespan and reproduction. However, in many species, including humans, fruit flies, rodents and monkeys, dietary restrictions lengthen lifespan, while for instance in *Drosophila* a protein-rich diet reduces it (Lee et al. 2008, Hoedjes et al. 2017). This link between diet and lifespan involves the nutrient sensitive IIS and TOR pathways, which are conserved from yeast to humans (Fonseca et al. 2016). The inhibition of those pathways through dietary restriction inhibits cell growth and had a negative effect on fecundity, but improves stress resistance. Those observations suggest that in social insects i) certain pathways or biological processes may be differently regulated compared to *Drosophila* (Rodrigues et al. 2016), and/or ii) that queens receive processed food from workers with a specific composition that modifies the effects of nutrients on lifespan (Honda et al. 2011, LeBoeuf et al. 2016). In contrast to rodents and *Drosophila*, caloric restriction has a negative effect on both lifespan and reproduction in *Temnothorax rugatulus* queens (unpublished results).

The link between longevity and investment in immunity might also differ between social insects and other species. In *Cardiocondyla* ants, queens that activated their immune system are shorter lived (Schrempf et al. 2015), whereas across *Caenorhabditis* species longevity and immunocompetence seem to be positively linked (Amrit et al. 2010). Recent studies focussing on gene expression differences between castes and variation with age point towards a complex link

between molecular damage, repair mechanisms and lifespan, which are potentially species-specific, and suggest that investments in fecundity and body maintenance may shift during an ants' life (Lucas et al. 2017 a, Lucas et al. 2017 b, Tasaki et al. 2017, Kohlmeier et al. 2018).

Here, we compare tissue-specific gene expression between old queens (several years old) and young founding queens (a few weeks old) of the ant *T. rugatulus* (Figure 4.1). Our aim was to investigate which genes and pathways change their expression over the lifetime of ant queens. We selected brain and fat body as focal tissues because i) the brain is the production site of several physiological important hormones and is prone to numerous age-related degenerative diseases (Min et al. 1997), ii) the fat body is a physiologically very active organ where most of the haemolymph proteins are synthesized and processed (Corona et al. 2007).

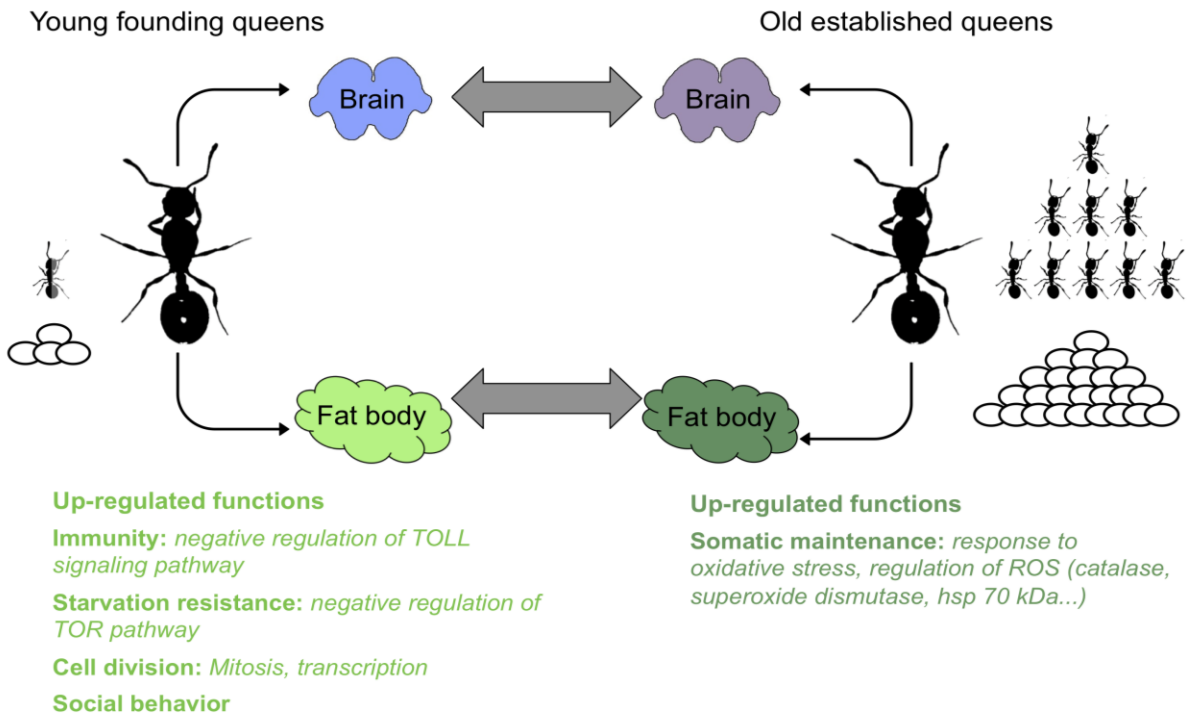

Figure 4.1: This figure illustrates the experimental design in our investigation of tissue-specific changes in gene expression with age and fecundity in ant queens. RNA was extracted from the brain and the fat body of N = 8 young founding queens and N = 8 old established queens and two different differential expression analysis were conducted within-tissues / between-age classes comparison (grey double arrows); The young founding queens were found in the field with 0 to 8 workers and had much less developed ovaries compared to the old established one for which the colony size ranged between 197 and 337 workers. The eggs illustrate relative fecundity of queens, and worker number illustrate colony size. The coloured boxes summarize the findings about differential investment in traits and function out of the comparison between young and old queen fat bodies (ROS = radical oxygen species).

Independent from age, we expected young queens to invest more into immunity and starvation resistance, as they were likely to be exposed to pathogens during the mating flight and consequent colony foundation, and are in the process of raising their first worker generation solely based on their body reserves. Conversely, we predicted old queens, which experience a low extrinsic mortality as they are well cared for by their large workforce, both in terms of food provisioning and social immunity, to concentrate on fecundity and body maintenance mechanisms (Cremer et al. 2007, Heinze et al. 2012, Negroni et al. 2016).

Materials and Methods

Field collection and ant maintenance

Temnothorax rugatulus is a small North American ant widely distributed throughout the western part of the continent. Colonies live in small crevices or under stones in high elevation oak and pine forests. Two queen morphs occur in this species: the large macrogynes and the small microgynes (Rueppell et al. 1998, Rueppell et al. 2001 a). Here, we focussed on the large macrogynes that can start their colony independently and live mostly in monogynous societies. Queens of monogynous *Temnothorax* species can live more than a decade (Plateaux 1986, Keller et al. 1997).

Ant colonies were collected in August-September 2015 in the Chiricahua Mountains, Arizona at seven sites, approximately 2-3 months after the mating flight starting in June (Rueppell et al. 1998). Thus, founding queens were only 2-4 months old and had zero to eight minim workers (Plateaux 1986). For the old queen cohort, we selected the largest monogynous colonies from our collections, which contained between 197 - 337 workers. Based on their colony size and the slow growth rates of *Temnothorax* colonies, we estimated that these queens were at least several years if not a decade old (Plateaux 1986, Keller et al. 1997). We selected eight queens and their colonies from each of the two age categories (Figure 4.1) and those 16 colonies were kept individually in artificial nests as described in (Kohlmeier et al. 2018).

Dissection, fertility measurements and RNA extraction

After 11 weeks under stable conditions (22°C, 12L:12D), the 16 queens were killed by decapitation, and brain, fat body and ovaries were immediately dissected on ice. Tissue dissection took less than 10 min for each queen. Order of dissection was randomized over age classes to control for potential confounding effects. For each queen the brain and fat body were separately and individually homogenized in 50µL of TRIZOL for storage at -20°C before RNA extraction. RNA was extracted from fat body and brain samples separately using the RNeasy mini kit (Qiagen), resulting in total in

32 RNA samples (16 brains and 16 fat bodies, of 8 young and 8 old queens). Library preparation was conducted according to standard protocols at BGI Hongkong, which also sequenced 100bp paired reads on an Illumina HiSeq 2000/2500. The ovaries were further dissected and a photo was taken for fertility measurements (magnification x20; camera *Leica DFC425*; measurements: Leica software *LAS version 4.5*). To analyse differences in fecundity between age classes, we used a linear model with ovary length (in mm) as a dependant variable, and we used a generalized linear model with a *quasipoisson* distribution (link function = log; overdispersion = 1.6) with the number of maturing (white) eggs (count data) as a dependent variable. Age class was implemented as an explanatory variable in both models. Statistical analyses were conducted in R v. 3.0.2 (R Dev. Core Team 2008).

Gene expression analysis and annotation

After trimming the raw reads (filtering out the low-quality reads coming from sequencing artefacts) with *Trimmomatic-v0.36* (Okada et al. 2017), and quality checking using *FastQC-v0.11.5* (Yang et al. 2013), all paired reads were *de novo* assembled. We used different assemblers, *Soap* (Li et al. 2008), *Bridger* (40), *Trinity* (trinityrnaseq-Trinity-v2.4.0) (Okada et al. 2017), and also constructed a meta-assembly of the three assemblies with *MIRA* (Chevreux et al. 1999). Using the program *TransRate* (Smith-Unna et al. 2016), we conducted a quality comparison between the four assemblies. Especially based on the contig length (set of overlapping sequences rebuilding a transcript sequence), and back-mapping rate (percent raw sequences mapping to the *de novo* assembled contigs), we decided to choose the Trinity assembly for further analysis (back mapping rate of 87%).

We used *RSEM*-v1.3.0 with the implemented *Bowtie2* aligner in order to obtain read count estimates per contig and sample. The read count consists of the number of sequence reads that align to a given contig, reflecting the expression level of the corresponding gene. In order to visualize sample similarity and detect putative outliers, we built hierarchical clustering dendrograms of

samples based on the Euclidean distance (using *average* as agglomeration method, with the R command *htclust* from the package *stats* (Henningsen et al. 2011)), based on the entire dataset, but also on a subset only including brain or fat body data, respectively. The differential gene expression analysis performed with pairwise contrasts with the R package *Deseq2-v1.2.10* (*contrast* function). Venn diagrams were built using the online available tool Venny (*http://bioinfogp.cnb.csic.es/tools/venny/*). Based on the top 15,000 contigs with the highest across sample variance in expression, we assessed the overall variance across samples within groups and compared old versus young queens (across all tissues), and brain versus fat body (across both age classes), using a permutational multivariate analysis of variance (PERMANOVA, 999 permutations) based on the Bray-Curtis similarity in the software Primer 6.0 and PERMANOVA (Primer-E Ltd.).

To annotate the contigs we conducted a *BlastX* homology search (based on alignment and sequence similarity) (Altschul et al. 1990) against the non-redundant invertebrate protein database (state June 2016). Nucleotide sequences were translated into amino-acid sequences with *Transdecoder-v3.0.1* (Okada et al. 2017), before conducting the gene ontology (GO) and the Kyoto encyclopaedia of genes and genomes (KEGG) term annotation using *InterProScan-v5.25-64.0* (Quevillon et al. 2005), assuming evolutionary conservatism of gene functions across species. For this within tissue, between age classes comparison, we furthermore manually annotated differentially expressed genes (FDR-p < 0.05), with Log2FoldChange > 2 (at least four times more expressed in one age class relative to the other), using the *Uniprot* database (*www.uniprot.ong*), with *Drosophila* annotations when available, or other organism if not (*Drosophila*: 35.2 %; other insects: 4.2 %; other non-insects: 60.6%).

In order to check whether contigs showing the same age-specific expression pattern in the two tissue types (upregulated contigs in a given age class, both in brain and in fat body) were housekeeping genes, we made use of *Busco v.3* (Simão et al. 2015) using the conserved insect database as backbone.

To identify gene networks, a weighted gene co-expression network analysis (WGCNA) was performed using the R package *WGCNA* (Langfelder et al. 2008) on the top 15,000 contigs with the highest variance in expression across samples. For these analyses we used the following parameters soft thresholding power of 5 for brain and 10 for fat body data (following the manual instructions), with the minimal number of contigs per cluster set to 220 and a dissimilarity threshold of 0.2. No obvious outliers were detected from the clustering analysis of both tissues, so that all samples remained in the analysis.

In order to investigate which biological processes were differentially activated in the two different tissues of each age group, we performed a GO term enrichment analyses based on the subsets of differentially expressed contigs using the R package *TopGo -v-3.6* (Alexa and Rahnenfuhrer 2016), with the "weight01" algorithm. This was done separately for contigs upregulated in the brain and fat body of old and young queens. A GO enrichment was also performed on the co-expression modules. The enrichment analysis revealed the over-representation of functional categories in our test group (the differentially expressed genes in young/old queens and fat body/brain) in comparison to the reference set (all contigs in the transcriptome). The p-values for each GO term were obtained by using a *Fishers exact* test. Additionally, we conducted a functional enrichment based on the list of unique isoforms (with duplicates filtered out based on the gene identity), which revealed qualitatively similar results compared the one performed on the entire list of differentially expressed contigs.

Results

Fertility

Queens of the two age classes strongly differed in fecundity (Figure 4.2 a, b). Old queens had longer ovarioles (F_1 = 46.23, P < 0.001), and about five times more white eggs in their ovaries (X^2_1 = 74.7, P < 0.001) compared to young queens.

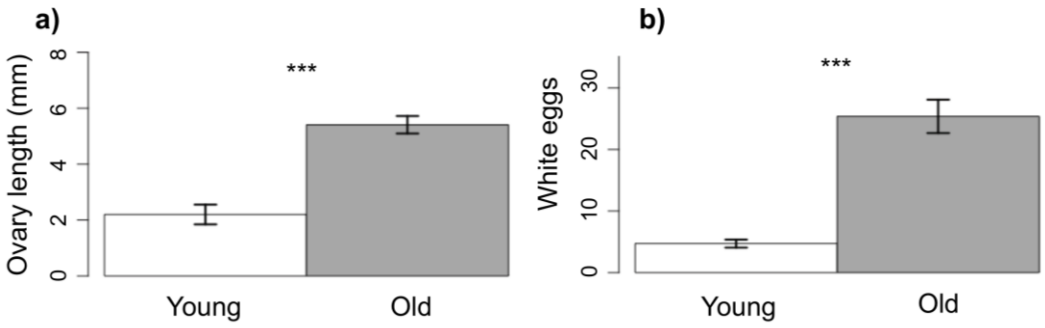

Figure 4.2: Young queens are less fecund than older queens. Differences in ovary development include (a) length of ovarioles and (b) the number of eggs in development. Old queens have significantly longer ovaries and a higher number of eggs than young ones (respectively: F_1 = 46.23, P < 0.001; X^2 = 74.7, P < 0.001).

Transcriptome analyses

The *Trinity* assembly resulted in 128,764 number contigs, of which 39% were annotated with *BlastX* against the non-redundant insect database. The hierarchical sample clustering analysis revealed that gene expression differed more strongly between tissue types than between age classes. However, old queens differed more in their gene expression than young queens (PERMANOVA, 999 permutations; t = 2.37, P = 0.018). Clustering by age classes were more pronounced in the fat body than in the brain (Figure 4.3). Moreover, the between-sample overall dispersion in gene expression was higher in the fat body compared to the brain (PERMANOVA, 999 permutations; t = 3.38, P = 0.012).

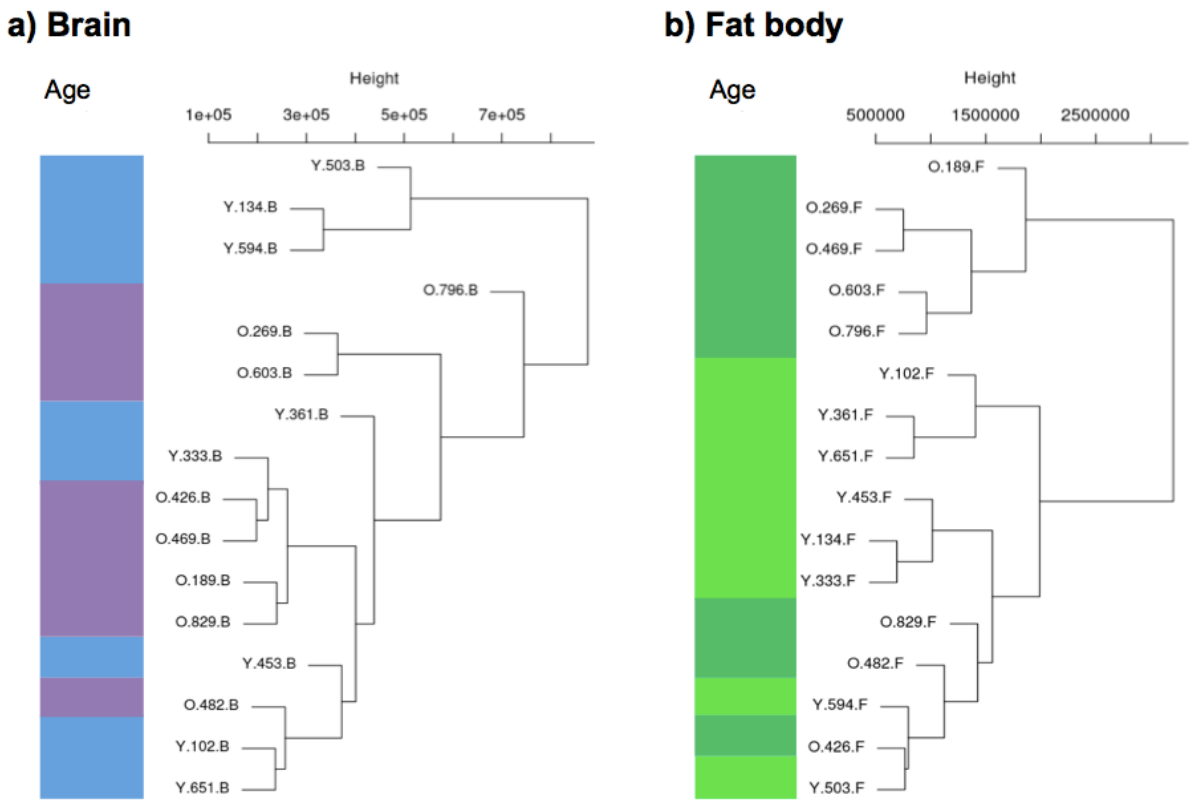

Figure 4.3: Dendrograms depicting sample similarity based on read counts per contig (a) for the brain and (b) for the fat body. The colors summarize the clustering per age with blue or light green young queens and purple or dark green the old queens respectively.

i) Differential gene expression

Differential gene expression analysis per tissue and between age classes (Figure 4.4a) revealed many more differentially expressed genes between old and young queens in the fat body compared to the brain (Pearson's X^2 test: $X^2 = 1154.6$, df = 1, $P < 0.001$; Figure 4.4a), although the absolute LogFoldChanges were higher in the brain (Figure 4.4b). In total, 1,597 contigs were differentially expressed in the fat body between the two age classes, compared to only 169 between the brain of young and old queens (FDR-p < 0.05). Only few (N= 11) upregulated contigs were shared across tissues (Figure 4.4a), with one of them *40S ribosomal protein S2*, being involved in oogenesis in *D. melanogaster* and consistently upregulated in old queen fertile queens both for brain and fat body. Among these 11 overlapping contigs we found that two of them matched the conserved insect

database (*Busco*) and might thus represent housekeeping genes. In both tissues, old queens upregulated many more genes than young ones (Pearson's X^2 test; brain: $X^2 = 55.7$, df = 1, $P < 0.001$; Pearson's X^2 test; fat body: $X^2 = 173.9$, df = 1, $P < 0.001$), and those genes also had a significantly higher change in expression (linear-mixed model: X2= 509.85, df= 1, p-value < 0.001; Figure 4.4b, read count per sample available online at NCBI's Short Read Archive (SRA) under study accession N° GSE111415, see data accessibility section).

