Defying Senescence

The causes of death and the costs of living of ant queens

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES DER
NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR
BIOLOGIE UND VORKLINISCHE MEDIZIN DER
UNIVERSITÄT REGENSBURG

vorgelegt von
Luisa María Jaimes Niño

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Bogotá, Kolumbien

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Cardiocondyla obscurior queen, by LMJN.

Abstract

Understanding how superorganisms achieve a highly productive and long life compared to solitary species adds a new facet to gerontology. But it is not only the pace of aging, the absolute time of an organism's lifespan, but also the shape of aging that is interesting in social insects. The shape of aging, i.e., life mortality and fertility trajectories, is determined by how much and how often an organism invests in reproduction. In iteroparous organisms, maturity characterizes the peak of fitness, after which selection against senescence becomes weaker, marking the onset of senescence. In contrast, the strength of selection is maintained in semelparous organisms until the only reproduction bout occurs.

In Chapter 3, I show that queens increase the production of sexuals in late life regardless of their absolute lifespan or worker investment. This means that selection strength against senescence needs to be maintained after the peak of sexual production. Young and middle-aged queens, before experiencing their maximum investment into sexuals, experience no classic signs of senescence (Chapter 2). However, old queens that pass the fitness peak exhibited a dramatic breakdown of their entire physiology, even though queens were still fertile. We propose that the evolution of superorganismality is accompanied by "continuusparity", a life history strategy that is distinct from other iteroparous and semelparous strategies across the tree of life. It combines continuous reproduction and maintenance of selection strength, a fitness peak late in life, and reproductive death.

Quite unexpectedly, I found that workers exhibit a shape and pace of aging that mirrors that of the queens (Chapter 4) while completely lacking reproductive potential. This indicates that programmed aging occurs similarly in both castes. Lastly, how ant queens show high variability in life-history traits is still unclear. Therefore, I investigated the genetic contribution of fertility and longevity traits in the offspring's quality and could not find evidence of trait heritability (Chapter 5). Taken together, this thesis provides a framework for the study of aging in social insects.

Work arising from this thesis

This thesis is composed of the following four manuscripts, two of them are published, one of them is submitted to a repository and is currently under consideration, and one is soon to be submitted.

A. Harrison, MC, **Jaimes Nino, LM**, Rodrigues, MA, Flatt, T, Oettler, J and Bornberg-Bauer, E (2021) Gene co-expression network reveals highly conserved, well-regulated anti-ageing mechanisms in old ant queens, Genome Biology and Evolution, DOI: https://doi.org/10.1093/gbe/evab093 (Chapter 2)

B. **Jaimes-Nino, LM**, Heinze, J, Oettler, J (2022) Late-life fitness gains and reproductive death in *Cardiocondyla obscurior* ants, eLife 11:e74695 DOI: https://doi.org/10.7554/eLife.74695 (Chapter 3)

C. **Jaimes-Nino, LM**, Oettler, J (Manuscript) Fitness variation in queens of the ant *Cardiocondyla obscurior* (Chapter 4)

D. **Jaimes-Nino, LM**, Süß, A, Heinze, J, Schultner, E, Oettler, J (preprint) The indispensable soma of *Cardiocondyla obscurior* ants, bioRxiv DOI: https://doi.org/10.1101/2022.10.02.510526 (Chapter 5)

During this thesis, I also contributed to manuscripts and projects (most of them from my Master's period) which are not part of this work:

E. Brehm, G, Niermann, J, **Jaimes Nino, LM**, Enseling, D, Juestel, T, Axmacher, JC, Fiedler, K (2021) Moths are strongly attracted to ultraviolet and blue radiation, Insect Conservation and Diversity, DOI: http://dx.doi.org/10.1111/icad.12476

F. Backhaus, L, Albert, G, Cuchietti, A, **Jaimes Nino, LM**, et al. (2021) Shift from trait convergence to divergence along old field succession, Journal of Vegetation Science, DOI: https://doi.org/10.1111/jvs.12986

G. **Jaimes Nino, LM**, Moertter, R, Brehm, G (2019) Diversity and trait patterns of moths at the edge of an Amazonian rainforest, Journal of Insect Conservation 23: 751 – 763 DOI: 10.1007/s10841-019-00168-4

Author contributions

Manuscript A

M.C.H., E.B.B. conceived and initiated the project. M.C.H., E.B.B., and J.O. designed the study. M.C.H. wrote the manuscript and carried out most analyses. J.R. assisted with dN/dS analyses. M.C.H. and J.O. interpreted ant data, all authors interpreted comparative data. **L.M.J.N.** assisted in the interpretation of GO term enrichment analyses. T.F. and M.A.R. generated fly data and helped analyze them. M.C.H. wrote the manuscript which was revised and approved by all the authors.

Manuscript B

L.M.J.N., J.H., and J.O. conceptualized the project, J.H. and J.O acquired the funding, managed and supervised the project, **L.M.J.N**. collected, curated, analyzed, and visualized the data, J.O. and **L.M.J.N.** wrote the original manuscript which was revised and approved by all the authors.

Manuscript C

L.M.J.N., and J.O. conceptualized the project, **L.M.J.N.** analyzed and visualized the data, J.O. and **L.M.J.N.** wrote the original manuscript which was revised and approved by all the authors.

Manuscript D

J.H., E.S., J.O. conceptualized the project, **L.M.J.N.,** A.S., E.S., J.O. collected the data, **L.M.J.N**. curated, analyzed, and visualized the data, **L.M.J.N.**, and J.O. wrote the manuscript which was revised and approved by all the authors.

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„Ein Zaun (könig) währt 3 Jahr, ein Hund 3 Zaunalter, ein Ross 3 Hundsalter, ein Mann 3 Rossalter, macht 81 Jahre. Der Esel erreicht 3 Menschenalter, die Schneegans 3 Eselalter, die Krähe 3 Gänsealter, der Hirsch 3 Krähenalter, die Eiche 3 Hirschesalter“

- Mittelhochdeutscher Spruch

Chapter 1

1 Introduction

Social insects are extraordinary in terms of aging (Keller and Genoud, 1997). Reproductives of social insects seem to defy senescence by exhibiting extremely long lifespans compared to other insects. Known it is the case of a *Lasius niger* queen living in the laboratory for ~30 years and laying eggs until her last year of life (Kutter, 1969). Social insects not only have extreme longevity but are also very fertile. Queens of the termite *Macrotermes* can live up to 15-20 years and lay up to 20,000 eggs per day (Traniello and Leuthold, 2000). Workers of the same species, on the contrary just live around 2-3 months. This thesis aims to understand how and why aging occurs in social insects, and if ant queens do show signs of senescence.

1.1 Definition of senescence

Senescence is defined in its broadest terms, as a loss in vitality and fertility functions with advancing age (Hughes and Reynolds, 2005). Specifically, it can be translated as an increase in the mortality rate (i.e. ‘demographic aging’, or ‘actuarial senescence’ (Gaillard and Lemaître, 2017)), a decrease in the fertility rate (i.e. reproductive senescence), or how abrupt and sharp the changes in these rates are (Jones et al., 2014). This contrasts with the definition of ‘aging’ as the neutral process of time passing by (Medawar, 1952).

It is generally believed that although senescence has a detrimental effect on fitness, it is a nearly universal feature of multicellular organisms (Hughes and Reynolds, 2005), and single-cell organisms too (Herker et al., 2004; Proenca et al., 2018). Yet, demographic age

trajectories of a broad range of metazoan species (11 mammals, 12 other vertebrates, 10 invertebrates, 12 vascular plants, and a green alga, Jones et al., 2014) have shown that aging patterns are highly diverse, and that senescence is not a widespread phenomenon. While deterioration with age is common in mammals, negligible deterioration or negative senescence is mostly observed in amphibians, reptiles, and some plants (Jones et al., 2014). Invertebrates show a diverse range of aging patterns, from bdelloid rotifers and water fleas exhibiting a high actuarial and reproductive senescence similar to mammals, and the red gorgonian *Paramuricea clavata*, exhibiting an increase of reproduction and a slight decrease in mortality with age (Jones et al., 2014). These data suggest aging is to some degree phylogenetically constrained, but further specific aspects of the life history will determine the aging of each species. For example, species known for eluding senescence generally exhibit a high modularity (Franco and Silvertown, 1996), regenerative cell capacity (Martínez, 1998), and/or lack of germ-line sequestration from the soma (Martínez and Levinton, 1992).

1.2 Ultimate causes of senescence

Several theories have tried to explain the ultimate evolutionary reasons for the occurrence of senescence. The notion of 'wear and tear' of the body, proceeding the time of the industrial revolution, was formally introduced by August Weissman. He stated that, compared to inanimate objects, organisms' cells also wear out through use and function causing the shortening of lifespan (Weismann, 1882). Later aging was explained as a deteriorative process in which the mechanical wear of the body, like any other object (e.g. car or tool), accumulated chemicals or oxidated (Weismann, 1882).

However, it was not until 1952, that a more formal explanation of senescence was developed. In a lecture, Medawar explained the hypothesis that the force of natural selection declines with time (Medawar, 1952). He made an analogy with test tubes in the laboratory. In the absence of senescence (intrinsic mortality), the tubes do not deteriorate, but after a certain amount of time they break, and broken tubes are replaced by new ones of age 0. This leads to an exponential decline in time in the number of older test tubes. Similarly, a constant percent of individuals in a population die in any given period by extrinsic mortality (due to

e.g. predation, disease, starvation). Excluding senescence, an individual will produce the same amount of progeny from maturity until death, increasing its total progeny linearly with time. Medawar called the 'reproductive effect' the amount of progeny produced per individual by age. The contribution of offspring declines per age group, and so does the evolutionary impact and the force of natural selection (Hamilton, 1966). Late-expressed deleterious genes and mutations have a negligible effect on the reproductive potential of an individual if it has already produced most of its offspring. These genes are then beyond the reach of effective negative selection, leading the organism to accumulate them (i.e. 'mutation accumulation theory', Medawar, 1952).

Postzygotic (or *de novo*) mutations could lead to genome mosaicism, i.e. soma heterogeneity within tissues. Even when studied in depth mostly in humans, *de novo* mutations appear to be more frequent with increasing age (reviewed in Vijg and Dong 2020). One of the main problems with this theory is that such mutations are predicted to appear and accumulate randomly. However, changes in lifespan have been linked to specific molecular signaling pathways (e.g. insulin/insulin-like growth factor and target of rapamycin signaling pathway) across a broad range of taxa (Alic and Partridge, 2011; Fontana et al., 2010), and senescence to the development of similar pathophysiologies (López-Otín et al., 2013), suggesting that a systematic process is taking place.

Williams stated how the decrease in the force of natural selection (also known as 'selection shadow') created an opportunity for genes with deleterious effects late in life to fixate in the population if they had a beneficial effect early in life (i.e. 'antagonistic pleiotropy theory' APT, (Williams, 1957). He also predicted that senescence should start at the time of adult maturation and that after that point, the extrinsic mortality rate and senescence rate should exhibit a positive correlation. Mathematical models have shown that age-independent extrinsic mortality does not affect the senescence rate (Hamilton, 1966; Moorad et al., 2020a, 2020b; Wensink et al., 2017). Similarly, Williams stated that young mortality rates do not affect senescence, but Hamilton's formulae show that age-specific mortality (including juvenile mortality) does affect selection and senescence (Hamilton, 1966; Moorad et al., 2020b).

The proximate causes of aging, under APT, could occur due to energy and/or functional trade-offs. The former depicts a limitation of resource allocation between reproduction and somatic maintenance (disposable soma theory, DST) (Kirkwood, 1977; Kirkwood and Austad, 2000), the latter a suboptimal gene regulation after maturation (developmental theory of aging, DTA) (Maklakov and Chapman, 2019). While the DST predicts that changes in investment in lifespan affect reproduction outcome, the DTA states that age-specific optimization of gene expression could increase lifespan without fitness costs (Lind et al., 2021). Support has been found for a negative correlation between early-life reproduction, and both late-life reproduction and survival (for *Drosophila melanogaster* reviewed in Maklakov and Chapman, 2019). Nevertheless, several studies on different organisms have uncoupled the trade-off between reproduction and longevity, challenging the main predictions of the energy trade-offs (Lind et al., 2021).

In hand with the AP theory, the Hyperfunction theory states that aging is caused by biological processes that are optimized for early-life function but become harmful when they continue to perform in late-life (Gems and Partridge, 2013). As selection late in life is too weak, suboptimal regulation could cause some of the following hallmarks of aging: genomic instability, telomeres attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (López-Otín et al., 2013).

1.3 Aging in social insects – ultimate explanations

Two phenomena have drawn scientists' attention to aging in social insects: their extended lifespan, and their lack of senescence. These two traits make up two different dimensions of aging. The first one is related to the extension of the life of an organism (either mean or maximum lifespan) and is also described as the pace of aging. The second is the shape of aging, i.e. the mortality and fertility of an organism for each time point, standardized by age (Baudisch, 2011). Social insects seem to be remarkable in both pace and shape. Reproductives of social species exhibit a long lifespan compared to other similar-sized

solitary insects and their non-reproductive counterparts social insects (Carey, 2001; Keller and Genoud, 1997), and senescence appears to be absent [e.g. having a high reproductive outcome at old ages (Kramer et al., 2015)].

The peculiarities of social insect aging can only be understood under the concept of superoganismality. It refers to species of social insects that have irreversibly transitioned into morphologically differentiated reproductive and nursing castes (Boomsma and Gawne, 2018; Wheeler, 1911). Here, natural selection acts on different hierarchical levels; from the individual level to the superorganism level, i.e. the colony. This is of great relevance as any selection for individual lifespan is ultimately selected in terms of benefit for the entire colony. Reproductives of social insects exhibit a positive correlation between fertility and longevity (Blacher et al., 2017; Kramer et al., 2015). It has been argued that such simultaneous optimization of two-life history traits could be only possible if some of the costs are transferred to the workers (Kramer et al., 2015). This argument has been used as an explanation for the common belief that "all ants", and other eusocial insects, show divergence in lifespan among castes, with long-lived queens (or kings) and shorter-lived workers (Hölldobler and Wilson, 1990; Kramer et al., 2022). Such divergence intensifies as the colony increases in size (Kramer and Schaible, 2013a).

The most common explanation found in the literature for the low rate of aging and long lifespan of reproductives is the evasion of extrinsic mortality, due to their sheltered life in well-protected nests (Heinze and Schrempf, 2008; Keller and Genoud, 1997; Rueppell et al., 2004). However, differences in caste-specific extrinsic mortality are not sufficient nor necessary to explain the longevity of reproductives and the divergence among castes (Kramer et al., 2022). Instead, the delay of sexual offspring production and the monopolization of reproduction (monogynous vs polygynous colonies, colonies with sterile workers vs workers capable of some degree of reproduction) could explain the long lifespans in the reproductive caste (Kramer et al., 2022). Mathematical models based on superorganismality show that extrinsic mortality can positively affect lifespan divergence only in colonies with multiple queens and fertile workers, yet this effect tends to be small (Kramer et al., 2022).

Ant queens in monogynous colonies live much longer than in polygynous colonies (Keller and Genoud, 1997). It was argued that ant queens in polygynous colonies evolved shorter lifespans due to their higher risk of extrinsic mortality (Keller and Genoud, 1997). Queen founding in monogynous colonies is typically independent and perilous, but once the colony has been established in a complex sheltered nest, the queen experiences low levels of extrinsic mortality. In contrast, queens of polygynous ant colonies frequently change nest locations and live in less protected nests (Hölldobler and Wilson, 1990). However, as pointed out before, models show that the lifespans of polygynous queens are shorter independently of the extrinsic mortality (Kramer et al., 2022). Another explanation could be that the new cohorts of workers produced by the newly arrived queens (in polygynous colonies) can outnumber the older workers and decrease the relatedness to the previous generation of queens. Under this scenario, the risk of queens being "dismissed" earlier by younger worker cohorts increases, and selection for shorter lifespans could occur (Boomsma et al., 2014). Additionally, the reproductive task in a polygynous colony is shared among several queens and not by a single individual, possibly relaxing selection on the queen's lifespan (Kramer et al., 2022).

Considering aging in the worker caste, it has been shown that lifespan can be extended by reproduction in species in which the workers retain the ability to reproduce (Hartmann and Heinze, 2003; Tsuji et al., 1996), contrary to the common phenomenon in which reproduction imposes a cost over survival in other organisms (Harshman and Zera, 2007). Senescence has been studied in a few cases in social insect queens and workers, in most cases in the honeybee. Studies have shown that the worker lifespan is task dependent in *Apis mellifera*. In-hive workers (nurses and winter bees) live longer compared to foragers (Rueppell et al., 2004), and the age of onset of foraging is key in determining the workers' lifespan (Rueppell et al., 2007a). Moreover, honey bee workers show a decline in immune response with age, i.e. functioning hemocyte/immunocyte reduction, associated with the switch to the foraging task (Amdam et al., 2004). As workers revert to nurses, they also recover their past immune response with high levels of functional hemocytes (Amdam et al., 2006). Workers show no signs of functional senescence matching the chronological age (Rueppell et al., 2007b).

In the case of ants, few studies have captured the shape of aging (age-specific reproduction and mortality, reviewed in Cole, 2009) and are based on punctual periods of growth and death of colonies. On ant queens of a few species (mostly monogynous species) data has been recorded for less than 20% of their estimated lifespan (Keeler, 1993; Perfecto and Vandermeer, 1993; Porter and Jorgensen, 1988; Tschinkel, 2017), failing to capture the end of the queen's lifespan. Worker lifespan data is also incomplete because some studies lack age-controlled cohorts (Chapuisat and Keller, 2002; Gordon and Hölldobler, 1987; Modlmeier et al., 2013; Negroni et al., 2021; Schmid-Hempel and Schmid-Hempel, 1984), surveyed marked individuals in the field without distinguishing between extrinsic and intrinsic mortality (Calabi and Porter, 1989; Gordon and Hölldobler, 1987; Schmid-Hempel and Schmid-Hempel, 1984), or monitored the lifespan of temperate species with artificial hibernation (Kramer et al., 2016), and lacking comparable queen data for the species. Still, a study on minor workers of the ant *Pheidole dentata* showed no signs of senescence in the sensorimotor functions after 86% of their laboratory lifespan (i.e. 120-days, Giraldo et al. 2016), pointing to a non-linear occurrence of senescence following chronological age.

1.4 *Cardiocondyla obscurior* as a social insect model for aging research

Known populations of *Cardiocondyla obscurior* derive from two phenotypically and genetically differentiated lineages: a New World and an Old World lineage (Errbii et al., 2021; Schrader et al., 2014). *C. obscurior* ant colonies are typically small (around 20-30 workers to a few hundred) and polygynous, with several queens present. Workers possess no ovaries and are completely sterile (Heinze et al., 2006), making them one of the few ant genera (11[1] out of 283 genera, Bourke and Franks, 1996) with a complete major evolutionary transition and highest reduction of conflict across castes (Bernadou et al., 2021; De Menten et al., 2005).

Recent research in this species has shown that the queen has control over caste determination and is determined in the early embryo (Schultner et al., 2021). Queen mating occurs only

[1] *Eciton, Anochetus, Leptogenys, Solenopsis, Monomorium, Tetramorium, Pheidole, Pheidologeton, Cardiocondyla, Hypoponera*, and *Wasmannia* (but also *Carebara, Brachyponera, Vollenhovia, Strumigenys* in Bernadou et al., 2021).

once, usually inside the nest with a non-dispersing wingless male, i.e. 'ergatoid' or work-like male (Figure 1-1, (Heinze, 2017; Schmidt et al., 2016). Ergatoids are longer lived compared to winged males and have a constant spermatogenesis (Heinze and Hölldobler, 1993). They possess long toothless and sickle-shaped mandibles (Oettler et al., 2010) and engage in lethal fighting with other wingless males as soon as they eclose from their pupal stage (Cremer et al., 2012; Seifert, 2003). Winged males, on the contrary, are rarely seen in the field. Their appearance has been correlated to stressful environmental conditions such as temperature drops, starvation, and/or the reduction in worker numbers (Cremer and Heinze, 2003; Schrempf and Heinze, 2008).

Figure 1-1. Ergatoid male (left) and C. obscurior queen (right). The long mandibles of the ergatoids are used for a male to male competition, even against ergatoid male pupae (Cremer et al., 2012).© LMJN.

This myrmicine ant is arguably the best-known social insect aging model (Oettler and Schrempf, 2016). Queens collected in Una, Brazil (from the New World lineage population) showed an increase in egg laying rate during the queen's life. Additionally, and contrary to what is expected for senescent organisms, queens show no signs of decreasing fecundity until 1-2 weeks prior to death (Heinze and Schrempf, 2012). This reflects *terminal investment* in this species, i.e. an increase of egg investment with age and lack/minimal reproductive senescence. In this species, the positive correlation between longevity and reproduction in the queens (Heinze and Schrempf, 2012; Kramer et al., 2015; Schrempf et al., 2017) is

apparently not causal. Manipulations to induce an increase in investment into egg production in single queen colonies did not show any negative effect on lifespan.

Other social conditions determine the queen's longevity, showing that lifespan is plastic in this species. Among them, the mating status has the largest positive effect on the queen lifespan (2-fold increase), even when mated to sterilized males (n=18, Schrempf et al., 2005a). Such an increase in lifespan does not relate to differential treatment by workers in the colony, while it is also exhibited by mated queens in the absence of workers (n=6, Rueppell et al. 2015). Furthermore, the cuticular hydrocarbon profile, which is thought to signal the nestmates for the queen`s fertility, is undistinguishable from the mating status (Will et al., 2012).

The transcriptomic expression profile change with age and after mating (Wyschetzki et al., 2015). Whole body transcriptomes of young and old queens (4 and 18 weeks old respectively), showed significant overlap in expression to age-related genes in *Drosophila melanogaster*, e.g. genes related to the generation of neurons (Wyschetzki et al., 2015). More interestingly, most of such overlapped genes were expressed in the opposite direction compared to old fruit flies. From these genes, GO-term enrichment analysis suggested that processes involved with cell division and reproduction were upregulated, while the development and contraction of muscles were downregulated in old queens in contrast to *D. melanogaster* (Wyschetzki et al., 2015). Based on earlier studies, 18-week-old *C.obscurior* queens have approximately 50% survival chance and are therefore middle-aged (Schrempf et al., 2005). This dataset implies that while signals of senescence in the gene expression are evident at the time point of 65% survival for the fruit fly, such signals are not evident, or are differently regulated in *C. obscurior* queens (Wyschetzki et al., 2015).

Lastly, mating can also have a negative effect on longevity. Queens lived shorter and produced fewer sexuals when mated to ergatoid males than to winged males, while the onset of egg laying and the egg laying rate are unaffected (n=22, Schrempf and Heinze, 2008). Mating with an allopatric male also decreases the lifespan and reproduction capability due to

embryonic death by cytoplasmic incompatibility in the presence of the symbiont *Wolbachia* (Schrempf et al., 2015; Ün et al., 2021).

Different aspects of the life history of *C. obscurior* make it an ideal model for aging research. A new generation of offspring can be obtained in the short time of 3 months (Schrempf and Heinze, 2006), and queens have a maximum lifespan of about a year (Jaimes-Nino et al., 2022). Queen aging seems plastic and responds to different social and physiological cues. Easily mated and reared in the laboratory, this small-sized ant (2.5-3mm) offers the possibility to test for ultimate and proximate explanations for the evolution of aging. In terms of a mechanistic approach, *C. obscurior* offers the possibility to explore pathways related to aging, e.g. insulin/insulin-like growth factor (IGF) signaling (IIS)/TOR pathway. Recently, a recent version of the genome of *C. obscurior* has been released (Errbii et al., 2021), describing a special genome architecture with an unusual distribution of transposable elements in this ant (Errbii et al., 2021; Schrader et al., 2014). Future research on aging will benefit from the use of this species as an insect model.

1.5 Outstanding questions

This thesis aims to understand why and how ants age and senesce, using the model *Cardiocondyla obscurior*. To this end, I aimed to answer the following questions:

1) How are the pace and the shape of aging in ant queens and workers?
2) Which trade-offs at the colony and individual level are evident in terms of fertility and longevity?
3) How can one ultimately explain the extraordinary long lifespan and the lack of (or minimal) reproductive senescence in ant queens?
4) Is there a heritable component in the longevity and fertility trait of queens?

1.6 Outline of this thesis

This thesis contains four results chapters.

The first results chapter (**Chapter 2**) arises from a collaboration with Dr. Mark Harrison at the WWU Münster and is the reanalysis of transcriptomic data of 14 *C. obscurior* mated ant

queens (4- and 18-week-old). We found that not only 18-week-old queens do not exhibit signs of senescence, but that several antiaging mechanisms are taking place, e.g. old-biased genes are under stronger purifying selection compared to young-biased genes and older queens exhibit higher connectivity in their gene-coexpression network.

Subsequently, I studied the effect of investment into the maintenance of the colony (by manipulating the colony size) on the queens' longevity and total productivity (**Chapter 3**). This study tried to assess if trade-offs between fertility and longevity at the level of the colony and the individual occur. I then analyzed the pace and the shape of aging in queens and workers. Additionally, I analyzed the transcriptomes of middle-aged queens and *prope mortem* queens (queens close to death), to evidence signs of senescence after the onset of reproductive senescence. This chapter resulted in the development of a life history framework "continuusparity" for social insect aging.

In **Chapter 4** I used the first generation of queens (F_0) obtained in the study described in the third chapter and established mating pairs to rear a new generation of mated queens (F_1) and to test the heritability of the traits: lifespan and fertility. Additionally, as the F_0 generation lived under controlled colony size conditions, I tested for environmental maternal effects in the F_1 life-history traits.

Finally, in **Chapter 5** I explored the pace and shape of aging in workers in a standardized set-up without marked workers, to complement our understanding of aging in the species *Cardiocondyla obscurior*. We found that aging in workers mirrors the pace and shape of the queen, calling for a programmed mechanism, or *Zeitgeber*, acting in workers and queens alike and dictating the onset of senescence.

