Sociality and Ageing: Longevity and Reproductivity in a Social Insect

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Thesis Abstract

Sociality and ageing represent major evolutionary features but how they interact remains relatively little understood. Eusocial insects (i.e. those with a worker caste) provide highly informative systems in this context because reproductive forms (queens and reproductive workers) exhibit unusual features such as extended longevities and positive relationships between fecundity and longevity. This thesis therefore presents a set of experiments using the eusocial bumblebee *Bombus terrestris* to elucidate the relationship between sociality and ageing. *B. terrestris* is especially suited to this goal because workers can be reproductive depending on social context and reproductive workers exhibit a condition-dependent positive fecundity-longevity relationship. An experiment to test the hypothesis that greater larval nutrition leads to high-quality adult workers able to express this relationship revealed, as predicted, significantly positive associations between body size, reproductivity and longevity, with larval nutrition affecting worker quality via its positive connection to adult body size. A reciprocal transfer experiment to test the relative influence of individual and social factors on ageing and longevity in social organisms simultaneously at the phenotypic and transcriptomic (gene expression) level revealed workers' longevity and age-related gene expression were affected in a similar manner by an interaction of individual and social factors. A novel phenomenon uncovered by this thesis was within-colony, within-cohort multimodality in the frequency distribution of worker longevity. An experiment showed that isolated workers did not express such multimodality, supporting the role of social factors in longevity determination in this context. It also showed that, surprisingly, isolation significantly increased worker longevity, possibly connected to workers' reproductivity. Lastly, RNA sequencing suggested that, as predicted, the molecular basis of ageing differs between reproductive and non-reproductive workers, and characterized the gene expression changes associated with workers becoming egg-layers. In sum, this thesis helps provide new understanding of how social evolution affects the evolution of ageing and longevity.

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Author Contributions

Chapter 2 | An Experimental Test of the Effect of Larval Nutrition on Reproductivity, Longevity and Body Size in *Bombus terrestris* Workers

Liliana Fischer designed the study along with Andrew Bourke and Jenny Livesey. LF and JL conducted the experimental work and data collection. Undergraduate students Justyna Sawa, Phoebe Eddon and Rebecca Welch joined the experimental data collection. LF performed all analyses and produced all figures, infographics and tables. LF wrote the manuscript under the supervision of AB.

Chapter 3 | Individual versus Social Influences on Ageing in Social Organisms: a phenotypic and transcriptomic test in bumblebees

Liliana Fischer designed the study along with Andrew Bourke, with input from Alexei Maklakov and Tracey Chapman. LF conducted the experimental work and data collection. Undergraduate students Alex Stapleton, Alex Quinlan, Georgiana Godawatta Liyanage and Rosie Thompson joined the experimental data collection. LF performed all analyses and produced all figures, infographics and tables. LF wrote the manuscript under the supervision of AB.

Chapter 4 | An Experimental Test of the Effect of Social Isolation and Reproductivity on Longevity in *Bombus terrestris* Workers

Liliana Fischer designed the study along with Andrew Bourke, with input from Alexei Maklakov and Tracey Chapman. LF conducted the experimental work and data collection. LF performed all analyses and produced all figures, infographics and tables. LF wrote the manuscript under the supervision of AB.

Chapter 5 | Gene Expression associated with Ovary Activation in *Bombus terrestris* Workers

Experiment A: Liliana Fischer designed the study along with Andrew Bourke, with input from Alexei Maklakov and Tracey Chapman. LF conducted the experimental work and data collection. Experiment B: David Prince, Anders Wirén, Tamas Dalmay and Andrew Bourke designed the study. DP and Tim Huggins conducted experimental work and data collection. LF performed all analyses and produced all figures, infographics and tables. LF wrote the manuscript under the supervision of AB.

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Chapter 1 | Sociality and Ageing - A general Introduction

1.1 The Evolutionary Theory of Ageing

Ageing is defined as 'progressive physiological changes' that result in a decline in biological functions of an organism that will eventually lead to its death (Rose, 1994; Kirkwood and Austad, 2000). This process is also described as 'senescence' . Demographically, ageing manifests in individuals as an increase in the risk of mortality per unit time as chronological age increases (Kirkwood and Austad, 2000). Historically, the fact that most higher animals age was regarded as one of the big puzzles in evolutionary biology. Most individuals function well while they are young but cease to do so with age. However, one might assume, on general grounds, that perpetual reproduction throughout a limited lifespan would increase fitness and should therefore be favoured by selection (Partridge and Barton, 1993; Kirkwood and Austad, 2000), since one should expect natural selection to favour increasing fitness rather than decreasing it. To assume that ageing is purely explainable by wear and tear over time and therefore the accumulation of damage, as with abiotic objects, does not solve this issue. The propagation of unicellular organisms over billions of years, as well as the survival of germlines of multicellular organisms over extreme timespans, demonstrate the capacity of biological systems to repair and maintain functionality continuously (Charlesworth, 2000). Nowadays, however, the evolutionary theory of ageing (ETA) in its current form has provided a set of empirically-supported, ultimate (evolutionary) explanations for ageing, although, as this introductory chapter shows, substantial puzzles remain.