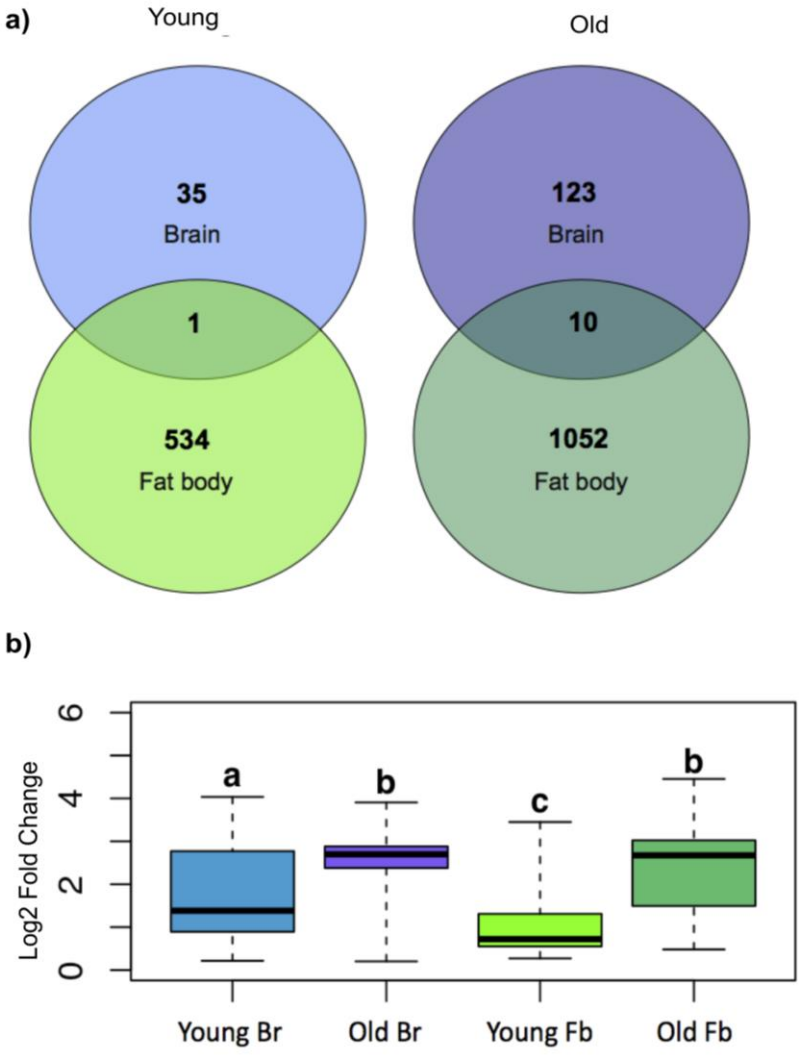

Figure 4.4: Summary of the within tissue differential gene expression analysis between old and young queens: (a) Venn diagrams depict the number of upregulated genes per tissue, between age classes with overlaps for contigs shared across tissues; (b) Relative expression level (Log2 Fold Change) of upregulated genes per tissue and age class. Upregulated contigs in young queen brains compared to old ones are in light blue; up-regulated contigs in old queen brains compared to young ones in purple; up regulated contigs in young queen fat bodies compared to old ones in light green; up regulated in old queen fat bodies compared to young ones in dark green. Test of the effect of tissue in interaction with age class reveled a significant effect of both tissue, age class and interaction (respectively: X^2=9.77, df=1, P < 0.002; X^2=509.85, df=1, P < 0.001; X2=9.77, df=1, P = 0.008), and the results from the post-hoc pair wise comparison are summarized with letters (at the threshold of 0.05 after Bonferroni correction).

We identified interesting candidates among the differentially expressed genes involved in longevity or fecundity regulation, or associated with the social environment (Table 4.1). Indeed, we detected six contigs upregulated in old queens annotated to genes involved in longevity pathways of humans and *Drosophila*, and positively associated with lifespan (see KEGG database, *Longevity regulating pathway*). In old queens, *Superoxide dismutase*, and *heat shock 70 kDa protein cognate 4* showed a higher expression in both tissues, while *catalase* and *heat shock 70 kDa protein IV* were specifically upregulated in the fat body. These genes play a major role in the regulation of oxidative stress and protection against reactive oxygen species (*ROS*) (Orr et al. 1994, Sun et al. 1999, Sørensen et al. 2002 Sampayo et al. 2003). Surprisingly, only few contigs associated with fertility were detected among the age-specific genes expressed in both tissues (2% in the fat body versus 3% in the brain, LogFoldChange > 2, FDR-p < 0.05). No obvious candidates were discovered in the pathway analysis.

Table 4.1. Candidate genes up-regulated per tissue and age class comparing young and old queens. We list only the genes likely involved in the regulation of longevity and/or fecundity or associated with the social environment. Shown are the blast annotation with corresponding species, the logFoldChange (LFC) from the comparison between old and young within tissue, the corresponding corrected P-value, the functional annotation made on UniProt as well as the corresponding species (*D. melanogaster* for *Drosophila melanogaster*)

		Blast				Uniprot	
Age	**Tissu**	**Annotation**	**Species**	**LFC**	**P-val**	**Function**	**Species**
Young queens	**Fat body**	collagen alpha-1(IV) chain	*Trachymyrmex septentrionalis*	2.37	0.011	Oviduct morphogenesis	*D. melanogaster*
		alkylated DNA repair protein alkB	*Solenopsis invicta*	0.48	0.003	DNA repair	*Homo sapiens*
		mitotic spindle assembly checkpoint protein MAD1	*Wasmannia auropunctata*	0.47	0.023	Sister chromatin cohesion	*Homo sapiens*
Old queens	**Fat body**	catalase isoform X2	*Metaseiulus occidentalis*	4.62	0.001	Determination of adult lifespan	*D. melanogaster*
		heat shock 70 kDa protein IV	*Strongylocentrotus purpuratus*	4.21	0.004	Negative regulation of apoptotic process	*Homo sapiens*
		heat shock 70 kDa protein cognate 4-like	*Polistes dominula*	4.14	<0.001	Cellular response to topologically incorrect protein	*D. melanogaster*
		heat shock 70 kDa protein 4-like	*Parasteatoda tepidariorum*	3.68	0.017	Negative regulation of apoptosis	*Homo sapiens*
		superoxide dismutase Cu-Zn	*Athalia rosae*	3.60	0.022	Age-dependent response to oxidative stress	*D. melanogaster*
		catalase-like	*Trichogramma pretiosum*	3.49	0.028	Determination of adult lifespan	*D. melanogaster*
		40S ribosomal protein S2	*Copidosoma floridanum*	3.36	0.035	Oogenesis	*D. melanogaster*
		heat shock 70 kDa protein cognate 4	*Trachymyrmex cornetzi*	3.13	0.005	Cellular response to topologically incorrect protein	*D. melanogaster*
		translationally-controlled tumor protein	*Apis cerana*	2.71	0.029	DNA repair	*D. melanogaster*
		ataxin-2 homolog	*Solenopsis invicta*	2.81	0.038	Oocyte differentiation	*D. melanogaster*
	Brain	superoxide dismutase Cu-Zn-like	*Rhagoletis zephyria*	2.68	0.04	Age-dependent response to oxidative stress	*D. melanogaster*
		C. briggsae CBR-SODH-1 protein	*Caenorhabditis briggsae*	3.32	<0.001	Defence response to Gram-positive bacterium	*Caenorhabditis briggsae*
		heat shock 70 kDa protein cognate 4	*Linepithema humile*	3.09	<0.001	Cellular response to topologically incorrect protein	*D. melanogaster*
		40S ribosomal protein S2	*Copidosoma floridanum*	3.21	0.003	Oogenesis	*D. melanogaster*

ii) Functional enrichment

In the list of enriched functions in upregulated genes (Table 4.2) in young queens' fat bodies, we found the *TOLL pathway*, which is known to play a major role in insect immunity (Valanne et al. 2011). Also the *negative regulation of the TOR pathway*, a universal nutrient sensitive pathway, could be explained by food deprivation of young queens with no or only few workers during the founding phase. Interestingly, *tryptophan catabolism into kynurenine*, which is negatively associated with lifespan in *Drosophila*, was also enriched in the fat bodies of young queens (Frick et al. 2004). Finally, mechanisms ensuring DNA integrity seem to be activated in young queens' fat bodies, such as *mitotic cell division checkpoint* or *sister chromatin cohesion*.

In old queen's fat bodies (Table 4.2) we found functions associated with translation, but without any transcription or RNA degradation in contrast to the young queens, which may indicate a less intense protein turnover and a less dynamic protein composition. Interestingly, our results suggest an activation of mechanisms involved in regulation of oxidative damage such as *regulation of response to ROS* or *response to oxidative stress*. The upregulation of genes involved in the *S-adenosylmethionine biosynthetic process* was surprising as an increase of this molecule is linked to aging in *Drosophila melanogaster* (Obata et al. 2015). Finally, the enrichment of *acetyl-CoA metabolism* in both tissues is also relevant as acetyl-CoA plays a role in the epigenetic regulation of stress response genes through histone acetylation (Eisenberg et al. 2014).

Table 4.2: Results from the functional enrichment of upregulated contigs per age class for brain and fat body. Significantly enriched functions are indicated in bold for the most relevant ones. The indicated p-value was calculated with a Fisher exact test.

Age	Tissue	GO ID	Biological Process	P-value
Young Queens	Fat Body	GO:0046416	D-amino acid metabolic process	**< 0.001**
		GO:0006367	**transcription initiation from RNA pol. II promoter**	**0.002**
		GO:0000398	**mRNA splicing, via spliceosome**	**0.004**
		GO:0043504	**mitochondrial DNA repair**	0.007
		GO:0006351	**transcription, DNA-templated**	**0.011**
		GO:0006499	**N-terminal protein myristoylation**	**0.013**
		GO:0006482	**protein demethylation**	**0.013**
		GO:0046294	formaldehyde catabolic process	0.013
		GO:0000387	spliceosomal snRNP assembly	0.017
		GO:0006402	**mRNA catabolic process**	**0.020**
		GO:0008063	**Toll signaling pathway**	**0.020**
		GO:0048011	neurotrophin TRK receptor signaling pathway	0.020
		GO:0006278	RNA-dependent DNA biosynthetic process	0.020
		GO:0030163	**protein catabolic process**	**0.028**
		GO:0051603	**proteolysis involved in cellular proteins**	**0.032**
		GO:0006479	**protein methylation**	**0.032**
		GO:0006401	**RNA catabolic process**	**0.033**
		GO:0019441	**tryptophan catabolic process to kynurenine**	**0.033**
		GO:0007064	**mitotic sister chromatid cohesion**	**0.033**
		GO:0035176	**social behavior**	**0.033**
		GO:0006412	**translation**	**0.033**
		GO:0016567	**protein ubiquitination**	**0.037**
		GO:0032007	**negative regulation of TOR signalling**	**0.039**
		GO:0007094	**mitotic spindle assembly checkpoint**	**0.039**
		GO:0043066	**negative regulation of apoptotic process**	**0.046**
		GO:0000122	**negative regulation of transcription from RNA pol. II promotor**	**0.046**
	Brain	GO:0055114	oxidation-reduction process	0.009
Old Queens	Fat body	**GO:0006412**	**translation**	**< 0.001**
		GO:0015986	ATP synthesis coupled proton transport	0.003
		GO:0006556	**S-adenosylmethionine biosynthetic process**	**0.003**
		GO:0006979	**response to oxidative stress**	**0.006**
		GO:0009082	branched-chain amino acid biosynthetic process	0.007
		GO:0045901	**positive regulation of translational elongation**	**0.007**
		GO:0045905	**positive regulation of translational termination**	**0.007**
		GO:0006452	**translational frame-shifting**	**0.007**
		GO:0006754	ATP biosynthetic process	0.010
		GO:0006084	**acetyl-CoA metabolic process**	**0.016**
		GO:0019878	lysine biosynthetic process via aminoadipic acid	0.021
		GO:0006685	sphingomyelin catabolic process	0.021
		GO:0006414	**translational elongation**	**0.031**
		GO:0006099	tricarboxylic acid cycle	0.036
		GO:0003333	amino acid transmembrane transport	0.038
		GO:1901031	**regulation of response to reactive oxygen species**	**0.041**
		GO:0006075	(1->3)-beta-D-glucan biosynthetic process	0.041
	Brain	GO:0006412	translation	**< 0.001**
		GO:0006596	polyamine biosynthetic process	0.003
		GO:0006075	(1->3)-beta-D-glucan biosynthetic process	0.008
		GO:0046168	glycerol-3-phosphate catabolic process	0.026
		GO:0006084	acetyl-CoA metabolic process	0.038
		GO:0006096	glycolytic process	0.042

iii) Weighted gene co-expression network analysis

Co-expression network analysis (WGCNA) for the fat body revealed that two, of the 15 modules were significantly positively associated with young age and none with old age. Of the 11 co-expression modules of the brain data, one was positively associated with young age and none with old age. Among modules positively associated with young age, we found a significant overrepresentation of genes with functions in *regulation of cell growth,* and *lipid catabolic process* in the fat body.

Discussion

With their extreme lifespan and seemingly circumventing the widespread trade-off between longevity and fecundity, social insect queens provide the unique opportunity to investigate the molecular basis of senescence. We studied tissue-specific changes in gene expression with age in queens of the ant *T. rugatulus.* Old and young queens strongly differed in ovary length, which was weakly reflected by a differential expression of fecundity-associated genes in the two tissues investigated. We show age-related changes in the expression of longevity and immunity genes and pathways, both in the brain and in the fat body (Figure 4.4a). Moreover, we provide evidence for regulatory changes of candidate genes and pathways compared to *Drosophila* and other solitary organisms, making *T. rugatulus* a good candidate to investigate the atypical positive association between lifespan and reproduction in social insects (Table 4.3).

Table 4.3: List of candidate genes and pathways associated with longevity and potentially involved in the reshaping of the trade-off between lifespan and reproduction in social insects. Additional information comprises the according biological process, trait association in other organisms with reference as well as information on the respective expression pattern in the solitary species in comparison to *T. rugatulus*.

Candidate gene	**Biological process**	**Trait association in solitary organisms**	**Age-related expression pattern in solitary organism**	**Age-related expression pattern in *T. rugatulus* queen**
catalase isoform X2 / *catalase-like*	Reduction of oxidative stress (longevity pathway, mammals and *Drosophila*)	Positively associated with longevity in *Drosophila* (Orr et al. 1994)	Decreasing in the rat brain and in *Drosophila* (Semsei et al. 1991, Klichko et al. 2004)	Increasing in the fat body
superoxide dismutase (Cu-Zn)		Positively associated with longevity in *Drosophila* (Orr et al. 1994)	Decreasing in the rat brain (Klichko et al. 2004)	Increasing in the fat body and in the brain
SAM synthase-like / *SAM synthase isoform X5* / *SAM synthase isoform X2* / *SAM synthase isoform X1* / *SAM synthase isoform X3*	SAM biosynthesis (SAM metabolism)	Negatively associated with longevity in *C. elegans* (Ching et al. 2010) (SAM degradation extends lifespan in *Drosophila* (Obata et al. 2015))	NA	Increasing in the fat body
kynurenine/alpha-aminoadipate aminotransferase, mitochondrial-like	Tryptophan catabolism into kynurenine (kynurenine pathway)	Positively associated with aging (Frick et al. 2004, Capuron et al. 2014, Belkacem et al. 2017) and negatively associated with longevity in *Drosophila* (Navrotskaya et al. 2016)	Increasing in human serum (Frick et al. 2004)	Decreasing in the fat body

By an order of magnitude more genes changed their expression with age in the fat body compared to the brain. Moreover, typical longevity genes such as heat shock proteins *70 kDa* (*hsp70*), *superoxide dismutase* (*SOD*) or *catalase* (*CAT*) (Flatt et al. 2004, Tower 2011) were found among the differentially expressed genes in the fat body in higher number compared to the brain,

suggesting that aging or the fight against senescence rather takes place in the fat body (Table1). Furthermore, *T. rugatulus* may employ additional genes for longevity regulation, compared to the known candidates from model species (Table 4.1), plus additional candidates might be hidden among the non-annotated differentially expressed contigs. Detailed functional analyses to elucidate their specific function will be necessary in the future.

Only few genes (N = 11) showed the same age-specific expression shifts in the two tissue types, indicating little across-tissue consistency. Among these genes, two may represent housekeeping genes, and none of the eleven genes were linked to longevity. In old, fertile queens, *40S ribosomal protein S2*, being involved in oogenesis in *D. melanogaster*, was consistently upregulated in both tissues, making it a good fertility candidate gene in our species. The general picture indicates a low consistency in expression pattern across tissues, which is in agreement with a recent study on the ant *Lasius niger* (Lucas et al. 2017 b), stressing the necessity of conducting tissue-specific expression analyses.

The relative expression level of upregulated genes in old queens was at least twice higher in both tissues compared to upregulated genes in young queens. As gene expression by itself is energy consuming (Lane et al. 2010), this finding might be related to established queens having a larger work force, and thus more resources available than founding queens, and may thus be able to invest more in elevated gene expression levels. In line with this hypothesis the co-expression network analysis reveals that young founding queens have a more active lipid catabolism as they utilize more body reserves than the old queens.

The overall variance in expression was higher in older queens compared to young ones, which may be explained by a higher across-sample variance in chronological age among established queens compared to rather similar aged founding queens. On the other hand, we found a much stronger change in gene expression related to worker number in young, compared to old queens. Taken together these results might be due to a stronger effect of worker number on social

environment in young queens with few workers (N workers = 0-8), compared old ones with relatively large established colonies (N workers = 197-337).

Fecundity and immunity signatures

Despite significant differences in ovary development, relatively few genes related to fertility were differentially expressed between the highly fecund old queens and the young ones. Indeed, more than five times as many eggs were in development in the ovaries of old queens compared to young ones. Possibly the fat body is the wrong tissue to look for differential expression of fertility genes, which might be more likely to differ in their expression in the ovaries (Cong et al. 2015). Another not mutually exclusive hypothesis is that young queens have already activated fertility genes, which are not yet reflected in their ovary development. Consistent with our observations, *Cardiocondyla obscurior* ant queens have been shown to increase their fecundity with age independently of the number of workers in the nest (Heinze et al. 2012).

In the fat body, young queens activate the TOLL signalling pathway, which plays an important role in insect immunity (Valanne et al. 2011). This fits to our prediction, and might reflect a higher pathogen exposure during the mating flight and the colony-founding phase. Older queens live in a highly protected environment where pathogen pressure is very low and social immunity strategies of workers shield queens from parasites (Corona et al. 2007). An example of plastic regulation of the immune system according to age and environmental conditions (colony age) has been evidenced in bumblebee workers (Moret and Schmid-Hempel 2009). Immunocompetence is costly and may be traded-off with fecundity and longevity (DeVeale et al. 2004, Badinloo et al. 2018, Adamo et al. 2001). Old queens might thus be able to avoid the costs of upregulating immune genes by delegating the immune defence to workers (Stroeymeyt et al. 2018), and potentially invest these resources into other functions such as body maintenance (e.g. antioxidant production) and in egg production (Buchanan et al. 2018).

Candidate genes and altered regulation in T. rugatulus

We detected a number of candidate genes potentially involved in the reversal trade-off between fertility and longevity in social insect queens (Table 4.3). In both tissues, old queens upregulated multiple genes involved in longevity pathways in *Drosophila* and mammals. These candidates, such as *hsp70*, *SOD* and *CAT*, play an important role in the reduction of oxidative stress-related molecular damage (Flatt et al. 2004, Tower 2011). In *Drosophila* the decrease in stress resistance with age is considered a consequence of an age-related reduction of *hsp70* expression (Sørensen et al. 2002, Doroszuk et al. 2012). SOD and CAT are known as powerful antioxidants and in *Drosophila* and *C. elegans* the overexpression of CAT reduces oxidative stress and extends lifespan (Sampayo et al. 2003, Badinloo et al. 2018). Moreover, the expression and the activity of *SOD* and *CAT* is known to decline naturally with age in solitary species including *Drosophila* (Orr et al. 1994, Semsei et al. 1991, Klichko et al. 2004), but also in the brain and abdomen of honeybee queens (Corona et al. 2005). In *T. rugatulus* queens we observe the opposite pattern with stronger expression in old queens. This is similar to termites, where *CAT* is up-regulated in queens compared to workers, which correlates with a lower rate of protein oxidation and may thus be involved in the lifespan differences between the two castes (Tasaki et al. 2017). This finding is corroborated by the enrichment of *stress response to reactive oxygen species* as well as *regulation of response to oxidative stress* mechanisms in fat bodies of old established queens, indicating that established queens activate mechanisms dealing with oxidative stress-related damage commonly associated with aging, suggesting a higher investment into body maintenance (Finkel et al. 2000, Tasaki et al. 2017, Doroszuk et al. 2012). However, a higher investment in body maintenance through an overproduction of antioxidants may not be the only way for living long in every social insects, but maybe species-specific. For example, honeybee workers upregulate *CAT* and *SOD* compared to queens (Corona et al. 2005) but live shorter. Alternatively investing in molecular repair may also contribute to queen longevity as in the ant *Lasius niger* queens upregulate somatic repair genes rather than antioxidant, compared to workers (Lucas et al. 2016). Currently, we cannot distinguish whether the higher

expression of anti-oxidants in older, established queens is due to a higher age-related generation of ROS or whether it reflects a preventive mechanism independent of ROS level. Oxidative stress measurements of individuals with and without stress (e.g. using paraquat), would allow to identify the cause and effect of the observed age-related increase in stress response genes in *T. rugatulus* queens. While our results indicate a higher investment into anti-aging mechanisms in old queens through oxidative stress reduction, the enrichment of the *SAM biosynthetic process* in the fat body of older queens suggests that not all longevity pathways are upregulated to prevent aging in ant queens. In *Drosophila*, the best understood insect aging model, S-adenosylmethonine (SAM), the first metabolite of methionine, increases under a methionine-rich diet which negatively affects lifespan, but positively affects reproduction (Obata et al. 2015, Lee et al. 2014). Moreover, the total amount of SAM in the fat body is associated with aging (Obata et al. 2015) while blocking SAM biosynthesis or enhancing SAM degradation extends lifespan, in *Caenorhabditis elegans* (Ching et al. 2010) and *Drosophila;* (Obata et al. 2015), respectively. In *T. rugatulus* the high production of SAM could facilitate egg production, whereas the life-shortening effects might be overcome by other somatic maintenance mechanisms, for example through the production of anti-oxidants. This hypothesis could be experimentally tested by sequentially knocking down downstream genes of SAM synthesis, and studying longevity and fecundity and associated gene expression including *CAT* and *SOD*.

