Reversal of the fecundity/longevity trade-off across social transitions in ants

Dissertation

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“*Nadakkum neram kokkum naavum neetikonde koodum*
koottar kandaaltheerum jeevan ayyo! thanthaane;
(Predators come at us with their beaks and tongues as we walk along, we are finished if we are seen)

Nadakkunne ee irukaalikal ee muthuke chavituum odikkum
othukkum munne pothiyum kadikkum idanjaal thanthaane;
(The two-legged creatures step on us but we bite them before they break our backs)

Maalathil cherum munne, marikkan pedimunde
Madakkam chorillathillee;
(Though we are deathly afraid, we will not return to our nest without food)

Porikkum choodum konde, kilirkkum maarim kondee
Nadathum yudham theera mannil odunnunde njangal;
(In the blistering heat and bursts of rain, we are still engaged in the never-ending battle in the soil)

Paavam urumbe thanathaane thanathaane
Thaalam paranjaane ponathaane;
(Poor ants marching to their own beat)
Aanee urumbe thanathaane thanathaane
Thaalam paranjaane ponathaane;
(We are ants marching to our own beat)”

-Excerpt from the Malayalam song *Urumbu* (Ant) by Thaikkudam Bridge

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Note: Supplementary material not presented due to the nature or size of the files are available online: Supplementary files for all chapters

Zusammenfassung

Die Begriffe „Alterung" oder „Seneszenz" beziehen sich auf die fortschreitende Verschlechterung der physiologischen Funktionen eines Organismus im Laufe der Zeit. Erklärungen für das Altern finden sich in Evolutionstheorien und beschreiben einen Kompromiss zwischen Fruchtbarkeit und Langlebigkeit. Dieser Kompromiss wird durch die Beobachtung gestützt, dass die Fruchtbarkeit eines Individuums negativ mit seiner Langlebigkeit korreliert ist. Soziale Insekten wie Termiten, einige Bienen und Wespen sowie Ameisen stellen einen wichtigen Übergang in der Evolution dar, der durch Gesellschaften mit ausgeprägter Arbeitsteilung bei der Fortpflanzung gekennzeichnet ist. Ein oder einige wenige Individuen, die "Fortpflanzungsfähigen", haben das Monopol auf die gesamte Nachkommens Produktion in einer Kolonie, während die nicht fortpflanzungsfähigen Arbeiterinnen andere Aufgaben wie Brutpflege, Nahrungssuche und Nestverteidigung übernehmen. Im Gegensatz zu anderen klassischen Alterungsmodellen leben bei sozialen Insekten die fruchtbarsten Individuen am längsten. Je nach Spezies bedeutet das eine Lebensspanne von einigen Jahren bis zu über zwei Jahrzehnten. Im Gegensatz dazu weisen die unfruchtbaren Arbeiterinnen eine kurze Lebensspanne von einigen Wochen, Monaten und in seltenen Fällen Jahren auf. Bei vielen sozialen Insektenarten sind die Arbeiterinnen jedoch trotzdem in der Lage sich fortzupflanzen und leben, wenn sie dies tun, in der Regel länger.

Ameisen weisen eine große Artenvielfalt auf, die sich auch hinsichtlich des Fortpflanzungspotenzials der verschiedenen Kasten unterscheidet. In der Regel ist die potenzielle Fruchtbarkeit der Königinnen viel höher als die der Arbeiterinnen. Darüber hinaus werden das Fortpflanzungspotenzial und die Restlebensdauer der Arbeiterinnen unter anderem durch ihre Fortpflanzungsart, ihr Alter, die von ihnen ausgeführten Aufgaben und die Anzahl der Fortpflanzungsorgane in ihrer Kolonie beeinflusst. Viele der oben genannten Faktoren lassen sich beobachten und manipulieren. Dies macht Ameisen zu einem idealen Modell, um zu untersuchen, ob die vorhergesagte Umkehrung des Kompromisses zwischen Langlebigkeit und Fruchtbarkeit bei Ameisen existiert und wie sie je nach Fortpflanzungspotenzial der Königinnen und Arbeiterinnen variiert.

Ich habe getestet, ob die Entfernung der Königin aus der Kolonie, die bekanntermaßen die Fortpflanzung der Arbeiterinnen einiger Arten anregt, auch dazu führt, dass sie widerstandsfähiger gegen oxidativen Stress werden. Die Arbeiterinnen von *Atta colombica* und *Acromyrmex echinatior* aus Kolonien ohne Königin überlebten oxidativen Stress besser als jene, die mit ihren Königinnen („queenright") gehalten wurden. Im Gegensatz zu den Arbeiterinnen der Ameisenart *Temnothorax rugatulus*, bei denen kein signifikanter Unterschied zwischen den beiden Gruppen festgestellt wurde (Kapitel 1). Ein ähnliches Experiment wurde mit der invasiven Art *Lasius neglectus* (Kapitel 2) durchgeführt, bei der die Arbeiterinnen obligatorisch steril sind und sich daher nicht fortpflanzen können. Hier hing die Überlebensrate der Arbeiterinnen nicht von der Anwesenheit der Königin ab. Dieser fehlende Unterschied zwischen Arbeiterinnen mit und ohne Königin wurde auch durch die Transkriptionsaktivität in den Fettkörpern bestätigt, die nicht von der Anwesenheit der Königin abhing. Ich zeige, dass das

Fortpflanzungspotenzial der vier Arten, das durch ihre Fortpflanzungsweise zusammen mit anderen naturgeschichtlichen Merkmalen beeinflusst wurde, erklären kann, ob sie aktiv in das Überleben unter oxidativem Stress investieren konnten.

Ich nutzte die klonale Ameisenart *Platythyrea punctata* (Kapitel 3), um Alter und Kaste voneinander zu trennen. Die reproduktiven Tiere zeigten weniger Transkriptionsveränderungen als die nicht fortpflanzungsfähigen Tiere, was auf ein verlangsamtes Altern bei den reproduktiven Tieren hindeutet. Ich habe Königinnen von *L. neglectus* (Kapitel 2), *A. colombica* (Kapitel 4) und *A. echinatior* (Kapitel 5) verwendet, um weitere Erkenntnisse über Gene mit altersabhängiger Expression in reproduktiven Tieren zu gewinnen. Überraschenderweise war bei keiner der Königinnen ein signifikanter Rückgang der Fruchtbarkeit mit dem Alter festzustellen. Die meisten Veränderungen wurden bei der Untersuchung von über 15 Jahre alten *A. echinatior* Königinnen beobachtet, bei denen viele Anzeichen der Seneszenz, wie z. B. eine gestörte Proteostase und eine geringere Insulinsignalisierung, zu beobachten waren; allerdings eher im Gehirn als in den Reproduktionsorganen (Ovarien) oder deren Regulator, dem Fettkörper. Daher wird das fruchtbare Individuum nach seiner Fruchtbarkeit selektiert, welche während der gesamten Lebensdauer optimiert wird. In dieser Arbeit werden auch andere Merkmale des Alterns und Kandidaten für künftige Studien identifiziert.

Summary

‘Ageing’ or ‘senescence’ refers to the progressive deterioration of physiological function over time. Evolutionary theories explain ageing with a life-history trade-off between fecundity and longevity. This is supported by the observation that an individual’s fertility is negatively linked to its longevity. Social insects such as termites, some bees and wasps, and the ants represent a major transition in evolution characterized by societies with distinct division of reproductive labour. One or a few individuals, the ‘reproductives’, monopolise all offspring production in a colony, while the ‘non-reproductive’ workers undertake other tasks such as brood care, foraging and nest defence. Contrary to other classical ageing models, in social insects, it is the most fertile individuals that live the longest: a few years to over two decades. In contrast, workers exhibit short lifespans of a few weeks, months, and in rare cases years. In many social insect species workers occasionally reproduce and then typically live longer.

Ants showcase a diversity of species with variation in reproductive potential of the different castes. Typically, the fecundity of queens is much higher than that of workers. Additionally, the reproductive potential and residual life span of workers is affected by their mode of reproduction, their age, tasks they perform, the number of reproductives in their colony among others. Many of the above-mentioned factors can be observed and manipulated. This makes ants ideal models to study whether the predicted reversal of the trade-off between longevity and fecundity exists in ants, and how it varies according to the reproductive potential of queens and workers.

I tested whether queen removal, known to induce reproduction in workers of some species, would also make them invest into longevity by becoming resilient to oxidative stress. *Atta colombica* and *Acromyrmex echinatior* workers from queenless colonies survived oxidative stress better than when they were kept with their queens (queenright), unlike *Temnothorax rugatulus* workers, which showed no significant difference between the two groups (Chapter 1). A similar experiment was carried out with the invasive species *Lasius neglectus* (Chapter 2), in which workers are obligatorily sterile and thus completely unable to reproduce. Here, worker survival rate did not depend on queen presence. This lack of difference between queenright and queenless workers was also supported by transcriptional activity in the fat bodies, which did not vary with queen presence. I show that the reproductive potential of the four species, which were affected by their mode of reproduction along with other natural-history traits, can explain whether they could actively invest in survival to oxidative stress.

I made use of the clonal ant species *Platythyrea punctata* (Chapter 3) to disentangle age and caste. The reproductives showed fewer transcriptional changes than non-reproductives indicating reduced ageing in reproductives. I used queens *of L. neglectus* (Chapter 2), *A. colombica* (Chapter 4) and *A. echinatior* (Chapter 5) to gain further insights into genes with age-related expression in reproductives. Surprisingly, there was no significant reduction in fecundity with age in any of the queens. The most changes observed were in the study of *A. echinatior* queens over 15 years old where many indications of senescence such as dysregulated proteostasis and lower insulin signalling were observed; albeit in the brain rather than the reproductive ovarian tissue or its regulator, the fat body. Therefore, the reproductive is selected for its fecundity to be optimised over its lifetime. This thesis also identifies other hallmarks of ageing and candidates on which to base future studies.

General Introduction

Ageing: What, why and how

‘Ageing’ or ‘senescence’ refers to the progressive deterioration of physiological function with the passage of time (chronological age). It is demographically characterized by an age-dependent increase in mortality and decline in fecundity (da Costa et al., 2016; López-Otín et al., 2013; Rose, 1991). It is a phenomenon that has fascinated researchers and non-scientists alike. Ageing appears to be universal although the rate at which it occurs and the types of effects it has vary across the web of life (Jones et al., 2014). There are a wide range of theories that attempt to explain the evolution of ageing: Why would something that reduces the fertility and survival probability of an organism persist through evolutionary time in the first place?

Many researchers, chief among them Peter Medawar (Medawar, 1946) and George Williams (in 1957), reasoned that ageing might not be a target of natural section but a by- product of it. In the wild, extrinsic rather than intrinsic mortality is the cause of an organism’s death. Death due to extrinsic factors reduces the likelihood for the propagation of beneficial alleles, but also reduces the likelihood of removal of detrimental alleles which reduce reproductive fitness later in life. This **selection shadow** (Fig 1 a) introduced by JBS Haldane and Medawar is based on the insight that the strength of natural selection on a population declines with increasing age. The genes with deleterious effects only late in life can thus get fixed in a population because there is no selection against them. This allows such late-in-life deleterious mutations to accumulate; an idea engendered by the **mutation accumulation (MA) theory** (Fig 1 b). MA explains the occurrence of ageing due to the random accumulation of deleterious mutations over generations. Some alleles might even be advantageous earlier in life and increase fitness but have deleterious effects later in life. The theory of **antagonistic pleiotropy (AP)** (Fig 1c) acknowledges that single genes often have multiple functions and are interconnected. Genes would still be favoured and propagated if it provided a fitness advantage over its competitors albeit linked to deleterious effects later in an individual’s life (*Reviewed in*: Kirkwood, 2005; Ljubuncic & Reznick, 2009; Maklakov & Chapman, 2019; Roget et al., 2022; Williams, 2001). A mechanistic explanation for physiological changes involved with ageing comes from Thomas Kirkwood’s **disposable soma theory (DST)** (Fig 1 d**)**. Inspired in part by August Weisman’s ideas, DST envisions organisms as being limited in the resources available to them. To assure fitness, these resources must be invested into somatic maintenance and into growth and reproduction. Reproduction rather than somatic repair and maintenance is more likely to be optimized by selection due to its implications in fitness which is favoured over longevity. Therefore ageing is a result of allocating fewer resources into repair and maintenance in favour of reproduction (Johnson et al., 2019; T. B. Kirkwood, 1977; Ljubuncic & Reznick, 2009).

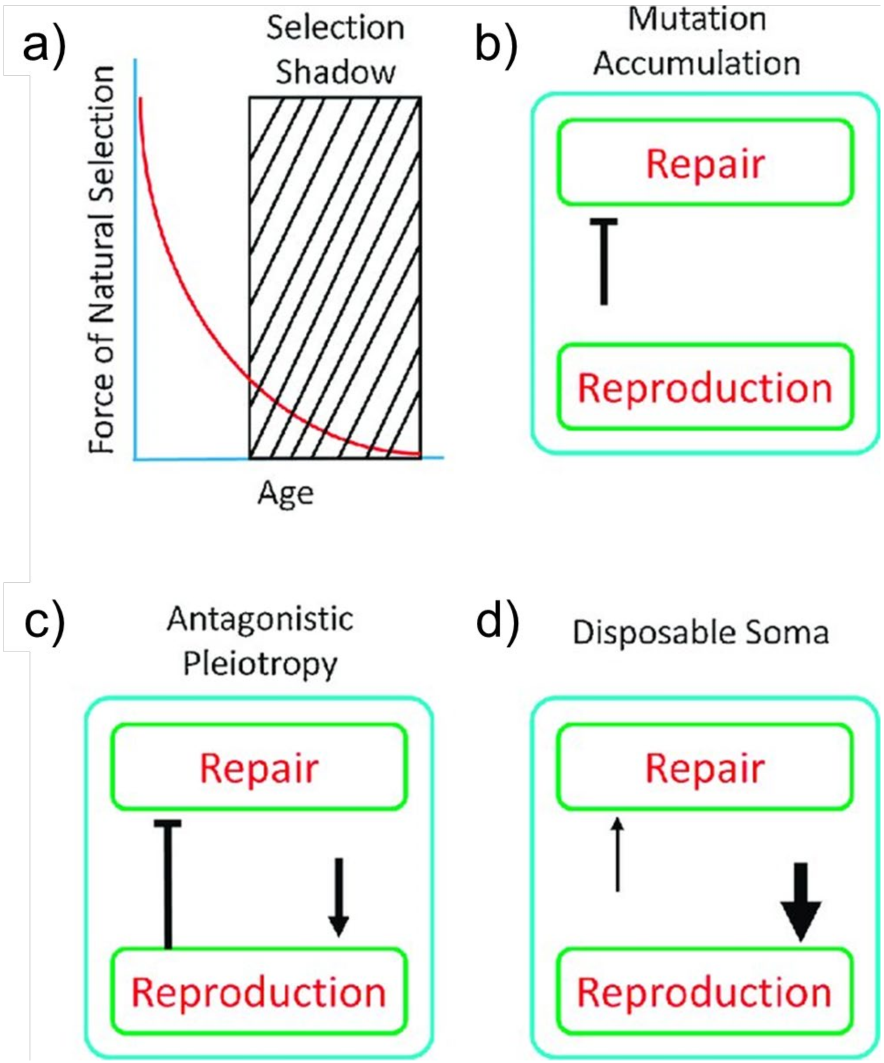

Figure 1: Figure from Johnson et al., 2019 summarizing the classic theories that explain the evolution of ageing. A) shows the **selection shadow** model of Haldane and Medawar, which explains the force of selection on a population reducing with age. This results in traits or alleles detrimental at later ages to not be removed by selection. B) The **Mutation accumulation** (MA) theory continues that such deleterious mutations that affect only late life fitness can accumulate in a genome and get fixed over time because of the lack of selection on them C) The **Antagonistic pleiotropy** (AP) theory predicts that some detrimental deleterious mutations may remain in a population and get selected for because those very mutations/variants have positive effects on fitness early in life of an organism. D) Thomas Kirkwood's **Disposable Soma Theory** (DST) provides a mechanism by which ageing might occur; because organisms are limited by energy/resources and have to allocate resources into either reproduction or maintenance, and since reproduction and not long life is the main determinant of fitness the allocation of more resources to reproduction rather than repair leads to the breakdown of the organism's physiology over time.

Fertility and longevity: The trade-off and a lack thereof

Direct support of the trade-off predicted by DST can be found in studies which show early-life fecundity that comes at the cost of an increased rate of senescence in various mammal and bird species as well as in *Drosophila melanogaster* (Flatt & Heyland, 2011; Maklakov & Chapman, 2019). The most extreme case was John Maynard Smith's discovery of the sterile *Drosophila subobscura* mutant '*grandchildless',* which outlived the wild-type reproducing flies. Similarly artificial selecting *Drosophila* to have a higher survival as adults had negative consequences on their early reproduction, while reproduction at late stages did increase lifespan but reduced early-life fecundity (Flatt, 2011). Germline ablation in the nematode *Caenorhabditis elegans* also increased their lifespan (Patel et al., 2002). These studies, along with many others support the idea of a trade-off between reproduction and somatic repair and body maintenance. Large scale crowdsourced genealogy data from around 300 years of 16 European countries show that higher fertility had a significantly negative impact on longevity for both men and women (Hsu et al., 2021) although the presence of a post-reproductive phase in women also leads to more confounds and covariates, which should be considered in such studies (Gagnon et al., 2009).

Social insects have garnered a lot of interest in the past few years due to their peculiar life-histories. Social insects, including the blattoid termites and the social hymenopterans (wasps, bees and ants) represent a major evolutionary transition of group-living characterized by aligned fitness interests amongst members and a division of labour (Bourke, 2011; Sumner et al., 2023). The division of labour entails the presence of a reproductive and a non-reproductive caste; the former responsible for all reproductive output of the group while the latter undertake non-reproductive tasks for the nest and gain indirect fitness benefits from raising the reproductives' offspring. What makes them interesting models in ageing studies is that the reproductive caste is often much more long-lived than the non-reproductive counterparts while also being the most fecund individuals (Keller & Jemielity, 2006). The reproductives also do not leave their nest once they found their nests/colonies which is not the case for solitary organisms that risk predation or mortality from abiotic factors. Honeybee queens can live for up to two years whereas the workers may live for a maximum of one year. More extreme examples are ants where the queens can live for decades while her workers survive for a few months or a few years (Keller, 1998; Keller & Genoud, 1997). Scientists also observed that in species where workers were able to activate their ovaries and lay eggs, the reproducing workers too could outlive their non-reproducing worker counterparts (Amarasinghe et al., 2014; A. Hartmann & Heinze, 2003; Negroni et al., 2021). This was correlated with higher investment in oxidative stress resistance, immunity and accompanied by behavioural changes indicating conserved molecular mechanisms which could be investigated to understand the proximate mechanisms of ageing(Choppin et al., 2021; Kennedy et al., 2021; Korb et al., 2021; Majoe et al., 2021; Rau & Korb, 2021).

Proximate causes of ageing: insights from various taxa

The aforementioned trade-off between fertility and longevity involves key pathways that are interconnected. They have over the years been implicated in many a life processes including growth, repair, reproduction and determination of lifespan. The **Insulin/Insulin-like growth factor signaling** (**IIS**) pathway regulates metabolism, growth, and development as well as longevity in relation to an individual's nutritional status. It is strongly linked to another nutrient sensing **TOR (Target of rapamycin)** pathway (mTOR in mammals) which also has emerged as a regulator of ageing via its role in protein synthesis and autophagy. Inhibiting TORC1 (TOR complex 1) in *Saccharomyces cerevisiae* (baker's yeast) and *C. elegans* increased their lifespan as did dietary restriction in the latter. Rapamycin or dietary restriction increases lifespan as well as does the inhibition of the IIS by inhibiting the Insulin receptor, *InR* (Gáliková & Flatt, 2010; Partridge et al., 2011; Razquin Navas & Thedieck, 2017; Sarbassov et al., 2005). In the social insects, life-history related polyphenisms, between castes and among workers, are linked to **Juvenile Hormone (JH)**, which also plays a role in fecundity and regulates longevity in solitary *D. melanogaster* (Rodrigues & Flatt, 2016; Tu et al., 2005). High JH titres correspond to normal physiological changes in aging bees and reduced *Vg* expression in workers, while queens have high *Vg* titres and high JH (Corona et al., 2007; Remolina & Hughes, 2008; Rutz et al., 1976). *Vg* is a downstream element of the **Insulin/Insulin-like growth factor signaling** (**IIS) cascade.** It is through the regulation and potential rewiring of these pathways or their relationships that are predicted to account for the reversal of the fecundity-longevity trade-off observed in social insects(Rodrigues & Flatt, 2016). This is supported by observations in bees, ants and termites (Abrisqueta et al., 2014; Amdam et al., 2012; Chandra et al., 2018; Korb et al., 2021; Libbrecht et al., 2018; Münch et al., 2008) [Figure 2].

The rate of ageing can also be influenced by other factors and may be universal across taxa. Nine hallmarks of cellular and molecular ageing have been described (López-Otín et al., 2013) including age-associated marks of deterioration, which fulfil the following criteria: 1) they manifest during the normal ageing process 2) their experimental aggravation increases the rate of ageing 3) their experimental amelioration reduces the effects of ageing and thus increases chances of a longer life. The nine hallmarks are as follows: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. These hallmarks are by no means independent and experimental manipulation of one may affect others as well (Fig 2). DNA damage accumulation occurs over time and is hastened by random factors such as DNA replication errors, Reactive Oxygen Species (ROS) etc. ROS are natural by-products of cellular processes and at lower-levels, ROS play roles in cellular signalling. However, their accumulation can be detrimental if not promptly and regularly cleared out by antioxidants present. In *D. melanogaster,* higher expression of *superoxide dismutase* enzyme gene caused an extension of lifespan (Sun et al., 2002). In the social insects, the role of oxidative stress in aging, and the production of anti-oxidants differs based on the study specimen and method (de Verges & Nehring, 2016; Kramer et al., 2021). The role of *vitellogenin (Vg),* a yolk-protein commonly associated with higher fertility also seems to protect sterile honeybee workers from oxidative stress (Corona et al., 2007). This life-extending effect of *Vg* is in contrast with *C. elegans* where it shortens the lifespan (Seehuus et al., 2006)

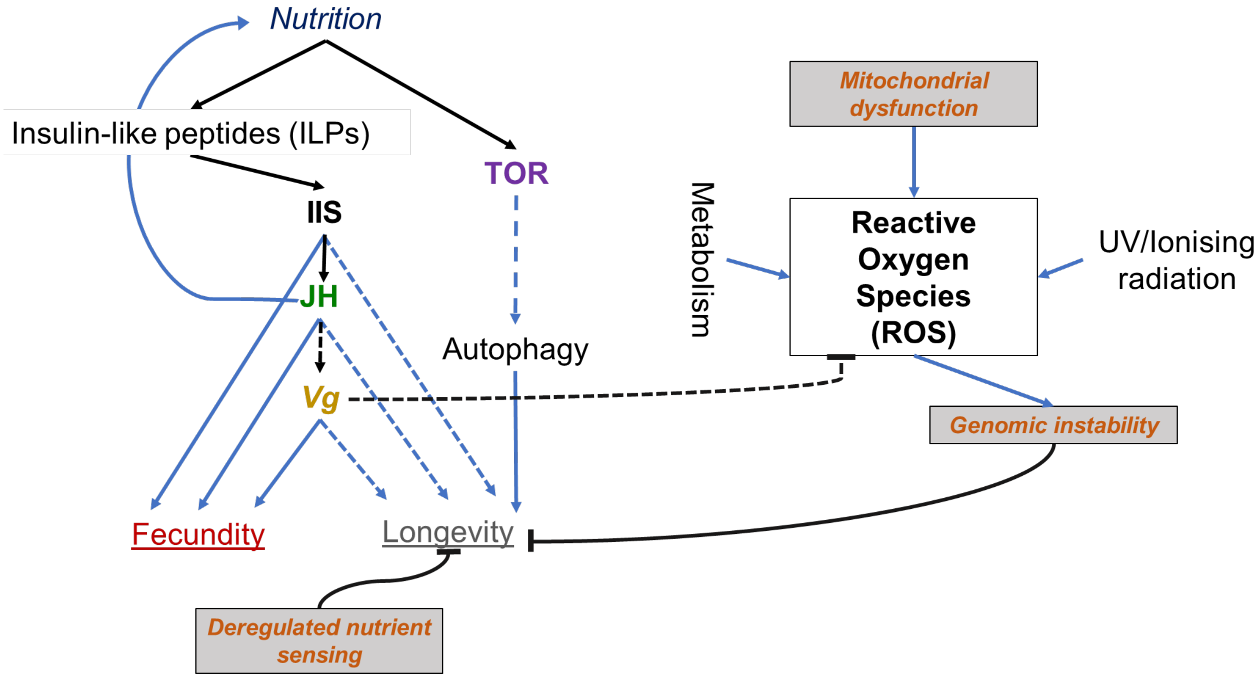

Figure 2: A schematic illustrating typical hallmarks of ageing (grey boxes with italicized font) which can negatively impact longevity observed in social insects. *Vitellogenin (**Vg**)* shielded honeybee workers against induced oxidative stress from ROS though its anti-oxidant role in other social insects is unknown. The three signalling pathways- **IIS, TOR and JH** and their connection to ***Vg*** is implicated in ageing in *D. melanogaster* as well as other insects. A rewiring of these pathways and their connections may enable highly reproductive individuals to also be long-lived while a deregulation especially in the IIS and TOR pathways are a hallmark of ageing. Arrows indicate activation and lines with bars on the end indicate repression. Dotted lines indicate that the relationship is not fully supported across taxa or untested. Figure based on (Lin et al., 2021; López-Otín et al., 2013; Rodrigues & Flatt, 2016)

Ant sociality and senescence: variety and complexity

There are around 15,000 species of ants, encompassing diverse social structures, colony dynamics and maximum lifespans (Hölldobler & Wilson, 1990; Keller, 1998; Keller & Genoud, 1997; Schultheiss et al., 2022) . The most striking divergence of lifespans exists between the castes of some ant species, with queens that live for more than two decades and their workers that perish within a few months. Queens and workers are closely related but queen-worker differentiation is nutritionally determined at the larval stage and is irreversible beyond that time point. However, workers of different ant species differ in their ability and opportunity pertaining to reproduction. In the presence of a queen, workers might exercise self-restraint in terms of egg laying (Dijkstra et al., 2005). Alternatively, the queen or workers might police egg-laying and reproduction in workers (Brunner & Heinze, 2009; Holman et al., 2010; Ortius & Heinze, 1999; Schmid et al., 2013). In most ant species, workers eclose with functional ovaries which degrade with time. Although they cannot store sperm due to the lack of spermathecae, they can still lay haploid eggs resulting in male offspring via arrhenotokous parthenogenesis. In this thesis, these ants are referred to as 'regular' in terms of their mode of reproduction (Fig 3). In some species, workers are obligately sterile, incapable of reproduction even in the absence of a queen. These 'sterile' workers may still have ovaries but may be used to produce trophic (non-viable) eggs and cannot lay viable eggs even in the absence or a queen (C. Perry & D. Roitberg, 2006; Gobin et al., 1998). Lastly, in some species workers are 'totipotent', in such that they can either mate within the nest and/or produce female offspring without mating. These species still have a dominance hierarchy that is established to determine one or a few individuals that monopolise reproduction while the subordinates undertake non-reproductive tasks. Though the castes here are not determined at larval stages and are reversible, there still exists a positive correlation between reproduction and longevity in these species. The two castes are also often morphologically quite similar and the lifespan differences between them are not as stark (Hartmann et al., 2005; Hartmann & Heinze, 2003; Tsuji et al., 2011; Yan et al., 2022)

Even among the workers of the same species and physical morph, those that carry out intranidal tasks have a higher survival probability than workers engaged in extranidal tasks, which has been linked to their ability to reproduce (Choppin et al., 2021; Kohlmeier et al., 2017; Lee et al., 2017; Negroni et al., 2021). The older, more dispensable workers engage in the risky tasks outside of the nest, so that age and task are often linked (Blanchard et al., 2000; Chapuisat & Keller, 2002; C. Hartmann et al., 2019). These older workers also contain less fat and have less developed ovaries (Bernadou et al., 2020). While the performance of tasks can be plastic and task-switching has been observed based on availability of workers, context and colony size, age and task/ social-role of a worker are still strongly correlate (Iwasa & Yamaguchi, 2020; Quque et al., 2023).

Amongst the different ant species, the queens too differ in their ability or opportunity to reproduce based on a few factors. This is correlated with their lifespan. Monogynous ant colonies contain one single queen and are their colony's sole reproductive individual and often live for decades. Queens of polygynous ant species have been observed to have shorter lifespans compared to monogynous queens, especially in species such as *C. obscurior* (Jaimes-Nino et al., 2022; Schrempf et al., 2011)*, Solenopsis invicta* (Klobuchar

& Deslippe, 2002) and *Linepithema humile.* Fertility of the queens may also be linked with their chronological age. Honeybee queens have been shown to reduce egg production over time (Tarpy et al., 2000) and the univoltine nature of bumble bee colonies is characterized by aggression towards the queen whose fertility is reduced with time followed by worker-reproduction (Amsalem et al., 2017). In the ant species *Cardiocondyla obscurior*, egg-laying fecundity reduced with time implying reproductive senescence but it was accompanied by high investment into the production of sexuals (Jaimes-Nino et al., 2022). *Temnothorax rugatulus* ant queens that established their colony 2-3 months prior to collection showed higher investment into immunity genes whereas older, established queens had more genes related to antioxidant activity. The younger queens had shorter ovarioles indicating lower fecundity than older, presumably non-senescent queens (Negroni et al., 2019) although these results may be due to the confounding effect of recent colony-founding by the younger queens. The above study also indicated differences in the changes between the two tissues brain and fat body. Such tissue specific age-related changes have also been observed in model organisms such as *D. melanogaster* (Tain et al., 2021)*, C. elegans* (Wang et al., 2022) and humans (Yamamoto et al., 2022).

Objectives

In this thesis, I employ a broad taxonomic approach to investigate the apparent reversal of the fecundity/longevity trade-off observed and theorised in social insects. I make use of the natural differences in reproductive potential between species, between castes and within the worker-caste, to investigate if and howthese difference affect their survival ability and their transcriptional activity. The diversity of species from different subfamilies differs in their natural habitats, their colony sizes, number of queens and the ‘mode of reproduction’ of their workers (summary of the scope Fig. 3). In **Chapter 1 and 2**, I manipulated social dynamics of a colony, leaving some workers queenless and others still with their queens. The reproductive potential and residual lifespan also vary between younger and older workers which take up different tasks and are present in spatially separate positions with respect to the nest. After giving the workers enough time for ovarian activation, I tested their susceptibility to oxidative stress since oxidative stress is a threat to longevity. In **Chapter 1,** I used three Myrmicine ant species, differing in their mode of reproduction to answer this question. *Acromyrmex echinatior* and *Temnothorax rugatulus* workers can produce haploid male offspring (‘regular’), whereas *Atta colombica* workers were much less likely to do so (‘sterile’).

In **Chapter 2** I focus on the Formicine species *Lasius neglectus,* where the workers are ‘obligately sterile’, with no observed egg-laying and unicolonial colony structure in introduced habitats. I contrasted transcriptional activity in the fat body of queenright and queenless workers, each collected inside and outside the nest. Queens in these species get readopted after mating and thus do not need to undergo claustral founding. This eliminated the potential confound of colony-founding behaviour when testing for age effects in the transcriptomic signals in queens of different ages of *Lasius neglectus* in **chapter 2.**

It is difficult to directly compare queens and worker because they differ so much: Not just in reproduction but also in lifespan, development, behavior, nutritional needs, and morphologies. In addition, the chronological age of the two castes does not reflect in their rate of ageing. I used the Ponerine clonal ant species *Platythyrea punctata* in **chapter 3**. All individuals in the colony are clones, thus highly related. They are also all 'totipotent', capable of producing female offspring without mating. I was able to disentangle age from caste in this experimental set up to investigated age- and caste-related changes in abdominal fat body transcriptomic data. I additionally enquired whether the two genetically identical showed differences in ageing patterns.

Tests for age effects in long-lived reproductive castes often makes use of comparisons between queens that have recently founded colonies with those who have established colonies, but their chronological age is unknown. I used brain-RNA-seq data from *Atta colombica* queen of known chronological ages, in the early years after colony establishment in **chapter 4**. In this ergonomic phase of the colony, prior to production of sexuals, I investigated whether age-related changes can occur in the brain, which might indicate ageing down the line. **Chapter 5** gave me the opportunity to not just use queens of the ergonomic phase of colony life, but to contrast transcriptional activity of very old queens (10 to little over 15 years) and young queens (< 1 to around 3 years). I was also able to use transcriptomic data from three different tissues: the brain, the fat body and the ovaries. This helped understand changes in different tissues over the 15-year period I could track using this Myrmicine species *Acromyrmex echinatior.* This chapter also includes a thorough investigation of natural history information as well as potential correlates and confounds of age.

A summary of the scope of this thesis is presented in Figure 3. My quest to investigate the fecundity/longevity trade-off aims to shed light on whether and how differing reproductive potential may ameliorate some of the effects of ageing. I identify biological and cellular markers and candidates for ageing in different tissues which may be used to track tissue-specific ageing in other organisms.

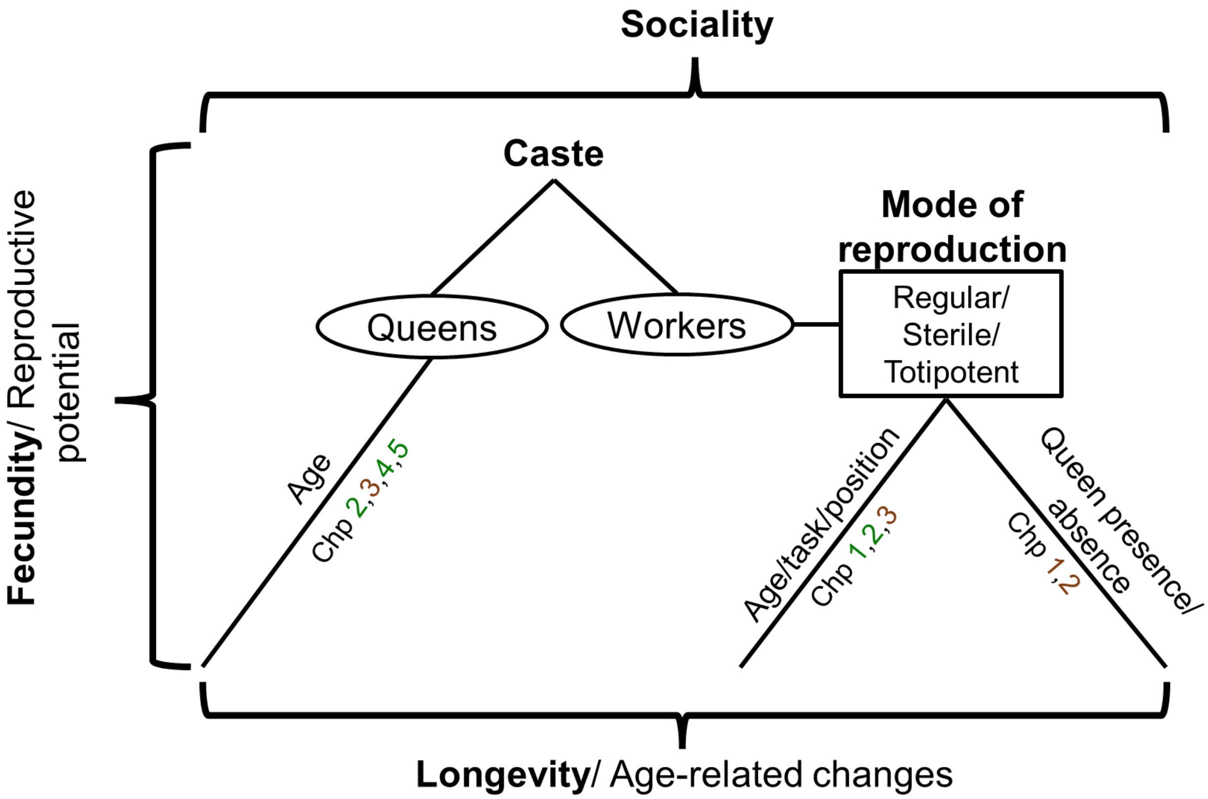

Figure 3: Diagrammatic representation of the scope of this thesis. I investigated the relationship between fecundity and longevity in two castes, the queen and the workers, which naturally differ in their reproductive division of labour as well as lifespans. Workers of different species also differ in their mode of reproduction, some capable of male-destined egg-production (regular), some obligately sterile (completely incapable of reproduction), and some that can produce female offspring thelytokously (totipotent). Worker lifespan is also affected by their age and the task they perform, both of which can be correlated. This affects their reproductive potential as does the absence of a queen, which gives some workers an opportunity to reproduce if they are still physiologically capable. Worker reproduction in turn is also correlated with their longevity. Chapters where naturally occurring effects were investigated are presented in green, whereas chapter numbers in brown entailed manipulation of the natural social structure of a colony (of workers in chapter 1,2 of totipotent reproductive and non-reproductive clonemates in chapter 3).

Chapter 1

Queen loss increases worker survival in leaf-cutting ants under paraquat-induced oxidative stress

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Abstract

Longevity is traded off with fecundity in most solitary species, but the two traits are positively linked in social insects. The most fecund individuals (queens and kings) live longer than the non-reproductive individuals, the workers. In many species, workers may become fertile following queen loss, and recent evidence suggests that worker fecundity extends worker lifespan. We postulated that this effect is in part due to improved resilience to oxidative stress and tested this hypothesis in three Myrmicine ants: *Temnothorax rugatulus,* and the leaf-cutting ants *Atta colombica* and *Acromyrmex echinatior*. We removed the queen from colonies to induce worker reproduction and subjected workers to oxidative stress. Oxidative stress drastically reduced survival, but this effect was less pronounced in leaf-cutting ant workers from queenless nests. We also found that, irrespective of oxidative stress, outside workers died earlier than inside workers did, likely because they were older. Since *A. colombica* workers cannot produce fertile offspring, our results indicate that direct reproduction is not necessary to expand the lives of queenless workers. Our findings suggest that workers downregulate resilience to oxidative stress in the presence of the queen and raise questions on the proximate and ultimate mechanisms underlying socially mediated variation in worker lifespan.

Keywords: life-history evolution, fecundity, longevity, workers, ants, survival

Introduction

Advanced eusocial insects live in societies that contain many closely related individuals of several generations that exhibit negligible within-group conflict (West et al., 2015). Queens (and kings in termites) reproduce, whereas workers perform all other tasks necessary to raise the brood. In solitary insects, reproduction is typically traded off with lifespan (Attisano et al., 2012; Flatt, 2011; Mockett & Sohal, 2006), whereas the opposite is true in social insects: The reproductive castes live much longer than workers in all social insects (Page & Peng, 2001). Yet, workers of many social insects retain the ability to reproduce under certain circumstances. Most of the ant workers are unable to mate, but can lay haploid eggs, which develop into males. However, worker reproduction in queenright colonies is rare, as there are fitness incentives for workers to refrain from reproduction in the presence of a fertile queen (Bourke, 1988; Korb & Heinze, 2016; Wenseleers & Ratnieks, 2006). Workers that attempt to develop their ovaries and lay eggs are policed by other workers who physically attack them or destroy their eggs, and these fights reduce colony efficiency (Dijkstra et al., 2010; Ratnieks et al., 2006; Stroeymeyt et al., 2007a; A. K. Tsuji et al., 2011). Worker life histories are thus optimized for their lifetime contribution to colony fitness rather than for direct reproduction – which has led to extreme forms of self-sacrifice (Bourke, 2008; Shorter & Rueppell, 2012; Sobotnik et al., 2012; Tofilski et al., 2008)

In case the queen dies, the fitness landscape of a worker changes drastically. Since no more female brood can be produced, the residual lifespan of a colony is now limited to that of the remaining workers. After queen loss, remaining female larvae that have not reached the point of caste-differentiation in their development will typically be raised to become queens instead of workers, as a form of terminal investment (Dijkstra & Boomsma, 2007; Fletcher & Ross, 1985; Strätz & Heinze, 2004). Rearing these larvae might not exhaust the lifetime work capabilities of the remaining workers. In this case, policing is relaxed in many ant species and workers turn to direct reproduction. However, this option is not available for ant workers of all species. In advanced eusocial species, typically with extremely large colonies and strong morphological caste differentiation, workers are invariably sterile in an evolutionary sense, as they cannot lay eggs that develop into fertile males (e.g. army ants, *Atta* leaf-cutting ants, and many invasive ants (Aron et al., 2001; Dietemann & Peeters, 2000; Dijkstra & Boomsma, 2006; Korb & Heinze, 2016; Kronauer et al., 2010; Montague & Oldroyd, 1998; Shimoji et al., 2017)).

The ability of workers to develop their ovaries is also influenced by age and task, as younger workers are more likely to develop their ovaries than older workers (Bourke, 1988). Age often predicts worker behaviour as well: Young workers typically start out as nurses inside the colony and then later transition to outside behaviours such as foraging (Giraldo & Traniello, 2014a). This age polyethism is thought to be caused by variation in the extrinsic mortality among tasks; workers that carry out risky tasks, such as leaving the colony or defending the nest, are more likely to be killed in the process. If young workers performed these risky tasks, they would endanger losing their long residual life- and workspan, during which these workers could still contribute to the colony's fitness. Old workers, in contrast, have a reduced residual lifespan so their death impacts the colony's fitness less than the young workers' (Kramer & Schaible, 2013a; Tofilski, 2002).