1.1.1 Extrinsic Mortality

Organisms are under constant threat of extrinsic mortality. This arises from events that cause death independently of ageing, such as predation, disease, or abiotic threats. Therefore, the fitness benefit of current reproduction is higher than that of possible future reproduction (Medawar, 1952; Williams, 1957; Kirkwood, 1977). The first to investigate ageing in a theoretical way was Fisher (1930). In his theory he allocated a 'reproductive value' to an individual, corresponding to the mean future reproductive output to be expected by individuals of a given age and sex in the population. He also introduced the 'Malthusian Parameter' r, now known as the intrinsic rate of increase of a population (Fisher, 1930; Charlesworth, 2000). Natural selection will favour life histories with the optimal balance of maintenance, growth and reproduction that maximises reproductive value of an individual throughout its lifespan, linking the fitness of a distinct life history to the intrinsic rate of increase of a population, r (Charlesworth, 2000; Edward and Chapman, 2011).

The presence of extrinsic mortality ineluctably leads to the fact that there is a weaker selection for genes that have a positive effect on survival and fecundity only late in life compared to genes that show these effects early in life. In general this means that extrinsic mortality leads to a decline in the strength of natural selection with age (Hamilton, 1966; Charlesworth, 1980, 2000). Beginning with maturity (Williams, 1957), this in turn leads to a 'selection shadow' that arises because mutations that have deleterious effects within the shadow, so at a greater age, will have already been passed on to offspring of the individual bearing the mutation produced before this age (Hamilton, 1966; Medawar, 1952; Williams, 1957). However, the role of extrinsic mortality in the evolution of ageing, originating in verbal models of Williams (1957), has recently been challenged (Moorad et al., 2019). Some empirical studies have even documented a reverse connection between extrinsic mortality (for example predation risk) and ageing (e.g. in guppies *Poecilia reticulata*, Reznick et al. (2004)). Moorad et al. (2019) argued that intrinsic and extrinsic mortality cannot be considered separately, because an internal condition is possibly causing susceptibility to extrinsic factors leading to mortality (Williams and Day, 2003), and propose a focus on how selection intensity changes age-dependently due to the connection between a susceptibility to environmental mortality pressure and age. As a potential resolution to this argument, de Vries et al. (2023) concluded that the diversity in modes of population regulations among species plays an important role, explaining why in some cases extrinsic mortality can be a driving factor of ageing and in other cases not. Furthermore, models show that the decline in strength of selection, the selection shadow, is not necessarily driven by extrinsic mortality, but rather by how many offspring stem from parents that have reached a certain age, thereby making it density-dependent (Hamilton, 1966; Moorad et al., 2019, 2020; Kramer et al., 2022; Jaimes-Nino et al., 2022). In corroboration, in eusocial insects (those with reproductive and non-reproductive or less-reproductive phenotypes, or castes) , recent models have found that caste-specific mortality risks are not required to explain the evolution of the extreme longevities of queens (reproductives) compared to workers (non-reproductives) (Kramer et al., 2022) (**Section 1.3.1**). Instead, assuming worker sterility, Kramer et al. (2022) propose, it is rather queens' monopolisation of reproduction and delayed production of sexual offspring (typically produced only after a phase of worker production) that lead to selection for increased queen longevity.

1.1.2 Evolutionary Theory of Ageing: Specific Theories

Within the ETA, a number of specific theories have been proposed to describe the detailed evolutionary basis of ageing, as now described.

Mutation Accumulation Theory

Medawar (1952) postulated the '*Mutation Accumulation*' theory of ageing. This predicts that there is little selection against mutations with deleterious effects late in life because the majority of a population would not live to the point when the effect of these mutations begins to appear. Such mutations therefore accumulate in the genome, so causing physiological and functional degradation with age (e.g. Kirkwood and Austad (2000)). Generally, this theory makes no presumptions about what the underlying proximate (mechanistic) basis involved might be, such as which signalling pathways are involved, because the postulated mutational effects could possibly occur randomly across the genome (Maklakov and Chapman, 2019).

Antagonistic Pleiotropy Theory

Following Medawar (1952), Williams (1957) proposed that deleterious effects could accumulate in a population via another means, in which pleiotropic genes have opposing effects on fitness, mainly on survival and fecundity, at different ages or somatic conditions. Specifically, the '*Antagonistic Pleiotropy*' theory proposes that such genes would be selected if they positively affect an individual's fitness at a young age, despite having negative effects on an individual's fitness later in life (Maklakov and Immler, 2016). In accordance with this theory, alleles that show antagonistic pleiotropic effects have been found in multiple species (Maklakov and Chapman, 2019). An example of a phenotype resulting from such pleiotropic effects can be found in high levels of the human sex hormone testosterone, which lead to increased fitness in male humans early in life and during sexual maturation, but decreased fitness later in life as high levels of testosterone increase the risk of prostate cancer (Gann et al., 1996).

Disposable Soma Theory

The theory of the '*Disposable Soma*' (Kirkwood, 1977) proposes a general physiological and mechanistic explanation for the Antagonistic Pleiotropy theory discussed above (Maklakov and Chapman, 2019). Natural environments offer limited amounts of resources. The Disposable Soma theory supposes that an over-investment of resources into somatic maintenance and genetic repair, resulting in longer lifespans

at the expense of reproduction, should be counter-selected, because, as already described, the pressure of extrinsic mortality results in current reproduction having a higher fitness value than potential future reproduction (Kirkwood, 1977). In other words, there should be a constant 'competition' for resources and energy between somatic maintenance and reproduction, i.e. a trade-off. Since natural selection favours an increase in fitness, in most scenarios this process would favour reproduction at the expense of survival, although when reproductive chances are low a longer lifespan would be promoted.