Tryptophan (TRY) metabolism and kynurenine (KYN) pathway of TRY degradation are powerful regulators of age-related disorders and lifespan in many organisms (54,62). In humans and rats an increasing ratio of KYN/TRY is associated with aging and is shown to be a strong predictor of age-related diseases and mortality (Table 4.3; (Frick et al. 2004, Capuron et al. 2014, Belkacem et al. 2017). In *Drosophila*, inhibition of TRY catabolism into KYN extends lifespan, and elevation of *kynurenic acide*, an immediate metabolite of KYN, induces aging (Navrotskaya et al. 2016). The function *tryptophan catabolism into kynurenine* was enriched in young queens. This is in agreement with our predictions of a lower investment into somatic maintenance in young queens that have a

higher extrinsic mortality and other investment priorities. Moreover, those results point again to an opposite age-related expression pattern compared to other solitary organisms that could underlie modification in the KYN and related pathways, specific to our species, and potentially involved in the remodelling of the trade-off between lifespan and reproduction in social insect (Table 4.3). Alternatively, this finding could also reflect a higher stress level of founding queens leading to the activation of immune defences (Capuron et al. 2014). Moreover, the existence of other mechanisms involved in the extreme longevity of queens could escape our analysis if their activity remains stable over queen lifetime. Investigating how workers and queens differ in gene expression and how they change the expression of longevity genes with age could allow to identify these mechanisms.

Conclusions

Gene expression changes in response to age were much more pronounced in the fat body compared to the brain. Among these, we were able to identify a number of longevity candidates, but only few fecundity associated genes and pathways despite strong differences in fecundity between the two queen types. Young queens rather invest in immunity, starvation resistance, and prevention of mitotic replication mistakes despite a physiological rearrangement associated with the founding phase. In contrast, highly fertile, established queens that have a reduced extrinsic mortality rate and the support of many workers, seem to invest more in somatic maintenance mechanisms through the overproduction of antioxidants. This observation suggests a plastic regulation of somatic maintenance in ant queens. Finally, we identify two candidate genes (*CAT* and *SOD*), and one candidate pathway (TRY/KYN pathway), which are potentially involved in the reversed trade-off between lifespan and fecundity in *T. rugatulus* queens (Table 4.3).

CHAPTER 5

Intrinsic worker mortality depends on behavioral caste and the queens' presence in a social insect

Philip Kohlmeier *, Matteo Negroni *, Marion Kever, Stefanie Emmling, Heike Stypa, Barbara Feldmeyer, Susanne Foitzik

* Authors contributed equally to the study.

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Abstract

According to the classic life history theory, selection for longevity depends on age-dependant extrinsic mortality and fecundity. In social insects, the common life history trade-off between fecundity and longevity appears to be reversed, as the most fecund individual, the queen, often exceeds workers in lifespan several fold. But does fecundity directly affect intrinsic mortality also in social insect workers? And what is the effect of task on worker mortality? Here, we studied how social environment and behavioral caste affect intrinsic mortality of ant workers. We compared worker survival between queenless and queenright *Temnothorax longispinosus*nests and demonstrate that workers survive longer under the queens' absence. *Temnothorax*ant workers fight over reproduction when the queen is absent and dominant workers lay eggs. Worker fertility might therefore increase lifespan, possibly due to a positive physiological link between fecundity and longevity, or better care for fertile workers. In social insects, division of labor among workers is age-dependant with young workers caring for the brood and old ones going out to forage. We therefore expected nurses to survive longer than foragers, which is what we found. Surprisingly, inactive inside workers showed a lower survival than nurses but comparable to that of foragers. The reduced longevity of inactive workers could be due to them being older than the nurses, or due to a positive effect of activity on lifespan. Overall, our study points to behavioral caste-dependent intrinsic mortality rates and a positive association between fertility and longevity not only in queens but also in ant workers.

Introduction

Although in nature, organisms mostly die due to external factors such as diseases, accidents, or predation (Wachter and Finch 1997), those individuals that escape this extrinsic mortality ultimately face death through the intrinsic process of senescence. Hence, mortality has been attributed either to intrinsic or extrinsic causes (Sparkman et al. 2007, Shattuck and Williams 2010). Senescence, a decline in fitness with age, is an evolutionary paradox, which can be explained by an increase in extrinsic mortality and a decrease in selection with age (Medawar 1952, Williams 1957, reviewed by Kirkwood and Rose 1991). In contrast, metabolic theories consider aging to be a physiological process that largely evolved independently from ecological factors, pointing to an association of the metabolic rate with a progressive intrinsic deterioration of the body (Sohal 1986, Finch 1990).

Evolutionary theories of aging explain the tremendous variation in lifespan between queens and workers in social insects (Keller and Genoud 1997, Kramer and Schaible 2013, Kramer et al. 2015). The high mortality early in life during mating and colonization of new nest sites and later the low extrinsic mortality risk for adult queens due to the safety of the nest plus intensive worker care are thought to have led to the evolution of the extraordinary life spans of ant and termite queens (Keller and Genoud 1997, Keller 1998, reviewed by Negroni et al. 2016). Differences in extrinsic mortality also occur among workers of different castes and may vary depending on their tasks and the environment associated with it (Solis and Strassmann 1990). For instance, exposure to external risks such as desiccation and predation is more pronounced in foragers than in nurses that remain in the nest (Schmid-Hempel and Schmid-Hempel 1984, O'Donnell and Jeanne 1992). This contributed to the evolution of age-dependent division of labor, whereby young workers mostly perform intra-nest tasks, whereas old workers take over the risky tasks outside the nest, such as foraging (Jeanne 1986, Bourke and Franks 1995, Wakano et al. 1998, Woyciechowski and Moroń 2009). Alternatively, evolution could also have lead to caste-specific modulation of intrinsic mortality associated with variation in risk exposure (Rueppell et al. 2007). This prediction has rarely

been tested (Chapuisat and Keller 2002), and a recent review found little evidence for demographic senescence in worker ants (Giraldo and Traniello 2014). However, differences in risk exposure connected to division of labor allow testing evolutionary theories of aging in social insects (Calabi and Porter 1989, Chapuisat and Keller 2002, Graeff et al. 2007).

Activity levels of workers strongly vary although they share the same social environment (Jaisson et al. 1988). While some workers are proverbially busy, direct observations reveal that a large proportion of workers are inactive and that this behavior is consistent over time and across individuals (Charbonneau and Dornhaus 2015, Charbonneau et al. 2015). The adaptive role of these inactives remains poorly understood, though a number of hypotheses have been proposed (Charbonneau and Dornhaus 2015). Some consider inactive workers as reserves for sudden demands of additional workforce, or as food storage, as proposed but not tested by Charbonneau and Dornhaus (2015). Inactivity may also constitute a selfish behavior to save energy for reproduction (Jandt and Dornhaus 2011). Finally, activity levels might be related to age. Immature workers are young and inexperienced and thus inactive, or alternatively senescing workers might become inactive towards the end of their life (Corbara et al. 1989, Klein et al. 2008, Fresneau 1984, Johnson 2008, Retana and Cerdá 1990). At any rate, independent of age, metabolic rates of inactive workers might be lower than that of active ones, potentially lengthening their lifespan compared to active workers (Trout and Kaplan 1970, Lighton et al. 1987, Sohal 1986, Schmid-Hempel and Wolf 1988, Wolf and Schmid-Hempel 1989, Calabi and Porter 1989).

In most animal taxa, investment in reproduction is negatively associated with lifespan (Roff 1992, Stearns 1992, Holliday 2006). In social insects, however, this common life history trade-off is reversed with the queen being both, long-lived and highly fecund, whereas the infertile workers live significantly shorter (Schrempf et al. 2005, Heinze and Schrempf 2008). Sterility in workers can be explained by colony-level fitness costs of worker reproduction (see Cole 1986, Bourke 1988). However, although most social Hymenopteran workers cannot mate, they are able to lay unfertilized, male-destined eggs in the absence of the queen, which otherwise inhibits their reproduction

(Hölldobler and Wilson 1990). Fertile workers in queenless nests stay inside the nest and take over the queens' status (Cole 1986, Van Doorn and Heringa 1986, D'Ettorre et al. 2004, Harrison et al. 2015). As the comparison between queens and workers suggests that fecundity has a positive rather than a negative impact on longevity in social insects, it raises the question whether this positive association between fecundity and longevity is queen-specific or whether fecundity also affects worker longevity. Indeed, in ant queens, high fecundity is often associated with old age (Heinze and Schrempf 2012) and in the clonal ant *Platythyrea punctata* workers that become dominant and take over reproduction live longer than their subordinate sisters (Hartmann and Heinze 2003). However, whether workers in species without totipotent workers live longer irrespective of extrinsic factors when being reproductively active, remains unclear.

In social insects, the extreme divergent life histories of queens and workers and the resulting reproductive division of labor are not genetically determined (Keller 1998, Libbrecht et al. 2013), but are the result of phenotypic plastic responses to variation in larval nutrition mediated via changes in gene expression (Wheeler 1986 , Wurm et al. 2010, Colgan et al. 2011, Ferreira et al.2013, Feldmeyer et al. 2014). Social insects are ideal models to investigate how ecological, social, and/or physiological factors interact and affect intrinsic mortality (Jemielity et al. 2005, Chapuisat and Keller 2002). However, as collection of intrinsic mortality data, even for workers, requires work-intense long-term observations, variation in lifespan related to division of labor has rarely been studied (Chapuisat and Keller 2002). In this long-term study, we focus on the ant *Temnothorax longispinosus*, a species in which workers are monomorphic though organized in behavioral castes and have a low reproductive potential, compared to the queen. Nevertheless, in the queens' absence, *Temnothorax* workers compete over dominance and dominant workers lay male-destined eggs (Alloway et al. 1982, Choe 1988, Konrad et al. 2012). Here, we question how the presence of the queen influences worker longevity. Moreover, we test whether worker mortality varies between workers that take over different roles. We investigate intrinsic mortality of workers (i) from queenless and queenright colonies and (ii) those focussing on different tasks including inactive workers. In

particular, we expect the older foragers to survive less well than nurses, even independent of external sources of mortality.

Materials and Methods

For our experiments, we used 29 monogynous *T. longispinosus* colonies (mean 47.7 ± S.E. 2.9 workers per colony) collected in summer 2013 at the E.N. Huyck Preserve in Rensselearville, New York, USA. In our laboratories, ant colonies were transferred to glass slide nests within plastered, three-chambered nest boxes, maintained at 20 °C and a 12 h dark/12 h light cycle and fed twice a week with honey, crickets, and water. In January 2014, after 4 months of hibernation at 5 °C, temperature was step-wisely increased to 25 °C (12 °C for 1 week and then increased 4 °C per week) and kept for 1 month. During this time, the experiments were set-up.

In each nest, all workers were categorized according to their behavior: (i) foragers, workers found outside the nest; (ii) nurses, workers interacting with the brood when the nest was opened; and (iii) workers found inside of the nest, but remaining inactive upon disturbance. All workers were then labeled with a thin metal wire (0.02 mm, Elektrisola). Workers of the same caste and colony were marked with a wire of the same color, and wire colors were randomized over colonies. We categorized workers into different castes based on a single observation and their spatial position in the nest. We did so because behavioral castes occupy different locations within an ant colony. For example, a strong link between spatial location and worker behavior was shown for *Myrmica ruginodis* (Pamminger et al. 2014). To confirm this association also for our focal species, we observed the behavior of individually labeled foragers and brood carers in 16 *T. longispinosus* colonies. The following 3 days, these workers were scanned 10 times a day by recording their contribution to brood care or foraging. Workers previously classified as foragers spent more time outside than previously classified brood carers (GLMM*: $\chi^2_1 = 4.5$, $P < 0.03$), and brood carers exhibited more nursing behavior than foragers* (GLMM: $\chi^2_1 = 13.3$, $P < 0.0002$).

These data confirm that categorization of workers into different behavioral castes reflects division of labor in the nest.

Colonies were equally split, ensuring that each colony fragment received the same number of workers of each behavioral caste, eggs, larvae, and pupae. One of the two nest fragments received the queen, resulting in a queenright and a queenless subcolony. After 1 month at 25 °C to initiate ovary development in workers, all colonies were maintained at 20 °C and 12 h dark/12 h light. Colonies were inspected weekly for 200 days and the number of workers of each behavioral caste still alive was recorded. Note that this species has a synchronized brood production and larval development takes about 1 year. Hence, workers were at least 6 months old at the time when they were marked. Workers of *Temnothorax* species from temperate climates have a mean life expectancy of 2–3 years and a maximum of 7 years (Plateaux 1986) and brood carers are about 1 year old when they are replaced by the new worker generation and start contributing more to foraging duties (Kohlmeier et al. 2018). Moreover, the brood was equally split at the onset of the experiment, so that similar numbers of new workers emerged in each nest part in summer 2014 (see the "Results" section). We stopped our analyses before hibernation, as *Temnothorax* ants form tight clusters and would have to be strongly disturbed to accurately determine individual worker survival during winter. However at the end of the observation period, colonies were prepared for hibernation by step-wisely reducing the temperature until +5 °C. *Temnothorax* queens and workers cease laying eggs in preparation for hibernation (Foitzik and Heinze 1998). Therefore, and because the potential impact of fertility on survival could bias our analyses, we did not compare worker fertility between the two treatments. However, an earlier study on the same *T. longispinosus* population showed that workers in queenless nests develop their ovaries and lay eggs within a few weeks after separation from the queen (Konrad et al. 2012). Indeed, average ovary development was clearly increased in workers of queenless nests.

Caste-dependent differences in survival were analyzed using the *coxme()* function of the *coxme* package using *R*, Version 3.3.2 loaded with the packages *coxme***,** *car*, and *lme4*.

Survival model included right censored *number of days until death* per individual, *behavioral caste* (Forager, Inactive, Nurse) and *presence of queens* (Yes, No), and their interaction as response variables. As colonies were split and multiple workers per colony were observed, *colony ID* and *fragment ID* were added as random factors.

To test whether the amount of brood differed between queenless and queenright colonies at the end of the observation, a generalized linear mixed model (GLMM) was run including *number of larvae* as response variable and *presence of queen* as explanatory variable and *colony ID* as a random factor. As a measure of colony productivity, the number of newly emerged workers in summer 2014 were counted and compared between queenless and queenright colonies using a GLMM including *the number of newly emerged workers* as response variable, *presence of queen* as an explanatory variable, and *colony ID* as a random factor.

Results

Worker survival was mainly affected by behavioral caste (Cox model: $\chi^2_2 = 30$, $P < 0.001$, Figure 5.1) and the queens' presence in the nest (Cox model: $\chi^2_1 = 5.0$, $P < 0.025$; Figure 5.2), but not by an interaction between the two factors (Cox model: $\chi^2_2 = 1.6$, $P = 0.44$). Nurses showed a higher long-term survival than foragers (Cox model summary: $z = 4.52$, $P < 0.001$) and inactive workers (Cox model summary: $z = 2.78$, $P < 0.006$), but there was no difference between foragers and inactives (Cox model summary: $z = 0.66$, $P = 0.51$). After 200 days of observation, 32.6% of the foragers, 31.1% of the inactives, and 46.8% of the nurses were still alive. Workers survived longer under the queens' absence (32.7% survival) than when the queen was present (28.1% survival). At the end of the observations, the number of larvae did not differ between queenless and queenright colonies, indicating that the fertile workers of a colony together reproduced at comparable rates as the queen (GLMM: $\chi^2_1 = 0.9$, $P = 0.33$). Additionally, the number of newly emerged workers in summer 2014 did not differ between the two colony types

(GLMM: $\chi^2_1 = 0.7$, $P = 0.42$), showing that queenless nests are as effective in raising worker brood to adulthood as queenright nests.

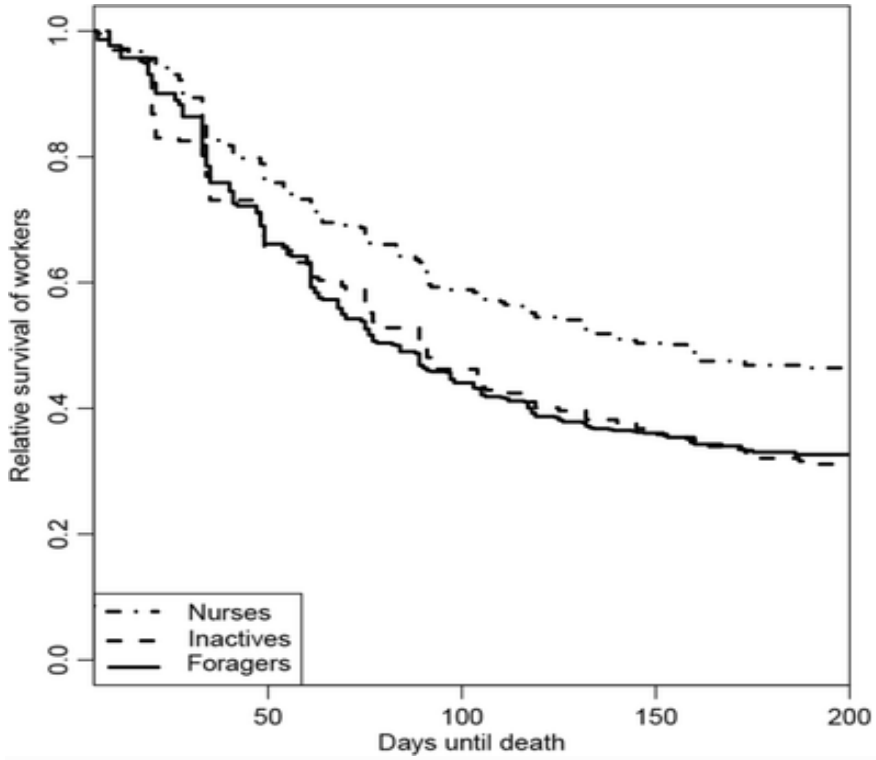

Figure 5.1: Survival of *T. longispinosus* ant workers over 200 days depending on their behavioral caste at time of the onset of the experiment. Foragers are depicted with a *solid line*, inactives with a *simple dashed line*, and nurses with a *complex dashed line*

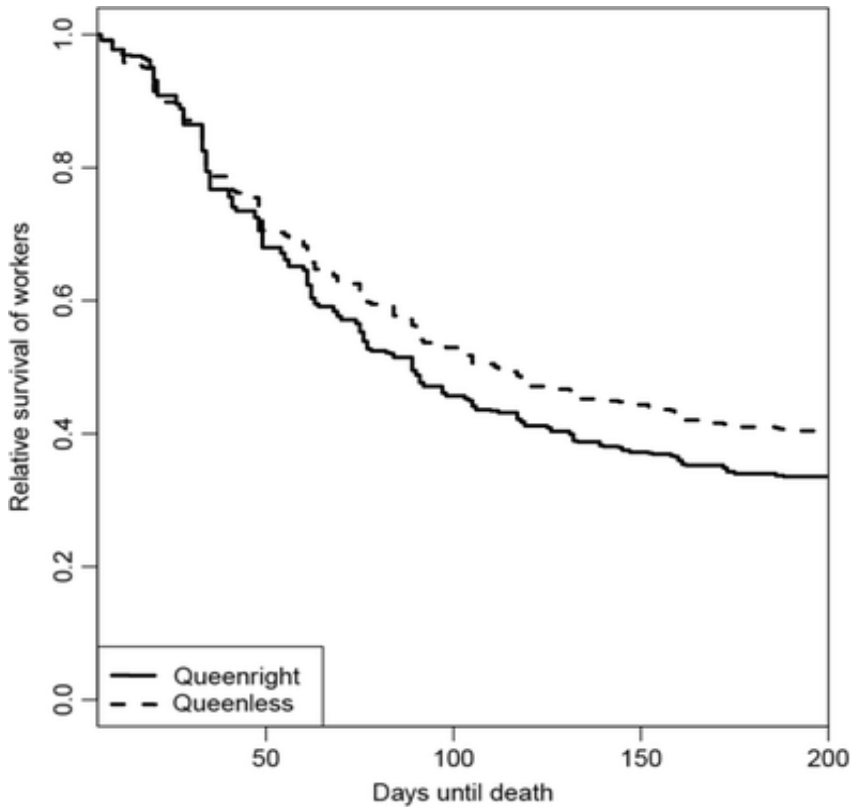

Figure 5.2: Worker survival in colony fragments with the queen (*black line*) and in queenless fragments (*dashed line*)

Discussion

Within insect societies, workers of different behavioral castes do not only differ in their task but also in age, risk exposure, and activity level, factors which could affect caste-associated intrinsic mortality. Moreover, the unique positive association between fecundity and longevity in social insects raises the question whether and how fertility induced by queen removal influences intrinsic mortality in workers. Here, we show that long-term survival of *T. longispinosus* workers on both behavioral caste and presence of the queen but not on an interaction of these two factors. We found that foragers exhibit a lower survival than nurses, even in the absence of external sources of mortality. Moreover, survival of nurses was elevated compared to inactive, inside workers. Finally,

workers, independent from their behavioral caste, survived longer in queenless compared to queenright colonies.