Interestingly, recent studies on termites, bees and ants have shown that worker reproduction can have an effect on worker lifespan as well: When the queen is removed and queenless workers begin to reproduce, they live longer than workers in colonies with a queen (Dixon et al., 2014; dos Santos Conceição Lopes et al., 2020; A. Hartmann & Heinze, 2003; Kohlmeier et al., 2017a; Kuszewska et al., 2017; A. K. Tsuji et al., 2011). This makes sense from an evolutionary perspective, because as soon as direct reproduction is an option for workers, living longer increases the period in which they can reproduce, and hence increases their lifetime fitness (Usher et al., 1979). In accordance with this reasoning, totipotent workers of wood-dwelling termites live long and invest in anti-ageing pathways (Monroy Kuhn et al., 2019a; Rau & Korb, 2020). The hypothesis that selection on the individual level has caused the evolution of longer worker lifespans at the cost of the colony has not been tested so far. It would predict that only the lives of those workers that can indeed reproduce are prolonged, but not of workers that are too old for egg production, or of workers from species with sterile workers. There are also alternative explanations for the observed patterns: Colony lifespan is limited by the lifespan of the remaining workers – and if the latter is prolonged, the colony will survive for longer as well. The longer the colony lives, the higher the chance that of any sexual offspring raised from the remaining queen brood live to adulthood, long enough to take part in the next mating flight. This would predict that all workers prolong their lives, not just the fecund ones.

The idea that reproductive workers have longer lifespans than sterile ones is in line with the finding that highly fecund social insect queens live much longer than the typically sterile workers (Keller & Genoud, 1997a; Korb, 2016). One way to prolong lifespan is investment into body maintenance. There is no consistent evidence of whether and how queens invest more in body repair or resilience than workers (Corona et al., 2005a; Flatt & Partridge, 2018; E. Lucas et al., 2016; E. R. Lucas & Keller, 2014a), but they can handle some stressors better than workers (Corona et al., 2007b; Schneider et al., 2011) One potential stress factor is oxidation. Imperfect mitochondrial respiration leaks reactive oxygen species (ROS) into cells, where they damage macromolecules such as proteins and lipids. Such damage can be avoided if organisms produce antioxidants that neutralize ROS before they do any critical damage, or they repair the damage after it has occurred (Münch et al., 2008; Ray et al., 2012; Schieber & Chandel, 2014; Seehuus et al., 2006b).

Here, we investigated resistance to oxidative stress as a potential mechanism by which ant worker lives are prolonged when they become fertile. We subjected queenright and queenless workers to paraquat-induced oxidative stress. We predicted that queenless workers that activate their ovaries are better able to withstand oxidative stress. We further investigated whether the consequence of oxidative stress differs between workers collected from inside the colony and to those performing outside tasks: Usually, only inside workers can become fertile and would thus be able to profit from the benefits of a longer life through direct reproduction. We studied three ant species of the sub-family Myrmicinae: *Temnothorax rugatulus* and the two leaf-cutting ants *Acromyrmex echinatior* and *Atta colombica*. *A. colombica* workers are sterile, which allowed us to test whether it is indeed direct fitness prospects that caused selection for the longer lives of orphaned ant workers.

Material and Methods

To test whether fertility increases worker resistance to oxidative stress, we split colonies of three Myrmicine ant species (*Acromyrmex echinatior, Atta colombica, Temnothorax rugatulus*) into queenright and queenless half-colonies for 10-15 weeks. From these half-colonies, we created small sub-colonies consisting of equal numbers of marked inside and outside workers. Workers in each subcolony were subjected to either paraquat-induced stress or a control treatment for two weeks (Fig. 1).

Study Species, ant collection, and maintenance

We selected three Myrmicine species with known reproductive potential of workers. *A. echinatior* and *A. colombica* are neotropical leaf-cutting ants that typically have a single, multiply-mated queen (Nehring et al., 2018; Villesen et al., 2002). *T. rugatulus* is a nearctic species with small monogynous or polygynous colonies and singly inseminated queens (Rüppell et al., 1998).

Acromyrmex and *Temnothorax* workers rarely lay eggs in the presence of the queen, but queen loss triggers in young workers ovary development and the production of eggs that remain unfertilized and develop into haploid male offspring (Dijkstra et al., 2010; Dijkstra & Boomsma, 2007; Giehr et al., 2020; Kohlmeier et al., 2017a). In contrast, *Atta* workers are sterile in an evolutionary sense, as worker groups rarely produce any males even months after queen loss, and the few males that mature are smaller and likely infertile (Dijkstra & Boomsma, 2006). To confirm these species-specific differences in worker reproduction in response to queen removal, we dissected the ovaries of 30 *A. echinatior* workers and 46 *A. colombica* workers from inside the fungus gardens of the experimental half-colonies at the time of the experiment. We found that queenless workers of *A. echinatior* were more likely to have developed eggs in their ovaries (13/15) than queenright workers (7/15; Fisher's exact test $p = 0.05$). In contrast, virtually no *A. colombica* workers developed eggs (1/22 and 0/24 for queenright and queenless conditions, respectively; Fisher's exact test $p = 0.48$). In *T. rugatulus*, queen removal stimulated ovarian development and egg production in nurses (4 weeks after queen removal, M. Choppin, unpublished; 53 weeks after queen removal, (Negroni et al., 2020)).

The *A. echinatior* and *A. colombica* colonies were collected in Gamboa, Panama in the years 2017 and 2018. In the lab, the leaf-cutting ants' fungus gardens grew in beakers. These were covered by upturned plastic flowerpots placed in fluon-lined plastic boxes (57x51x28cm), the latter acting as a foraging arena. They were housed in a climate chamber at 25-27°C, 65-70% humidity with a 12-hour light-dark cycle and provided with bramble, rice, and apple slices twice a week, but received no additional water.

The *T. rugatulus* colonies were collected in the Chiricahua Mountains in Arizona, USA in 2018. All the colonies were habituated to laboratory conditions. The *T. rugatulus* colonies nested in plastic boxes (52x30x18mm) with plaster at the base and an aluminium foil-lined lid. This box was placed in a (95x95x65mm) closed square plastic container with small holes in the lid. All these boxes were placed in a 22-25°C climate chamber at ambient humidity in a 12-hour light-dark cycle. They had ad libitum access to water and were fed with mealworms and honey once a week.

Queen removal experiments

All source colonies were equally split into experimental colonies that either contained a queen ("queenright") or no queen ("queenless") and maintained this way for 10-15 weeks. *A. echniatior* and *A. colombica* colonies are very large containing many thousands of workers and live inside a fungus gardens (Dijkstra & Boomsma, 2008; Hölldobler & Wilson, 1990; Mueller, 2002). Thus, it is difficult to count the exact number of workers. Therefore, we broke the 1.5-2 L of fungus garden into smaller pieces and redistributed the parts while keeping the vertical structure of the garden intact, distributing workers and brood equally within both halves. We split two source colonies of *A. echinatior* and four source colonies of *A. colombica*, creating from each colony a queenright and a queenless half-colony. After 13-15 weeks, we set up between two and four subcolonies from each half-colony, resulting in 12 subcolonies for *A. echinatior* and 32 subcolonies for *A. colombica*. Workers of half of the subcolonies were subjected to a paraquat treatment, the other half to a control treatment. Each subcolony contained 10-12 inside and 10-12 outside workers. All workers used in this experiment were of median size (head width 1.36±0.06 mm for *A. echinatior* and 1.35–2.69 mm in *A. colombica* based on (Clark, 2006; Dijkstra et al., 2005; Holman et al., 2011)). We collected inside workers from the fungus garden and picked outside workers from the foraging area, but not from the trash pile. The cuticle colouration - light for inside and dark for outside workers - was used to collect *A. echinatior* workers according to age, as shown before for *Acromyrmex octospinosus* (Armitage & Boomsma, 2010). Inside and outside workers were marked with randomly selected colours (Edding 750 paint markers). Subcolonies were housed in petri dishes (90 mm diameter). They were provided with some fungus bereft of any eggs, larvae or workers, one single, size-standardized blackberry leaf, and a moist cotton pad to sustain the fungus that the workers can access ad-libitum. Workers that died before the onset of the oxidative stress treatment were removed from the analysis. Our total sample size comprised 248 *A. echinatior* workers (12 subcolonies x 10-12 inside and 10-12 outside workers) and 641 *A. colombica* workers (32 subcolonies x 10 inside and 10 outside workers).

We split six *T. rugatulus* source colonies with each half-colony receiving exactly the same number of eggs, larvae and workers. These half-colonies were maintained for 10 weeks. Thereafter, we created two sub-colonies from each of them, each sub-colony composed of about five inside workers (collected near the brood-pile) and five outside workers (collected from the feeding arena). *T. rugatulus* workers are monomorphic. Subcolonies were housed in plaster lined petridishes (55mm diameter), capable of holding moisture. Each sub-colony was provided with honey-soaked pieces of sponge on the first day and subsequently had only access to water through the wet sponge. In total, 208 *T. rugatulus* workers were included in the analysis after some mortality between marking and treatments (24 subcolonies x 5 inside and 5 outside workers).

Oxidative stress treatment

One day after subcolony set up, each ant in half of the sub-colonies was subjected to a 0.46 M solution of Paraquat dichloride ($CH_3(C_5H_4N)_2CH_3 \cdot 2Cl$) dissolved in millipore water (oxidative stress treatment). Paraquat is a herbicide that induces oxidative stress through superoxide formation (Cousin et al., 2013). Since injecting paraquat might lead to injury, and administration by food cannot be controlled, especially in fungus-consuming leaf-cutting species, we applied the paraquat solution to each worker's head with a size 2 Vernisage^{TM} paintbrush such that it covered the entire surface. Based on preliminary experiments using different paraquat concentrations, we chose a dose that would kill approximately half of the workers within 6-8 days. The procedure ensured that the amount of solution the ants received was roughly equivalent to the size of the ant as workers of the three species varied in size. To avoid transfer of the liquid through trophallaxis and to facilitate self-grooming and ingestion, each ant was placed in an isolated well of a Falcon^{TM} cell-culture plate. All workers were isolated from each other for 3-4 hours, after which all survivors were returned to their petridishes (Fig. 1). Workers of the remaining half of the subcolonies were treated in the same manner, but here we applied pure millipore water instead of the paraquat solution (control treatment).

We noticed that ants would groom themselves and consume the solution during isolation. We did not observe any attacks or special interest by the nestmates following the return of the treated workers into the subcolonies. We found no dead individuals with signs of fights (e.g. missing appendages) inside the subcolonies. Mortality was noted once before the treatment and once after the isolation period on treatment days. During the isolation, 0.01% - 10% of the workers died in the control treatment and 10% - 22% in the paraquat treatment, depending on species. The paraquat treatment was repeated six more times on every other day. The final treatment was administered on day 13 and the final survey of the sub-colonies happened at the end of this day.

Statistical analyses

We used the coxme version 2.2-16 package (Therneau, 2020) in R 3.5.2 (RStudio Team, 2016) to build cox-regression mixed-effect models for each species separately. The presence of queen (queenright vs queenless), treatment (paraquat vs water), and the location of workers (outside vs inside), as well as all possible interactions, were entered as fixed factors, and source colony ID as a random factor. Hypothesis testing was done with the 'Anova' function from the package car (Fox & Weisberg, 2019) for each species separately (supplementary table 1). The function 'ggsurvplot_facet' built under the ggplot2 version 3.3.0 package (Wickham, 2016) was used to plot the Kaplan-Meir survival curves. As a post hoc analysis, we also ran the same model without the treatment factor for both treatments separately (oxidative stress, control) in each species to illustrate the interactions of other factors with treatment (supplementary Table 1). If workers lost their colour marks, they were not included in the analyses (NA). A few workers escaped during the treatment procedure (in total n = 2, 4 and 27 individuals in *A. echinatior, A. colombica* and *T. rugatulus,* respectively); these data were censored in the analyses.

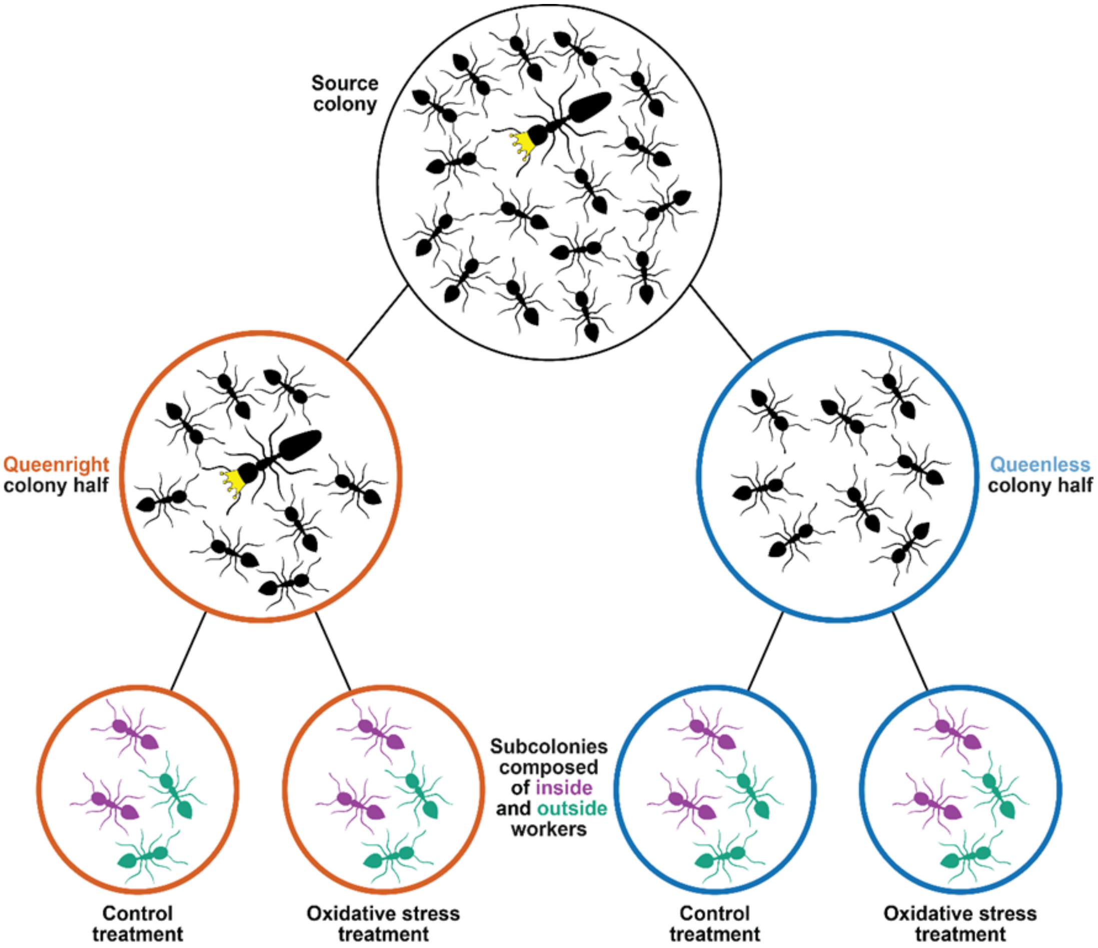

Fig 1: Overview over the experimental design. Source colonies were split into queenright and queenless half-colonies. 10-15 weeks later, we created sub-colonies from each half-colony subjected either to paraquat-induced oxidative stress and a control treatment. Each sub-colony contained equal numbers of 'outside' and 'inside' workers. Treatments were administered to individual workers that were isolated in wells of cell-culture plates for four hours and then returned to the sub-colonies.

Results

Workers of all species died earlier when subjected to paraquat-induced oxidative stress compared to the control treatment ($p < 0.001$ for each species, Fig. 2 and 3; statistical details: Supplementary Table 1 a, b, c). The effects of the other factors varied with species.

In *A. echinatior*, worker survival depended on two two-way interactions. First, the effect of queen removal depended on the treatment (queen absence : oxidative stress treatment $\chi^2{}_1 = 5.242$, n = 248 $p < 0.05$, supplementary table 1a). A post-hoc analysis revealed that the survival of control workers was not affected by the queen's absence (Fig 2a, supplementary table 1a), but inducing oxidative stress caused disproportionally high mortality in queenright workers when compared to queenless workers (Fig 2b). Second, the effect of location also depended on treatment (location : oxidative stress treatment, $\chi^2{}_1 = 10.745$, n=248, $p < 0.001$ Supplementary Table 1a). Outside workers died earlier than inside workers in control subcolonies (Fig. 3a, supplementary Table 1a), but this effect disappeared when workers were under oxidative stress (Fig 3b Supplementary Table 1a).

In *A. colombica* the queen's absence (Queen absence $\chi^2{}_1$= 9.093, n = 641, p < 0.01) and location (location $\chi^2{}_1$=57.499, n = 641, p < 0.001) had independent effects on worker survival (see supplementary Table 1b for details). There was no clear evidence for an interaction (queen absence : treatment $\chi^2{}_1$=2.664, p = 0.102; Supplementary Table 1b). However, post-hoc tests revealed that workers without the queen survived better than queenright workers under oxidative stress (Supplementary Table 1b, Fig 2c), but this was not the case in the control treatment (Supplementary Table 2b, Fig 2d). Inside workers survived better than outside workers under both control (Fig.3c) and oxidative stress treatments (Fig.3d; Supplementary Table 1b).

In *T. rugatulus*, inside workers outlived outside workers (location $\chi^2{}_1$= 9.093, n = 208, p = 0.037; supplementary table 1c), but queen absence had no effect on worker survival (queen absence $\chi^2{}_1$, p = 0.322 Fig 2e, supplementary table 1c) and there were no significant interactions between any of the factors (Supplementary Table 1c; p > 0.29 for all possible interactions in the full model). Post-hoc tests showed a tendency of inside workers to survive better than outside workers under control treatment (Fig 3e, supplementary table 1c) and this trend was more visible under oxidative stress (Fig. 3f; supplementary table 1c).

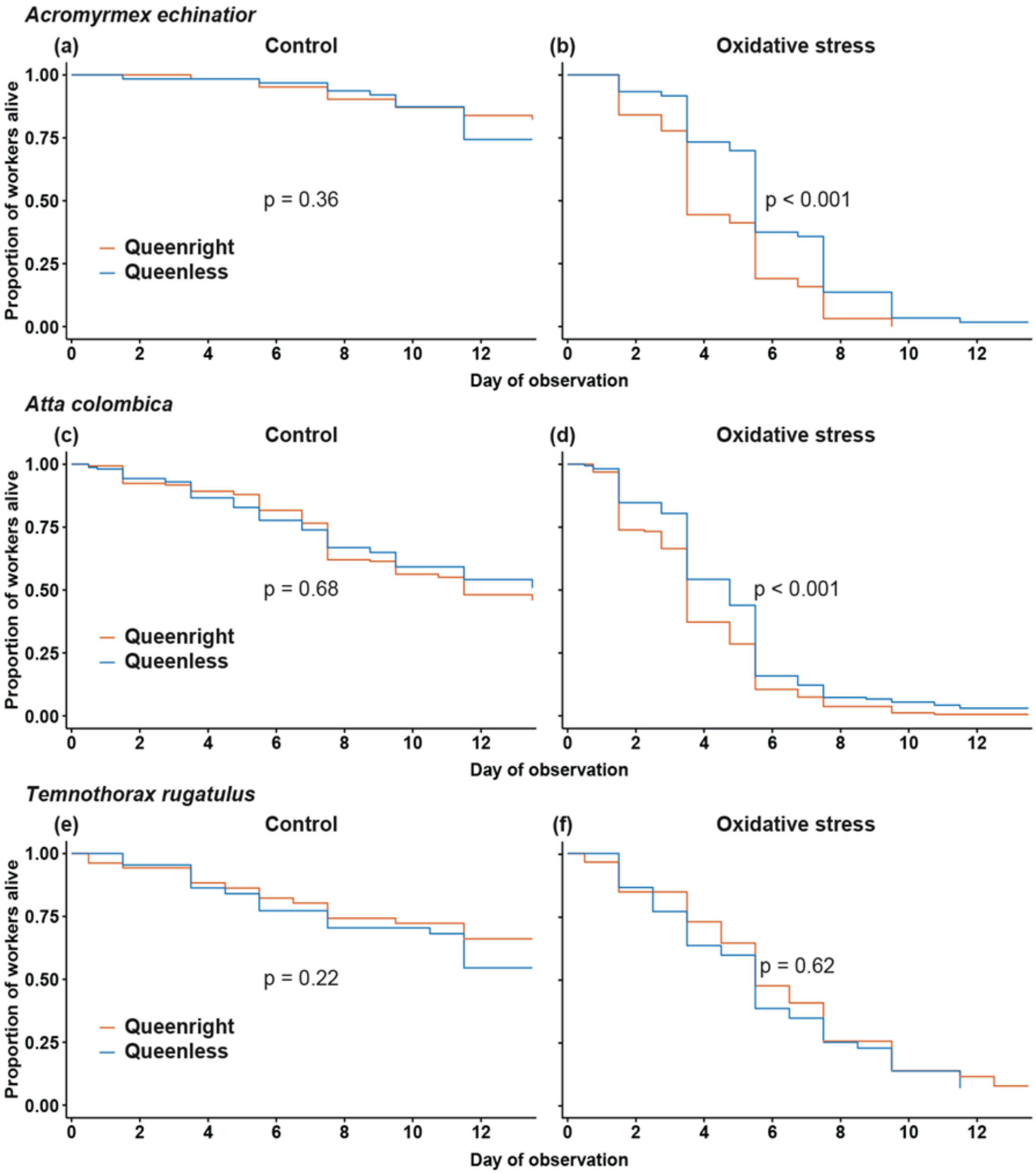

Figure 2: Survival of ant workers of three Myrmicine species (a-b: Acromyrmex echinatior, c-d: Atta colombica, e-f: Temnothorax rugatulus), under control (a, c, d) and oxidative stress treatment (b, d, f). Under oxidative stress (b, d), queenless A. echinatior (total N = 248, evenly distributed over the groups) and A. colombica (total N = 641 workers survive better than queenright workers do. This effect is absent in the control treatment (a, c) and in T. rugatulus (e-f; total N = 208). Significance values shown in the plots are for comparisons of queenright with queenless workers, calculated post hoc from a reduced dataset limited to the treatment (control, oxidative stress) in question (supplementary table 1).

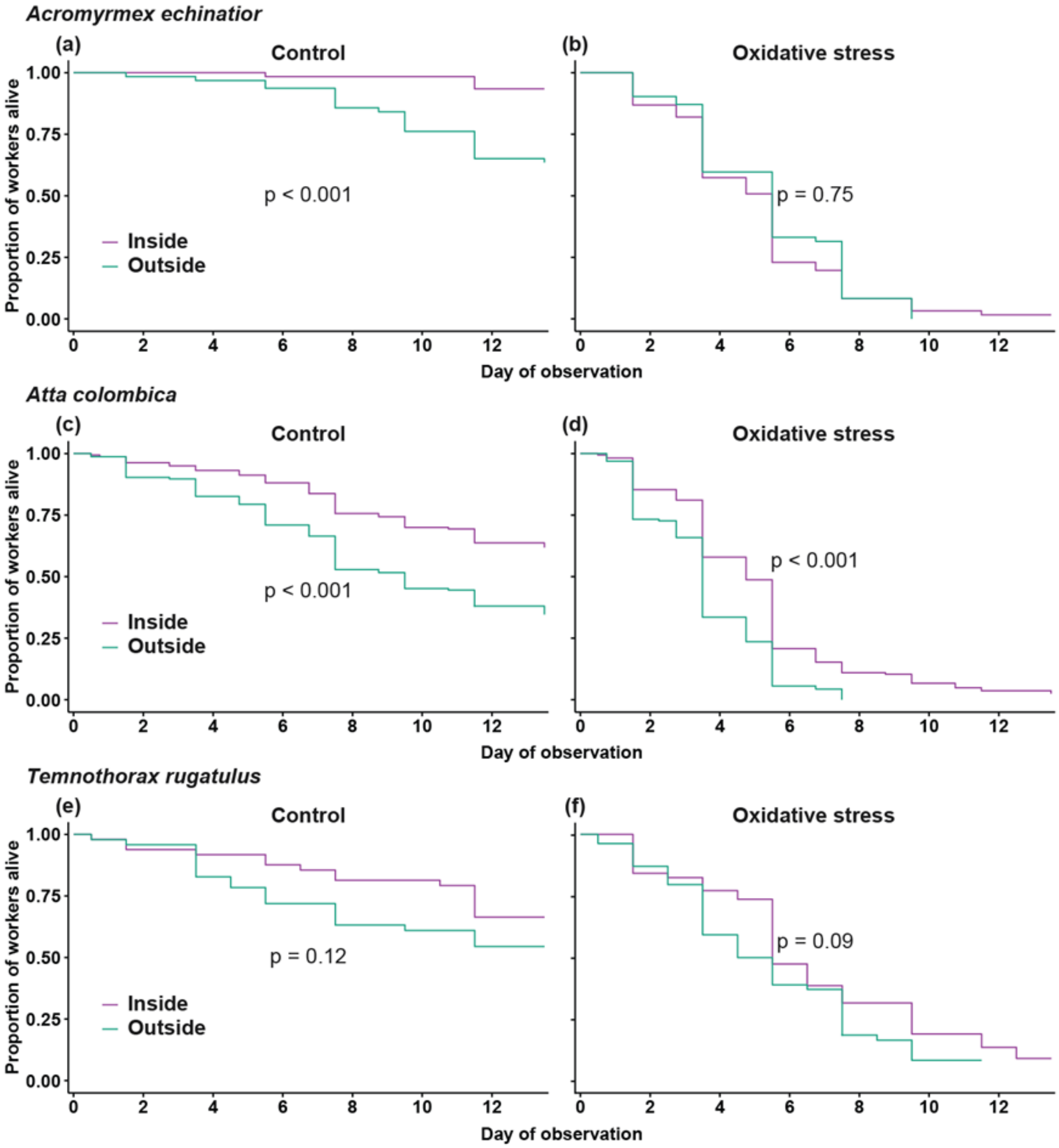

Figure 3: Survival of ant workers of three Myrmicine species (a-b: *Acromyrmex echinatior*, c-d: *Atta colombica*, e-f: *Temnothorax rugatulus*), under control (a, c, e) and oxidative stress treatment (b, d, f). Inside workers from *A. echinatior* (survive better than outside workers under control conditions (a), but not under oxidative stress (b; total N = 248, evenly distributed over the groups), while in *A. colombica*, the same effect is visible independent of treatment (c,d; total N = 641). The effect is weak or absent in *T. rugatulus* (e,f; n = 208). The figure is based on the same dataset as Fig. 2. Significance values in the plots are for a comparison of inside with outside workers and were calculated post hoc from a reduced dataset limited to the treatment (control, oxidative stress) in question (supplementary table 1).

Discussion

We experimentally analysed the influence of queen presence, oxidative stress, and their interactions on worker survival in three Myrmicine ant species. We found queenless workers of two of the species to be more resilient to paraquat-induced oxidative stress than queenright workers. An increase in resistance to oxidative stress in orphans either via the production of anti-oxidants or by repairing molecular damages induced by ROS could potentially explain how queen removal can prolong the lives of workers. The higher survival rate of inside workers is likely due to their younger age and was independent of paraquat-induced oxidative stress, suggesting that resilience to oxidative stress is not changing with age.

Queenless workers deal better with oxidative stress

In all three species, paraquat-induced oxidative stress caused a higher mortality than the control treatment and killed most workers in all experimental groups, indicating that paraquat and the dose we used is suited to induce stress and mortality. Queenless workers have previously been found to live longer than queenright workers in some ant species (Dixon et al., 2014; A. Hartmann & Heinze, 2003; Kohlmeier et al., 2017a; Morandin, Hietala, et al., 2019; Negroni et al., 2020; A. K. Tsuji et al., 2011) and we had hypothesized that this effect may in part be caused by individuals being more resilient to stressors such as ROS. We found evidence for this in the two leaf-cutting ant species we tested. In *A. echinatior* and *A. colombica*, workers survived the oxidative stress treatment better when they came from queenless colonies.

Proximate and ultimate causes of long-lived orphans

Our results demonstrate that increased resilience to oxidative stress could be responsible for the prolonged lives of orphaned workers in ants. A simple explanation would be that orphaned workers upregulate their investment into the production of antioxidants (de Verges & Nehring, 2016), which totipotent termite workers do (Monroy Kuhn et al., 2019a; Rau & Korb, 2020) and which has also been shown in *Temnothorax* ants, where workers can only have male offspring (Negroni et al., 2021). Alternatively, the oxidative stress resilience and short-term increase in survival of orphaned workers might be a mere by-product of a physiology modified for reproduction. Evidence from honeybees suggests that the lipoprotein vitellogenin could cause such effects. This yolk precursor required for egg production also has antioxidant properties in bees (Havukainen et al., 2013; S. C. Seehuus et al., 2006b). Vitellogenin is preferentially oxidized and thus protects other proteins from ROS in many tissues, including the honeybee brain (Münch et al., 2015), where involvement in reproduction is unlikely. Honeybees with high vitellogenin titres survive experimentally induced oxidative stress better, and *vitellogenin* knockdown leads to a lower oxidation tolerance [69]. High expression of *vitellogenin* in queens and to a lesser extend also in young nurses can explain their decreased intrinsic mortality compared to foragers. This may mean that any egg-producing individual is also less susceptible to oxidative stress, resulting in a longer life. It is as of yet unclear whether vitellogenin plays a similar role in ants. (Corona et al., 2007b; de Verges & Nehring, 2016; Salmela & Sundström, 2017; Seehuus et al., 2006), where the *vitellogenin* gene underwent sub-functionalization after duplication with different copies taking over different functions in reproduction and division of labour (Corona et al., 2013; Kohlmeier et al., 2018; Morandin et al., 2014a).

If worker resilience is not a mere by-product but an adaptive trait, it may be caused by a shift in the evolutionary interests of workers. In our focal species, there is little incentive for workers to reproduce in the presence of their queens (Meunier et al., 2010; Wenseleers & Ratnieks, 2006). However, this situation drastically changes when queen reproduction is no longer an option. Then, producing their own offspring will be the most efficient way of gaining fitness (Boomsma, 1996). In this scenario, increased resilience, and the longer lives of queenless workers demonstrated by other studies (Morandin, Brendel, et al., 2019; Rüppell et al., 1998), may have been selected for in workers because this would give them more time to produce offspring.

Selection for long worker lives should be restricted to those workers that can still lay eggs. In most social insects, workers transition from inside to outside tasks throughout their lives. Incidentally, worker reproduction is typically restricted to younger workers, while older workers often resorb their ovaries, as in our focal species (Bourke, 1988; Dijkstra et al., 2005; Fletcher & Ross, 1985; Heinze, 2008). Such a pattern should then lead to young workers, but not older workers, prolonging their lives after queen loss. In none of our focal species did we find any indication of such an effect. There were no interactions between location, which is a proxy for worker age and queen presence, which would be needed to support such a hypothesis (Fig. 3, suppl. Tab 1). In particular, within the oxidative stress treatment, the effect of queen presence was not restricted to inside workers. However, a potential effect might have been obscured, because we found mortality of outside over inside workers to be increased similarly in both the oxidative stress treatment and the controls. In addition, in *A. colombica*, where workers were more resilient in the absence of the queen, workers are sterile in an evolutionary sense. Increased lifetime fecundity can thus not be the reason for longer lives in this species, but an onset of ovarian development even only for the production of trophic non-viable eggs can.

If increased resilience is not restricted to potentially fecund individuals but applies to all orphaned workers, it may be caused by a different effect: Upon queen loss, a colony's workforce cannot be replenished because workers can only lay haploid eggs that develop into males, but not into workers. At the same time, leaf-cutting ant colonies are always full of queen-produced brood in different stages, many of which can still develop into queens that can go on to found daughter colonies. This development will take a few months (Dijkstra et al., 2005; Verza et al., 2017) and it is thus crucial that enough workers are available throughout to take care of the brood and to forage. Further, the young queens cannot simply leave the nest and found a colony whenever they have eclosed, but they depend on mating flights that occur only once or twice a year. Thus, it would mean a great increase of fitness if workers were able to extend their lives long enough to sustain the colony until the next mating flight. This reasoning based on indirect fitness gains would also explain the life-prolonging effect of queen loss in sterile *A. colombica* workers and of older workers.

Finally, a potential proximate reason for sterile *A. colombica* workers to increase oxidative stress resistance in response to queen loss might be that the physiological changes associated with queen loss can prolong lifespan without realised fecundity. The molecular networks that translate the social environment into reproductive activity may be conserved, and perhaps cannot be rapidly modified by evolution. Such physiological changes upstream of egg production, for example linked to the insulin signalling, juvenile hormone, and vitellogenin pathways (Libbrecht et al., 2013a, 2018; Rau & Korb, 2020)may result in an extended lifespan via higher resistance to oxidative stress (Eyer et al., 2017; Flatt, 2011; Flatt & Kawecki, 2007b; Rodrigues & Flatt, 2016; Rueppell et al., 2015; Smedal et al., 2009).

Inside workers survive better

Overall, inside workers outlived outside workers in all three species and this was largely independent of the oxidative stress treatment. The exception was *A. echinatior*, where oxidative stress seemed to eradicate the survival difference. The observation that no induced oxidative stress was required for outside workers to die earlier indicates that control workers also experienced some sort of stress during their daily lives or due to our handling. The most likely cause for the survival difference between inside and outside workers is that the former are younger than the latter, and that older workers have a higher intrinsic mortality rate. In many advanced eusocial insects, workers undergo a temporal polyethism. They start out as young workers conducting inside tasks such as brood care, and only later move outside the nest, e.g. to forage (Camargo et al., 2007; Giraldo & Traniello, 2014a; Kohlmeier et al., 2018; Larsen et al., 2016; Wilson, 1980). Since old workers are more likely to leave the nest and forage, they are also more exposed to predators and parasites than the inside workers. Such a higher risk of extrinsic mortality would select for a lower investment into body maintenance in old workers and consequently a higher intrinsic mortality. Any investment into body repair processes are less likely to pay off with increasing chances that an individual will be killed (Corona et al., 2007a; de Verges & Nehring, 2016; Giraldo & Traniello, 2014a; Kramer & Schaible, 2013b; Tofilski, 2002).

No effect of queen loss on Temnothorax rugatulus workers

Our result that queen loss did not improve the resilience of *T. rugatulus* workers to oxidative stress is surprising, because unchallenged workers have been previously shown to indeed live longer in queenless colonies of *T. longispinosus* and *T. rugatulus* (Kohlmeier et al., 2017c; Negroni et al., 2020). It is possible that the prolonged lives of *T. rugatulus* workers are caused by a different mechanism than in the leaf-cutting ants, or that the differences in lifestyle cause the leaf-cutting ants to be more sensitive to oxidative stress in general (e.g. the fungus garden resource(Villesen et al., 2002) or metabolic rates (Keller, 1998a)). Leaf-cutting ant workers exhibit much shorter lifespans (around 3-7 months (Dijkstra et al., 2005; Verza et al., 2017); Simon H, Majoe M, Nehring V. unpublished data) than *Temnothorax* workers, which can live for 2-3 years (Kohlmeier et al., 2017a) supporting the notion that *Temnothorax* workers are more resilient. Indeed, both *Acromyrmex* and *Atta* workers typically die within a few days without food (Nehring V. unpublished data), while *T. rugatulus* workers can live for months without nutrition (Rueppell & Kirkman, 2005).

Conclusions

We show here that queenless leaf-cutting ant workers are more resistant to paraquat-induced oxidative stress than queenright workers are. This indicates that queen loss leads to physiological changes in orphaned workers that increase their resilience to oxidative stress, and potentially lengthens their lifespan. These changes may be directly caused by fecundity, e.g. because vitellogenin synthesized for egg production can act as an antioxidant, as it does in honeybees. Workers may also upregulate their body maintenance in other ways to increase their chances of future direct reproduction. However, given that one of the species we tested has sterile workers, it is possible that the effect is ultimately caused by workers becoming more valuable, because they cannot be replaced anymore, or by fundamental physiological constraints. Queen loss had no effect on the resilience of *T. rugatulus* workers, although these are known to live longer when orphaned. To elucidate the causes of these interspecific differences, comparative studies including more species (Korb, 2016) detailed research into the physiological and molecular mechanisms underlying worker lifespan are needed.

Ethics

Ant collection and export permits were obtained for the leaf-cutter ants from the Autoridad Nacional del Ambiente (ANAM) in Panama by JJ Boomsma, and for *T. rugatulus* by S. F. from the Coronado National Forest via the SWRS. Import and export licences are not required for the transport of our study species. We followed the DFG Animal welfare guidelines and the local laws.

Authors' contribution

All authors designed the experiment. *T. rugatulus* ant collection was done by S.F. with help of Barbara Feldmeyer and Marina Choppin. M.M. conducted the experiments and survival analyses. V.N. and R.L. helped M.M. in the statistical analyses. M. M. wrote a first draft and all authors revised it. V.N., S.F. and R.L. co-supervised the project.

Competing interests

We have no competing interests.

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Supplementary

The following are available online:

Supplementary table 1: Results of the Type II ANOVA results of the coxme survival models for each of the three Myrmicine species. The whole models are followed by post-hoc survival analyses of each species under control and oxidative stress treatments.
Supplementary table 2: Raw data for the experiment with *Acromyrmex echinatior*
Supplementary table 3: Raw data for the experiment with *Atta colombica*
Supplementary table 4: Raw data for the experiment with *Temnothorax rugatulus*
Supplementary document 5: The R script containing the analyses associated with this study.

Chapter 2

Functional decoupling of queens and workers in an invasive unicolonial ant *Lasius neglectus*

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In prep

Abstract

Social hymenopteran societies consist of reproductive queens and non-reproductive workers. Their caste-fate is typically determined early in development and is irreversible in adulthood. Among ant species, workers have usually lost their sperm storage organ, but un-inseminated workers can still produce haploid males from unfertilized eggs upon absence or removal of their queen. In a few evolutionarily derived genera, workers have completely lost functionality of their ovaries and become obligately sterile, a characteristic often found in workers of unicolonial invasive species. These ants always have many queens per colony, such that budding colony fragments are essentially never queenless for any substantial period of time and workers have no possibility of gaining direct fitness benefits. In such cases, queen removal should no longer affect worker physiology as it typically does in species where workers do develop their ovaries after queen loss. We hypothesized that worker sterility combined with excessive queen abundance should have removed important aspects of queen-worker-interdependence. We investigated this hypothesis by measuring fat body gene expression and overall worker resistance to oxidative stress in queenright and queenless colonies of the Formicine ant *Lasius neglectus* which is unicolonial in its invaded habitats. We additionally compared queens and workers of different ages. We found age-specific differences in gene expression for both castes, but no difference in gene expression or stress resistance between workers with or without queens. Our results imply that invasive unicoloniality appears to be driven by aworker force that is functionally independent of the actual presence of the queen caste, because this social condition never occurs in the wild.

Keywords: Unicolonial ant, *Lasius neglectus*, sterile workers, gene expression, fat body

Introduction

In many animal societies, reproduction is unevenly distributed among individuals. For instance, in non-human primates, one or a few individuals are socially dominant and monopolise reproduction (Balasubramaniam et al., 2012; Beisner et al., 2016; Brent, 2015; Cords, 2007; Qi et al., 2009; Thierry, 2007). In some species of mole rats, reproduction is also completely monopolised by few individuals. The offspring stay with their parents as helpers. The helpers do not ovulate, have low sperm counts, and die much younger than their parents (Burda et al., 2000; Jacobs et al., 2021). Helpers can become reproductively active upon the death of the dominant individuals, which also triggers changes in their morphology, physiology, and behaviour (Faulkes & Bennett, 2001).

In contrast, the social insects represent a major transition in evolution (MTE) with obligate, permanent division of reproductive labour leading to the formation of the castes-reproductive and non-reproductive. Such an evolution of morphologically and physiologically differentiated castes evolved convergently over evolutionary time in the hymenopteran corbiculate bees,vespine wasps and ants (Boomsma & Gawne, 2018). A similar MTE probably also occurred in the isopteran ancestors of termites, (Revely et al., 2021). In all these lineages, the queens are always specialized as colony's-germline, laying all or almost all of the eggs, while the workers have primarily somatic colonial functions, such as brood care, upkeep of the nest, foraging, and defence. Such a germline-soma analogy never applies to the open societies of vertebrates, insects, and some other arthropods.

Obligate reproductive division of labour in colonies of closely related individuals is often reflected not just with specialized caste-specific morphology but also lifespan. In perennial superorganisms with a single inseminated queen (ants, bees, wasps) or royal pair (termites), the reproductives can live for decades while the non-reproductive workers often fail to survive for much more than a year (Keller & Genoud, 1997b; Korb, 2016; E. R. Lucas & Keller, 2020; Parker, 2010). Compared to workers, the highly fecund queens have upregulated molecular gene regulatory networks for body maintenance, which somehow appear to mediate an investment into a longer life span (S. Lin et al., 2021; Monroy Kuhn et al., 2021; Negroni et al., 2019). However, this contrast is smaller in annual superorganisms (bumblebees and yellowjacket wasps) and in perennial superorganisms that have either queen replacement procedures or polygyny in the form of multiple coexisting queens (Boomsma et al., 2014). The queens usually signal their presence and fecundity with queen pheromones, which are remarkably similar across the Hymenoptera. Queen pheromones exist in several social insect lineages, consistent with these chemical compounds likely having their origin in a common solitary wasp ancestor (Grüter & Keller, 2016; Holman et al., 2010; Keller & Nonacs, 1993; Shimoji et al., 2017). As long as the queen pheromones are present in a colony, the workers typically do not develop their ovaries.

Worker ovary development is usually conditional on the queen's capacity for egg-laying, which varies over time (Monnin, 2006). Workers may thus interpret a reduced or terminated flow of queen pheromones as an incentive to produce male-destined haploid eggs, as usually happens in haplodiploid hymenopteran lineages of ants, bees and wasps (Khila & Abouheif, 2010; Meunier et al., 2010), or by leaving the nest and starting their own colony, as in some lower termites (Rau & Korb, 2021). Across the superorganismal lineages, life-time unmatedness is therefore a better predictor of worker or soldier somatization than just their sterility, because diplo-diploid termite workers are automatically sterile when they cannot mate, while unmated hymenopterans cannot produce female offspring (Boomsma & Gawne, 2018). Upon queen loss, hymenopteran workers therefore typically develop their ovaries to lay eggs. Interestingly, when workers develop their ovaries and become reproductive; they can outlive their non-reproductive sisters. This may be a result of ovary development being accompanied by lifespan-extending physiological effects such as a higher resistance to oxidative stress and an upregulation of immunity and longevity genes (Giehr et al., 2020; Kohlmeier et al., 2017b; Majoe et al., 2021; Negroni et al., 2021; S. C. Seehuus et al., 2006a).