Chapter 2

2 Gene Coexpression Network Reveals Highly Conserved, Well-Regulated Anti-Ageing Mechanisms in Old Ant Queens

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2.1 Abstract

Evolutionary theories of ageing predict a reduction in selection efficiency with age, a so-called 'selection shadow', due to extrinsic mortality decreasing effective population size with age. Classic symptoms of ageing include a deterioration in transcriptional regulation and protein homeostasis. Understanding how ant queens defy the trade-off between fecundity and lifespan remains a major challenge for the evolutionary theory of ageing. It has often been discussed that the low extrinsic mortality of ant queens, that are generally well protected within the nest by workers and soldiers, should reduce the selection shadow acting on old queens. We tested this by comparing strength of selection acting on genes upregulated in young and old queens of the ant, *Cardiocondyla obscurior*. In support of a reduced selection shadow, we find old-biased genes to be under strong purifying selection. We also analysed a gene co-expression network (GCN) with the aim to detect signs of ageing in the form of deteriorating regulation and proteostasis. We find no evidence for ageing. In fact, we detect higher connectivity in old queens indicating increased transcriptional regulation with age. Within the GCN, we discover five highly correlated modules that are upregulated with age. These old-biased modules regulate several anti-ageing mechanisms such as maintenance of proteostasis, transcriptional regulation and stress response. We observe stronger purifying selection on central hub genes of these old-biased modules compared to young-biased

modules. These results indicate a lack of transcriptional ageing in old C. obscurior queens possibly facilitated by strong selection at old age and well-regulated anti-ageing mechanisms.
Key words: ageing, selection shadow, social insects, longevity/fecundity trade-off.

2.2 Significance

Understanding the exceptional longevity of ant queens and how they defy the trade-off between fecundity and lifespan remains a major challenge for the evolutionary theory and molecular biology of ageing. In this study, we offer several clues as to how this occurs on a molecular level in *Cardiocondyla obscurior* queens. Specifically, we believe a reduction in the selection shadow due to low extrinsic mortality, has allowed the evolution of well-regulated antiageing mechanisms. Consequently, we suggest several promising starting points for future research into the poorly understood phenomenon of extreme longevity in ant queens. Making progress in this field will not only allow us to better understand longevity and fertility in social insects but may also offer interesting research strategies for human ageing.

2.3 Introduction

Ageing, the progressive decline of physiological function with age, and thus of survival and fertility, is common to most multicellular species (Jones et al., 2014). Extensive genetic and molecular studies have illuminated several proximate mechanisms involved in the ageing process, allowing us to better understand how we age. The majority of these "hallmarks of ageing" can be attributed to the accumulation of cellular damage (Gems and Partridge, 2013; López-Otín et al., 2013) and an overall deterioration of regulation (Frenk and Houseley, 2018). One important hallmark of ageing, the loss of protein homeostasis, is caused by a reduction in quality control mechanisms such as chaperones that support correct folding and structure of proteins, as well as proteolytic pathways that ensure the removal of misfolded peptides (Calderwood et al., 2009; Koga et al., 2011; López-Otín et al., 2013; Rubinsztein et al., 2011; Tomaru et al., 2012). The result is an accumulation of toxic, misfolded proteins and an inefficient replenishment of correctly functioning proteins. Further hallmarks of ageing include deleterious changes in terms of cell cycle (a cessation of cellular replication),

intercellular communication, nutrient sensing, and epigenetic regulation (López-Otín et al., 2013), as well as a downregulation of mitochondrial and protein synthesis genes (Frenk and Houseley, 2018). Importantly, the ageing process is often accompanied by a dysregulation of transcription (Frenk and Houseley, 2018).

Several classic evolutionary theories of ageing aim to explain why organisms age (Flatt and Partridge, 2018; Kirkwood and Austad, 2000). These theories generally describe a reduction in selection efficiency with increasing age because the number of surviving individuals decreases due to extrinsic mortality. In the mutation accumulation theory, this "selection shadow" leads to an accumulation of mutations which have a deleterious effect later in life (Flatt and Partridge, 2018; Kirkwood and Austad, 2000). In support, empirical studies have found that genes with expression biased toward late life are less conserved than those highly expressed at young age across several tissues and mammalian species (Jia et al., 2018; Turan et al., 2019). Building on this, the antagonistic pleiotropy theory describes how genes with beneficial effects early in life can be maintained by selection even if they have pleiotropic negative effects later in life (Williams, 1957). In the disposable soma theory, the pleiotropic effect of more specific genes is described, that cause a trade-off between somatic maintenance and reproduction (Kirkwood, 1977), so that an increased, or early, investment in offspring is expected to come at the price of a shorter lifespan and vice versa (Kirkwood and Austad, 2000).

There are, however, exceptions to these expectations; possibly most notably within social insects, where reproductive castes exhibit relatively long lifespans compared with their sterile siblings (Keller and Genoud, 1997). This apparent lack of a trade-off between longevity and fecundity in social insects is at odds with expectations for the disposable soma theory. The longer life of queens compared with sterile castes might be explained by low extrinsic mortality due to the protection of a well-defended nest (Keller and Genoud, 1997; Negroni et al., 2016). The low extrinsic mortality of queens can in turn be expected to lead to a reduction of the selection shadow as more queens reach old age, allowing efficient selection on genes that are important for somatic maintenance late in life.

In an attempt to understand the relationship between fecundity and longevity in social insects, several studies have investigated caste and age-specific expression of putative ageing genes in honeybees (Aamodt, 2009; Aurori et al., 2014; Corona et al., 2007; Seehuus et al., 2013), ants (Lucas et al., 2016; Lucas and Keller, 2018; Negroni et al., 2019; Wyschetzki et al., 2015) and termites (Elsner et al., 2018; Kuhn et al., 2019). One of these studies, which compared gene expression between young and old queens of the ant *Cardiocondyla obscurior*, identified several overlaps with ageing pathways known from *Drosophila melanogaster* (Wyschetzki et al., 2015). However, surprisingly, for many genes, the ratio of expression level between old and young ant queens was reversed compared with *D. melanogaster*. Further studies comparing expression between castes and age groups highlight the importance of several gene pathways for longevity in social insects that have previously been implicated in ageing, such as antioxidants (Aurori et al., 2014; Corona et al., 2005; Kuhn et al., 2019; Negroni et al., 2019), immunity (Aurori et al., 2014; Kuhn et al., 2019; Lucas and Keller, 2018; Negroni et al., 2016, 2019), DNA and somatic repair (Aamodt, 2009; Kuhn et al., 2019; Lucas et al., 2016; Seehuus et al., 2013), respiration (Corona et al., 2005; Lockett et al., 2016), as well as the insulin/insulin-like growth factor (IGF) signaling (IIS) (Aurori et al., 2014; Kuhn et al., 2019), and the target of rapamycin (TOR) signaling pathways (Kuhn et al., 2019; Negroni et al., 2019). The IIS and TOR nutrient sensing pathways are of particular interest in this context, since their role in longevity and fecundity has been extensively studied in model organisms (Flatt and Partridge, 2018; Kenyon, 2010; Partridge et al., 2011; Tatar et al., 2003). These transcriptional studies offer insights into individual genes and their pathways that might be involved in ageing in social insects. However, a more holistic view of gene networks is likely to uncover further important genes as well as insights into transcriptional regulation. For example, a study of gene coexpression networks (GCNs) on mouse brains revealed that with age a decrease in the correlation of expression between genes occurred, showing that transcriptional dysregulation can lead to a significant reduction in gene connectivity (Southworth et al., 2009). These findings demonstrate the application of transcriptional studies for investigating whole pathways and gene networks and their wide-reaching implications for ageing. Furthermore, the extent at which a selection shadow may be reduced for old queens due to a reduction in extrinsic mortality has so far not been formally tested.

To address these questions, we investigated transcriptomic data available for young and old queens of the polygynous ant *C. obscurior* (Wyschetzki et al., 2015). These ant queens are relatively short lived compared with most ant species (median lifespan: 16–26 weeks; Kramer et al., 2015; Schrempf et al., 2005a), which is in accordance with expectations for polygynous species, where extrinsic mortality is higher than in monogynous colonies (Keller and Genoud, 1997). Nevertheless, as for most ant species, *C. obscurior* queens (up to 48 weeks) outlive sterile workers that are expected to live around 12–16 weeks (Oettler and Schrempf, 2016). Importantly, consistently high reproductive output throughout their lives until immediately before death indicates no apparent reproductive senescence in these ant queens (Kramer et al., 2015). To test for signs of ageing in transcriptional regulation, we carried out a GCN analysis, in which we identified gene modules related to young mated (4 weeks) and old mated (18 weeks) queens and compared overall network connectivity. We also tested the hypothesis that, due to low extrinsic mortality, selection efficiency should not decline with age in queens. We found evidence for an array of antiageing mechanisms that are more tightly regulated in old queens. We could find no evidence for a selection shadow, indicating stable selection efficiency throughout an ant queen life.

2.4 Results and Discussion

2.4.1 Old-Biased Genes Are Not under Weaker Selection

Evolutionary theories of ageing predict weaker selection on genes which are expressed in old individuals due to low effective population size and reduced fecundity (Flatt and Partridge, 2018; Kirkwood and Austad, 2000). In ant queens, we may expect a reduction of this "selection shadow" as low extrinsic mortality and lifelong, high fertility should lead to a stable effective population size up to old age. We tested this by estimating and comparing selection strength between three groups of genes. These were 1) old-biased genes n=46: significantly over-expressed in seven old (18weeks) compared with seven young (4 weeks) *C. obscurior* queens; 2) young-biased genes (n=96): significantly over-expressed in young compared with old queens; 3) unbiased genes (n=2616): no significant difference in expression between young and old queens. To estimate direction and strength of selection,

we measured dN/dS (ratio of nonsynonymous to synonymous substitution rates) for one-to-one orthologs with a set of 10 ant species (see Materials and Methods). A dN/dS ratio ≈ 1 indicates neutral evolution, whereas values <<1 signify purifying selection. We find no evidence for weaker purifying selection in old-aged queens, since dN/dS in old-biased genes (median: 0.084) is in fact significantly lower than in young-biased genes (median: 0.127; P value = 0.016; Mann–Whitney U test; Fig. 2-1), indicating increased purifying selection with age. This is in contrast to published results for age-biased genes in humans, in which old-biased genes had a significantly higher dN/dS (median: 0.22) than young-biased genes (median: 0.09, P-value = 1.4×10^{-50}), as would be expected for a reduction in purifying selection with age (Jia et al., 2018). This was confirmed by a further study on several mammalian tissues, in which an adjusted dN/dS metric correlated more strongly with expression in young compared with old individuals (Turan et al., 2019). Interestingly, dN/dS in young-biased genes is also significantly higher than in unbiased genes (median: 0.100; P-value = 2.2×10^{-4}; Mann–Whitney U test), as has previously been reported for the ant, *Lasius niger* (Lucas et al., 2017). To further test the ability of this method to detect a selection shadow in insects, we repeated the analysis for *D. melanogaster*. Age-biased gene expression was measured for a novel data set containing expression data for young (10 days) and old (38 days) female flies across two tissues (head and fat body) and different feeding regimes. Evolutionary rates were obtained for these genes from published analyses based on alignments of 12 *Drosophila* species (Clark et al., 2007). In contrast to our results for ant queens but in agreement with expectations for a selection shadow, we find significantly higher dN/dS levels in old-biased fly genes (median: 0.060) compared with young-biased genes (median: 0.047; P-value= 5.1×10^{-8}; Mann–Whitney U test).

We also investigated the numbers of ant genes that are under significant positive selection within old-biased compared with young-biased and unbiased genes, using a site test of the codeml suite (Yang, 1997). Contrary to expectations for weaker selection strength on old queens, we found no difference in the proportion of genes under positive selection between the three groups of genes (old biased: 21.7%; young-biased: 21.9%; unbiased: 16.0%; $chi^2 = 3.3$; P-value = 0.19). The effect size of the observed difference in proportions of genes under positive selection between youngand old-biased genes is so low (Cohen's *h*: 0.003), that we

assume the lack of significance is not due to a lack of power. The genes under significant positive selection in old-biased genes contain two regulatory genes (transcription factor and methyltransferase), an electron transport protein, a member of the COPI coatomer complex (important for protein transport), and *Notch* (Table 2.1). The latter is the central signaling protein within the Notch signaling pathway which is involved in tissue homeostasis and age-related diseases (Balistreri et al., 2016).

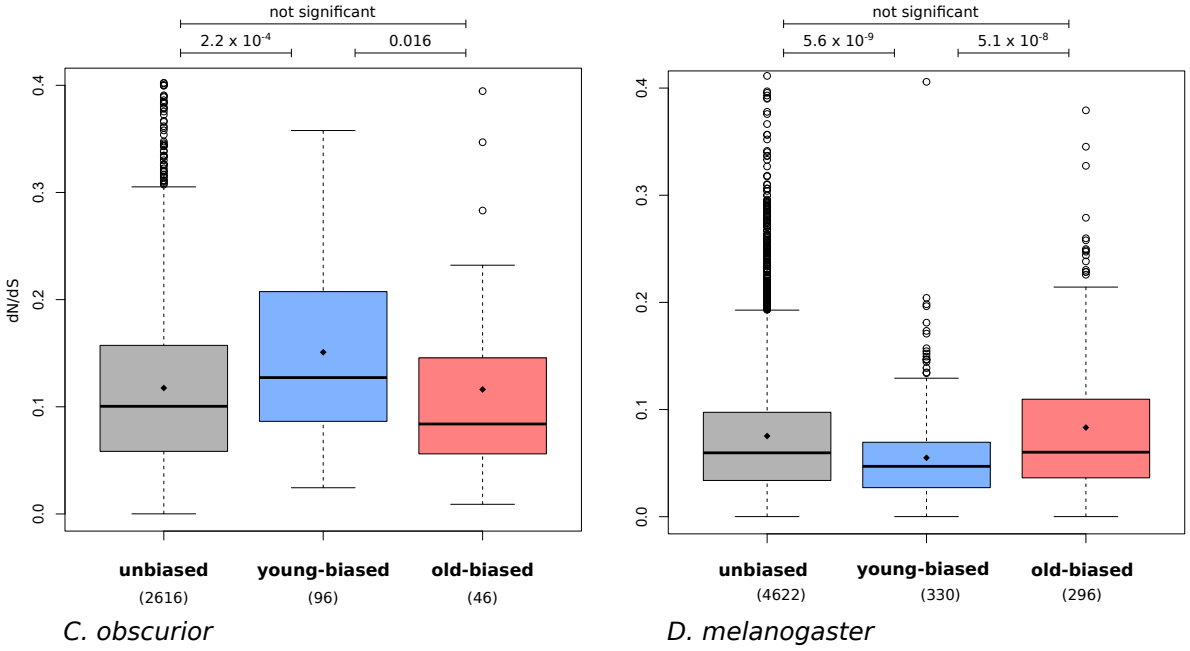


Figure 2-1. Evolutionary rates (dN/dS) in genes with unbiased expression, young-biased and old-biased expression in C. obscurior queens and D. melanogaster adult females. Significance was tested with Mann-Whitney U test.

Contrary to expectations based on evolutionary theories of ageing, these results suggest selection is not weaker on genes expressed mainly in old queens. We speculate that high fertility in old queens, coupled with an overall low extrinsic mortality, which is typical for social insects (Keller and Genoud, 1997; Negroni et al., 2016), may reduce the selection shadow in *C. obscurior* queens, leading to stable selection strength throughout their fertile life.

Table 2-2-1. Old-biased genes under significant positive selection.

Gene	Ortholog	Putative Function
Cobs_01221	uncharacterised	unknown
Cobs_04278	FBgn0002121 (l(2)gl)	polarity of neuroblasts and oocytes
Cobs_06663	FBgn0085424 (nub)	transcription factor
Cobs_08231	FBgn0004647 (Notch)	tissue homeostasis
Cobs_08620	FBgn0027607 (Dymeclin)	organisation of Golgi apparatus
Cobs_09212	FBgn0033686 (Hen1)	methyltransferase, methylates siRNA & piRNA
Cobs_11651	FBgn0036714 (CG7692)	unknown function
Cobs_12452	FBgn0008635 (βCOP)	subunit of the COPI coatomer complex, transport from Golgi to ER
Cobs_16420	FBgn0034745 (CG4329)	unknown
Cobs_16765	Cytochrome b561 domain-containing protein 1 (Q8N8Q1)	electron transport protein

2.4.2 Increased Connectivity in Old Ant Queens

In old queens, we expected to find little evidence for age-related transcriptional dysregulation in the form of reduced correlation of gene expression, as previously reported for ageing mouse brains (Southworth et al., 2009). We investigated this by measuring gene connectivity separately within old queens and within young queens, using the softConnectivity function of the WGCNA package (Langfelder and Horvath, 2008). This connectivity describes the total strength of correlations that a gene possesses with all other genes in a GCN (Langfelder and Horvath, 2008) and is thought to correlate positively with gene essentiality (Carlson et al., 2006). In fact, we find gene expression connectivity to be significantly higher in older queens (median: 145.3) than within young queens (median: 142.5; effect size: 0.255; P-value=4.3 x 10^{-29}; Wilcoxon signed-rank test), suggesting an increased regulation of gene networks in older queens.

Those genes which are more highly connected in older queens (1,471 genes with connectivity fold change > 2) are enriched for GO term functions (FDR < 0.1) related to protein synthesis, transcription, purine synthesis, cellular respiration, and ATP metabolism (supplementary table S1, Supplementary Material online). Most of the 20 genes with the strongest increase in connectivity in old queens (4.8–7.1-fold increase) compared with young queens are involved in transcriptional regulation (7 genes) or protein homeostasis (6 genes; supplementary table S2, Supplementary Material online). For example, a member of the 26S proteasome complex, important for the degradation of misfolded proteins, is the gene with the highest increase in connectivity in old queens. As has been shown for several organisms, including humans (Lee et al., 2010), yeast (Kruegel et al., 2011), and *C. elegans* (Vilchez et al., 2012), increased proteosome activity can extend lifespan by reducing proteotoxic stress (López-Otín et al., 2013). An increase in connectivity of *fatty-acid synthase 3* may have implications for colony communication (Yan and Liebig, 2021). Further highly connected genes include ribosomal proteins or genes involved in the correct folding, post-translational modification, or transport of proteins. The genes with highly increased connectivity in old ant queens, which are involved in transcriptional regulation, include two transcription factors, a transcritional coregulator (*taranis*), and four mRNA regulators. These results suggest that, contrary to expectations for ageing individuals, increased transcriptional regulation and protein homeostasis takes place in old queens.

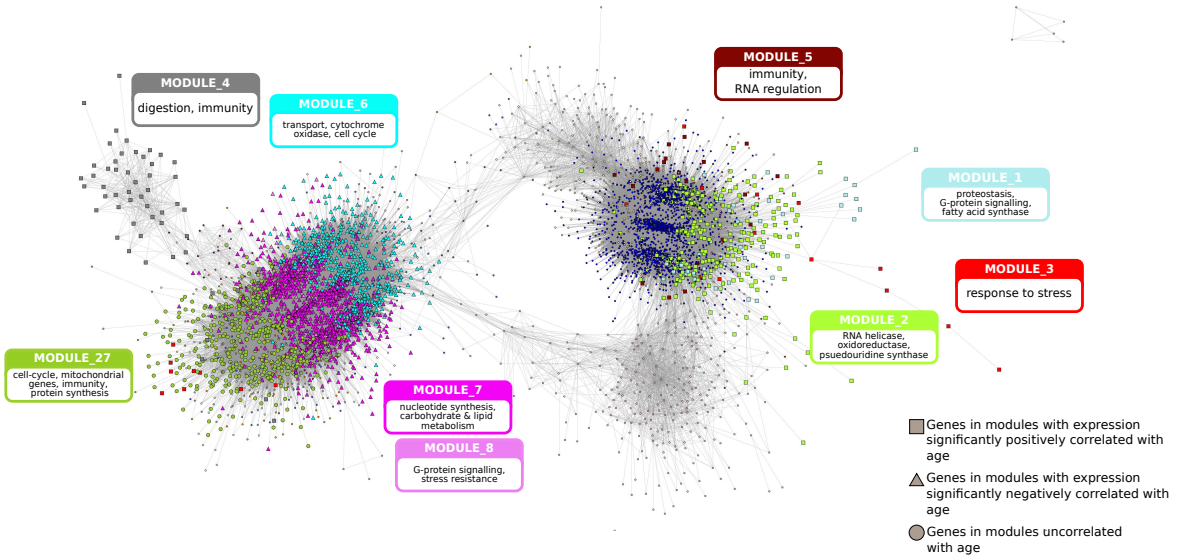
2.4.3 Coexpression Modules Related to Age

We constructed a signed, weighted GCN (Langfelder and Horvath, 2008) based on the correlation of normalized gene expression across all 14 samples (7 young queens and 7 old queens). Within the GCN, genes could be grouped into 27 modules, within which gene expression was especially strongly correlated (Fig. 2-2). To determine the importance of these modules for old and young queens, we first calculated eigengene expression based on the first principal component of each module. We then correlated eigengene expression of each module with the binary trait “age” (young and old). Five of the modules were significantly, positively correlated with age ($P < 0.05$; FDR < 0.1), indicating an overall higher expression of these modules in old compared with young queens.

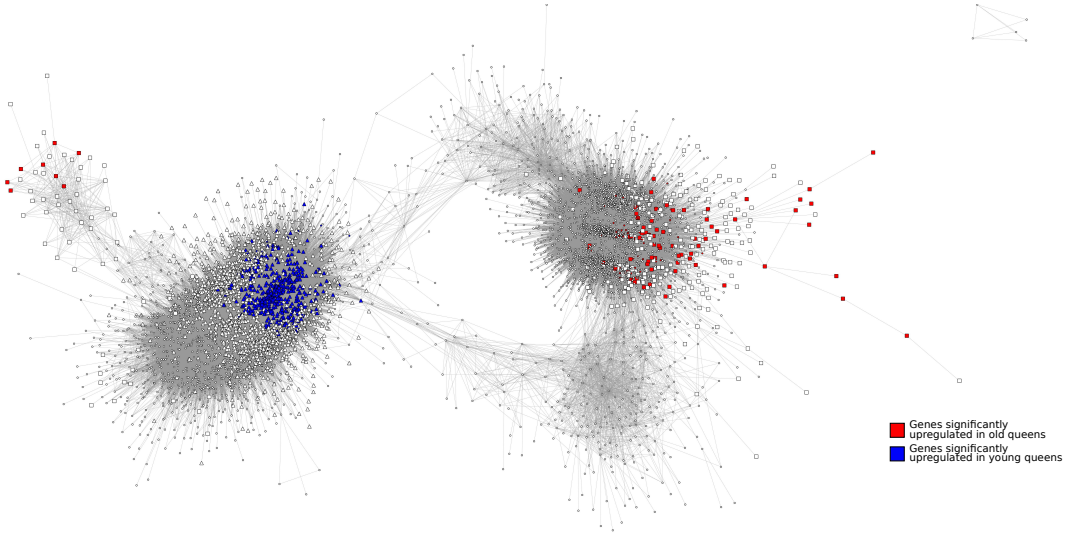
Three modules were significantly, negatively correlated with young queens, indicating a downregulation in old queens. To validate these correlations, we analyzed difference in expression of genes between old and young queens ($\log2[\text{expression}_{old}/\text{expression}_{young}]$) within each of these modules. Accordingly, the median log2-fold-change in expression was greater than zero in each of the old-biased modules (0.148–0.340) and less than zero within the young-biased modules (-0.376 to -0.249; supplementary fig. S1, Supplementary Material online). Four of the five old-biased modules (1, 2, 3, and 5) belonged to a larger cluster within the GCN, which is quite distant from the cluster containing the young-biased modules (6, 7, 8; Fig. 2-2). Module_4 (old biased), on the other hand, forms a more distinct cluster, adjacent to the young-biased cluster. The old-biased modules contained several genes that had previously been identified as upregulated in old queens via standard differential expression analysis (Wyschetzki et al., 2015) but contained no genes that were upregulated in young queens. The opposite was true for young-biased modules, thus confirming the validity and compatibility of both methods (Fig. 2-2b).

However, importantly, the GCN analysis also allowed the identification of many additional age-related genes that cannot be identified by standard differential expression analyses. For example, module_1, which has the strongest association with old queens (ρ=0.96; P-value=$5.3\text{x}10^{-8}$; FDR1=$1.4\text{x}10^{-6}$; Pearson correlation), contains 109 genes, of which only 41 are individually significantly differentially expressed between old and young queens. Similarly, module_6, which is strongly negatively associated with old queens (ρ=-0.90; P-value= 9.4×10^{-6}; FDR = 1.3×10^{-4}; Pearson correlation), contains 970 genes, of which 240 were identified as individually significantly upregulated in young queens (Wyschetzki et al., 2015). In the following section, we describe these eight age-biased modules in terms of their functional enrichment and detail the top hub genes (genes with the highest intramodular connectivity) within these modules.

(a) GCN module



(b) Differentially expressed genes



(c) Clustering of modules

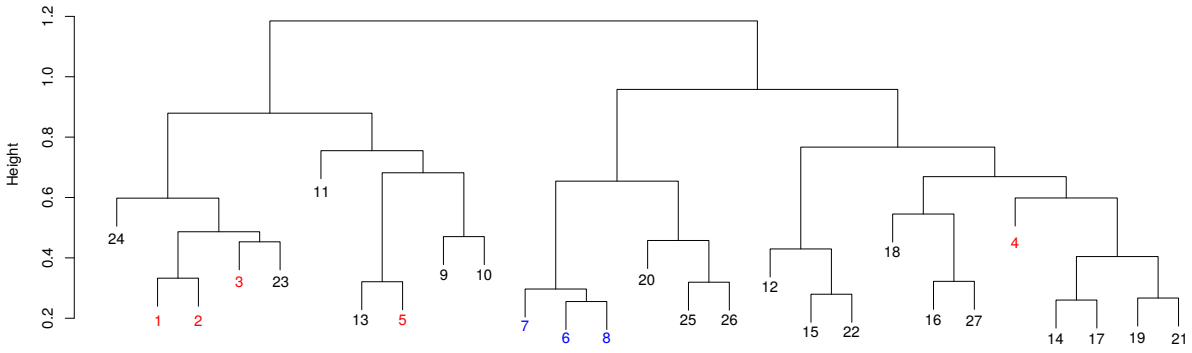


Figure 2-2. Caption on next page.