Cast-specific survival

As predicted, we found that nurses survived better than foragers even under the absence of external threats. The most likely explanation for this finding is that foragers were older than nurses at the onset of the experiment and in-line with the typical age polyethism of social insects (Wilson 1971, Jeanne 1986, Woyciechowski and Moroń 2009). Indeed, in *T. longispinosus*, young workers care for the brood and older workers take over the risky tasks outside of the nest, such as foraging (Kohlmeier et al. 2018). Caste-specific differences in survival could be in part caused by differential investment in body maintenance as an evolutionary response to differences in risk exposure (Schmid-Hempel and Schmid-Hempel 1984, O'Donnell and Jeanne 1992). Indeed, foragers of the desert ants of the genus *Cataglyphis*, which often survive only a few days after the onset of foraging, have less developed immune defenses (Bocher et al. 2007, Helft et al. 2012). Yet, in the ants *Lasius niger* and *P. punctata***, s**horter lifespan of foragers seems to be rather linked to less body reserves (Dussutour et al. 2016) and honeybee workers appear to senesce independently from behavioral caste (Rueppell et al. 2007). Further experiments, potentially including automated tracking (Mersch et al. 2013) and colony manipulations, will show whether age differences alone or—independent from that—also caste-specific investment in body maintenance explain the increased survival of nurses (Bernadou et al. 2015).

Interestingly, inactive workers showed an intrinsic mortality comparable to the foragers and clearly higher than that of the nurses. If not caused by age differences between the two groups of inside workers, for which we have no evidence, this unexpected finding contradicts both the predictions of evolutionary aging and metabolic theories (Prothero and Jürgens 1987, Promislow and Harvey 1990). By remaining inside the nest, inactive workers have a similar low exposure to external threats than nurses, and due to their inactivity, their metabolic rate should be lower than

that of both foragers and nurses (O'Donnell and Jeanne 1992). Therefore, our observations suggest that worker inactivity is related to age and may thus be non-adaptive (Corbara et al. 1989, Klein et al. 2008), a pattern also found in ponerine ants. In these ants, inactive workers have been described to be too old to work and senesce inside their mother nests (Fresneau 1984). In contrast, aging in workers of honeybees and *Pheidole dentata* ants not appear to result in functional senescence (Rueppell et al. 2007, Giraldo et al. 2016).

Queen presence and worker lifespan

In addition to differences between castes, the presence of the queen clearly affected worker survival, but interestingly not in interaction with caste. As predicted, ant workers lived longer in queenless nests than in the queenright ones, confirming a positive link between longevity and fecundity in social insects found before for queens and gamergates of the clonal ants *Diacamma* cf. *rugosum* and *P. punctata* (Tsuji et al. 1996, Hartmann and Heinze 2003, Heinze et al. 2013), and more recently in honeybee workers (Dixon et al. 2014). This finding also indicates that intrinsic mortality in workers can be modulated even in the adult stage irrespective of social behavior, providing a good example for aging plasticity.

The death of the queen leads to fights over reproductive dominance among workers in many *Temnothorax* ants including *T. longispinosus* (Konrad et al. 2012, Choe 1988, Heinze et al. 1997). The winners of these fights develop their ovaries and start laying eggs. Most often, these reproductive workers are the younger ones, which even in the presence of the queen have more developed ovaries (Bourke 1988, Tsuji 1990, Higashi et al. 1994, Tsuji et al. 1996, Heinze et al. 2002). As a consequence, we would have expected that the effect of the queens' absence should depend on the behavioral caste of the workers, hence to be stronger in young nurses. However, we found no interaction between behavioral caste and the queens' presence. This could be due to all workers developing their ovaries to a certain extent as dominant workers are less able to suppress ovary development in their worker sisters (Heinze et al. 2002, Cuvillier-Hot et al. 2004). Indeed,

dissections in queenless *T. longispinosus* nests revealed that not only reproductive workers become fertile but that, on average, ovary development is more pronounced in workers (Konrad et al. 2012). The overall increase in survival in queenless worker groups may reflect a global increase in worker fertility, which does not appear to only affect a few top-ranking reproductive workers of a specific caste.

As worker fertility after queen removal has been shown before in this species (Konrad et al. 2012), we did not directly confirm worker fertility after queen removal. The fact that queenless nests contained as much brood after 200 days as queenright ones indicates that egg-laying rates and brood survival was not reduced in nests without a queen. Also, queen removal did not negatively affect colony productivity as queenless nests raised as many worker pupae to adulthood as queenright ones (Kramer et al. 2014). This likely indicates that queen absence weakly impacts colony functioning. Nevertheless, reproducing individuals might allocate less energy or time into other tasks. Thus, possibly the absence of productivity differences between queenright and queenless nest indicates that infertile workers increase their workload to compensate for reproducing individuals.

Potential mechanisms underlying the increase in worker survival could non-exclusively include (i) a reduction of activity, (ii) better care (including food availability and grooming) for fertile workers, and (iii) physiological changes positively linked to fecundity. Our results contradict the first hypothesis as we found that inactive workers do not live longer than active ones and that queen removal does not reduce colony productivity. Secondly, an increased care for fertile workers is also unlikely to affect overall worker survival, as it should only increase longevity of the dominant workers, for which we have no evidence. This leaves the third hypothesis—a remodeling of the molecular pathways in workers after queen removal potentially linked to fertility induction—as the most likely explanation. However, an interaction of the different hypotheses and age-dependent effects of physiological remodeling cannot be entirely excluded. One way to proceed here is to study the proximate basis of this reversed trade-off in social insects (e.g., Amdam et al. 2007), which is

currently a hot topic in social insect biology (e.g., Corona et al. 2007, Von Wyschetzki et al. 2015, Rueppell et al. 2015, Negroni et al. 2016, Oettler and Schrempf 2016).

To conclude, we demonstrated caste-dependant differences in worker survival in the laboratory indicating lower intrinsic mortality rates in nurses compared to foragers and inactive workers, which likely are the consequence of caste-specific age differences. Despite their different activity levels and environmental risks, foragers survived as well as inactive inside workers, suggesting that activity per se is not the cause of the caste differences in survival. Furthermore, a modulation of intrinsic mortality in workers in response to social conditions, such as the queens' presence, reveals adult phenotypic plasticity for life history traits and raises the question of the cost of body maintenance.

CHAPTER 6

Immune-challenge diminishes gut microbiome diversity and triggers fertility- and longevity-dependent transcriptomic responses in an ant

Matteo A. Negroni*, Francisca H.I.D. Segers*, Fanny Vogelweith, Susanne Foitzik

* Authors contributed equally to the study.

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Abstract

In most organisms, longevity is traded-off with reproduction, but in social insects they are positively associated: the highly fertile queens live longest, while their non-reproducing workers are short-lived. Yet, once fertility is induced in workers, e.g. by queen removal, worker lifespan increases. It is currently unknown how this positive link between fecundity and longevity is achieved on a molecular level and thus social insects provide a unique opportunity to investigate intrinsic regulators of lifespan. Here, we investigate the role of immunity and gut microbiome in the extension of worker lifespan in the ant *Temnothorax rugatulus*. We subjected fertile workers to an immune challenge to investigate responses in fat body gene expression and gut microbiome composition. Fertile workers upregulated repair mechanisms, potentially explaining their extended longevity. The immune challenge altered the expression of several thousand genes in the fat body, including many immune genes, and, interestingly, this transcriptomic response depended on worker fertility: Only fertile, immune-challenged workers upregulated genes involved in alpha-ketoglutarate synthesis. This important immune regulator extends lifespan by down-regulating the TOR pathway and reducing oxidant production in *Caenorhabditis elegans*. Additionally, we observed a dramatic loss in bacterial diversity in ant guts 24h after the immune challenge. The expression of immune genes was linked to the microbiome composition. We demonstrate a negative effect of immune flare-ups on gut microbiome stability in insects, suggesting a previously underestimated cost of immunity. Moreover, our results help to unravel the molecular basis of the positive association between fertility and longevity in social insects.

Introduction

Understanding why and how organisms age is a long-standing scientific question. One of the most popular theories considers senescence as a consequence of the progressive accumulation of molecular damages due to incomplete somatic repair (Finkel and Holbrook 2000), which results in a decline in physiological functions including immunity and homeostasis (Finkel and Holbrook 2000). Endogenous causes of molecular damage are diverse, and include the production of reactive oxygen species (ROS) through metabolic activity and spontaneous errors in replication, transcription, translation (Finkel and Holbrook 2000, Rattan 2006). Hence, in an environment free of extrinsic risks (e.g., starvation, predation, pathogens), lifespan ultimately depends on two antagonist processes that determine the somatic maintenance ability: the rate of molecular damage and the efficiency with which such molecular damage is repaired (Finkel and Holbrook 2000, Partridge and Gems 2002). The well-conserved *insulin/insulin-like* growth factor (IIS) and the *target of rapamycin* (TOR) signalling pathways are important regulators of longevity that underlie the negative association between somatic maintenance and reproduction that is observed in most organisms (Narasimhan and Tissenbaum 2009, Flatt et al. 2013)

Still, the mechanisms that regulate longevity are not entirely understood, and the importance of some factors may have been underestimated. For instance, while numerous studies have investigated the influence of nutrition on longevity (Fontana and Partridge 2016), less attention has been paid to the gut microbiome, which is tightly linked to the host physiology, as a potential regulator of host lifespan (Ottaviani et al. 2011). The composition of the gut microbiome community results from a balance between the immune system of the host and bacterial proliferation (Kwong and Moran 2016). The role of immunity *per se* or in interaction with the gut microbiome, in the regulation of longevity has been rarely considered (Moret and Schmid-Hempel 2000, Fabian et al. 2018).

In most organisms, longevity is traded-off with reproduction, but in social insects, these two life-history traits are positively associated: the most fertile individuals in insect societies, the queens,

live the longest (Heinze and Schrempf 2008, Kohlmeier et al. 2017). Longevity is a phenotypic plastic trait as the large differences in lifespan between the reproductive queen and the non-reproductive worker castes likely arise from the same genetic background (Keller and Jemielity 2006). The positive association between fecundity and longevity does not only hold when queens and workers are compared, but is also observed within castes. Indeed, shifts can occur not only during development, but also during the adult life of a social insect. For example, in *Temnothorax* ants, queen removal induces ovary development and egg-laying in workers, which extends their lifespan by 13% (Kohlmeier et al. 2017). However, it remains unclear how this lifespan extension as well as the general positive link between fecundity and longevity is achieved in social insects on a molecular and physiological level. Social insects provide thus a unique opportunity to investigate intrinsic and environmental regulators of lifespan (Rodrigues and Flatt 2016).

We propose here three non-mutually exclusive hypotheses to explain the increase in worker longevity with fertility. First, the lifespan extension of fertile workers could be explained by a higher investment into body maintenance. Possibly, fertile workers extend their lifespan by investing more energy into i) a reduction of ROS production, ii) a better resistance to oxidative stress (e.g., via production of antioxidants) (Sun and Tower 1999, Tasaki et al. 2017), and/or iii) better repair mechanisms. The uncoupling or positive link between longevity and fecundity in social insects suggests a reshaping of those pathways and/or modifications in their regulation and downstream effects compared to solitary organisms (Rodrigues and Flatt 2016).

Second, the increase in worker longevity could stem from changes in the gut microbiome community (O'Toole and Jeffery 2015). IIS and TOR signalling pathways are nutrient sensitive, thus the gut bacteria could affect the regulation of longevity pathways depending on the digestion and absorption of certain nutrients by the host (Zheng et al. 2017). In line with this hypothesis, the microbiome diversity changes with biological age, and in *Drosophila* gut microbiome composition has been causally linked to aging and lifespan (Clark et al. 2015). Moreover, gut bacteria can also be beneficial for immune defence. In *Caenorhabditis elegans*, inoculation through feeding on non-

pathogenic bacteria *Lactobacilli* and *Bifidobacteria* not only extends lifespan but also improves pathogen resistance (Ikeda et al. 2007, Komura et al. 2013). In honeybees, the positive effect of gut bacteria on growth and pathogen resistance is related both to improved digestion and absorption of nutrients, and to a higher production of antimicrobial substances (Zheng et al. 2017).

Finally, fertility could be linked to an upregulation of the immune system, so that increased pathogen resistance could extend lifespan. However, immunity is typically costly for body maintenance, and impairs survival (Garschall and Flatt 2018). For instance, a non-lethal immune challenge reduces lifespan in bumblebee workers (Moret and Schmid-Hempel 2000), likely through the production and activity of radical oxygen species (ROS). If the lifespan extension in ant workers would involve a change in immunity at the molecular level, fertile workers should alter their expression of immunity genes, an effect that may only be detectable when the immune system is provoked.

In this study, we test these three hypotheses by investigating the molecular regulation of longevity, and its link to immunity and gut microbiome composition in workers of the ant *Temnothorax rugatulus*. First, we show in a long-term survival experiment that worker fertility induced by queen removal extends worker lifespan also in the ant *T. rugatulus*, our focal species. Then we subjected these fertile and non-fertile workers to a non-lethal immune challenge and compared gut bacterial diversity as well as their transcriptomic responses in the fat body (Figure 6.1), the primary organ of systemic immunity in insects (Hoffmann 2003). systemic immunity in insects (Hoffmann 2003).

If fertile workers live longer because of an increased investment in body maintenance, we predict an upregulation of genes involved in molecular repair mechanisms or antioxidant production. If changes in gut microbiome composition, are contributing to the increased longevity of fertile ant workers, we would expect an effect of worker fertility on the gut microbiome. Finally, worker lifespan extension may be explained by an improved immune-competence. If so, we envisage that i) the baseline expression of immune genes differs between fertile and non-fertile workers and ii) the

immune response following an immune challenge depends on worker fertility. We show below that fertile workers invest more into repair mechanisms and other somatic maintenance functions, especially when immune-challenged, which might explain their lifespan extension, in particular when exposed to pathogens. Gut microbial composition is not involved in the increase in longevity following fertility induction. However, the immune challenge had a severe impact on the gut microbiome by inducing a drastic loss of microbial diversity, which was linked to the expression of immune genes.

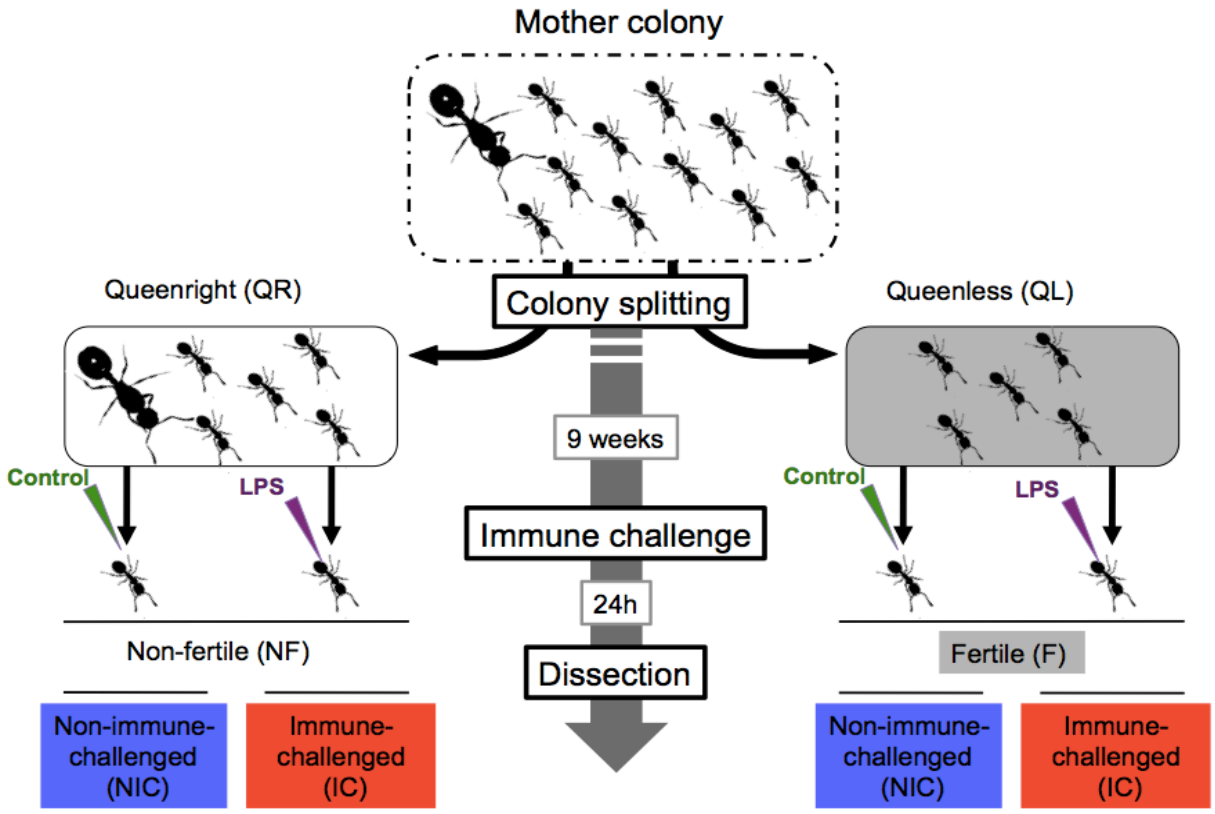

Figure 6.1: Overview of the experimental design. Eight monogynous mother colonies were equally split in two fragments: one containing the queen (queenright: QR) and the other being queenless (QL). After 9 weeks, two workers were picked out from each nest. One was immune-challenged (IC) by pricking with a needle dropped into a 10 % lipo-polysaccharide solution (LPS), while the other non-immune-challenged worker (NIC) received the same manipulation, but the needle only touched the cuticle. 24h after the manipulation, the fat body was dissected for RNA extraction, the gut for 16S microbiome sequencing and the ovaries for size measurements. QL workers had longer ovaries than workers from the QR nests (fertile workers F; non-fertile workers NF).

Materials and Methods

Colony collection

Colonies of the species *Temnothorax rugatulus* were collected in the Chiricahua Mountains, Arizona USA in August-September 2015. A total of 52 queenright monogynous colonies were used for our experiments, with colony size varying between 85 and 304 workers (mean = 143.5; SD = 68.4). After collection, colonies were kept individually in boxes (9 x 9 cm) with a humid plaster floor containing an artificial nest-site, which consisted of a Plexiglas perimeter (2 mm high) sandwiched between two microscope slides and an entry hole. Colonies were fed twice per week with honey and crickets and had access to a constant water supply. The colonies were maintained in controlled climatic conditions simulating field conditions.

Queen removal and worker survival

In order to confirm the positive effect of queen removal on worker survival in our focal species, we conducted a survival experiment, for which we used 44 colonies. Each colony was split in two equal fragments (worker number: mean = 39.5; SD = 13.8), while we ensured that each fragment had the same number of workers and similar proportions of brood, nurses and foragers, with the only difference being the queen. We labelled 2 to 6 nurses from each fragment with two coloured wire loops around the petiole (0.02 mm, Elektrisola, combination of red, green, blue or silver). Colonies were then transferred to new artificial nest boxes and kept at 22°C, 12h/12h light/dark.

The survival of the labelled workers was recorded every two weeks following colony splitting for a total period of 30 weeks. Differences in survival according to queen presence was analysed using Cox proportional hazards model, specifically the *coxme* function of the *coxme* package implemented in *R*, Version 3.2.1. Survival model included number of days until death per individual

as response variable, queen presence (Yes, No) as explanatory variable and colony identity and fragment identity as random factors.

Queen removal prior immune challenge, gut and fat body dissection

To obtain non-fertile and fertile workers to subject to an immune challenge, we used eight colonies, with a colony size varying between 34 - 93 workers (mean = 62.3; SD = 23.7). To induce worker fertility, each colony was split in two (queenright "QR" and queenless "QL"). In each fragment four nurses, which are workers caring for the brood, were individually labelled with a coloured wire loop around the petiole (0.02 mm, Elktrisola, red or green). Young *Temnothorax* workers take over brood care and are most likely to become behaviourally dominant and start reproduction following queen removal (Heinze et al. 2002). Colonies were then transferred to new artificial nest boxes and kept at 25°C, 12h/12h light/dark for a period of nine weeks. During this time, workers in the QL fragment could respond to queen removal by developing their ovaries and laying eggs, which we determined by dissection (Figure 6.1).

Fertility induction in response to queen removal

Queen removal led to a 28.6% increase in worker ovariole length (mean ± SD; queenright: 0.49 ± 0.14 mm; queenless: 0.63 ± 0.19 mm; $F_1 = 5.55$, $P = 0.027$). The number of eggs in development in the ovaries did not differ between the two groups (mean ± standard error; queenright: 0.35 ± 0.13; queenless: 0.38 ± 0.14; $F_1 = 0.03$, $P = 0.86$). *Temnothorax rugatulus* workers can lay male-destined, haploid eggs following queen removal and we observed worker-laid eggs in all queenless colonies during the nine weeks following queen removal. Due to their enlarged ovaries, we refer to workers from the queenless treatment as fertile (F; Figure 6.1), and those from the queenright as non-fertile (NF; Figure 6.1).

Immune challenge

Nine weeks after colony splitting, each nest was placed for 10 min at -20°C in order to reduce worker activity. Immediately afterwards, the nest-site was opened and each labelled worker was removed with forceps and individually isolated in a Petri dish. From each fragment, two of the four labelled workers received an immune-challenged (IC) by pricking them with a needle (diameter = 0.15 mm, length = 40 mm) dipped in a 10% lipo-polysaccharide (LPS) solution. Workers were carefully pricked through the thin cuticle between the 2nd and 3rd segment of the gaster. The non-immune-challenged workers (NIC) received the same manipulation, but the needle only touched the cuticle, but not penetrated it. Thereafter, all labelled workers were returned to their colony.