Not all hymenopteran workers are equally likely to reproduce after queen loss. Newly hatched workers carry out tasks inside the nest, such as brood care, and older workers have progressed to riskier tasks outside the nest, such as foraging or nest defence. Across a variety of lineages, young workers are more likely to activate their ovaries and start laying eggs than older workers (Dijkstra et al., 2005; Leonhardt et al., 2016; H. Lin et al., 1999; Nakaoka et al., 2008). Nurses and foragers also tend to take up spatially distinct positions within the nest, with nurses staying closer to the queen and brood, and older workers being more peripheral (Richardson et al., 2021).

The potential for worker reproduction appears beneficial when the queen becomes sick or dies, because it allows the colony to pass on more genes to future generations before it collapses. However, worker reproduction also implies a risk of intra-colony conflict when it would happen prematurely. This is because the fitness interests of haplo-diploid workers and queens, and among different workers, are typically not aligned. In many, but not all species studied, these conflicts are suppressed by policing when workers attack their own egg-laying sisters or when they destroy worker-laid eggs (Brunner & Heinze, 2009; Dijkstra et al., 2010; Helanterä & Sundström, 2007; Schmid et al., 2013; Stroeymeyt et al., 2007b; Van Oystaeyen et al., 2014).

However, none of this applies in the various ant lineages where worker ovaries are lost or have become otherwise non-functional. This evolutionary derived development in some peripheral branches of the ant phylogeny renders workers sterile for life. These species tend to be overrepresented among the invasive ants that are almost invariably characterized by high degrees of polygyny (multiple queens per nest), low relatedness among the workers often approaching zero, and a highly polydomous colony structure without hostility between neighboring nests (Helanterä et al., 2009). Unicolonial ants with sterile workers thus represent the most distinct analogy between colonial superorganisms, with queen-germlines and worker-soma, and metazoan organisms with segregated cell-lineages with germline and soma functions.

To our knowledge, no studies have addressed whether and how workers of unicolonial ants interact in terms of fertility signaling. Sterile workers of *Atta* and *Acromyrmex* leaf-cutting ants are known to become more resistant to oxidative stress when they lose their queen, indicating that queen presence is still important here (Majoe et al., 2021). However, *Atta* colonies have a single irreplaceable queen and *Acromyrmex* colonies are at best weakly polygynous (Nehring et al., 2018). *Atta* species have fully sterile workers, while the large worker caste of *Acromyrmex* does develop its ovaries without a queen. In spite of this difference, the queenless colonies of both genera appear to prioritize raising the remaining sexual offspring of the queen over worker reproduction (Dijkstra & Boomsma, 2007). When the only queen in the monogyne form of the red imported fire ant *Solenopsis invicta* dies, all but one of the virgin unmated sexuals are promptly executed by the workers of the colony. The surviving sexual then produces maximally related male offspring in a final reproduction attempt (Wurm et al., 2010). In this species, queen loss, even in monogynous colonies had only subtle effects on whole-body gene expression of workers (Manfredini et al., 2014).

In contrast to monogynous and weakly polygynous ant species, queen loss is effectively impossible in unicolonial ants whose sterile workers normally experience no boundaries between nests. Queens freely migrate across nests according to circumstances, and where newly hatched virgin queens are inseminated without needing a dispersal flight (Helanterä et al., 2009; Silverman & Brightwell, 2008; Tsutsui et al., 2000). This implies that queen mortality has become decoupled from the survival of both local nests and the entire polydomous colony, and that any form of selection for physiological responses to queen loss in the worker caste is expected to have disappeared.

We tested these expectations using the invasive species *Lasius neglectus*, a unicolonial formicine ant that was discovered rather recently (Van Loon et al., 1990)and is native to Central Asia (Stukalyuk, 2020). The species has been introduced to Europe via the Black Sea area (Cremer et al., 2008) and spread further across most of Europe through human-assisted dispersal (Boomsma et al., 1990; Seifert, 2000). *Lasius neglectus* is a suitable model species because a high-quality sequenced genome is newly available and workers have never been observed to lay eggs, which is consistent with their ovaries regressing completely within the first four months after hatching (Espadaler et al., 2004; Espadaler & Rey, 2001; Gotoh et al., 2016; Stukalyuk & Radchenko, 2018). Other *Lasius* species are known to have workers with active ovaries (Bourke, 1988) and reproductively active *Lasius neglectus* queens still produce the same fertility pheromone as other *Lasius* ants (Holman et al., 2013). This suggests that a possible decoupling of worker physiology from queen mortality is evolutionary recent, consistent with the existence of a sibling species, *Lasius turcicus*, that has not become invasive and unicolonial albeit it too has been observed to use intranidal mating and colony budding (Cremer et al., 2008).

We examined fat body transcriptomes because the fat body is an important general metabolic organ, analogous to the human liver and adipose tissue, and it plays an important role in regulating fertility (Partridge et al., 2011; Tatar et al., 2003; H. Yan et al., 2022). We investigated whether queen removal increases oxidative stress resistance, as has been found previously for workers of other ant species (Majoe et al., 2021) and the honey bee (Kennedy et al., 2021; S. C. Seehuus et al., 2006a). For *L. neglectus* workers, we examined workers collected inside nest by the brood pile, and outside the nest, near the food. Their positions with respect to the nest was used as a cue for their age. For queens, we obtained a series of age-specific gene expression profiles, which allowed us to identify sets of genes that changes over time in the two female castes. Consistent with our hypothesis, we found that the queen's presence or absence had no effect on the workers' susceptibility to oxidative stress or on their fat body gene expression profiles. However, we observed strong differences between 'inside-workers' and 'outside-workers', both in terms of overall gene expression profiles and oxidative stress susceptibility, consistent with rapid aging in these obligately-sterile workers.

Material and Methods

Study site, collection, and laboratory maintenance

All ant colonies used for the study were collected from the Jena Botanical Garden in Germany on three collecting trips in July 2019, September 2019, and July 2020. Alates, brood, workers, and queens were found close to the surface near flowerbeds and in sand piles in disturbed, slightly damp areas of the garden. We assigned ants collected from different sand piles or areas that were about 5-10 m apart to independent 'parent' colonies and kept them separate once brought into the laboratory. The ants could place their brood and queens in glass tubes half-filled with water and stoppered with cotton wool. These tubes were covered with aluminum foil to provide a darkened environment. The tubes were placed in Fluon™-coated boxes (10 x 10 x 5 cm), which acted as foraging arenas. The ants could access an agarose-based jelly-mixture containing honey and egg, ad-libitum. All boxes were placed in a climate cabinet with a temperature set to 25°C and a 12h light:12 h dark cycle.

Creating subcolonies

Dealate queens collected within colonies were later assigned to be 'old' queens in our transcriptome study. 'Young' queens were those collected with wings - presumably still unmated. We separated the dealate and winged queens arising from the same parent colonies and set each up with half of the workers from that colony. Then we added males to the sub-colonies with the winged queens, which could so become inseminated. Typically, after copulation, the queens shed their wings and become reproductive.
To investigate the effect of queen removal on workers, we established queenless sub colonies from young-queen (de-alated) and old-queen colonies. These sub colonies were composed of about 80 workers and larvae. They were separated for four weeks, which was sufficient in other ant species to elicit ovary development in workers (Kohlmeier et al., 2017b). Since our ants were in smaller nest boxes with low activity, we starved them for three days prior to collection. We then marked individuals that were the first to arrive at the food on two consecutive days as 'outside-workers' and those that stayed by the brood pile as 'inside-workers'.

Oxidative stress experiment with workers

The marked workers from each sub colony (queenright or queenless), some brood, and water were used to produce two experimental units consisting of five outside-workers and five inside-workers. Each experimental colony was randomly assigned to control or treatment. Over the next twelve days, 196 workers from five colonies (three old-queen and two young-queen colonies) were subjected to paraquat, which induced oxidative-stress, or water as a control treatment. Every two days, the solutions were applied with a brush on the heads of an ant, followed by a period of 2 hours of isolation allowing each ant to self-groom and ingest the solution. Their survival was tracked every day. The treatment and observation of survival followed the protocol of a previous study on susceptibility of myrmicine workers to oxidative stress (Majoe et al., 2021).

Transcriptome analyses

Six weeks after the division into queenright and queenless sub-colonies, in November 2021, we sacrificed workers between 12:00 and 16:00, and dissected their fat bodies. We pooled the fat bodies of four workers into a single sample. All samples were stored in Trizol at -80°C. The queens were sacrificed on two consecutive days in November 2021 as described for the workers, but each sample contained the fat body of only a single queen (Supplementary table 1). At the time of sampling, the young queens of five colonies were about six months old. Because the old queens were reproducing when we collected them in the field, we assumed that the colonies had produced them at an earlier reproduction event than the cohort of winged queens that we collected at the same time. Since *L. neglectus* colonies have been observed to produce winged sexuals only once a year (Espadaler & Rey, 2001; Stukalyuk, 2020; Stukalyuk & Radchenko, 2018), we inferred that the old queens were one year or older when we sacrificed them. We also had two queens that were 2.5 years or older according to the same inference. The ovaries of five young and five old queens were dissected to ascertain that they were inseminated. We could confirm that all queens had sperm-filled spermathecae and that mean ovariole lengths were not significantly related to age ($n = 10$; $\chi^2=0.02$, $p=0.89$; Supplementary fig 1).

Genome assembly and annotation

Lasius neglectus specimens, which were collected in 2015 in Jena and since then reared in the laboratory, were provided by the Sylvia Cremer group and used for genome sequencing as part of the Global Ant Genomics Alliance (GAGA) initiative (Boomsma et al., 2017). High-quality DNA was extracted from workers using a phenol/chloroform phase separation DNA extraction protocol. Single-tube long fragment read (stLFR) libraries were prepared and sequenced by BGI using the BGISEQ-500 platform. In addition, tTotal RNA from whole individual queens, males and brood was extracted using the RNeasy Plus Mini Kit from QIAGEN. The quality of extracted RNA was assessed with the Agilent 2100 Bioanalyzer. RNA-seq libraries were prepared with >1µg total RNA using the MGIEasy RNA Library Prep Kit (BGI) and sequenced on the BGISEQ-500 platform.

Raw stLFR reads were first cleaned from adaptors and PCR duplicates, and the barcode IDs were assigned in the read names using the stlfr2supernova_pipeline (https://github.com/BGI-Qingdao/stlfr2supernova_pipeline). The clean reads were then assembled using MaSuRCA v3.3.0 (Zimin et al., 2013); "JF_SIZE = 2500000000") and the resulting assembly was further scaffolded using the barcoding information from stLFR reads to assemble contigs into scaffolds with SLR-superscaffolder pipeline (https://github.com/BGI-Qingdao/SLR-superscaffolder). Putative duplicated scaffolds were identified and filtered using Funannotate "clean" pipeline v1.8.3 (Palmer & Stajich, 2022). The genome assembly was screened for putative contaminations from other organisms using a pipeline established and optimized for the ant genomes in the GAGA project. In brief, we compiled separate databases containing 1908 complete bacterial genome sequences, 43 complete insect genome sequences, as well as databases containing corresponding bacterial or insect CDS sequences. We divided the *L. neglectus* genome assembly in 2000 bp sliding windows (overlap 500 bp) and searched each window against the different insect and bacterial databases using mmseqs (release_12-113e3) and identified the single best hit (according to bitscore) for each sliding window against each database. For each scaffold, we calculated the ratio of windows showing higher similarity to bacterial than to eukaryotic databases and used this, along with coverage and GC content information, as three lines of independent evidence to identify contaminant scaffolds. Genome assembly quality was evaluated using contiguity metrics, gene completeness with BUSCO v5.1.2 (BUSCO citation), and consensus quality (QV) and k-mer completeness using Merqury (Rhie et al., 2020) shown in Supplementary Table A . Finally, some mitochondrial (*CO1* and *CytB*) and autosomal gene markers (*Wingless*, *LwRh*, *AbdA*, *ArgK*) were annotated in the genome assembly using BITACORA (Vizueta et al., 2020) to confirm the species identity from *Lasius neglectus* sequencing data.

Homology-based and *de novo* methods were conducted in combination to identify the transposable elements (TEs). The genome sequences were aligned against the Rebase TE library (v25.03) (Roberts et al., 2015) and TE protein database using RepeatMasker and RepeatProteinMask (version 4.1.2) (Smit et al., 2013). In addition, RepeatModeler v2.0.2 (Smit & Hubley, 2021) was used to build a *de novo L. neglectus* repeat library, which was subsequently used to annotate repeats using RepeatMasker. TRF v4.10.0 (Benson, 1999) was used to find tandem repeats with parameters: "Match = 2, Mismatch = 7, Delta = 7, PM = 80, PI = 10, Minscore = 50". Finally, we combined all evidence resulting in 26.82% of the assembled genome being repeat sequences, with a total length of 68 Mb (Supplementary table A).

General genome annotation was conducted by combining gene annotation from several sources using a pipeline optimized for the ant genomes generated by the GAGA project. First, publicly available RNA-seq from *L. neglectus* workers and queens (NCBI Bioproject PRJDB4088, Morandin et al., 2016), as well as the GAGA generated RNA-seq were aligned to the reference repeat soft-masked genome assembly using STAR v2.7.2b default options (Dobin et al., 2013). In addition, we retrieved the publicly available gene annotations from the fruit fly *Drosophila melanogaster*, the red flour beetle *Tribolium castaneum*, the parasitoid wasp *Nasonia vitripennis*, the honeybee *Apis mellifera*, the clonal raider ant *Ooceraea biroi* and the Florida carpenter ant *Camponotus floridanus* (Hoskins et al., 2015; H. S. Kim et al., 2010; McKenzie & Kronauer, 2018; S. Richards et al., 2008; Shields et al., 2018; Weinstock et al., 2006; Werren et al., 2010) . The annotations from these insect species were used to conduct homology-based gene predictions using GeMoMa v1.7.1, which also incorporated the RNA-seq evidences for splice site prediction (Keilwagen et al., 2019). Second, the independent RNA-seq alignments were merged creating a consensus GTF (Gene transfer format) using Stringtie v2.1.5 (Kovaka et al., 2019), and BestORF (Molquest package, Softberry) was used to identify open reading frames (ORF) in the transcript sequences, after which the transcripts with incomplete ORFs were filtered out. Third, we randomly selected ~1,000 high–quality genes from the GeMoMa prediction to train Augustus v3.2.2 (Stanke et al., 2008). The *de novo* gene prediction was then performed using Augustus with the repeat-masked genome, filtering out genes with lower length than 150 bp or incomplete ORFs. Finally, gene annotations from the three independent sources of evidence were combined, generating a final gene annotation for the *L. neglectus* genome, from which we also generated an annotation with a single representative isoform per gene (i.e.: the longest isoform was kept as the representative one). Transposon-related proteins were identified and filtered using a BLASTP search against the Swissprot database and the transposable element protein database from RepeatMasker. The quality of the genome annotation was evaluated in terms of completeness based on the BUSCO hymenoptera dataset (Supplementary table A).

Data Deposition: The genomic data of *L. neglectus* have been deposited into the CNGB Sequence Archive (CNSA) of the China National GeneBank DataBase (CNGBdb) with accession number CNP0003995.

RNA extraction and analysis

RNA from queens and workers was extracted using a Trizol: Chloroform: Isoamylacohol mixture based on an in-house protocol (adapted from Lin, Werle, & Korb, 2021) followed by cleaning steps employing the Qiagen RN-easy Mini Kit (Supplementary info 1). The concentration and quality of the isolated RNA was checked on an Agilent Bioanalyzer (Agilent RNA 6000 Nano Kit) at the Beijing Genomics Institute (BGI). The libraries were prepared and sequenced by BGI using the Illumina HiseqXTen sequencing platform, yielding 150 bp paired-end reads with a sequencing depth of 23.85+/-1.97 (mean+/-sd) million reads.

Raw reads for 47 samples (9 old queens, 9 young queens, 8 queenright old workers, 8 queenright young workers, 6 queenless old workers, 7 queenless young workers; Supplementary 1) were trimmed using fastp (version 0.2), such that all reads that passed had a minimum length of 70 bp. The trimmed results were checked with MultiQC-1.7 and the quality of the reads was assessed with FastQC (version 0.11.8). HISAT2 (version 2.1.0) was used to align the trimmed sequences to the *L. neglectus* genome. All samples were assigned to the genome with an alignment rate of 83.7-91.66%. The SAM files generated by HISAT2 were first converted to BAM files and then sorted by name using SAMtools (version 1.9). The sorted BAM files were used to create gene count tables with Htseq-count (Htseq version 0.11.2; settings: -f bam, -i ID, -t gene, -m union, -r name, --stranded=no). A single gene count table consisting of all samples was then compiled. In addition, a metadata table was assembled that contained information about each sample. The data were further analyzed using R-Studio (R version 4.0.4). We used the DESeq2 package (version 1.30.1) to analyse differential gene expression. Since we wanted to explore different predictors for queens and workers, we decided to analyze the data sets for the two castes separately. In each dataset, we removed genes that had low read counts in more samples than could potentially be expected even if only one of the experimental groups would express the gene. We thus only kept genes with at least ten

reads in at least five worker samples (out of 14059 genes in the genome, 10691 remained) or at least eight queen samples (10022 genes remained).
For the queen analysis, we used "age" (young vs. old) and "parent colony" as factors and determined the effect of age using the Likelihood Ratio Test (LRT), while controlling for the effect of parental colony. The worker-specific model included the factors "parent colony", "queen presence" (present/absent), "worker position" (inside/outside), and the interaction between the latter two variables. Since the interaction did not reveal any differentially expressed genes (DEGs), we built an additive model with the three factors and used the LRT to test the effects of location and queen presence separately, while controlling for the other two factors. Adjusted p-values of 0.05 were used as a cut-off to determine all genes whose expression was significantly explained by the factor of interest. By default, this adjusted p-value was a result of correcting p-values for false discovery rates using the Benjamini-Hochberg procedure.

Gene annotation, functional enrichment and comparison to known candidate gene lists

We ran Interproscan (v5.54) on the *Lasius neglectus protein coding genes* to obtain functional annotations and process GO (Gene Ontology) IDs associated with our differentially expressed gene (DEG) lists. We extracted proteins associated with age- and position-related DEGs from the queen and worker analyses respectively. The GO terms from the Interproscan results were used to perform enrichment analyses using topGO (v 2.4.2) on R-Studio (R version 4.0.5). We used the 'weight01' algorithm to determine the nodes and Fisher's test with $p<0.05$ after Benjamini Hochberg correction to determine the significant GO terms from our lists by comparing them to a dataset of 8255 unique proteins with GO IDs present.

We ran a local BLASTp (BLAST v 2.5.0+) of the entire proteome against a database of nine insect species: *Drosophila melanogaster, Apis mellifera, Lasius niger, Linepithema humile, Solenopsis invicta, Ooceraea biroi, Acromyrmex echinatior, Atta colombica* and *Temnothorax curvispinosus*. An e-value of 1e-5 was used to filter and a maximum of 10 hits were displayed, the best hits were chosen based on the highest bit-score. The functional annotation of genes associated with specific GO terms could be determined by retrieving the selected *Lasius neglectus* genes from the full list (Supplementary table 2).

We also specifically investigated some candidate genes. We used Orthofinder (v.2.5.4) to find orthogroups of similar *L. neglectus* and *D, melanogaster* proteins. We then extracted those orthogroups associated with 387 proteins coded by 123 candidate genes implicated in ageing in *D, melanogaster* and other social insects. Referred to as 'TI-J-LiFe' genes (Korb et al., 2021), this list features genes in IIS (Insulin/Insulin Like Growth factor), TOR (Target of Rapamycin), and Juvenile hormone signalling in *Drosophila melanogaster.* Similarly, we extracted orthogroups associated with 85 proteins from 33 known *D. melanogaster* derived oxidative stress genes (Kramer et al., 2021).

We additionally searched specifically for the expression of any *Vitellogenin* (*Vg*) genes in the *L. neglectus* genome because of the role of *Vg* and their copies in determining fecundity, regulating oxidative stress, and division of labor. The *Vg* genes were identified by conducting a reciprocal BLASTP search of the *L. neglectus* proteome against a database of annotated Vitellogenin amino acid sequences with an e-value of 1e-5. The sequences were from the 33 species used in (Kohlmeier et al., 2018).

Results

Position, but not queen presence, affects worker resistance to oxidative stress

Survival was significantly lower under paraquat-induced oxidative stress compared to the control (treatment, n = 196, $\chi^2 = 26.1$, $p < 0.001$). The outside (foraging) workers had a lower survival in general (foragers *versus* nurses, n= 196, $\chi^2= 17.6$, $p<0.001$), and we did not detect a significant effect of queen removal on worker survival (queen presence *versus* absence, n=196, $\chi^2=0.53$, $p=0.46$), neither for inside (nursing) nor for outside workers (interaction queen presence/absence : inside/outside worker position, n= 196, $\chi^2 = 0.19$, $p = 0.66$). Similarly, there was no interaction between paraquat treatment/control and queen presence/absence (n= 196, $\chi^2= 1.8$, $p = 0.18$), or between paraquat treatment/control and inside/outside position n=196, $\chi^2= 0.32$, $p = 0.57$).

To make sure that we did not miss potentially weak effects of worker position or queen presence/absence because they were overshadowed by the strong treatment effect, we additionally ran two separate models for paraquat treatment and control. Queen removal still had no effect on worker survival; neither in the control (queen presence/absence, n = 97, $\chi^2= 2.1$, $p = 0.15$), nor in the paraquat treatment (queen presence/absence, n = 99, $\chi^2= 0.07$, $p = 0.78$). There was no significant interaction between queen presence and worker position on worker survival in either the control treatment (queen presence/absence x inside/outside position, n= 97, $\chi^2= 0.05$, $p=0.82$) or the paraquat treatment (queen presence/absence x inside/outside position, n= 99, $\chi^2= 0.63$, $p=0.42$). This implied that the inside/outside sampling position of workers explained most of the variation in worker survival, both in the control experiment (inside/outside position, n = 97, $\chi^2= 5.95$, $p=0.015$) and in the paraquat-treatments (inside/outside position, n= 99, $\chi^2= 11.58$ $p<0.001$; Fig. 1, Supplementary table 3).

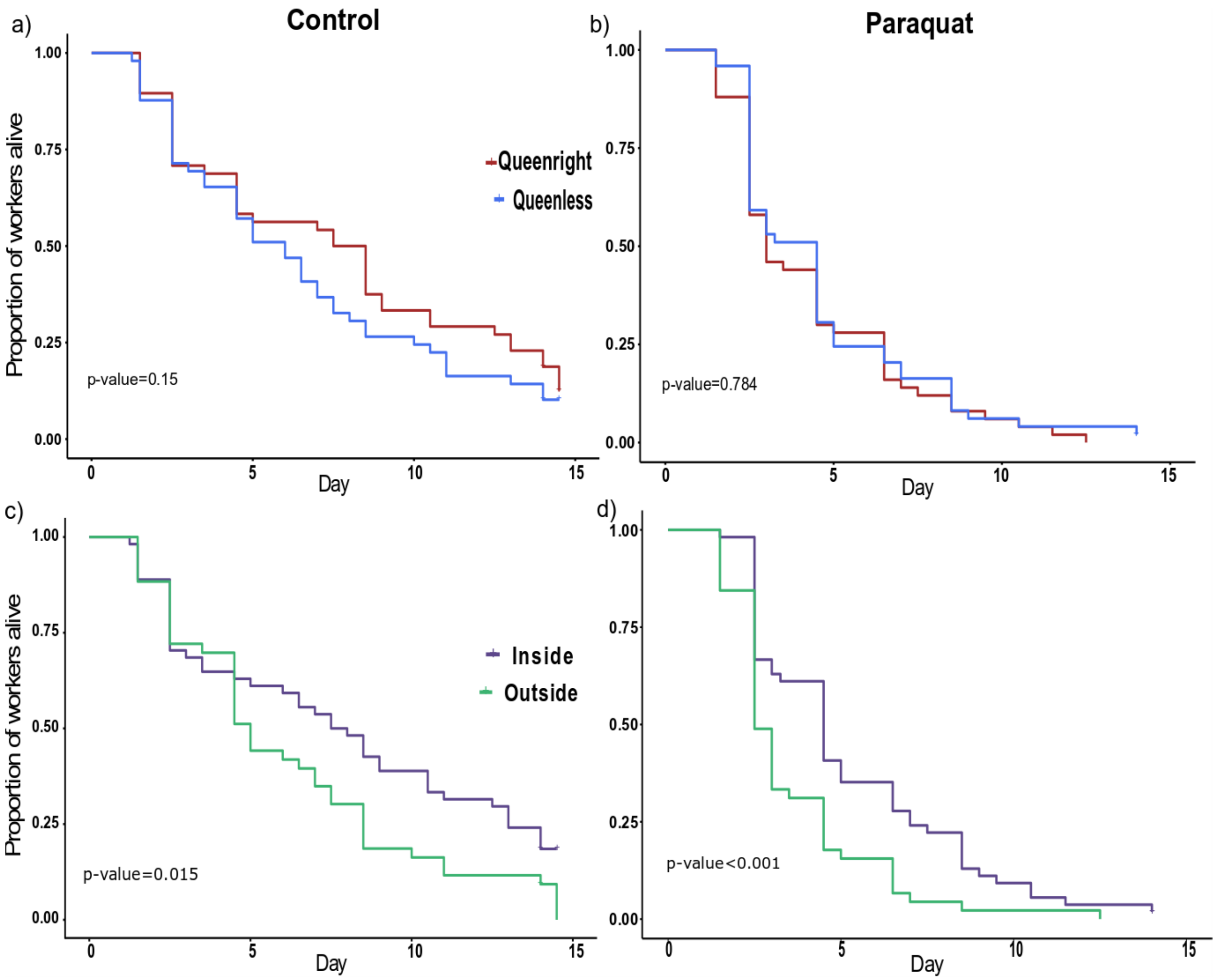

Figure 1: The Kaplan-Meier curves show the survival of *Lasius neglectus* workers over 14 days. Queen presence did not affect worker survival under control (a) or paraquat-induced oxidative stress conditions (b). Inside (nursing) workers tended to survive better (c,d), in particular under paraquat treatment (d).

Gene expression analyses

There was no effect of queen presence/absence on worker gene expression, neither for outside nor for inside-workers after correcting for false discovery rate ($p_{adjusted}$ >0.05 for both queen presence alone and for the interaction term inside/outside location versus queen presence/absence; Fig. 2). There were 2743 differentially expressed genes (DEGs) between inside and outside-workers, with 1444 of these overexpressed in inside workers and 1229 in outside workers. DESeq2 analysis of the queen samples identified 165 genes that were differentially expressed between young and old queens (Fig 2d, Supplementary Table 4). Of these, 80 genes were significantly overexpressed in younger queens while 85 genes were significantly overexpressed in older queens (Fig 2). There is thus an order of magnitude difference in DEGs between young and old queens compared to young and old workers.

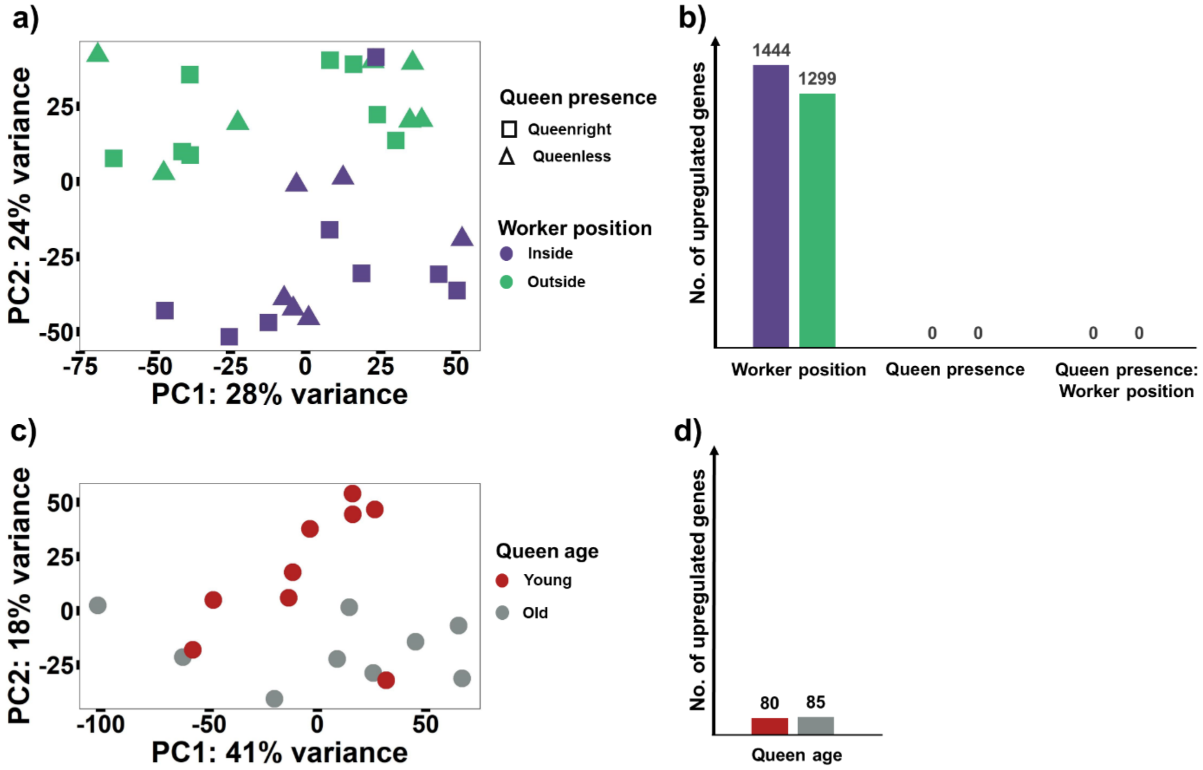

Figure 2: Principal component analyses based on the expression of: a) all genes expressed in workers and (c) all genes expressed in queens, after filtering out genes with low read counts (see methods). The expression of many genes was affected by the inside/outside position of workers, likely reflecting their age (b: inside/outside; Deseq2 $p_{adjusted} < 0.05$), but no genes were affected in their expression by queen presence/absence alone or by the interaction between queen presence/absence and inside/outside position. d) Queen age also affected gene expression. The colours in panels b and d match those of panels a and c.

Differentially expressed genes were significantly enriched in six GO terms for workers and in four GO terms for queens (padjust < 0.05 after correcting for multiple testing; Figure 3; Supplementary Table 4). The genes upregulated in inside workers were related to DNA replication (Figure 3a, Supplementary table 4). Interestingly, the GO term ‘proteolysis involved in cellular protein catabolic processes’ and ‘cholesterol metabolic process’ were enriched in both the outside workers (Fig 3b) and the old queens (Fig 3c). This first common term contained four ‘proteasome subunits’ related genes that were overexpressed in both outside workers and older queens (*Lneg_g05162* BLASTp match protein XP_011339939.1, *Lneg_g05171* match protein XP_011167410.1.1, *Lneg_g13954* match protein XP_024875312.1, *Lneg_g16342* match protein NP_525092.1). Similarly, two hormone sensitive lipases associated with genes *Lneg_g10026* – BLASTp match against XP_012229728.1 and *Lneg_g10677*- BLASTp match against XP_012229728.1 were upregulated in both the outside workers as well as the old queens (Supplementary table 4)

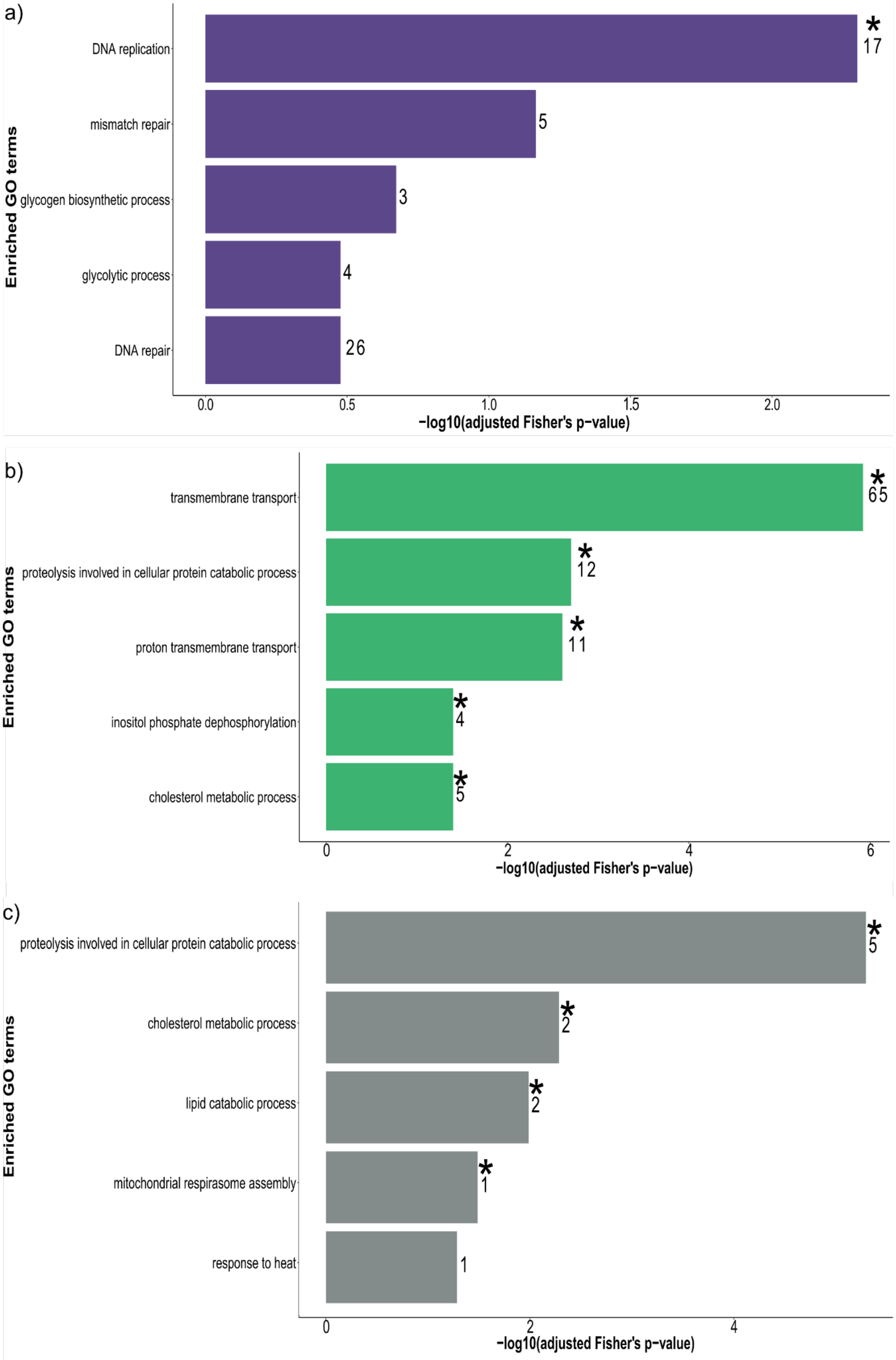
a)
Enriched GO terms
DNA replication
mismatch repair
glycogen biosynthetic process
glycolytic process
DNA repair
*
17
5
3
4
26
0.0
0.5
1.0
1.5
2.0
−log10(adjusted Fisher's p−value)
b)
Enriched GO terms
transmembrane transport
proteolysis involved in cellular protein catabolic process
proton transmembrane transport
inositol phosphate dephosphorylation
cholesterol metabolic process
*
65
*
12
*
11
*
4
*
5
0
2
4
6
−log10(adjusted Fisher's p−value)
c)
Enriched GO terms
proteolysis involved in cellular protein catabolic process
cholesterol metabolic process
lipid catabolic process
mitochondrial respirasome assembly
response to heat
*
5
*
2
*
2
*
1
1
0
2
4
−log10(adjusted Fisher's p−value)

Figure 3: Enriched GO terms (Biological Process) based on genes upregulated in: a) inside-workers vs outside-workers (likely younger nurses), b) outside-workers vs inside-workers (older foragers), and c) in older queens compared to young queens. Go terms are presented from top to bottom in their order of p-value significance in Fisher's exact tests, and all significant terms are displayed. Terms with $p < 0.05$ after Benjamini-Hochberg correction for multiple testing are marked with * next to the bars. The number of DEGs associated with each of the GO Terms is indicated next to each bar. The x-axis shows the -log10 of the adjusted Fisher's test p-value such that the longest bars lowest adjusted p-values (Supplementary Table 3). Bar colors are the same as in Figure 2.

Ageing and oxidative stress related genes

We found 66 common orthogroups when extracting those orthogroups containing the proteins associated with the 123 candidate genes involved in IIS (Insulin/Insulin Like Growth factor I), TOR (Target of Rapamycin) and Juvenile hormone (JH) signalling in *Drosophila melanogaster.* These three pathways have been implicated in lifespan regulation and ageing in social insects (referred to as 'TIJ-LiFe' by Korb et al., 2021). We then compared this list with our lists of DEGs ($p_{adjusted} < 0.05$) for both the workers and the queens. Seven unique orthogroups containing 10 of the 1444 genes upregulated in inside-workers were found (X^2=1.4 e-25, p=0.99). They contained genes such as four copies of *jhamt*, the TOR complex 1 regulator *gig* and the gene *thor* which encodes a translation initiation factor *4- E binding protein* which is in turn regulated by *tor.* The genes overexpressed in outside workers contained 11 of all 66 orthogroups containing 'TIJ-LiFe' genes (X^2=3.99, p=0.04). They contained 17/1229 DEGs. Among these genes were two copies of the gene *NLaz* and the gene *ImpL2* inhibitors of IIS signaling. Similarly, of the orthogroups present in young queens as well as in the 'TIJ-LiFe' genes list was one gene *pdk1* out of 80 DEGs. One copy of a *Lipase* gene and one copy of a *jhamt* were found among the 85 DEGS associated with old queens (Supplementary table 5).

For the analysis of oxidative stress genes, we employed the same method as above and found 31 unique orthogroups containing the 33 genes related to oxidative stress (85 proteins) in *D. melanogaster* and previously tested in other social insects (Kramer et al., 2021). Of these genes, the inside workers had upregulated expression of two copies of *Catalase* , one *Grx5* and one *gfzf* gene*.* whereas outside foraging workers had upregulated expression of 7 oxidative stress-associated genes in 7 unique orthogroups. These included: *Grx1*, *MsrA* , *Trx-2 and Trxr2*. None of these oxidative stress genes occurred in the list of queen DEGs (Supplementary table 5).

Finally, we found four matches when we conducted a reciprocal BLASTp of the whole *L. neglectus* proteome against the Vitellogenin database (Kohlmeier et al., 2018). Two genes *Lneg_Vitellogening002* and *Lneg_g15702 (both Dseq2 padj < 0.0001)* were overexpressed in inside workers relative to outside workers. They encode proteins that showed BLASTp matches in 'Vitellogenin' of *Lasius niger* (KMQ94142.1) and 'Vitellogenin-like protein A' of *Formica exsecta* (AII96913.1). Both these vitellogenins have been classified as Vg-like A copies according to (Kohlmeier et al., 2018; Supplementary Table 4). The expression of *Vg-like C* (encoded by the gene *Lneg_g03782 Dseq2* padjust = 0.0504), with a BLASTp match of AII96915.1 with Vitellogenin-like Protein C in *Formica exsecta*, tended to be higher in outside workers relative to inside workers. The protein of gene *Lneg_g11491* which matched against ‘vitellogenin-precursor’ of *L. niger* was classified as the Conventional-Vg/C-Vg. Its expression did not yield a p-value in workers signifying its expression was zero or contained outliers. In the queens, *C-Vg* was marginally overexpressed in younger queens with respect to older queens but it’s expression was not significantly explained by queen-age (*Lneg_ g11491 Dseq2* padjust = 0.105) (Supplementary table 5).

Discussion

In this study, we aimed to investigate whether and to what extent sterile *Lasius neglectus* workers respond to experimental queen removal. Our results demonstrate that the presence/absence of queens does not affect the susceptibility of workers to oxidative stress or alter their gene expression in the fat body. On the other hand, we found large differences in transcriptional activity and survival between younger inside workers and older outside workers and, to a much smaller extent, also between young and older queens.

Queen loss does not affect worker survival and fat body gene expression

Our experiments demonstrate that the experimental queen removal manipulation had no effect on the susceptibility of *L. neglectus* workers to stress induced by paraquat or on their fat body gene expression. There were, in fact, no genes that differed in expression between queenright and orphaned workers of *L. neglectus.* This is in sharp contrast with many studies reporting that queen removal triggers a cascade of phenotypic and regulatory changes in the otherwise fully somatic worker caste. In the ants and honeybees studied, queen loss affected the expression of hundreds if not thousands of genes, especially of genes encoding proteins associated with oxidative stress (Amsalem et al., 2017; Giehr et al., 2020b; Kennedy et al., 2021a; Negroni et al., 2021; Seehuus et al., 2006a; Tsuji et al., 1996). Queenless workers were also typically longer lived or more stress resistant (Majoe et al., 2021).

In most ant species, workers respond to queen loss by activating reproductive pathways and by laying male-destined eggs (Alaux et al., 2006; Dijkstra et al., 2010), while at the same time their lifespan increases (Choppin, 2022; Negroni et al., 2021). It has been proposed that reproduction itself may automatically lead to longer life in social insects because molecular pathways required for egg production also affect physiological mechanisms important for body maintenance. For instance, the egg yolk precursor vitellogenin also protects honeybees workers from oxidative stress (Münch & Amdam, 2010). In contrast, we were unable to observe any differences in the stress resistance of *L. neglectus* workers after queen removal. This is consistent with the hypothesis that reproduction itself prolongs worker lives and with that effect being absent when workers are permanently sterile.