Figure 2-2 (Previous page). Gene co-expression network (GCN). (a & b) Graphical representation of the gene co-expression network, containing only the most strongly connected genes (n = 5442). In (a) genes are coloured according to the modules to which they belong. The main enriched functions (based on hubs and GO terms) of the 9 discussed modules are labelled (see text for more details). In (b) genes are coloured according to their differential expression; red: over-expressed in old queens; blue: over-expressed in young queens; white: not differentially expressed. In both representations, genes in modules significantly related to old queen expression are depicted as squares, and those significantly related to young queens are triangles; all other genes are represented by circles. (c) Clustering dendogram of modules; height represents dissimilarity based on topological overlap. Modules significantly related to age are highlighted in red (positive correlation) and blue (negative correlation). Higher resolution image available in the online version.

2.4.4 Old-Biased Modules

The most highly connected hub genes in module_1, the module most strongly upregulated with age (ρ= 0.96; P = 5.3 x 10^{-8}; FDR = 1.4 x 10^{-6}; 109 genes; Fig. 2-3), include three genes with functions related to maintaining and restoring proteostasis in old queens (supplementary table S3, Supplementary Material online), the loss of which has been described as one of the hallmarks of ageing (López-Otín et al., 2013). These are: a member of the TRAPP complex, important for protein transport, Socs44A, a gene involved in ubiquitination and GRXCR1, responsible for the post-transcriptional S-glutathionylation of proteins, a modification which is often triggered as a defence against oxidative stress (Dalle-Donne et al., 2009). The top hubs of this module also include two genes which encode integral members of the G-protein signaling pathway, namely, a Rho guanine nucleotide exchange factor and a G-protein α-subunit. The most connected gene within this hub is a fatty-acid synthase which may play an important role in colony communication. This module is enriched for a GO term related to the regulation of transcription (supplementary table S4, Supplementary Material online).

Module_2 (596 genes; upregulated with age: ρ= 0.65; P = 0.012; FDR = 0.080) contains hub genes coding for proteins with diverse functions, including an RNA helicase, a maternal protein, a protein with oxidoreductase activity and a pseudouridine synthase (supplementary table S3, Supplementary Material online).

Module_3 (433 genes; upregulated with age; ρ = 0.63; P = 0.017; FDR = 0.080) is particularly interesting since it is not only upregulated with age but, on average, gene members of the module are more strongly connected within old than in young queens (Fig. 2-3). Hub genes

indicate this module is important for responses to age-related stress, especially processes related to a maintenance of proteostasis (supplementary table S3, Supplementary Material online). For instance, the top 10 hubs contain the endoplasmic reticulum (ER) stress protein, disulfide-isomerase, which reacts to protein misfolding and oxidative stress (Laurindo et al., 2012), as well as fringe, which modulates Notch signaling, a pathway important for regulating tissue homeostasis and implicated in ageing related diseases (Balistreri et al., 2016). A further hub is a trehalose transporter, orthologous to tret1-2, indicating that the transport of trehalose (the main haemolymph sugar in insects) from fat body to other tissues is well regulated in old queens (Kanamori et al., 2010). This may have a positive effect on survival, since trehalose treatment increases longevity in *C. elegans* (Honda et al., 2010).

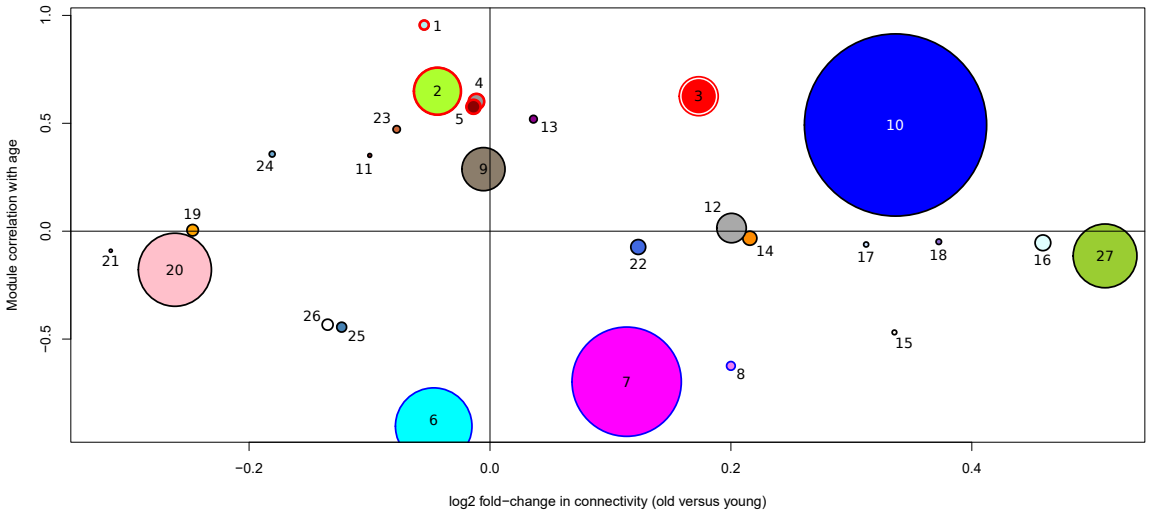


Figure 2-3. Correlation of GCN modules with age and their change in connectivity between old and young queens. A positive correlation with age (y-axis) signifies an upregulation of a module in old queens. A positive log2foldchange in connectivity (x-axis) represents a higher connectivity in old queens. Modules are labelled with their assigned module numbers. Sizes of dots represent relative number of genes within modules. Modules with red outlines are significantly upregulated and modules with blue outlines are significantly downregulated in old queens compared to young queens.

The top 10 hub genes in module_4 (186 genes; ρ =0.61; P = 0.021; FDR = 0.080) fulfil various functions, such as the digestive enzymes alpha glucosidase and chymotrypsin-1, indicating a possible modification in diet with age (supplementary table S3, Supplementary Material online). The third most connected gene within this module is orthologous to pirk in *D. melanogaster* (involved in the negative regulation of the immune response; Kleino et al.,

2008), indicating the immune system may be downregulated with age in *C. obscurior*. Interestingly, long-lived flies also tend to downregulate the induction of immune effector genes (Fabian et al., 2018; Loch et al., 2017). This module is enriched for the GO term "transmembrane transport" (supplementary table S4, Supplementary Material online).

Module_5 (169 genes; $\rho = 0.58$; P = 0.028; FDR = 0.095) may be important for controlling the immune system since two hub genes (supplementary table S3, Supplementary Material online), coding for the COMM domain containing protein 8 (COMMD8) and the WD40 domain containing angio-associated migratory cell protein, are both known to inhibit the transcription factor NF-κ-B (Bielig et al., 2009; Burstein et al., 2005). An upregulation of NF-κ-B occurs with ageing and its inhibition, as apparently occurs within this module, can reduce the effects of senescence (Tilstra et al., 2012). Interestingly, COMMD8 is also characterized by a strong increase in connectivity (1.68-fold change), indicating its heightened importance in old queens. Further functions of this module may be related to RNA regulation, as evidenced by the hub gene eyes_absent, a transcription factor with importance in embryonal eye development in *D. melanogaster* (Bonini et al., 1998). Based on the 10 nearest neighbours in the *C. obscurior* GCN, eyes_absent may regulate several enzymes involved in post-transcriptional processes, such as mRNA export from the nucleus (*sbr*, Cobs_03187), and tRNA modification (*Tgt*: Cobs_16650; *HisRS*: Cobs_01013; CG3808: Cobs_18201).

2.4.5 Modules Downregulated with Age

Module_6 (970 genes) is the module most strongly downregulated with age ($\rho = -0.9$; P= 9.4 x 10^{-6}; FDR = 1.3 x10^{-4}) and is enriched for the GO terms "transmembrane transport" and "potassium ion transport" (supplementary table S4, Supplementary Material online). Interestingly, the top 10 hubs contain three genes with no detectable homology to any protein in the uniprot arthropod database (supplementary table S3, Supplementary Material online). Otherwise, the functions of hub genes in this module span various functions, such as cell–cell interactions, cytochrome oxidase, an odorant receptor and a negative regulator of the cell cycle.

Module_7 (1385 genes; ρ =-0.7; P=0.006; FDR = 0.050) has several enriched functions in the nucleotide synthetic process, oxidoreductase activity, carbohydrate and lipid metabolism, ATP metabolic processes, cofactor, and coenzyme binding (supplementary table S4, Supplementary Material online). Accordingly the top hubs in this module contain a thioredoxin, a proteasome subunit (α6) and two genes involved in ubiquitination (*STUB1* and *Ubc6*; supplementary table S3, Supplementary Material online).

Module_8 (103 genes; ρ =-0.62; P=0.018; FDR = 0.080) is enriched for the function "G-protein coupled receptor activity" (supplementary table S4, Supplementary Material online). The top hub gene in this module (intraconectivity 0.90), Cobs_08138, is orthologous to the methuselah-like receptors in *D. melanogaster* (Friedrich and Jones 2016). Interestingly, mutant flies, carrying P-element insertions in one of these *methuselah* genes, live 35% longer and are significantly more resistant to stresses than wild-types (Lin et al., 1998). There are indications that these effects on lifespan and stress response may represent the ancestral function of methuselah receptors in *Drosophila* (Araújo et al., 2013). A similar function of the *methuselah* ortholog in *C. obscurior* would explain how a reduction in expression within older queens may facilitate life extension and greater stress resistance.

We also examined module_27 (808 genes) in more detail since it shows the strongest increase in connectivity in old compared with young queens (1.47 fold) of all modules (Fig. 2-3), suggesting an increased regulation of this module with age. The functions connected to this module, based on hubs (supplementary table S3, Supplementary Material online), increases in connectivity (supplementary table S5, Supplementary Material online) and GO terms (supplementary table S4, Supplementary Material online), indicate that in old queens an increased regulation of cell cycle, mitochondrial genes, immunity genes, transcriptional genes, and members of the protein synthesis machinery takes place, which is in stark contrast to the expected gene expression hallmarks of ageing in multicellular eukaryotes (Frenk and Houseley, 2018).

2.4.6 Robustness of GCN

Since our sample size of 14 is one lower than the recommended minimum of 15, we confirmed the robustness of our results by adding further samples from the same study (Wyschetzki et al., 2015). For this, we incorporated expression data from seven old queen samples that had mated with sterile males ("sham-mated") and then created eight further GCNs, seven of which contained one sham-mated queen (total n = 15) and one GCN containing all seven sham-mated queens (n = 21). We used preservation statistics (Langfelder and Horvath, 2008) to compare the modules of our GCN with these larger GCNs. Within each module, correlation, adjacency, connectivity, and variance explained by the eigen-node are compared between all nodes, and for each statistic a z-score is calculated based on 200 permutations. A composite z-summary of these statistics is calculated, whereby a threshold of 2 is deemed as necessary for classing a module as preserved, whereas a score greater than 10 offers strong evidence for module preservation. In each comparison against the 8 additional, larger GCNs, our age-biased modules scored at least 10, offering strong support that our GCN is not affected by a limited sample size.

2.4.7 Old-Biased Module Hubs Are Highly Conserved

We investigated evolutionary rates of the most connected genes within the old- and young-biased modules. Hub genes (intraconnectivity > 50%) of the five old-biased modules have significantly lower rates of protein evolution (dN/dS median: 0.081) than hubs in young-biased modules (median: 0.118; $P = 6.0 \times 10^{-4}$) or compared with all lowly connected genes (intraconnectivity < 50%; median: 0.101; P = 0.017; Fig. 2-4). We investigated the influence of expression levels on these results, since highly expressed genes are often found to be under stronger purifying selection (Drummond et al., 2005). However, expression levels, based on mean normalized read counts among all 14 samples, do not differ between hub genes of old-biased (mean: 291.5) and young-biased genes (mean: 326.8; W = 3160, P value = 0.18). These results suggest the hub genes of old-biased modules are highly constrained by strong purifying selection.

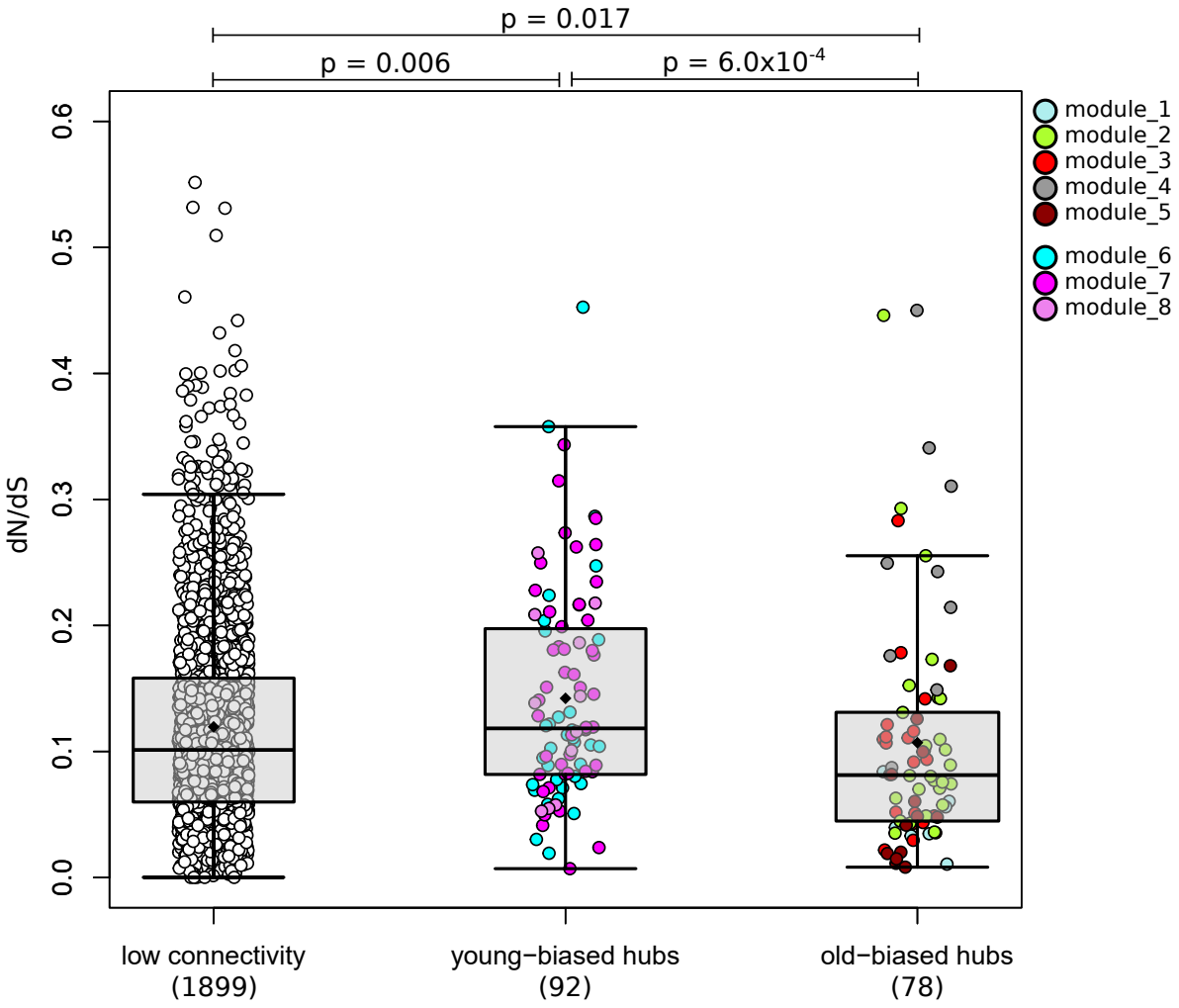


Figure 2-4. dN/dS rates in hub genes within young- and old-biased modules compared to lowly connected genes. Each dot represents a gene, which are coloured by the module membership. Whiskers of the boxplots represent up to 1.5 times the interquartile range. Black diamonds are means, and horizontal bars within the boxes are medians. Hub genes have an intraconnectivity > 50%; lowly connected: < 50%.

2.5 Conclusions

Evolutionary theory of ageing predicts a selection shadow on genes expressed late in life due to a reduction in effective population size with increasing age caused by extrinsic mortality (Kirkwood and Austad, 2000). We expected to find a reduced selection shadow in *C. obscurior* queens, as ant queens generally experience low extrinsic mortality. In support, we find compelling evidence for strong purifying selection on old-biased genes (significantly upregulated in seven old compared with seven young queens), for which evolutionary rates (dN/dS) are significantly lower than young-biased genes. In contrast, we find evidence of a

selection shadow in *D. melanogaster* where dN/dS is significantly higher for old-biased genes. Our results suggest, therefore, that *C. obscurior* queens are not affected by a selection shadow, so that genes important at old age cannot be expected to accumulate deleterious mutations at an increased rate compared with early-acting genes. This offers an explanation for the apparent lack of ageing and the high reproductive output of old ant queens.

Furthermore, we were interested in understanding whether *C. obscurior* queens show signs of ageing, especially within transcriptional regulation. This is a particularly intriguing question since the reproductive fitness of these ant queens remains high until old age, although they outlive their sterile siblings (Oettler and Schrempf, 2016). In fact, our analysis of coexpression networks in *C. obscurior* queens uncovers a significant increase in gene connectivity in old queens. This result offers evidence for an increased transcriptional regulation, especially in genes that are themselves involved in transcriptional regulation, as well as several genes involved in protein synthesis and degradation, which are important mechanisms for counteracting symptoms of ageing (Frenk and Houseley, 2018). Also, the analysis of old-biased modules (clusters of highly correlated genes, upregulated with age) within the GCN revealed an increase in expression and connectivity of genes involved in proteostasis, stress response, and transcriptional regulation (Fig. 2-2a), offering further support for well-regulated antiageing mechanisms. The hub genes within these old-biased modules are more highly conserved than hubs of young-biased modules, indicating strong purifying selection acting on these important central regulators.

In summary, we find no evidence of ageing in transcriptional regulation in *C. obscurior* queens. Low extrinsic mortality may allow selection to shape genes important at old age, which is evident in low divergence rates (dN/dS) of the hubs of old-biased modules. Well regulated molecular mechanisms likely allow the ant queens to counteract any symptoms of ageing, thus maintaining high reproductive fitness through-out life. We suggest further transcriptional studies into the short period directly before death when the reproductive out-put of *C. obscurior* queens decreases (Heinze and Schrempf, 2012; Kramer et al., 2015), which we expect to illuminate processes of transcriptional ageing. Transcriptional studies of other ant species are necessary to investigate the generality of our findings. In monogynous

ants, for example, in which individual queens are less dispensable, we would expect to observe an even weaker selection shadow. Also, *C. obscurior* queens are relatively short lived compared with other ant species. Selection strength on age-biased genes of extremely long-lived queens may be less affected by reductions in effective population sizes due to longer generation times. Further detailed research on individual pathways is important to understand how an upregulation of antiageing mechanisms occurs; especially proteomic analyses may reveal the true relationships between pathway members.

2.6 Materials and Methods

2.6.1 Data Set

Genome and proteome sequences of the *C. obscurior* genome, version 1.4, were obtained from the hymenopteragenome.org website (Elsik et al., 2016; accessed July 2018). We estimated gene functions based on orthology, primarily to *D. melanogaster*, as well as PFAM domains and GO terms. Putative protein functions were based on descriptions in the flybase (Thurmond et al. 2019) and UniProt (The UnitProt Consortium 2018) databases, unless otherwise stated. We calculated orthology to *D. melanogaster* with the method of reciprocal best BLAST hit (Rivera et al., 1998). For this, the proteomes of *C. obscurior* and *D. melanogaster* (v. 6.21; obtained from ftp://ftp.flybase.net/releases/current/dmel_r6.21/fasta/, accessed June 2018) were blasted against each other using BLASTp (BLAST 2.7.1+; (Camacho et al., 2009) and an e-value threshold of $1e^{-5}$. Reciprocal best BLAST hits were extracted from the output files using a custom perl script. Where no orthology could be detected using this method, protein sequences were blasted against the swissprot database with BLASTp (version 2.7.1+; Altschul et al., 1990) and the best hit was retained with an e-value < 0.05. Protein sequences were annotated with PFAM domains using pfamscan (Mistry and Finn, 2007), to which GO terms were mapped with pfam2GO (Mitchell et al., 2015).

Published RNAseq data were obtained for seven old (18weeks) and seven young (4weeks) ant queens from NCBI (Wyschetzki et al., 2015). These queens had each been individually reared from pupal stage in separate experimental colonies, each containing 20 workers and 10 larvae, originating from the genome reference population in Bahia, Brazil (Schrader et al.,

2014; Wyschetzki et al., 2015). Fastq files were mapped to the *C. obscurior* genome (version 1.4) with hisat2 (Kim et al., 2019) using default parameters. We then indexed and sorted sam files using samtools (version 1.7; Li et al., 2009) and generated counts per gene using htseq-count (Anders et al., 2015). All statistical analyses on these counts were carried out in R (version 3.5.1; R Core Team 2018). Where necessary, we corrected for multiple testing with the p.adjust function, using the fdr method (Benjamini and Hochberg, 1995). A total of 10,339 genes were expressed in at least two individuals with a read count of at least 10. This subset of genes was used for all analyses.

2.6.2 Determining Age-Biased Expression

Within this subset of 10,339 genes, we identified genes with age-biased expression by comparing expression in the seven old to the seven young samples. This was carried out with the R package DESeq2 at default settings (Love et al., 2014). Genes with an adjusted P value < 0.05 were deemed either old or young biased. All other genes were classified as unbiased.

2.6.3 Molecular Evolution and Selection Analyses

In order to carry out evolutionary analyses, we first determined orthology between the proteomes of *C. obscurior* and nine further ant species, which we either downloaded from the hymenopteragenome.org website (Elsik et al., 2016; accessed August 2020): *Atta cephalotes, Pogonomyrmex barbatus, Solenopsis invicta* and *Wasmannia auropunctata*; or NCBI (accessed August 2020): *Monomorium pharaonis, Temnothorax curvispinosus, Temnothorax longispinosus, Vollenhovia emery*. Data for *Crematogaster levior* were obtained from the authors of the genome publication upon request (Hartke et al., 2019). Orthology was determined with OrthoFinder (Emms and Kelly, 2015) at default settings. We chose orthologous groups that contained single gene copies within each of the 10 species. Protein sequences of each ortholog set were aligned with prank (version 170427; Löytynoja, 2014) at default settings. The corresponding CDS sequences were aligned using pal2nal (Suyama et al., 2006). CDS alignments were trimmed for poorly aligned codon positions with Gblocks (version 0.91b) with the following parameters: -t=c -b2=6 -b3=100000 -b4=1

-b5=h. We calculated dN/dS ratios using the null model of codeml in the PAML suite (Yang, 1997), using the following tree based on a published ant phylogeny (Ward et al., 2015):

(((((((Tlon,Tcur),Clev),Veme),Cobs), (Waur,Acep)),(Mpha,Sinv)),Pbar)

dN/dS ratios were used for analyses only if dS < 3. dN/dS ratios were compared between old-biased, young-biased, and unbiased genes using the Mann–Whitney test with the R function wilcox.test. In order to detect genes that contain codon sites under positive selection, we performed a likelihood-ratio test between models 7 (null hypothesis; dN/dS limited between 0 and 1) and 8 (alternative hypothesis; additional parameter allows dN/dS > 1) of the codeml program within the PAML suite (Yang, 1997). For this we used runmode 0, model 0, and set "NSsites" to 7 and 8.

2.6.4 Gene Coexpression Analysis

The expression counts data were normalized using the built-in median of ratios method implemented by default in DESeq2 (version 1.22.2; Love et al., 2014) and then transposed to a matrix containing genes in columns and samples in rows. With the reduced set of 10,339 genes, we created a signed weighted GCN using the WGCNA package (version 1.68; Langfelder and Horvath, 2008) that incorporated expression values from all 14 queen samples (7 young and 7 old). We followed the standard stepwise protocol (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/; last accessed March 2021), using a soft power of 14 and the biweight midcorrelation function for calculating coexpression similarity. Minimum module size was set at 30 and resulting modules with a correlation of at least 0.75 were merged. Hub genes within modules were determined based on the intramodular connectivity, which we calculated with the *intramodularConnectivity* function on the adjacency matrix, that was produced during the WGCNA pipeline. Age-biased modules were identified by correlating (Pearson) the eigengene of each module with the binary trait young/old. FDRs were calculated with the p.adjust function, and modules with an FDR < 0.1 were considered significantly related to age. To compare connectivity between young and old queens, we calculated connectivity with the *softConnectivity* function separately within the young and the old queen expression data. We used the same soft power value of 14 and the biweight midcorrelation function.

To create a visualization of the GCN, the topological overlap matrix was reduced to only contain genes with a topological overlap of at least 0.1 to at least one other gene. Edge and node files were created with the WGCNA function *exportNetworkToCytoscape*, using a threshold of 0.1. All further visualizations of the network were conducted in Cytoscape (v. 3.7.2, Shannon et al., 2003). To test the robustness of our GCN, we created seven additional GCNs each with one extra sample taken from the sham-mated queens previously published within the same data set as our main data used here (Wyschetzki et al., 2015). We also created one larger GCN containing all 7 sham- mated queens, therefore containing 21 samples. Each additional GCN was created with the same parameters as our original GCN and then compared with our original GCN with the built-in WGCNA function, *modulePreservation* and the *Zsummary* statistic was calculated. This composite *z*-score combines several comparative statistics, such as adjacency, connectivity, and proportion of variance explained, with a score of 10 suggested as a threshold for strong evidence of module preservation (Langfelder and Horvath, 2008).

2.6.5 GO Enrichment

GO term enrichment analyses were carried out with topGO (version 2.34.0; Alexa and Rahnenfuhrer, 2018) on the "biological process" category, using the classic algorithm. Node size was set to 5, Fisher statistic was implemented and we only kept GO terms that matched at least three genes and with a P value < 0.05. An FDR was added using the R function p.adjust and the method "fdr" (Benjamini and Hochberg, 1995); GO terms with an FDR < 0.1 were described in the text.

2.6.6 Drosophila melanogaster Data Set

To estimate evidence of a selection shadow in *D. melanogaster*, we accessed a recently compiled, but so far unpublished, RNAseq data set (SRA accession: PRJNA615318). This data set comprised RNAseq of 34 samples of five pooled flies. We used y1, w1118 mutant flies (full genotype: yw; +/+; +/+). These flies were maintained in laboratory conditions at 25°C, 12h:12h light:dark and 60% relative humidity.