Fat body dissection and sequencing

Twenty-four hours after the immune challenge, all labelled workers (Figure 6.1) were removed from their nest with soft forceps, immediately killed by decapitation and dissected on ice in 1% PBS. The fat body attached to the two segments of the gaster was crushed into 50µL of TRIZOL and then stored at -80°C until RNA extraction. Dissection of each worker took less than 5 min. Ovaries were dissected and photographed with a Leica DFC425 camera at 20x magnification under a stereomicroscope. Ovary length was measured and the developing eggs were counted using Leica software (LAS version 4.5). RNA was extracted using the RNAeasy mini extraction kit (Qiagen).

Gene expression analysis

Library preparation and sequencing of 100bp paired reads was done according to standard protocol on an Illumina HiSeq 4000 (StarSeq, Mainz, Germany). In total, we sequenced the fat body transcriptomes of eight workers from each of our four treatments, resulting in 32 sequenced samples of 45 million reads pairs each (Figure 6.1). The raw reads were trimmed using *Trimmomatic-v0.36* (Bolger et al. 2014), and quality checked using *FastQC-v0.11.5*. Subsequently, all paired reads were assembled *de novo* using *Trinity* (trinityrnaseq-Trinity-v2.4.0) (Haas et al. 2013). Due to the large amount of data (± 45 million read pairs per sample) we could not assemble all paired reads at once.

Thus, we generated two assemblies, each with half of our samples, ensuring an equal amount of each treatment in each set of the samples. We subsequently merged the two assemblies by first removing the identical contigs with the program *CD-HIT-EST* (Li and Godzik 2006) and then merging contigs with CAP3 (Huang and Madan 1999). The initial two assemblies encompassed 156.371 and 151.820 contigs, while the merged assembly had 150.423 contigs (mean length of 1128 bp, back mapping rate of 70.65 % on average).

We conducted a *blastX* search against the non-redundant invertebrate protein database (state September 2017) to annotate the contigs. About 50.42% were annotated in *Blast* with 24.15% having a unique Blast hit. After translation of nucleotide sequences into amino-acid sequences with *Transdecoder-v3.0.1* (https://github.com/TransDecoder), the gene ontology (GO) and the Kyoto encyclopaedia of genes and genomes (KEGG) term annotation were performed using *InterProScan-v5.25-64.0* (Jones et al. 2014).

Read count per contig and sample was obtained by using RSEM-v1.3.0 (Li and Dewey 2011), and we did a differential gene expression analysis with the R package of Deseq2-v1.2.10 (Anders 2010). We separately tested the main effects of fertility and immune challenge, and their interaction, while controlling for colony identity. Additionally, among the contigs, which were significantly influenced by the fertility *x* immunity interaction, we compared the different subgroups (any combination of two factors): i) fertile versus non-fertile workers, separately and successively, within the immune-challenged workers, and within the non-immune-challenged ones; and ii) immune-challenged versus non-immune-challenged workers, separately and successively, within the fertile workers, and within the non-fertile ones. Among the list of differentially expressed genes we searched for candidate genes associated with fertility, longevity and immunity with help of the literature, by searching for gene names and traits. Using the online tool *KEGG Mapper* (http://www.genome.jp/kegg/tool/map-pathway1.html) we conducted a pathway analysis for the upregulated contigs of interest.

To investigate which biological processes are associated with differential gene expression we found in response to the immune challenge and fertility treatment, we performed a functional enrichment analysis with all upregulated contigs. The enrichment analysis was done for the contigs that were affected by the interaction of fertility and immune treatment by doing separate tests for each level of each factor. The contigs that were upregulated in response to one treatment irrespective of the other treatment (for example upregulated in fertile workers compared to non-fertile ones irrespective of the immune challenge). For the enrichment analysis we used the R package *TopGo* (Alexa and Rahnenfuhrer 2010) and for each GO term the ps were obtained with a ***Fisher's exact*** test.

Gut dissection and DNA extraction

Immediately after removing the fat bodies we separated the guts without the crop from the rest of the abdomen with clean dissection tools. The guts were stored individually at -80°C in 10µl 1% PBS until DNA extraction. To control for bacterial contaminants, we included three samples from the 1% PBS, in which the ants were dissected: one with only the PBS, another one in which we dipped the tools used for the dissections and one in which we dipped several intact ants to control for bacteria present on the cuticle. It was not possible to wash the ants before dissection, because this might have interfered with their gene expression. As a fourth control, we took a sample from the food of the ants.

Before DNA extraction the guts were frozen in liquid nitrogen and subsequently crushed with sterile plastic mortars. For DNA extraction we used an industrial kit (MasterPure™, from EpiCentre, Wisconsin, USA). We added an extra lysozyme step to the manufacturer's protocol to ensure the lysis of gram-positive bacterial cell walls: we added 2µl of ready-to-lyse lysozyme (250U/µl TES buffer) to each sample and subsequently they were incubated for 30 min. at 37°C. The resulting DNA samples were stored at -80°C until sequencing.

Gut microbiome analysis

The amplification and sequencing of the 16S V4 region was performed by StarSEQ GmbH, Mainz, Germany. Amplification was done with the 515f-806rB primer pair. Overlapping, paired-end reads of 250bp were generated with Illumina MiSeq. De-multiplexing, adapter trimming and quality filtering was done following the normal Illumina MiSeq workflow by StarSEQ. The initial data set contained 505.050 read pairs (available online see data accessibility section).

To determine Operational taxonomic units (OTUs) we mainly used the Uparse 9.2 pipeline (Edgar 2013): Paired-end reads were merged (on average 86.2% ± 2.7 SD could be merged per sample) and subsequently filtered (maxEE cut-off set to 1). The “-fastx-uniques” step was used to de-replicate filtered reads and afterwards we used the “-cluster-otus” step to cluster the sequences into OTUs with a threshold of 97%. This latter step also removes chimeras. Next, the merged, unfiltered reads were mapped to the OTUs with a 97% identity cut-off with the “usearch-global” algorithm (on average 97.7% ± 1.1 SD were mapped to the OTUs per sample). Using Qiime 1.9.1. (Caporaso et al. 2010), we allocated taxonomic groups to the OTUs, with as a reference the Greengenes database gg-13-8. Unassigned OTUs and OTUs classified as chloroplasts or mitochondria were removed from the OTU table, but archaeal OTUs were left in. If an OTU was not at least five times more abundant in any experimental sample than in a control sample, it was discarded. Additionally, we excluded OTUs that did not make up at least 0.1% of the community in at least one sample, to further eliminate contaminations. The resulting OTU table contained 378 taxonomic units.

Total bacterial 16S copy numbers per ng DNA were quantified by quantitative realtime PCR (qrt-PCR) with universal 16S primers (Forward: 5’-ACTCCTACGGGAGGCAGCAGT-3’; Reverse: 5’-TATTACCGCGGCTGCTGGC-3’) using a Biozym Blue S'Green qPCR Kit (separate ROX) on a micPCR (Bio Molecular Systems). Reactions took place in a total volume of 20µl containing 6.4µl of ddH2O, 0.8µl of each primer (10µM), 10µl of Sybr mix and 2µl of the extracted DNA per sample. All

runs consisted of an initial 2 min. initiation step at 95°C, 40 cycles of 95°C for 10 s, 64°C for 15 s (initially 6 touchdown steps starting from 70°C) and 72°C for 10 s. Each run was concluded with a dissociation curve analysis. All samples were replicated three times in the same run. Each run also included two to three negative controls with 2µl of ddH_2O added instead of DNA. We excluded replicates with double peaked melting curves and which had Cq values that exceeded 0.5 from the other two replicates. The DNA concentration of each sample was deducted from a standard curve made with a tenfold dilution series (10^4 to 10^{10}) of a mixture of 16S DNA copies of the bacterial associates of a firebug (*Pyrrhocoris apterus*). Due to a depletion of some samples for the Illumina MiSeq sequencing and the exclusion of samples based on double peaked melting curves, we were left with 11 samples from non-immune-challenged individuals and 13 samples of immune-challenged ants.

The total DNA concentration of each sample in ng/µl was determined with a Qubit Fluorometer (Thermofisher). For each replicate we calculated the amount of bacterial 16S copies per ng of DNA in a sample. The means of the replicates were log transformed and subsequently compared between non-immune-challenged and immune-challenged individuals with linear mixed-effects (LME) models from the R package nlme, including colony as random effect.

The OTU table was rarefied to a depth of 1,500 reads. Using the rarefied table, we calculated the pairwise Bray-Curtis dissimilarities between the samples in Qiime. Samples were clustered based on their Bray-Curtis dissimilarities (average linkage method, 1,000 bootstraps) with the pvclust package in R (Suzuki and Shimodaira 2006). Clusters that get assigned AU values higher than 98.5 are highly supported. The OTUs were binned according to microbial family and the relative abundance of each microbial family was calculated for each sample. This data was used to generate a stacked bar plot which was combined with the pvclust tree plotted with the R package ggtree (Yu et al. 2017), to visualize the differences in community composition between the gut samples.

To compare the bacterial diversity between the treatments we calculated for each sample the Shannon's diversity index with the alpha-diversity.py script of Qiime using the rarefied table. We tested for an effect of fertility, immune challenge and their interaction on the Shannon's diversity index with LME models: The fragment and the colony of the ant were included as a nested random effect term. Model residuals were visually checked for deviation from normality.

To identify taxonomic groups that differed in relative abundance between treatments we binned the OTUs into their respective microbial families. To restrict the analysis to the more important taxa, the microbial families that were present in less than 15% of the samples were removed from the OTU table. For the differential abundance testing we performed permutation tests as implemented in the *fitLogNormal* function of the metagenomeSeq R package (Paulson et al. 2013), using 100 permutations. The ps were adjusted with the "FDR" method. Taxonomic features with adjusted p-values smaller than 0.05 were regarded as significantly different in relative abundance between the treatments.

We were interested whether variation in *T. rugatulus* gut communities could be explained by the expression of effector genes involved in antimicrobial immunity. Therefore we looked for the presence of such genes among the contigs and checked whether these contigs were significantly differentially higher expressed in immune-challenged compared to non-immune-challenged individuals. If multiple contigs were annotated with the same effector gene name we chose the contig with the highest average expression (TPM). With the resulting set of contigs we did a constrained ordination analysis with the *capscale* function of the R package *vegan* (distance-based redundancy analysis, Bray-Curtis) (Oksanen et al. 2009): To test for an association between the expression of the antimicrobial effector genes on the gut communities we compared for each contig separately two models with an ANOVA: one model with only fixed factors and one model with next to the fixed factors the expression of the contig (TPM).

Results

Queen removal, fertility induction and lifespan extension

More than inducing fertility in workers (see above in the Materials and Methods section) the long-term survival experiment, confirmed that queen removal also increases worker longevity in *T. rugatulus* (cox survival mixed-effects model: X^2 = 10.2; df = 1; P = 0.0014). Worker survival increased by 12.5% in QL colony fragments over 30 weeks (Figure 6.2).

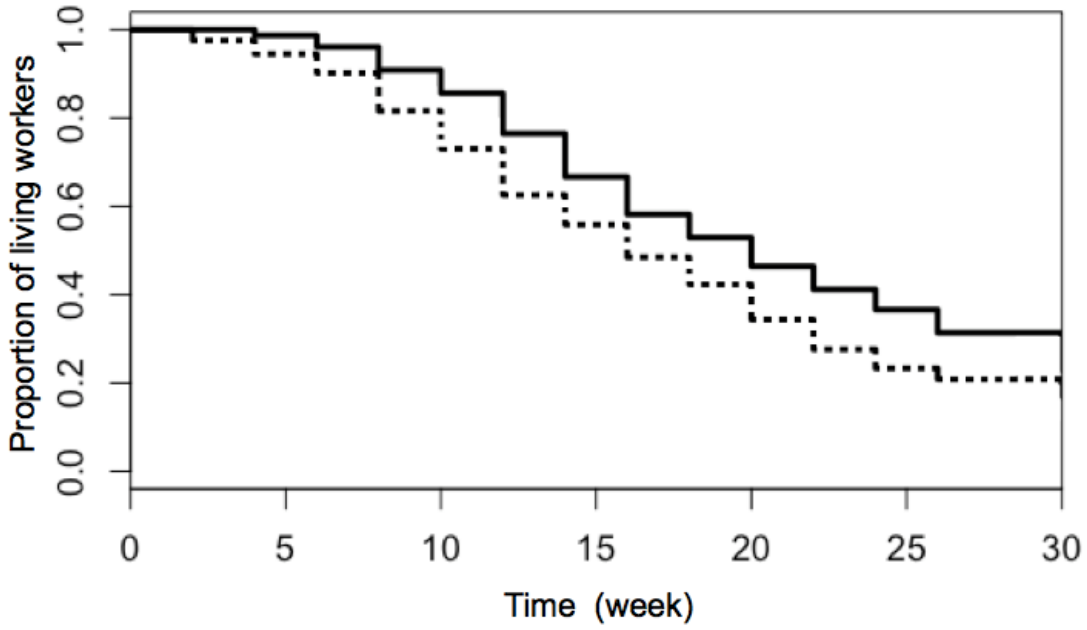

Figure 6.2: Worker survival over time in queen presence (dashed lines) and absence (solid line). Survival analysis revealed a higher survival in the absence than in the presence of the queen (X^2 = 10.2; df = 1; P = 0.0014).

Effect of fertility induction and immune challenge on gene expression

The *de novo* transcriptome assembly resulted in 150,423 contigs. Fertility induction and immune provocation interactively altered the expression of 2,387 contigs, and about 55% of them displayed an opposite expression pattern in response to the two factors, queen presence and immune challenge. Thus we compared the following subgroups: i) fertile *vs.* non-fertile workers, separately for the immune and non-immune-challenged workers, and ii) immune-challenged *vs.* non-challenged

workers, separately for the fertile and non-fertile workers (Palakurty et al. 2018). Fertility induction was associated with differential expression of 1,466 contigs within the immune-challenged workers and 1,101 contigs within the non-challenged workers (Figure 6.3a). Conversely, the immune challenge resulted in the altered expression of 2,422 contigs in non-fertile workers, whereas 1,518 contigs changed their expression in the fertile workers (Figure 6.3b).

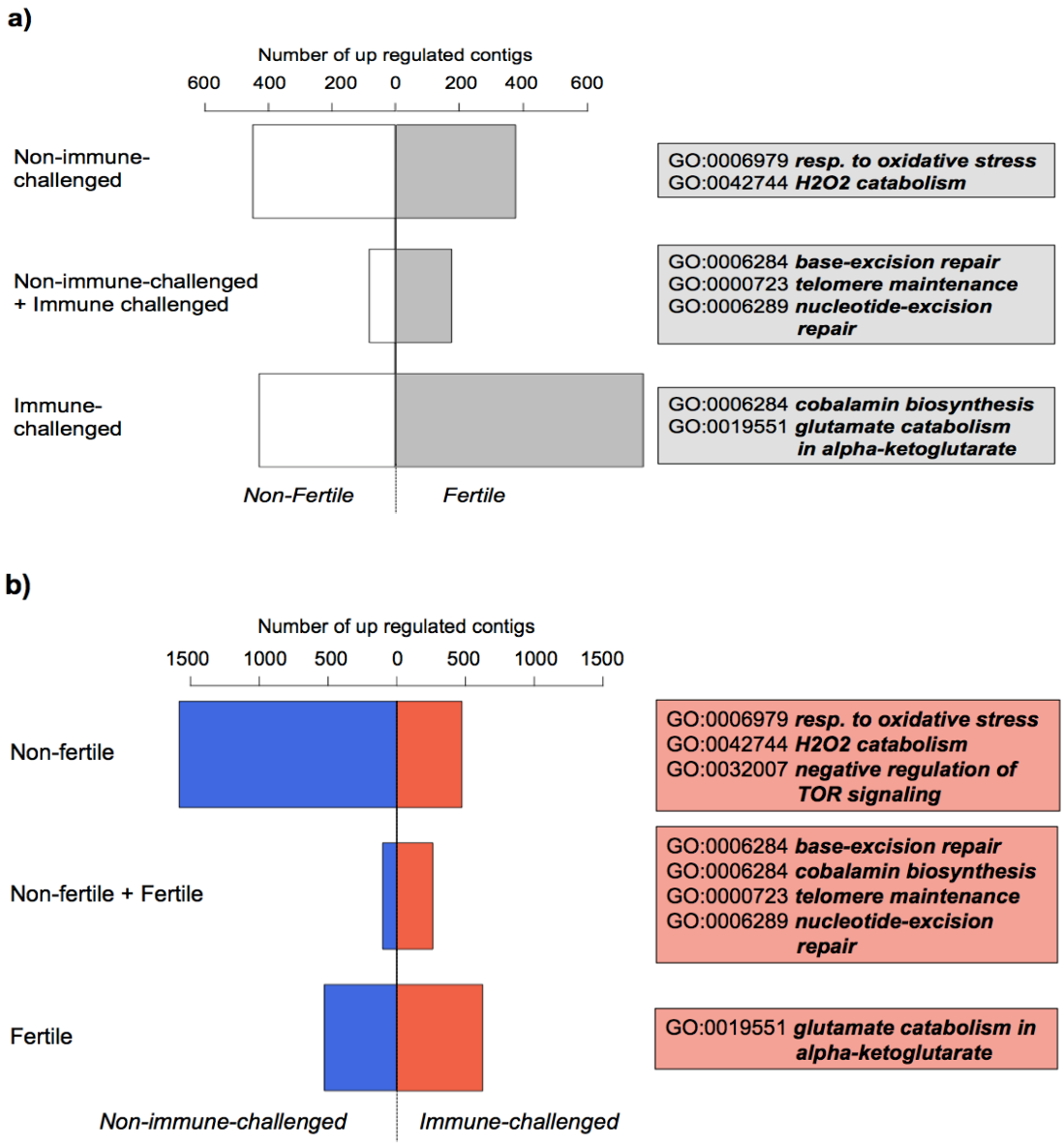

Figure 6.3: Barplots illustrating the results from the expression analysis based on contigs, whose expression was influenced by an interaction between the immune challenge and the fertility induction: a) from the comparison between non-fertile (NF) and fertile (F) workers, the number of upregulated contigs in NF (white bars), the number of upregulated contigs in F (grey bars), specifically within non-immune-challenged (non-immune-challenged), irrespective from the immune-challenged (non-immune-challenged + immune-challenged), or specifically within immune-challenged workers. b) from the comparison between non-challenged (NIC) and the immune-challenged (IC) workers, the number of upregulated contigs in NIC (blue bars) and the number of upregulated contigs in IC (red bars), specifically within NF (non-fertile), irrespective from the immune-challenged (non-fertile + fertile), or specifically within immune-challenged workers (fertile). A summary from the functional enrichment on the right represents candidate functions upregulated in a) F

workers compared to NF ones and b) IC compared to NIC ones. No function was associated with fertility, longevity, or immunity among the genes upregulated in NF and NIC compared to F and IC.

The functional enrichment analysis based on the list of upregulated contigs in each factor level separately. Both immune-challenged and non-immune-challenged workers upregulated molecular repair mechanisms in response to fertility induction (Figure 6.3a). Conversely, both non-fertile and fertile workers upregulated molecular repair mechanisms in reaction to the immune challenge (Figure 6.3b). Analysis of influence of the immune challenge on gene expression within fertile workers revealed that fertile workers activated genes of the *glutamate degradation into alpha-ketoglutarate* pathway in response to the immune challenge (Figure 6.3a, 3b). Instead, non-immune-challenged workers specifically activated mechanisms involved in oxidative stress reduction (*response to oxidative stress, H_2O_2 catabolism*) in response to the fertility induction (Figure 6.3a). Non-fertile workers only upregulated these functions following the immune challenge (Figure 6.3b).

In general, the transcriptomes clustered more strongly depending on whether the ants had undergone an immune challenge than whether they were fertile or not (Figure 6.4), which was reflected in the main effects of the expression analysis. Fertility induction changed the expression of 4,268 contigs independently of the immune challenge. Specifically, 3,129 contigs were upregulated in non-fertile workers and 1,139 in fertile ones, among the latter is a *vitellogenin receptor*. The function *S-adenosylmethionin biosynthetic process*, enriched in upregulated contigs of fertile workers is important for protein synthesis. It also plays a role in the molecular regulation of longevity, as a knockdown of *S-adenosylmethionin synthase* extends lifespan in *Caenorhabditis elegans* (Chin et al. 2014).