An alternative hypothesis is that longer worker life spans are not a direct result of worker reproductive ability but a means to increase the colony’s reproductive output despite the queen’s absence. Orphaned workers in some ant species might have been selected for longer life spans, so that they have more time to raise the remaining queen-laid brood before the colony dies. This has been documented in *Acromyrmex* leaf-cutting ants where orphaned workers raise “emergency” sexuals that should be able to disperse and spread the colony’s genes, rather than workers that would be of little use in a queenless colony that is dying anyway (Dijkstra et al., 2005; Dijkstra & Boomsma, 2007). Only after the excess queen brood matured did workers’ sons get raised in laboratory colonies kept under optimal conditions. The authors suggested that this likely implies that the actual reproductive success of worker sons in the field is very small, which could then possibly explain why workers became completely sterile in the sister genus *Atta*.

Positive effects of oxidative stress on worker longevity had previously been observed in both *Acromyrmex echinatior* and *Atta colombica,* and particularly for young workers inside the nest for *Atta colombica* (Majoe et al., 2021), consistent with also orphaned *Atta* workers raising emergency sexuals as their sterility leaves them no alternative option. In monogynous *Solenopsis invicta* colonies, a species with permanently sterile workers and a unicolonial lifestyle in the polygynous form (Krieger & Ross, 2005), queen removal eliminated the otherwise substantial difference in gene expression between foraging and non-foraging workers, but the effect of queen removal itself was very minor (Manfredini et al., 2014). Whether orphaned fire ant workers live longer is unknown, but the specific strategy of culling virgin offspring followed by raising the sons of an unfertilized sister gyne (Wurm et al., 2010) implies that enhanced longevity of orphaned workers may also have evolved in monogynous fire ants. Because there is limited, but non-negligible gene flow between monogynous and unicolonial populations of *Solenopsis invicta* (J. S. C. Chen et al., 2006; Fletcher et al., 1980; Krieger, 2005), it would be interesting to investigate whether the polygynous unicolonial form of is ant species has lost these responses to queen loss similar to what we find in *L. neglectus.*

Overall, our finding that queen removal in *L. neglectus* does not affect worker physiology matches our expectation outlined in the Introduction that a predictable overabundance of queens in unicolonial ants must have selected against maintaining responses to queen loss. In particular, sterile workers of highly polygynous colonies might have evolved to be so specialized that they have lost their sensitivity to queen fertility signals, because the information encoded in such signals is irrelevant for sterile workers. Our interpretation of previous studies suggests that similar forms of functional decoupling between colony germline and colony soma may have convergently evolved in other unicolonial ants. However, it remains to be shown that effects of queen loss are also absent in the gene expression profiles of other tissues, particularly the brain.

Inside and outside workers differ in gene expression and susceptibility to stress

We investigated the fat body gene expression of workers collected near the brood (‘inside workers’) and those from the foraging arena (‘outside workers’). The patterns we found are consistent with the expectation that inside and outside workers perform different tasks, namely brood care and foraging. *Vg-like A* was overexpressed in inside workers while *Vg-like C* was overexpressed in outside workers. Similar gene expression patterns were found in the fat bodies of the ant *Temnothorax longispinosus* and a *Diacamma* species, and in whole-body transcriptomes of *Formica fusca* (Kohlmeier et al., 2018; Miyazaki et al., 2021; Morandin, Hietala, et al., 2019). Vitellogenins are proteins typically involved in egg production, but several homologs arose after a series of duplications of the ancestral ‘conventional’ *Vg* gene. These homologs might share some functional similarity with the *Conventional Vg* but tend to be differentially expressed depending on caste and task (Morandin et al., 2014b; Salmela et al., 2016; Wurm et al., 2011). Overexpression of *Vg-like A* in nursing inside workers of *L. neglectus* is in line with studies indicating that *Vg-like A* may be more related to behavior than fertility (Kohlmeier et al., 2018).

Inside and outside workers differed in more than just the task they were performing. Although there are no data on temporal polyethism in *L. neglectus*, it is very likely that the outside workers were older than the inside workers. This is typically the case in related ant species and in social insects in general (Bernadou et al., 2015; Giraldo & Traniello, 2014b; Pamminger et al., 2014; Ravary et al., 2007). The main pattern of polyethism is that workers initially work inside the nest (e.g., with brood care and intranidal sanitation) and that older workers move outside to take on more hazardous tasks such as foraging and defense. The results of our gene expression analysis confirmed the assumptions that inside and outside workers differed in age. They varied in the expression of over 2000 genes, among them several genes typically involved in aging. Relative to inside workers, outside workers overexpressed genes associated with proteolysis, such as proteasome subunits. This seems consistent with individuals being suspected to accumulate more carbonylated proteins as they age, which are then degraded by proteasomes. Although proteostasis typically decreases with age in many organisms (Hipp et al., 2019), upregulation of proteasomal subunits with age was also observed in *C. elegans*, similar to foraging workers of *L. neglectus*.

When we tested the effects of oxidative stress, we found that inside workers survived better than outside workers. This difference was visible not only in the individuals exposed to oxidative stress using paraquat, but also in the control workers treated with water. More than half of the control workers died in the 15-day survival experiment. This suggests that the experimental protocol with isolation in relatively small experimental colonies followed by manipulation every other day was very stressful even for control ants. We cannot disentangle the potential effects of task and age in our study, so part of the reason that outside workers died earlier may have been that mortality is naturally higher at old age. However, although the average lifespan of *L. neglectus* workers is unknown, ages over seven months have been recorded (Espadaler & Rey, 2001), making it unlikely that enhanced natural age-specific mortality alone can explain the ca. 50% death rate in outside control workers. Our data for *L. neglectus* thus suggest that outside workers are more susceptible to stress than inside workers, which is consistent with studies on other species (e.g. leaf-cutting ants, (Majoe et al., 2021); honeybees, (Alqarni et al., 2019; Amdam et al., 2005; S. C. Seehuus et al., 2006a).

Age-related effects on queen gene expression

Fat bodies of older *L. neglectus* queens differed in the expression of 165 genes from those of young queens, which is considerably less than the age effects reported for queens in other ant species (Negroni et al., 2019; von Wyschetzki et al., 2015). We also kept all factors other than queen age constant. This was not the case in another study where young queens had not yet completed colony establishment and thus lived in a different social environment than older queens (Negroni et al., 2019), implying that behavioural differences might explain a significant proportion of the observed gene expression differences there. Similar potential confounds may apply to our *L. neglectus* gene expression results for inside and outside workers with different age-related behavioural phenotypes, which involved an order of magnitude more genes than the gene expression differences between young and old queens. However, it is difficult to compare queen and worker results because we did not know their age relative to average caste-specific lifespan. Also, our sampling strategy differed between the castes, as multiple workers were pooled per replicate, which could potentially average out individual variation, but queen samples consisted of single individuals. Nevertheless, we could detect interesting genes overexpressed in both the old queens as well as outside workers, such as GO terms relating to proteolysis and cholesterol metabolism that were enriched in old queens. This indicates that older queens, similar to outside workers need an increased effort to keep their enzymes and cell membranes functional.

Although we do not know the exact age of the older queens, there was no indication that young queens were healthier than old queens, or that the older queens in our experiments were close to the natural end of their lives. In *Cardiocondyla obscurior,* young and old queens have fewer changes in gene expression compared to the queens are about to die (Jaimes-Nino et al., 2022; von Wyschetzki et al., 2015). The typical excess abundance of queens in unicolonial ants may have implied selection for faster aging and shorter life span in *L. neglectus* as is generally the case in polygynous ants (Keller & Genoud, 1997b), but old and young queens did not appear to differ in fecundity as their ovaries were of similar size (Supplementary Figure 1) and the fat body transcriptomes did not differ in the expression of fecundity genes such as *Conventional vitellogenin (C-Vg* padjust = 0.105). Our results thus support the general view that social insect queens do not age linearly over their reproductive lifetimes, but that they deteriorate very rapidly toward the end of their lives, both reproductively and somatically (Elsner et al., 2018; Jaimes-Nino et al., 2022, Chapter 5)

Conclusion

As in the social wasps and bees, haplodiploidy normally allows ant workers to develop their overies and produce male offspring from unfertilized eggs, particularly after queen death when any conflicts over male production have become moot. This implies that fertility pheromones have remained deeply conserved not only to regulate colonial life in general but also as a tool to reset worker phenotypes via major changes in gene expression when their reproduction becomes a common interest for all colony members. However, in some species worker ovaries have become rudimentary for unrelated reasons. Selection on workers to remain receptive to these pheromone cues may still be maintained for other reasons, e.g., because queen loss changes the optimal economy of brood rearing. Excessive queen numbers as found in unicolonial ant species remove that constraint under natural conditions, so that the performance of the sterile worker soma is expected to become fully decoupled from queen fertility information. The results on

L. neglectus reported here are broadly consistent with this conceptual expectation and suggest that a similar decoupling may have evolved in other unicolonial ants.

Ethics

Lasius neglectus ants have been introduced via potting soil and are considered as a pests/tramp species in Germany. Therefore, we did not require any licenses to collect and transport the species from the Jena Botanical Garden in Germany to our laboratory in Freiburg. Also in all other respects we followed the guidelines of the Study of Animal Behaviour and the legal and institutional rules.

Data accessibility

The raw data behind the Figures presented here are provided in the electronic supplementary material. The genome assembly and annotations used for L. neglectus have been generated as part of the Global Ant Genomics Alliance (GAGA) project - the data can be accessed upon request.

Authors' contribution

VN, SF, RL and MM designed the experiment. The collection of *L. neglectus* was done by VN and MM. JVM, ZX and LS from the GAGA consortium coordinated by JJB conducted the genome assembly and JV and ZX conducted the gene annotation. NS, supervised by SF and co-supervised by MM, cleaned and primed the transcriptomic data for mapping and the analyses that MM conducted. VN, RL and SF helped MM in the statistical analyses and interpretation. MM wrote a first draft, and all authors helped to revise it. VN, SF, and RL co-supervised the project.

Competing interests

We have no competing interests.

Funding

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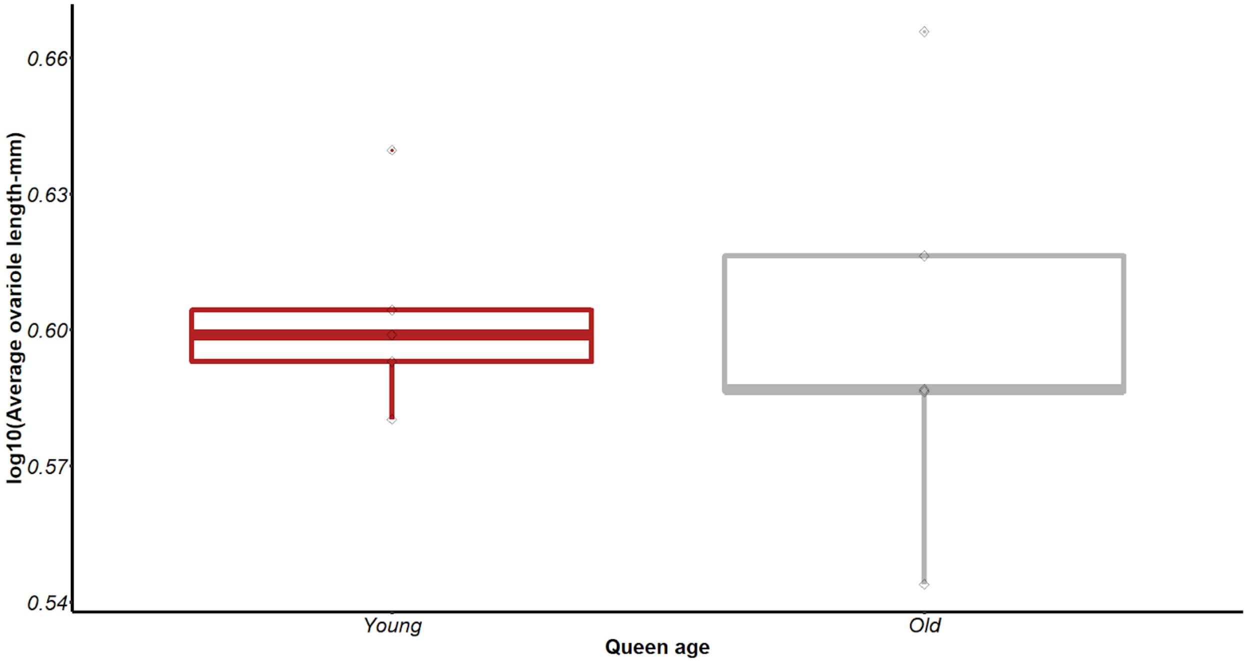
We thank the Jena Botanical Garden for allowing us to collect ants on their property. We are indebted to Julius Rombach and Anna Waffender for the upkeep and maintenance of the colonies and for conducting the oxidative stress experiments. Thanks to Anna Lenhart for her insights during the writing phase.

Supplementary



Supplementary figure 1: Box plots indicating the log10 values of mean ovariole length measured in five young and five old *Lasius neglectus* queens. The ovarioles of older queens were more varied but not significantly longer on average than those of young queens (χ2=0.02, p=0.89).

Supplementary table 1 a) Information on all queen samples used for RNA-seq analysis

Sample	**Colony**	**Caste**	**Age**
Lne23QY1	Lne23	Queen	Young
Lne23QY3	Lne23	Queen	Young
Lne24QY1	Lne24	Queen	Young
Lne25QY1	Lne25	Queen	Young
Lne25QY2	Lne25	Queen	Young
Lne25QY3	Lne25	Queen	Young
Lne26QY1	Lne26	Queen	Young
Lne26QY3	Lne26	Queen	Young
Lne26QY4	Lne26	Queen	Young
Lne12QO2	Lne12	Queen	Old
Lne13QO1	Lne13	Queen	Old
Lne13QO3	Lne13	Queen	Old
Lne22QO1	Lne22	Queen	Old
Lne22QO3	Lne22	Queen	Old
Lne23QO1	Lne23	Queen	Old
Lne26QO1	Lne26	Queen	Old
Lne26QO2	Lne26	Queen	Old
Lne26QO3	Lne26	Queen	Old

Supplementary table 1 b) Information on all worker samples used for RNA-seq analysis

Sample	Colony	Caste	Queen-presence	Age
Lne22NQIn1	Lne22	Worker	Queenless	Young
Lne22NQIn2	Lne22	Worker	Queenless	Young
Lne23NQIn1	Lne23	Worker	Queenless	Young
Lne23NQIn3	Lne23	Worker	Queenless	Young
Lne24NQIn1	Lne24	Worker	Queenless	Young
Lne26NQIn1	Lne26	Worker	Queenless	Young
Lne26NQIn2	Lne26	Worker	Queenless	Young
Lne22NQOut	Lne22	Worker	Queenless	Old
Lne23NQOut1	Lne23	Worker	Queenless	Old
Lne23QNQOut2	Lne23	Worker	Queenless	Old
Lne24NQOut1	Lne24	Worker	Queenless	Old
Lne26NQOut1	Lne26	Worker	Queenless	Old
Lne26NQOut2	Lne26	Worker	Queenless	Old
Lne22QIn2	Lne22	Worker	Queenright	Young
Lne22QIn3	Lne22	Worker	Queenright	Young
Lne23QIn1	Lne23	Worker	Queenright	Young
Lne23QIn2	Lne23	Worker	Queenright	Young
Lne23QIn3	Lne23	Worker	Queenright	Young
Lne24QIn1	Lne24	Worker	Queenright	Young
Lne24QIn2	Lne24	Worker	Queenright	Young
Lne26QIn1	Lne26	Worker	Queenright	Young
Lne22QOut1	Lne22	Worker	Queenright	Old
Lne22QOut2	Lne22	Worker	Queenright	Old
Lne23QOut1	Lne23	Worker	Queenright	Old
Lne23QOut2	Lne23	Worker	Queenright	Old
Lne24QOut1	Lne24	Worker	Queenright	Old
Lne26QOut1	Lne26	Worker	Queenright	Old
Lne26QOut2	Lne26	Worker	Queenright	Old
Lne26QOut3	Lne26	Worker	Queenright	Old

All other supplementary tables: Supplementary files for all chapters

Chapter 3

Age of clonal *Platythyrea punctata* explains more variation in fat body gene expression than their social type

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Abstract

Exploring the effects of age on an individual in a social insect colony is often confounded by their reproductive caste (fertile queen/king or infertile worker) and non-reproductive tasks undertaken. The reproductive caste seems to have a divergent life-history trajectory accompanied by molecular mechanisms that support the extension of lifespan. But the division labour can affect and are affected by genetic background, physiology and metabolism. Within the worker caste too, worker age is intertwined with their physiology, residual lifespans, and likelihood to undertake certain tasks which in turn also affects their lifespan. Comparing the two castes directly is riddled with confounds as they can diverge a lot in their actual age as well as their rate of ageing. The effect of mating might play a role in the ageing trajectory of reproductives; impossible for non-reproductives that have lost the ability to store sperm. The clonal ponerine ant, *Platythyrea punctata* offers the advantage of being a clonal species where all individuals can produce female offspring via thelytoky although still organizing themselves into an egg-laying hierarchy despite their clonality. We can artificially create age-controlled colonies in which these clonemates organise themselves such that one (or some) individuals become principal egg-layers (dominants) and others perform non-reproductive tasks for the colony (subordinates). We used fat body tissue from four- and seven-month-old *P. punctata* clonemates to disentangle the effect of reproduction from actual age and explore genes that changed with age a) regardless of social type b) specifically in each social type. More than 100 genes were influenced by age regardless of social type whereas only nine genes were explained by a clonemate's social type when age was controlled for. More genes changed with age in subordinate individuals compared to the genes that changed with age in dominant individuals. This study disentangles the effect of chronological age and caste. We saw that dominant individuals which are analogous to the queen-caste, vary less over the same time period when subordinate individuals go through more changes the genes expressed in their fat body. Differing transcriptomic signals in the fat body between dominants and subordinates indicates a life-trajectory divergence as observed in between two castes of other social insects.

Keywords: ageing, clonal ant, *Plathythyrea punctata,* fat body, gene expression

Introduction

Division of labour; characterized by functional specialization amongst units is observed at a cellular level in multicellular organisms and at an individual level in organisms that live in social groups (Simpson, 2011). But the social insects, termites, bees, wasps and ants, represent a major evolutionary transition, with a characteristic extreme division in reproductive labour between castes and negligible conflict (Cooper & West, 2018; West et al., 2015). The reproductive caste includes queens (as well as kings in termites) which are highly fecund compared to the functionally non-reproductive workers (Amdam & Page, 2005; Chapuisat, 2014; Hölldobler & Wilson, 1990; Wilson, 1971). This reproductive division of labour is linked to the evolution of two distinct ‘ageing-phenotypes’ in such that although closely related to each other, the reproductive individuals always outlive the non-reproductives, reaching adult lifespans of several decades while workers live for a few months or years (Keller, 1998b; Keller & Genoud, 1997b; Keller & Jemielity, 2006; Shilovsky et al., 2018). The reproductives of an established colony are shielded from extrinsic causes of mortality protected deep in the nest from predation and weather. They also display intrinsic molecular and transcriptomic signals indicating that they are shielded from senescence, typically by investing into life-prolonging immunity or anti-oxidant genes (Bernadou et al., 2021; Corona et al., 2005b, 2007a; Gräff et al., 2007; Korb & Heinze, 2021; Negroni et al., 2016, 2019).

The workers display a further division of non-reproductive labour in the presence of the queen. Behavioural tasks such as the intranidal (inside the nest) nursing tasks and extranidal (outside the nest) foraging tasks are often dictated by an age polyethism where workers graduate from performing safer intranidal to riskier extranidal tasks over the course of their lives (Camargo et al., 2007; Hölldobler & Wilson, 1990; Tofilski, 2002). In many social insects (as well as in eusocial mammals such as naked mole-rats), the removal of the reproductive individual brings about changes in the colony’s dynamics (Almond et al., 2019b; Buffenstein et al., 2021; Kronauer et al., 2010). The workers typically receive indirect fitness benefits from raising their queen’s offspring. However, the workers of some species still retain the ability to reproduce and can try to reproduce to gain direct fitness benefits even though most social hymenopteran workers cannot mate or store sperm and only produce haploid, male offspring (Bourke, 1988). Apart from, and in concert with worker age, many factors such as a worker’s genetic background, physiology (Barie & Amsalem, 2022; Schwander et al., 2010; Smith et al., 2008) and residual lifespan may all contribute to its task-performance and in turn its propensity to reproduce in the absence of a queen (Azevedo et al., 2011; Constant et al., 2012; Guidugli et al., 2005; Kuszewska & Woyciechowski, 2013; H. Lin et al., 2004; O’Donnell & Jeanne, 1995). In workers that do reproduce, studies note increased longevity compared to their non-reproducing counterparts although the increase in lifespan is not as much as seen in the original reproductive. The reproducing workers survive oxidative stress better and express molecular signals typically associated with extended lifespan (Cardoso-Júnior et al., 2021; Dixon et al., 2014; Kennedy et al., 2021; Majoe et al., 2021; Negroni et al., 2021; O’Connor et al., 2002).

Therefore, reproduction seems to be positively implicated in lifespan extension in social insects regardless of caste. This relationship is in contrast to solitary insects and many other organisms which display a 'trade-off' between fecundity and longevity (Blacher et al., 2017; De Loof, 2011; Keller & Jemielity, 2006; E. R. Lucas & Keller, 2014b; Rueppell et al., 2015). Workers can be used to study the evolutionarily conserved mechanisms and molecular pathways that account for this lack of fecundity-longevity trade-off observed in other organisms since reproducing workers do outlive their non-reproducing counterparts (Negroni et al., 2021; K. Tsuji et al., 1996). However drawing conclusions about ageing in reproductive caste by exploring proximate mechanisms of ageing in non-reproductive caste/workers is fraught with the following confounds: Firstly, caste may be predetermined genetically and is irreversible, influenced by time-sensitive provisioning of care, and food, differing in quality and/or quantity for the different castes (Barie & Amsalem, 2022; Schwander et al., 2010; Smith et al., 2008). Secondly, due to divergent shape and pace of ageing, in part depending on their differences in morphology, physiology, behaviour immunity etc, a decade old queen could still be in the prime of her life while a typical worker with a lifespan of less than a year would be considered extremely 'old'. Additionally, mating too is a confounding factor. Whereas queens of social insect colonies are mated or mate throughout their lives (as in termites) workers cannot. Mated queens are longer lived compared to unmated sexuals; inseminated ant queens upregulate their invested into immunity compared to unmated sexuals (gynes) (Gálvez & Chapuisat, 2014; Rueppell et al., 2015; Schrempf et al., 2005; von Wyschetzki et al., 2015).

Social flexibility in some ant lineages have been used to disentangle the irreversible caste fate from task and longevity (Gospocic et al., 2017; Libbrecht et al., 2018; Rabeling & Kronauer, 2012; Ulrich et al., 2016). *Platythyrea punctata* is a clonal species where all individuals can produce female offspring using a form of parthenogenesis– thelytoky giving rise to female offspring which happen to be clones of the parent. Some colonies have been discovered to be obligatory thelytokous. Despite the morphological and genetic similarities, the clonemates organize themselves into two categories; the dominant individual/s are principal egg-layers and behaviourally distinct from the subordinate individual which do not reproduce in the presence of the dominant despite all individuals being capable of egg-production (Hartmann et al., 2003; Heinze & Hölldobler, 1995; Schilder et al., 1999). Additionally, as in the other social insects, the reproducing dominant caste/social type of *P. punctata* outlive their non-reproductive subordinate clonemates; the former with an average lifespan of around 800 days and the subordinates around 250 days. (A. Hartmann & Heinze, 2003) Their clonal nature allows the *P. punctata* colonies to be easily manipulated, allowing investigations into the division of reproductive (Bernadou, Schrader, et al., 2018; Bernadou et al., 2020; Korb et al., 2021) as well as non-reproductive labour (Bernadou, Czaczkes, et al., 2018; Bernadou et al., 2015) avoiding the confound of chronological age and genetic diversity. We thus used *P. punctata* to pose the following questions a) what molecular signals change with age and which change with caste/ social type? b) Do dominants and subordinates; akin to the two castes, experience the effects of age differently? We made used of abdominal fat body tissue for our study due to the variety of roles it plays in energy storage, as an endocrine organ as well as a metabolic regulator and sensor (Casano & Sixt, 2018). We found 109 genes changing with age independent of source colony and social caste. Meanwhile only nine genes differed between dominant and subordinate individuals. We found support in our prediction that the effect of age differs on dominant and subordinate individuals despite the clonal nature of these individuals. This also explains the divergence in lifespan in the species and strengthens the case for making use of this species to identify and manipulate social and molecular elements that may be implicated in ageing across other social insects.

Material and Methods

Collection and colony maintenance

All *Platythyrea punctata* stock colonies were collected at the Yunque National Forest, Puerto Rico in 2012. They have since been maintained in the lab at the University of Regensburg. Colonies were kept in climate chambers under near-natural conditions (12 h 26.8°C/12 h 22.8°C, 70% humidity). All colonies are housed in plastic boxes with plaster on the bottom and fed diluted honey, cockroaches, and *Drosophila* 2-3 times a week which they can access ad libitum. Six source colonies were selected to prepare ‘Old’ and ‘Young’ sub colonies respectively. In June 2020, 6-8 newly eclosed individuals (callows) were collected from each of the parent colonies so we could. control for the age of the individuals within the sub-colonies. In September 2020, another set of 6-8 callows that emerged from the parent colony were collected and placed into separate boxes to create the ‘Young’ sub colonies. The age-cohorts were maintained by removing newly emerging callows from the parent colonies. Due to travel restriction in place during COVID, the 12 boxes (6 ‘Old’ and 6 ‘Young’) containing live ants were delivered to Mainz in January 2021 and placed in our climate chamber (25-26°C, 65-70% humidity). After about 10 days of acclimatization, all individuals were sacrificed by selecting boxes in a random order. There had been some mortality since the sub colonies had been set up in Regensburg and some possibly during travel to Mainz, but we could obtain 3-5 live individuals from each box.

RNA extraction, social type determination, and analyses

The individuals were sacrificed on ice. The last segment of the abdomen was removed and set aside. The abdomen was cleaned as much as possible to avoid contamination of fat body with parts of the digestive tract. Finally, the anterior leftover segments of the abdomen were scraped with clean forceps and broken to ensure all the fat body could be collected. This crushed abdomen was placed into 100 μl of cold Trizol in an Eppendorf tube and stored in -80°C until RNA could be extracted. The dissection and extraction were also randomized in their order. Processing of fat body sample took only about two minutes until they were placed in Trizol. The last segment that had been set aside was examined and the ovaries photographed since those with longest and most developed ovarioles are usually the ‘Dominant’ egg-layers in that sub colony. In this chapter, we use “social type” to divide individuals as ‘dominant’ or ‘subordinate’ egg-layers. Due to the mortality and time-sensitivity, we could not conduct behavioural assays to confirm social status by observing consistent and stereotypical dominance or subordinate behaviour although ovarian development and dominance behaviour are correlated (Bernadou, Schrader, et al., 2018). The Leica stereomicroscope with the LAS v4.5 software was used to capture and process images of the dissected ovaries. In the younger colonies, the social type was not immediately apparent based solely on ovarian development. The pictures were subjectively assessed by all authors who then reached a consensus regarding which individuals were dominant and which were subordinate. We later obtained measures for mean ovariole length of our samples using Fiji ImageJ v5 software. These measurements were used to confirm that our categories of social type rather than age could significantly explain the mean ovariole length ($p<0.001$; Supp fig 1).

We extracted RNA from the fat body tissue along with cuticle stored in Trizol at the time of dissection. RNA was extracted using an in-house protocol adapted from (S. Lin et al., 2021) followed by cleaning steps employing the Qiagen RN-easy Mini Kit (Supplementary 1). All samples were sent to Beijing Genomics Institute (BGI) in China. The concentration and quality of the isolated RNA were checked with an Agilent Bioanalyzer (Agilent RNA 6000 Nano Kit). The 25 samples were sequenced using the Illumina platform HiseqXten (150bp Paired end reads). The index sequences from the machine reads were removed by BGI before sending the raw sequences to Germany for further analyses. Raw reads were trimmed using fastp (version 0.20.0), such that all reads that passed, had a minimum length of 70 bp. The quality of the reads was assessed with FastQC (version 0.11.15). SortMeRNA (v 4.3) was used to eliminate possible non-coding RNA sequences from our samples. All resulting samples were visualised with MultiQC-1.7. All trimmed and cleaned reads were aligned to the genome file made available by GAGA (Global Ant genomics Alliance) with an alignment rate 88.17% to 93.87% using HISAT2 (version 2.2.1). The SAM files generated by HISAT2 were first converted to BAM files and then sorted by name using SAMtools (version 1.7). The sorted BAM files were used to create gene count tables with Htseq-count (Htseq version 0.13.5; settings: -f bam, -i ID, -t gene, -m union, -r name, --stranded=no). A single gene count table consisting of all samples was then compiled. In addition, a metadata table was assembled that contained information about each sample.

The data was further analyzed using R-Studio (R version 4.0.4). Genes that had more than one read in one or more samples were retained for further analyses. We used the DESeq2 package (v 1.32.0) to analyse differential gene expression depending on age (old vs young) and dominance type (dominant vs subordinate). We transformed the raw reads via the function 'varianceStabilizingTransformation'. This enabled us to summarise the variance of all 10038 expressed genes (of the 11338 genes in the genome) and visualize the spread of 25 samples along 2 Principal Components using the function 'plotPCA'. For the calculation of the differentially expressed transcripts the full model Colony+Type+Age was used compared against two simpler models using a Likelihood ratio test (LRT). The first reduced model to determine the effect of age (young and old); and the second reduced model to determine the effect of social type (dominant and subordinate) on gene expression.

Secondly, we aimed to determine what kind of genes change over three months in dominant and subordinate individuals. To this effect, we separated the dataset such that we still had nine dominant (three young, six old) and 12 subordinate individuals (six young, six old). Each dominant individual came from a unique sub colony. However, we had 10 young subordinate individuals; some which were not unique to a sub colony. Therefore, we chose individuals with the lowest mean ovariole-length amongst other subordinates from their sub colony (Supplementary table 1). This ensured a similar subset for subordinate individuals such that only one subordinate individual per colony was represented. For both social type models (Dominant-Model, Subordinate-Model), the full model contained ‘Age’ and the reduced model obtained the gene expression differences explained by age in dominant and subordinate individuals respectively. In all tests conducted using DESeq2, adjusted p-values less than 0.05 were the cut-off used to determine the genes whose variation in expression was significantly explained by the factor of interest. Adjusted p-values are determined by DESeq2, which corrects the p-value of each gene for multiple testing by employing the Benjamini-Hochberg method.

Functional annotation and enrichment

All the lists of differentially expressed genes (DEGs) were separated by age ('old', 'young'), and dominance type ('dominant', 'subordinate'). We retrieved their amino acid sequences from the proteome produced by GAGA and ran a local BLASTp (version 2.6.0) against a database of the following fourteen proteomes available on NCBI: *Drosophila melanogaster, Apis mellifera, Polistes dominula, Nasonia vitripennis Harpegnathos saltator, Odontomachus brunneus, Lasius niger, Formica exsecta, Linepithema humile, Ooceraea biroii, Solenopsis invicta, Acromyrmex echinatior, Atta colombica and Temnothorax curvispinosus.* An e-value of 0.00001 was chosen and a maximum of 10 hits were displayed from which the best hits were chosen based on the highest bit-score.

We ran Interproscan (v5.54) on the available *Platythyrea punctata* proteome to obtain protein annotations and process GO (Gene Ontology) IDs associated with our differentially expressed gene (DEG) lists. We used a proteome produced by GAGA, containing one single representative isoform per gene. We extracted proteins associated with age and position related DEGs from the queen and worker analyses respectively. The GO terms from the interproscan results were used to perform enrichment analyses using topGO (v 2.4.2) on R-Studio (R version 4.0.5). We used the 'weight01' algorithm to determine the nodes and Fisher's test with values <0.05 to determine the significant GO terms from our lists by comparing them to a universe of 7341 proteins with GO IDs present. A Benjamini-Hochberg correction was applied to the Fisher's p-values to account for multiple testing.

Vitellogenin (*Vg*) and their copies are implicated in fecundity, regulating oxidative stress as well as determining task-division (Corona et al., 2013; Guidugli et al., 2005; Morandin et al., 2014b). We conducted a reciprocal BLASTp search of the *P. punctata* proteome against a database of annotated Vitellogenin amino acid sequences of 34 species used in Kohlmeier et al., 2018 with an e-value of $1e^{-5}$. Genes associated with these were determined to be *Vitellogenins* and classified as per their closest match from the above paper. We additionally searched specifically for the expression of any *Vitellogenins* in the DEGs. We performed a local reciprocal BLASTP search comparing the proteins associated with the DEGs against a list of 33 known *D. melanogaster* derived candidate genes associated with oxidative stress (Kramer et al., 2021). All reciprocal BLASTP were performed with the e-value $1e^{-5}$.

Results

Age explains more variation than social type of individuals

When accounting for all 10038 genes expressed in the fat body of 25 *Platythyrea punctata* individuals, PC1 explained 19% of the variance while PC 2 explained 15% of the variance but did not clearly distinguish amongst the different groups (Supp Fig 2a). Since we were also interested in changes with age in each social type, we ran separate PCAs for the dominant (Supp fig 2b) and subordinate (Supp fig 2c) social types with more apparent clustering based on age.

From the additive DESeq2 model, we controlled for the effect of source-colony and age yielding 9 genes whose expression could be significantly explained by the social type of the individual. Eight of these were explained by dominant and 1 single gene's variation explained by subordinate social type (Fig 1, Supplementary table 2). The dominant individuals had relatively higher expression of the gene *PlpuOBP14* (BLASTp match against XP_011148183.1, pheromone-binding protein Gp-9-like; *Harpegnathos saltator*) The subordinate individuals all had a higher expression of *Plpu_g00761*; a gene whose protein's best BLASTp match was against XP_019697160.1 (ras-like protein family member 10B isoform X1; *H. saltator*).

From the same additive DESeq2 model, we controlled for the effect of source-colony and social type. The expression of 109 genes was then significantly explained by age (48 overexpressed in young individuals with respect to the older individuals; 61 genes in older; Fig 1). BLASTp results attributing functions to the proteins associated with young individuals and those with old individuals are detailed in Supplementary Table 2. For the 109 fat body genes whose expression was significantly explained by the age of the sub colony, we obtained GO terms associated with their biological processes. Of the GO terms, only GO:0006457 associated with the biological process 'protein folding' was significantly enriched (BH $p_{adjust} < 0.01$). Genes such as *Plu_g06181* and *Plpu_g0791* were two of 6 genes involved in 'protein-folding' and BLASTp revealed the genes to code for two chaperone proteins: XP_032677613.1, dnaJ homolog shv/Hsp 40 [*Odontomachus brunneus*] and XP_029668495.1, endoplasmin/Hsp_90 [*Formica exsecta*]. (Supplementary table 2)

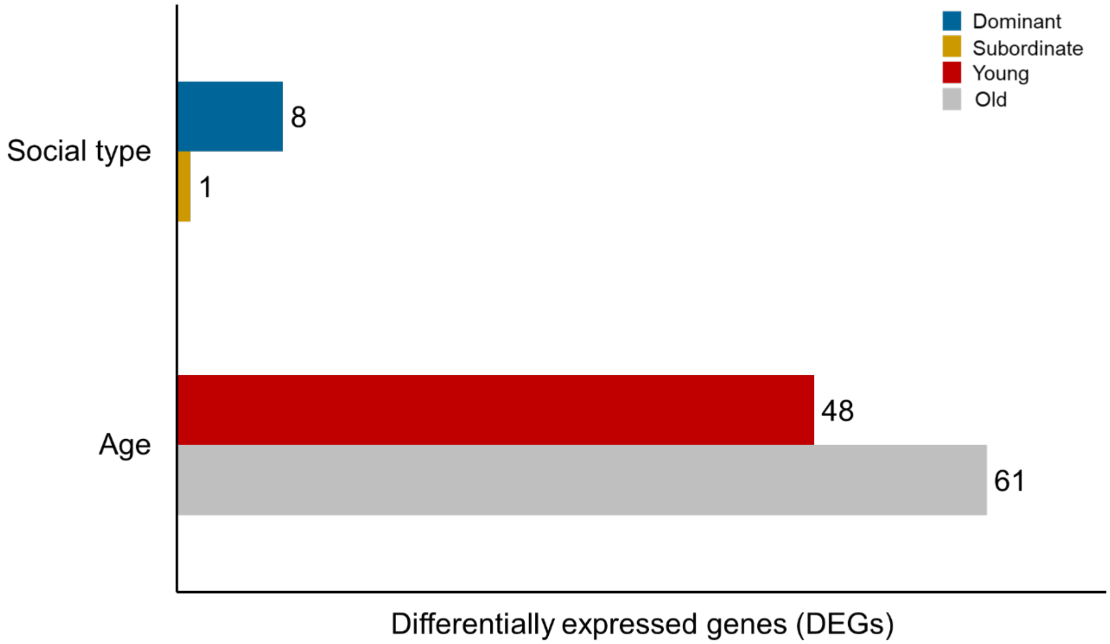

Figure 1: Two separate likelihood ratio tests (LRT) compared reduced models against the full model (Colony+Social type +Age) to determine the effects of social type (top panel) and of age (bottom panel). The number of genes whose expression was significantly explained by the aforementioned factors of interest (DEGs) are displayed using the bars. The colours of the bars and the numbers next to them refer to the genes whose variation is most explained by dominant individuals or subordinate individuals regardless of age and colony. Similarly, the lower panel contains those genes best explained by the young, 4-months-old individuals and the older 7-months-old individuals.

Genes changing with age in dominant and subordinate individuals differ

The ‘dominant model’ contained the subset of nine dominant individuals: three young and six old. Of the 9818 genes expressed in these individuals, age significantly explained the variation in expression of 24 genes. Young individuals explained the variation of 9 of these genes while 15 of the genes were attributed to the older individuals (Fig 2). The ‘subordinate model’ consisted of 12 individuals. Age explained the gene expression of 44 of the 9932 genes expressed. Of these 20 genes were overexpressed in 7-months-old individuals, whereas 24 genes were associated with the 4-months-old individuals (Fig 2). The proteins associated with all the genes were obtained and the annotations compiled using a local BLASTp against 14 species (Supplementary table 3).

The dominant and subordinate individuals differed in the genes that were affected by age except for three genes overexpressed in 7-months-old individuals of both dominant and subordinate social types. *Plpu_g03215* (protein BLASTp against XP_026829554.1, cytochrome P450 4C1-like, *O. biroi*), *Plpu_g05448* (protein BLASTp against XP_024874066.1, uncharacterized protein LOC112456025, *T. curvispinosus*), *Plpu_g05048* (protein BLASTp against XP_029660876.1, calumenin-B, *F. exsecta*) [Supplementary fig 2]. Additionally, eight genes from the 48 DEGs overexpressed in young clonemates were also observed in young subordinate individuals. Similarly, three of 61 DEGs linked to older clonemates was overexpressed in old-dominant individuals, nine in younger-dominant individuals and one was common amongst all three models tested (Supp fig 2, Supplementary table 4).

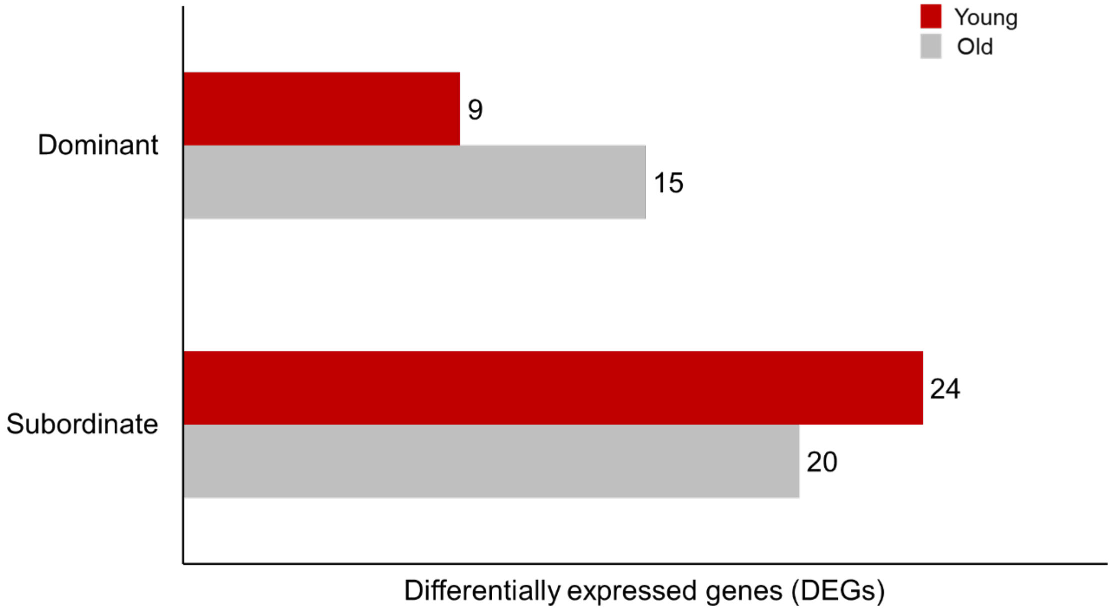

Figure 2: The original dataset was split based on social type; the dominant model containing nine individuals and the subordinate model with 12 individuals. Both models were then compared to reduced models using an LRT (likelihood ratio test) to obtain the effect of age on the gene expression of dominant individuals (top panel) and on subordinate individuals (bottom panel). The numbers next to the bars refer to the number of genes whose variance is most explained by young or by old individuals in their respective social types.

Comparing gene lists to known genes of interest- vitellogenins *and oxidative stress*

We also classified the v*itellogenins* based on their closest BLASTp match from a known database of 34 species (Kohlmeier et al., 2018) but the expression of none of the three Vitellogenins found in the *P. punctata* proteome were significantly explained by age or by social type (Fig 3 , Supplementary Table 4). We carried out reciprocal BLASTp to detect *Platythyrea punctata* genes which find matches amongst the known candidate genes related to oxidative stress (Kramer et al., 2021). The gene *Plpu_g02155*, overexpressed in the older 7-months-old clonemates compared to their 4-months-old ‘young’ counterparts matched against the protein Jafrac1 (FBpp0073594, padjust=0.046, Supplementary table 4).