2.6.7 Experimental Setup

Adult virgin females and males were collected separately, and 3 days later they were pooled together to freely mate. Eggs were laid in a controlled density (50–100 eggs per bottle) and developed until the adult stage in the same conditions as mentioned above. After eclosion, the offspring adult flies matured for one day. On the second day after eclosion, female and male flies were collected and transferred to a demographic cage. Each cage contained 130 females and 70 males. Once cages were set up, they were divided into four groups, which consisted of four different diet treatments. The diet treatments differed only in the content of yeast (20, 40, 80 or 120g) present in the fly food; the other ingredients were added in the same quantities in all diets (1 l water, 7 g agar, 50 g sugar, 10 ml 20% nipagin, and 6 ml propionic acid). All cages were maintained in the same conditions as described above.

2.6.8 Sampling and RNA Extractions

Female flies were sampled at two time points: 10 days (young) and 38 days (old). For each time point, sampling and dissections were done between 1 and 6 PM. Two groups of five females each (two replicates) were anesthetized in the fridge (approximately 4°C), and afterwards fat bodies were dissected in ice-cold 1xPBS. To guarantee that we sampled the entire fat body, we decided to use in this experiment fat bodies still attached to the cuticle—usually referred to as fat body enriched samples—because the cuticle is transcriptionally inactive. In ice-cold PBS, the female fly abdomens were opened, and the organs were carefully removed. Once the fat body tissue was clean, the abdomen cuticle was separated from the thorax. The fat body-enriched tissues were transferred into Eppendorf with 200 µl of homogenization buffer from the RNA isolation kit (MagMAXTM-96 Total RNA Isolation Kit from Thermo Fisher). The tissues were homogenized and stored at -80°C until RNA extraction. To sample head transcriptomes, flies were transferred to Eppendorfs and snap frozen with liquid nitrogen. Then the Eppendorfs were vigorously shaken to separate the heads from the bodies. The heads were then transferred into an Eppendorf containing 200 µl of homogenization buffer, from the RNA isolation kit. As described above, tissues were homogenized in the solution and kept at -80°C until RNA extraction. All extractions were

done using the MagMax robot from Thermo Fisher and the MagMAX^{TM}-96 Total RNA Isolation Kit. In this experiment there is a total of 34 samples: 2 time points x 4 diet treatments x 2 tissues = 16 groups, for each group we have 2–3 replicates (all groups have 2 replicates except for the second time point for 2% yeast diet, where we have 3 replicates). The sequencing of the RNA samples was done in BGI, Hong Kong, China. The samples were sequenced (paired end, 100 bp) on an Illumina HiSeq 4000 platform. Gene counts were generated in the same manner as for *C. obscurior* using genome version 6.21 (obtained from ftp://ftp.flybase.net/releases/current/dmel_r6.21/fasta/, accessed June 2018).

Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

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Author Contributions

M.C.H., E.B.B. conceived and initiated the project. M.C.H., E.B.B., and J.O. designed the study. M.C.H. wrote the manuscript and carried out most analyses. J.R. assisted with dN/dS analyses. M.C.H. and J.O. interpreted ant data, all authors interpreted comparative data. L.M.J. assisted in the interpretation of GO term enrichment analyses. T.F. and M.A.R. generated fly data and helped analyze them. M.C.H. wrote the manuscript which was revised and approved by all the authors.

Data Availability

Ant queen data are already published (Wyschetzki et al., 2015) and available at SRA under accessions: PRJNA293450 and PRJNA284224. *Drosophila* data are deposited on SRA under accession: PRJNA615318. Scripts are available on the github: https://github.com/MCH74/AgeingInCardiocondyla.

Chapter 3

3 Late-life fitness gains and reproductive death in *Cardiocondyla obscurior* ants

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3.1 Abstract

A key hypothesis for the occurrence of senescence is the decrease in selection strength due to the decrease in the proportion of newborns from parents attaining an advanced age – the so-called selection shadow. Strikingly, queens of social insects have long lifespans and reproductive senescence seems to be negligible. By life-long tracking of 99 *Cardiocondyla obscurior* (Formicidae: Myrmicinae) ant colonies, we find that queens shift to the production of sexuals in late life regardless of their absolute lifespan or the number of workers present. Furthermore, RNAseq analyses of old queens past their peak of reproductive performance showed the development of massive pathology while queens were still fertile, leading to rapid death. We conclude that the evolution of superorganismality is accompanied by "continuusparity", a life history strategy that is distinct from other iteroparous and semelparous strategies across the tree of life, in that it combines continuous reproduction with a fitness peak late in life.

Keywords: aging, selection shadow, senescence, social insects.

3.2 Introduction

The phenomenon that social insect queens live exceptionally long compared to solitary insects is widely recognized (Carey, 2001; Keller and Genoud, 1997). Given how prominent this is, however, only patchy information exists about the proximate mechanisms that are involved with the regulation of senescence, i.e., a phase marked by an increase in relative

mortality and a decrease in relative fecundity with age. Even less is known about the ultimate causes of social insect aging.

The classic trade-off between reproduction and maintenance shapes the life history strategy of species along a continuum between iteroparity (repeated events of reproduction) and semelparity (single event of reproduction) (Hughes, 2017). These strategies shape the way species age, i.e., how resources are allocated to maximize fitness. In iteroparous species, fitness decreases after the first reproductive peak(s). Thus, the strength of selection against age-specific mortality decreases with age, as the proportion of offspring that come from parents surviving to a specific age becomes smaller with time (Hamilton, 1966; Moorad et al., 2020a). This is known as the selection shadow, which begins with maturity (Williams, 1957) and may negatively affect reproductive performance and survival (i.e. senescence). Classic model systems in aging research, such as *Drosophila*, *Caenorhabditis* (but see the discussion of quasi-semelparous hermaphrodites, (Gems et al., 2021), mice, and humans, are of the iteroparous type, and a plethora of studies have revealed common mechanisms associated with senescence rate (Gems and Partridge, 2013). One prominent evolutionary theory of aging explains senescence by genes with antagonistic pleiotropic effects early and late in life (Williams, 1957). Semelparity instead predicts that organisms optimize their resources to one fitness peak, after which reproductive death occurs, i.e., allocation of remaining resources into fecundity and not into maintenance. Thus, selection acts strongly against senescence before the single reproductive event. To understand how investment in reproduction of ant queens changes with chronological age and how they age, it is vital to understand where they sit in this parity continuum.

Two dimensions are necessary to understand aging from an evolutionary perspective: the pace and the shape of demographic trajectories (Baudisch, 2011; Baudisch and Stott, 2019). The pace refers to factors that describe the time-scale (e.g., life expectancy), and the shape refers to time-standardized measures of the distribution of mortality and fertility across a life history. Studies that capture the shape of aging (age-specific reproduction and mortality) of ants are scarce (Cole, 2009), and based on punctual periods of growth and death of colonies, mostly of long-lived species in which colonies have a single queen (monogyny). Often such

field data correspond to less than 20% of the estimated lifespan of the species (*Atta cephalotes* (Perfecto and Vandermeer, 1993); *Atta colombica* (Wirth et al., 2003); *Pogonomyrmex owyheei* (Porter and Jorgensen, 1988); *Pogonomyrmex occidentalis* (Keeler, 1993); *Pogonomyrmex badius* (Tschinkel, 2017). These studies have generally failed to capture the end of the queen's lifespan and thus did not document senescence and lifetime reproductive investment. More complete yearly census data from *Pogonomyrmex barbatus* showed no relation between reproductive success (number of successfully established offspring colonies) and age (Ingram et al., 2013), but an increase in the production of male and female sexuals with age (Wagner and Gordon, 1999). In contrast, a study on a related species, *Pogonomyrmex occidentalis*, showed no correlation between sexual production and colony size (as a proxy for age) once colonies had initiated sexual reproduction (Cole and Wiernasz, 2000), suggesting that it is difficult to infer the dynamics of age, colony growth, and reproduction from field data. To better understand how aging, senescence, and reproductive investment are related in ants, complete lifetime production data of individual queens are needed.

To study aging patterns and senescence of social insect queens it is helpful to consider the colony as a superorganism (Boomsma and Gawne, 2018; Wheeler, 1911), analogous to a soma- (i.e., workers) and a germline (i.e., queens), where the investment into both castes is related and affects overall fitness (Bourke, 2007; Kramer and Schaible, 2013a). Ant species such as *Cardiocondyla obscurior*, where workers are completely sterile and seemingly without any direct reproductive power, exhibit an extreme case of superorganismality. By manipulating the colony size, we expected to find trade-offs (i.e., lifespan and investment in queen/worker/male offspring). We monitored the lifetime production of individual queens in 99 single-queen colonies maintained with 10, 20, or 30 workers each (Figure supplement 1A-B). Worker number corresponds to the colony size variation observed in the field (Schrader et al., 2014) Figure supplement 2) and was standardized weekly. Furthermore, queens whose egg production declined below a rate of ~10 eggs/week exhibited lethargic behavior, were less mobile, left the nest and/or were harassed by workers, and died within a few days to weeks. To assess if senescence was restricted particularly to the end of life, we compared RNAseq data of 18 of such *prope mortem* (Lat. near death) queens (between 28-29 weeks

old) and 18 middle-aged queens (between 19-21 weeks), which were in their peak of fertility (Figure supplement 3A-B). To compare queen and worker mortality, we tracked the survival of 40 workers kept in 40 colonies with either 10 or 20 nestmate workers.

3.3 Results

3.3.1 Reproductive strategy

The treatment (varying worker number) did not affect total production of eggs (Package "generalized linear mixed models using template finder" v. 1.1.2.3 in R) (Figure 3-1A, 10 vs. 20 workers: glmmTMB z-value = -0.38, p = 0.70 and 10 vs. 30: z-value = -0.96, p = 0.34) or worker pupae (Figure 3-1B, 10 vs. 20 workers: glmmTMB z-value = 0.09, p = 0.93 and 10 vs. 30: z-value = -0.39, p = 0.70). The treatment did also not affect the lifespan of queens (Figure supplement 6A, Cox proportional hazard regression model, Likelihood ratio test, X^2=1.57, p = 0.46), which was highly variable across treatments (Variation coefficient: 32.2%, Figure supplement 6B).

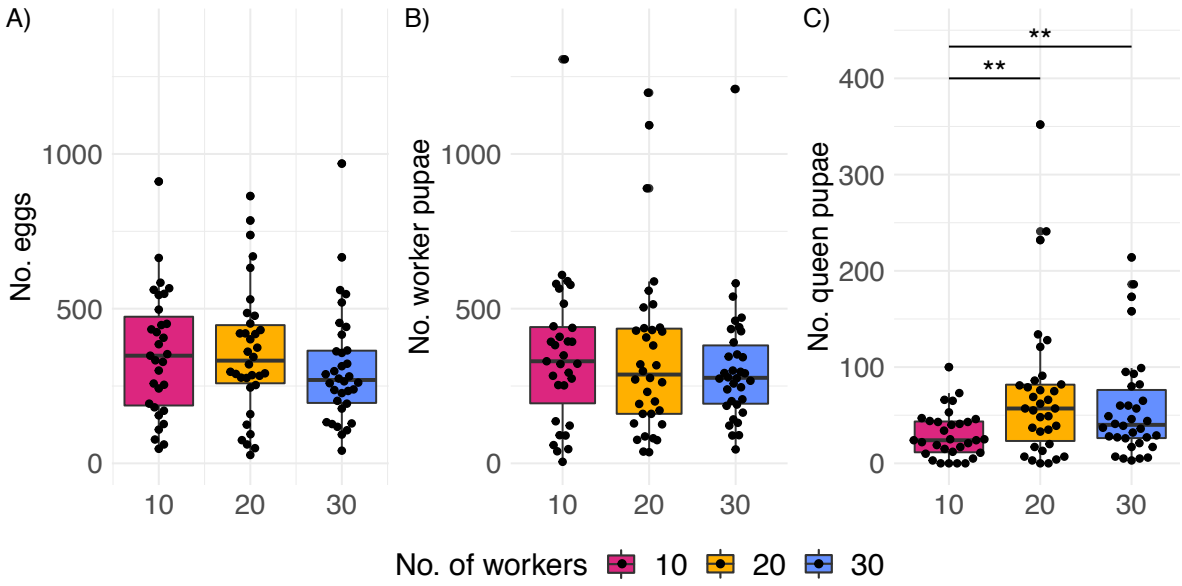

*Figure 3-1. Productivity of C. obscurior colonies across treatments. A) Total number eggs, B) worker pupae, and C) of queen pupae (N = 31, 34 and 34 for 10, 20 and 30 worker colonies, respectively). Significant differences are given with ** for p<0.01 and *** for p<0.001. Boxplots depict upper and lower quartile plus 1.5 IQR.*

We hypothesized that colonies that experienced a worker shortage would compensate by investing less into the production of new queens as these are larger and therefore more costly to produce. Indeed, queens with 10 workers (n = 31) produced significantly fewer queen pupae than queens with 20 (n = 34) (glmmTMB z-value = 2.81, effect size=1.97, p=0.005) and 30 workers (n = 34) (glmmTMB z-value = 2.58, effect size=1.78, p=0.009, Figure 3-1C) with no significant differences between 20 and 30 workers (glmmTMB z-value = –0.49, p=0.877). Similar results were obtained when accounting for the differences in biomass between workers and queens (Figure supplement 7, Supplementary file 1A). Probably due to difficulties assessing precise egg numbers which are reared in piles, and extremely worker-biased caste ratios (average pupae developed into workers = 0.86), egg counts do not reflect these subtle but significant differences. The median sex ratio (Queen/Queen + Male pupae) across treatments was 0.85 (25% and 75% quantiles = 0.79 and 0.90), and total production of male pupae (two types of males occur in *C. obscurior*: winged and wingless) was unaffected by the treatment (10 vs. 20: glmmTMB z-value =1.94, p =0.05 and 10 vs. 30: glmmTMB z-value =1.52, p = 0.13, figure supplement 8). Queens produced very low numbers of winged males during their lifetime (mean = 0.36, median = 0, N = 99).

A first peak in the investment in queen pupae occurred around 15 weeks after the colonies were established (Figure 3-2A), followed by an increasing queen-bias with age (Figure 3-2B). In general, new queens, which start a new colony, invest first in growing numbers of workers (ergonomic phase) and subsequently in the production of new sexuals, when the colony has reached the threshold required to enter the reproductive phase (Beekman et al., 1998; Macevicz and Oster, 1976; Oster and Wilson, 1978). This shift in caste ratio does not result from a drop of the production of pupae at the end of life. In contrast, pupa production is at its highest just before death (Figure supplement 9). Importantly, in *C. obscurior* this caste ratio shift appeared to be a fixed trait, independent of colony size and queen lifespan. Both, queens with short and long lifespans (below and above the mean lifespan of 25 weeks, Figure 3-2C and D respectively), equivalent to queens with low and high productivity, exhibited late life investment into queens.

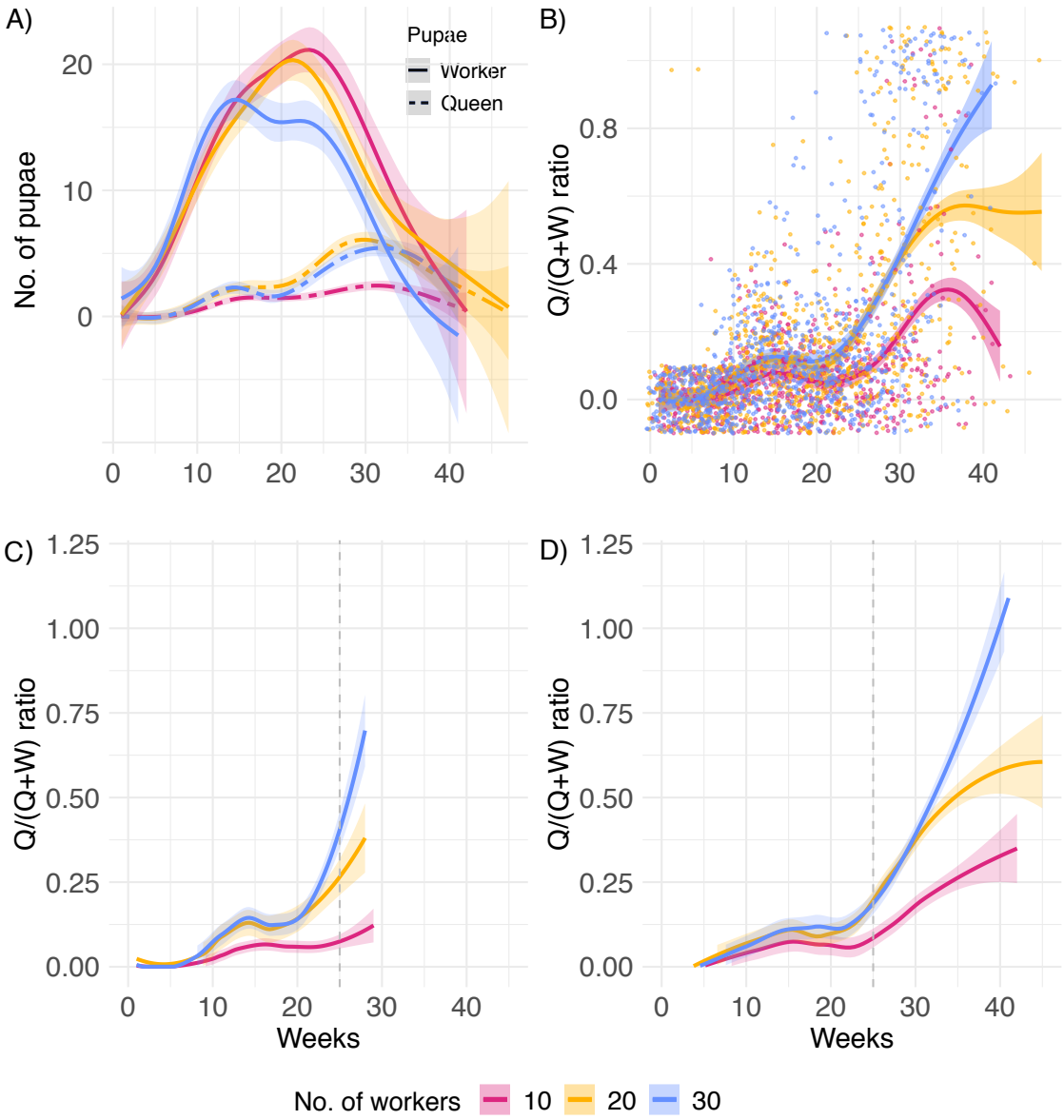

Figure 3-2. Life-time caste ratio. A) Numbers of worker and queen pupae produced over time, B) Queen/(Queen + Worker) pupae caste ratio produced by queens (n = 31, 34 and 34 for 10, 20 and 30 worker colonies respectively), C) Pupae caste ratio for queens with lifespan below (n = 44), and D) above the mean lifespan of 25 weeks, indicated by the dashed line (n = 55). After queen death, eggs and larvae were allowed to develop into pupae for a final count. Therefore, smooth splines extend ca. 4 weeks after queen death.

In addition to the effect on the caste ratio, the treatment had an effect at the colony level. We explored whether the quality of workers was affected by measuring the head width of workers produced over months 3 to 6 of the queen's lifetime (~5 workers per month). Head width of workers was 2% and 3% significantly smaller in small colonies with 10 workers than in

colonies with 20 (glmmTMB z-value = 2.22, p = 0.026) and 30 workers, respectively (glmmTMB z-value = 2.68, p = 0.007, Figure 3-3), but not different between colonies with 20 and 30 workers (glmmTMB z-value=0.22, p=0.97). This suggests that small colonies lack sufficient numbers of nurse or forage workers, and indeed colonies collected in the field have worker numbers closer to 20 or 30 (Schrader et al., 2014), Figure supplement 10).

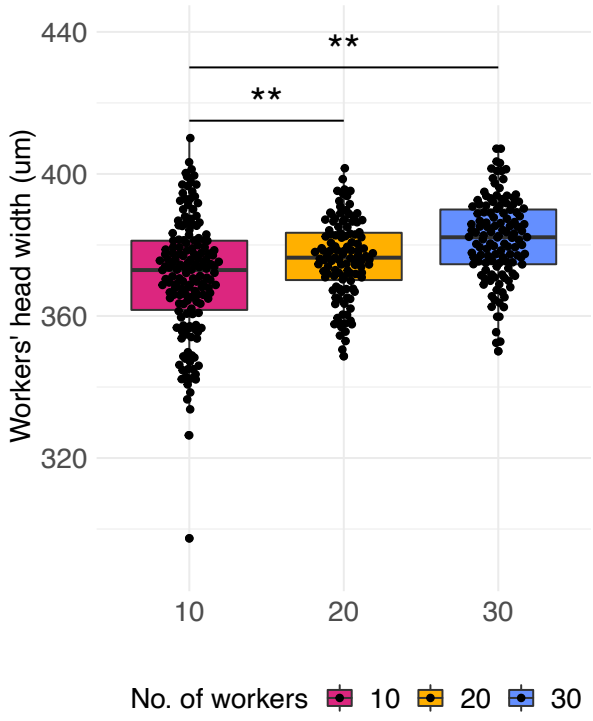

*Figure 3-3. Worker quality across treatments. Head width measurements of workers produced by queens of colonies with 10, 20 and 30 workers (n = 160, 112 and 68 respectively). Significant differences are given with * for p < 0.05 and ** for p < 0.01. Boxplots depict upper and lower quartile plus 1.5 IQR.*

After mean-standardizing queen age-specific mortality and fecundity (following Jones et al., 2014), we found that relative fecundity reached its maximum after ~16 weeks, before completion of the median lifespan (~25 weeks), and then decreased (Figure 3-4). Production of workers tightly followed the curve of egg production. Importantly, relative investment in queen and male pupae reached its maximum late in life (~28 weeks). This pattern is not due to the delay in development from egg to pupa, because queen and male development only lasts ~5 and ~3 weeks, respectively (Schrempf and Heinze, 2006). Furthermore, *C. obscurior* ant queens exhibited a below average level of adult mortality until week 30, after which

mortality increased above the average level (Figure supplement 11). This indicates maintenance of selection until after the peak of relative investment in sexual offspring. Therefore, queens continue to experience strong selection even at high ages, i.e., weeks after they reached the mean lifespan. Monitored workers in colonies with 10 or 20 nestmates did not differ in survival (Cox proportional hazard regression model, Likelihood ratio test, X^2=0.06, p = 0.8). Therefore, the mean-standardized age-specific mortality was calculated for the 40 workers. Note that regardless of the differences in time scale, the shape of mean-standardized mortality of workers was similar to that of queens (Figure supplement 12). This suggests that aging is a genetically fixed trait expressed by queens and workers alike.

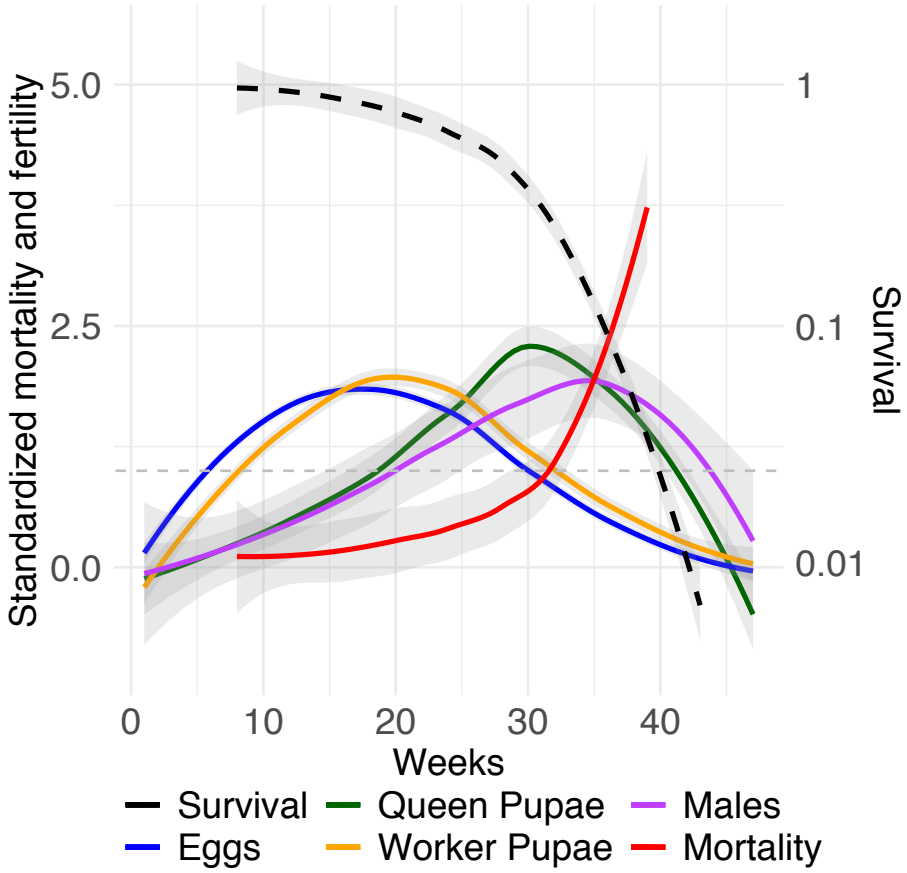

Figure 3-4. Relative mortality and fecundity as a function of age. Mean-standardization of age by dividing age-specific mortality and fecundity of queens (n = 99) by their mean after maturation (Jones et al. 2014). Survivorship (black dashed line) is depicted on a log scale. The graph uses a Loess smoothing method (span = 0.75) and a confidence interval of 95%. The dashed grey line at y = 1, indicates when relative mortality and fertility are equivalent to mean mortality and fertility.

3.3.2 RNAseq of prope mortem queens

To determine if queens show signs of reproductive senescence and loss of physiological function, we analyzed gene expression data of *prope mortem* queens exhibiting decreasing egg laying rates, and middle-aged queens that were at their peak reproductive performance. To account for possible effects of fertility we sampled queens with low, medium, and high egg productivity at 18 weeks of age (Figure supplement 13A-B). We subjected the head plus thorax and the gaster (see methods for terminology, Figure supplement 5) to RNAseq separately to assess if reproductive tissue shows a different physiological wear and tear than head-thorax tissue. The analyses revealed that head-thorax and gaster tissues showed similar mapping rates to the genome (Figure 14A-B), but that gaster samples had a lower GC content on average and more duplicated reads (Figure 14C-F) in *prope mortem* queens compared to middle-aged queens.