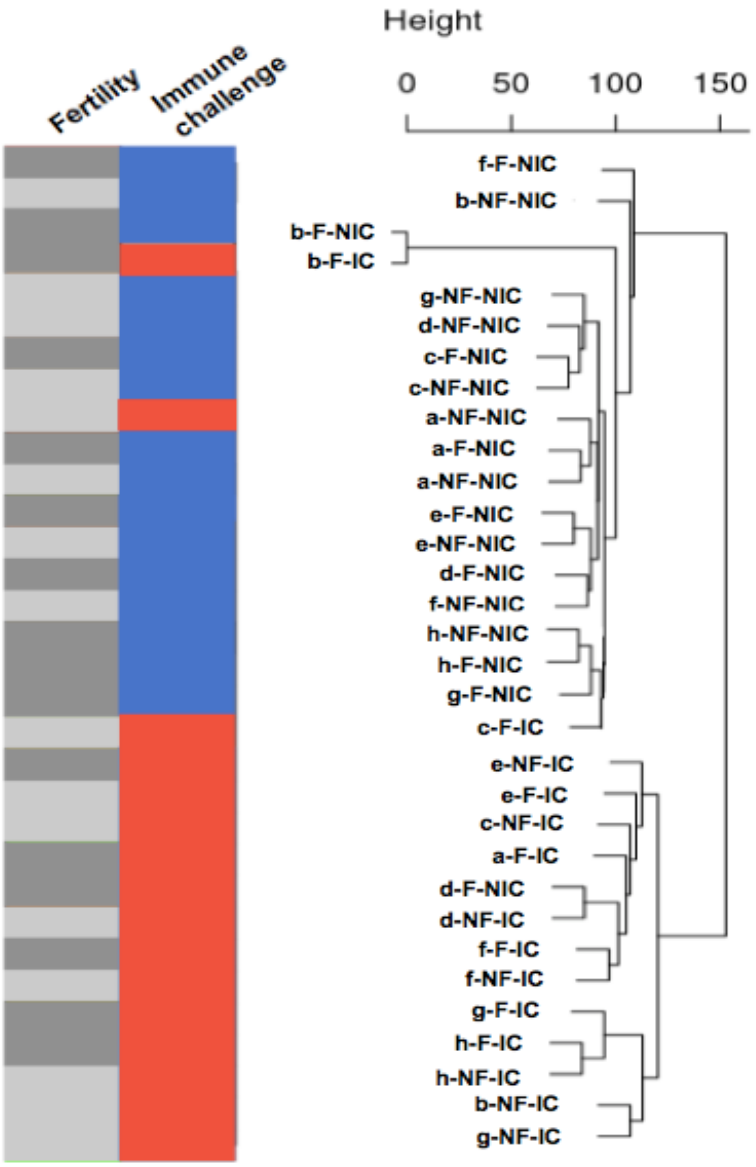

Figure 6.4: Dendrograms showing clustering of samples according to fertility (non-fertile: NF and green; or fertile: F and red), and treatment (non-immune-challenged: NIC and light grey; or immune-challenged: IC and dark grey).

In comparison, more than double as many contigs, 10,717, altered their expression in response to the immune challenge irrespective of the fertility state: In immune-challenged workers 6,136 contigs were upregulated, while 4,581 were higher expressed in non-immune-challenged workers. The enrichment analysis based on the list of upregulated contigs revealed functions related to immunity, such as *peptidoglycan catabolic process*, *defence response*, *de novo IMP biosynthetic process* and

innate immune response, or stress responses such as *response to oxidative stress* (Figure 6.5). Interestingly, in non-immune-challenged workers the functions *innate immune response* and *social behavior* were enriched among the upregulated contigs, whereas *immune response* was marginally enriched in immune-challenged workers (P = 0.056).

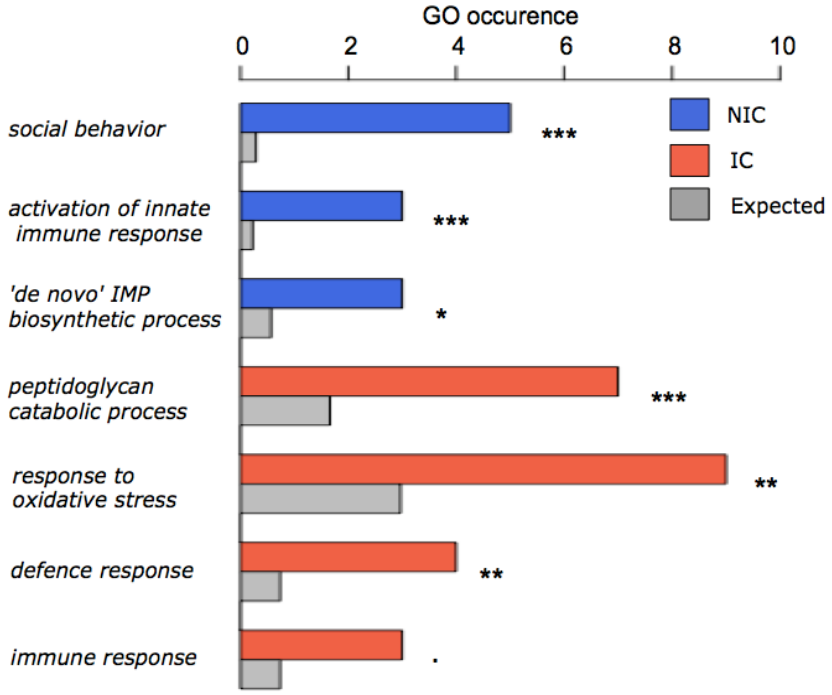

Figure 6.5: Gene ontology term (GO) representation of relevant functions from the functional enrichment analysis (significance code: *: <0.05 ; **: <0.01 ; *** : <0.001; the dot represents marginal enrichment for a P = 0.056). The number of times a function occurred in the list of upregulated contigs for the non-immune-challenged (NIC) workers, immune-challenged (IC) workers, and the expected number (following a random distribution) are coloured blue, red and grey, respectively.

Effect of fertility induction and immune challenge on the gut microbiome

The analysis of pairwise Bray-Curtis dissimilarities between samples, revealed no clustering of gut communities based on worker fertility (Figure 6.6) and Shannon's diversity between the gut communities of fertile and non-fertile ants did not differ (Linear mixed-effects model, Fertility: F = 1.75, P = 0.23; Fertility × Immune challenge: F = 1.72, P = 0.21). In contrast, most of the gut communities from immune-challenged ants clustered separately from the ants that did not undergo

this treatment (Figure 6.6). In agreement with the results of the clustering, the constrained ordination analysis indicated that only the immune challenge and not fertility induction explained gut community variance (Immune challenge: F = 4.64, P = 0.01; Fertility: F=0.72, P = 0.71; Fertility × Immune challenge: F = 0.78, P = 0.63).

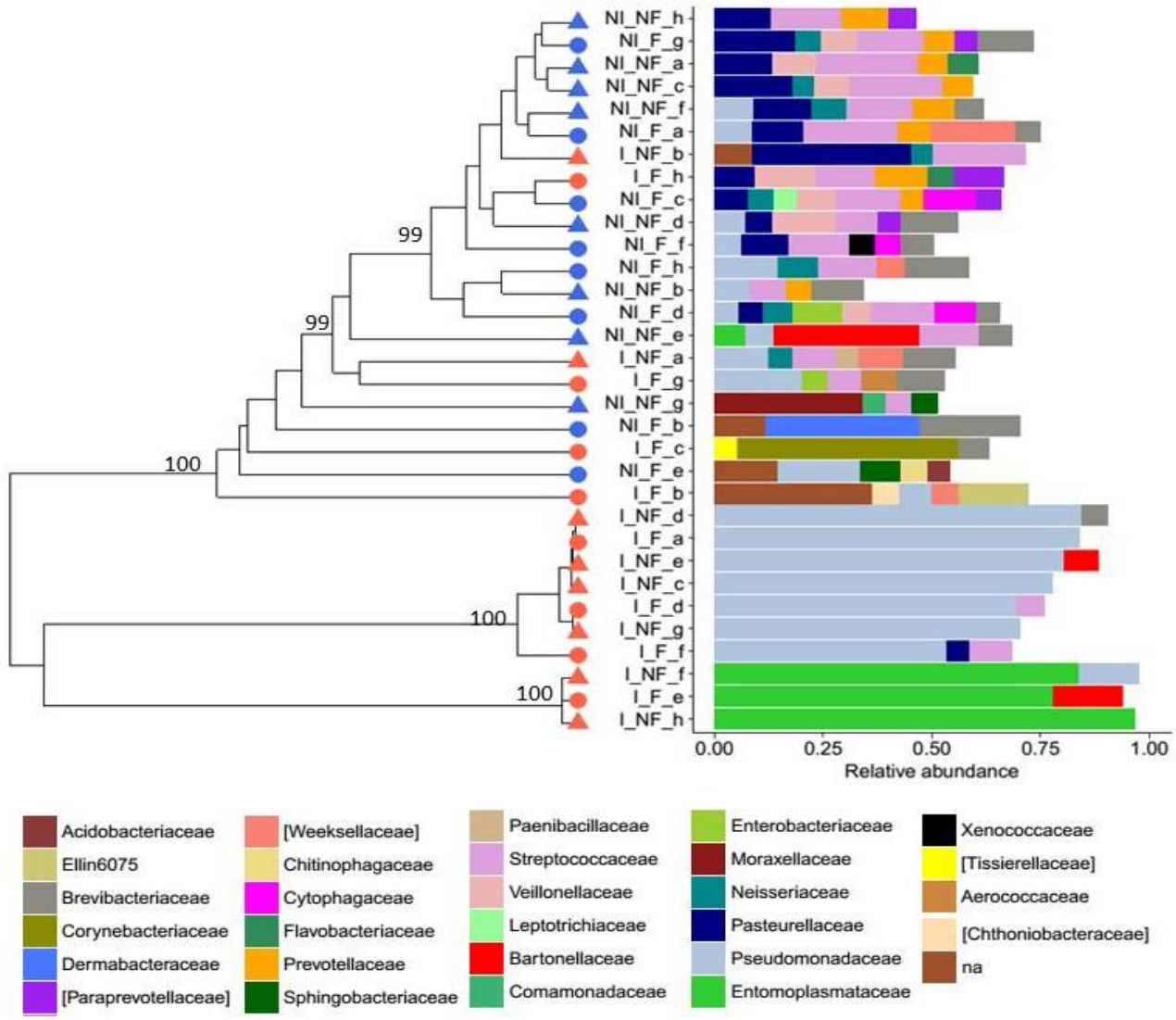

Figure 6.6: Samples were clustered based on their Bray-Curtis dissimilarities using the average linkage method. Clusters with AU values > 98.5 are highly supported clusters and are indicated on the tree with their respective AU values. Red and blue symbols at the tree tips represent the samples from immune-challenged and non-immune-challenged ants, respectively. Fertile and non-fertile workers are indicated by, respectively, circles and triangles. Samples that share the same letter (a to h) originate from ants belonging to the same colony. The relative abundances (≥0.05) of microbial families are plotted next to the samples. The OTUs which did not have a family assigned were binned in the category “na”.

Remarkably, the gut communities of immune-challenged ants generally lacked the diversity observed in non-immune-challenged ants (Figure 6.7). In fact, the immune challenge greatly reduced Shannon's diversity of the gut communities (Linear mixed-effects model, $F = 30.12$, $P < 0.001$; Figure 6.7). Three gut communities of immune-challenged ants consisted mainly of one OTU assigned to the bacterial family Entomoplasmataceae and the intracellular genus *Entomoplasma*, while seven of those gut communities were dominated by the Pseudomonaceae, mainly belonging to one OTU from the genus *Pseudomonas* (Figure 6.6).

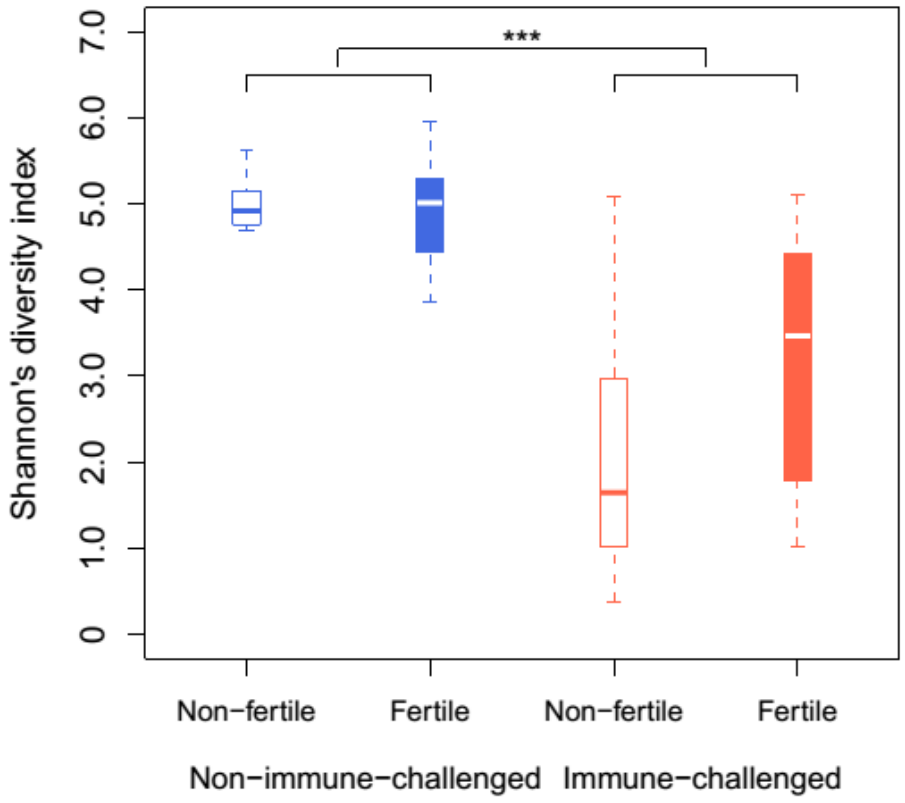

Figure 6.7: The Shannon diversity indexes grouped by treatment. The horizontal bars in the boxplots represent the medians and the boxes indicate the first and third quartile. Immune-challenged and non-immune-challenged ants harboured gut communities that differ significantly in Shannon's diversity, as indicated by the asterisks on the plot.

In line with the results of the cluster analysis, worker fertility did not affect the relative abundance of microbial families, suggesting that fertility induction did not alter gut community composition. In contrast, 20 of the 64 examined bacterial families contributed less to the gut

communities of immune-challenged workers compared to the non-immune-challenged ones. The two families that made up the largest parts of the gut communities of immune-challenged individuals (Figure 6.6), Entomplasmataceae and Pseudomonaceae, are among the few families with a positive log-fold change. However, of those two families only the increase in the Pseudomonaceae approached significance (adj. P = 0.09). Gram-negative and gram-positive bacteria did not differ in their response to the immune challenge (Pearson's Chi-squared test, $\chi^2 = 0.16$, P = 0.66).

Suggesting a coevolution between bacteria and host, the relative abundance of the Aerococcaceae was very similar in both immune-challenged and non-immune-challenged workers. Four OTUs were assigned to the Aerococcaceae, but only one of them was common. An online BLAST search of this OTU sequence against the 16S bacterial/archaeal NCBI sequence database gave a 100% identity hit with a lactic acid bacterium, *Dolosigranulum pigrum*. Lactic acid bacteria are regularly found in insect guts, although they are most often from the genus *Lactobacillus* (Engel and Moran 2013).

One operational taxonomic unit (OTU) of the Pseudomonadaceae was present in every gut community, belonging to the genus *Pseudomonas*. The genus *Pseudomonas* is well-known for its metabolic diversity and ability to colonize many niches, including guts. The omnipresence of the genus makes it also likely to be unintentionally sequenced. However, the respective OTU was on average 23 times more abundant in our experimental samples than in our controls and thus we deem it unlikely to be a contaminant. A BAST search of its 16S rRNA sequence gave hits with a wide variety of *Pseudomonas* species, but no other known gut inhabitants.

Bacteria belonging to the order of the Rhizobiales are commonly found in the guts of Hymenopterans and have been hypothesized to recycle nitrogen for their host (Van Borm et al. 2002, Segers et al. 2017). Among the *T. rugatulus* gut communities we found twelve rhizobial OTUs and five rhizobial families were included in our differential relative abundance tests (Bartonellaceae, Brucellaceae, Methylobacteriaceae, Methylocystaceae and Rhizobiaceae), but only the

Methylocystaceae were significantly less abundant in immune-challenged samples. The Bartonellaceae and Brucellaceae had a positive log-fold change. Only one OTU belonged to the Bartonellaceae and this OTU was not determined to the genus level. A BLAST search gave no identical hits, but a close hit was found in the NCBI Nucleotide collection: the Bartonellaceae OTU shared most 16S rRNA sequence similarity with a bacterium found in a study on symbionts in myrmecine ants (Liberti et al. 2015), a subfamily to which our focal species also belongs. The family Brucellaceae was solely represented by an OTU assigned to the highly diverse genus *Ochrobactrum*, which encompasses mammalian pathogens, plant and soil associated bacteria, but is also regularly found as part of insect gut communities (e.g. Mathew et al. 2012, Aksoy et al. 2014).

The immune challenge had no significant effect on the number of 16S rRNA gene copies, although there was a trend that immune-challenged ants harboured more rather than less bacteria (linear mixed-effects model, N=24, LR=2.87, P = 0.09, Figure 6.8). The three samples that had the most 16S rRNA copies were from three immune-challenged ants that had a high relative abundance of *Entomoplasma* in their gut community (Figure 6.6).

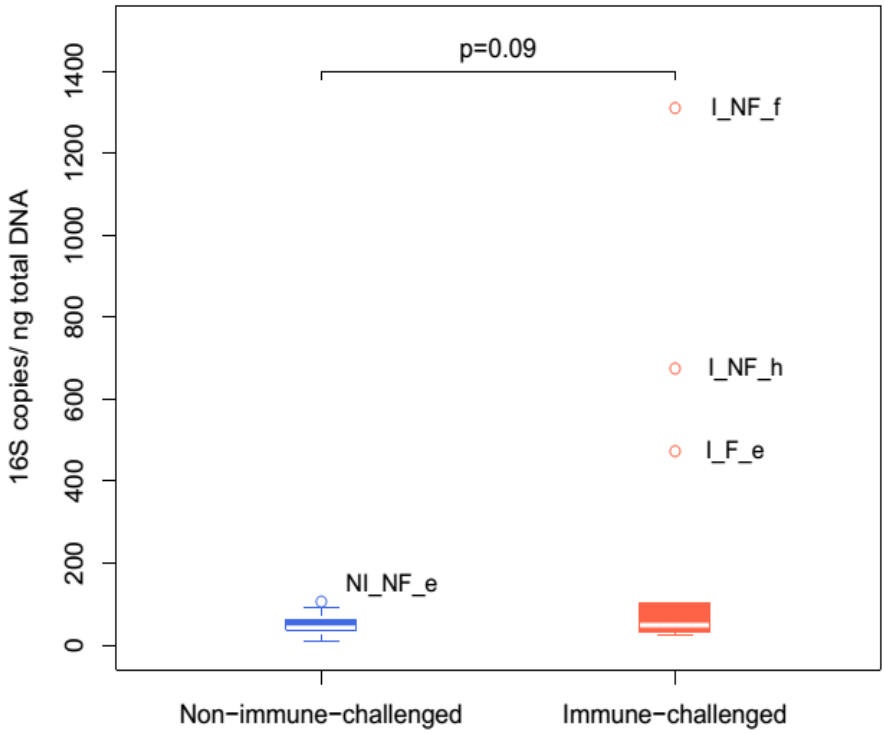

Figure 6.8: The number of 16S rRNA copies per ng of total DNA grouped by immunity treatment. Boxplots as in Figure 6.7. Samples originating from immune-challenged ants tended to have more copies of the 16S rRNA gene. The samples outside the third quartile are represented by dots and their names, corresponding to those used in Figure 6.6, are written on the plot.

Expression of immune genes and the gut microbiome

From contigs that were annotated as antimicrobial effector genes, seven were significantly upregulated in immune-challenged individuals. However, only *abaecin*, *hymenoptaecin* and a *defensin* contig were associated with the Bray-Curtis distances between the gut communities (Table 6.1). The constrained ordination analysis only including the gut community composition, the immune challenge, and the expression (TPM) of *abaecin*, *hymenoptaecin,* revealed 31.19% of the variation in gut communities explained by the model, while *hymenoptaecin* and *defensin* remained significant (CAP, *hymenoptaecin*: F = 6.21, P = 0.01; *defensin*: F = 2.79, P = 0.01; Figure 6.9), immune challenge and *abaecin* were no longer significant in this model (backwards elimination, F = 1.59, P = 0.13 and F = 1. 25, P = 0.18, respectively).