Developmental Theory of Ageing

Where the theory of the disposable soma considers energy trade-offs, the '*Developmental Theory of Ageing*' (de Magalhães and Church, 2005), invokes functionality trade-offs, stating that ageing is an extension of development (Comfort, 1964). It proposes ageing to be caused by physiological processes that are optimized for development and growth early in life but are suboptimal during adulthood and as such can be seen as an extension of the Antagonistic Pleiotropy theory. One phenomenon of human ageing that could be explained by the Developmental Theory of Ageing is neurodegeneration that can eventually lead to different forms of dementia (de Magalhães and Church, 2005). According to the theory this stems from the continuation of processes in early life, like the decrease of brain plasticity before adulthood (de Magalhães and Sandberg, 2005). This theory argues that it is genetic factors which drive development that also regulate the rate of ageing, making ageing an unintended side effect of well-programmed developmental processes, which could explain the proportionality of adult longevity to development times in mammals (de Magalhães and Church, 2005).

Hyperfunction Theory

Lastly, the '*Hyperfunction Theory*' (Gems and Partridge, 2013; Gems and de la Guardia, 2013) makes a connection between the Antagonistic Pleiotropy theory (Kirkwood, 1977) and the Developmental Theory of Ageing (de Magalhães and Church, 2005). The Hyperfunction Theory proposes that pathways and mechanisms that are essential for growth and development in early life will continue to run, or 'hyperfunction' even if not necessary, as the individual grows older, causing detrimental physiological effects leading to ageing (Blagosklonny, 2012; Maklakov and Chapman, 2019). This theory is based on in vitro studies of cell cultures that showed under arrest of cell proliferation, cells can still transition into a senescent state when confronted with high levels of growth signalling (Blagosklonny, 2012; Demidenko and

and Blagosklonny, 2008). The theory argues that an over-stimulation can lead to increased functions (or hyperfunction) which inevitably leads to cellular senescence as a continuation of cellular growth (Blagosklonny, 2012).

1.1.3 The Fecundity – Longevity Trade-off

A common outcome of the specific theories considered above (**Section 1.1.2**), explicitly so in the case of the Disposable Soma theory, is that a trade-off inevitably exists between reproduction and survival, and hence between fecundity and longevity (Stearns, 1992; Edward and Chapman, 2011). As predicted, in most solitary (non-social) species there seems to be such a trade-off between the rate of offspring production (fecundity) and lifespan (longevity) (Partridge and Harvey, 1988; Stearns, 1989). For example, a higher rate of reproduction, early life mating and greater total number of offspring are associated with shorter lifespans, especially for females, in Columbian ground squirrels (Neuhaus and Pelletier, 2001), the fruit-fly *Drosophila melanogaster* (Fowler and Partridge, 1989; Travers et al., 2015) and humans (Westendorp and Kirkwood, 1998). In addition, many studies on birds and mammals have shown that, when fecundity is experimentally increased, there are negative effects on future reproduction and/or survival of the breeding individuals or their offspring and its quality, suggesting that such effects are caused by the increased allocation of resources into reproduction (reviewed inMaklakov and Chapman (2019)). Such a trade-off could arise from reproduction causing damage to tissues and the genome as well as from the allocation of resources to reproduction rather than somatic maintenance (Edward and Chapman, 2011). Especially the latter leads to trade-offs between reproduction and immunity and between reproduction and stress resistance, resulting in an overall trade-off between fecundity and longevity (Zuk and Stoehr, 2002; Li-Byarlay and Cleare, 2020). Overall, a negative relationship between reproducing and maintaining other body functions, hence creating general costs of reproduction, seems to be the rule rather than the exception throughout the animal kingdom (Ball, 1986; Flatt and Heyland, 2011; Flatt, 2011; Harshman and Zera, 2007; Stearns, 1992; Williams, 1957, 1966).

1.2 Mechanistic Basis of Ageing

1.2.1 Molecular Pathways involved in Ageing

On a proximate level, ageing and the associated deterioration of bodily functionality must be caused on a molecular level. Several molecular signalling pathways

have been proposed to play key roles. In many invertebrates, one such key regulating pathway of ageing is the insulin/insulin-like growth factor 1 signalling (IIS)/target of rapamycin (TOR)/juvenile hormone (JH) network (reviewed in Korb et al. (2021)). The availability of nutrients, especially carbohydrates, is sensed by the IIS- and TOR-pathways and leads via a signalling cascade to the production of JH (Korb et al., 2021). The nutrient sensing TOR-pathway is involved in growth and development, though if proliferation is blocked in a cell culture, by arresting the cell cycle, it remains active. This then drives the cells into senescence without any molecular damage (reviewed in Blagosklonny (2012)). Additionally, a downregulation of this pathway results in a prolonged lifespan in both sterile (Bjedov et al., 2010) and fertile flies (*D. melanogaster*) without reducing their fertility (Mason et al., 2018). Interestingly, it seems that in *Apis mellifera*, high activity of the TOR encoding gene amTOR in larvae is associated and necessary for the development of the queen phenotype, which is the longest-lived in this species (Patel et al., 2007). The TOR-pathway is one of the growth pathways proposed by the Hyperfunction Theory (Gems and Partridge, 2013; Gems and de la Guardia, 2013) to continue to run even after the developmental growth of an organism has stopped. There should be strong selection for a functioning TOR pathway because it is essential in early life and development. However, as predicted by the ETA (Hamilton, 1966; Medawar, 1952; Williams, 1957), this might occur even if there are harmful effects (by hyperfunction) later in life as well (Blagosklonny, 2012).