Of the 20,006 expressed genes in head-thorax tissue, 3,565 (17.8%) genes were differentially expressed between middle-aged and *prope mortem* queens (after FDR adjustment $p < 0.001$, DESeq2, Source data 9). Of these, 1,725 genes (48%) were upregulated, and 1,840 genes (52%) were downregulated in *prope mortem* queens compared to middle-aged ones. GO-term enrichment revealed signs of rapid physiological decay of *prope mortem* queens, such as reduced translation, proteasomal, ribosomal, and mitochondrial function (Fisher test using the weight01 algorithm, $p<0.05$, Supplementary File 1B, Figure 3-5), increased splicing and transcript processing (Supplementary File 1C, Figure supplement 15). Such processes have previously been related to aging in several model organisms (López-Otín et al., 2013); e.g., the loss of protein homeostasis (Hipp et al., 2019), the decrease in ribosomal proteins (Walther et al., 2015), alterations in the mitochondrial function (Green et al., 2011), disruption of splicing (Bhadra et al., 2020), and others (Harries et al., 2011). Another characteristic of aging, changes in gene connectivity among gene expression networks found in mice (Southworth et al., 2009), was not affected by age in *C. obscurior* (calculated using the softConnectivity and the biweight midcorrelation functions on gene networks for middle and *prope mortem* queens using WGCNA, and modeled using glmmTMB, Z-value =-1.7, $p = 0.09$). PCA ordination of the head-thorax tissue separated middle-aged and *prope mortem* queens by age (PERMANOVA test, F-value= 7.59, $p < 0.001$), but not by fertility (F-value

= 1.09, P = 0.26) or duplication percentage (Figure supplement 16A-B).

In the gaster tissue, 4,832 (24.3%) of 19,925 expressed genes were differentially expressed between age groups (after FDR adjustment $p < 0.001$, DESeq2, Source data 10). Of these, 2,306 genes were upregulated (48%) and 2,526 downregulated (52%) in *prope mortem* queens compared to middle-aged queens. GO-term enrichment likewise showed that many fundamental processes were affected in *prope mortem* queens, such as DNA damage, telomere maintenance, and enrichment of transcription processes (Supplementary File 1D, Figure supplement 16), and among others processes related to protein processing, glycolytic processes, and the Notch signaling pathway were downregulated (Supplementary File 1E, Figure supplement 17). In contrast to head-thorax tissue, gene connectivity among gene co-expression networks in the gaster was significantly different (*prope mortem* queens: median = 69.45, and middle-aged queens: median = 52.56) (glmmTMB, Z-value =-19.5, $p < 2e\text{-}16$), but contrary to what was found for aged mice (Southworth et al., 2009).

The PCA of the 500 most variable expressed genes in gaster tissue shows that the samples group according to age (PERMANOVA test, F-value= 13.91, $P < 0.001$), fertility level (F-value= 1.95, $p = 0.04$), and also the percentage of duplicated reads in the libraries (F-value= 4.83, $P = 0.002$, Figure supplement 18A-B). This is not a typical technical artefact (no correlation to sequencing lane, RNA concentration or quality). Spike-in reads were used as a control for library preparation and showed a positive linear relationship between expected and observed reads independent of age group, tissues, and lanes (Figure supplement 19A-C). However, this linear relationship has different slopes among age groups in the gaster samples (Figure supplement 19B), indicating biological changes with age pertaining specifically to the gaster.

Given these discrepancies between tissue types, 104 GO terms were significantly enriched in both tissues, of these 44 in *prope mortem* queens (Supplementary File 1F) and 60 in middle-aged queens (Supplementary File 1G). Thus, signs of similar physiological pathologies occur in reproductive and non-reproductive tissue.

Figure 3-5. Caption on next page.

Figure 3-5.(Previous page) Enriched GO-terms downregulated in prope mortem queens compared to middle-aged queens the head-thorax tissue. Functional annotation and enrichment analysis using topGO (version 2.46.0) and the weight01 algorithm to calculate significance for A) biological processes, B) cellular components, and C) molecular functions.

3.4 Discussion

The near-ubiquitous occurrence of senescence has been explained by two classic prevailing evolutionary theories, mutation accumulation and antagonistic pleiotropy (Williams, 1957). These theories have in common the basic assumption of the existence of a "selection shadow:" a decrease in the force of natural selection with age. The selection shadow leads to loss-of-function and senescence, i.e., an increase in relative mortality and a decrease in relative fecundity with age (Maklakov and Chapman, 2019). Originally explained as a consequence of extrinsic mortality, models have shown that the strength of selection is in fact influenced by the proportion of offspring coming from parents that survived to a certain age (Hamilton, 1966; Moorad et al., 2020a). Extensive demographic data show a huge diversity of aging patterns across metazoan species, ranging from 20 times the average mortality at terminal age to less than a half in other species (Cohen, 2018; Jones et al., 2014). In some cases a short phase of senescence is self-evident, for example in semelparous species such as salmon, where death follows reproduction to provision the next generation with resources.

Here we show for the first time the shape of aging in a social insect. While fecundity decreases in the ant *Cardiocondyla obscurior*, reflecting reproductive senescence, investment into sexuals reaches a maximum late in life, regardless of individual fitness (queen lifespan or total egg productivity) and colony size. Males in this species usually transfer an excess amount of sperm (Schrempf and Heinze, 2008), and only one queen showed signs of sperm depletion and produced only males at the end of her life. Therefore, reproductive senescence cannot be explained by sperm depletion. The magnitude of the investment (i.e., number of queen pupae produced) is affected by the number of workers available. In *C. obscurior*, most new queens were produced by queens older than the mean queen lifespan, indicating that queens continue to experience strong selection at high ages.

This is in line with the hypothesis that the strength of selection against age-specific mortality is proportional to the probability for any offspring in the population to be produced by parents of that age and older.

Strikingly, relative mortality did not increase directly after maturity or after total egg production started decreasing, but after the production of sexual pupae had reached its maximum. Indeed, transcriptome profiles of *prope mortem* queens shortly after this investment peak, which produced decreasing numbers of eggs, revealed signs of a broad range of physiological pathologies. The changes seemed stronger in the gaster (e.g., percentage of duplicated reads), which contains the reproductive organs and most of the digestive system, but to a similar extent occurred in head and thorax, containing most neuronal and muscle tissue. Such a systemic breakdown is expected assuming that the entire physiology is optimized towards a fitness peak. Strikingly, a comparative transcriptomic study of 8 week young queens with fully mature *C. obscurior* queens at or close to their peak fecundity (18 weeks old) did not find signs indicative of aging, but in comparison to aged *Drosophila* flies an opposite regulation (e.g. cellular ketone, carbohydrate, and organic acid metabolic processes) and genes (e.g. *ref(2)P*, *emp*, *P5cr-2*, *CCHa2*, *NLaz*, *Sirt6*) involved in aging (Wyschetzki et al., 2015). Furthermore, a gene co-expression network study using the same data showed higher connectivity in old queens indicating increased transcriptional regulation with age (Harrison et al., 2021). Together, this suggests that the physiology of queens is maintained until the fitness peak is reached, at which time they undergo physiological deterioration, while still being reproductively active. This pattern is reminiscent of semelparous species with reproductive death rather than that typical of iteroparous species in which selection against age-specific mortality decreases after a first reproduction event and actuarial senescence unfolds under the selection shadow.

3.5 Conclusion

Superorganismality is a major evolutionary transition, and this transition is accompanied by a change in the mode of reproduction. We propose that the evolution of “continuusparity” (Lat.: “continuus” meaning incessant/successive; and “parere” meaning giving birth), i.e., the

combination of life-long continuous reproduction and increasing fitness returns late in life, underlies the delay of the selection shadow, the maintenance of selection strength against age-specific mortality, a brief phase of senescence late in life, and finally reproductive death. This is not to be confused with the meaning of the term negligible senescence, as actuarial and reproductive senescence clearly occur at the end of life.

"Continuusparity" emerges as a combination of iteroparous and semelparous characteristics: reproduction resembling continuous iteroparous species but without the inter-parous non-reproductive breaks, during which nests are built, mating occurs, resources are acquired, etc. The iteroparous solitary ancestor of ants is thought to be related to mud dauber wasps (Sphecidae) and cockroach wasps (Ampulicidae) (Ward, 2014), parasitoid wasps with mass provisioning. This combines with aging/resource allocation patterns of semelparous species (which are in contrast mostly short-lived), optimized towards one reproductive episode at the end of life, followed by reproductive death. Continuous reproduction is possible because no extra time or energy is necessary for further acquisition of resources, brood care, territoriality, etc., because of the extended phenotype, the colony. With time the colony increases in size, and resources increase accordingly, analogous to solitary organisms with life-long growth (Keller, 1998). Continuous reproduction is further facilitated by the presence of a spermatheca in female insects, which allows for a single mating event and lifelong sperm storage. Thus, the costs of additional matings are zero.

We propose that continuusparity and its effect on the shape of queen aging is a property of superorganismality, and that this life history strategy ultimately underlies the evolution of long lifespans of social insects. For the pace of aging, it is important whether queen and worker interests are aligned, and whether direct and indirect reproductive investments of queens and workers are optimized. With respect to which proximate mechanisms regulate aging in social insects, this framework predicts that there are no genes/pathways with antagonistic pleiotropic effects, because there is no "later in life" past the fitness peak. Under this perspective, many questions remain open. Queens appear to have different properties that underlie both lifespan and fertility (Kramer et al 2015), probably determined during larval development (Schultner et al., 2017), and which are key to understand why some

individuals live longer than others. What is this, and how is it determined, maintained, and terminated? Queens do not senesce until shortly before death, then how is the trade-off between somatic maintenance and reproduction resolved? Using this framework, we can now start to study the proximate regulators that maintain the homeostasis in *C. obscurior* ant queens, which remain hidden in the excess number of associated processes.

3.6 Materials and Methods

3.6.1 The species

Cardiocondyla obscurior is probably the best studied ant species with respect to aging due to the relatively short lifespan of queens (~6 months). Colonies comprise a few queens (body length ~3mm), a few dozen workers (~2mm), and nest in small cavities in dead twigs, aborted fruits, rolled leaves, under bark etc. in trees and shrubs (Schrader et al., 2014). Virgin queens usually mate once with related wingless males inside the natal colony (Heinze and Hölldobler, 2019; Schmidt et al., 2016), generally stay in the nest, and new colonies are formed by budding of colony fragments. This mode of reproduction from small propagules allows for successful colonization of disturbed habitat in warm climates around the world (Heinze, 2017; Heinze and Delabie, 2005). Various social, environmental, and biotic factors affect the lifespan of queens (Oettler and Schrempf, 2016). Queens that lay more eggs (total output and weekly rate) live longer than less fecund queens, irrespective of body size (Kramer et al., 2015), and thus seem to evade the common trade-off between reproduction and maintenance.

3.6.2 Reproductive strategy

We set up 138 freshly eclosed queens from stock colonies of a Japanese population (OypB, from the Oonoyama Park in Naha, Okinawa) established in the laboratory since 2011. The experiment took place between January 2019 and January 2020. Queens were allowed to mate with a single wingless male and were placed in nest boxes with either 10, 20 or 30 workers from the maternal colony to establish monogynous colonies (n = 46 each). These numbers of workers represent the naturally occurring number in the field and correspond to

the first, median and third quantile of number of workers of this population (n = 62, median = 28.5, Figure 1 – figure supplement 2). The colony was set up with half of the workers selected from inside of the nest near the brood (younger nurses) of the stock colonies, and the other half from outside the nest (older foragers) in order to minimize a putative effect of worker age on the queen (Giehr et al., 2017). Colonies were kept under a 12 h dark 22°C/12 h light 26°C cycle and fed ad libitum three times per week with diluted honey (0.6:1 honey: distillated water), cockroaches and flies. Once per week workers, eggs, and all pupae (worker, queen, winged and wingless male) were counted and queen survival was monitored. Additionally, the number of workers was standardized to the assigned treatment, and newly produced sexual pupae produced were removed. *C. obscurior* workers are sterile, and all produced offspring originated from the focal queen. Queen control over caste fate was assumed, as caste fate can be determined as early as the last embryonic stage, 7 days after egg laying (Schultner et al., 2021). The number of counted eggs correlates with the production of workers, queens, and the workers and queens together (Figure 2 – figure supplement 2A-C, Kendall's rank correlation test, $p < 0.001$: eggs-worker pupae, $\tau = 0.70$; eggs-queen pupae, $\tau = 0.59$; eggs-worker and queen pupae, $\tau = 0.73$). Pupae might have been counted more precisely than eggs, especially when larger number of eggs were produced. Pupae are hardly missed, compared to eggs which tend to cluster together. Eggs and worker pupae might have been counted more than once, as development lasts a median of 8 and 18 days for eggs and worker pupae, respectively. Colonies were counted after ca. 4 weeks after the queen's deaths, until the last eggs had developed into pupae. Finally, three colonies (10 worker treatment) were not considered in the analysis as they were accidentally killed, leaving a total of 99 colonies for life-long tracking and 36 colonies for RNA-seq analysis.

3.6.3 Worker aging

To examine worker aging, 40 focal unmarked worker pupae were set up in individual colonies with 10 or 20 marked workers (n=20 each). These two treatments were selected, because colonies with 20 and 30 workers did not differ in queen productivity. Marking of non-focal workers was done by clipping the tarsus of the middle right leg. Colonies were set up with brood (5 larvae in the 10 workers colonies, and 10 in the 20 workers colonies), and

two wingless queens to avoid a queenless period in case one died. The survival of the focal worker was monitored, and the number of marked workers, queens and larvae was standardized weekly to the assigned treatment. Newly produced pupae were removed. Dead marked workers were replaced with fresh worker pupae, which were marked one or two days after eclosion to avoid confusion with the non-clipped focal worker. Dead queens were replaced with adult ones.

3.6.4 Offspring investment

360 freshly eclosed adult workers from the queen's colonies were sampled monthly for head width measurements (from the 3rd to 6th month of the queen's life, and up to five workers depending on availability). Workers were dried, pinned, and blindly measured using a Keyence Microscope 200X. A single worker was chosen randomly and measured 10 times to obtain a proxy for measurement error (Mean = 383.61 μm, standard deviation=5.05 μm).

3.6.5 Statistical tests

To test for significant differences between treatments, we used generalized linear mixed effects models within the R package glmmTMB (R version 3.5.2, (Pinheiro et al., 2011)) and a negative binomial distribution for count data. If the count data and caste investment ratios were log transformed, a Gaussian family distribution was used. The dependent variable was analyzed as a function of the fixed effects: treatment (Number of workers as a factor), and random effects: stock nest and box of origin, box of set up, set up date. All models were also graphically checked for consistency and model diagnostics were performed using the DHARMA package (R version 0.3.3.0, (Hartig, 2020)). Caste ratio was calculated as queen over the total caste investment (as Queen*c / [Queen*c + Worker]). The coefficient or correction factor *c* is used, as the dry average weight measurements of queen over workers to the power conversion factor of 0.7 assuming differences in metabolic rates between queens and workers adopting the logic for sex ratio investment (Boomsma, 1989). As this is an assumption, we used different values of c. The results are robust power conversion values of 0.6-1 (Supplementary File 1A). To test for differences in head width, we used the average of the head width measurements of the workers per time point (each month). Predictions of

the data were visualized using the loess method with the geom_smooth function and default span (ggplot2 v.3.3.2). Relative mortality and fecundity as a function of age were mean-standardized by dividing age-specific mortality and fecundity by their mean after maturation, following (Jones et al., 2014). In contrast to Jones et al 2014, the whole life range was considered until death, since removing the last gap of age (where the 5% of survivorship occurs) showed similar results. Age-specific mortality without the mean-standardization was also estimated for the 135 queens (99 and 36 ant queens for RNAseq) using a survival Bayesian trajectory analysis (Figure 4 – figure supplement 1). Data is available as Source data files 1 - 7, and the R-script used as file Source Code File 1.

3.6.6 Prope mortem queens selection

To obtain samples of low, medium, and highly productive queens, 18 queens at age 19-21 weeks were sacrificed for RNAseq based on egg productivity until week 18. Values of weekly egg productivity below the first quantile for the treatment group (colony size) were considered as low, values between the first and the third quantile as medium, and values greater than the third quantile as high. Other 18 queens were monitored until they showed decreasing fertility (Figure supplement 3) and one or more of the following signs of senescence: lethargy, loss of mobility, presence outside the nest and/or harassment by workers. These senescent queens were also selected based on low, medium, and high fertility, and then sacrificed (28-49 weeks old). Queens were snap-frozen in liquid nitrogen, after the head and thorax was separated from the gaster with a blade between the petiolus and post-petiolus in a drop of PBT 0.3% (Phosphate Buffered Saline and Tween 20). During this procedure, queens were manipulated for less than 1 minute.

3.6.7 Terminology

What we refer to as “thorax” actually refers to the thorax plus the fused first abdominal segment, together making up the “mesosoma” in the Hymenoptera. The “metasoma” in Hymenoptera comprises the segments making up the constriction plus the hind end. In the ant subfamily Myrmicinae, this constriction is made of two segments: the petiole corresponds to the second, constricted, abdominal segment, while the post-petiole refers to the third,

constricted, abdominal segment. The "gaster" refers to the bulbous posterior part (Figure supplement 5).

3.6.8 RNA-seq

Total RNA was extracted using the ReliaPrep kit (Promega) from the 72 samples (36 queens, two samples per queen: head-thorax and gaster). Spike-In RNA Variant Controls (SIRV-Set 3 Lexogen #05101, Lot 5746/001492) were spiked to a 2% fraction of the total RNA (measured using Bioanalyzer – Agilent Technologies). Eight of the 72 samples showed RIN values below 7 (gaster samples from older queens which seemed more degraded). For those samples, the concentration of RNA was estimated based on the mean value of the non-degraded gaster samples. Total RNA was amplified using SPIA (single primer isothermal amplification, Ovation RNA-seq System V2, Tecan) prior to cDNA generation. The library preparation and sequencing (100bp PE) was performed at the Cologne Center for Genomics, using Nextera XT sequencing on a NovaSeq6000 platform. Reads were trimmed with fastp v.0.20.1 to a minimum length 70 and from Nextera adapters. Then SortMeRNA version 4.2.0. was used to discard undesired rRNA reads using the default data base (smr_v4.3_default_db. fasta). Remaining reads were aligned using hisat2 (version 2.1.0) to the newest version of the genome (Cobs.2.1., Errbii et al. 2021). Putative splice sites were obtained using gffread (version 0.12.1) and the extensive transposable elements annotation v.2.1 (Errbii et al., 2021) was considered for the mapping procedure. Samtools (version 1.9) was used to sort and convert .sam into .bam files. Raw sequencing data will be deposited in NCBI.

3.6.9 Gene expression analysis

After filtering genes with 0 values, we used a gene set of 20.006 genes for the head-thorax analysis and 19.925 genes for the gaster, respectively. PCA plots were produced to visualize the samples after variance stabilizing transformation. An analysis of the homogeneity of group dispersions (variances) was performed (multivariate analogue of Levene's test for homogeneity of variances, with the function permutest and 999 permutations (vegan Package v. 2.5-7) to test for differences in the variance among the age groups (middle aged and prope mortem queens) (betadisper, vegan package).

Subsequently, a nonparametric multivariate ANOVA (PERMANOVA) test was performed (999 permutations) with the design model Head-Thorax_expression ~ AgeGroup + Fertility, with two (middle-aged, old) and three levels (low, medium and high fertility) to test for statistical differences in the transcriptomic profiles due to age group (senescent or not) and level of fertility (low, medium and high) using the adonis function (vegan Package), with the default bray distance method. Age and fertility (average number of laid eggs per week) were scaled and centered. Then, differential expression was analyzed using the design = ~ Eggs per week + Age group, with the R package DESeq2 (v. 1.28.1). Age group was used as categorical variable with two levels (middle-aged and *prope mortem*), and log2FC were calculated as log2 [*prope mortem* / middle-aged]. The cut-off threshold of statistical significance (alpha parameter) was set as 0.001 after p-value adjustment with FDR. Functional annotation and enrichment analysis was done using topGO (version 2.46.0) and the weight01 algorithm implemented in the package.

A signed weighted co-expression network was constructed using the WGCNA package (v. 1.70-3), and the count data transformed using variance-stabilizing transformation from the DESeq2 package after excluding genes with 0 read values. For the head-thorax tissue network, the total set of 20.006 genes was used, and the WGCNA was performed with default parameters and a soft threshold power 14. We compared the connectivity of the two separate networks, one for middle aged and one for older queens, with the softConnectivity and the biweight midcorrelation functions. The soft threshold power selected was 14 based on the scale-free fit index as recommended by the manual.

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Chapter 4

4 Fitness variation in queens of the ant *Cardiocondyla obscurior*

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In preparation.

4.1 Abstract

Fitness variation between individuals of a population is common and is caused by genetic and non-genetic (epigenetic and environmental) differences. Queens of the ant *Cardiocondyla obscurior* show high variability in lifespan and fertility. From life-long monitored queens exhibiting either high or low productivity, we set up inbred lines and tested whether fecundity and lifespan are correlated with those of F_1 daughter queens. We found no correlation between maternal and offspring traits, indicating low heritability of life-history traits. In contrast, some lines had lower fitness, suggesting background variation, signs of inbreeding depression, or variation in male quality.

Keywords: ants, productivity, reproductive success, trait heritability

4.2 Introduction

Evolution by natural selection is possible if traits have a variable and a heritable component (Stearns, 1992). In 1930 Fisher (edited in Fisher, 1999) predicted low heritability of fitness traits (or life-history traits) in equilibrium. Fitness traits or life-history traits, i.e., traits that affect fitness closely such as fertility, show high variability in natural populations. They are presumed to have low additive genetic variance due to rapid fixation in a population. Additionally, life-history traits might depend on several genetic loci hindering their heritability. This is in contrast to traits more distantly associated with fitness, such as morphological ones, that have a higher heritable component (Merilä and Sheldon, 2000).

This idea has been challenged as traits closely related to fitness show in some species a disproportionate large unexplained variance due to environmental and nonadditive genetic sources (Merilä and Sheldon, 2000). For instance, two studies in *Drosophila melanogaster* (a within-generation paternal half-sib and a cross-generation parent-offspring assay) showed that much of the phenotypic variation in adult female fitness was attributable to genotype (Long et al., 2009; Pischedda and Chippindale, 2006). Interestingly, such a relationship does not occur in sons (Pischedda and Chippindale, 2006). Other effects (not ascribed to inheritance) of the parental phenotype into the offspring phenotype occur, such as developmental plasticity. In that case, they are referred to as parental effects (Uller, 2008). Parental-induced plasticity might occur if there is a strong correlation between the parent and the offspring environment or if the offspring environment is a direct consequence of the parental phenotype (Uller, 2008).

Heritability and parental effects could explain some of the variability observed in life-history traits. In social insects, longevity and fecundity are positively correlated, in the way that reproductives exhibit a long and very fertile life compared to other organisms (Lucas and Keller, 2017). Data are lacking to understand why and how queens show high variability in these both traits. A possible explanation is that the offspring inherit genetic components in fertility and longevity. Here we use the ant species *Cardiocondyla obscurior* to investigate the heritability of life-history traits, as ant colonies show high variability in egg productivity and lifespan within the same population (Jaimes-Nino et al., 2022). We test if maternal (F_0) traits such as egg/queen pupae lifetime productivity and lifespan are good predictors of the offspring (F_1) traits productivity and longevity. Additionally, we study if environmental maternal effects are present after one generation, testing for F_1 productivity patterns depending on colony size experienced by the F_0.

We selected maternal single-queen colonies based on their egg productivity as high or low productive. Daughter queen pupae of such colonies were mated with brothers, as typical for the species (Heinze, 2017) and their productivity was monitored throughout life. We tested for effects on the offspring quality in terms of F_1 queen lifespan, and productivity. The colony size of maternal queens was standardized to 10, 20, and 30 workers throughout the whole

experiment. As queens in colonies with 10 workers produce significantly fewer queen pupae than in 20- or 30-workers colonies (Jaimes-Nino et al., 2022), we tested if the colony conditions (environment) that affected the productivity of the maternal queen could have an effect on the offspring quality (i.e. maternal effect). Additionally, we controlled for maternal age as maternal age is known to affect the offspring's pre-adult and adult performance in other organisms (Ivimey-Cook and Moorad, 2020; Priest et al., 2002). Specifically, we tested if maternal age explained the F_1 queen productivity/lifespan.

4.3 Material and methods

Complete reproduction data of 99 queens in individual colonies were recorded for their whole lifespan, and 36 other queens were sacrificed shortly before death after egg laying had ceased for RNA sequencing (Jaimes-Nino et al., 2022). From this batch, 16 queens showing high and low productivity were selected as F_0 queens, based on their total productivity at 15 weeks of age (Figure 4-1A). 15 weeks is a good predictor for lifetime productivity (Pearson's r =0.74, df= 96, P-value <0.001, Figure 4-S1).

To minimize confounding putative effects of the age of F_0 queens on the fitness of the offspring, F_1 queens were selected when F_0 queens were between 25-43 weeks old (median = 31) at the moment of F_1 eclosure.

The offspring of the maternal colonies (F_1) was allowed to mate between siblings and then transferred to a new nest, together with 10 workers. The colony size was kept constant throughout the experiment, and monthly standardized. Between 1 to 6 new queens (F_1) per maternal queen were successfully raised and monitored for their whole lifespan (except for two F_1 queens out of 56). The productivity of the F_1 queens (egg, workers, and pupae production) was recorded starting from the 10^{th} week after eclosion. Newly produced queen pupae (F_2) were counted and removed weekly. The age at death of the F_0 queen was recorded for 10 of the F_0 queens. The remaining F_0 queens (n=6) were sacrificed for RNAseq after egg laying ceased (in Jaimes-Nino et al., 2022) between 36 to 49 weeks after eclosure. This means that most of the F_0 egg production was censored.

To test for an environmental effect of colony size on fitness, the 16 queens were chosen from three different colony sizes which have been shown to affect F_1 worker head-width (used as a proxy of body size). Their colony size was weekly standardized to 10, 20, or 30 workers. Colonies were kept under a 12 h dark 22°C/12 h light 26°C cycle, 75-80% humidity, and fed ad libitum three times per week with diluted honey (0.6:1 honey: distilled water), cockroaches and flies.