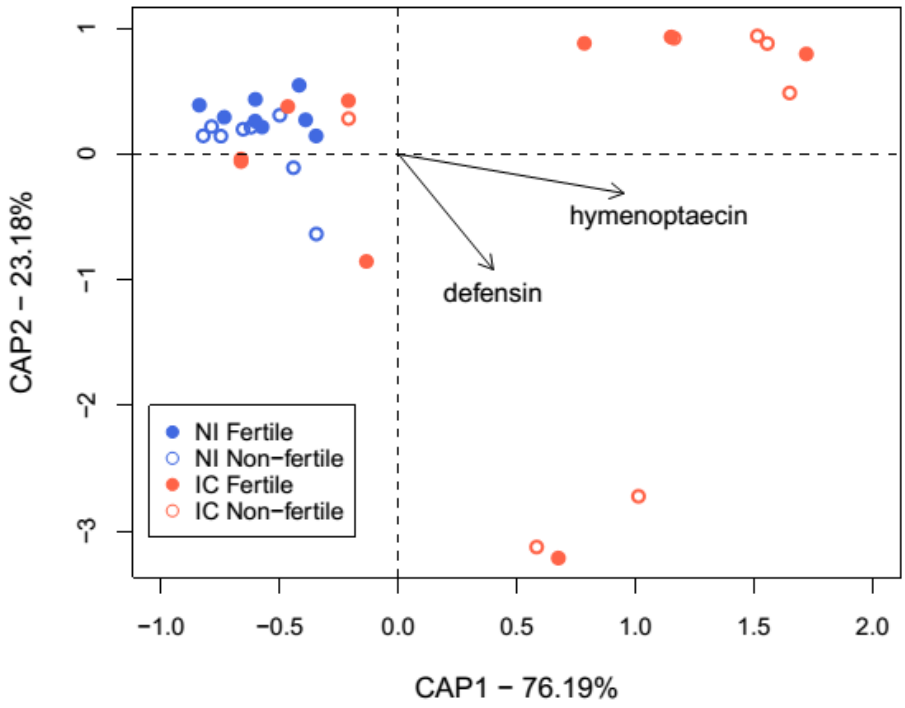

Figure 6.9: Constrained ordination biplot of the T. rugatulus gut communities, showing the association between the expression of the antimicrobial effector genes hymenoptaecin and defensin (FPKM) with the bacterial community variance. The length of the arrows reflects the strength of the correlation of each contig with the gut communities.

Table 6.1: Results of the models testing the effect of antimicrobial gene expression on gut community composition.

Effector gene	F	P
Abaecin	1.90	0.04*
Defensin	2.01	0.03*
Defensin-2	1.73	0.06
Hymenoptaecin	2.42	0.01*
Lysozyme 2	1.56	0.08
Phormicin	1.61	0.08
Sapecin-C	1.66	0.07
Transferrin	1.37	0.15

Discussion

In *Temnothorax* ants, queen removal induces fertility and a lifespan extension in workers (Kohlmeier et al. 2017). In this study we investigated whether immunity or changes in the gut microbiome composition play a role in the regulation of these life history traits. Our gene expression and gut microbiome analyses showed that an immune challenge induced by an injury from a lipopolysaccharide coated needle had a drastic impact on fat body gene expression in *T. rugatulus*, while fertility induction by queen removal had a much smaller effect. A look into functional enrichment revealed that fertile workers invest more into body maintenance, in particular in molecular repair mechanisms. Transcriptomic responses to the immune challenge depended on worker fertility, but even so many immune genes were upregulated in both fertile and non-fertile immune-challenged workers. The immune challenge caused a dramatic drop in gut microbiome diversity, while fertility induction had no effect on the gut microbiome in *T. rugatulus*.

Interactive effects of fertility and immune challenge on gene expression

Our transcriptome analysis revealed that many genes altered their expression in response to an interaction between the fertility and immunity treatments. In the absence of an immune challenge, fertile workers focussed on degrading ROS (*hydrogen peroxide catabolism*). Yet, following the immune challenge, these fertile workers produced more of *alpha-ketoglutarate* (*alpha-KG*), an immune-enhancing molecule that reduces the accumulation of ROS in the mitochondria by inhibiting the ATP-synthase (sub-unit V) and reduces energy consumption (Chin et al. 2014, Fu et al. 2015, Wu et al. 2016). Moreover, this molecule is suggested to be an endogenous tumour-suppressor and has been shown to downregulate TOR and to extend lifespan in *C. elegans* (Chin et al. 2014, Fu et al. 2015). In addition, in response to the immune challenge, fertile workers upregulate *degradation of glutamate for alpha-KG*, while non-fertile workers activate stress response mechanisms, such as degradation of reactive oxygen species, but also downregulate TOR. TOR signalling was proposed

to have evolved in response to temporal variation in the environment by facilitating life history trait plasticity, in particular by varying investment in somatic maintenance (*somatic maintenance polyphenism*, Flatt et al. 2013). Stress causes a downregulation of TOR signalling in *Drosophila*, which in turn induces increased survival and stress resistance (Flatt et al. 2013). Thus, a downregulation of TOR in response to an immune challenge could increase stress resistance in *T. rugatulus* and it is interesting that only non-fertile workers do so, whereas fertile workers appear to use another pathway, that of *alpha-KG.*

The independent effect of fertility on gene expression

No obvious candidate genes or enriched functions associated with immunity were detected in the transcriptomic changes following fertility induction. However, fertile workers upregulate a *vitellogenin receptor.* These receptors facilitate the vitellogenin up-take of eggs during maturation. In *Solenopsis invicta,* highly fecund ant queens overexpress *vitellogenin receptors* in their ovaries (Lu et al. 2009). Also in *T. longispinosus,* this *vitellogenin receptor* is upregulated in queens *vs.* workers and in nurses *vs.* foragers, albeit interestingly its expression does not differ between fertile and non-fertile workers (Feldmeyer et al. 2014). Here, we found fat body expression of the *vitellogenin receptor* to be linked to worker fertility. Moreover, fertile *T. rugatulus* workers upregulate genes with the enriched function *S-adenosymethionine biosynthetic processes*. S-adenosylmethionin (SAM) accumulates during aging in *Drosophila* and lifespan is extended when blocking its synthesis in the nematode *Caenorhabidtis* or increasing its catabolism in the fly (Lee et al. 2014, Obata and Miura 2015). The analysis of the interaction between immune challenge and fertility revealed that fertile workers invest more into molecular repair mechanisms such as *telomere maintenance*, *base-excision repair*, *nucleotide-excision repair* than non-fertile workers do. Since especially telomere maintenance ability is a strong predictor of longevity (Heidinger et al. 2012), we interpret these findings so that fertile workers invest more into body repair, potentially explaining their extended lifespan.

In general, our results show weak transcriptomic changes of fertility genes in the fat body and only few candidate genes or biological pathways altered their activity, potentially indicating that the fat body is not the tissue of the molecular regulation of fertility. Instead, many fertility genes might be directly expressed in ovarian tissues (Cong et al. 2015). We also found no evidence for changes in immunity allocation associated with fertility. However, irrespective from the immune challenge, fertile workers invest more into somatic maintenance or longevity than non-fertile ones, pointing to candidates genes or biological processes involved in the lifespan extension of fertile workers.

The independent effect of immune challenge on gene expression

Compared to the fertility induction, the immune challenge resulted in a stronger transcriptomic response as the expression of more than double as many genes was altered. Perhaps not surprisingly *peptidoglycan catabolism, defence response* and *immune defence* were enriched in immune-challenged workers. Peptidoglycan is a main component of bacteria cell membrane and its catabolism represents a major step in the defence against pathogenic bacteria (Steiner 2004). Unexpectedly *activation of innate immune response*, and the IMP (inosine monophosphate) synthesis was higher in non-immune-challenged workers. Supplementation of IMP, a ribonucleotide monophosphate, positively affects survival after an immune challenge in fish (Song et al. 2012). Overall, our data reveal a downregulation of some aspects of the innate immune system in response to a strong immune challenge. This challenge seems to have induced oxidative stress or increased the production of radical oxygen species. We observed a similar pattern in the interaction analysis, as immune-challenged workers activated molecular repair mechanisms (*base-excision repair, nucleotide-excision repair, telomere maintenance*), which indicate a physiological cost of the immune response. Indeed, an immune reaction is costly and generates reactive oxygen species and molecular damage that has to be compensated (Garschall and Flatt 2018, Navrotskaya and Oxenkrug 2016). Moreover, an immune response is mostly accompanied by immune cell proliferation

(hemocytes), which shortens telomeres (Sequeira 1996, Wong and Collins 2003), and thus could explain the enrichment of telomere maintenance (Wong and Collins 2003).

Gut microbiome changes with immune challenge

Animals generally have intimate relationships with their gut microbes. Yet, the physiological, behavioural and hormonal changes that come with an increase in fecundity in ants, as evidenced also by the strong transcriptomic changes, did not affect the gut microbiome in *T. rugatulus* workers. Although gene expression of fertile and non-fertile immune-challenged ants differed markedly, this did not seem to result in differences in antimicrobial peptide production, as the microbiomes in the gut of immune-challenged ants did not differ depending on fertility. Thus, our study did not provide evidence for a role of gut microbial diversity in the observed lifespan extension with worker fertility, albeit experimental inoculation with specific gut microbes increased survival and pathogen resistance in *C. elegans* and *A. mellifera* (Kwong and Moran 2016, Ikeda et al. 2007, Komura et al. 2013).

However, we found that the injury and LPS exposure caused a drastic decrease in the diversity of the gut bacterial community. In *Drosophila* NF-κB/Relish-driven antimicrobial peptide expression is repressed to maintain a normal gut microbiome (Dantoft et al. 2013, Dantoft et al. 2016). Consistent with this, an immune hyperactivity impacts the healthy gut microbiome in *Drosophila* and humans, resulting in disease and reduced lifespan (Dantoft et al. 2016, Garrett et al. 2010, Mistry et al. 2017). Additionally, parasites seem capable of modulating antimicrobial peptide synthesis in their host to impair the growth of certain gut bacteria species that would otherwise have prevented the parasite's establishment (Vieira et al. 2016). Our results demonstrate that a single immune challenge can cause a sharp decline in gut microbiome diversity in ants, suggesting that spill-over effects of unspecific immune flare-ups can have negative consequences for gut homeostasis.

There is overwhelming evidence that gut bacteria benefit their insect hosts in various ways, for example, by aiding in digestion (Salem et al. 2013, Hu et al. 2018), protecting against pathogens (Ryu et al. 2008) and increasing fecundity (Rosengaus et al. 2011). In another *Temnothorax* species, *T. nylanderi*, (Segers et al., under review) found a positive correlation between the diversity of the colony gut microbiome and productivity. Thus, eradication of the normal gut microbiome following a provocation of the immune system somewhere else in the body could present an additional cost to immune responses in insects, next to the direct energetic costs of immune activity. To the best of our knowledge, this is the first study to demonstrate negative effects of a systemic immune response on gut bacteria in insects. If the individual survives the injury or infection that triggered the immune flare-up, the gut community may be able to re-establish by re-inoculation of bacterial strains through frequent mouth-to-mouth and mouth-to-anus contact ("trophallaxis") with nestmates (e.g. Grüter et al. 2006) and contact with nest material (Powell et al. 2014). It is possible that these options to re-establish a diverse microbiome enable ants to respond so drastically to an immune challenge. The recovery time of the normal gut bacterial community after an immune flare-up should be investigated as a start to assess the costs of gut bacteria diversity loss after immune activity.

Tests for associations between the gut microbiome composition and the expression of several antimicrobial effector genes revealed some genes that may have killed gut bacteria after the immune challenge. Although we did not measure the expression of *hymenoptaecin, defensin* and *abaecin* in the gut tissue directly, antimicrobial peptides are both locally produced by epithelial cells and systemically in the fat body in *Drosophila*, after which they are secreted into the hemolymph (Zhang and Gallo 2016). Thus, the expression of *hymenoptaecin*, *abaecin* and *defensin* in the fat body may reflect the systemic concentrations of their respective proteins and have a measurable effect on the gut microbiome. Insect defensins are mainly active against gram-positive rather than against gram-negative bacteria (Bulet et al. 1999), while abaecins and hymenoptaecin are active against both (Bulet et al. 1999, Casteels et al. 1993). Thus, although LPS is a component of the cell wall of gram-negative bacteria, its use did not result in an immune response that was specific to

gram-negative bacteria. However, the piercing of the cuticle itself may have kicked off an unspecific immune-flare-up.

The lack of difference in absolute bacterial abundance between the samples of immune- and non-immune-challenged ants suggests that after the host immune response wipes out most bacterial species in the gut, unaffected bacteria species can rapidly increase in population size due to a decrease in competition. Similarly, inhibition of the intestinal homeobox gene *Caudal* in *Drosophila* led to an overexpression of antimicrobial peptide genes, resulting in the disappearance of the normal commensal bacteria population and the subsequent proliferation of a pathogen (Ryu et al. 2008).

An increase in relative abundances of bacterial taxonomic groups in *T. rugatulus* after an immune flare-up may indicate special relationships between the ant host and these bacteria, not necessarily mutually beneficial ones. Bacteria from the families Aerococcaceae, Bartonellaceae, Brucellaceae, Entomplasmataceae and Pseudomonaceae did not decrease in relative abundance in the gut communities of immune-challenged ants, suggesting that these endosymbionts can withstand the immune defences of *T. rugatulus*. How these bacteria escaped the immune onslaught from their host and whether this is the result of host-symbiont co-evolution are interesting topics for future research.

Conclusions

Our investigation of the proximate mechanisms regulating fertility and longevity supported some of our earlier predictions, as fertile workers invested more in somatic maintenance and responded differently to an immune challenge. Other expectations such as a role of the gut microbiome in the lifespan extension of fertile workers were not met. Instead, we uncovered an unpredicted dramatic reduction in microbiome diversity caused by the immune challenge and associated with an upregulation of important immune effectors. Indeed, the injection of lipopolysaccharide challenged the immune system of ant workers so severely, that fat body gene expression responded to it much more than to the induction of fertility. The challenge induced physiological stress such as oxidative

stress and molecular damage, which was counter-acted by the activation of stress response and molecular repair mechanisms. The expression of additional genes was linked to an interaction between the two factors. For example, only fertile workers responded to the immune challenge by producing alpha-KG, an important immune-regulator that reduces the accumulation of reactive oxygen species and extends lifespan through the downregulation of the TOR signalling pathway. Fertility induction only weakly altered immune investment, but fertile workers invested more into somatic repair, possibly explaining their extended lifespans. Finally, worker fertility did not alter the composition of the gut microbiome, suggesting that the latter is not involved in the lifespan extension of fertile workers. Yet the single immune challenge caused a drastic loss in gut microbiome diversity and as evidence from other *Temnothorax* ants indicate beneficial effects of a high microbiome diversity in the gut, our study revealed another potential intrinsic cost of an immune reaction, which is the loss of beneficial symbionts.

General Discussion

Matteo Negroni

“What's been gratifying is to live long enough to see molecular biology and evolutionary biology growing toward each other and uniting in research efforts.“

--Edward O. Wilson

About a hundred years after Darwin proposed his general theory of evolution (1859), the development of modern evolutionary theories of aging were great steps forward in our understanding of the how senescence can evolve (Medawar 1952; Williams 1957; Kirkwood, 1977). Based on a decline in the power of selection with age due to extrinsic death, they imply that the evolution of lifespan is modulated by the rate of extrinsic mortality (Medawar 1952). These theories (the **mutation accumulation**, the **antagonistic pleiotropy**, and the **disposable soma**) are not mutually exclusive and all of them have gained experimental support, but the importance of one relative to the other is difficult to quantify. On a proximate level, the underlying cause for senescence and intrinsic death are not fully understood. One possibility is that it is due to an accumulation of physiological somatic damage (**somatic molecular damage** theory) with age such as molecular damages on the DNA or proteins (Kirkwood et al. 1979, Kirkwood and Austad 2000, Lombard et al. 2005, Schulz et al. 2007, Sun et al. 2016). Another possible cause of senescence could also be the accumulation of the excessively biosynthesized molecules at old age, which although beneficial at an earlier stage are causing aging later in life (**hyperfunction** theory; Blagosklonny 2008; Gems and de la Guardia 2013, de Verges and Nehring 2016). The difficulty in identifying the key proximate causes of senescence can be due to a general deterioration of interconnected biological processes, leading to convergent phenotypical changes. At the organismal level, lifespan can be regulated by and is susceptible to numerous physiological traits and environmental factors including nutrients, reproduction, immunity, metabolic rate, or the gut microbiome, but the mechanisms involved are poorly understood. Often interpreted as a trade-off, lifespan is negatively linked to reproduction in solitary species (Reiznick 1985; Westendorp and Kirkwood 1998; Sgro and Partridge 1999, Partridge et al. 2005). In social insects, these two traits appear positively associated as the most fertile individuals live longer, but mechanisms involved remain however largely unknown.

Due to their social life, social insects display a number of life history and genetic characteristics, which makes of them a unique system for studying the molecular bases of senescence and life history trait-associations. Queens have been recorded as the longest-lived

insects that we know of. The remarkable plasticity in longevity and fecundity brings the opportunity for identifying the molecular bases of their regulation (Keller and Jemielty 2006, Lucas et al. 2016). The reshaping of life history trait associations, such as the one between lifespan and reproduction may contain keys for understanding the physiological constraints resulting in a trade-offs between these two traits in solitary species including us (Rodriguez and Flatt 2016, Kramer et al. 2016). Intriguingly, researches focussing on the ultimate and proximate bases of senescence, have for a long time ignored the potential of social insects for this purpose.

In this thesis, I investigated the ultimate and proximate bases of the regulation of lifespan and reproduction in social insects. A first step consisted in identifying the evolutionary drivers of life-history evolution of female castes (**chapter 1**). In a second phase, I experimentally tested the influence of different factors, including some of these, on life-history strategies in *Temnothorax ants* (**chapter 2-6**). I investigated the link between fertility and somatic maintenance within castes, as well as the role of other factors or traits such as nutrient intake, immunity, or the gut microbiome on the regulation of lifespan and reproduction (**chapter 2-6**). By connecting phenotypic results to gene expression data, I shed light on the molecular basis of this regulation as well as the proximate mechanisms underlying the reshaping of the longevity/fecundity trade-off in social insects (**chapter 2-4 and 6**).

Ultimate and proximate factors influencing life-history strategies and evolution in social insects

Testing the effect of the main evolutionary drivers of social insect life history traits, on *T. rugatulus* revealed that those also impact life-history strategies at the individual level. Our results point two colony-level traits, which are colony size and social structure, as main determinants of queen fertility. At a more proximate level I show that social environment, food availability and immunity can play an important role in the plastic regulation of lifespan and reproduction in queens or workers. Although

my study does not indicate an important role of gut microbiome in the regulation of lifespan and reproduction, I demonstrate a strong interaction between gut bacteria composition and the host immune system. Finally, I point to a more complex link between metabolic rate, body size and lifespan than that described by the **rate of living** theory.

The role of colony size

I highlight colony size as a major evolutionary driver affecting both individual level and colony level life-history evolution (**chapter 1**). Colony size strongly impacts colony fitness, as larger colonies have a better colony survival rate, higher resilience to environmental variation and reproductive output (Elmes and Wardlaw 1982, Kaspari and Vargo 1995, Changizi 2002, Franks et al. 2006, Shik 2008). Other colony-level or individual-level traits associated with colony size may thus be under a strong selection. Indeed, with increasing colony size life-history traits of queens and workers, such as body size, longevity and fecundity diverge more and more between the two castes (Kramer and Schaible 2013, Bourke 1999, Ferguson-Gow 2014). In larger colonies the reproductive potential of worker decreases which consequently is expected to lower selection for fecundity in this caste. Although colony size is determined by worker turnover, data are lacking to establish the importance of colony size in the evolution of worker lifespan (Giraldo and Traniello 2014). In a larger colony, the queen is better protected from extrinsic risks, which should favour selection for her longevity. As colony size reflects colony workforce, fitness benefits of a larger colony size may favour selection for a higher egg-laying rate in queen, or alternatively multiply the number of reproductives in the colony (*polygynou*s; Keller and Genoud 1997, Boulay et al. 2014).

My results on *T. rugatulus* reveal the determinant effect of colony size on queen and colony egg production. I show in **chapter 4** that *T. rugatulus* queens increase their investment into fertility and anti-aging mechanisms, via the overproduction of antioxidants, not only with age but also with increasing colony size. While queen number had no influence on the total colony egg production, I

additionally demonstrate in **chapter 2** a causal positive effect of colony size on queen and colony egg production.

The role of social structure and founding strategy

In social insects, social structure is not only associated with queen founding strategy but also with queen body size lifespan and fertility (Bourke and Franks 1995, Keller). Queens that start new colonies independently commonly do not accept additional queens later on (monogynous) in the colony, while species in which the queen start a new colony with the help of workers from the mother nest (dependent colony founding) are mostly polygynous. Furthermore, queens of polygynous species are typically shorter-lived, less fecund and smaller compared to queens from monogynous ones (Bourke and Franks 1995). With the occurrence of two queen morphs differing in body size, associated with founding strategy and loosely with social structure, *T. rugatulus* (Rueppell et al. 1998, Rueppell et al. 2001 a, b) seems to be the perfect system for investigating the origin of this *syndrome of polygyny* while controlling for any species-specific confounding effects.

As predicted in **chapter 1,** my results **chapter 2** indicate that differences in queen body size in *T. rugatulus* may be rather explained by associated differences in founding strategy than by differences in social structure as small queen laid as many eggs as large ones (Schmid-Hempel 1998, Gordon and Kulig 1996, Hölldobler and Wilson 1990, Schmid-Hempel 1998, Peeters and Ito 2001, Cronin et al. 2013). In contrast to a dependent colony-founding mode, the independent colony founding success is higher for larger queens as body size reflects larger body resources available for the production of the first generation of worker. In the presence of workers, queen resources and extrinsic risks rather depend on colony size and queen number than body size that may not have such a direct causal link with queen fertility and survival. Variation in adult to juvenile mortality associated with difference in founding strategy, may mainly explain why polygynous queens live shorter than monogynous ones in general (**chapter 1**, Schmid-Hempel 1998, Gordon and Kulig 1996, Hölldobler and Wilson 1990, Schmid-Hempel 1998, Peeters and Ito 2001, Cronin et al. 2013).