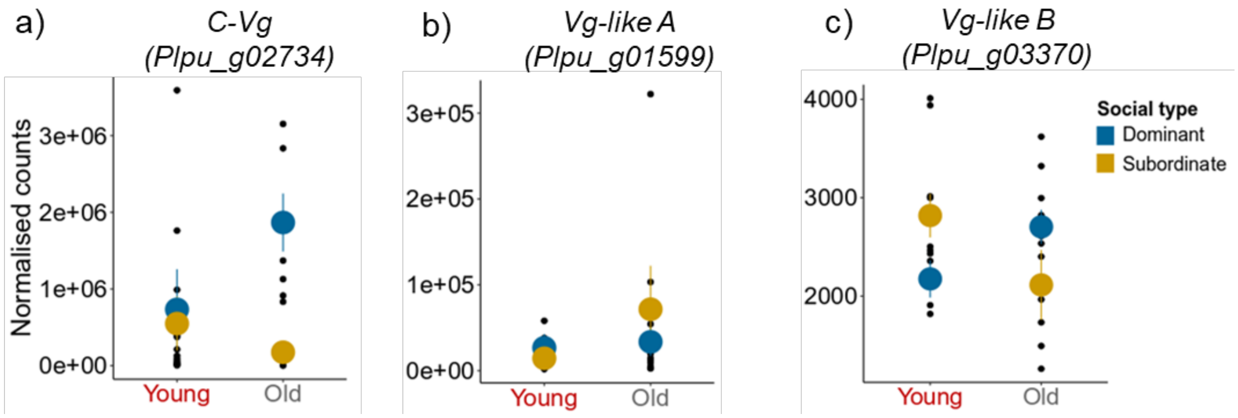

Figure 3: Normalised counts used to plot genes associated with the three Vitellogenin proteins annotated in the *P. punctata* proteome (see methods for details). Neither age nor social type could significantly explain the expression of a) *conventional vitellogenin* b) *Vg-like A* or c) *Vg-like B* in the fat body of the clonemates. The black dots represent each individual and the blue (dominant), and ochre (subordinate) circles represent the mean normalized read count with standard error of each subgroup.

Discussion

Age, reproductive potential, and behavioural aspects are strongly intertwined in many social insects. Through this study, we attempted to disentangle the genes affected by age and those associated with reproductive dominance in the clonal ant species *Platythyrea punctata.* We were able to create 4-months and 7-months-old sub colonies wherein all individuals (clonemates) of the same age could organize themselves into dominant and subordinate social type. When the effects of source colony and social type were controlled for, age significantly explained the variation in expression of 109 genes. We also tested if the same genes changed with age in dominant and subordinate indviduals. In dominant individuals, 24 genes differed with age whereas 44 genes differed with age in subordinate individuals. Three genes were common, expressed in old-dominant and old-subordinate individuals. Genes whose expression was significantly explained by age regardless of social type showed minor overlap with genes explained by age in different social types.

Disentangling, age and reproductive division of labour

The passage of time affects all members of a social insect colony although not in the same way. Therefore, we invariably contend with many confounds studying the effects of age on colony members that vary in morphology, metabolism, reproductive capacity and residual lifespans; all despite their genetic similarities (Cohen, 2018; Jaimes-Nino et al., 2022; Mugel & Naug, 2020; Page & Peng, 2001). Some ant lineages allow to control for the confounds of morphology, and the irreversibility of caste-fate; wherein caste (whether an individua will be queen/king or worker is determined at a relatively early stage in larval development and is fixed until they die. (Collins et al., 2021; Lo et al., 2019; Penick et al., 2021). In obligately thelytokous colonies of *P. punctata,* all individuals can produce female offspring without mating. Despite this, the individuals naturally organise themselves after bouts of non-lethal antennal boxing such that there is one (or a few) dominant individuals which lay eggs developing that in turn develop as female offspring. The other members perform non-reproductive tasks, further dividing into intranidal and extranidal workers. (Bernadou, Czaczkes, et al., 2018; C. Hartmann et al., 2019, 2020; Schilder et al., 1999).

Since such a reorganization leading to ‘dominant’ and ‘subordinate’ individuals also emerges in manipulated colonies containing of age-matched individuals, we used this system to disentangle known age from social type- an indicator of reproductive division of labour without the confound of mating.

A recent study containing *P. punctata* data explored principal components most associate with age, social type (akin to ‘caste’- dominant versus subordinate) and their possible interaction when explaining the variance of the 500 most differentially expressed transcripts (Korb et al., 2021). Upon analysing whole heads’ transcriptomic signals, age more so than social type explained the variance of the top 500 most differentially expressed transcripts between young (17-days old) and old (112-days old) *P. punctata* individuals when looking at dyads containing a single dominant and subordinate clonemate each. In the same study, understanding the effect of age and social type was harder for gasters of these individuals though there was a signal of age even when both tissues were combined. We used larger sub colonies, starting with around 8 individuals instead of just dyads containing one dominant and one subordinate individual. Our ‘young’ colonies were 4-months/120-days-old, older than the old workers of the aforementioned study. We used tissue-specific transcriptomic data from the fat body. The fat body is the metabolic hub of the insect, acting as production centers and/or regulators of nutrient sensing and storage, and growth and reproduction (Arrese & Soulages, 2010). By not taking the entire abdomen, we have excluded the confounding effects of ovary gene expression. Despite the differences in set-up, actual age of the workers and tissues/body parts, we observed similar patterns in that age independently explained more of the variation in gene expression than social type when controlling for other factors (Fig 1 a).

Changes in genes of interest

Vg expression is higher in the fat body of dominant egg-layers/ gamergates of *Harpegnathos saltator* and *Diacamma sp.* compared to others. Gamergates in these species also live longer than their non-reproductive counterparts, similar to dominant and subordinates in *P. punctata* (A. Hartmann & Heinze, 2003; Liebig & Poethke, 2004; Miyazaki et al., 2021; K. Tsuji et al., 1996; H. Yan et al., 2022). Cuticular hydrocarbons (CHCs) are known to signal ovarian development to clonemates which then police and attack excess reproductives (Hartmann et al., 2005). We could not detect any of the three *Vg* genes’ expression to be significantly explained by age or by social type. We also did not see any of the *Vgs* in the fat body to significant change with age within the dominant or the subordinate individuals. It is possible that though egg-laying is policed, *Vg* expression is reflective of the species wherein all members of the colony are able to produce female offspring if the opportunity arises. In fact, another study on different populations in the species note the occurrence of colony fusions which would allow for more egg-laying opportunities for subordinate individuals since non-nestmate discrimination seems to be very low in the species (Hartmann et al., 2003; Kellner et al., 2010). The *Vgs* may also play different roles in dominants and subordinates. *Conventional Vg (C-Vg)* was highly expressed in the heads of only older-dominant clonemates in (Korb et al., 2021) although this copy of Vg the brain may serve to shield it from oxidative stress as observed in the honey bees (Corona et al., 2007a). Normalised counts of *C-Vg* expression from old dominant individuals compared to the subordinates also hints at a similar pattern (Fig 3 a). Our results also saw age, independent of social type significantly explaining the variation in expression of *Jafrac 1*, a *D. melanogaster* homolog of the gene *Peroredoxin II* in humans. The gene codes for the enzyme which can degrade the Reactive Oxygen Species (ROS) hydrogen peroxide. It is a downstream effector of JNK/FOXO signalling enhancing stress resistance in *Drosophila sp.* brains (DeGennaro et al., 2011; Lee et al., 2009). We saw a higher expression of this gene in older clonemates. Another study using exploring the same oxidative stress markers we used also found age and not social type/caste affecting the expression of anti-oxidant genes ; with higher expression of the genes in older clonemates relative to their younger counterparts despite them using whole abdomens for their analysis (Kramer et al., 2021).

Social organization and distinct ageing trajectories in social types

Dominant *P. punctata* individuals can live for ~400-500 days whereas the subordinates live for ~200 days (Bernadou, Schrader, et al., 2018; A. Hartmann & Heinze, 2003). We had a fair bit of mortality in our colonies and individuals may have undergone more stress than usual due to lower frequency of maintenance and habituation to a new laboratory condition (see methods for details). Although we couldn't make behavioural observations prior to sacrificing individuals, we confirmed our categories with mean ovariole length as a cue for social type. Earlier studies have correlated dominance behaviour to larger oocyte length and higher fat content (Bernadou et al., 2020) Considering that we collected subordinates from older (7-months-old) colonies we may have inadvertently picked those higher fitness considering they outlive the average 200 days lifespan of a subordinate. In some younger sub colonies, we were unable to find clear differences amongst individuals which we decided to keep in the data increasing the number of subordinates in the full model. Despite this, we still saw nine genes differing between the two social types (Fig 1 a, Supplementary table 1). The dominant individuals had relatively higher expression of the odorant binding protein *Gp-9* (*PlpuOBP14* significant blastp match against XP_011148183.1, pheromone-binding protein Gp-9-like [*Harpegnathos saltator*]). This was also one of the nine genes implicated in division of labour in social insects and also previously found expressed in whole heads of *P. punctata* (Bernadou, Schrader, et al., 2018).

The queens and kings of the termite *Macrotermes bellicosus* can live for decades the sterile workers don't survive past a few weeks. While the gene expression variation between young and old reproductives was negligible, the workers showed more differences, with the old major workers showing increased expression of transposable elements implicated in ageing (Elsner et al., 2018). In the ponerine *Harpegnathos saltator*, the senescence or removal of the queen induces a caste switch in adult workers all of whom can mate. This gives rise to several gamergates (pseudo-queens) that mate within the nest and lay eggs. The caste switch is accompanied by change in CHC correlated with their ovarian activity (Liebig et al., 2000). Additionally observed is also increased *Insulin-like peptide* (*Ilp2*) expression in the brain. The gamergates also show an increased brain *Impl2* expression. The aforementioned patterns are associated with increased lifespans known from other species (H. Yan et al., 2022). Our interest lay in exploring whether the transcriptomic signals associated with age in the fat body differed between the two social types; one which monopolized all reproductive tasks- the dominant and the other which performed non-reproductive tasks- the subordinate. We thus saw it fit to subset our data to analyse two models, one containing all dominant individuals, and one containing all subordinate individuals such that there were no biological replicates from the same source colony. Indeed, the effect of age was different on the two social types (Fig 1 b, Supplementary table 3) affecting 20 genes in dominant individuals and 44 genes in subordinate individuals, with only three genes in common; those overexpressed in older dominant and older subordinate individuals.

We expected subordinate social type to have a more varied gene expression, considering a) they would be more susceptible to change with age and b) subordinate individuals we collected would also have been individuals engaging in different non-reproductive tasks, which we could not ascertain because of lack of behavioural data. Corroborating our hypothesis, we found more genes differing with age in the subordinate than the dominant individuals. Except one gene *Plpu_g05048* (blastp match calumenin-B [*Formica exsecta*]) commonly seen in old dominant and old subordinate individuals, all other genes affected by age were unique to the social types. Dominant individuals expressed genes associated with fatty acid metabolism such as *Plpu_g12957, Plpu_g01300* overexpressed in younger dominant individuals (best protein BLASTp hits against acyl-CoA Delta(11) desaturase [O. biroi] and fatty acyl-CoA reductase wat-like [*T. curvispinosus*] respectively) and *Plpu_g12345* in older dominant individuals (protein BLASTp hit against long-chain-fatty-acid--CoA ligase [O. biroi] ; Supplementary table 2). The amount of fatty acid and lipid concentration is associated with CHC production, perhaps signaling fecundity or dominance, division of reproductive and non-reproductive labour as well as longevity (Bernadou et al., 2020; Bustos & Partridge, 2017; Pei et al., 2019). Additionally, unlike other species of social insects with irreversible caste-fate, social type in *P. punctata* is reversible, affected by stress, diets, natural inter-individual variation etc. (Bernadou, Schrader, et al., 2018). This could explain the few genes that do change with age in dominant individuals although the genes the dominants invest in may be implicated in their divergence of life-history compared to their subordinate clonemates. Therefore, despite age-matched sub colonies which had ad-libitum access to similar quality and quantity of food, age-related changes in the two social types iterate previous behavioural and empirical studies on the species and well as reflect the different genes and pathways changing with age in queen and worker castes in other social insects.

Conclusion

We made use of one single tissue, the fatbody, to obtain tissue-specific transcriptomic signals of age in this species where reproductive division of labour is flexible and decided by dominance-displays. By using this thelytokously clonal species with age-matched sub colonies we could disentangle age from social type, analogous to caste in other typical social insect colonies. While age was artificially controlled for, the speceis' clonality provide a natural control for caste or reproductive division of labour, since it is not fixed, and is no longer confounded with the effect of differing patrilines and mating status. We see that despite clonality in the species and reversibility of social type, dominant and subordinate individuals still differed in the number and the kinds of genes they upregulated; reflective of caste-based differences in other social insects which differ vastly in their life-trajectories. In the future, manipulating the social hierarchy to see which genes are affected the most when individuals switch from subordinate to dominant or vice-versa and whether this is reflected in their lifespans would help ascertain the role of some of the genes from this study with respect to how it affects ageing. Additionally, investigating the same effects in other tissues would add further nuance since some tissues may be shielded from ageing more than others (see Chp. 5 of this thesis).

Ethics

The colonies used were raised in the laboratory since 2012. Collection of source colonies in Puerto Rico was permitted by USDA Forest Service and Departamento de Recursos Naturales y Ambientales, 2012-IC-036.

Authors' contribution

A.B, V.N,S.F,R.L and M.M designed the experiment. The experiment was set up and cared for by A.B in Regensburg. M.M dissected the specimen and extracted RNA. N.S primed the data and created the count tables. M.M conducted further analyses and wrote a first draft revised by V.N,S.F,R.L and A.B. V.N., S.F. and R.L. co-supervised the project.

Competing interests

We have no competing interests.

Funding

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Acknowledgements

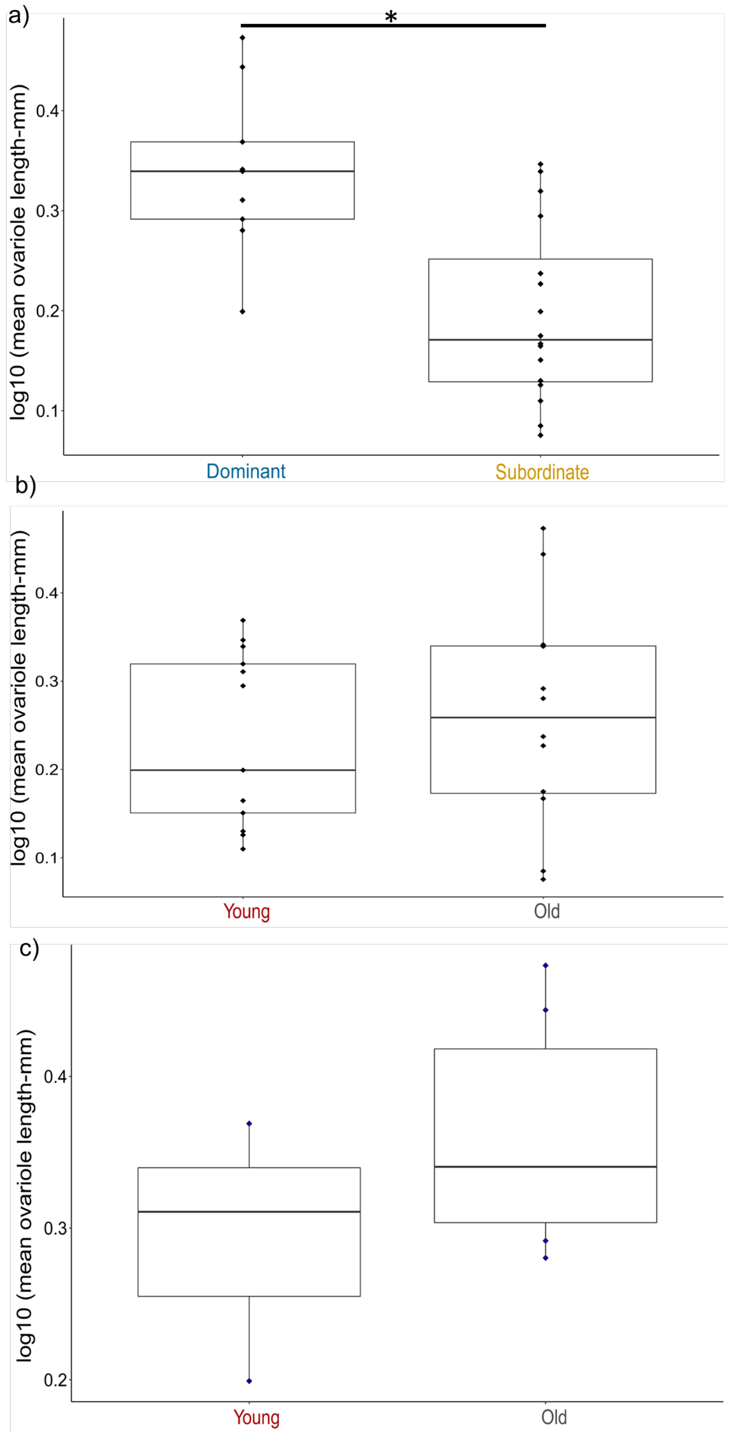
We are indebted to Dr. Luisa Maria Jaimes-Nino who helped transport the ants from Regensburg.

Supplementary

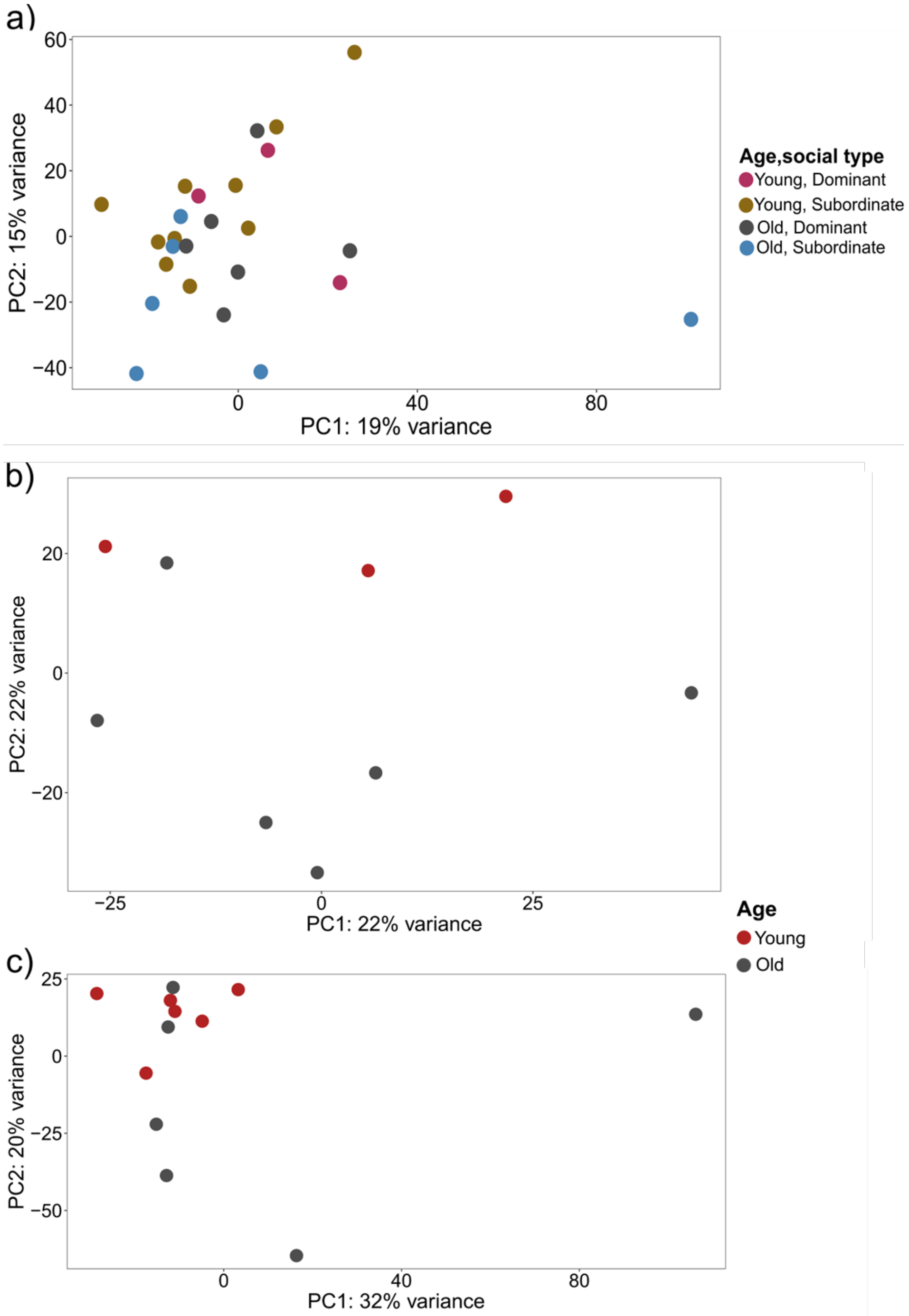
Supplementary table 1: All 25 samples used for the analysis to determine the effect of age and the effect of social type on gene expression. DeSeq2 analyses on the subset of dominant individuals and of subordinate individuals made use of only the chosen samples. This was to ensure a more even distribution of samples. Normalised counts of the three *Vitellogenins* is also displayed. Their expression was not significantly explained by age or by social type.

Sample Name	Source Colony	Social Type	Age	Mean ovariole length(mm)	Chosen	Count Cvg	Count VgA	Count VgB
PP11	Pr11	Sub	Young	2.184	Yes	15627.71	37914.5	2841.291
PP12	Pr11	Dom	Young	2.338	Yes	33346.87	58019.24	1818.971
PP14	Pr46	Sub	Young	1.971	No	989096.9	16155.7	2357.676
PP17	Pr46	Sub	Young	1.349	Yes	130225	31019.61	2999.093
PP19	Pr46	Sub	Young	2.087	No	3591400	4884.48	4010.815
PP21	Pr54	Sub	Old	1.727	Yes	833170	4947.713	1261.417
PP22	Pr54	Dom	Old	1.907	Yes	2834492	103286	2996.225
PP25	Pr94	Sub	Young	2.221	No	374117.7	5041.927	2431.836
PP26	Pr94	Sub	Young	1.461	Yes	90770.53	21563.15	3013.794
PP28	Pr54	Dom	Young	2.045	Yes	1760646	5223.831	2464.264
PP29	Pr54	Sub	Young	1.582	Yes	3596.081	11100.73	2182.254
PP33	Pr23	Dom	Old	2.194	Yes	1370238	2969.831	2822.158
PP35	Pr23	Sub	Old	1.216	Yes	99217.02	21068.14	3620.078
PP36	Pr93	Dom	Young	1.582	Yes	405333.5	16580.84	2242.038
PP40	Pr93	Sub	Young	1.288	Yes	18999.1	11404.19	2502.839
PP42	Pr93	Sub	Old	1.19	Yes	57254.28	12240.99	2609.144
PP43	Pr93	Dom	Old	2.185	Yes	1128169	63250.71	2401.657
PP44	Pr23	Sub	Young	1.336	Yes	58874.56	3236.039	3938.639
PP45	Pr23	Sub	Young	1.415	No	212465.7	1758.74	1907.969
PP46	Pr94	Sub	Old	1.496	Yes	2514.39	54297.7	1733.355
PP47	Pr94	Dom	Old	2.972	Yes	3152579	2522.065	3321.435
PP4	Pr11	Sub	Old	1.469	Yes	4018.701	322118.1	1493.68
PP5	Pr11	Dom	Old	1.957	Yes	1801432	9377.538	2534.732
PP6	Pr46	Dom	Old	2.778	Yes	913586.1	19800.24	2150.094
PP8	Pr46	Sub	Old	1.686	Yes	52877.72	14649.9	1965.768

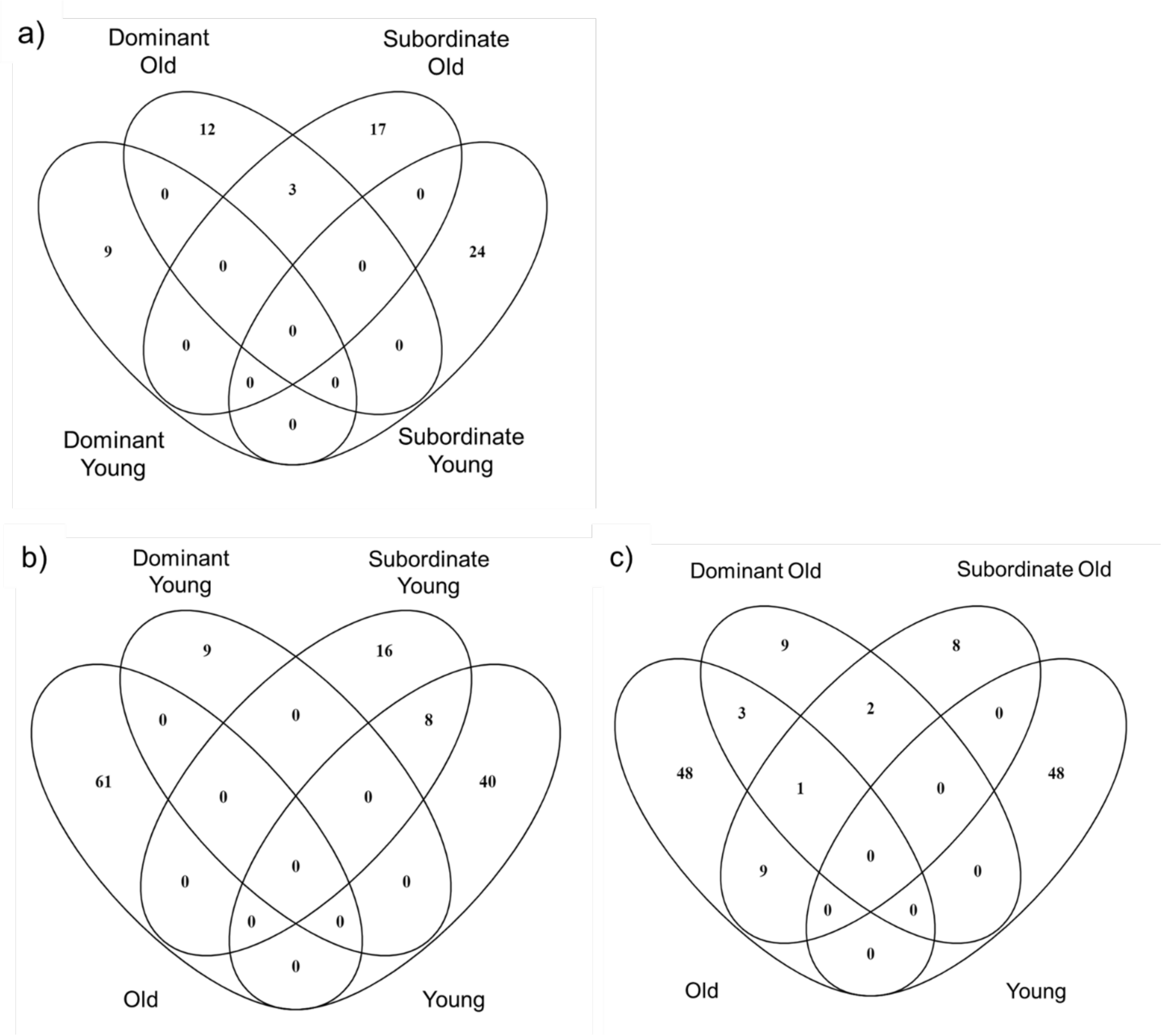
Supplementary Figures



Supplementary figure 1: *Platythyrea punctata* samples were assigned as 'dominant' or 'subordinate' based on subjective assessment of ovarian development; with the former group containing individuals with better developed ovaries than the latter. Post-hoc, we could test mean ovariole length of the samples. This confirmed a) social type to significantly influence mean ovariole length rather than b) age (X^2=17.47, p<0.001). Pairwise test between the two social types also indicated them to significantly differ from each other (z-ratio =3.65, p<0.001). c) Additional model to showed no significant difference in mean ovariole lengths of young (n=3) and old (n=6) dominant individuals.



Supplementary figure 2: Principal component analyses plotting the variation in *Platythyrea punctata* fat body gene expression when a) considering 100038 genes in 25 individuals. The coloured dots specify the age and social type of each individual. b) PCA plotting the variance of 9818 genes expressed in the 9 dominant individuals c) shows the 9932 genes expressed in 12 subordinate individuals. The 4-months-old individuals (young) and 7-months-old individuals (old) are indicated in red and grey respectively.



Supplementary figure 3: Venn-diagrams (generated by Venny2.1) of DEGs associated with age in dominant individuals and those significantly explained by age in subordinate individuals. Three genes were commonly overexpressed in older individuals of both dominant as well as subordinate social types. No other genes varied similarly with age in the two social types. The DEGs from dominant and subordinate models which were associated with age groups b) young and c) old were compared to the 109 genes associated with age regardless of social type and colony. One single gene *Plpu_g05048* (protein BLASTp against XP_029660876.1, calumenin-B, *F. exsecta*) was obtained from 7-months-old individuals in all three models.

All other supplementary tables: Supplementary files for all chapters

Chapter 4

Brain transcriptomes indicate very few age-related changes in young established *Atta colombica* queens

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Unpublished

Abstract

Hallmarks of ageing have been identified across several animal taxa. They refer to physiological aspects that change with age, and which when manipulated, can change the rate of ageing. We investigated whether we could detect such age-related changes in eusocial ant queens known for their high fecundity and exceptionally long lifespans. We focused on shifts in brain gene expression with age in nine leaf-cutting ant *Atta colombica* queens in their early years: one, two and three years after colony establishment. All queens had successfully raised workers and established a fungus garden, so that potential transcriptomic differences could not be explained by recent mating flights or colony foundation. In contrast to other studies on ants, which also included founding queens, we detected only three genes that were differentially expressed by age when controlling for fungus volume (a cue for colony size). Though we express caution in interpreting our results owing to the strong positive correlation between age and fungus volume, this correlation is also reflective of the species' colonies in the wild. The queen need not be sensitive to fungus-volume once she has set up her colony. Especially since all the fungus gardens were healthy with enough workers to tend to the queen. Age when tested by itself, could not explain the variation in the expression of any of the genes in the brain. Even the model accounting for effect of fungus volume yielded only three genes changing with age in the brain of these nine queens. Our results thus indicate that transcriptomic activity in the brains of young queens does not change drastically over these three years, though the genes affected correspond to known hallmarks of ageing and hold promise as future candidates of neuronal degeneration in the species.

Keywords: aging, senescence, social insects, brain physiology, leaf-cutting ants

Introduction

Ageing refers to the decline in biological functions with chronological age, resulting in a reduction in fecundity, health, and survival (Kirkwood & Austad, 2000). The rate of such a breakdown varies across the web of life, albeit involving ubiquitous pathways and molecular regulators (Baudisch, 2011; López-Otín et al., 2013). Evolution of ageing is theoretically explained as a consequence of competing energy demands between reproduction and somatic maintenance (Maklakov & Chapman, 2019). This is based on the observation that highly fertile individuals or species are often short-lived, while those with long lives often produce only few offspring. In recent years, the universal mechanistic trade-off between fecundity and longevity has been questioned because in social insects, the reproductive caste (queens and sometimes kings) is both the most fecund as well as the most long-lived compared to their non-reproducing workers. The reproductive caste of social insects is an interesting model to study the molecular pathways that enable high fecundity of long-lived individuals (Keller & Genoud, 1997; Keller & Jemielity, 2006; Korb, 2016; Lin et al., 2021).

Investigations into queen physiologies are limited by two factors. First, many monogynous species have extraordinarily long-lived queens, reportedly reaching 30 years of age in some ant and termite species (Keller, 1998). Conducting longitudinal studies in standardized laboratory settings is thus typically restricted to species with short-lived queens that could be followed throughout their lifetime. (Almond et al., 2019a; Anderson et al., 2018; Jaimes-Nino et al., 2022). At the same time, exceptionally short-lived queens are naturally not a good system to understand extraordinarily long lives; genes expressed, and molecular pathways employed by shorter and longer lived reproductives may vary.

Sampling presumably old queens from established field colonies is an alternative to studying short-lived species, but the exact chronological age of the queen is difficult to ascertain. The age is thus estimated based on how long a nest has persisted, or the size of the nest and/or number of workers. Often, studies then contrast old queens with young foundresses that do not have workers yet, or with queens from very small colonies that are less than one year old. These studies tend to find differences between old and young queens (e.g. Negroni et al. 2019), but queens that are collected soon after mating or during colony-establishment can introduce confounding effects that entail colony founding such as foraging behaviour, or consequences of the mating flight (exposure to parasites and adverse biotic conditions) (Cronin et al., 2013).

Mating too causes changes, detectable in the brain (Nanfack-Minkeu & Sirot, 2022). Behaviour-modulating biogenic amines differed between mated and unmated *Formica japonica* (Aonuma & Watanabe, 2012). This was especially true for dopaminergic amine (DA), increasing in its levels from day one to ten post-emergence. The brain also shrinks in size considerably once ants are inseminated (Julian & Gronenberg, 2002; Nagel et al., 2020). Colony founding may thus cause a particular physiology in founding queens that is much different from that of young but established queens, which are cared for by workers. Once her colony is established, the queen is typically protected against effects of extrinsic mortality posed by predators, temperature extremes, or lack of food, because she usually tended to by the workers. As a (or the sole) reproducing member of the colony, the queen would be prioritized to receive care since her offspring are highly related to the sterile workers who derive indirect benefits from tending to the queen and raising her brood. A few studies using queens nearing the end of their known lives has helped reported the sudden changes in fertility, investment into sexuals, unstable or impeded protein repair etc (Alaux et al., 2006; Jaimes-Nino et al., 2022; Monroy Kuhn et al., 2021).

The brain is an organ of interest due to its many functions: cognition, memory, sensory perception, hormone production, and behavioral control. Many of these functions do change with age (Bloch et al., 2000; Dassati et al., 2014; Herman et al., 1971; Koch et al., 2021). Though most parts of the rat brain show no changes with age, neuron-loss was reported in the pre-frontal cortex with advancing age (Stranahan et al., 2012). Mazin et al., 2013 reported mRNA splicing changes related to neuronal functions across the lifespan of an individual human being. Some genes that are expressed in the early years after the colony's establishment may later on contribute to the queen's senescence. López-Otín et al., 2013's seminal paper identified nine 'hallmarks' of ageing mechanistic reasons for the loss of fitness in ageing across many taxa. These hallmarks ideally fulfilled three criteria: i) manifested during the normal ageing process ii) accelerated ageing when experimentally manipulated iii) retarded/ impeded ageing when experimentally ameliorated. Some of these hallmarks, especially with relation to epigenetic alterations (Choppin et al., 2021; Libbrecht et al., 2016), loss of proteostasis (Hipp et al., 2019; Jaimes-Nino et al., 2022; Monroy Kuhn et al., 2021), and deregulated nutrient sensing (Vafopoulou, 2014; H. Yan et al., 2022) have already been observed and manipulated in social insect ageing.

We investigated whether we could detect change associated with chronological age in the brain of nine established queens of the leaf-cutting ant *Atta colombica* aged one, two, and three years old. Using established queens ascertained that we did not confound our results with the effect of recent mating. We also wanted to consider the potential effect of colony size in the laboratory setting. We did so by observing the fungus-garden volume for each queen's colony. The workers cultivate and tend to fungus gardens in which the colony resides and upon which the ants feed. Fungus-garden-volume is a proxy for colony size; how many workers has the queen produced which can tend to the fungus garden and allow more workers to be reared from the queen-brood. However, fungus-garden-volume is also intrinsically linked to the queen's age. With various analyses, some taking fungus-volume into account, we could find a maximum of only three genes; related to protein modification and immunity whose expression in the brain was affected by age. Therefore, the first three years after colony-establishment following a tumultuous period of colony-founding reflect a period of stability where the queen's brain does not undergo drastic changes.

Material and Methods

Species, collection and colony maintenance:

Atta colombica is a fungus-growing Myrmicine ant species of the tribe Attini. It is well documented in terms of social structure, worker task-partitioning, fungus-culture, as well as reproductive behaviour of the queens and reproductive potential of the workers (Baer & Boomsma, 2006; Brown et al., 2006; den Boer et al., 2009; Holman et al., 2011; Majoe et al., 2021; Shutler' et al., 1990). Incipient colonies of *Atta colombica* were collected at STRI field station in Gamboa, Panama in the years 2017, 2018, and 2019, when they were around one year old. Such nascent colonies in the field are recognized by small fresh mounds with funnel-like entrances on the top and little to no activity outside the nest. The small fungus gardens (<100ml) containing the young queen and her workers were excavated from the soil for transport. In the laboratory, the fungus gardens were placed under upturned plastic beakers which were kept moistened. These beakers were covered with upturned flowerpots and the entire set-up was housed in plastic boxes coated with Fluon™. All colonies could access cut bramble leaves, apple-slices, and parboiled uncooked rice ad libitum. The colonies were maintained in a 25°C climate chamber with humidity ranging from 65-70%.

RNA extraction and RNA-seq analyses

The queens were sacrificed on ice and their brains dissected and placed in PBS and stored in -80°C. We sacrificed nine queens in September of 2019. Three of these queens were from colonies collected in 2017, three from 2018, and three from the year 2019 (the month of May). When we collected the colonies in the field, they were relatively small (size <50 gm), indicating that they had been founded in the previous year. At time of sacrifice, the nine focal ant colonies contained between 50ml and 3000 ml in fungal volume (Supplementary table 1).

We followed a Trizol:Chloroform:Isoamylalcohol based RNA extraction protocol (as described in (S. Lin et al., 2021)). All samples were sent to Beijing Genomics Institute (BGI) in China. The concentration and quality of the isolated RNA were checked with an Agilent Bioanalyzer (Agilent RNA 6000 Nano Kit), low-input Tn5 RNA libraries were prepared and sequenced using the Illumina platform HiseqXten (150bp Paired end reads). The index sequences from the machine reads were removed by BGI before sending the raw sequences to Germany for further analyses. Raw reads were trimmed using fastp (version 0.2), to remove known adapters; and filtered such that only reads that had a minimum length of 70 base pairs (bp) were retained. The trimmed results were checked with MultiQC-1.7 and the quality of the reads was assessed with FastQC (version 0.11.8). All trimmed reads were aligned to the available NCBI genome of *Atta colombica* (RefSeq GCF_001594045.1) with an alignment rate of 76.51-87.64% using HISAT2 (version 2.1.0). The SAM files generated by HISAT2 were first converted to BAM files and then sorted by name using SAMtools (version 1.9). The sorted BAM files were used to create gene count tables with Htseq-count (Htseq version 0.11.2; settings: -f bam, -i ID, -t gene, -m union, -r name, --stranded=no). A single gene count table consisting of all samples was then compiled. In addition, a metadata table was assembled that contained information about each sample. The data was further analyzed using R-Studio (R version 4.0.4). Genes that had fewer than 1 read in 2 or more samples were removed (754 genes out of a total of 10691 genes in the genome).

We used the DESeq2 package (version 1.30.1) to analyse differential gene expression explained by age and fungus-garden volume at the time of queen sacrifice. The fungus garden of a colony is an indication of the colony health which is affected by and in turn may affect the queen. The 1-year-old colonies had been brought to the lab only 6 months prior to sacrifice and varied widely in their fungus-garden volume compared to the older colonies. The size of the larger colonies depended mostly on how much space we provided them with, and less on the intrinsic health of the colonies. We found a correlation between fungus volume and age of the queens (cor=0.88, $p < 0.01$; Supplementary fig 1).

We first transformed the raw reads via the function 'varianceStabilizingTransformation'. Raw reads were used to carry out a Principal Component Analysis (PCA) to visualise all nine samples using the function 'plotPCA'. Then, we carried out a differential gene expression analysis with a likelihood ratio test (LRT) for hypothesis testing. Our predictor of interest was age, which was confounded by fungus-garden volume of the colony. We thus calculated different models to make sure any genes whose expression we explained with age could not actually have been an effect of fungus-garden-volume. The full DEseq2 model consisted of age and fungus volume, both as continuous predictors. We compared the full additive model against two separate models: one to determine those genes whose expression was significantly explained by the effect of age when controlling for fungus volume, and the other to find the genes whose expression changes were significantly explained by fungus volume when controlling for age. We additionally ran two DEseq2 models exclusively containing either age or fungus volume as continuous predictors to find how many genes vary with known age (comparing age-containing model to one without) or with fungus volume (by comparing it to a reduced model with just the intercept). Adjusted p-values of 0.05 were used as a cut-off after correcting for multiple testing correction using the Benjamini-Hochberg method. This determined all differentially expressed genes (DEGs) significantly explained by each factor of interest in the models considered.

Gene annotation

We retrieved a functional annotation and information from the NCBI database for all genes in our DEG lists. The RefSeq Protein sequences from these genes were used to conduct BLASTP searches online (https://blast.ncbi.nlm.nih.gov/ , last accessed 20thFebruary 2023) against a database of the following species: *Drosophila melanogaster*, *Apis mellifera*, *Acromyrmex echinatior*, *Temnothorax curvispinosus*, *Ooceraea biroi*, *Lasius niger*, *Linepithema humile*, *Solenopsis invicta*. Further information about the proteins and similar genes were obtained by using the text mining online portal PaperBLAST (Price & Arkin, 2017) (last accessed 20thFebruary 2023).

Results

When accounting for all 9937 genes expressed in the nine brain samples of *Atta colombica* queens, PC1 explained 59% of the variance while PC 2 explained 12% of the variance. Clustering was not based on age groups or fungus volume (Fig 1).

The DESeq2 results indicated that age explained the expression change of three genes when the fungus-garden volume of the colony was controlled for: *LOC108686192* (unchracterised in *A. colombica;* BLASTp hit against XP_024878006.1, putative aminopeptidase 2, *Temnothorax curvisopinosus*) (Fig 2a), *LOC108689025* (transmembrane protease serine-9-like) (Fig 2b) and *LOC108695106* (*15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like*) (Fig 2c). [Supplementary table 2]. Upon controlling for the effect of queen age, fungus volume could not significantly explain the expression of any genes expressed in the brains of these queens (padjust>0.05). Testing the effect of each predictor separately, without taking the other factor into account, resulted in zero genes being explained by the age of the queen. Only one single gene *LOC108689145* (*endochitinase*, BLASTp match against XP_011056666.1, endochitinase isoform X2 *A. echinatior*) varied with fungus volume of the colony (Fig. 3; Supplementary table 3).