4.4 Statistical tests

For comparing the productivity of the F_0 queens a linear model with normal distribution was used, with productivity categorized at week 15 as a predictor (either Low or High). To assess differences in lifespan between F_0 queens, a Kaplan-Meier log-rank test was used with the *survdiff* function (survival R package, v. 3.4-0).

To test for a lineage effect on the productivity (eggs, worker, queen pupae, and caste ratio) of the F_0 queens a generalized linear mixed model (R package 'generalized linear mixed models using template model builder v. 1.1.2.3; R version 3.5.2, Pinheiro et al., 2011) was used, under a negative binomial distribution (nbinom2). We used the *glht* function (multcomp R package, v.1.4-20) as a post hoc test for comparing multiple means. Differences in survival depending on maternal lineage were tested fitting a proportional hazards regression model (function *coxph*, survival package).

We tested if maternal productivity influenced the daughter's productivity (eggs, worker, and queen pupae production), and also for a correlation between F_0 and F_1 lifespans. We used the maternal colony ID as a random effect in the model. The explanatory variable F_0 productivity (High or Low) was used as a factor and F_0 colony size as a continuous variable. As aging is a non-linear process in *C. obscurior* (Jaimes-Nino et al., 2022) we included a binary categorical variable in the model for the mother's age as senescent or not. This meant that in case the maternal queen exhibited reproductive senescence at the moment of producing the daughter queen, and scored her as senescent or not senescent. Reproductive senescence refers to a decrease in egg laying with age. All models were graphically checked using the

DHARMA package (R version 0.3.3.0, Hartig, 2020). We modeled the survival probabilities of the F_1 queens using the *coxme* function (coxme R package v. 2.2-16) and used the random variable as described before.

4.5 Results

The selected maternal queens (F_0) differed in productivity (eggs, lm T-value=-8.58, $p < 0.001$, Figure 4-1A). While lifespan and productivity are positively correlated in the maternal 99 queens (Figure 4-1B), the 16 selected F_0 queens did not differ in their longevity (Kaplan-Meier log-rank test, $X^2_1=0.3$, $p = 0.6$).

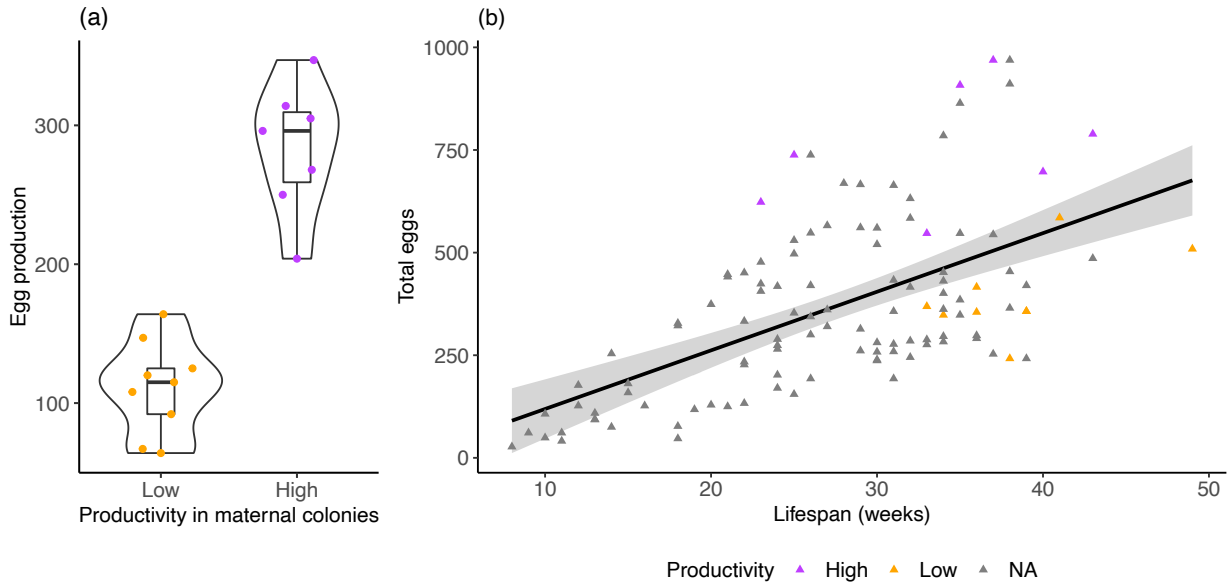

Figure 4-1. F0 Maternal colonies (n = 16) productivity and lifespan. A) Egg productivity of maternal colonies at week 15 after eclosure. The median egg production of all maternal colonies is 155.5, and B) relation between recorded lifespan and total egg produced by the maternal queens (F_0). The 99 queens that were monitored for their whole lifespan are depicted in grey, the F_0 queens in color. High productive F_0 queens are indicated in magenta and low productive queens in yellow.

The lineages differed in productivity (Table 4-1, Figure 4-2A, Figure 4-S2) but not in caste ratio produced and not in lifespans (caste ratio, Figure 2B; lifespan, *coxph* Likelihood ratio test=10.87, df = 15, p=0.76, Figure 4-S3). As we used queen pupae as the fitness currency

(workers are completely sterile in this species), all queen pupae were weekly removed and censored. Worker pupae and egg numbers were censored with less frequency (once per month) and are not precise estimates of fitness.

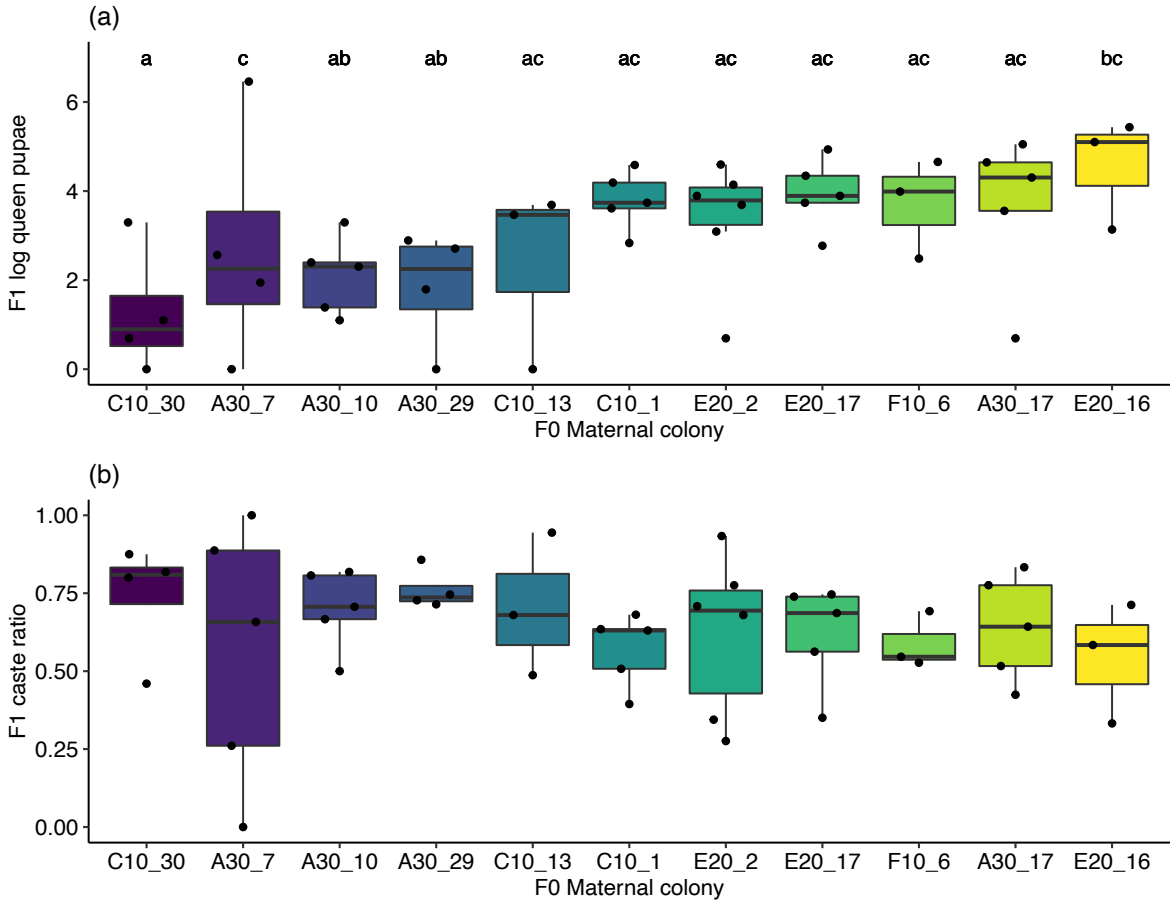

Figure 4-2. Daughter colonies (F_1) production per maternal lineage (F_0). A) F_1 total queen pupae produced in natural log scale, and B) F_1 caste ratio (queen pupae / female pupae). Different letters indicate significantly different mean estimates at P-value<0.05.

The F_0 queen productivity had no effect on the productivity of the F_1 queens (glmmTMB, eggs: z-value = -0.06, p=0.95, Figure 4-3A; worker pupae: z-value = -0.06, p = 0.95, Figure 4-3B; and queen pupae: z-value =1.22, p=0.22, Figure 4-3C). Likewise, lifespans of F_0 and F_1 queens do not correlate (coxme, z-value=-0.88, p = 0.38). The size of the maternal colony

had no effect on the total F_1 productivity (glmmTMB; eggs: z-value = 0.35, p=0.72; worker pupae: z-value=0.16, p=0.87; and queen pupae: z-value=0.41, p=0.68) or F_1 lifespan (coxme model, z-value = -0.75, p = 0.45). Finally, the maternal age F_0 did not affect the F_1 productivity (glmmTMB, eggs: z-value = -0.19, p = 0.85; worker pupae: z-value =0.13, p=0.90; and queen pupae: -0.48, p=0.63) or survival (coxme, z-value = 1.22, p = 0.22). In this setup, maternal age was skewed to old ages (mean = 27 weeks, Figure 4-S4).

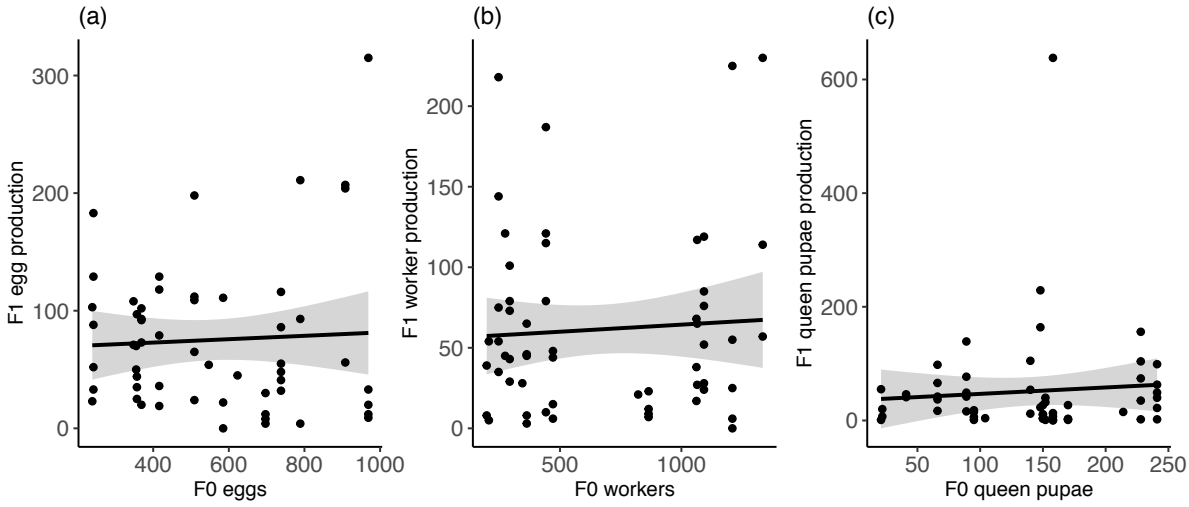

Figure 4-3. Correlation between maternal (F_0) and daughter productivity (F_1, n=56). Effect of the maternal productivity in A) eggs B) worker pupae, and C) queen pupae F1 productivity.

4.6 Discussion

Overall, there was no correlation between maternal and daughter productivity. We cannot exclude that the methodological design has a certain bias on the results. As sexuals from very low productive maternal lines are difficult to obtain, these are underrepresented in the F_1 lines. The F_1 productivity (eggs and worker pupae) was in general lower than the F_0 productivity. This could be explained by a lower monitoring frequency, meaning that they are not directly comparable (Figure 4-3A, B).

We can also not rule out a genetic effect. Some maternal lines produced consistently more productive daughter queens than others. Studies in *Linepithema humile* have shown that the

maternal lineage influences the total offspring production, and the paternal lineage the total female caste ratio (Libbrecht et al., 2011). Is still unclear why while there is a genetic effect in the maternal lines we do not observe a correlation between maternal and daughter productivity. Fitness variation could be explained by differences in the quality of the paternal lines used in the F_0 crossing. A study in *D. melanogaster* showed that high-fitness mothers produced low-fitness sons, and high-fitness males produced low-fitness daughters (Pischedda and Chippindale, 2006), which could be explained by intralocus or interlocus sexual conflict (Schenkel et al., 2018). If this is the case in *C. obscurior*, this could disrupt the correlation in productivity across female generations.

Additionally, the wingless males used here may not represent the naturally occurring variation. Wingless males compete over access to females ferociously. While most of the queens produce less than ten males during their lifetime (70% of all queens, Figure 4-S5A), around 50% of all males are laid during the lapse of a week (Figure 4-S5B). They may live for several weeks (Heinze, 2016), and differ in size probably because their development is less canalized than that of the winged males (Oettler et al., 2019). In multiple-queen colonies, more than one queen may produce males simultaneously (Cremer and Heinze, 2002). This means that most males will encounter a rival, and the weakest will be outcompeted. It might be beneficial for a queen in a polygynous colony to synchronize the wingless male production with other queens to outcompete rivalling males. However, it is unclear why fighting between siblings in single-queen colonies is beneficial. A certain combination of chromosomes might be more favorable than others, and in lack of recombination in the production of the haploid male zygotes, sib fighting could act as genetic purging. In this study, males used in the F_0 crossing were selected before they had a chance to meet any other males. Theoretically, this could explain why some maternal lines vary in F_1 productivity. Further studies should focus on male morphological traits (size, weight, sperm count and length, etc.) and trait heritability.

Maternal age can be detrimental to offspring quality in several species (Ivimey-Cook and Moorad, 2020; Priest et al., 2002). In contrast, we found no effects of maternal age on the

offspring fertility. Due to the low sample size, we do not have the power to test for an interaction effect between the maternal lineages and the maternal age at the moment of the egg laying. However, in this species, selection against senescence is maintained after the majority of the sexuals have been produced, meaning that age should not affect the quality of sexuals. The paternal age (F_0) was unknown, but did not affect F_1 life expectancy and productivity in a previous study in *C. obscurior* (Heinze et al., 2018). Further, it is unlikely that the age of the male at the moment of mating affects queens because wingless males have life-long spermatogenesis (Heinze and Hölldobler, 1993).

Lastly, the F_1 queens differed in productivity but not lifespan suggesting that these two paramount life-history traits are not strictly correlated in *C. obscurior,* as has been shown previously (Jaimes-Nino et al., 2022; Schrempf et al., 2017). Interestingly, the caste ratio did not differ between low and high-productive F_1 queens. Could this be an indication that the costs to produce the two female castes are the same? A limitation in the workforce can lead queens to produce fewer sexuals implying a cost of producing gynes (Jaimes-Nino et al., 2022). One possibility is that cost of producing both castes is similar, but rearing costs differ as queens have different nutritional requirements (Csata and Dussutour, 2019; Dussutour and Simpson, 2009) and developmental time than workers (Schrempf and Heinze, 2006).

Acknowledgments

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Competing interests: Authors declare no competing interests.

4.7 Supplementary material

Table 4-1. Tukey multiple comparisons of means of egg and queen pupae production in different maternal lineages. P-values<0.1 are shown from all 55 possible combinations. Bold letters indicate significant p-values.

Comparison	Estimate	Std. Error	Z-value	P-value
Total egg production				
C10_30-A30_17	-2,02E+00	5,25E-01	-3,85	**0,011**
C10_30-C10_1	-1,97E+00	5,25E-01	-3,76	**0,015**
F10_6-C10_30	2,03E+00	5,94E-01	3,42	**0,048**
C10_30-A30_7	-1,75E+00	5,25E-01	-3,33	0,060
C10_30-A30_10	-1,73E+00	5,26E-01	-3,29	0,070
E20_17-C10_30	1,73E+00	5,26E-01	3,29	0,070
Total queen pupae				
C10_30-A30_7	-2,77	0,72	-3,87	**<0,01**
A30_7-A30_10	2,48	0,67	3,71	**0,017**
A30_7-A30_29	2,58	0,71	3,62	**0,024**
E20_16-C10_30	2,82	0,81	3,28	**0,038**
E20_16-A30_10	2,53	0,77	3,30	0,068
E20_16-A30_29	2,63	0,81	3,25	0,078

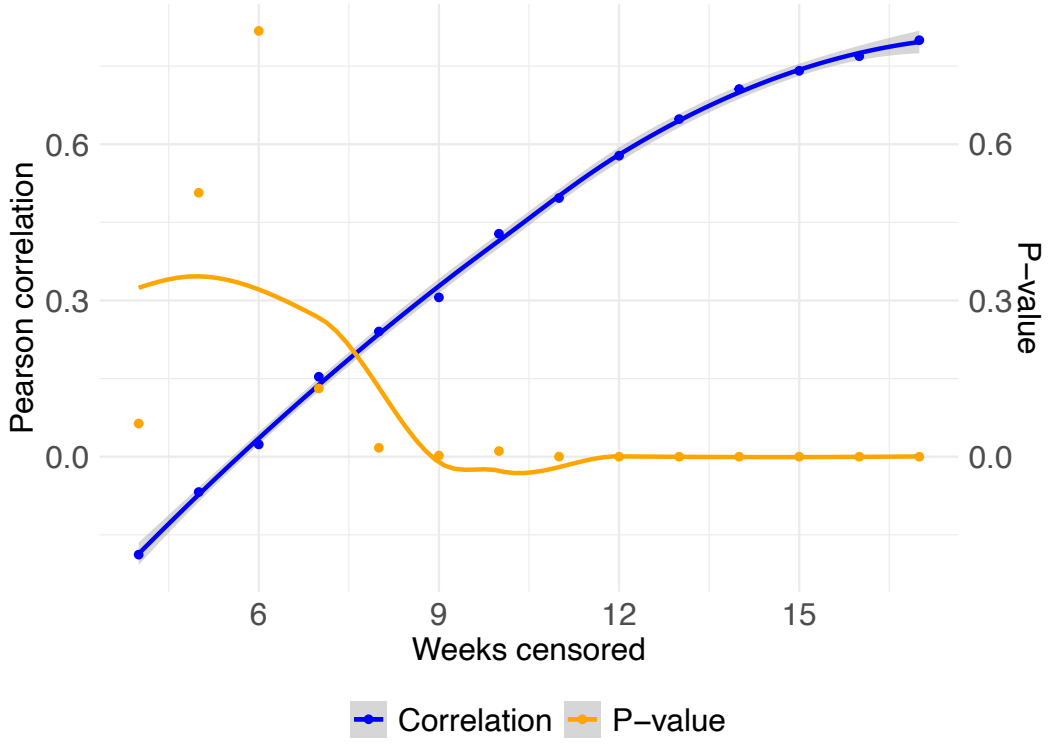

Figure S 4-1. Estimate of total queen productivity based on the eggs recorded from 4 to 17 weeks. Correlation between the total egg production of 99 single queen colonies (Jaimes-Nino et al., 2022) and the censored cumulative number of eggs after 4 weeks, and up to 17 weeks. In blue are the Pearson correlation and in orange the P-value of the correlation depicted. The graph uses a loess smoothing method and a confidence interval of 95% for the Pearson correlation.

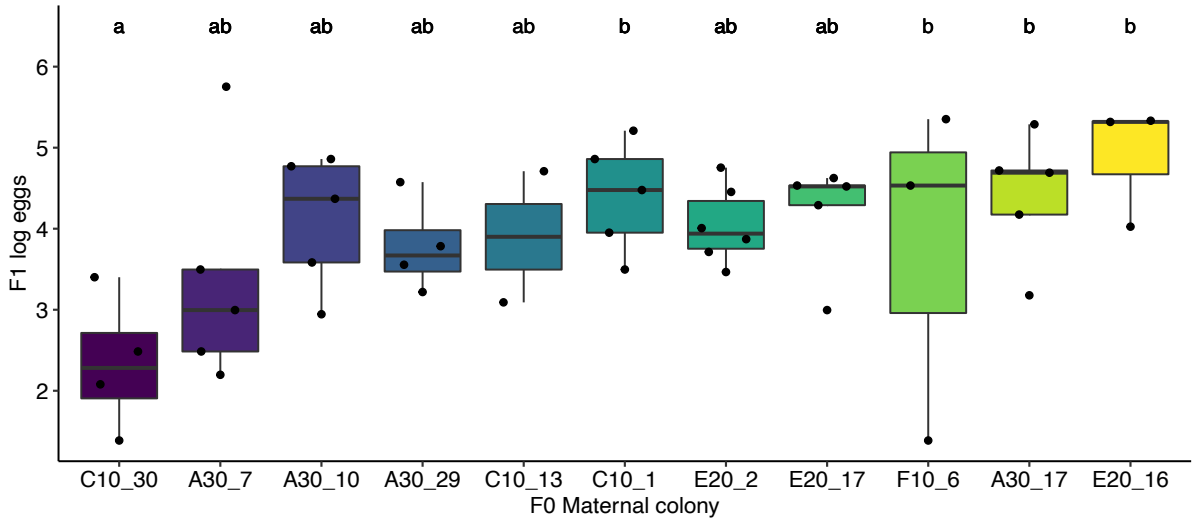

Figure S 4-2. Daughter colonies (F_1) total production per maternal colony (F_0). F1 total egg production in log scale. Different letters indicate significantly different mean estimates at P-value<0.05.

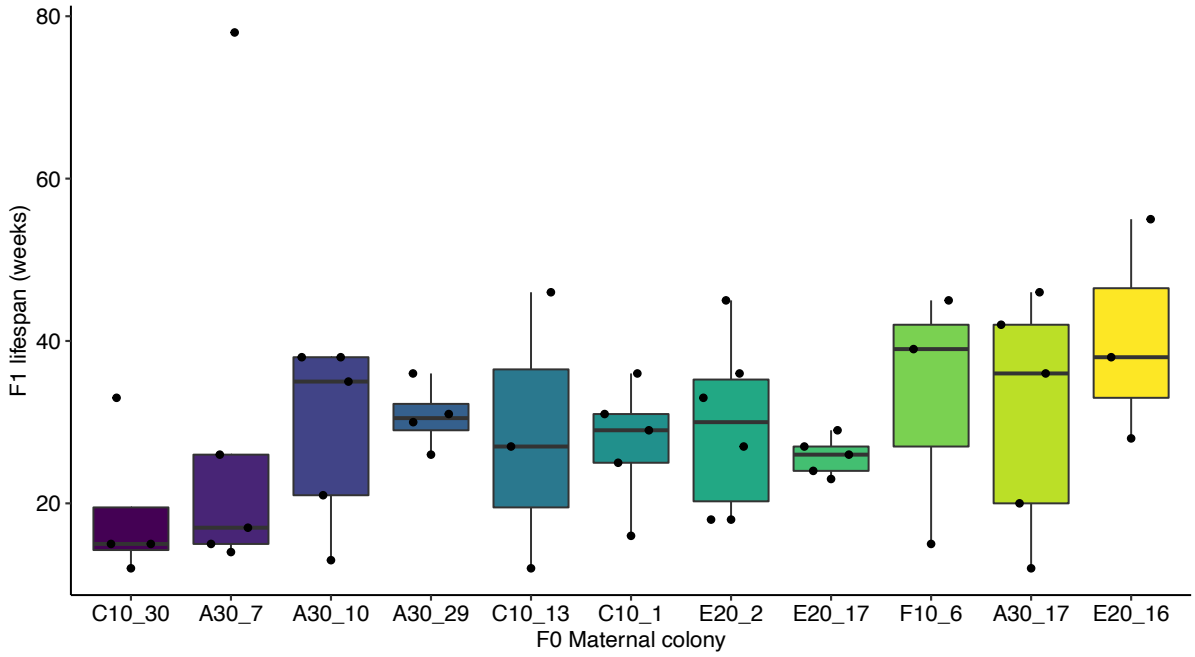

Figure S 4-3. Daughter colonies (F_1) lifespan per maternal colony (F_0). No statistically significant differences were detected between maternal lines.

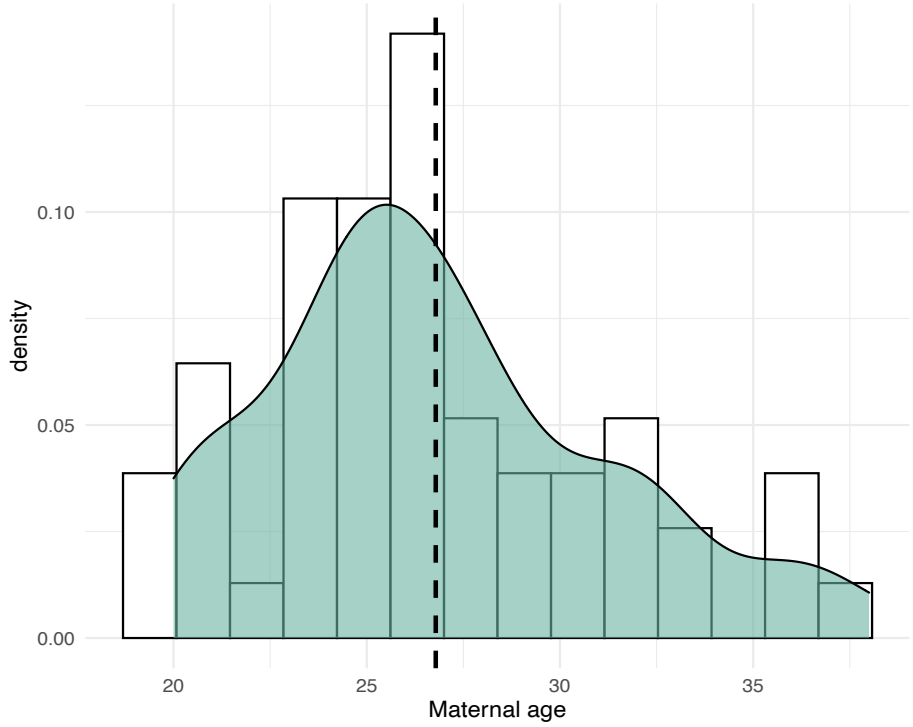

Figure S 4-4. Maternal age at the moment of laying the daughter's egg (n=56). The maternal age at the moment of the daughters' mating was censored. As the development of a queen from egg to adult lasts ~4-5 weeks (Schrempf and Heinze, 2006), we estimated maternal age as 5 weeks prior to the adult daughter's appearance.