The IIS pathway responds to the availability of nutrients and most likely many other environmental cues (Regan et al., 2020). Within this cascade, several kinases are activated that have specific transcription factors as their substrate for phosphorylation (Russell and Kahn, 2007). In a phosphorylated state these transcription factors are released into the cytoplasm where they are degraded. Without phosphorylation, the transcription factors are translocated to the nucleus where they induce the transcription of genes elongating lifespans (reviewed in Russell and Kahn (2007)). This pathway seems to be conserved for nematodes, flies and mammals, although the detailed effects vary. For example , in the nematode *Caenorhabditis elegans*, the IIS pathway seems to regulate longevity and reproduction independently of one another (Dillin et al., 2002) and its down-regulation can lead to increased parental longevity and offspring fitness (Lind et al., 2019) when the down-regulation is initiated during adulthood but to a decrease in fitness when initiated during development (Carlsson et al., 2021). By contrast, studies in *D. melanogaster* describe a connection between down-regulating the IIS signalling pathway and, on the one hand, elongated lifespans and increased stress resistance, and, on the other hand,

reduced growth and fertility (Finch and Ruvkun, 2001; Tatar et al., 2003; Russell and Kahn, 2007; Toivonen and Partridge, 2009).

The *corpora allata* (endocrine glands in insects) respond to IIS by producing JH, which has multiple effects, including on development, fecundity, stress resistance, immunity and lifespan (Finch and Ruvkun, 2001; Tatar et al., 2003; Flatt et al., 2005; Toivonen and Partridge, 2009; Rodrigues and Flatt, 2016). In insects, JH is also a gonadotropin controlling vitellogenesis and hence the production of the egg-yolk precursor protein vitellogenin (Vg) and of yolk proteins (YP), which are produced in the fat body and secreted to the haemolymph and then taken up by developing oocytes (Robinson and Vargo, 1997). Overall, therefore, the IIS-JH-Vg/YP network triggers pro-reproduction and pro-ageing effects in non-social insects and vertebrates (Flatt et al., 2013; Rodrigues and Flatt, 2016). Because of its downstream effects on longevity and reproduction, this network has also been termed the TI–J–LiFe network (TOR/IIS–JH–Lifespan and Fecundity network) (Korb et al., 2021).

Oxidative Stress and Ageing

Another important factor potentially influencing ageing on the proximate level is molecular damage caused by oxidative stress (**Table 1.1**). Oxidative stress accrues from reactive oxygen species (ROS), including free radicals, created during vital metabolic reactions, that have the ability to cause damage to macromolecules. Such damage includes DNA mutation, protein oxidation and the peroxidation of membrane lipids (Li-Byarlay and Cleare, 2020). Organisms are equipped with a large network of antioxidant genes to prevent or counteract the damage caused by ROS. An imbalance between this antioxidant ability and the pro-oxidant manifestation of ROS results in oxidative stress (Finkel and Holbrook, 2000). The '*Free Radical Theory*' of ageing proposes that, mechanistically, much of the accumulation of defects in macromolecules underpinning ageing is caused by oxidative stress (Harman, 1956b,a; Kirkwood and Holliday, 1979). Some studies in eusocial Hymenoptera support this concept, showing that experimentally induced oxidative stress increases the ageing of individuals (**Table 1.1**; *A. mellifera*: Seehuus et al. (2006); Corona et al. (2007), ants: Schneider et al. (2011); Majoe et al. (2021)).

Specifically, ROS can cause the oxidisation of amino acids in proteins (reviewed in Levine (2002)). A protein with such an oxidation not only loses its catalytic function, but also becomes marked for proteolytic degradation, affecting basic cellular functionality (reviewed in Levine (2002)), which could promote ageing. There are three ways in which organisms might counteract ageing caused by oxidative stress: they could either avoid or reduce the production of ROS, or mitigate the effects

of already-present ROS using antioxidants, or repair oxidative damage after it has been caused (Kramer et al., 2021). The differential expression of genes encoding antioxidants therefore possibly provides a mechanism to explain differences in lifespans within eusocial insect species (Heinze and Schrempf, 2008; Rueppell et al., 2016). Examples of key enzymes with an antioxidant function include peroxidases (POXs), catalases (CATs) and superoxide dismutase (SOD) (reviewed in Li-Byarlay and Cleare (2020)).