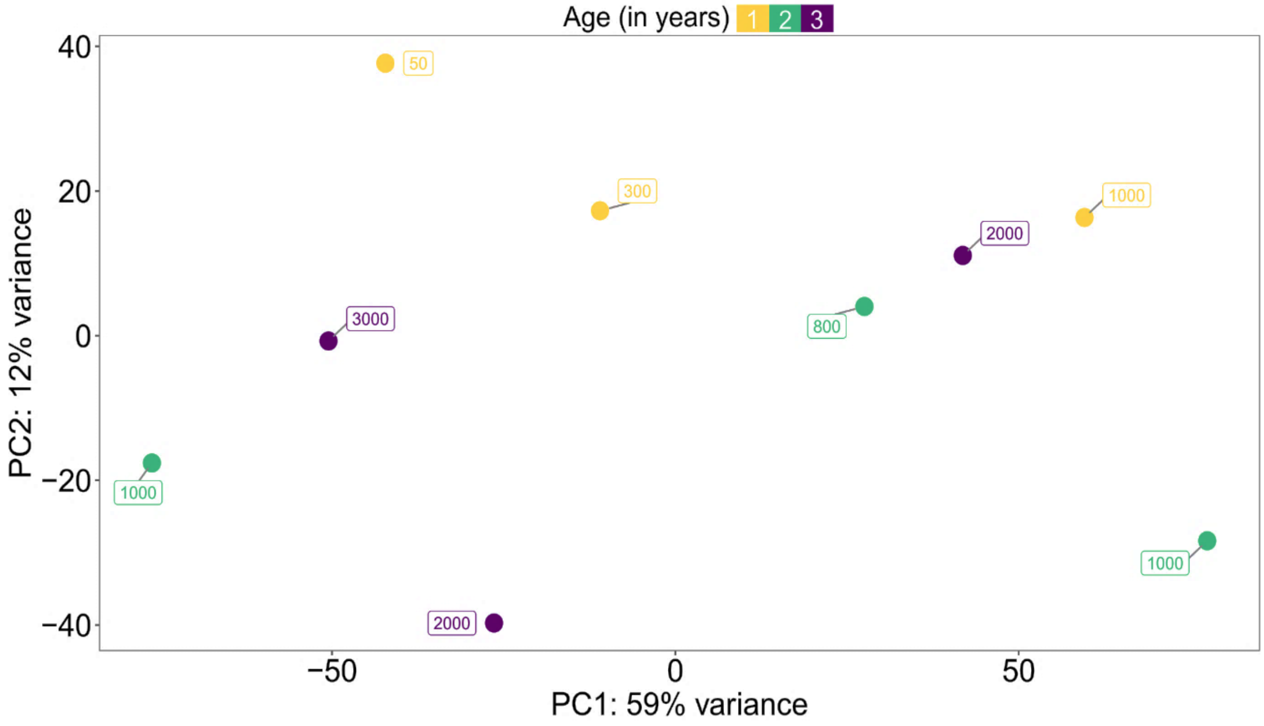
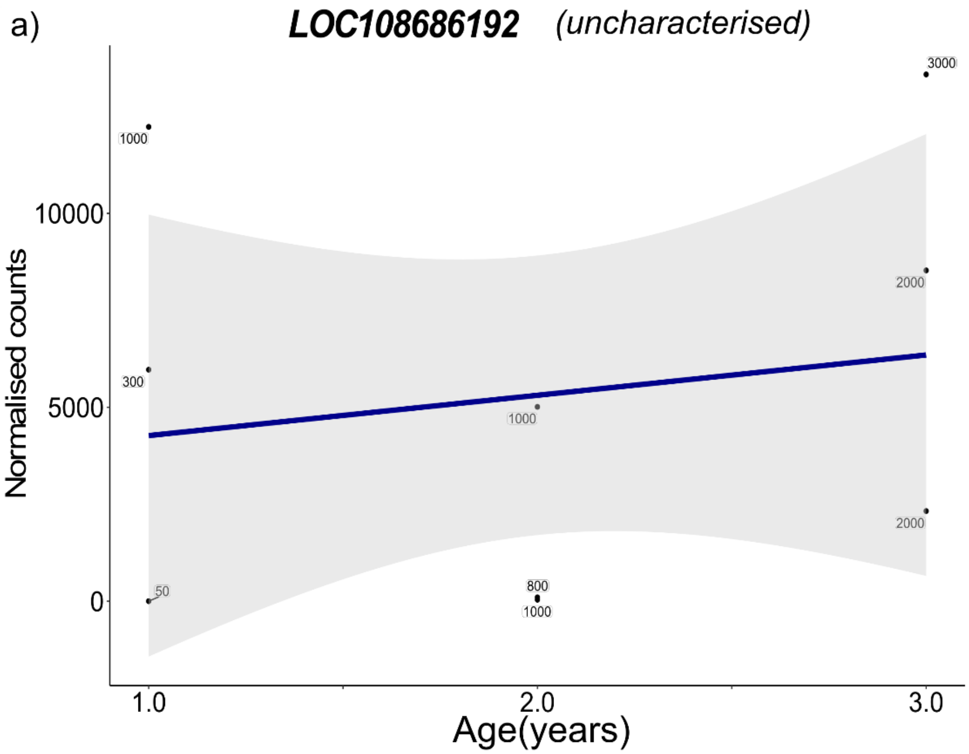


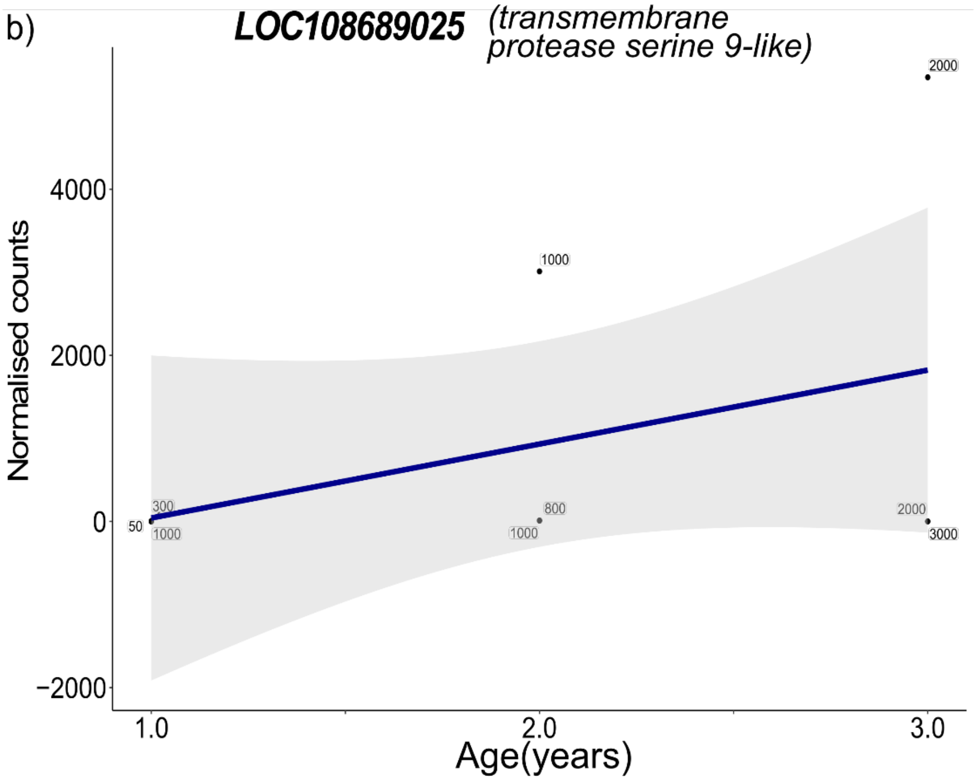
Figure 1: Each point in the PCA (Principal Component Analysis) represents the expression profile consisting of 9937 genes of each sample (age specified by different colors and the colony's fungus-garden-volume indicated by numbers (in milliliters) at the time of queen-sacrifice)

a)



LOC108686192 *(uncharacterised)*

b)



LOC108689025 *(transmembrane protease serine 9-like)*

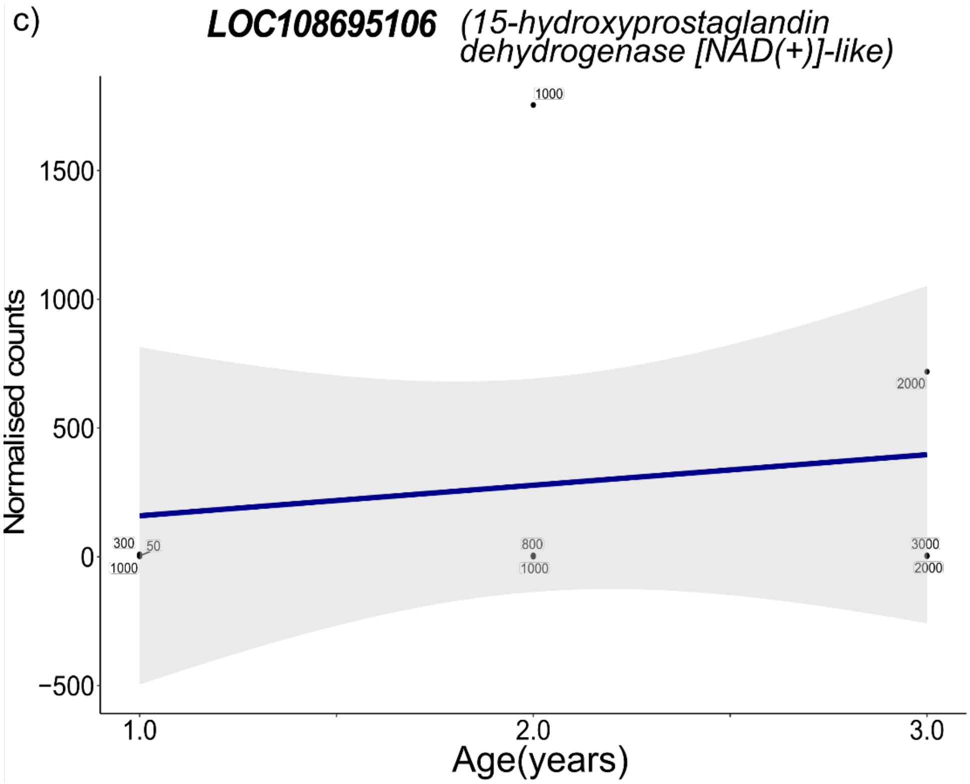


Figure 2: Age of the queen significantly explained the variation of three genes after accounting for the effect of fungus-garden-volume (in mililitres is displayed next to the data points). Normalised counts of the genes: a) *LOC108686192* (unchracterised in *A. colombica;* BLASTp hit against XP_024878006.1, putative aminopeptidase 2, *Temnothorax curvisopinosus*) b) *LOC108689025* (transmembrane protease serine-9-like) and c) *LOC108695106* (*15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like*). [Supplementary table 2].

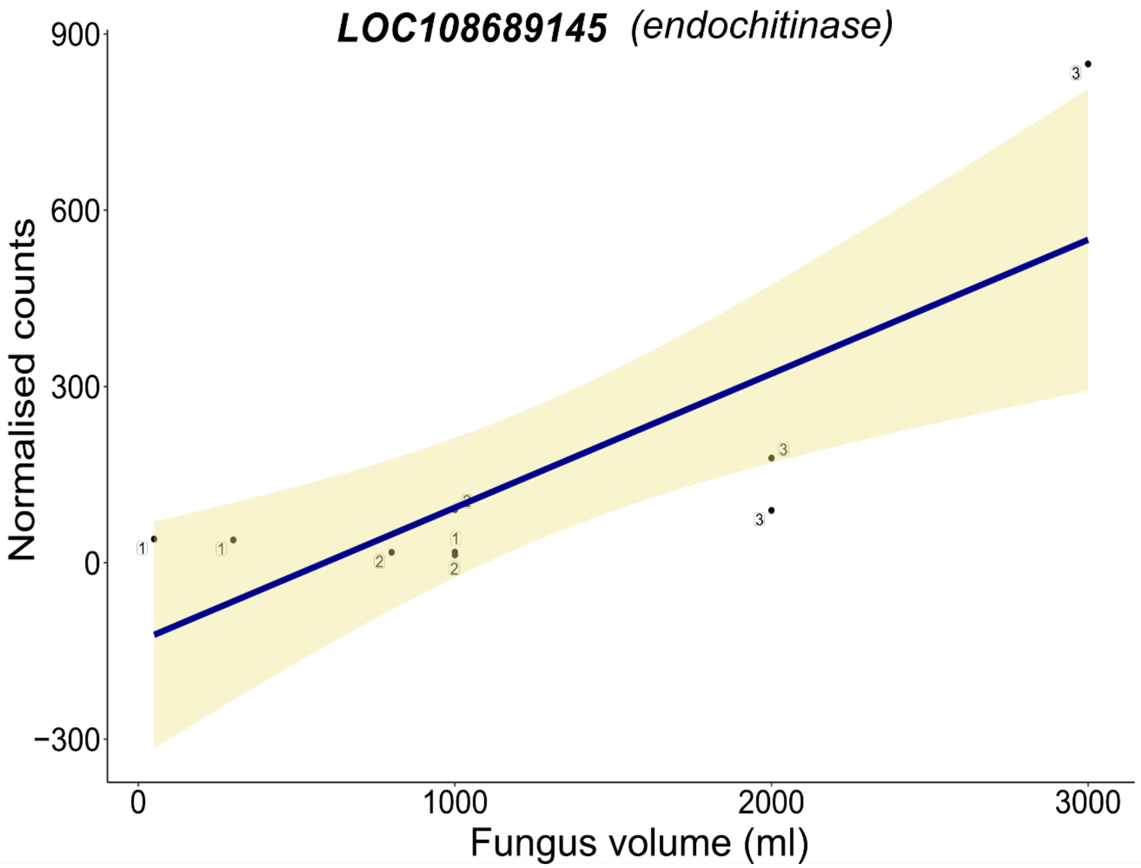


Figure 3: In the model where only the effect of fungus- volume was tested. This factor explained the variation of only one single gene's expression: *LOC108689145*- endochitinase. The figure plots fungus volume (in ml) on the x-axis while the numbers in the plot refer to the ages of the queens (in years). [Supplementary table 3]

Discussion

We investigated queens of the leaf-cutting ant *Atta colombica* for changes in brain gene expression in the early years after colony establishment. The expression of only three genes changed with age. All queens had managed to raise workers and to establish a healthy fungus garden, and colony size did not affect gene expression.

Queens of the genus *Atta* can live up to 10-20 years (Boer et al., 2009; Keller, 1998; Weber, 1972). Since the oldest queens in our study were only three years old, they were not close to their death. We also controlled for the effect of fungus-garden volumes. Age and fungus volume were correlated (cor-0.88, $p<0.01$; Supplementary fig 1) although all queens assuredly passed the colony-founding stage and had enough workers tending to them in our set-up. Our results showed only three genes that changed their expression with age in the brain (Supplementary table 2). Though the exact functions of the three genes in the brain of our ants are unknown, they all code for enzymes. Two genes - *LOC108686192* (Fig2 a) and *LOC108689025* (Fig 2 b) - code for an aminopeptidase and a protease, both of which break down proteins and polypeptides, and can regulate response to infections (Harrell et al., 2021; Viljakainen et al., 2018). PaperBLAST indicated similarity of protein XP_018051105.1 (*LOC108689025)* with protein CG7142 in *D. melanogaster* (40% identity, 22% coverage of protein sequence). CG7142in *D. melanogaster* is a protease which regulates proteostasis. The loss of proteostasis is one of the hallmarks of ageing (Rai et al., 2022) and has also been documented in ant (Jaimes-Nino et al., 2022) and termite queens (Monroy Kuhn et al., 2021) nearing the end of their lives. The third gene, *LOC108695106* (Fig 2 c), codes for 15-hydroxyprostaglandin dehydrogenase NAD+ (15HPGD) and can inhibit the activity of prostaglandin E2 (PGE2), thus inhibiting uncontrolled tumor growth in humans and mice (M. Yan et al., 2004). 15-HPGD can also generate oxo-fatty acid products, which inhibit proinflammatory cytokine responses (Wendell et al., 2015).

Our result that only few genes change in expression over the early years finds support in other studies. Reproductives of the highly social termite *Macrotermes bellicosus* that had set up their colony one year prior to collection differed very little in head gene expression from reproductives known to be at least 6 years old (Elsner et al., 2018). Similarly, in a study on another Myrmicine ant species, *Temnothorax rugatulus*, more changes attributed to age were present in the fat body of the queens than in their brains. The pattern persisted despite the fact that the exact ages of the queens were unknown and the ‘young’ queens only had 0-8 minim (1st set of smaller-sized) workers (Negroni et al., 2019). Our study of established queens of another leaf-cutting ant species, *Acromyrmex echinatior* ranging from 0.75-16 years old instead showed that the brain changes considerably more than the fat body and the ovaries over those long years (Chapter 5). This too provides indirect support to our observation that very few changes occur at younger ages.

Colony size is strongly linked with chronological age in the wild since older queens typically have more workers tending to them and their brood than younger queens. Similarly, the size of the fungus garden of *Atta* species is an indicator of how many workers a queen has produced to tend to her, her brood, and to the fungus garden. Age and fungus volume were positively correlated in our study (pearson’s correlation=0.88, p=0.002; Supplementary figure 1) making it harder to disentangle the effect of one factor from the other. Though the one-year-old queens have smaller fungus gardens than older colonies in the wild, all our colonies were set up in the laboratory where fungus-garden-growth is limited for ease of maintenance. Thus, while the one-year-old colonies varied significantly after being in the laboratory for 6 months since collection, the two- and three-year-old colonies' garden size could also be an artefact of how much they were allowed to grow in the laboratory. Thus, we expected fungus volume to be a somewhat artificial source of variance amongst our queens. Additionally, all our queens already had healthy gardens and had assuredly succeeded in overcoming their founding-phase and may not be as sensitive to fungus-garden size once that stage has passed. Age could not explain the variation in any of the genes expressed when tested as the only factor of interest in the model. Fungus-garden volume by itself failed to explain the variation in any of the genes when the effect of age was controlled in an additive model. When tested as a factor by itself, the fungus volume significantly explained the expression of one single gene *LOC108689145* (Fig3, Supplementary table 3)*. Endochitinase* maybe associated with digestion of chitin present in the fungus cultivated by Attine ants (Ješovnik et al., 2016; Nygaard et al., 2016) though its expression in the brain warrants further investigation. In another leaf-cutting ant species, *Acromyrmex echinatior,* we made a more thorough assessment to disentangle the effect of age and fungus volume. When we tested for the effect of fungus volume on the brain's gene expression in 12 established queens ranging from 0.75 to 16 years of age, we only found one single gene (*LOC105144601; acetylcholinesterase-like*) that varied with changing fungus-garden volume.

Since we only tested the effect of age within brain, we may have missed more dynamically changing tissues such as fat body or ovaries, which often act as sites of physiological changes associated with nutrient modification, fertility, and digestion (Arrese & Soulages, 2010; Choppin et al., 2021; Kohlmeier et al., 2018; Koubová et al., 2019; Negroni et al., 2019). However, we did detect more changes in the brain than in the fat bodies or ovaries when investigating age-related changes in another leaf-cutting ant *Acromyrmex echinatior* (Chapter 5). Future studies should consider other tissues when feasible, especially if the interest lies in tracking differences in fecundity of queens with age. The few genes that we found changing expression over the three years in *A. colombica* queens' brains play diverse roles in protein modification and regulation of growth and reproduction. Despite the queens' young age, we may already be able to detect and track genes related to the hallmarks of ageing albeit it is only 0.03% (3/9937) of all expressed genes that varied. Future studies could use these genes as candidates to map a neuronal trajectory in brains to ascertain if they can indeed predict ageing in the species' brains. Tracking the trajectory of these genes will assist in establishing 'genetic lifespan calendars' as in mice and humans (Ezra et al., 2023; Skene et al., 2017) suggesting critical windows of time which most affect the process of ageing.

Conclusion

The first three years after colony establishment represent the early years of the queen's life in her role as the reproductive caste of a colony. Our sampling and set-up help disentangle the confound presented by colony-establishment related gene expression changes from those of age-related changes in other studies. We did not detect many changes in brain gene expression associated with age, despite its strong correlation with fungus volume which may contribute to some of the variation in gene expression. These early years after a relatively tumultuous colony-founding period seems to be a phase of relative molecular stability in the queen's brain. Studies conducted on older queens in the species, or on workers, could use the genes from this study as candidates to ascertain their role as markers and regulators of the hallmarks of ageing in ants.

Ethics

Ant collection and export permits were obtained for the leaf-cutter ants from the Autoridad Nacional del Ambiente (ANAM) in Panama by Jonathan Shik. Import and export licences are not required for the transport of our study species. We followed the DFG Animal welfare guidelines and the local laws.

Authors' contribution

All authors designed the experiment. M.M. and V.N assisted with queen dissection. M. M conducted the analyses and wrote a first draft and all authors revised it. V.N., S.F. and R.L. co-supervised the project.

Competing interests

We have no competing interests.

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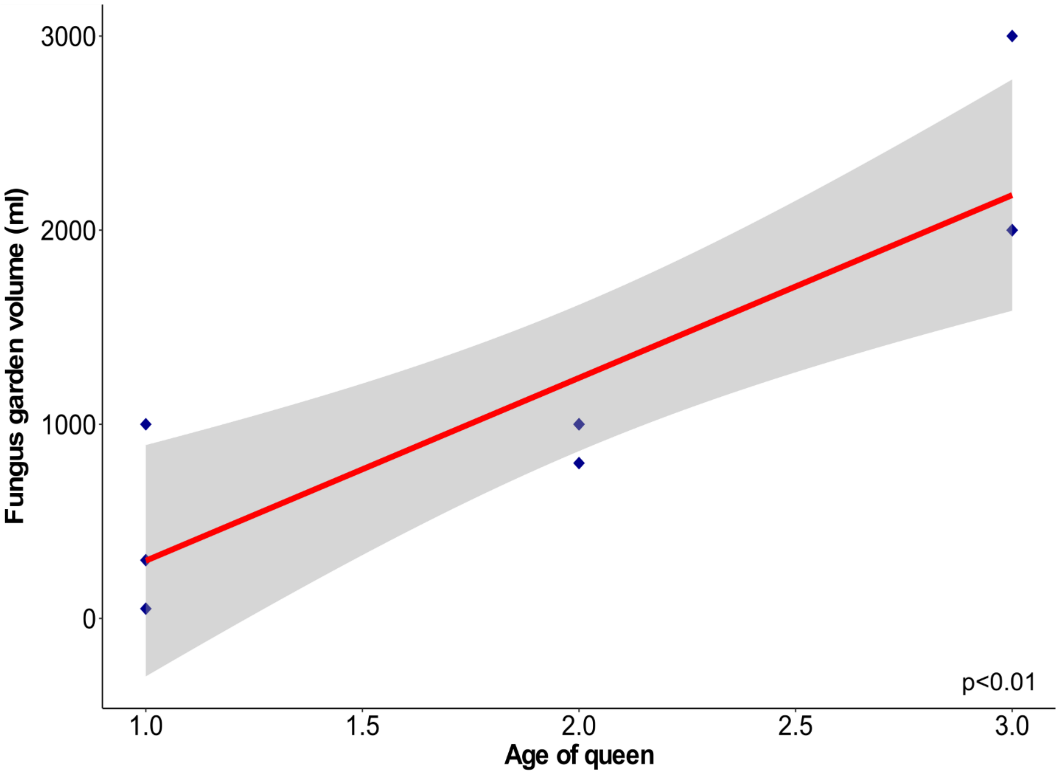
Supplementary

Supplementary table 1: Metadata table used to find correlation between age of queens and volume of their colonies' fungus gardens. The same information was used for DEseq2 analyses.

Colony	Tissue	Collection date	Age(years)	Fungus(ml)	Dissection date
Acol12	Brain	3.5.2019	1	1000	27.9.2019
Acol16	Brain	12.5.2019	1	50	27.9.2019
Acol17	Brain	12.5.2019	1	300	27.9.2019
Acol10	Brain	25.5.2018	2	1000	27.9.2019
Acol11	Brain	24.5.2018	2	1000	27.9.2019
Acol8	Brain	20.5.2018	2	800	27.9.2019
Acol1	Brain	23.5.2017	3	2000	27.9.2019
Acol3	Brain	24.5.2017	3	2000	27.9.2019
Acol5	Brain	25.5.2017	3	3000	27.9.2019



Supplementary figure 1: Plot of the fungus-garden volume (in ml) at time of sacrifice (y-axis) associated with the age of the nine queens in years (x-axis). Pearson's correlation coefficient = 0.88, p<0.01.

Supplementary table 2: The expression of three genes was significantly explained by the age of the *A. colombica* queens when fungus-volume was held constant. The RefSEq protein IDs as well as the best BLASTP hits of the proteins against a predetermined database is displayed.

Gene name:	**LOC108686192**	**LOC108689025**	**LOC108695106**
RefSeq protein:	**XP_018046833.1**	**XP_018051105.1**	**XP_018060355.1**
baseMean:	5307.885	931.645	277.228
log2FoldChange:	**-2.37318**	**6.13384**	**2.03245**
lfcSE:	1.9532	4.10977	3.94335
Stat:	463.0074	340.5397	31.1159
p-value:	1.07E-102	4.87E-76	2.43E-08
Adjusted p-value:	**1.07E-98**	**2.45E-72**	**8.14E-05**
NCBI annotation-gene:	**uncharacterized**	**transmembrane protease serine 9-like**	**15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like**
Best BLASTP match (highest bit score& e-value <1e-5):	**XP_024878006.1, putative aminopeptidase 2, *Temnothorax curvisopinosus***	**XP_011063330, polyserase-2 like *Acromyrmex echinatior***	**XP_011062502, 15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like, *Acromyrmex echinatior***

Supplementary table 3: The expression of the only gene explained by fungus-volume of the *A. colombica* colonies. The RefSEq protein IDs as well as the best BLASTP hit of the protein against a predetermined database is displayed.

Gene name:	***LOC108689145***
RefSeq protein:	**XP_018051274.1**
baseMean:	**147.8758**
log2FoldChange:	**0.001562**
lfcSE:	**0.000409**
Stat:	**22.49147**
p-value:	**2.11E-6**
Adjusted p-value:	**0.020496**
NCBI annotation-gene:	***endochitinase***
Best BLASTP match (highest bit score& e-value <1e-5):	**XP_011056666.1, endochitinase isoform X2 *Acromyrmex echinatior***

Chapter 5

Tissue-specific ageing in long-lived queens of the leaf-cutting ant *Acromymex echinatior*

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In prep

Abstract

All organisms age, but the rate of ageing varies greatly between species and tissues. Natural selection can reduce the average rate of ageing, but what allows the caste differentiated social insects to show extreme variation in lifespans is poorly understood. As queens specialise on reproduction, it seems obvious to investigate the mechanisms that protect their reproductive tissues against aging. However, just as colony-level (family) selection has shaped the superorganismal integration of the germline-analogous queens and soma-analogous workers, analyses should also differentiate between reproductive and somatic ageing. We investigated the transcriptomic signatures of ageing in three tissues of leaf-cutting ant queens after they had been reproductively active for <1 years to a little over 15 years. We found that age affected gene expression almost exclusively in the brain. Ovary development and the proportion of live stored sperm remained constant across the years, but gene expression changes in queen brains were ageing- and maintenance-related, affecting IIS/TOR signaling, autophagy, and JH production. Vitellogenin pathway genes were mainly expressed in the brains and not in the fatbodies or the ovaries, suggesting that the regulation of reproduction follows a different scheme than is suspected in other insects. Our results support the view that superorganismal family selection has produced queen germlines that reproductively perform at a constant rate until they die from accumulated damage in the neuronal control tissues rather than in the reproductive system.

Keywords: tissue-specific ageing, leaf-cutting ant, queens, brain senescence, germline- soma

Introduction

The evolution of caste-differentiated colony life has repeatedly produced degrees of intraspecific within-family variation in life span that is never found in solitary organisms (Keller & Genoud, 1997b; Kramer et al., 2016; Monroy Kuhn et al., 2021; Münch et al., 2008; Schrempf et al., 2005). Reproductives (queens in Hymenoptera, and queens and kings in termites) often achieve astonishingly long lifespans with constantly high fertility, which contrasts with their non-reproductive worker offspring that are short-lived (Keller & Genoud, 1997b). This is puzzling because we typically assume that reproduction is costly and should thus shorten lifespan. In spite of considerable research efforts, we still know very little about how natural selection has produced reproductive castes in ants and termites that combine extreme fecundity with very high longevity (de Verges & Nehring, 2016; Korb, 2016). Considering the analogies between caste-differentiated superorganismality and obligate multicellularity may offer some conceptual clarification of this conundrum (Boomsma & Gawne, 2018; Elsner et al., 2018). Somatic metazoan cell lineages diverge and specialize on multiple support functions, and their life spans vary between days (e.g. gut-lining) and decades (e.g. neurons). These specializations are a collective somatic effort to maximize germline survival and ultimately achieve a high inclusive fitness of all cells within the organism (Boomsma, 2022). In parallel, lifetime unmated workers support their queen germline to similarly maximize inclusive fitness via sibling production and careful maintenance of the colonial-body infrastructure (Boomsma, 2013).

One way to prolong the lifespan of germline tissues and reproductive ‘colonial germline’ individuals is to delegate the wear-and tear to the somatic cells and workers as much as possible(Elsner et al., 2018; Schrempf et al., 2005). Indeed, superorganismal colonies always hide their reproductive germline individuals from potential predators in the deep interior of the nest, and the perennial colonies of ants and termites have evolved extreme degrees of social immune defenses so that microbial challenges can be unambiguously met and only require sacrificing somatic workers (Cremer & Sixt, 2022). However, a crucial mismatch that remains unresolved is that germ-soma differentiated bodies ultimately die from somatic failure (e.g. cancer) while caste-differentiated colonies apparently die from germline failure, that is, the death of the queen, unless germline replacement procedures have evolved secondarily (Boomsma, 2022; Boomsma et al., 2014). This implies that superorganismal lineages that maintained the ancestral irreplaceable single-queen germline (cf. Hughes & Boomsma, 2008) needed to evolve fundamental adaptations for maximizing colonial reproductive success that are fundamentally different from those that maximize reproductive success in bilaterian metazoan individuals.

Previous studies have revealed that the molecular regulation of fecundity and longevity changes between young and older ant queens, albeit differently across tissues (e.g. Korb et al., 2021). However, only a few studies have been able to sample queens at the end of their natural lifespans, because such samples require obtaining colonies of known age from the field and maintaining them under controlled conditions for decades. Most studies therefore compared founding queens with queens of at best middle age, but such comparisons conflate the possible trade-offs between fecundity and ageing with effects of colony foundation. Colony-founding queens have no or very few workers and their colonies have not yet transitioned from the ergonomic to the iteroparous reproductive stage (Oster & Wilson, 1978), while middle-aged queens are in their reproductive and physiological prime; there is then little reason to assume they should already show signatures of senescence when they still have a decade or longer to live. There are only two apparent exceptions, none of which really resolves the conflation problem. One study documented that *Cardiocondyla obscurior* gene expression in head and thorax versus the abdominal gaster segments containing the reproductive organs showed parallel signatures of aging shortly before the natural end of queen (Jaimes-Nino et al., 2022). However, *Cardiocondyla* is an evolutionary derived ant lineage, where queens have lifespans of only ca. one year because colonies regularly produce replacement queens (Heinze, 2017). The other study investigated transcriptomes of *Macrotermes bellicosus* termites and found that heads of ca. six year old queens showed no signs of ageing while worker heads did (Elsner et al., 2018).

The present study is the first to address questions of queen aging by sampling ant queens of known old age. We exploited the almost three-decade long routine rearing of colonies of the Panamanian leaf-cutting ant *Acromyrmex echinatior* in temperature- and humidity-controlled rooms of the Copenhagen Centre for Social Evolution (CSE). While workers of this species have typical lifespans of less than six months (unpublished data; see Camargo et al., 2007 for a related species), the maximal lifespan of queens is ca. two decades and colonies are, with few exceptions, headed by a single multiply inseminated queen (Nehring et al., 2018). This allowed us to sample queens of established mature colonies with known ages varying between less than two and 16 years, of which the older queens had passed their median species-specific lifespan. We investigated age-related gene expression and related transcriptomes to a sequenced reference genome of high quality (Nygaard et al., 2011). We analysed transcriptomes from three different tissues: (a) the brain as key organ for sensory integration, hormonal and behavioural coordination, and canalized superorganismal germline development (e.g. Qiu et al., 2022); (b) the fat body: a highly conserved insect organ for peripheral endocrine regulation and the synthesis of proteins that affect both fecundity and somatic longevity (Arrese & Soulages, 2010; Knight, 2020); and (c) the ovaries, the key organ for queen fecundity that mediates germline functionality for the entire colony (den Boer et al., 2009; Y. Wang et al., 2011). We also obtained colony-level resource covariates that could independently affect gene expression: (1) fungus garden size, as a proxy of colony-wide resource availability reflecting both worker number and foraging capacity; (2) mean ovariole length in queen ovaries, reflecting her fecundity at the moment of sampling; and (3) the viability of the sperm stored in the queen's spermatheca.

After adjusting for potentially confounding resource and fecundity variables, we found that age-related differences in gene expression were almost exclusively restricted to the queen brain, while the functionality of her ovarioles and fat body tissues remained unaffected. This finding matches the common notion that old ant colonies tend to be highly reproductively successful and suggests that germline failure is a cliff-edge phenomenon of terminal collapse rather than a process of gradual decline. Functionally, neuronal efficiency and tissue maintenance decline simultaneously in the aging queen brain, even though no effects on fecundity and fat body functioning were apparent. IIS/TOR signaling and the expression of autophagy inducers and protectors against neurodegeneration decreased in aging queen brains, suggesting that routine removal of dysfunctional structures is compromised. Also, juvenile hormone expression appeared to be lower in the brains of aging queens, even though their fertility remained unaffected. Finally, we found that genes in the vitellogenin pathway were expressed in the brains rather than in the fat bodies, suggesting an unusually remote regulation of fertility that may be instrumental in maintaining extreme and age-independent fertility until the very end of a queen's life. As we detail in the Discussion, these findings change the way we have traditionally been thinking about aging in caste-differentiated social insect colonies.

Material and Methods

Ant collection, colony maintenance, and sampling

Acromyrmex echinatior queens used in this study were collected in Gamboa, Panama, between 2003 and 2017. When collected, all but three queens had small fungus gardens (approx. volume 100 ml), indicating they had been established ca. one year before collection (collection took place around the time of the mating flights). The three remaining queens were collected as newly mated queens with no workers. All the queens were subsequently transported to the Department of Biology, University of Copenhagen, where they were maintained at 25°C and 70% relative humidity. Soon, the founding queens established full colonies. All colonies were provided with bramble leaves, apple slices, and rice ad libitum. The queens were sacrificed by decapitation, and their brains, fat bodies, and ovaries dissected out on ice in February 2018 and stored at -80°C. The fungus-garden volumes were noted and the ovaries were photographed to measure the mean ovariole length using Fiji ImageJ software (v5).

Sperm motility assays

To record *A. echinatior* sperm motility, we punctured the queen spermatheca with forceps and collected samples of outflowing sperm using 20 µl pipette tips. For each sperm sample, the pipette tip was previously loaded with 3 µl Hayes saline (9 g NaCl, 0.2 g CaCl2, 0.2 g KCl and 0.1 g NaHCO3 in 1000 ml H2O, adjusted to pH 8.7 and sterilized by filtration through a 0.22 µm syringe-filter, Membrane Solutions) containing 375 µM of SYTO 13 (Molecular Probes) fluorescent dye. Sperm suspensions were then pipetted into a counting chamber (SC-20-01-04-B, Leja) and immediately observed with a spinning-disc confocal microscope (Revolution XD, Andor) using a 20X dry objective. The fluorescent dye was excited with a 488 nm laser and motility recorded with an Andor iXon DU-897-BV EMCCD camera, setting the EM gain to 60 and the exposure time to 32 ms, allowing us to record videos of sperm movements at 30 frames per second. We obtained a 5 s recording of sperm motility from each of eight males, while for the queens we obtained two to five such recordings, between which we changed the field of vision within the same counting chamber. We analysed recordings with the computer-assisted sperm analyser (CASA) plugin (Wilson-Leedy & Ingermann, 2007) for ImageJ (http://imagej.nih.gov/ij/). To do so, we used the same parameter settings as were previously determined (Liberti et al., 2016, 2018), which are specific to *A. echinatior* and the microscope system we used: a = 20, b = 250, c = 30 d = 12, e = 3, f = 10, g = 10, h = 5, i = 1, j = 10, k = 10, l = 10, m = 80, n = 80, o = 50, p = 60, q = 30, r = 683.3728, s = 0, t = 1, u = 1. We then measured the proportion of motile sperm as the proportion of tracked sperm identified as exhibiting motility during the 5 s of analysis.

Sperm survival assays

To quantify sperm survival we used a Live/Dead sperm viability kit (Molecular Probes), consisting of the membrane-permeate stain SYBR-14 (staining live sperm cells in green), and the cell stain propidium iodide (staining dead sperm cells in red). For each measurement, we collected outflowing sperm from the queen spermatheca or male ATs with a 20 µl pipette tip previously loaded with 3 µl Hayes saline. The sperm suspensions were incubated with 3 µl of SYBR 14 working solution (2 µl SYBR 14 stock in 98 µl Hayes saline) at room temperature in the dark on the surface of a microscope slide placed inside a humidified box to avoid desiccation of the sperm-containing droplets. After 10 min of incubation we added 1 µl of propidium iodide and incubated the samples for an additional 7 min before loading 3 µl of these mixtures into a chamber of a SC-20-01-04-B microscope slide (Lejca). Sperm were imaged with a fluorescent microscope. We used a GFP filter to visualize green (live) sperm and a N2.1 filter for red (dead) sperm.

RNA extraction and sequencing

RNA was extracted using a Trizol:Chloroform: Isoamyl alcohol-based protocol (Lin et al., 2021). The libraries were prepared and sequenced by the Beijing Genomics Institute (BGI Shenzhen, China). The concentration and quality of the isolated RNA were checked with an Agilent Bioanalyzer (Agilent RNA 6000 Nano Kit). The brain and fat body samples had lower RNA concentration than the ovaries, so we used low-input Tn5 RNA libraries for those two tissues, and standard library preparation for ovary samples. Library preparation failed for one fat body sample. All other libraries were sequenced using the platform BGISEQ-500 (100-bp Paired End reads) to produce 6-12 Gigabases of raw data. We obtained between 55 and 169 million reads per sample.

Transcriptomic analyses

Raw reads of the 35 samples (3 tissues from 12 queens, barring one fat body sample that failed library preparation) that did not meet a minimum length threshold off 70 base pairs were removed using fastp (version 0.2, Chen et al., 2018). A summary of the above were visualised using MultiQC-1.7 and the quality of reads post-filtering were assessed with FastQC (version 0.11.8, Andrews & others, 2010). HISAT2 (version 2.1.0, Kim et al., 2019) was used to map trimmed sequences against the annotated Ref-seq assembly of the *Acromyrmex echinatior* genome GCF_000204515.1 (version 3.9), obtained from NCBI. All samples mapped against the genome with 65-89% alignment rate regardless of library preparation method. SAM files generated by HISAT2 were first converted to BAM files and then sorted by name using SAMtools (version 1.9, (H. Li et al., 2009)). The sorted BAM files were used to generate gene count tables using Htseq-count (Putri et al., 2022; Htseq version 0.11.2; settings: -f bam, -i ID, -t gene, -m union, -r name, --stranded=no). A gene-count table consisting of all the samples was produced. We also produced a meta-data table consisting of information for each sample. The data were further analysed using R-Studio (R version 4.0.4, R Core Team, 2021). Genes that had fewer than ten reads in more than 5 samples were removed (1902 genes of a total of 12292 genes in the genome) from the overall dataset. For a first overview, we transformed the raw counts using a variance stabilizing transformation (function vst of the DESeq2 package (version 1.30.1, Love et al., 2014) to account for differing sequencing depths and tissue-specific expression; then visualised using a Principal Component Analysis (PCA).

We then focused on the effect of age on gene expression in each tissue separately. We estimated how much influence each of the three predictors age, fungus volume, and ovariole length had on the variance in the expression of the filtered genes, using the variancePartition package (version 1.20.0, Hoffman & Schadt, 2016) with a procedure adapted from Santos et al., 2022. After excluding the genes that were not expressed in all samples of the tissue in question, we analysed the relative variance explained by the combination of the three factors (without interactions) in each tissue separately (8886 genes in the brain, 8347 genes in the fat body, and 8631 genes in the ovaries). We then reduced the dataset to only those “focal” genes 50% of whose variation was explained by a combination of these three predictors (2461 genes in the brain, 713 in the fat body, and 1579 in the ovaries; a threshold of 80% yielded the same patterns in the subsequent analyses). Finally, we ran three separate models for each tissue, one with each predictor separately, to calculate the maximum variation each factor could explain (Supplementary Fig 1). This procedure circumvents the problem of variation that could be explained by more than one factor, which would arise in additive models.

We also tested for each gene whether it was differentially expressed by age, fungus volume, or ovariole length, using DESeq2. This analysis was also done for each of the three tissues separately. We excluded genes that had fewer than 10 reads in 5 or more of the samples. DESeq2 accounts for variation in sequencing depth and uses transformed read counts for each gene. The full model contained all three factors (age, fungus volume, and mean ovariole length) as continuous predictors. Hypothesis testing was done by removing one factor at a time from the full additive model and comparing the full with the reduced model using the Likelihood Ratio Test (LRT). Thus for each tissue, this procedure tests for the effect of one factor at a time on gene-expression, while controlling for the other two factors in the reduced model. Only those genes whose variation is explained solely by one predictor and not a combination of two or more will thus be considered for testing. While this procedure is less sensitive than testing each factor in isolation, it may find effects that would otherwise be obscured by stronger effects of other factors. Genes that had an adjusted p-value; padj < 0.05 after FDR/Benjamini-Hochberg multiple testing were considered to vary significantly with the factor in question; all p-values mentioned in the manuscript that relating to the DESeq2-analysis are adjusted p-values.