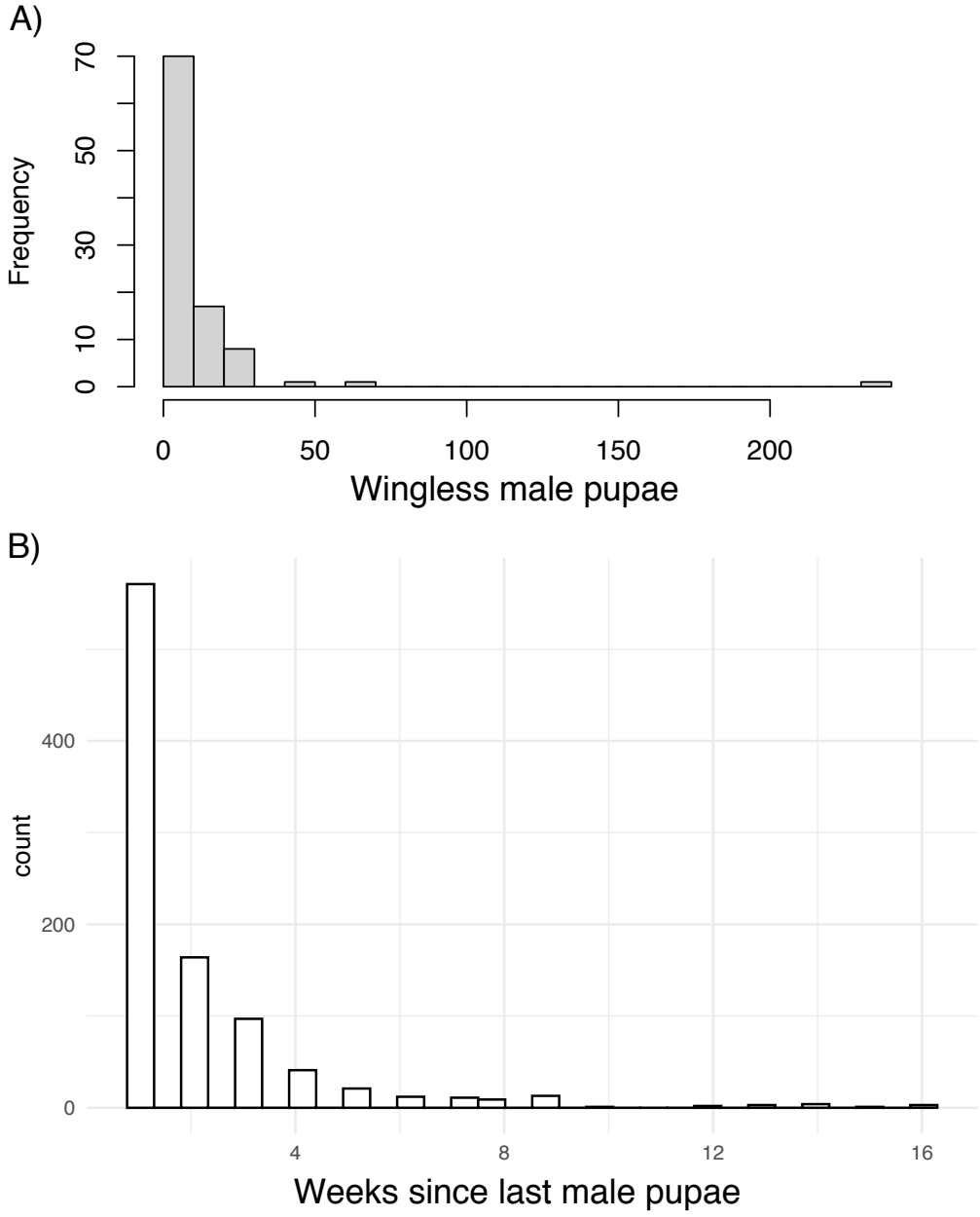

Figure S 4-5. Wingless male (ergatoid) production in 99 single-queen colonies (in Jaimes-Nino et al., 2022). A) Quantity of single-queen colonies producing a certain number of total wingless male pupae. 70 queen colonies produced less than 10 ergatoids in their lifetime. B) Time in weeks between each male pupa appearance. Records for 1075 male pupae in 99 independent colonies. Most of the male pupae are laid in the span of one week since laying the last male pupa.

Chapter 5

5 The indispensable soma of *Cardiocondyla obscurior* ants

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5.1 Abstract

The evolutionary mechanisms that shape aging in social insects are not well understood. It is commonly assumed that queens live long and prosperous, while workers are regarded as a short-lived disposable caste because of their low reproductive potential. Queens of the ant *Cardiocondyla obscurior* gain high fitness late in life by increasing investment into sexual offspring as they age. This results in strong selection against senescence until shortly before death. Here, we show that workers have the same lifespan and shape of aging as queens, even though workers lack reproductive organs and cannot gain direct fitness. Under consideration of the prevailing aging theories and the biology of the species, we hypothesize that programmed aging has possibly evolved under kin selection.

Keywords: aging, Continuusparity, social insects, worker lifespan.

5.2 Impact statement

Morphologically distinct fertile queen and sterile worker castes in the model ant *Cardiocondyla obscurior* show the same pace and shape of aging, contradicting the paradigm of queen/worker lifespan divergence in social insects.

5.3 Introduction

Queens of some social Hymenoptera (ants and bees) live long while being highly fertile, seemingly avoiding a trade-off between lifespan and reproduction (Hartmann and Heinze,

2003; Korb, 2016; Korb et al., 2020; Parker, 2010). Using the tiny ant *Cardiocondyla obscurior,* a model for social insect aging (reviewed in Oettler and Schrempf, 2016), we recently identified molecular processes associated with queen aging (Wyschetzki et al., 2015), and demonstrated that the strength of selection on age-biased genes differs between social and solitary insects (Harrison et al., 2021). Based on these insights and lifespan data from *C. obscurior* queens, including transcriptomic data from queens shortly before death, a life history framework was proposed (Jaimes-Nino et al., 2022).

The concept of "continuusparity" is grounded in a pattern observed in many ants, namely that resources are first invested into workers, and only when colony size has reached a certain threshold, resource investment is diverted to the production of sexual offspring (males, queens) (Oster and Wilson, 1978). Thus the strength of selection against senescence does not decline with age despite the life-long reproduction (Jaimes-Nino et al., 2022). Irreversible reproductive division of labor between queens and workers is a pre-requisite for the evolution of continuusparity, however, not all social insects must exhibit continuusparity, for example, species with only one caste. In respect to senescence, continuusparous social insects should sit between iteroparous and semelparous species, the former experiencing decreasing selection strength after the first reproductive bout, while the latter undergo strong selection against senescence until a single, and final, reproductive bout, followed by reproductive death.

As progress is made in understanding aging in ant queens, the aging patterns of workers remain largely unknown. Anecdotal references such as "mother queens live much longer than workers in all groups of ants" (Hölldobler and Wilson, 1990), and "all eusocial taxa show a divergence of long queen and shorter worker life spans, despite their shared genomes and even under risk-free laboratory environments" (Kramer et al., 2022) have been made. However, studies of worker lifespan lacked age-controlled cohorts (Chapuisat and Keller, 2002; Gordon and Hölldobler, 1987; Modlmeier et al., 2013; Negroni et al., 2021; Schmid-Hempel and Schmid-Hempel, 1984), surveyed marked individuals in the field without distinguishing between extrinsic and intrinsic mortality (Calabi and Porter, 1989; Gordon and Hölldobler, 1987; Schmid-Hempel and Schmid-Hempel, 1984), or monitored lifespan of

temperate species with artificial hibernation (Kramer et al., 2016). Furthermore, it is difficult to find reliable lifespan data of queens for comparison (Kramer and Schaible, 2013b). Here, we present the first empirical study of worker lifespan under controlled conditions in an ant with morphologically distinct castes and show that the paradigm of lifespan divergence between ant castes is not true.

5.4 Methods

Cardiocondyla obscurior (Formicidae: Myrmicinae), the two-colored heart node ant, is a small tramp species (queens: 3mm, workers: 2mm, (Seifert, 2003), which forms colonies comprising a few queens and several dozen workers in trees and shrubs around the tropics and subtropics (Heinze, 2017; Oettler, 2021). Queens and workers differ in discrete traits such as the presence of wings and ocelli, and, importantly, workers in this species lack reproductive organs and are thus fully sterile. Queens produce on average 300 (± 198 SD) offspring over 25 (± 8 SD) weeks (Jaimes-Nino et al., 2022) and have control over caste and sex allocation (De Menten et al., 2005; Schultner et al., 2021).

We obtained lifespan data for worker ants by setting up colonies containing 10 dark-colored worker pupae (a few days before hatching into adults) from independent stock colonies of a laboratory population collected in 2011 in Japan ("OypB", (Errbii et al., 2021; Schrader et al., 2014; Ün et al., 2021). Two virgin queens were added to the colonies to help the workers eclose and were removed after two weeks. Colonies were kept in climate-controlled conditions (26°C/22°C, 12h/12h light, 75-80% humidity) in nests made of acrylic glass with narrow slits, sandwiched between two microscope slides, and covered with a dark foil, placed in a square petri dish half-filled with plaster. The ants always had access to water and were fed with honey, *Drosophila,* and pieces of cockroaches twice per week. To test for the effect of workload on worker lifespan, colonies were subjected to one of three treatments: 1) no larvae (NL), 2) with two second instar larvae (low workload, LW), and 3) with ten second instar larvae (high workload, HW) (n=20 each). To keep workload constant, in the two treatments containing brood, colonies were checked every few days and larvae that had pupated were removed and replaced by new second instar larvae.

After 18 weeks there was no difference in survival between workers with or without larvae (see results). This led us to adjust treatments to provoke more variation in lifespan. Colonies were standardized to five workers, and those containing fewer than five workers were excluded (n after exclusion: NL=18, LW=20, HW=16). The remaining replicates from each treatment were split into two groups; in one group, workers were kept without larvae while in the other, two second instar larvae were added to simulate low workload (n after split: NL=9, NL-LW=9, LW-NL=10, LW=10, HW-NL=8, HW-LW=8). Larvae were replaced as described above. As after 36 weeks still no effect of workload treatment was apparent (see results), we removed all larvae and continued to monitor worker survival weekly until all workers had died.

Differences in survival across worker treatments and between workers and queens (using queen data from Jaimes-Nino et al., 2022) were tested. A Cox proportional hazard mixed-effect model was implemented (coxme package in R, v. 2.2.-16) using colony as a random factor; post-hoc tests using multiple comparisons of means were run where appropriate (Tukey contrasts, *glht* function in multcomp package in R, v. 1.4-19). Relative mortality as a function of age was mean-standardized by dividing age-specific mortality by its mean (following Jones et al., 2014). We considered mortality trajectories from the age at eclosion to the age at which 5% survivorship from eclosion occurs. Predictions of the data were visualized using the loess method with the geom_smooth function and default span (ggplot2 v.3.3.2). The mean life expectancy of all workers was calculated with the *survfit* function (survival package in R, v.3.3-1) using Kaplan Meier survival analysis.

5.5 Results

After 18 weeks, there was no difference in worker survival between the treatments with and without larvae (Figure 4-1, coxme, NL vs. LW, z-value=-1.35, p=0.37, NL vs. HW, z-value=1.35, p=0.37), but the survival of workers with low workload was higher than the survival of workers with high workload (coxme, LW vs. HW, z-value=2.7, p=0.02). Shortly after colonies were standardized to five workers and split into groups without larvae or with

low workload (week 18), survival differed between treatments (coxme ANOVA, X^2_5=14.24, p<0.05), but this difference was not significant after correction for multiple testing. After 36 weeks there was still no effect of the presence of larvae on worker survival (coxme, NL vs. NL-LW, z-value=-1.08, p=0.89, NL vs. LW, z-value=-0.79, p=0.97, NL vs. HW-LW, z-value=1.58, p=0.61), so we stopped replacing larvae, removed any remaining brood items and continued to monitor survival weekly. As some larvae were potentially overlooked and may have developed into adult workers, 14 replicates (NL-LW=8, LW=3, HW-LW=3) were discarded at this point. The remaining replicates were monitored until the last worker was recorded alive after 50 weeks (Figure 4-2A).

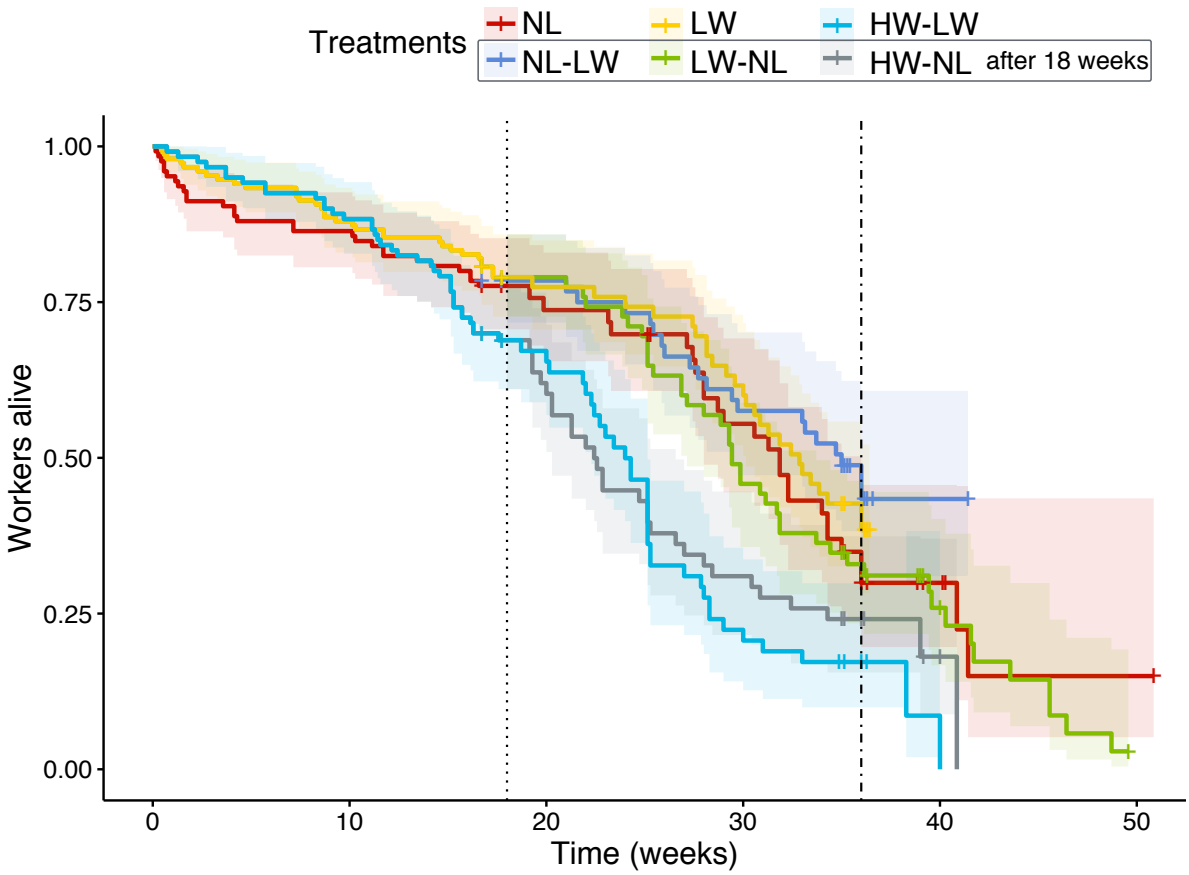

Figure 5-1. Survival probability curve of C. obscurior workers depending on treatment (NL: No larvae; LW: Low workload; HW: High workload) estimated using Kepler-Meier analysis. At week 18, indicated by the dotted line, colonies were standardized to 5 individuals, and half of the colonies from the NL and LW treatments, and all HW colonies, were subjected to a treatment change (from NL to LW: NL-LW, from LW to NL: LW-NL, from HW to NL and LW: HW-NL, HW-LW). After 36 weeks, indicated by the dot-dashed line, no more larvae were added. The NL-LW and LW curves are truncated after 36 and 42 weeks due to the dismissal of replicates in which larvae may have eclosed.

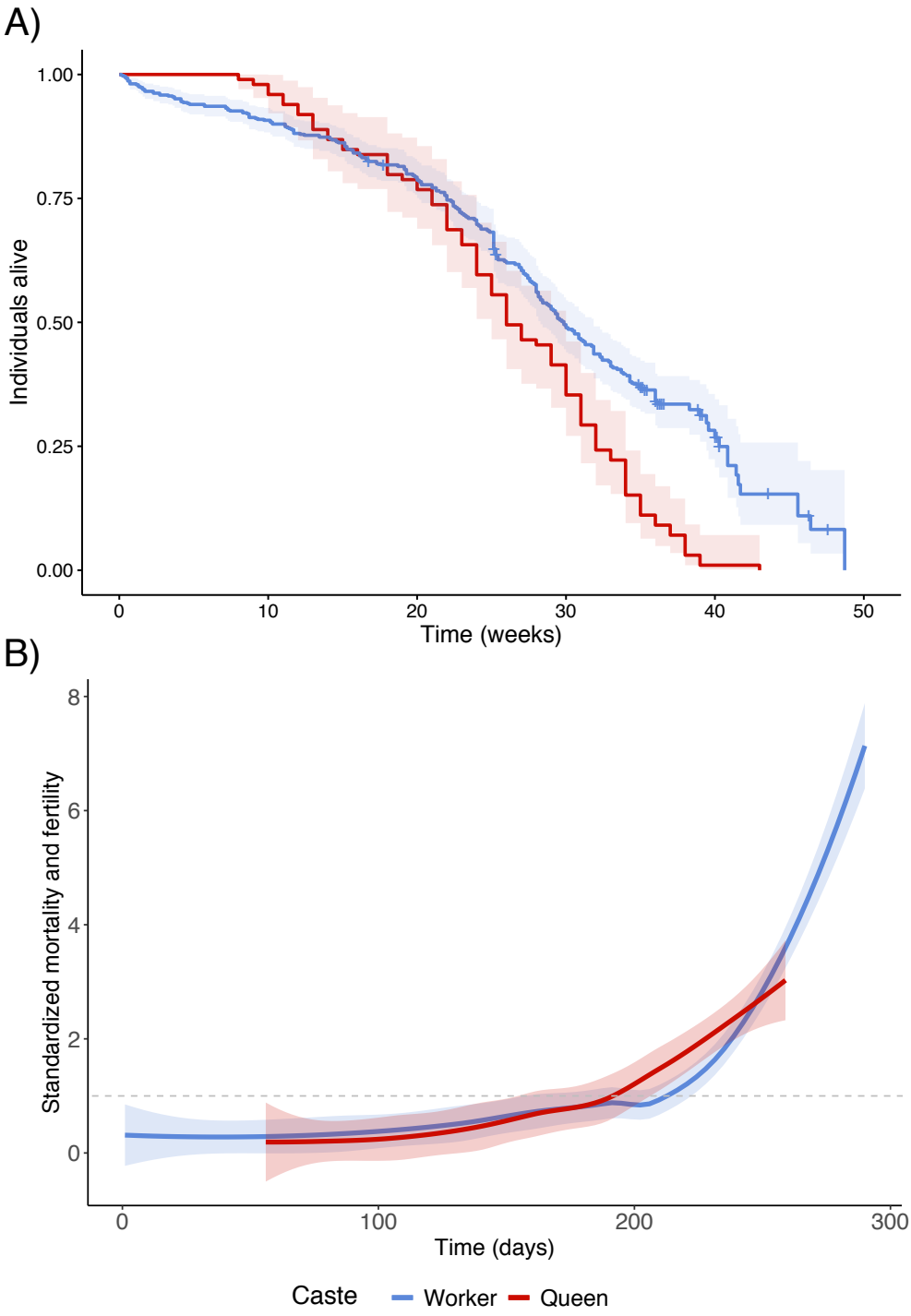

Figure 5-2. Worker and queen survival and standardized mortality. A) Survival probability curve of queens (n = 99; from Jaimes-Nino et al. 2022) and workers (n=530). B) Relative mortality as a function of age. Mortality is standardized by dividing age-specific mortality of queens and workers by their means after eclosion (following Jones et al., 2014). The graph uses a Loess smoothing method (span = 0.7) and a confidence interval of 95%. The dashed gray line at y = 1 indicates when relative mortality is equivalent to mean mortality.

The median worker lifespan over all treatments was 29 weeks (209 days, CI 95%=198-223 days). The lifespans of workers were similar to those of queens obtained in a recent study using similar laboratory conditions (Jaimes-Nino et al., 2022). Workers had a lower hazard rate compared to queens (Hazard ratio = 0.55, coxme ANOVA, X^2_1=56.95, p<0.001). Strikingly, the shape of aging, i.e. how mortality increases with time, was similar in the two castes, with mortality below average before day 200, and thereafter continuously increasing (Figure 4-2B).

5.6 Discussion

5.6.1 No causal relationship between fertility and lifespan

Aging is linked to metabolic rate, growth, and reproduction, and is determined by life-history optimization (White et al. 2022). The shape of *C. obscurior* queen aging is characterized by increasing investment into sexual reproduction with age, and thus a higher probability for (sexual) offspring to come into existence (Hamilton, 1966). After this bout of sexual production, mortality increases. Queens die soon after they cease egg-laying, suggesting that reproduction is optimized and intrinsic resources are depleted (Jaimes-Nino et al., 2022). Finding a similar pace and shape of aging in workers is surprising because queens and workers differ in morphology (Figure 4-3, (Oettler et al., 2019), holobiome (Klein et al., 2016), presumably extrinsic mortality, and, perhaps most importantly, reproductive physiology, behavior and their associated energy expenditures.

Fertility is the most common proximate explanation for long lifespans in social Hymenoptera. Several studies have suggested causality between lifespan variation and core gene pathways and proteins involved in reproduction such as TOR, insulin-like signaling, juvenile hormone, and vitellogenines (honeybees: (Corona et al., 2007; Münch and Amdam, 2010; Rueppell et al., 2016; Seehuus et al., 2006); ants: (Korb et al., 2020; Negroni et al., 2021; Yan et al., 2022). In *C. obscurior*, queen fertility is positively correlated with lifespan (Heinze and Schrempf, 2012; Jaimes-Nino et al., 2022; Kramer et al., 2015), but the two traits are not causally linked: mated queens and queens mated to sterilized males lived longer

than unmated queens, although the latter two both had low fertility and only produced unfertilized, haploid male eggs (Schrempf et al., 2005). Mated and sham-mated queens also differed from unmated queens in cuticular hydrocarbon composition and gene expression (Will et al., 2012; Wyschetzki et al., 2015). Together, this points toward a causal relationship between the aging rate and mating status, rather than fertility. Fertility and fecundity are certainly not linked to the lifespan of workers because *Cardiocondyla* workers have no ovaries. This demonstrates that reproduction and lifespan are regulated independently in this ant, and supports a growing number of proximate and pathway-oriented studies in solitary animals showing that these two fundamental life history traits can be uncoupled (Dillin et al., 2002; Lind et al., 2021; Mason et al., 2018).

Figure 5-3. A virgin C. obscurior queen (left) and a worker (right) tending to some brood. © Lukas Schrader.

5.6.2 Queen and worker lifespans are similarly long and variable

Worker lifespan is probably as variable as the queen lifespan, which in addition to mating status responds to the social environment (Schrempf et al., 2011a, 2005), mating partner (Schrempf and Heinze, 2008), and immunity (Schrempf et al., 2015; Ün et al., 2021). The lifespans of queens reported by Jaimes-Nino et al. (2022) exceeded those in previous studies likely because laboratory conditions changed after 2015 (nest architecture, temperature,

humidity, feeding rate). In the present study, workers also lived considerably longer than in the previous experiment in which focal workers were kept with 10 or 20 workers (each marked by tarsal clipping), and a fertile queen, presumably making life more demanding (Jaimes-Nino et al., 2022). In this study, the removal of larvae after 18 weeks led to a temporary drop in the survival curves. This could point to a social or physiological factor that affects worker behavior, leading to more search activity ("where is the brood?"), and resulting in premature death (Koto et al., 2015). The slightly lower hazard rate in workers compared to queens may be explained by different energy requirements at the end of life.

The presumed universality of queen/worker lifespan divergence in ants, if true (~130MYR evolution, ~14.000 species), provokes the question of why queens do not live longer (or workers shorter) in *C. obscurior*. While it seems futile to speculate without more data from more species for perspective, one explanation may be that this ant does not display traits thought to be associated with the evolution of lifespan divergence between castes, including extreme size polyphenism, colonies headed by single, highly fertile queens, and large colony sizes (Kramer et al., 2022; Kramer and Schaible, 2013a). It has also been argued that in species with multiple queen colonies such as *C. obscurior*, new cohorts of workers produced by young queens can outnumber older workers, decreasing the average relatedness of workers to the previous generations of queens. This increases the risk of queens being "dismissed" earlier by younger worker cohorts and may select for shorter lifespans in queens of polygynous species (Boomsma et al., 2014; Kramer et al., 2022). However, *C. obscurior* shows no signs of conflict between castes over sex or caste allocation (De Menten et al., 2005; Schultner et al., 2021) in contrast to many other ants (Heinze, 2004), speaking against this adaptationist explanation.