However, evidence is increasing that suggests the accumulation of macromolecular damage due to free radicals and hence oxidative stress is not a universal proximate cause of ageing (Parker, 2010; Gladyshev, 2014). For example, studies suggest that an efficient reduction of oxidative stress mediated by antioxidants can shorten lifespans, whereas a slight increase in ROS can prolong them (Radak et al., 2005; Gems and Partridge, 2008). Blagosklonny (2008) also proposed that, although ROS do induce molecular damage, this is of little consequence because TOR-driven ageing leads to death via hyperfunction (**Section 1.1.2**) before the 'lethal threshold' of molecular damage is reached (Blagosklonny, 2008). This idea is based on the concept that the TOR-pathway is involved in many processes, such as inhibition of autophagy, stimulation of translation and ribosomal synthesis and general cell growth processes, that, while important during the developing and growth phase of an organism, over time lead to cellular hyperfunction, causing cellular damage and organ failure rather than molecular damage (Blagosklonny, 2008, 2012). In turn, this would mean that an accumulation of molecular damage is not the cause of ageing but is rather the result of it via the hyperfunctioning of metabolic or signalling pathways (Blagosklonny, 2012) (**Section 1.1.2**). Generally, then, the role of oxidative stress and ROS in ageing remains highly debated.

1.3 Sociality, Ageing and Longevity

The ETA was developed largely on the basis of considering processes of ageing in solitary (non-social) organisms (e.g., Hughes and Reynolds (2005); Rose (1994)). This means that, for example, trade-offs between reproduction (fecundity) and somatic maintenance (longevity) (**Section 1.1.3**) are deemed to act on the level of the individual organism. More generally, all factors impacting ageing are considered to act on individuals in isolation from social effects. However, in social organisms, such factors need to be considered not only on the level of the individual but also on the level of its social partners or the group as a whole, including in terms of potential effects on social partners. Factors like resource availability and the risk of extrinsic

mortality may differ between a solitary individual and an individual in a group and can potentially also differ for each group member (e.g. in the context of social hierarchies). Furthermore, the life history and mortality schedule of each group member may affect those of other group members, creating an interaction between ageing in individual group members and in other members of the group (e.g., (Alexander et al., 1991; Bourke, 2007)). Because sociality, ageing and longevity are therefore strongly intertwined and affect one another's evolution (Lucas and Keller, 2020), and because sociality in its various forms is widespread across taxa (e.g. Wilson (1975); Bourke (2011)), it is necessary for a full understanding of the evolution of ageing across all organisms to consider ageing in a social context.

A well known effect of a social trait influencing ageing is the 'grandmother effect' in humans, whereby selection for care to grandoffspring by grandmothers has been shown to promote prolonged post-reproductive life in females (Hawkes et al., 1998; Lahdenperä et al., 2004), with analogous lifespan extension being proposed to occur in a few other mammals as well (e.g. whales Nattrass et al. (2019)). More generally, several aspects of sociality have been proposed to affect ageing and longevity. Firstly, sociality seems to positively affect longevity such that social organisms often show extended longevities compared to closely related solitary species (Korb and Heinze, 2021). Examples include the eusocial vertebrate, the naked mole-rat (*Heterocephalus glaber*) (Kim et al., 2011), and the cooperatively-breeding Seychelles warbler (*Acrocephalus sechellensis*) (Hammers et al., 2019). The most extreme examples of sociality being associated with increased longevity, in which the scale of the effect can amount to a hundred-fold increase, occurs in queens of eusocial insects compared to their solitary relatives (Keller and Genoud, 1997; Kramer and Schaible, 2013). Similarly, in absolute terms, queens of eusocial insects show some of the longest maximum lifespans found among insects (up to 30 years in some ant species; (Keller and Genoud, 1997; Keller, 1998). Eusociality, in which there is a reproductive division of labour by which only one or a few individuals (queens or kings) produce offspring while an overlapping generation of helpers (workers) cooperatively take care of the young (Batra, 1968; Wilson and Hölldobler, 2005), is widespread, particularly in insects. It is found in the order Hymenoptera (bees, wasps and ants; Hölldobler and Wilson (1990); Cardinal and Danforth (2011)), Isoptera (termites; Costa-Leonardo and Haifig (2014)) and occasionally in other orders of insects (Hemiptera, Thysanoptera, Coleoptera; Day (1992); Stern (1994); Aoki and Imai (2005)), as well as in crustaceans (snapping shrimp; Duffy et al. (2000)) and mammals (Naked mole-rat; Burda et al. (2000)).

Secondly, within a social group there may be a disruptive selection on longevity,

meaning that the life histories of different group members are impacted differently by sociality (Alexander et al., 1991; Lucas and Keller, 2020). In eusocial insects, reproductive queens and kings are typically very long-lived compared to the less reproductive or sterile workers (Keller and Genoud, 1997; Bourke, 2007; Lucas and Keller, 2020; Lopez-Vaamonde et al., 2009; Kramer et al., 2015; Southon et al., 2015; Rodrigues and Flatt, 2016). Queens may outlive workers many times over, with some ant queens living 60 times longer than their non-reproducing sisters, although they carry the same genome (Hölldobler and Wilson, 1990; Keller and Genoud, 1997; Rueppell et al., 2015). Generally, therefore, eusocial insect species seem to be an exception to key concepts within the ETA, because greater reproductivity appears to be associated with greater longevity (Keller and Genoud, 1997; Korb, 2016; Blacher et al., 2017). For these reasons, eusocial organisms have become a major focus of attention in the study of the effects of sociality on ageing and longevity (e.g. Alexander et al. (1991); Keller and Genoud (1997); Bourke (2007); Korb and Heinze (2021)).