Gene annotation, functional enrichment, and comparison to known candidate gene lists

We obtained the functional annotation of differentially expressed genes (DEGs) related to age in each tissue from DAVID (2021 Update,Huang et al., 2009; Sherman et al., 2022; Supplementary table 1). We ran BlastKOALA (Kanehisa et al., 2016) on the entire proteome available on NCBI (GCF_000204515.1_Aech_3.9_protein.fasta) using “family eukaryotes as the KEGG genes database. We obtained KO ID annotations . For the 366 genes significantly affected by queen age (p-adj <0.05) that also had KO terms, we performed a KEGG enrichment analysis using clusterProfiler v3.10.1 (Yu et al., 2012) in R-Studio (R version 4.0.5, (R Core Team, 2021)) on a background of 5414 unique KO terms expressed in the queens’ brains. We removed all pathways related to human disease (map05*) or drug response (map07*) from the pathway database and tested the remaining 292 pathway maps for enrichment.

We used OrthoFinder (version 2.5.4), (Emms & Kelly, 2015, 2019) to identify orthologous proteins in the proteomes of Drosophila melanogaster and Acromyrmex echinatior. We then searched the list of shared orthogroups for 123 candidate genes involved in ageing process. These ‘TI-J-LiFe’ genes consist of genes related to the Insulin/Insulin-like Grwoth factor 1 Signalling (IIS), Target of Rapamycin (TOR) and Juvenile Hormone (JH) pathways implicated in lifespan (‘Li’) and fecundity (‘Fe’), derived from genes in *Drosophila melanogaster*. These and additional genes were selected due to their known or suspected involvement in signaling pathways associated directly with IIS, TOR, JH, Vitellogenin, or ageing in *D. melanogaster, C. elegans* and/or social insects (Korb et al., 2021). The genes were visualized across tissues by using the DESeq2 function ‘plotcounts’ which normalized and plotted the reads. In a similar fashion, we also used the orthogroups to search for oxidative-stress-related genes expressed with age in queens based on 33 known oxidative stress genes across social insects (Kramer et al., 2021).

Results and Discussion

No indication of reduced fecundity of old queens

We directly investigated the integrity of the reproductive tissues of queens of up to 16 years of age. Queen age did not predict ovariole length (rho = 0.34, p = 0.27; Fig. 1.1a). nor the size of the fungus garden (spearman rank correlation rho = 0.44, p = 0.15 Fig 1.1 b) There was also no effect of age on the proportion of dead sperm in the spermatheca (p = 0.91; Fig 1.1c). Queens of all ages contained only very low proportions of dead sperm (under 10%). Interestingly, the stored sperm was largely immobile (Fig. 1.1d).

Only brain gene expression affected by age

The lack of age effects on the ovaries was mirrored in the gene expression of this tissue. The queens' gene expression patterns clearly clustered according to tissue, with low variation among brain and ovary samples, but more variation among fat body samples (Fig 1.3 a). We conducted a differential gene expression analysis with DESeq2 for each tissue separately. The analysis was designed to determine genes whose expression could be unequivocally explained by only one of the three predictors. Expression of most genes was explained by age rather than fungus volume or ovariole length, and these effects were largely restricted to the brain (Fig. 1.3 a). After accounting for ovariole length and fungus volume, we found 840 genes in the brain, six in the fat body, and six in the ovaries to have changed their expression with increasing age. In the ovaries, the expression of 39 genes was correlated with fungus volume, and in the fat body, that of 52 genes with ovariole length (list of genes with annotation from DAVID in Supplementary table 2).

Interestingly, gene expression in the ovaries was not best explained by ovary size but by fungus volume (Fig. 1.3 b), a factor correlated with ovary size (Fig. 1.3 b). Although the correlation makes it impossible to fully disentangle the effects of the two predictors, both analyses we employed suggest that there is a set of genes whose expression in the ovaries can best and only be explained by fungus volume and not any other predictor. Fungus size is a good proxy for a colony's worker number, and large colonies need more fecund queens to replace the dying workers, so what we observe may indeed be the regulation of egg production. There was no such unequivocal pattern for gene expression in the fat body, which may have to do with the fat body's central role in governing very different processes all over the body.

It is remarkable that the broader transcriptomic age-related changes were virtually restricted to the brain. The pattern indicates that ageing is tissue-specific in *Acromyrmex* queens, who might manage to stall senescence in tissues crucial for reproduction, such as the ovaries and fat body. In contrast, the brain clearly ages and perhaps accumulates all inevitable damage (see Fig 1.2 for indication of structural changes in the brain).

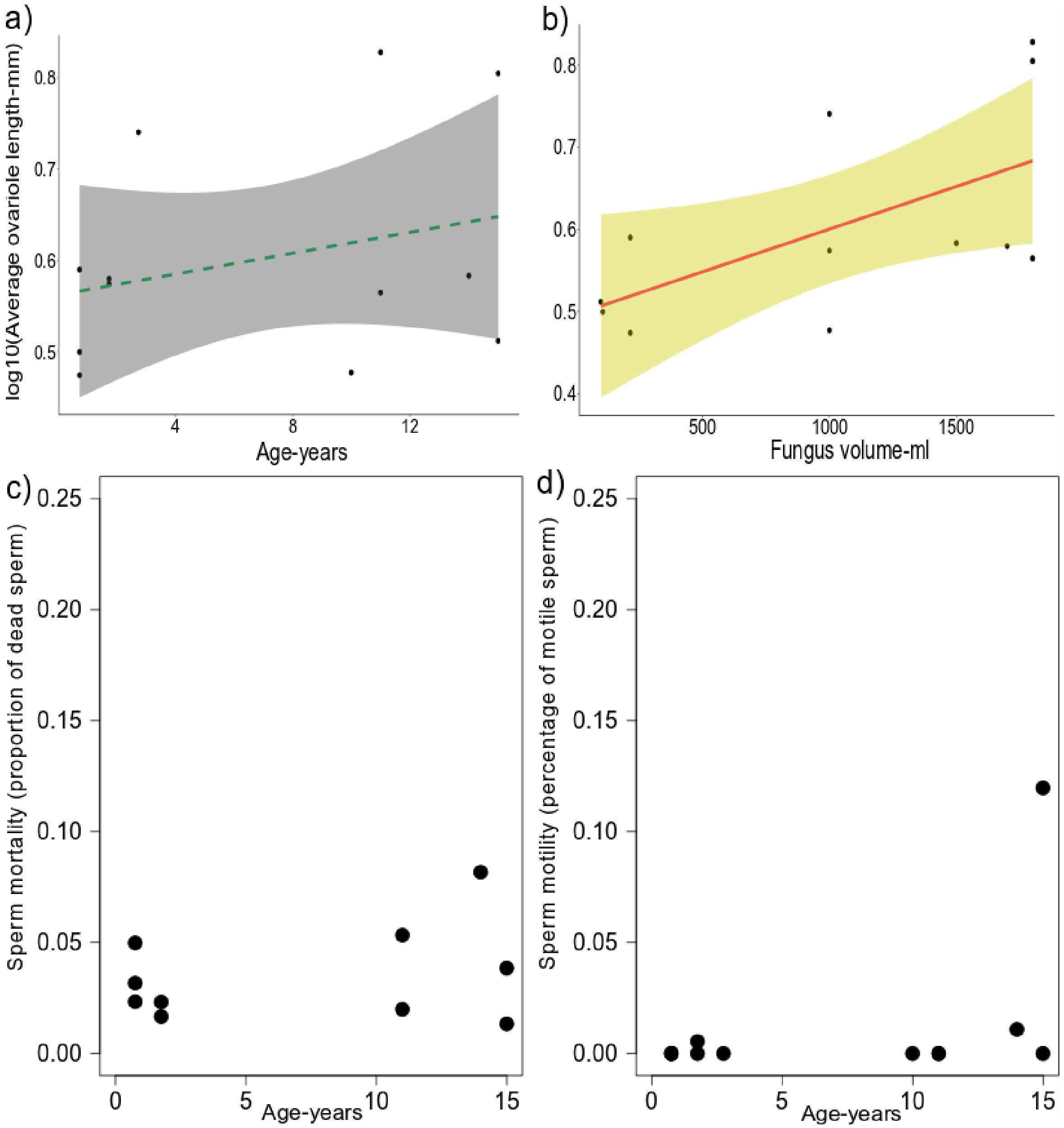

Figure 1.1: Our data indicate that queen fecundity and tissues associated with fecundity did not age over more than ten years of a queen's life. In contrast, the brain changed and potentially deteriorated. a) Age did not have an effect on mean ovariole length of queens (spearman rho = 0.34, p = 0.27) but b) there was a correlation between fungus garden size and ovariole length (spearman rank correlation test: rho = 0. 58, p = 0.05). Fungus garden size is a measure of colony size. When we investigated the sperm stored in the spermathecae of 11 out of the 12 queens, we did not find an effect of age, neither on the proportion of dead sperm (spearman rho = - 0.04, p = 0.91) nor on sperm motility (spearman rho = 0.43, p = 0.16). Sperm from queens of all ages was surprisingly immobile, which might be a strategy to ensure the survival of sperm cells by avoiding damage by reactive oxygen species produced as a byproduct of motility (Liberti et al., 2016, 2018)

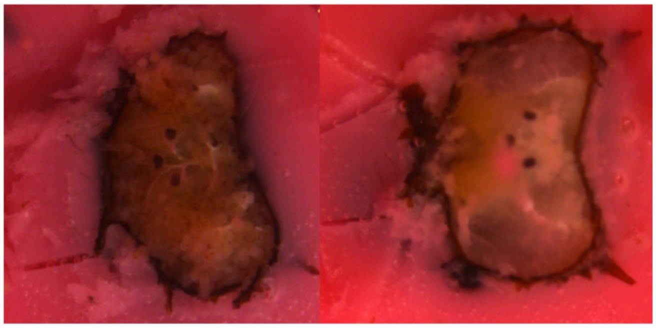

Figure 1.2: Representative images of dissected brains, showing that old queens (left) appeared to have more granular brains while young queens (right) had smooth surfaces. Old brains were also darker and had a brownish coloration.

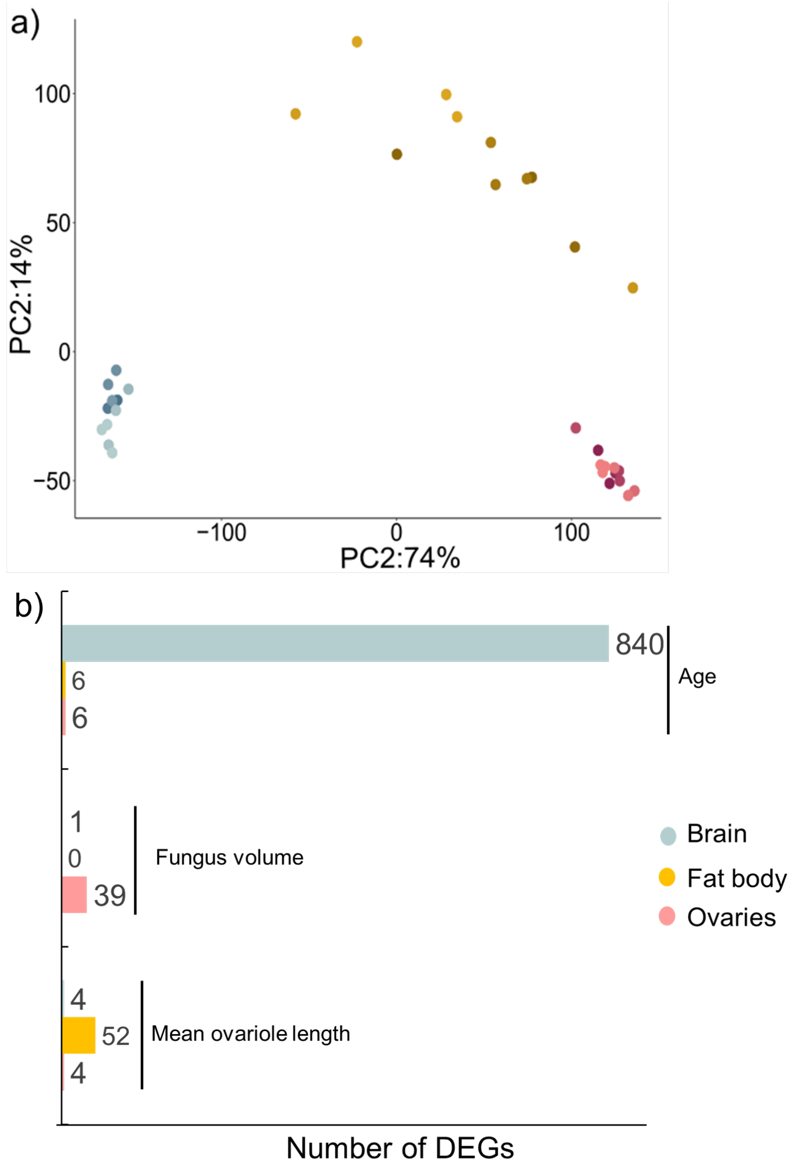

Figure 1.3: (a) Principal component Analysis (PCA) based on the variance-stabilised read counts of all 10388 expressed genes across all samples (PC1: 74% of variance explained; PC2: 14% of variance explained). Gene expression mainly clustered by tissue (brain, fat body, ovaries). Age effects (directional light-dark shifts) were observed for brain samples (albeit slight) but not for ovary and fat body samples where transcriptomes were most variable.(b) Age was the most powerful predictor for differential gene expression, and its effects were almost totally restricted to the brain. This indicates that fat body and ovary functions remain stable over more than a decade of a queen's lifespan, while there are considerable age-related changes in the brain. The proportion of differentially expressed genes varied among the three predictors (queen age, fungus-garden volume, mean ovariole length; χ^2 goodness of fit $p < 0.001$ in all cases) and the fraction of genes that were significantly differentially expressed showed similar variation (χ^2 homogeneity test $p < 0.001$ in all cases). The gene counts behind these analyses refer to genes that were significantly affected by each respective factor (p adj< 0.05) in Dseq2 analysis that adjusted for potential effects of the other two predictors in the same tissue.

Variance partitioning

We conducted a variance partitioning analysis to quantify the variation that each of the three predictors could explain of the total explained variation. In contrast to the Deseq2 analysis, the design allowed for multiple predictors to explain the same variation. The overall patterns, however, remained the same*:* In the brain, the three predictors age, fungus volume, and ovariole length explained more than 50% of the variance in expression of 2461 out of a total of 8886 genes expressed in the brain (28%; henceforth referred to as “focal genes”). Age was the most important predictor for gene expression (Fig 2a). Variation in the expression of many of the 2461 focal genes could be explained almost exclusively by age (in 798 of the 2461 focal genes, age was responsible for more than 80% of the total explained variance; upper triangle in Fig. 2 a). Fewer genes were mostly affected by ovariole length (104) and fungus volume (49) (Chisq goodness of fit $p < 0.001$).

For ovaries and fat body, the three predictors could explain more than 50% variation in the expression of relatively fewer genes (fat body: 713/8347; ovaries 1579/8631). In the fat body, age was again the dominant predictor (Fig. 2b): Out of 713 focal genes, age explained more than 80% of the expression of 155 genes. However, fungus volume was also important (fungus volume: 106 genes; ovariole length: 23 genes; $p < 0.001$). Ovary gene expression was mostly explained by fungus volume (351 out of 1579 focal genes), with lesser contributions of age and ovariole length (Fig 2c). Note that fungus volume and mean ovariole length are somewhat correlated (Fig 1b) so that we cannot fully disentangle their effects. In the variance partitioning analysis, fat body gene expression was mainly explained by age and fungus volume rather than by ovariole length (the latter was more important in the Deseq2 analysis). This was likely the case because there was variation caused by fungus volume that obscured smaller variance correlated with ovariole length. In the Deseq2 analysis, the fungus-related variation was removed before testing for ovary-related variation, which in this case increased the statistical power of the Deseq2 analysis.

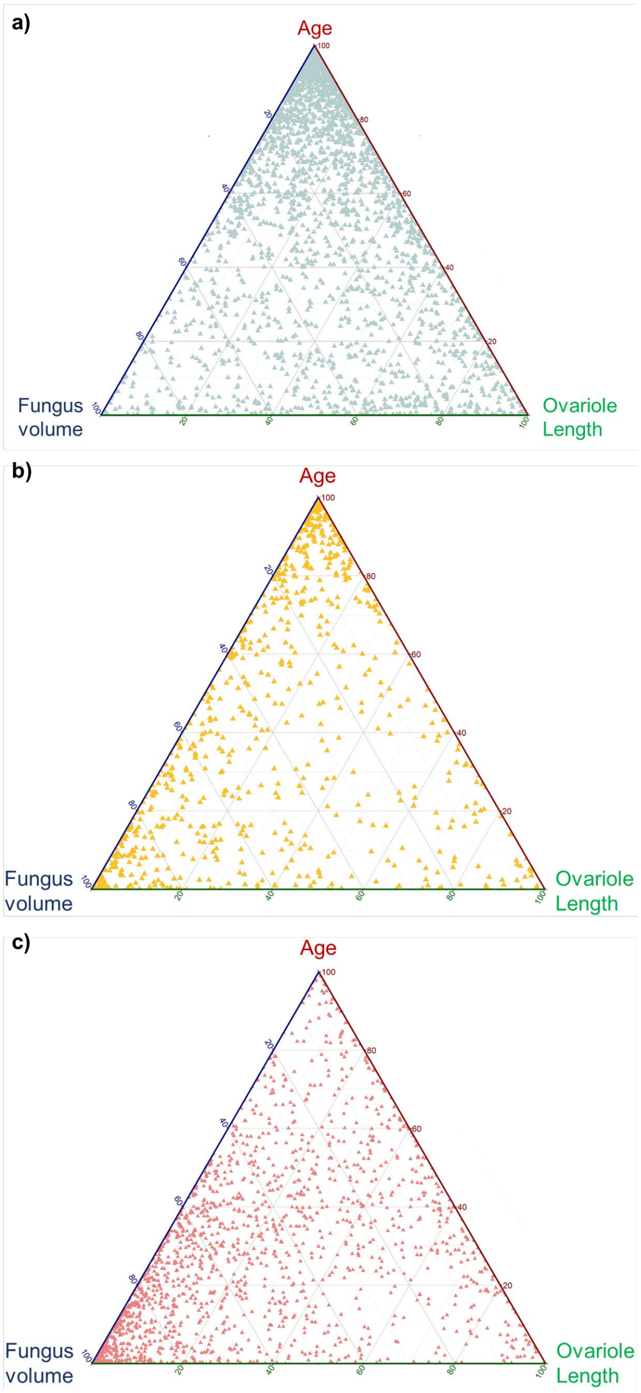

Figure 2: Ternary plots showing the relative explanatory power of the predictors age, fungus volume, and ovariole length on differential gene expression. Most differences in gene expression were related to age of queen brains. For fat body and ovaries, fungus volume also contributed to differential gene expression. These combined patterns (relative to the single factor analyses of Figure 1.3 b) confirm that age effects are strongest in the brain; a) and that differential gene expression in the other tissues b) fatbody and c) ovaries was mostly affected by other factors. Each dot represents the relative contributions of the three predictors to the expression of a single gene. The power of the three predictors variables increases from 0% to 100% of variance explained along the sides of the triangles. Only genes for which >50% of the expression variance could be explained by the three factors combined are shown.

Age affects Insulin/TOR gene expression in the brain

To analyse whether the age-related changes in gene expression were indeed signs of senescence, we subjected the 840 brain DEGs to a KEGG enrichment analysis that revealed 34 pathways, many of which stemmed from a set of pathways that constitute the insulin-TOR signalling network that has previously been implicated to be a central regulator of resource allocation and ageing (Korb & Heinze, 2021) . We then went through the IIS and TOR pathways manually (based on KEGG, Kanehisa et al., 2021; Kanehisa & Goto, 2000; Yan et al., 2022) to understand whether the directions of gene expression changes were consistent with our understanding of these pathways in other organisms, and what physiological effects they may cause. We found a lower expression of the gene locusta insulin related peptide (LIRP; padjust<0.0001) in old queens (Fig. 3). LIRP is orthologous to the *Drosophila* ilp1 gene and to *Ooceraea biroi* ilp2. It is one of only two genes for insulin-like peptides annotated in the *Acromyrmex echinatior* genome (the other being LOC105154102; insulin-like growth factor II, not affected by age; Nygaard et al., 2011). LIRP has previously been shown to be downregulated in workers relative to gynes in ten ant species, including *Acromyrmex echinatior* (Chandra et al., 2018; Qiu et al., 2018), and was shown to control reproductive variation in clonal raider ants (Chandra et al., 2018). Insulin-related peptides are involved in nutrient sensing and the regulation of cell growth, proliferation, and death, and are thus often responsible for variation in tissue function and lifespan (Partridge et al., 2011). In Drosophila, insulin takes its effects through pI3k and MAPK signalling, which both alter TOR complexes (Huang & Fingar, 2014; Rajak et al., 2018). Our results match this expectation, as both MAPK signalling (lower *SOS* and *rl* expression) and pI3k signalling (low expression of the regulatory subunit *p60*/*Pi3K21B*) were reduced with age (Fig. 3). The effect on pi3K signalling might be enhanced by an upregulation of both *Nlaz* and *convoluted* in old queens (Fig. 3), which have been shown to reduce the effect of insulin on pi3K in Drosophila (Arquier et al., 2008; Hull-Thompson et al., 2009; Pasco & Léopold, 2012). Both pathways affect TOR complex signalling (Laplante & Sabatini, 2009), and indeed we find reduced *tel2* (LOC105152500) expression (part of both tor complexes 1 and 2) and reduced expression of some TORC target genes (*mts*, *Rho*; Fig. 3; padjust < 0.05). The entire pattern is supported by trends in further genes within the pathway and TORC target genes (*MEK, PKC, atg1*; padjust < 0.1) and is fully in line with our understanding of the pathways from model organisms (Fig. 3).

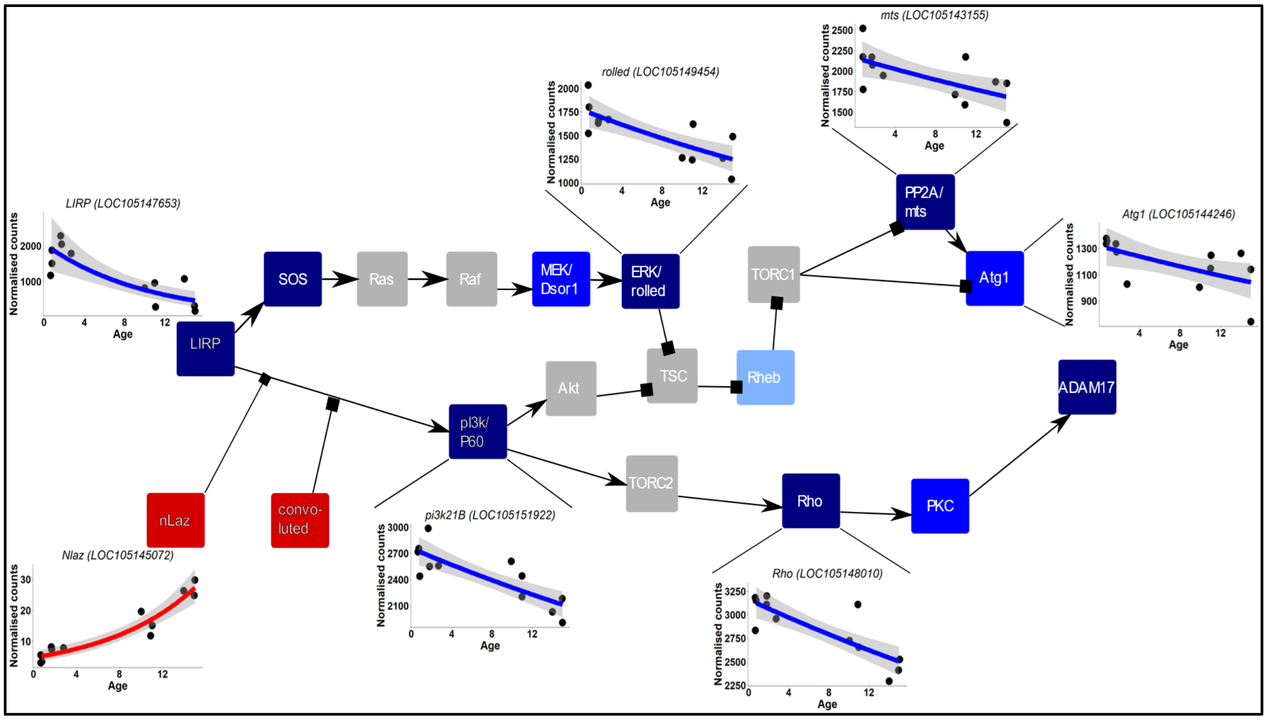

Figure 3: Gene expression analyses indicated a downregulation of insulin signaling leading to reduced autophagy and neuroregeneration in the aging brain, suggesting that queen brains age even though this does not appear to affect fecundity (see Figures 1 and 2). In more detail, the insulin-like peptide LIRP was downregulated and the insulin signalling antagonists *neural Lazarillo* (*NLaz*) and convoluted were upregulated with age, which explains lower PI3K expression and a possible TORC2 downregulation, leading to low levels of PKC and ADAM17, which are known inducers of neuroregeneration. Simultaneously, low insulin levels induced downregulation along the MAPK pathway (*SOS*, *MEK/Dsor1-like*, *ERK/rolled*), which might result in low TORC1 levels. TORC1 typically downregulates autophagy, consistent with our finding that the autophagy markers (*Atg1*, *PP2A/mts*) were downregulated in old queens. However, we did not find any other known effects of TORC1 modification (e.g., protein synthesis) and since *akt* and TSC were unaffected by queen age, it is also possible that TORC1 remains unaffected and that autophagy is regulated independent of the genes *TSC* and TORC1, perhaps directly by ERK/rolled. Genes significantly upregulated with the age of queen brains are highlighted in red, and downregulated genes in blue (adjusted $p < 0.05$). Lighter blue colours indicate trends in the same direction (adjusted $p < 0.1$, $p < 0.25$) that did not reach statistical significance. Grey genes were not affected (adjusted $p > 0.3$). The pathway plot was created based on the *Drosophila* mTOR pathway (Kegg dme04150 ; Kanehisa & Goto, 2000) with additions as explained in the text.

The effects of altered pI3k signalling are straightforward since it induces TORC2, which in turn induces Rho (Fig. 3) in actin cytoskeleton formation independent of Akt (Liu et al., 2010). Lowered pI3k/torc2/Rho signalling may further have caused the low expression that we observe of various *protein kinase C* genes in old queens (Fig. 3; Wullschleger et al., 2006). Some PKCs are known to induce ADAM17 (*LOC105143101*) in mammals (Domínguez-García et al., 2019; Geribaldi-Doldán et al., 2019), a metallopeptidase that is involved in the repair of neural structures - so that its downregulation that we observe in old queen brains (padjust < 0.05) may mean that the brain is less well maintained in old than in young queens. This focus on brain ageing integrates well with the recent findings that ensheathing glia is increased when females transition from being virgin queens to inseminated gynes in *Monomorium* ants (Q. Li et al., 2022).

The effects of pI3K and MAPK on the TORC1 complex are less consistent with expectations because we do not find any age effect on the expression of the intermediaries *akt* (no effect) and *TSC1/2* (not expressed in brain, no effect in fat body/ovaries). These intermediaries are typically thought to be altered by insulin signalling and to affect TORC1, although their involvement appears to depend on the specific physiological function and the details are still poorly understood (Huang & Fingar, 2014; Wullschleger et al., 2006). We do find some specific TORC1 targets that deal with autophagy to be downregulated in old queens *(mts*, *atg1, atg10*; Fig. 3; supplementary table 1; Omata et al., 2014).

Autophagy is used as a mechanism to re-purpose resources and invest them into newer proteins (Bánréti et al., 2012). Lower autophagy in queen brains may mean that damaged proteins are not cleared anymore but accumulate in the brain, which in the long run would inhibit brain function (Broughton & Partridge, 2009; Simonsen et al., 2008). *Drosophila* atg1 loss-of-function phenotypes include reduced autophagy, short life, and reduced dendritic growth (Maruzs et al., 2019). The notion that autophagy is reduced in old queens' brains is also supported by an age-related downregulation of *atg10* (*LOC105154559*, padjust = 0.057), coding for an E2-like enzyme involved in autophagosome expansion (Zirin & Perrimon, 2010).

Juvenile hormone signalling is an integral part of the Ti-J-LiFe-network and often affected by ageing. In our brain samples, an aldehyde dehydrogenase potentially responsible for JH synthesis was downregulated in old queens (padjust < 0.05), perhaps caused by an elevation in the expression of the JH synthesis blocker *allatostatin* (padjust = 0.08; Fig 4.1). The protein takeout-like was upregulated in old queens (padjust < 0.001). The expression of the gene *takeout* is induced by starvation and has been identified to be involved in lifespan extension in adult *D. melanogaster* (Bauer et al., 2010; Sarov-Blat et al., 2000). Takeout related proteins can bind to JH and its precursors with differing affinity, regulating their transport and preventing their uptake by non-target tissues (Hagai et al., 2007).

To challenge the hypothesis that queen fecundity remains unaffected by age, we specifically analysed the expression of genes directly involved with egg production. Vitellogenin is a protein that serves as an egg yolk precursor, but it is also a storage protein and protects against oxidative stress. The gene was repeatedly duplicated in the evolution of social hymenoptera (Corona et al., 2013; Morandin et al., 2014b; S. C. Seehuus et al., 2006a). In our *A. echinatior* queens, the 'conventional' vitellogenin (Kohlmeier et al., 2018) was not affected by age in any tissue. Interestingly, its expression was very low in the fat body and much higher in the brain (Fig. 4.2, cf. Kohlmeier et al., 2018). Three other vitellogenins were expressed in the fat body. While there was no statistically significant effect of age on the expression of these genes, the expression varied strongly among the fat bodies of young queens (Fig. 4.2). The gene coding for the vitellogenin receptors was overexpressed in brains of old relative to young queens (Fig. 4.2). Vg receptors are important for yolk uptake and its expression in the brain may be necessary for Vg transport to other parts of the body. Its expression was strongest in the ovaries, as would fit with its role, but age did not have any effect in the ovaries.

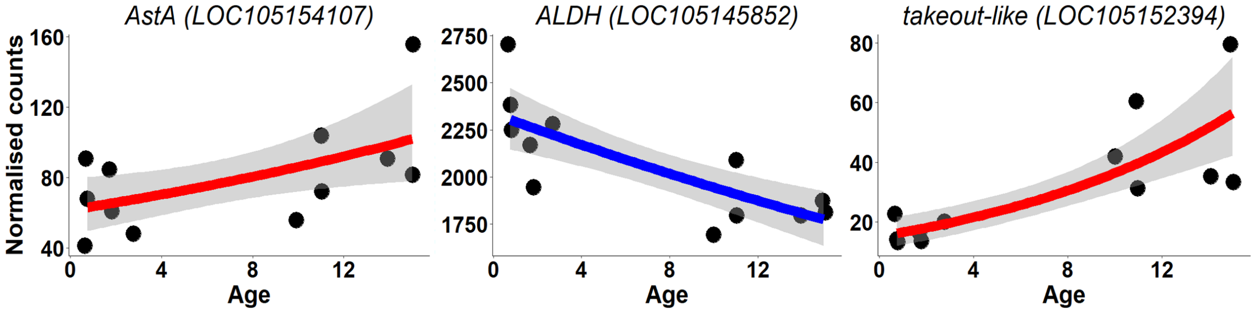

Figure 4.1: Age related brain gene expression for some genes involved in juvenile hormone signalling indicate that insulin signalling might be reduced in old queens. While there was merely a trend for the JH synthesis blocker *Allatostatin A* to be highly expressed in old queens (padjust < 0.08), they expressed significantly less of the gene coding for the JH synthesis gene *ALDH* (padjust < 0.05). Particularly the expression of *takeout-like* (*LOC105152394*), a protein that binds JH for transport or JH level regulation, increased in expression with increasing queen age (padjust < 0.001). Data points are brain samples of individual queens. The regression lines were obtained from a negative binomial glm and plotted with their 95% confidence intervals. Overall these observations suggest a reduction of JH expression in aging queen brains.

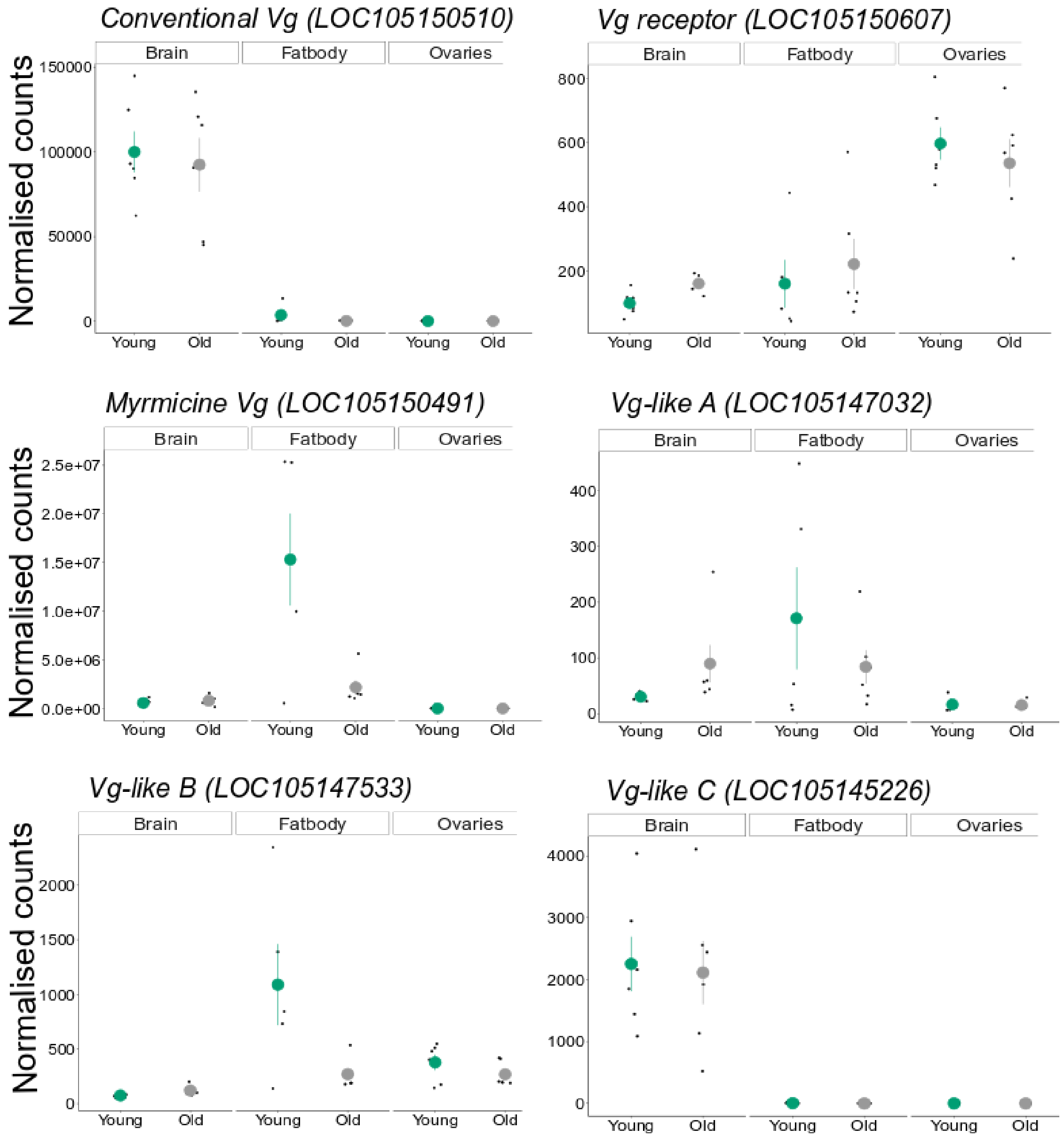

Figure 4.2:: Expression levels of some genes potentially associated with reproduction across ages and tissues do not support an age effect on reproduction. However, the pattern suggests that the functions of *Vg* genes differ from those of their homologs in other species. For example, conventional *Vg* was mostly expressed in the brain, unlike in other species, where it is expressed in the fat body as a yolk precursor. All six genes had their expression counted across tissues using the 'plotcounts' function of DESeq2 and the results were plotted by contrasting 'old' (> x years) and 'young' (< x years) queens. Significant changes occurred only in the brain (padjust values < 0.1 from the DESeq2 analysis are presented; all other comparisons produced padjust > 0.1 – see Supplementary table 4). Numerical queen ages, rather than the old/young categorization, were used in the statistical analysis.

Conclusion

We analysed reproduction from multiple angles in search for any effects of queen age on fecundity. Neither ovary morphology, sperm storage, nor gene expression data provided any indication that egg production, the main function of *Acromyrmex echinatior* queens, is affected by their decade-long duty. We sampled the queens from colonies of varied sizes which was partly mirrored in the queens' ovariole length. On the molecular level, the tissues directly responsible for reproduction (ovaries) or its regulation (fat body) stayed virtually unchanged over more than ten years of the queens' lives.

The only evidence for senescence we could observe were tissue-specific changes in brain gene expression with age. These changes followed a typical ageing pattern indicating failing maintenance of the tissue, with a reduction in autophagy to clear broken proteins, and a dysregulation of the repair of neural tissue. Other tissues, particularly those responsible for reproduction, seem to be shielded from senescence. Our results add to recent findings that tissues and organs age at different rates in humans and nematodes, but the ageing differential in the ant queens seems to be more extreme (Ezra et al., 2023; Nie et al., 2022; X. Wang et al., 2022).

In the context of social evolution, this pattern bears a striking resemblance to the reproductive division of labour among social insect queens and their workers. The presence of workers relieves queens of many tasks that are both risky and taxing, allowing them to fully optimise reproduction with little regard to any other function that would be crucial to the fitness of solitary organisms. In social insects as advanced as the leaf-cutting ants, this results in queens not at all interacting with the external environment once they are past the colony's founding phase. Our data are fully consistent with the notion that queens are purely optimised for long-lasting reproduction. The data demonstrate the success of outsourcing wear and tear to the workers, as the ovaries and fat body appear unphased from a decade of life and reproduction. While the queens maintain their ovaries very well, they appear to withhold the same level of maintenance from the brain, a tissue that is responsible for the interaction with the environment; this task is not the responsibility of reproductives in advanced eusocial insects. In conceptual analyses, the queens are often analogised with the germline of multicellular organisms (Boomsma & Gawne, 2018), with workers representing the soma. In the advanced eusocial *A. echinatior*, the germline in fact consists of little more than the queen's ovaries and some regulatory tissue; the queen's brain can at least in part be regarded as soma tissue from the perspective of the colony, and many of its functions can upon failure easily be taken over by the workers. By withholding physiological protection and repair from the relatively inconsequential brain tissue, the queens may manage to improve the maintenance of the reproductive tissues that are more important for the fitness of the entire colonies.

Our results help to explain how queen fecundity can be high until her death, which has been observed in other ant species (Heinze & Schrempf, 2012; Jaimes-Nino et al., 2022): They keep their reproductive tissues (which equals the entire colony's germline) intact at all cost, even at that of degrading function in other tissues. This observation suggests that social insect colonies die just as multicellular organisms do: By the eventual failure of somatic tissue.

Ethics

Ant collection and export permits were obtained for the leaf-cutter ants from the Autoridad Nacional del Ambiente (ANAM) in Panama by Jacobus (Koos) Boomsma. We followed the DFG Animal welfare guidelines and the local laws.

Authors’ contribution

J.J.B, G.Z and V.N designed the experiment. M.N, V.N and J.L dissected the queens and processed the brain, ovaries and spermatheca. M.S extracted RNA from all samples. M.M analysed all data supported by M.S, V.N, R.L and S.F. M.M wrote the first draft with V.N, which was then revised by J.L, G.Z, S.F, R.L and J.J.B at different stages of the final manuscript. V.N., S.F. and R.L. co-supervised the project.

Competing interests

We have no competing interests.