5.7 Conclusions

Regarding the different approaches to gerontology, what can we learn from this ant? A first approach focuses on aging from a damage-based perspective and asks why evolution, which managed to make life in the first place, is not capable of maintaining life for eternity (minus some entropy). Research is usually directed at mechanisms underlying lifespan variation

within species, and indeed there is overlap across taxa (e.g. dietary restriction, Fontana et al., 2010). *C. obscurior,* with its morphologically and physiologically distinct queen and worker castes, which nevertheless display a similar pace and shape of aging, is a unique model in this context (Jones et al., 2014), especially because proximate aging patterns contrast partly with those found in fruit flies (Harrison et al., 2021; Wyschetzki et al., 2015). While a senescent phase is only brief, this diphenic model will allow for disentangling how resources and energy are allocated into growth, aging, and reproduction.

A second approach adds an evolutionary perspective and considers life history strategies and putative associated metabolic or functional trade-offs (i.e. between lifespan and reproduction) to underlie senescence. Empirical evidence for this is found with mixed success (Dillin et al., 2002; Lind et al., 2021; Maklakov and Chapman, 2019; Mason et al., 2018). The results presented here do not support the idea that fertility-related trade-offs affect worker lifespan, simply because workers do not reproduce. There is also no evidence of fertility/lifespan trade-offs in queens (Jaimes-Nino et al., 2022; Schrempf et al., 2017), thus theories implying trade-offs do not apply here, including e.g., antagonistic pleiotropy, which postulates that genes with positive effects on the germline in early life can be selected for even if they cause senescence in the soma later in life. As antagonistic pleiotropy requires a phase after the point of strongest selection, it is unlikely to be effective in *C. obscurior* because there is no such phase (Jaimes-Nino et al., 2022). Along the same lines, metaphorically speaking, the results suggest that *C. obscurior* workers are not disposable soma.

In contrast to the first two approaches aimed at understanding senescence, a third controversial perspective considers whether death is not just a consequence of lifelong wear and tear, and lifespan is a trait under selection. The idea of programmed aging is strongly contested for insoluble problems with its logic (Kirkwood and Melov, 2011; Kowald and Kirkwood, 2016). First and foremost, how could selection ever favor death over reproduction, without invoking group selection? And why should aging be programmed in the first place, to shorten generation time for the betterment of the species? Programmed aging is also difficult to envision in iteroparous species exhibiting repeated reproduction and

increasing risks and costs of somatic maintenance, and a senescent phase that is rarely expressed in nature. This is partly a problem of semantics because "iteroparity" comprises a wide range of reproductive strategies, including humans with an exceptionally long post-reproductive and senescent phase. The concept of programmed aging seems easier to imagine in semelparous species, where reproduction is a single event. However, in semelparous species it is not lifespan that is determined. Instead, reproduction is optimized towards one episode, which is often triggered by season, probability of encountering prey or hosts, or other extrinsic factors.

Despite all valid counterarguments, including inconceivable proximate mechanisms, programmed aging can explain not only the shape of aging of *C. obscurior* queens but also the similarity of queen and worker aging patterns. All queens show increasing investment into sexuals with age, irrespective of overall fertility, lifespan or colony size, followed by increased mortality. This indicates some sort of Zeitgeber, informing the queen to increase investment into sexual production. As potential environmental signals are absent under controlled conditions in the lab, such a Zeitgeber must be linked to the organism's physiological condition, and it must act as an honest signal, immune to mutations and cheating. This Zeitgeber is likely a finite or limited resource. A putative mechanism may be related to cellular aging, such as a Hayflick limit, which describes the observation that a human cell can only replicate and divide a finite number of times (Chan et al., 2022). However, a Hayflick limit has not been described for insect cell cultures, to the best of our knowledge. Whatever the mechanism, it cannot be causally related to reproduction and caste and is thus likely rooted in the shared physiology and metabolism.

Kin selection is a powerful force and has overridden individual selection several times, resulting in major transitions in evolution (Bourke, 2011), including sterile organisms without direct fitness in the social insects (Hamilton, 1964). The discovery of continuusparous reproduction, coupled with the observation that queens and workers show overlapping patterns in the pace and shape of aging, lead us to hypothesize that kin selection underlies the evolution of programmed aging in *C. obscurior*. Our hypothesis is explicitly distinct from the idea of "phenoptosis" i.e. the removal of old individuals for the betterment

of the species. Instead, we propose that programmed aging has evolved to optimize investment into reproduction in a superorganism.

Glossary	
Aging	Time passing by. Neutral in terms of fitness – often confused with senescence.
Life expectancy	Species (mean) lifespan estimate based on mortality rates.
Lifespan	The maximum age an organism can reach – often confused with life expectancy.
Mortality rate (age-specific)	Expected probability to die at a given age in a particular population.
Pace of aging	Related to the extension of the life of an organism (either mean or maximum lifespan) See Baudisch, 2011b.
Senescence	When an organism shows signs of increasing mortality and decreasing fertility with age.
Shape of aging	Mortality and fertility of an organism for each time point, standardized by age.

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Chapter 6

6 Discussion

It is thought that superorganismality has reshaped aging in social insects by evading the reproduction-maintenance trade-off. But how can we explain the existence of a highly fertile and long-lived reproductive caste? While some research has focused on the proximate pathways involved with the regulation of senescence, nothing is known about the ultimate aspects of social insects' aging. This thesis aims to understand the ultimate causes of queen and worker aging in social insects using *Cardiocondyla obscurior* as a model. This thesis is the first comprehensive analysis of the life history of an ant species.

6.1 Pace and the shape of aging in ant queens and workers

In Chapters 2 and 5, I describe the pace and shape of aging of ant queens and workers. Queens exhibit an interesting aging shape, i.e. the reproductive investment into the next generation and mortality over time. The investment into egg production is bell-shaped, with a maximum around week 15. After 30 weeks, the maximum investment in sexuals is achieved. Those 15 weeks correspond to ca. 30% of an ant queen's lifespan (Fig 6-1).

Mortality differs in the standard model organisms used to study aging (e.g. *Caenorhabditis elegans* and *Drosophila melanogaster,* Jones et al., 2014). Instead of a continuous increase after the first reproduction bout, the mortality in the ant queens of *C. obscurior* is maintained below average until late in life, at ca. week 30. This indicates a delay in actuarial senescence until the end of the life of ant queens, specifically after the peak of sexual investment is reached. Evidence of this is shown in the reanalysis of transcriptomic data of 4-week and 18-week-old queens (Chapter 2). The 18-week-old queens (now referred to as middle-aged queens) do not exhibit signs of senescence. On the contrary, they show a stronger purifying selection on middle-aged-biased genes and higher conservation compared to younger-aged-biased genes. Generally, the opposite is observed, a weaker purifying selection in older-bias expressed genes in model organisms (Jia et al., 2018; Turan et al., 2019; Yıldız et al., 2022). Selection against senescence is maintained as indicated by the expression of several anti-

aging mechanisms, such as the maintenance of proteostasis, the regulation of the transcription, and the stress response.

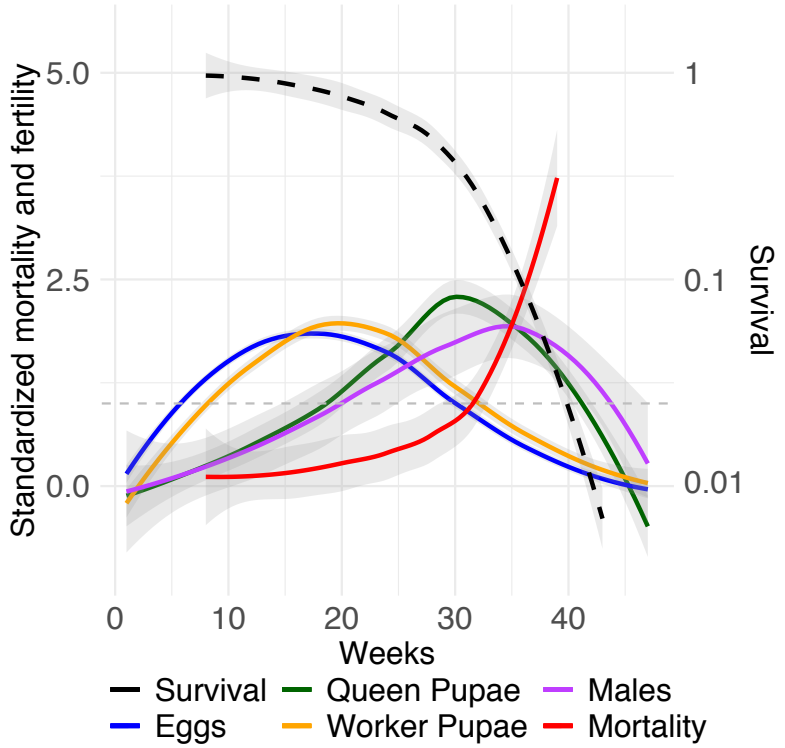

Figure 6-1. Relative mortality and fecundity as a function of age. Mean age standardization by dividing age-specific mortality and fecundity of queens (n = 99) by their means after maturation (Jones et al., 2014). Survivorship (black dashed line) is depicted on a log scale. The graph uses a Loess smoothing method (span = 0.75) and a confidence interval of 95%. The dashed gray line at y = 1 indicates when relative mortality and fertility are equivalent to mean mortality and fertility.

We demonstrated that the pace and shape of aging are similar in both castes. Workers do not live shorter than queens (Chapter 5); with a mean lifespan of 28 weeks, they live slightly longer than queens (26 weeks). Surprisingly, workers also show low intrinsic mortality until they have experienced ca. 75% of their lifespan. After this, mortality increases rapidly. It is intriguing to observe that workers exhibit the same mortality pattern as queens. In queens, it seems clear that a delay in the production of sexuals and senescence is beneficial. Colonies generally need to build a workforce before they can start producing sexuals (Hölldobler and Wilson, 1990). A parsimonious explanation is that selection is relaxed in workers because they are sterile and do not gain direct fitness; consequently, mortality mirrors the shape of the queens. This will also indicate that an intrinsic mechanism, independent of reproduction, determines when 75% of life has passed. In a sense, a type of programmed aging occurs in

queens and workers alike. It is adaptive because queens are optimized to produce the most sexuals at the end of their life. The onset of senescence in queens occurs after all the allocation into reproduction has been made, and plausibly the queens' internal resources start to deplete, if that is causal for death. One or two weeks before dying, queens cease egg production, are lethargic, and are commonly found outside the nest (Chapter 3).

Based on these results, we developed the term "continuusparity" to differentiate the reproductive strategy of *C. obscurior* from iteroparity and semelparity. Continuusparity is not an intermediate strategy along the continuum between iteroparity and semelparity. The end of life in a continuusparous organism is similar to the reproductive death experienced by semelparous organisms and the sudden detrimental change in physiology. However, reproduction has been continuous and multiple throughout the life of a mated ant. This newly developed framework might explain how superorganisms reproduce successfully at advanced ages.

6.2 Trade-off between fertility and longevity

Because of the positive relationship between longevity and fecundity in social insects, I wanted to test if trade-offs at the colony level or the individual exist in *C. obscurior*. In bumble bee workers, for instance, longevity and fecundity are considered to be positively related. However, a study showed that when all workers are forced to reproduce, reproduction has a negative effect on lifespan (Blacher et al., 2017). In this species, only the high-quality individuals choose to reproduce, those that can bear the costs of reproduction. It is necessary to point out that workers can still gain direct fitness benefits and that this species is annual. This could mean that while maximum lifespan is already determined by seasonality, workers' longevity is causally affected by reproduction.

In some ponerine species, where workers can completely replace the queens, reproduction is positively associated with longevity (Hartmann and Heinze, 2003; Tsuji et al., 1996). But in most ant species, workers commonly do not possess a spermatheca, and reproduction is limited to the asexual production of haploid males (Gotoh et al., 2016). Studies have shown that ant workers with the ability to produce haploid offspring display both a life extension

and activation of ovaries in the absence of the queen (Kohlmeier et al., 2017; Negroni et al., 2021). *C. obscurior* represents an extreme case of superorganismality with completely sterile workers. In addition, an egg removal experiment showed that the ant queens' fertility could be experimentally increased without any effect on longevity (Schrempf et al., 2017), suggesting that reproduction does not affect lifespan. This seemingly contrasts with the observation that the longest-lived queens are the most reproductive ones.

Table 6-1. The correlation between longevity and fecundity in superorganisms (i.e., lifetime physically differentiated caste fates) varies in terms of lifespan (annual vs. perennial) and the worker reproductive system (totipotency, male production, complete sterility).

	Superorganism type			
Lifespan	Annual	Perennial	Perennial	Perennial
Worker reproductive status	male production	totipotency (thelytokous parthenogenesis/ sexual reproduction)	male production	sterility
Studied species	*Bombus terrestris*	*Platythyrea punctata* and *Diacamma* cf. *rugosum*	*Temnothorax rugatulus*	*Cardiocondyla obscurior*
Studies	Blacher et al., 2017	Hartmann and Heinze, 2003; Tsuji et al., 1996	Kohlmeier et al., 2017; Negroni et al., 2021	Jaimes-Nino et al., 2022; Schrempf et al., 2017b
Correlation longevity / fecundity	Negative in workers	Positive in workers	Positive in workers	No causal correlation in queens

In my experiments, I did not find a causal relationship between the sexual production of ant queens and longevity (Chapter 3). In ant queens, mating may have a positive effect on lifespan but not reproduction *per se*, as queens mated with sterilized males show the same life expectancy as when mated with control males (Schrempf et al., 2005). That workers have an irreversible state of sterility in this species could indicate that conflict has diminished to a minimum and that longevity has been optimized to a maximum. Even when worker and queen survival data is scarce in Hymenoptera, it seems that different types of social

reproductive systems in superorganisms (Boomsma and Gawne, 2018; Wheeler, 1911) exhibit different correlations between longevity and fecundity (Table 6-1). I here use Wheeler's definition of superorganism (paraphrased in Boomsma and Gawne, 2018) as "all colony members having a single, morphologically differentiated caste phenotype for life".

C. obscurior queens are able to adjust egg laying rate when eggs are experimentally removed (Schrempf et al., 2017). It is unclear why the manipulation of colony size (Chapter 3) did not correspondingly lead to an increase in the number of eggs laid, nor in the number of worker pupae produced. Queens are clearly limited, as the manipulation affects the number of queen pupae produced.

6.3 Do ant queens defy senescence?

An organism that defies senescence would either experience negligible or negative senescence. Negative senescence indicates that mortality declines and that fertility increases with age, a case in which an "improvement" with age will be evident (Jones and Vaupel, 2017; Vaupel et al., 2004). Negligible senescence describes those organisms in which there is no improvement but also no notable deterioration with age. These terms do not lack ambiguity; it is more informative to refer precisely to the absolute mortality and fertility slope values.

Notable examples of organisms experiencing negative senescence (in terms of mortality) are the desert tortoise (*Gophezurs agassizii,* Turner et al., 1987), the white mangrove (*Avicennia marina*) and the netleaf oak (*Quercus rugosa*) (Jones et al., 2014). It has been calculated that 93% of all angiosperms show no senescence (Baudisch et al., 2013). Species in which relative mortality reaches an asymptote and remains constant at terminal age, e.g., collared flycatcher *Ficedula albicollis*, the great tit, *Parus major*, and *Hydra magnipapillata* (Jones et al., 2014), exhibit negligible senescence.

In the case of the ant queens of *Cardiocondyla obscurior*, it has been suggested that they experience terminal investment. This meant an increase in egg-laying rate with age (n=25, Heinze and Schrempf, 2012) in queens of a Brazilian population (New World lineage, Errbii

et al., 2021; Schrader et al., 2014). In the present study (Chapter 3), I used a different population of *C. obscurior*, originating from Japan and corresponding to the Old World lineage (Errbii et al., 2021; Schrader et al., 2014). Ant queens of this population do not show terminal investment (n=99, Jaimes-Nino et al., 2022). In contrast, the production of eggs, workers, and sexual pupae follow a bell-shaped trajectory, indicating that the ant queens in this experiment experienced reproductive senescence.

Regarding actuarial senescence, I report here for the first-time mortality trajectories for social insect ant queens (Chapter 3). Interestingly queens exhibit low relative mortality during 75% of their lifespan, and then mortality increases steadily, showing that queens experience actuarial senescence. Nevertheless, ant queens manage to delay the onset of actuarial senescence until very late in life. In support of these results, middle-aged queens show signs of an upregulation of anti-aging processes (Chapter 2).

In summary, I conclude that for most of their life, ant queens defy senescence, but senescence in this species is unavoidable. Furthermore, the worker's mortality trajectory resembles the one of the queens (Chapter 5). This suggests that workers also defy senescence for a relatively long period of their life.

6.4 Causes of death and costs of living

The causes of death of ant queens were investigated in Chapter 3. Middle-aged and near-to-death queens (*prope mortem*) were selected for RNAseq to analyze processes that occur after queens decrease/cease egg laying. Some of the processes resembled those regarded as hallmarks of aging for multiple taxa (López-Otín et al., 2013). The lower expression in translation, protein homeostasis, ribosomal machinery, and mitochondrial function are among the affected processes. All organisms need to preserve stability and functionality in their proteomes to keep healthy, for example by mantaining the correct folding of proteins. Aging is related to an altered proteostasis (Koga et al., 2011) and to the lack of degradation of damaged proteins which could lead to proteotoxicity (Balch et al., 2008; Murshid et al., 2013).

In the head-thorax of *prope mortem* queens, the processes of translation, single peptide processing, protein polyubiquitination, protein deneddylation, and proteolysis, among others, are less expressed. Most of these processes occur in the ribosome and the proteasome core complex. In the gaster, similar processes are downregulated with age, such as the protein dephosphorylation, proteolysis, protein glycosylation, and protein processing. This indicates that the protein machinery and degradation are downregulated in senescent queens, similar to what has been observed in model organisms (Francisco et al., 2020; Gao et al., 2020). In contrast, in the termite *Cryptotermes secundus*, the processes related to translation, protein folding, protein degradation, and protein synthesis are upregulated with age. The authors concluded that the upheaval of the proteasome machinery could respond to the old termite queens being under a state of stress, possibly causing their death (Monroy Kuhn et al., 2021), but this remains untested.

Another important hallmark of aging is the mitochondrial dysfunction, as the efficiency of the respiratory chain decreases with time (Green et al., 2011). The mitochondria's dysfunction could lead to increased ROS production (reactive oxygen species), causing cellular damage (i.e., free radical theory). This theory has been re-evaluated in the past years due to inconclusive data. For example, it has been shown that ROS prolong the lifespan of *C. elegans* (Raamsdonk and Hekimi, 2009), that manipulations in mice to increase ROS do not accelerate aging (Zhang et al., 2009), and an increase of antioxidants in mice do not extend lifespan (Pérez et al., 2009; reviewed in López-Otín et al., 2013). Nevertheless, there is consensus on the activation of compensatory homeostatic responses to stress by ROS and that these responses are consequences of aging, but probably not a cause of it (Hekimi et al., 2011). However, in other social insects, the association between oxidative stress and longevity remains inconclusive (Kramer et al., 2021). I found that *prope mortem* ant queens have a downregulation of the cell redox homeostasis and ATP synthesis.

The mitochondrial dysfunction could also alter intercellular communication, another known hallmark of aging (López-Otín et al., 2013; Raffaello and Rizzuto, 2011). I found that the potassium ion transport, the regulation of synaptic transmission, the integrin-mediated

pathway, and the cell communication are less expressed processes in *prope mortem* queens. Additionally, the genomic stability is altered with age as the DNA replication and the DNA damage checkpoint signaling are upregulated enriched GO-terms. I found lower expression of histone modification and upregulation in the histone lysine methylation. In conclusion, in the processes mentioned here, senescence in ant queens is not so dissimilar to senescence in other organisms, such as humans, mice, and flies.

Activating the insulin/insulin-like growth factor 1 signaling (IIS) pathway required for reproduction leads to a shorter lifespan in most animals (Partridge et al., 2011; Tatar et al., 2003). It is proposed that the nutrient-sensing regulatory network (comprising IIS / target of rapamycin (TOR) / Juvenile hormone (JH)) is differently wired in social insects compared to solitary organisms (Korb et al., 2020; Yan et al., 2022). What such rewiring implies is still unknown. Yan and co-authors proposed that while the mitogen-activated protein kinase (MAPK) branch of the IIS pathway is activated (leading to oogenesis) in gamergates of *Harpegnathos saltator*, the AKT/forkhead box O (FOXO) branch in the fat body is inactivated due to an ovarian anti-insulin (Imp-L2) (Yan et al., 2022). In most organisms, such deactivation leads to the dephosphorylation of the transcription factor gene FOXO. FOXO enters the nucleus, activating genes related to increased longevity (Gems and Partridge, 2013). In gamergates of *H. saltator*, the life-extension properties of the activation of Imp-L2 in the ovaries have not been corroborated. However, this is an outstanding candidate to test in other species for its function and relation to aging.

The costs of living for ant queens remain unknown. I expected to find trade-offs between the investment into workforce (by manipulating the colony size) and the longevity of the ant queens. I expected queens to produce more workers to compensate for the lack of workforce and observe an effect on the queen's lifespan. In Chapter 3, ant queens did not experience any differences in lifespan, even when they were constrained to produce smaller head-sized workers and fewer queen pupae in small-sized colonies. This shows that productivity has a cost and that queens in larger colonies, with a larger workforce, can be more productive and generate larger workers. Importantly, I could not find an effect on longevity.

6.5 Are longevity and fertility heritable?

In Chapter 4, I tested whether genetic and maternal effects contribute to longevity and fertility traits in the second generation of queens. The genetic component of such traits seems to be low. The maternal line determines productivity, but I could not directly link any life-history trait to the variability observed. What explains such large variability remains an open question. One possibility is that paternal contribution is essential. Ant queens of *C. obscurior* mate only once (Schmidt et al., 2016) and generally mate with an ergatoid male (i.e., wingless male) inside the nest. Ergatoid males engage in fierce combat with other wingless males until only one or few are found in the nest chambers of the colony. The variability observed across maternal lineages in Chapter 4 was likely due to a few F_0 male crossings, which were not highly productive. Under normal conditions, only a few ergatoid males will mate with most of the queens. In this manipulation, none of the males used for the F_1 crossing engaged in sibling fighting before mating. Therefore, males do not experience intrasexual selection. Additionally, around 70% of queens lay less than ten ergatoids during their lifetime, and they lay them mostly during a short time (one week) (Chapter 4). This could be a strategy to promote fighting among siblings. Otherwise, the older male in the colony likely has a survival advantage. How this occurs in multi-queen colonies still needs to be explored.

Likewise, some crossings between males and ant queens might be more productive than others. For example, after ten generations of sib-mating in *C. obscurior*, inbreeding depression signs appear as a shorter queen lifespan and higher brood mortality (Schrempf et al., 2006). Additionally, some lines exhibited strong male-biased sex ratios and 50% egg mortality. In the same study, sexual production was positively associated with maternal lifespan (Schrempf et al., 2006), but I could not find such an association (Chapter 4).

6.6 Outlook

One of the remaining open questions is whether other organisms/superorganisms exhibit continuusparity. There is evidence that primary reproductives of the lower termites *Cryptotermes secundus* have a non-gradual aging (Monroy Kuhn et al., 2021). Young and middle-aged reproductives have very low mortality rates. In the queens, a senescent signal in

gene expression appears after ten years of life (maximum lifespan 13 years), indicating the existence of a final period with massive pathologies similar to the one observed for *C. obscurior* queens. It is unknown if sexual productivity increases with age in this species, which should lead to the maintenance of selection against senescence strong in primary reproductives. Workers are totipotent and can develop into sexuals when the primary reproductive dies. This could be a case of an organism without completely committed castes for life, therefore non-superorganismal (*sensu* Wheeler, 1911), with a continuusparity reproductive strategy.

The non-gradual aging in social insects complicates comparative studies trying to understand the proximate mechanisms of social insect aging. Studies focused on comparing transcriptome profiles of young and old social insects, for example, generally lack an understanding of the reproductive strategy and the onset of senescence for each species. First, exploratory analyses should focus on obtaining better demographic data to determine senescent phases in queens and workers.

Early and middle-aged stages in reproductives of social insects could bring us valuable information about the molecular and physiological mechanisms that allow such organisms to defy aging. While we explore some of those mechanisms in Chapter 2, the open questions are numerous. Do queens have an age-specific expression that optimizes processes and maintains protein and metabolic homeostasis? The 'hyperfunction theory of aging' states that aging results from suboptimal gene function in later life, leading to excessive biosynthesis (Blagosklonny, 2008; Gems and de la Guardia, 2013; Gems and Partridge, 2013). As gene functioning is optimized for earlier growth, development, and reproduction, the decrease in selection strength later in life leads to a non-optimal biosynthesis. This could mean that hypertrophic and hyperplastic pathologies appear due to hyperfunction or hypofunction (Blagosklonny, 2008; Gems and de la Guardia, 2013; Gems and Partridge, 2013). Therefore, a future direction would be to test whether social insect reproductives optimize specific physiological processes.

Until now, our studies mainly focused on single-queen colonies, while the species is polygynous, and colonies usually contain half a dozen queens. We do not know whether competition in multi-queen colonies shapes investment in sexual reproduction. A study showed that *C. obscurior* queens had a shorter survival when co-occurring in 8-queen colony nests compared to single-queen colonies (mean lifespan 20 vs. 25 weeks, Schrempf et al., 2011). But it is unknown if all queens contribute equally to the brood and how strong the queen-queen conflict is. Some queens in other *Cardiocondyla* species actively bite and violently antennate other female sexuals (Yamauchi et al., 2007), leading to unequal contribution to reproduction. *C. obscurior* queens also engage in aggressive behavior (personal observation), but reproductive skew has not been tested due to the lack of genetic markers.

Furthermore, unmated queens constitute a considerable amount of all queens in a colony, e.g., 18% of all queens in the Brazilian field colonies collected in 2018 were presumably unmated winged gynes (unpublished data, Kramer B.). While virgin queens live shorter than mated queens (Schrempf et al., 2005), we have not modeled standardized mortality curves of virgins to test if they are similar to those of mated queens. They would likely show differences, as mating strongly affects the physiology and cuticular hydrocarbons of the queens (Will et al., 2012; Wyschetzki et al., 2015).

Lastly, a big open question is, how mated ant queens sense that 75% of their life has passed and that it is time to increase investment into female sexuals? Is there a way to assess the own physiological state? The onset of senescence could respond to sensing available resources, e.g., fat content. Future proteomic and metabolomic work at different time points of the queen's aging will elucidate any changes in the physiological profile related to the onset of senescence. This work lays the basis for future studies concerning the ultimate and proximate causes of aging in social insects.

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