1.3.1 Sociality, Ageing and Longevity: Ultimate Causes

Because eusociality involves only one or a few individuals reproducing while the (sterile or less reproductive) majority performs worker tasks, including risky ones such as foraging and nest defence, it reduces extrinsic mortality for those reproductive individuals (queens or kings), which remain well sheltered within the nest (Lucas and Keller, 2020). Additionally, queens seem to invest more highly in immunity than males, which reduces their risk of intrinsic mortality (Barribeau et al., 2015). Greater queen longevity is therefore consistent with the ETA (e.g. Alexander et al. (1991); Bourke (2007); Lucas and Keller (2020)), which proposes that decreasing extrinsic mortality increases the chance of survival and lowers the rate of ageing, therefore increasing longevity (Hamilton, 1966; Medawar, 1952; Williams, 1957), albeit the universal role of extrinsic mortality in bringing this about has been challenged (Kramer et al. (2022); Moorad et al. (2019, 2020); **Section 1.1.1**).

Recent work by Walton et al. (2024) theorises that where solitary animals need to tackle (oxidative) stress with costly processes of detoxification, tolerance and repair (Li-Byarlay et al., 2016), many members of a social insect colony experience shielding and buffering against stress from the colony (Fisher et al., 2019). They call this the '*Social Stress Protection Theory*', which describes any collective defences against stress (biotic and abiotic) that the individual would not be able to provide, including group-level defences such as nest construction (Walton et al., 2024). Differences in social stress protection for different members of the colonies (e.g. queens vs.

workers) but also between species, can help explain the plasticity in ageing rates of social insects (Walton et al., 2024).

Additionally, kin selection is expected to influence the evolution of extreme longevities of reproductive individuals in groups of high relatedness, such as in eusocial insect colonies, in which case the workers gain inclusive fitness benefits by promoting the queen's survival and reproduction (Alexander et al., 1991; Bourke, 2007). The concept of inclusive fitness and kin selection plays an important role in understanding the formation and evolution of eusocial insect societies (Hamilton, 1964; Crozier and Pamilo, 1996; Bourke, 2011). It is based on the realisation that an individual can gain fitness benefits by helping related individuals to reproduce because, like direct reproduction, this also ensures the passage of the individual's genes into the next generation. Other things equal, the gain in inclusive fitness is higher the more closely related the focal individual is to the offspring it helps to rear. Inclusive fitness theory is widely accepted as providing the ultimate explanation for why such highly social systems and a reproductive division of labour evolve and remain stable (West et al., 2007; Bourke, 2011). It also helps to provide an explanation, in eusocial systems, for the selection of increased reproductives' longevity, as the survival of reproductives ensures the gain of inclusive fitness for the (sterile) workers (Alexander et al., 1991; Bourke, 2007).

Simultaneously, an application of concepts from inclusive fitness theory to the ETA helps explain the reduced longevity found in eusocial workers (Alexander et al., 1991; Bourke, 2007; Lucas and Keller, 2020). In inclusive fitness theory, fitness consists of a direct component (via offspring production) and an indirect component (via rearing relatives) (Hamilton, 1964). Under the ETA, ageing in non-social organisms begins with sexual maturation (when development stops), because the age of first reproduction represents the onset of fitness accrual, fitness in this case being direct fitness (Hamilton, 1966). Since, by helping to raise the queen's brood, workers start gaining indirect fitness at an early age without reaching sexual maturity themselves (as helping does not require sexual maturity), the same argument predicts that ageing in workers should start earlier in their lives, at the age of first helping. That is, taking up brood rearing tasks at an early age may result in an overall reduction in worker longevity via an earlier onset of ageing (Alexander et al., 1991; Bourke, 2007). Overall, the combination of the early onset of ageing and the increased risk of extrinsic mortality is most likely to be the ultimate cause of the reduced longevities of workers in eusocial systems (Alexander et al., 1991; Bourke, 2007). Moreover, these circumstances lead to a self-reinforcing system that supports phenotypic specialisation of the helping caste (workers), since the early onset

of ageing in those individuals reduces the value of possible future offspring by them, making indirect fitness increase by helping to rear the closely-related brood of the reproductive caste more profitable (Alexander et al., 1991; Bourke, 2007).

Sociality, Ageing and Longevity: Positive Fecundity-Longevity Relationships in Eusocial Insects