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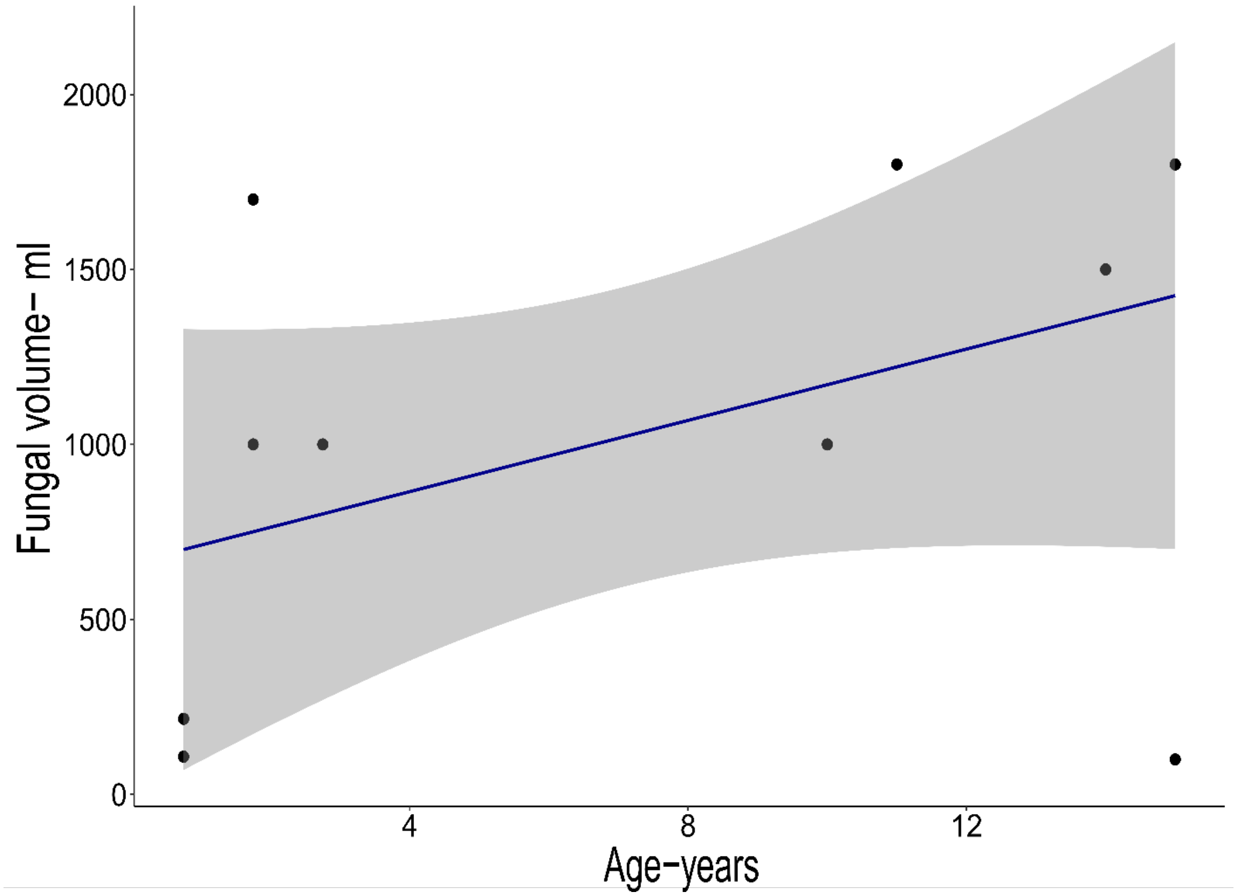
Supplementary

Supplementary table 1: Sample information table used to find correlation between age of queens and volume of their colonies' fungus gardens. The same information was used for DEseq2 analyses for the three tissues separately.

colony	**colony collection year**	**estimated age at collection (years)**	**date dissection**	**Age (years)**	**fungus volume (ml)**	**mean ovariole-length(mm)**
Ae226	2003	~1	24-02-18	15	100	3.25
Ae263	2003	~1	25-02-18	15	1800	6.38
Ae280	2004	~1	24-02-18	14	1500	3.83
Ae332	2007	~1	25-02-18	11	1800	3.67
Ae342	2007	~1	25-02-18	11	1800	6.73
Ae372	2008	~1	25-02-18	10	1000	3
Ae760	2016	~1	24-02-18	2.75	1000	5.5
Ae772	2017	~1	24-02-18	1.75	1000	3.75
Ae775	2017	~1	24-02-18	1.75	1700	3.8
LB51	2017	0(foundress)	25-02-18	0.75	108	3.16
LB53	2017	0(foundress)	25-02-18	0.75	216	3.89
LB57	2017	0(foundress)	25-02-18	0.75	216	2.98



Supplementary figure 1: The correlation between age of the queens and their colony's fungus garden volume was not significantly correlated (spearman rho=0.43, p=0.14)

All other supplementary tables: [Supplementary files for all chapters]

General Discussion

Main findings

In this thesis, I used natural and manipulated social structures in a range of ant species varying in their natural colony composition and life-histories to investigate the apparent positive correlation between fecundity and longevity observed in social insects. Workers typically undertake the non-reproductive tasks in the presence of a queen. When a queen is absent or removed, workers of different species naturally vary in their modes of reproduction in the absence of a queen- whether they can produce haploid male offspring- 'regular', produce female offspring- 'totipotent' or not reproduce at all because of loss of functional ovaries- 'sterile'. Their propensity to reproduce may also be affected by their age and/or task.

In **Chapter 1** and **Chapter 2** I created queenright and queenless sub colonies containing inside workers (expected to be younger) and outside workers (expected to be older). I subjected the workers from these sub colonies to oxidative stress, which challenged their survival. Workers of the two leaf-cutting Myrmicine species *Acromyrmex echinatior* ('regular') and *Atta colombica* ('sterile') collected from queenless nests were more resilient to oxidative stress compared to their queenright counterparts. Meanwhile the starvation and stress-resistant longer-lived workers of *Temnothorax rugatulus* ('regular') did not differ in survival although queen-absence is known to trigger reproduction and longer lifespan in the species. *Lasius neglectus* workers ('sterile') used in **Chapter 2** did not significantly differ in survival based on whether or not they had a queen. Transcriptomic signals in the fat body of queenless and queenless workers mirrored the same lack of queen-effect. The results confirm that in this species, there is no selection on longer worker-lifespan in the absence of a queen because workers in this polygynous system are never queenless in the wild unlike *A. colombica* (**Chapter 1)** that are monogynous and workers have been observed to produce male-offspring on rare occasions. In all the above species, regardless of their mode of reproduction, the workers collected inside the nest/closer to the brood survived oxidative stress better than workers that were collected outside the nest/at the foraging arena. This could be a result of higher reproductive potential of inside workers since young adults have ovaries that degrade over time.

Chapter 3, using *Plathythyrea punctata* ('totipotent') allowed me to disentangle age and task from reproductive potential due to the species' clonality and reversibility of caste-fate in adulthood. I found some genes that varied commonly with age in reproductively active (dominant) and inactive (subordinate) individuals while only nine genes varied in their expression between dominant and subordinate individuals. I could also show fewer age- related gene expression changes in dominants than in subordinates even though they are clonal and of the same age. Since reproduction is one of the few differences between the two social types, this further strengthens the positive link between fecundity and longevity.

Next, I focused on queens to detect age-related changes not confounded by colony-founding or reproductive senescence. The Formicine *L. neglectus* is unicolonial and new queens are readopted by colonies. I detected no significant difference in their fertility- either in terms of mean ovariole length or *Vg* expression in the fat body although there were significant differences in gene expression between the two age groups- young and old (**Chapter 2)**. *A. colombica* queens which are estimated to live 10-20 years used in **Chapter 4**. In this long-lived species, I detected barely any change in the brain's gene-expression in the first three years of colony-establishment. In **Chapter 5,** I used brains, fat body and ovaries from queens of another long-lived Myrmicine, *A. echinatior* ranging from less than 1 year to a little over 15 years. While the brain changed considerably with age, the fat body or ovary tissues did not. I observed signs of senescence such as dyregulation of proteostasis and insulin-signalling in the brains of these long-lived queens. The results propose that even within the reproductive caste, a differential allocation of resources allows stable fecundity over the long life of the individual while the somatic tissue, the brain ages.

In the following sections I summarise and compare the results of all the chapters addressing specific aspects of this work's objectives 1) fecundity and longevity relationship in the reproductive caste and in the non-reproductive caste. 2) comparing the different studies qualitatively to discuss how specific candidates and pathways implication in lifespan differ in the transcriptomic expression.

Reproductives: fecundity no significant changes over time

Ovariole lengths

Ovarian activation is one of the key differences between the longer-lived reproductive and shorter-lived non-reproductive castes. Even in species where workers can activate their ovaries, they are still not as productive as those of the reproductive castes. Studies use number and rate of eggs laid, size of oocytes in the ovaries, number and length of ovarioles as cues for fecundity (Cardoen et al., 2012; Hoover et al., 2003; Khila & Abouheif, 2010; Lu et al., 2009). I measured mean ovariole lengths as indicators of queen-fecundity in this thesis. I tested whether they changed significantly with age in queens of *Lasius neglectus* (Formicine, polygynous; **Chapter 2 Supplementary Fig 1**) and *Acromyrmex echinatior* (Myrmicine, monogynous in our set-up; **Chapter 5-Fig 1.1 a).** In both the above species despite their difference, I did not observe a significant change in mean ovariole length with age. I used ovarian development to assign social type in the clonal Ponerine ant, *Platythyrea punctata* (**Chapter 3**) as dominant (reproductive caste) and subordinate (non-reproductive caste) later confirmed using their mean ovariole lengths which differed significantly as predicted. Using the reproductives, I did not see a significant effect of age on the three 3-months-old individuals and six 7-months old individuals (**Chapter 3 Supplementary Fig 1 c)**. Typical ovary development of the aforementioned reproductives is summarized in Fig 1. Reproductives of all three species investigated show no significant effect of age on fecundity when using mean ovariole length as a cue for their fecundity.

Vitellogenin expression

Caste determination in the social insects involves differences in IIS (Insulin/insulin-like growth factor) and JH (Juvenile hormone) signalling which in turn affects Vitellogenin pathways (Corona et al., 2007a; Libbrecht et al., 2013b). The role of *Vitellogenin (Vg),* a gene coding for a yolk-precursor protein has been known for a long time and traditionally used as a parameter for evaluating female insect fertility(Dallacqua et al., 2007), a sub-functionalisation of the gene has occurred in some social insect lineages (Amdam et al., 2012; Corona et al., 2013; Morandin et al., 2014b; M. H. Richards, 2019). The traditional role of *Vg* in fertility is attributed to a *Vg* classified as '*conventional-Vg' (C-Vg)* by (Kohlmeier et al., 2018) and it does have a higher expression in the reproductive castes which are long-lived (Liebig & Poethke, 2004; Miyazaki et al., 2021; K. Tsuji et al., 1996). The expression of *C-Vg* was not significantly explained by age in fat body tissue of *A. echinatior* queens. *C-Vg* was marginally overexpressed in younger queens with respect to older queens but its expression was not significantly explained by queen-age (*Lneg_ g11491 DESeq2*padjust = 0.105) nor was it significantly changing with age in the ovarian tissue of *A. echinatior* queens (**Chapter 5 Fig 4.2**- comparison amongst tissues). Dominant (reproductive) *P. punctata* also did not have *C-Vg* expression that varied significantly with age in the fat body although its expression was higher than dominants than in the subordinates (**Chapter 3 Fig 3a**). The expression of *C-Vg* in *A. echinatior* brains was higher when compared to the other two tissues. Though it was not significantly explained by age, *C-vg* expression in the *A. echinatior* brains had reduced expression with increasing age (*LOC105150510*; padjust =0.102) while expression of *Vg-Receptor* was increased (**Chapter 5 Fig 4.2**). While the exact function of these two genes is unknown in the brain tissue, an overexpression of *Vitellogenin* (not classified) and *Vg-Receptor (VgR),* genes in the heads of both male and female subsocial burying beetle individuals was associated with social-parenting behaviour (Roy-Zokan et al., 2015). If *VgR* is sensitive to and binds to *C-Vg* for its transport, increased *VgR* may be a response to reduced *C-Vg* though whether or not the latter is directly linked to regulation of fecundity or has functions should be investigated.

Though the reproductive individuals used in this thesis are not close to death, I have tried to capture different windows of their lives to understand changes occurring with age without confounds of colony-founding or reproductive senescence. *P. punctata* reproductives used were around ¼ the age of their known average lifespan of 800 days (Hartmann & Heinze, 2003). Although the maximum lifespan of *L. neglectus* queens is unknown; the oldest queens used in the study had been alive in the laboratory for 2.5 years before sacrifice. Consequently, they were at least 2.5 years old or older. While strictly polygynous species with dependent colony founding have maximum lifespans of a few months to less than a year, *Solenopsis invicta* which has both monogynous and polygynous forms and dependent as well as independent colony founding was observed to have a longer mean lifespan (5-6 years) (Keller, 1998b). Perhaps the average lifespans of *L. neglectus* queens also similar to *S. invicta* though this needs to be formally tested. In queens of the leaf-cutting ant *A. echinatior* , maximum lifespans are also unknown but coud be around 20 years based on the maximum lifespan of other Attines (Keller, 1998b). The oldest *A. echinatior* queens in **Chapter 5** were over 15 years old while the youngest of our queens, collected as foundresses were less than a year old and had not produced any sexuals yet though they had fungus-gardens and enough workers to be considered established-queens.

L. neglectus queens live in polygynous colonies (Espadaler et al., 2004). *P. punctata* individuals are all clones of each other and social dominance and egg-laying can be manipulated in adulthood because caste-fate is not strictly fixed (Bernadou, Schrader, et al., 2018; Schilder et al., 1999). *A. echinatior* queens used in this thesis were from monogynous colonies. This thesis considers the two lines of evidence used to assess changes in fecundity over time in the reproductive caste: mean ovariole length and transcriptomic signals or reproductively active tissues. While there is no evidence reduction in fecundity by looking at ovariole length, fat body gene-expression of *C-Vg in L. neglectus* queens may indicate a reduction in their fecundity. Considering the marginal deterioration in fecundity despite the use of the three different species capturing different time-periods in their respective lives implies the crucial role of reproduction and high investment across species to ensure it is sustained at high levels. This specialised caste is considered the germline of the colony (Boomsma & Gawne, 2018).

Additional ways to assess of the lack of the fecundity-longevity tradeoff would be to investigate a) if the reproductive output is actually sustained in terms of quantity as well as quality of sexuals produced (fitness) b) whether the cost of maintaining the queen's high reproductive performance might also be borne by the workers and c) if the high fecundity of queens comes at the cost of lowered maintenance of some other functions which can affect queen's pathology and longevity. While a) is beyond the scope of this thesis, such fitness effects using transgenerational studies have been conducting on other organisms though a consistent pattern has not been observed across taxa (Maklakov & Chapman, 2019).

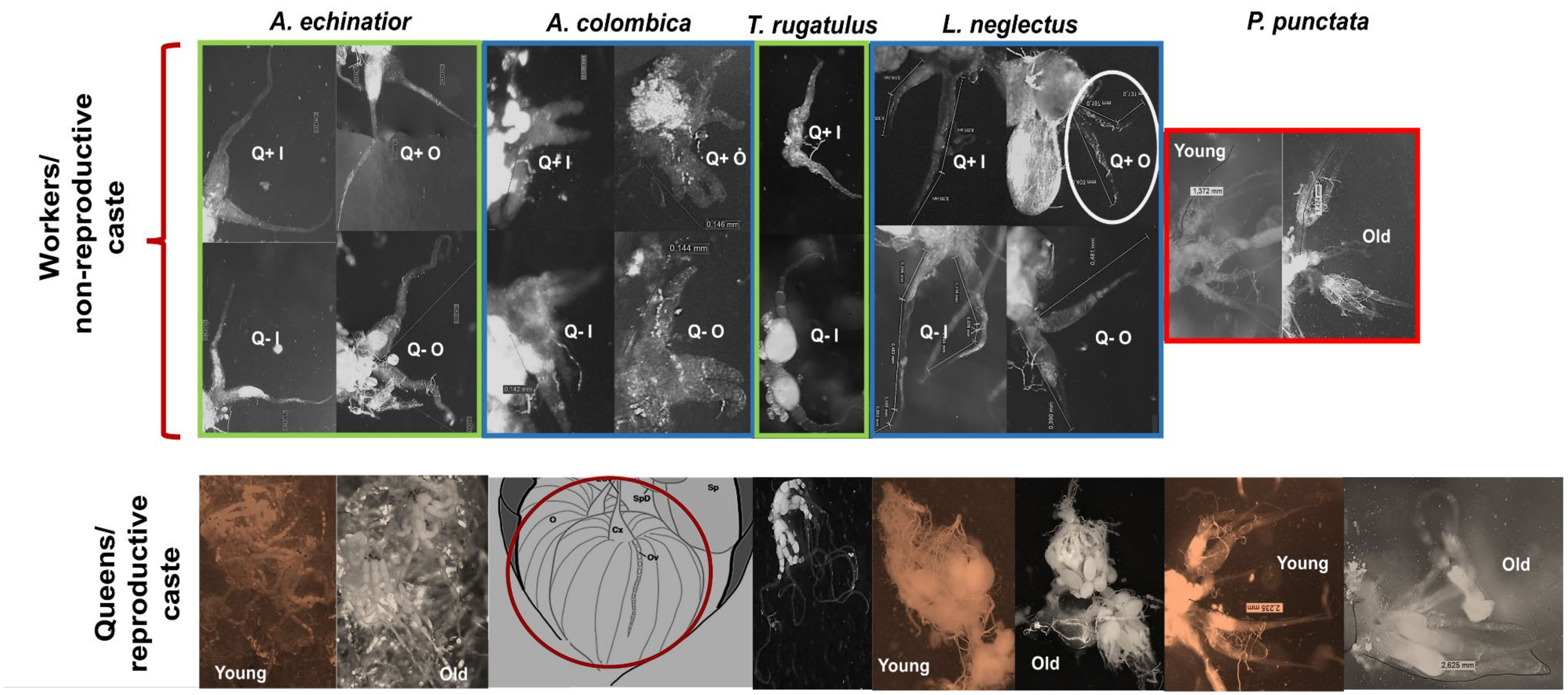


Figure 1: A brief overview of all species used in this study their ovary-morphology. The species names are presented on the top of each column. For the workers, in all species, except *T. rugatulus,* I dissected the ovaries of queen-right (Q+) and queenless (Q-) workers collected from inside (I) and outside (O) the nest. *A. echinatior* and *T. rugatulus* workers, covered by the bright green border are capable of producing haplodiploid male-destined eggs. *A. colombica* and *L. neglectus* can probably lay trophic eggs but the ovaries regress soon after eclosion as can be observed above in the thin wispy ovarioles of *L. neglectus* or short ovarioles of *A. colombica.* I used age-controlled manipulated colonies of *P. punctata* such that young 4-months-old colonies and older 7-months-old colonies both had one to afew dominants and subordinates. The subordinates are akin to the non-reproductive caste but totipotent and clonal (covered by red border) and less developed and shorter compared to their dominant clonemates. The other species, where I formally investigated the effect of age on mean ovariole length (as a cue for fecundity) included *A. echinatior* and *L. neglectus*. In neither of those species was mean ovariole length significantly explained by age. [Credits: *L. neglectus* worker images- Julius Rombach, Anna Waffender, *T. rugatulus* worker and queen ovaries images- Marina Choppin, schematic of ovaries in *A. colombica* – from (den Boer et al., 2013)]

Non-reproductives: Queen presence/absence based on mode of reproduction and age/task affect longevity

In social insect societies with obligate division of reproductive labour being a prerequisite to this major evolutionary transition, workers are typically functionally sterile in the presence of a queen. Instead, workers gain indirect fitness benefits from raising the offspring of the queens. However, in many social hymenopteran species, workers still retain their ovaries, though they have lost their ability to mate and store sperm. The ovaries can be used to produced trophic, non-viable eggs to feed the queen and/or brood and may switch to laying reproductive eggs (Crespi, 1992). Queens produce pheromones which act as an honest signal of their fertility and worker-reproduction is minimal in the presence of a queen although this is conditional, based on the queen's fertility (Monnin, 2006). In recent years, a growing number of studies have demonstrated queen loss leading to longer lifespans in workers with higher reproductive potentials (dos Santos Conceição Lopes et al., 2020; Kohlmeier et al., 2017b; Kuszewska et al., 2017). There are two factors contributing to the workers' reproductive potential in a colony addressed in this thesis.

A) Mode of reproduction

Upon the queen's declining fertility, untimely death or removal, whether or not a worker can and will reproduce also depends on their mode of reproduction. I used 'regular' workers from two Myrmicine species- *Acromyrmex echinatior* and *Temnothorax rugatulus*. Both of these species have been investigated to understand their egg-laying behaviour. They have been observed to lay viable eggs; the same size as queen-laid eggs, which hatch as males (*A. echinatior:* Dijkstra et al., 2005, 2010; Dijkstra & Boomsma, 2007; *T. rugatulus:* Choppin, 2022; Choppin et al., 2021; Negroni et al., 2021).

One way to prolong lifespan is investment into body maintenance. Oxidative stress is often a result of the production of Reactive Oxygen Species (ROS) in the cell. In large unmanaged quantities however, ROS induced oxidative stress can damage cells over time and is implicated in ageing (Finkel & Holbrook, 2000; López-Otín et al., 2013; Ray et al., 2012; Schieber & Chandel, 2014). I created queenless and queenright *A. echinatior* and *T. rugatulus* sub colonies in **Chapter 1** and the workers were subjected to paraquat induced oxidative stress. Queenless workers of *A. echinatior* survived oxidative stress significantly better than queenright workers (**Chapter 1 Fig 2b)** as predicted. Meanwhile such was not the case in the 'regular' workers of the other Myrmrcine *T. rugatulus* (**Chapter 1 Fig 2b)** despite the aforementioned studies confirming their ovary activation, general survival and upregulation of lifespan and immunity extending genes. Pertaining to our study, it is possible that in this species general survival as in the aforementioned study would vary significantly if continued longer. But perhaps oxidative stress along with the social stress of the set up was extreme and hid any nuanced differences in oxidative stress survival between queenright and queenless workers.

This thesis contains my investigation of two species of 'sterile' workers *A. colombica* (**Chapter 1**) and *L. neglectus* (**Chapter 2**). 'Sterile' workers ovaries are rudimentary, producing non-viable trophic eggs if they are present . Unexpectedly, queenless *A.*

colombica workers also performed better when subjected to paraquat-induced oxidative stress. *A. colombica workers* have been known to produce male offspring rarely though those males are smaller and less likely to gain mating success (Dijkstra & Boomsma, 2006). Thus, workers still have very low incentives or chance to gain direct fitness by reproduction. However, the pathways related to fecundity may still retain some of its lifespan extending properties though without actually increasing fertility of the worker. Such an effect has been observed in honeybees that where *C-Vg* in workers can protect them from oxidative stress which can be lethal (Corona et al., 2013; S. C. Seehuus et al., 2006a). In the obligately sterile *L. neglectus* egg-laying has never been observed (Espadaler & Rey, 2001). In this unicolonial species, workers never have the opportunity or the need to become reproductively active and in this extreme case of superorganismality, the workers are indifferent to queen signal. And this is not just in the case of survival under oxidative stress. There were also no transcriptional changes in worker fat body which could be attributed to queen presence/absence in **Chapter 2**. Future studies can test this hypothesis by observing worker and queen behaviour to queen-presence and queen-signals.

The above results help appreciate the different responses in workers based on their reproductive potential. While one 'regular' and one 'sterile' species of workers showed higher investment into their longevity under queenless conditions, two other species with 'regular' workers and 'sterile' workers did differ in their investment into longevity based on queen-presence. Bernadou et al., 2021 pointed out that 'superorganismality' is a trait of those eusocial insects where workers are exclusively and completely sterile and their fitness is realized exclusively via the germline-analogous reproductive caste. This seems to be the case especially in *L. neglectus* ('sterile' workers) and not *T. rugatulus* ('regular' workers). In the former, there were no survival benefits rendered by queen-lessness or queen-presence. This was also confirmed by the gene expression data which resulted in zero genes in the fat body tissue whose expression varied based on queen presence/absence (**Chapter 2).** While *T. rugatulus* in this study showed no differences in survival to paraquat-induced oxidative stress, gene expression data from other studies confirms the positive correlation between queenlessness-induced fertility and longer lifespan. Newly fertile individuals also live longer and have been observed to invest in lifespan-extending mechanisms as well as in fecundity related genes compared to individuals that were from queenright sub colonies. This effect was also sustained after workers were reintroduced to queenright colonies (Negroni et al., 2021).

B) Age and task

Worker physiology, including quantity and composition of fats and lipids, as well as metabolism can influence the susceptibility of workers to stress which can impact their lifespan (Bordier et al., 2017). It also predicts task-distribution in the colony wherein workers with highest lipid content engage in intranidal/ nursing tasks and workers with lower lipid content engage in foraging tasks (Bernadou et al., 2020; Blanchard et al., 2000). A reversion of tasks such that foragers engage in nursing tasks have been observed to reverse cellular immunosenescence in honeybees (Amdam et al., 2005), indicating the effect of task on longevity. Workers may display age-polyethism in task-performance, carrying out intranidal tasks when they emerge as adults and progress to more extranidal tasks as they near the end of their lives. However, workers are flexible in terms of task-performance. Foragers are known to revert to nursing successfully and intranidal workers can go foraging when needed (Camargo et al., 2007; Shimoji et al., 2020; Tofilski, 2002). Additionally, some species, including the leaf-cutter ant species *A. echinatior* and *A. colombica* have distinct physical worker morphs that differ in size and/or body morph. Such polyphenism comes with its own task-specialization which may or may not vary with worker-age (Okrutniak et al., 2020; Valadares et al., 2022; Westling et al., 2014). Age-polyethism was also observed in workers of *A. colombica*. In the same study, task division was also affected by body-size (Murakami et al., 2000). Norman et al., 2014 attempted to disentangle the effects of age, task and body-size in workers of *A. echnatior* workers by testing their threat-responses using mandible-opening-response. Interestingly, workers collected from the foraging arena performed significantly better at responding to threats than nurses collected at the fungus-gardens. Similarly, using cuticle colouration as a cue for age, three age classes were created and the oldest workers did significantly better at responding to threats. Body-size of age-controlled workers had no effect on threat response. To avoid the potential confounds of worker-morph and associated task-specialization, only medium-sized workers were used in the leaf-cutting ants in **Chapter 1***.* These are produced in large numbers and were observed inside the fungus garden as well as outside. Some workers in *A. echinatior* were marked and were observed to graduate from intranidal to extranidal tasks over their lifetime, accompanied by changes in cuticle colouring, behavioural responses and fatty acid composition (pers. comm., Volker Nehring). Additionally, workers present on or near the waste-disposal sites were avoided since they are a specialised and distinct behavioural-caste (Waddington & Hughes, 2010).

In both **Chapter 1** and **Chapter 2**, workers collected inside the nest (*A. echinatior*, *A. colombica*) and workers collected near brood inside the nest (*L. neglectus, T. rugatulus)* had a higher tolerance to oxidative stress. While studies with age-controlled worker cohorts can help disentangle the effect of age from task and the differences in reproductive potential, it is much harder to execute especially in species with large numbers of workers in the colony. Two genes overexpressed in the outside workers of *L. neglectus* (**Chapter** 3) found close matches to *Nlaz* (*Neural Lazarillo*, a homolog of the lipocalin *Apolipoprotein- D* in humans*).* The expression of this gene was also upregulated with increasing age in the brains of *A. echinatior* queens (**Chapter 5, Fig 3**). This lipocalin protein can play various roles due to its ligand-binding properties but has been implicated in stress-signaling via insulin as well as maintaining metabolic homeostasis. It shows an increased expression with age in humans, mice and *D. melanogaster* and diseases, including Alzheimers in humans (Dassati et al., 2014; Hull-Thompson et al., 2009; Pasco & Léopold, 2012; Ruiz et al., 2014).

Admittedly, results of survival and gene-expression between inside workers and outside workers cannot be attributed solely to the effect of worker-age without caution since their exact ages were unknown. When I collected leaf-cutting ants, they also differed in cuticle colouration such that workers collected from the fungus-garden were lighter relative to workers collected outside. This melanisation of the cuticle happens over time and is used as an indicator of age (C. Hartmann et al., 2019; Norman et al., 2014; Simon et al., n.d.; Waddington & Hughes, 2010). In **Chapter 3** *P. punctata* age was controlled and known. This revealed more genes changing with age regardless of social type (dominant vs subordinate) than with social type regardless of age. In the subordinate individuals which are non-reproductive and analogous to worker castes in the other species, more genes changed with age over three months than they did in the reproductives who were in the same nests as the subordinates. In *L. neglectus* workers, more than 2000 genes differed between the two groups. Some of the genes in the outside workers contributed to biological process such as 'proteolysis involved in cellular protein catabolic processes' and 'cholesterol metabolic process' which were similarly affected in old queens (**Chapter 2, Fig 3; Supplementary table 4**).

It was hard to confirm whether those workers surviving oxidative stress for longer were more fecund than those individuals that died earlier because ovaries are soft tissue and decay very quickly. The same problem persists with observing *C-Vg* in a survival study. However, independent of survival, *Vg* expression in fat body was noted in the workers/ non-reproductives of *L. neglectus* (**Chapter 2**) and *P. punctata* (**Chapter 3**) to understand the effect of these yolk-proteins in workers.

Candidates and hallmarks of ageing- a comparative exploration

While accumulation of damage by ROS and free radicals has been shown to cause ageing, the effect of oxidative stress on longevity and the expression of various proteins that may protect against oxidative stress do not show a consistent pattern; differing based on the species and caste (de Verges & Nehring, 2016; Kramer et al., 2021; Schneider et al., 2011). In this thesis too, candidate genes implicated in oxidative stress resistance in *D. melanogaster* compiled by (Kramer et al., 2021) were used to seek out any genes changing with age in queens and age/task in workers but there were no specific patterns in the kind of genes that were found. What is notable however is that no oxidative stress genes changed expression with age in the reproductive caste of *L. neglectus* (fat body), *A. colombica* (brain) or *A. echinatior* (brain, fat body, ovaries). However, *C-Vg* which was tended towards a low expression with age in long-lived *A. echinatior* (*LOC105150510*; padjust=0.102) may have anti-oxidant function in the brain as it does in functionally sterile soma/ workers of honeybee *Apis melifera* (S. C. Seehuus et al., 2006a).

In the aforementioned study, *C-Vg* acted as an antioxidant against paraquat-induced oxidative stress in functionally sterile workers and is evidence of the positive effects of fecundity-related genes on longevity. However, the sub-functionalisation of *Vg* in social insects means that the ancestral *Vg* has multiple copies which have different functions that are yet not ascertained via extensive experimental research (Corona et al., 2013; Dallacqua et al., 2007; Morandin et al., 2014b; Morandin, Hietala, et al., 2019; M. H. Richards, 2019). In **Chapters 1, 2,3 &5** all vitellogenins in the proteome were identified and classified based on the classification used by (Kohlmeier et al., 2018); for the first time in two of the species studied (*L. neglectus* and *P. punctata*). What has been observed in social insects is the preservation of the role of *C-Vg* directly in fecundity and egg-laying. It usually shows higher gene expression in reproductives (Nunes et al., 2013; S. C.

Seehuus et al., 2006a) Amongst all Vgs found in each species' proteome (summarized in Table 1), the only significant pattern was observed in the formicine ant *L. neglectus* wherein inside and outside workers showed an overexpression of *Vg-like A* and *Vg-like C* respectively as in another formicine ant *Formica fusca* (Morandin, Hietala, et al., 2019) along with other ant species (Kohlmeier et al., 2018; Miyazaki et al., 2021; Penick et al., 2011).

Vitellogenin does not act independently. In insects, much like in every other organism, molecular mechanism and pathways are linked and intricately so such that life-history aspects of sociality, fecundity and longevity are interwoven. I too conducted formal tests to find such genes in different castes and within the worker caste. The regulation of vitellogenin and its effect on fecundity is mediated by three main signaling pathways: The Insulin/Insulin-like growth factor 1(IIS), the Target Of Rapamycin (TOR) and the Juvenile Hormone (JH) signaling pathways (Abrisqueta et al., 2014; Chandra et al., 2018; Corona et al., 2007a; Flatt & Kawecki, 2007a; Gems & Partridge, 2013; Libbrecht et al., 2018). In recent years, studies investigating the biology of ageing have tried to test the connections between these pathways that were established based on studies in *D. melanogaster* and it's possible rewiring that explained the proximate mechanism for the reversal of the fecundity-longevity tradeoff in the social insects (Korb, 2016; Korb et al., 2021; S. Lin et al., 2021; Rodrigues & Flatt, 2016) [Fig 2]. A list of 123 candidate genes-dubbed the 'TIJ-LiFe' from the TOR, IIS, JH pathways related to lifespan and fecundity in social insects were tested in **Chapter 2, 3 and 5.**. *LIRP (Locusta Insulin-related Peptide)* in *A. echinatior* is orthologous to the *Drosophila* ilp1 gene and to *Ooceraea biroi* ilp2 and sits at the beginning of the IIS pathway, thus involved in nutrient-sensing, cell-proliferation, growth and lifespan (Broughton & Partridge, 2009; Partridge et al., 2011). *LIRP* expression was significantly downregulated with increasing age in the brains of *A. echinatior* (**Chapter 5 Fig 3**) indicating a possible dysregulation of the IIS-pathway which is strongly linked to lifespan. No such ill-effects of this deregulation could be detected in either the fat body or the ovaries of the same queens. The results indicate a tissue-specific ageing which has been observed in other taxa including in human tissue (Ezra et al., 2023) . The brain, a non-reproductive tissue was the one to display signs of senescence in >15 year old queens while the reproductive tissues (ovaries and their regulators-fat body) didn't. This implies a life- history strategy whereby the queen-reproduction, especially in monogynous colonies is stable and optimized in order to maximise colony-fitness.

Table 1: A summary of all the Vitellogenins found and classified for the species presented in this thesis. The classification of Vgs for *A. echinatior*, *A. colombica* and *T. rugatulus* were presented in (Kohlmeier et al., 2018). I used the same 33 species' protein sequence database to conduct reciprocal BLASTp and assign Vg based on their best match. I used gene expression data from both castes in *L. neglectus* and both reproductive (dominant) and non-reproductive (subordinate) in *P. punctata* while only gene-expression data of *A. echinatior* queen caste is presented in this thesis.

Species	Vgs found	Vgs classified	Vg-type	Tested gene expression	Sig. expression
A. echinatior typically monogynous "'regular" workers	5	Kohlmeier et al, 2018	*Conventional-Vg*	Yes (Queens)	Trend-Brain reduced with age
			Myrmicine Vg	Yes (Queens)	-
			Vg-like A	Yes (Queens)	-
			Vg-like B	Yes (Queens)	-
			Vg-like C	Yes (Queens)	-
A. colombica monogynous "sterile" workers	5	Kohlmeier et al, 2018	*Conventional-Vg*	No	NA
			Myrmicine Vg	No	NA
			Vg-like A	No	NA
			Vg-like B	No	NA
			Vg-like C	No	NA
T. rugatulus facultatively polygynous "regular" workers	4	Kohlmeier et al, 2018	*Conventional-Vg*	No	NA
			Vg-like A	No	NA
			Vg-like B	No	NA
			Vg-like C	No	NA
L. neglectus polygynous, unicolonial "sterile" workers	4	In this thesis	*Conventional-Vg*	Yes	Trend-Young Queens
			Vg-like A	Yes	Inside workers
			Vg-like A	Yes	Inside workers
			Vg-like C	Yes	Inside workers
P. punctata one or a few dominants "totipotent" workers	3	In this thesis	*Conventional-Vg*	Yes	-
			Vg-like A	Yes	-
			Vg-like B	Yes	-

No other previously studied regulators in the TIJ-LiFe network such as *Foxo* (Dong et al., 2021; Morris et al., 2015)*, tor* (E. J. Chen & Kaiser, 2003; Hands et al., 2009)*, Akt* (Haar et al., 2007; Wiza et al., 2012) or *Krueppel-homolog* (Hsieh et al., 2017; Ojani et al., 2018; Shpigler et al., 2010) were significantly affected in either caste. Neither were any of the three pathways significantly enriched in any caste over time. However, a few other candidate genes are noteworthy, especially with regards to their known effect on the IIS pathway and the aforementioned genes. Outside workers of *L. neglectus,* presumably older than inside workers, had a higher expression of a gene homologous to *ImpL2.* A higher expression of this gene was found in gamergate (pseudoqueens-reproductives) ovaries (H. Yan et al., 2022). The authors note that *ImpL2,* an antagonist of the IIS pathway, can regulate *Akt* and *FOXO* whose lowered expression is implicated in longer lifespans. And since gamergates live longer than their non-reproductives counterparts, maybe the proximate mechanism by which reproductive individuals live longer is mediated by *ImpL2*. In the sterile *L. neglectus* workers' fat body however, *ImpL2* overexpression may serve a different role albeit still as an antagonist of the IIS pathway. In *D. melanogaster*, an increase in the expression of *ImpL2* reduced Insulin/IGF1 signaling and caused tissue-wasting, an effect commonly observed in cases of starvation but also in cancer and other diseases. ImpL2 also plays a direct role against immune challenges (Gabriela et al., 2020; Kwon et al., 2015). Another gene, *Nlaz* a homolog of the human Apolipoprotein D was over expressed in outside *L. neglectus* workers as well as in the brains of *A. echinatior* (**Chapter 5, Fig 3**) and has been known to increase with age in humans and *D. melanogaster* (Dassati et al., 2014; Pasco & Léopold, 2012)*.* It has also recently also been implicated in caste differentiation of the termite *Zootermopsis nevadensis* with the sterile soldiers overexpressing *Zn-Nlaz* (Yaguchi et al., 2018)*.*

While many 'TIJ' genes were not significantly enriched, hallmarks of ageing were still detected. Biological processes enriched in older *L. neglectus* queens and outside workers indicated a change in protein metabolism; a higher expression of proteosomal sub-units similar to what has also been observed in *C. elegans* (Hipp et al., 2019) (**Chapter 2 Fig 3, supplementary table 3).** The deterioration of proteostasis leading to deregulated nutrient sensing is in fact of one the hallmarks of ageing observed in mammals (López-Otín et al., 2013) and also most negatively affected by age in the termite *Cryptotermes secundus* (Monroy Kuhn et al., 2019b) and *Cardiocondyla obscurior* ant queens that are close to death (Jaimes-Nino et al., 2022). It is noteworthy that one of the very few genes significantly affected by age in the brain of newly established *A. colombica* queens is a homolog of a serine-protease, which is known to regulate proteostasis in *D. melanogaster* (**Chapter 4,** Rai et al., 2022).

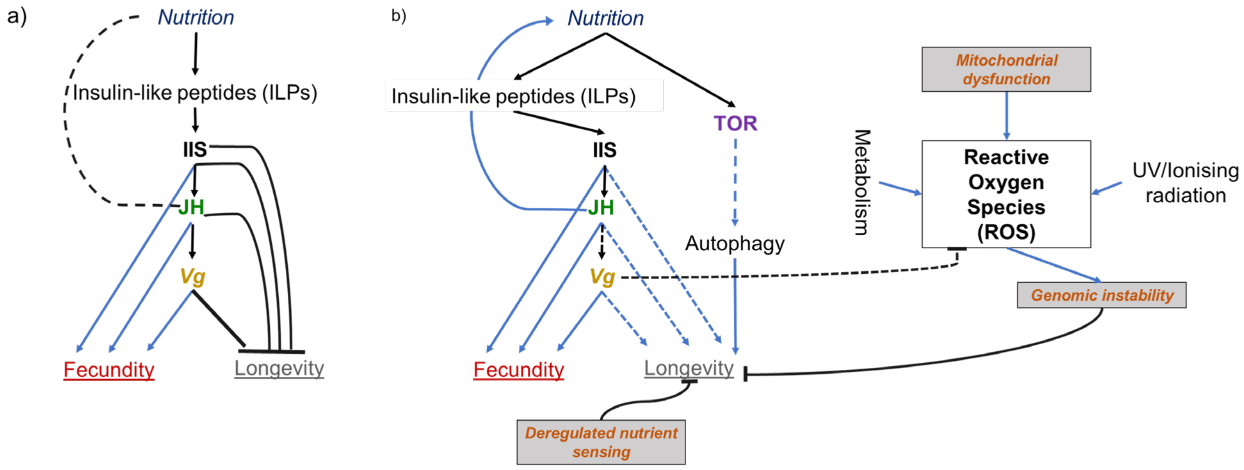


Figure 2: a) The three signalling pathways- **IIS, TOR and JH** and their connection to ***Vg*** is implicated in ageing in *D. melanogaster* b) A rewiring of the same pathways and their connections may enable highly reproductive individuals to also be long-lived (based on Rodrigues & Flatt, 2016, Lin et al., 2021; López-Otín et al., 2013)). Meanwhile a deregulation especially in the IIS and TOR pathways are a hallmark of ageing (‘deregulated nutrient sensing’). Other hallmarks of ageing found in this thesis which also negatively impacts longevity are ‘genomic instability’ and ‘mitochondrial dysfunction’, both a result of oxidative stress from excess ROS. Whether there is a direct amelioration of adverse effects of ROS due to increased fecundity is unknown although *Conventional-vitellogenin* (***C-Vg)*** has anti-oxidant properties in the face of paraquat-induced oxidative stress in honeybee workers (S. C. Seehuus et al., 2006a). Dotted lines indicate that the relationship is not fully supported across taxa or untested. The signalling pathways are connected with black arrows and their relationship to fecundity and longevity with blue arrows or black lines.

Future investigations

In this thesis, I investigated phenotypic traits and genes that are linked with longevity and fecundity in a range of ant species with varying degree of sociality/social structures. When qualitatively comparing the different studies, patterns that emerge do confirm the higher investment of reproductive castes into their fecundity as I detected very little change in this aspect of their life-history regardless of the species or their actual age. Meanwhile, age/task of the worker has a high impact on their susceptibility to oxidative stress and it explains more variation in gene expression compared to the reproductive caste's age. This work also identified candidates such as *LIRP, ImpL2, Nlaz* etc implicated in ageing in model organisms such as humans, *D. melanogaster, C. elegans* and mice.

While conducting manipulations and experimental knockdowns of these genes was well beyond the scope of this thesis, the genes of interest identified could be used as guides to explore proximate mechanisms that allow more fecund individuals to live longer. Indeed, genes identified in this thesis could be tested via RNA-i knockdown and their expression observed in other species at different time points of their lives to ascertain their role as potential markers of ageing in ants. Recent studies have also been able to compile age-related sex and tissue-specific changes in humans based on histology data (Ezra et al., 2023). Armed with the knowledge or a hypothesis with regards to the different candidate genes and their regulators and downstream effectors especially in other tissues will help ascertain the differential allocation of resources and whether they change over time. *Vg* and JH-signalling associated genes prove interesting candidates but the different chemical properties and range of functions of the *Vg* copies need to be ascertained via knockdowns and behavioural assays.

While previous work in social insects provided organ-level resolution in the functions of specific parts (Bressan et al., 2015; Chan et al., 2013), recent advances in single-cell transcriptomics have allowed for cell-type clustering and cell-specific expression including in ants (Q. Li et al., 2022; Sheng et al., 2020). Conducting more longitudinal studies using single-cell transcriptomics can help track cell-types from different tissues as workers and queens progress through time. Such 'genetic-lifespan-calendars', albeit with gene expression or histology data are present in mice (Skene et al., 2017) and can also help identify the trajectories occurring in the two castes.

We have tried to employ similar set-ups and analyses across multiple species which warrants in-depth meta-analyses with more species added to this foundation. This can involve various tissues, species and social dynamics. Results from such a comparison should shed a brighter light on commonalities and differences so that we can obtain a better understanding of the life-history tradeoff in non-social organisms too.

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Curriculum Vitae

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