If juvenile mortality exceeds adult mortality, which is the case of queen with an independent colony founding mode, life history theories predict an evolution toward a perennial iteroparous lifestyle, a late maturity and a longer lifespan (McGlynn 1999, Macevicz and Oster 1976). Nevertheless, social structure in itself should be considered as an additional evolutionary driver of queen lifespan and reproduction. I described in **chapter 1** why selection for these two traits may be relaxed in case of secondary polygyny (re-adoption of daughter queens) (Boomsma et al. 2014, Bourke 2007, Schrempf et al. 2011, Heinze and Schrempf 2008). I demonstrate **chapter 2** that, being limited by the per-queen worker number, individual queen egg-laying rate was reduced in polygynous compared to monogynous one. The high longevity of *Temnothorax* queens limits the recording of their long-term survival (Plateau 1986). I therefore cannot provide evidence that smaller and/or polygynous queens live shorter than monogynous ones or that independent-colony founding macrogynes live longer than microgynes that can only start their colony dependently (**chapter 1**). I nevertheless demonstrate that both microgynes and macrogynes survive similarly well oxidative stress, which appears to contradict my prediction. Yet, concluding that the two queen morphs do not differ in lifespan may be an over-interpretation of the data (**chapter 2**). The microgynes show a decline in fertility in response to oxidative stress induction, while macrogynes do not, and this could be interpreted as a sign of faster senescence (Vom Saal and Finch 1988). Due to differences in founding strategies and juvenile to adult mortality, the age class distribution of queens in the field possibly differed between the two queen morphs, resulting in small queens being potentially on average younger at the time of collection than the large ones.

The links between body size and metabolic rate, lifespan and egg-laying rate

The absence of a link between body size and survival to oxidative stress was unexpected given that the metabolic rate is twice higher in microgynes than macrogynes (**chapter 2**, Harman 1956; Speakman 2005, Finkel and Holbrook 2000). Assuming a direct link between metabolic activity, ROS production, oxidative damage and lifespan (Speakman 2005, Finkel and Holbrook 2000), I expected

microgynes to produce more antioxidants or better repair mechanisms to neutralize a higher production of ROS and limit oxidative damages. The lack of evidence for this by looking at the fat body transcriptome may indicate a more complex relationship between metabolic rate, ROS production and lifespan, than expected by the **rate of living** theory (Chown et al. 2007). The fact that macrogynes and microgynes differed in the activity of certain metabolic pathways such as the one of malate, known to also influence lifespan, may suggest that not only metabolic activity but also the nature of the metabolism should be taken into account regarding its influence on lifespan.

The role of immunity and the gut microbiome

Studies in social insects on the molecular bases of aging often ignore the role of immunity. My results suggest a role of the immune system in the regulation of lifespan and reproduction in *T. rugatulus*. Indeed, changes in fertility and body maintenance investment were consistently accompanied with changes in expression of immunity related genes (**chapter 3, 4** and **6**). Stimulation of egg production in queens that caused the upregulation of DNA repair genes also induced changes in the expression of genes involved in the major immunity pathway of insects: TOLL (Valanne et al. 2011). Long-lived fertile workers differed in their transcriptomic response to an immune challenge, from the shorter-lived non-fertile ones. With age, increasing fertility and antioxidant production, queens show a decrease in the activity of the TOLL pathway (**chapter 4**), suggesting a trade-off between immunity, lifespan and reproduction (Moret and Schmid-Hempel 2000). We found in **chapter 3**, that food availability increases fertility and somatic maintenance activity, but also the expression of immunity genes. In this experiment, queens may not have benefited from social immunity, which could have been the reason of this higher investment into personal immunity only when resources were not limited (Corona et al. 2007). Immune reaction generates ROS, susceptible to induce oxidative damages (Garschall and Flatt 2018; Navrotskaya and Oxenkrug 2016). In *T. rugatulus* the analysis of the transcriptome reveals that the immune challenge induced an immune response in workers accompanied by a response to oxidative stress. Recent studies have shown that while the

inflammatory reaction accelerates aging a better stability of the immune system delays it (Xia et al. 2016, Fabian et al. 2018). Our results in *T. rugatulus* suggest a link between lifespan and stability of the immune system. Fertile and longer-lived workers may specifically generate more alpha-ketoglutarate (alpha-KG), a stabilizer of the immune system homeostasis (Wu et al. 2016), in response to the immune challenge. As alpha-KG has also been shown to extend lifespan via the down regulation of TOR (Chin et al. 2014; Fu et al. 2015), fertile workers could, via this, compensate the oxidative damage induced by the immune response.

A higher production of ROS by the immune reaction is one of the physiological costs of immunity but its impact can be also less direct. I show that the immune challenge that triggered an immune response was followed by a drastic drop in gut microbiome diversity (**chapter 6**). Although I did not find any evidence for a role of the gut microbiome composition in lifespan extension of fertile workers, the loss of symbionts as unintended effect of the immune response is susceptible to have a negative effect on individual fitness and potentially on survival.

The role of food intake

As demonstrated repeatedly in numerous organisms our results suggest an important role of food intake on the regulation of fertility and body maintenance in *T. rugatulus* queens. We reveal a positive effect of food intake on queen fertility. However, instead of a negative effect on longevity, as expected in solitary species, I highlight in **chapter 3**, in *T. rugatulus* queens, a positive influence of nutrients on somatic maintenance via autophagy. The higher frequency of feeding could allow microgynes to be as fertile and as resistant to oxidative stress as macrogynes, despite their higher metabolic rate. Being established in a mature colony, queens receive more food from workers than during the founding phase, and I show in **chapter 4** that they also invest more into anti-aging mechanisms and fertility. The social structure influences egg-laying rate but not the frequency of trophalaxis (**chapter 2**). This may suggest that the quantity is not sufficient for an effect on at least fertility. Overall, we

reveal a reversal of the antagonistic effect of nutrients on somatic maintenance and reproduction in *T. rugatulus* compared to solitary organisms.

Molecular basis of the regulation of lifespan and reproduction

I report a number of factors influencing the regulation of fertility, lifespan and or somatic maintenance investment at the adult stage. Phenotypic changes in ovary development or activity were relatively weakly reflected on the transcriptomic level in the fat body of queens or workers, and not at all in queens' brains. I suggest further studies to look at the ovarian tissue directly to identify fertility genes (Cong et al., 2015). In contrast, transcriptomic analyses revealed important changes in the expression of somatic maintenance-related genes to occur in the fat body, pointing that regulation of lifespan or the fight against senescence occurs in this physiologically active tissue. These results bring a significant insight for our knowledge of the proximate bases of senescence in *T. rugatulus*. We moreover bring evidence for an alteration of certain pathways in this species compared to solitary one, and highlight their potential role in the reshaping of the trade-off.

Proximate causes of senescence in T. rugatulus

The different facets of investment in somatic maintenance reported in this thesis in queens or in workers include autophagy, the production of antioxidants, protection against molecular damages, and DNA repair mechanisms (**chapter 3, 4 and 6**). A higher investment into somatic maintenance, however, does not necessarily lead to an increase in lifespan. An elevated molecular repair activity may not affect the rate of molecular damage accumulation if at the same time the genesis of these damages is increased (Lucas *et al.* 2017). While established queens express more the antioxidants, *catalase* and *superoxide dismutase*, or a variety of heat chock proteins (*hsp 70 kDa*), they are also older, more fertile and maybe more exposed to oxidative damages due to a higher ROS production (Lucas et al. 2017). In queens we were unable to correlate the expression of somatic maintenance genes with survival data, but we demonstrate that an artificial induction of oxidative stress importantly

reduces the survival rate of queens, which support the **free radical** theory. In workers, the activation of DNA repair mechanisms including telomere maintenance correlates with lifespan extension, and suggests the accumulation of molecular DNA damages as a proximate cause of senescence (**molecular damage** theory). To test this hypothesis, I propose comparing the rate of damage in the fat body of workers of a specific age, between queen-right and queen-less nest. Something similar has been done in *Lasius niger*, where the queens that over express DNA repair genes compared to workers do not show a lower rate of accumulation of molecular damages (Lucas et al. 2016, Lucas et al. 2017a). However, the damage to the DNA was measured in the head and in the legs, where only few somatic cell divisions are expected to occur compare to the fat body (Abernethy 1998, Rockstein 1973).

Evidence for a reshaping in the molecular pathway compared to solitary species

In queens and workers, fertility was positively associated with the expression of somatic maintenance-related genes in the fat body (**chapter 3,4** and **6**), and also linked to a longer life of workers (**chapter 6**). This indicates that the positive association between reproduction and lifespan is also true within castes, and may underlie modification in molecular pathways compared to solitary species, allowing for a positive association between longevity and fecundity in social insects (Rodrigues and Flatt 2016; Figure disc.1).

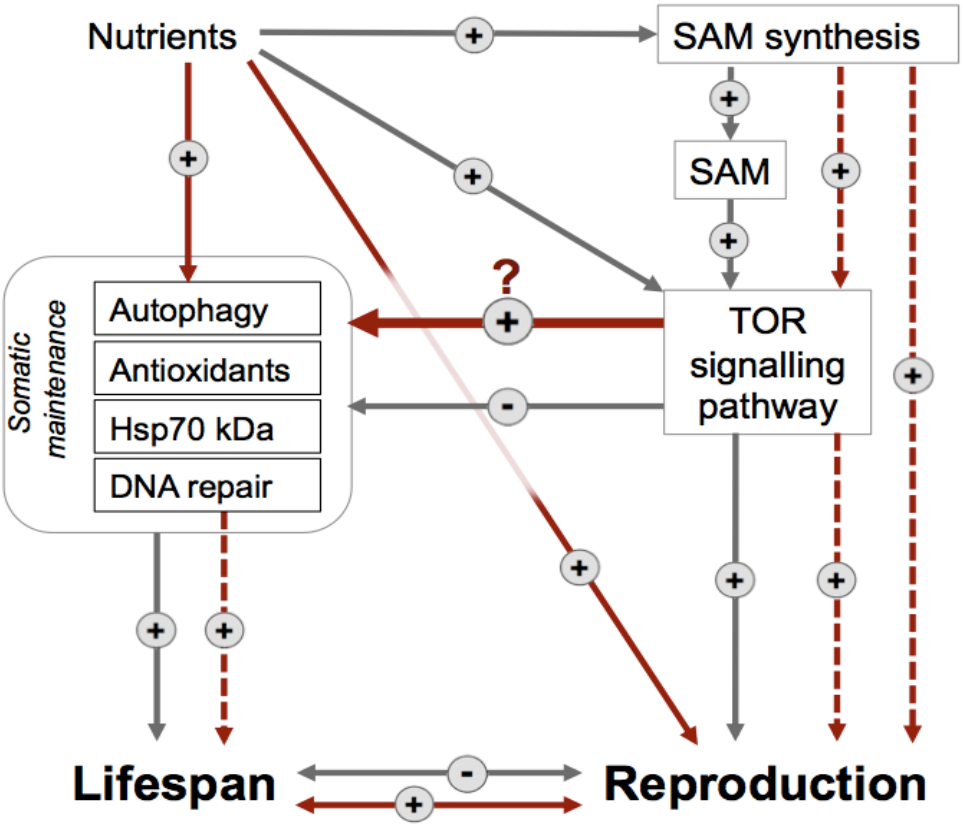

Figure disc.1: Flow chart scenario depicting the identified biological processes or factors influencing the regulation of lifespan and reproduction in *T. rugatulus (red arrows)* in comparison with what is known from solitary species (grey arrows). Arrows represent correlations (dashed lines) or causal (solid lines) positive (+) or negative (-) relationships; the large red arrow with the question mark illustrates a possibly modified interaction in our species compared to solitary one (i.e. *Drosophila melanogaser*), deduced from the different results of my dissertation; TOR: Target of rapamycin; SAM: S-adenosylmethionine. In solitary organisms the activation of the nutrient sensitive TOR signalling pathway in response to nutrient intake has a positive influence on reproduction, inhibits somatic maintenance mechanisms (autogphany, DNA repair activity, antioxidants and hsp70 kDa production) and has a negative effect on lifespan (Lee et al. 2008, Hoedjes et al. 2017, Kenyon 2005, Kenyon 2010, Flatt et al. 2013). This pathway importantly contributes to the negative association between lifespan and reproduction observed in solitary species (Flatt et al. 2013). My results in *T. rugatulus* reveal a positive and causal (**chapter 3**, **5** and **6**) association between lifespan, somatic maintenance and reproduction, and suggest an alteration of the negative effect of TOR on somatic maintenance, compared to solitary organisms. A higher synthesis of SAM, suggested to be an intermediate molecule of the activation of TOR by nutrient intake in *Drosophila melanogaster* (Obata and Miura 2015), correlates with reproduction and the activity of TOR in *T. rugatulus* (**chapter 4** and **6**). We show in *T. rugatulus* that nutrient intake has a positive effect on reproduction, but in contrast to solitary species (Madeo et al. 2015), has a negative effect on autophagy (**chapter 3**).

Indeed, some of my results suggest that such alteration occurred in our species, and point to some candidate genes and pathways. Among them I would like to focus on the expression pattern of genes related to S-adenosylmethionine (SAM) synthesis. SAM production in mammals and flies is associated with the positive effect of methionine-rich diet on fertility (Obata and Miura 2015, Lee et al. 2014), but consequently with its lifespan shortening effect. Indeed, SAM is the first metabolite of methionine utilisation, and is required for the activation of TOR signalling pathways (Gu et al. 2017; Figure disc.1), which has this antagonistic effect on longevity and fecundity. Moreover, the amount of SAM in the fat body is associated with aging in *D. melanogaster*, but the age–dependent elevation is delayed by methionine restriction (Obata and Miura 2015). I found SAM biosynthesis to increases with age and fertility in queens (**chapter 4**). I also found SAM biosynthesis higher in fertile workers compared to non-fertile ones (**chapter 6**). SAM production correlates with fertility in *T. rugatulus* queens and workers, similar to solitary species (Figure disc.1). The fact that the expression of genes related to SAM synthesis also correlates with lifespan extension in workers while they are associated with aging in the fruit fly (Obata and Miura, 2015) may indicate two things: i) the negative effect of SAM synthesis, through the activation of TOR on lifespan is compensated by other body maintenance related mechanisms including the production of antioxidant or the activation of molecular repair; ii) the negative effect of SAM synthesis on lifespan is avoided or reversed as a result of an alteration, in our species, of the related pathways likely downstream of SAM synthesis, thus including TOR signalling one (Figure disc.1). The fact that a lower synthesis of SAM was associated with the downregulation of TOR in **chapter 4** suggests that the activation of TOR by SAM was conserved in *T. rugatulus* and solitary species (Gu et al. 2017). Starvation-mediated lifespan extension requires the induction of autophagy as shown in solitary species (Jia and Levin 2007, Madeo et al. 2015). Autophagy is a cytoplasmic recycling process that counteracts the age-associated accumulation of damaged organelles and proteins, that is inhibited by TOR activity (Kamada et al. 2010, Sasaki et al. 2011). Contrasting with solitary organisms I show in *T. rugatulus*

queens (**chapter 3**) a positive effect of food availability on the expression of *sequestosome-1*, an essential cargo-protein in the process of autophagy (Figure dsic.1). This highly conserved protein from vertebrates to *D. melanogaster* and *C. elegans* is used as a proxy of autophagic activity (Sasaki et al. 2011, Pircs et al. 2012, Pietrocola et al. 2018). I could not connect the expression of *sequestosome-1* with queen survival but the positive effect of autophagy on lifespan has been shown in mice, flies and worms (Madeo et al. 2010, Bitto et al. 2014). Linking different results (**chapter 3, 4** and **6**) obtained in queens and workers, suggests thus that the antagonistic pleiotropic effect of TOR signalling pathways on body maintenance and reproduction is altered in *T. rugatulus* compared to solitary species (Figure disc.1). This may contribute to the observed positive association between lifespan and reproduction observed (Figure disc.1).

Perspectives for future researches

I identify in this thesis a number of candidate genes and pathways potentially involved in the reshaping of the trade-off between lifespan and reproduction, at least in our species. I suggest further studies to investigate in more details the gene regulatory network in *T. rugatulus* in comparison to what is known from *Drosophila* in order to identify which key changes are responsible for the positive link between lifespan and reproduction. Using a candidate gene and pathways approach, one could sequentially knock-down genes of interest and look at the consequence in the expression of the other downstream ones as well as associated phenotypic changes. I suggest having a particular focus at genes of the TOR signalling pathway (Figure disc.1). Moreover, my results point to a link between personal immunity and the regulation of lifespan and/or reproduction. Benefiting from social immunity, reproductives could delegate the physiological cost of personal immunity in term of lifespan and reproduction, to their non-reproductive helpers. I suggest to investigate the proximate role of the immune system on the longevity/fecundity trade-off reversal, in relation with social immunity.

One important aspect of this dissertation is that queens and workers can adapt their strategies of investment into reproduction, immunity and body maintenance according to their social environment, typically colony size, queen presence, queen number or egg presence (**chapter 2**, **3**, **5** and **6**). An important question to address for further research is by which mechanisms queens and workers change their strategy of investment in response to their social environment. Supported by the results, I propose modifications in their nutrition, mediated by non-reproductive workers, to trigger these physiological changes. In the colony queens or dominant-reproductive workers receive their food source from non-reproductive workers via trophalaxis. Possibly non-reproductive workers respond to the social information by modifying quantitatively and/or qualitatively the feeding of reproductives or future reproductives, resulting in changes in the regulation of immunity, reproduction and somatic maintenance. Reproductives receive more trophalaxy and maybe a trophalactic fluid enriched in certain nutrients or amino acids (Piper et al. 2017). In mice and *Drosophila* the ratio of protein and carbohydrate determines the effect of nutrient on lifespan and reproduction, and it has been shown in *Drosophila* that matching the amino acid balance from translated-exome optimizes growth and reproduction without cost to lifespan (Piper et al. 2017). In addition to nutrition, reproductives could receive certain components such as hormones, transcription factors, or siRNA, specifically present or enriched in the throphalactic fluid (Leboeuf et al. 2016). These components could directly influence gene regulation and potentially be responsible for the longevity/fecundity reversal trade-off. I suggest further studies to determine the composition of the trophalactic fluid transferred toward reproductive individuals, and explore its effect on the regulation of lifespan and reproduction. To test if certain candidate components specifically transferred to reproductive individuals are sufficient for a positive link between longevity and fecundity, one could look at their effect on the regulation of these two traits in a solitary species. Additionally, I suggest to characterize the effect of the diet on the regulation of longevity and fecundity by manipulating the composition in amino acid as well as the protein to carbohydrate ratio (Piper et al. 2017).

Higher selective pressure for lifespan in reproductive individuals compared to non-reproductive ones is a consequence of eusocial life, which led to the emergence of this positive association between longevity and fecundity specific to eusocial species (**chapter 1**). I bring evidence for molecular modifications in the studied species compared to solitary ones, potentially involved in this reversal link between these two traits (**chapter 3-6**). The rising questions that remain to be answered are: i) how conserved are the mechanisms allowing for this reversal of the trade-off across eusocial species? ii) is there a single or several ways to achieve a positive link between longevity and fecundity? iii) at which stage of the evolution of eusociality in ants, termites, wasps and bees, those different mechanisms emerged? I suggest further researches to investigate the regulation of lifespan and reproduction in different social species of ants, termites and bees. Comparing these data to the ones obtain in a solitary reference species would allow to indentify mechanisms that have been reshaped with the evolution of sociality. Finally, a comparative study of these mechanisms across social insect species would allow accessing for their evolution.

Conclusions

The review of the literature allowed me to identify several individual and colony level factors driving evolution of life history traits in social insects, whose reshaped the trade-off between lifespan and reproduction. Testing experimentally these factors, I could show that most of them impact life-history strategies not only on an evolutionary scale, but also at an individual one. Colony size and social structure strongly influence regulation of queen and worker life history traits. My results illustrate a plastic regulation of fertility and lifespan at the adult stage, where both queens and workers adapt their strategy of investment according to age and or environmental conditions. Connected to phenotypic results the analysis of gene expression allowed me to identify numerous candidate genes for the regulation of lifespan in workers and in queens with some being potentially involved in the longevity fecundity reversal trade-off in social insects. Moreover, I show that the positive link between the two traits was consistently verified even within caste. With some overlaps queens and workers

seem to differ in their use of molecular mechanisms for fighting against senescence, which leaves space for the deleterious mutation to accumulate within caste-specific expressed genes. Taken together my results suggest an important role of the food intake and the immune system in the particular regulation of lifespan and reproduction of social insects, which supports the hypothesis of a colony level trade-off in resources allocated to different type of colony members. Moreover, the absence of opposite effect of nutrient intake on somatic maintenance and fertility may indicate an avoidance of an antagonistic pleiotropic effect of growth on reproduction and lifespan likely due to a modification in the molecular pathways, as suggested by some our results. Further research should i) investigate the proximate impact of the immune system in the regulation of lifespan in social insects; ii) determine the composition of the trophalactic fluid received by the queen from workers and its importance for queen extraordinary longevity and fecundity and on the reversal of the trade-off in social insects**;** iii) looking at the conservation of the molecular mechanisms involved in the regulation of lifespan and reproduction across eusocial species.

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