As well as being particularly long-lived, and unlike the typical case in non-social species, eusocial insect queens show a positive relationship between fecundity and longevity across individuals within the queen caste. Many studies have shown that the longest-lived queens are also the most fecund or productive (Hartmann and Heinze, 2003; Lopez-Vaamonde et al., 2009; Heinze and Schrempf, 2012; Tsuji et al., 2012; Heinze et al., 2013; Kramer et al., 2015; Rueppell et al., 2015; Monroy Kuhn and Korb, 2016; Schrempf et al., 2017; Kennedy et al., 2021; Negroni et al., 2021; Jaimes-Nino et al., 2022; Collins et al., 2023). In eusocial insect queens, the otherwise ubiquitous trade-off between fecundity and longevity therefore seems to be absent, and the queens have seemingly overcome the costs of reproduction (Heinze et al., 2013; Monroy Kuhn and Korb, 2016; Schrempf et al., 2017). A positive fecundity-longevity relationship could potentially stem from the workers sharing the costs of reproduction with the queen (Lucas and Keller, 2020), given that workers perform many of the energetically costly tasks involved in brood rearing, such as foraging, feeding and (in some species) thermogenesis. This in turn means that the queen is possibly able to allocate more energy into somatic maintenance and survival than a solitary breeding individual would be able to, increasing her chances of survival (Lucas and Keller, 2020). However, a study by Rueppell et al. (2015) showed that, in the ant *Cardiocondyla obscurior*, a species in which queens exhibit a positive correlation between egg-laying rates and longevity, reproducing queens lived longer than non-reproducing queens even when isolated. Therefore positive fecundity-longevity relationships in queens do not appear to be solely the result of workers bearing the costs of reproduction on their behalf. Another study on *C. obscurior* queens by Schrempf et al. (2017) showed experimentally that queens of this ant species were insensitive to costs of reproduction. Costs of reproduction of queens were experimentally increased by removing queen-laid eggs, which causes queens to increase their egg-laying rates. Queens in the egg-removal treatment showed no decrease in longevity relative to control queens whose egg-laying rates were not increased (Schrempf et al., 2017). Overall, therefore, it appears that *C. obscurior* queens are able to enhance their reproductive outcomes without paying costs (Schrempf et al., 2017).

A later study of *C. obscurior* further showed that queens exhibit a peak of sexual production in late life, suggesting that the selection shadow is delayed for the long-lived queens of this species (Jaimes-Nino et al., 2022). The authors proposed that this was due to such species pursuing a breeding strategy that they termed 'continuusparity', defined as a 'combination of lifelong continuous reproduction and increasing fitness returns late in life', i.e. with the production of reproductive offspring occurring only towards the end of a queen's lifespan (Jaimes-Nino et al., 2022). This results in high selection strength maintained until late in life, leading to long lifespans without any apparent fecundity-longevity trade-off.

However, ants are considered 'advanced' eusocial species (showing a relatively high degree of queen-worker size dimorphism), and advanced eusociality is assumed to have evolved from the non-social state in stages (Wilson, 1971; Wheeler, 1986; Bourke, 2011, 2023). For less advanced eusocial species, such as bumblebees, Collins et al. (2023) hypothesised they might show positive fecundity-longevity relationships condition-dependently, with costs of reproduction being present but latent. Such a phenomenon would represent a state lying between the negative fecundity-longevity relationships found in non-social species and the apparently unconditional positive fecundity-longevity relationships found in advanced eusocial ants such as *C. obscurior*. Bumblebees (*Bombus* spp.) are regarded to be 'intermediately' eusocial, since the level of queen-worker reproductive dimorphism is intermediate between those found in 'primitively' and advanced eusocial species (Harrison et al., 2015). In addition, in bumblebees, a large number of genes are differentially expressed between the different castes, as in advanced eusocial insects, though reproductive workers still resemble queens quite closely in gene expression patterns, as in primitively eusocial taxa where one or more dominant, but otherwise equal, females take queen roles (Harrison et al., 2015). In addition, in *B. terrestris*, queens exhibit a positive relationship between sexual productivity and longevity (Lopez-Vaamonde et al., 2009). Collins et al. (2023) therefore tested for condition-dependence in this relationship in this species by repeating the experiment of Schrempf et al. (2017), i.e. by experimentally manipulating *B. terrestris* bumblebee queens to increase their egg-laying rate using egg-removal. The results were that treatment queens had reduced longevity relative to control queens. Additionally, expression levels of age-related genes in brain and fat body of treatment queens differed from those of control queens. These results suggested that, as hypothesised, costs of reproduction in *B. terrestris* queens occur but that they are latent, i.e. are masked in unmanipulated conditions because high-quality queens are able to overcome them, leading to the positive fecundity-longevity relationship shown by unmanipulated queens (Collins et al., 2023). The

findings of Collins et al. (2023) therefore further suggest that positive-fecundity-longevity associations in eusocial queens can be condition-dependent or unconditional, as a function of the degree of eusocial complexity.

Workers in many species of eusocial Hymenoptera have the potential to be reproductive, producing male offspring from unfertilised eggs via haplodiploidy (Bourke, 1988). Positive fecundity-longevity relationships also occur in reproductive workers in such species. For example, in the ant *Platythyrea punctata*, workers that become reproductively active show significantly longer longevities than non-reproductive workers (Hartmann and Heinze, 2003). Similar positive effects of reproductive activity on longevity in workers have been found in *A. mellifera* (Dixon et al., 2014; Kuszewska et al., 2017). In this case, reproductive activation is linked to greater protection from biotic and abiotic stressors, which suggests a potential proximate basis for the increased longevity of reproductive workers (Kennedy et al., 2021). There are also cases where worker reproduction has negative effects on worker longevity such as in the ants *Diacamma* sp, in which case workers that reproduced had a drastically reduced longevity compared to when they did not reproduce (Tsuji et al., 2012).

In *B. terrestris*, Blacher et al. (2017) tested how reproduction affects the longevity of workers experimentally. They found that, in unmanipulated conditions in whole colonies, workers exhibited a positive association between the level of ovary activation and longevity. Likewise, in these conditions, workers with activated ovaries showed increased longevity compared to reproductively inactive workers. Using a technique of Alaux et al. (2007); Blacher et al. (2017) then experimentally manipulated randomly-selected workers into activating their ovaries (by keeping them in queenless groups of three workers in which the two other workers were younger). These workers showed a negative association between the level of ovary activation and longevity, as well as decreased longevity compared to the control group (in which focal workers were each placed with two older workers, rendering them less likely to activate their ovaries). Blacher et al. (2017) therefore hypothesised that, in unmanipulated colonies, workers vary in quality and workers that become reproductive do so 'voluntarily' as high-quality workers able to overcome costs of reproduction. By contrast, workers experimentally forced to reproduce included low-quality workers unable to overcome these costs, leading to the negative fecundity-longevity association observed in these workers. In short, workers exhibit condition-dependence in the expression of costs of reproduction similar to that later reported in *B. terrestris* queens by Collins et al. (2023).

Positive fecundity-longevity relationships in eusocial insect queens and workers have, as a result of the light they shed on the fundamental issue of how sociality

affects ageing and longevity determination, become a major focus of interest (e.g. Korb and Heinze (2021)). However, other patterns found in the longevities in eusocial insects are also noteworthy in this respect. For example, a study by Holland and Bourke (2015) found that, in *B. terrestris*, which like almost all bumblebee species has an annual colony cycle, workers that eclose early in the colony cycle have significantly greater longevities (∼20 days on average) than those eclosing later on. Since this study was executed in the laboratory, such a difference in longevities could not be explained as the direct consequence of an increased risk of mortality associated with foraging for late-eclosing workers as has been proposed for other bumblebee species (Goldblatt and Fell, 1987; O'Donnell et al., 2000). At the ultimate level, it could be, however, that the queen's and workers' inclusive fitness interests are best served by not producing workers late in the colony cycle capable of living longer, given their increased foraging duties and the approaching end of the annual cycle of activity. More generally, an unresolved issue raised by the evolution of sociality from non-social ancestry, especially in euocial species, concerns the relative roles played by individual and social factors in detemining the ageing schedules and hence the longevities of social organisms. **Table 1.1** summarizes the studies discussed above, and related ones, evidencing the variety of the connections between sociality and longevity in eusocial species at different levels of eusociality.

Table 1.1. Summary of selected, key studies of possible ultimate causes of ageing in eusocial insects and the influence of level of eusociality.

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Ants (148 species)	Advanced eusocial	Longevity & life-history traits	Longevity positively correlated with eusociality; monogynous and independently founding queens had higher longevity	Keller and Genoud (1997)

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Acromyrmex echinator	Advanced eusocial	Longevity	Oxidative stress caused higher mortality in queenright than queenless workers; outside workers died earlier than inside workers	Majoe et al. (2021)
Atta colombica	Advanced eusocial	Longevity	Queenless workers survived oxidative stress better; inside workers outlived outside workers	Majoe et al. (2021)
Cardiocondyla obscurior	Advanced eusocial	Longevity	Workers in small colonies lived significantly longer than those in large colonies	Heinze and Giehr (2021)
Cardiocondyla obscurior	Advanced eusocial	Longevity & Reproductive rate	Egg removal increased queen fecundity; queen fecundity increased with and was positively correlated with longevity	Schrempf et al. (2017)
Oecophylla smaragdina	Advanced eusocial	Longevity	Minor workers more likely to reach old age; Lower longevity for major workers, with larger body mass	Chapuisat and Keller (2002)

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Platythyrea punctata	Advanced eusocial	Longevity	Reproductive individuals lived longer than non-reproductive workers	Hartmann and Heinze (2003)
Temnothorax rugatulus	Advanced eusocial	Longevity	Survival was much higher in queenless subcolonies; positive link between longevity and reproduction within castes	Negroni et al. (2021)
Apis mellifera	Advanced eusocial	Longevity	Oxidative stressors did not decrease survival in reproductively active workers; reproductive activation increased survival to virus and oxidative stressor	Kennedy et al. (2021)
Apis mellifera	Advanced eusocial	Longevity & Reproductive activity	Oviposition behaviour correlated with reduced mortality; reproductive workers outlived non-reproductive workers; early foraging correlated with lower longevity	Dixon et al. (2014)

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Apis mellifera	Advanced eusocial	Longevity & Reproductive potential	Workers developing under queenless conditions (high reproductive potential) lived 3–4 days longer; number of worker ovarioles correlated with longevity	Kuszewska et al. (2017)
Bombus terrestris	Intermediately eusocial	Fecundity	Queen longevity influenced workers' future reproductive success; queen fecundity declined later in colony cycle; aggressive workers more likely to activate ovaries	Almond et al. (2019)
Bombus terrestris	Intermediately eusocial	Longevity	Early-produced workers had greater longevity; Temperature extended colony longevity	Holland and Bourke (2015)
Bombus terrestris	Intermediately eusocial	Longevity & Fecundity	Worker ovarian activation positively associated with longevity; forced ovarian activation negatively associated with longevity; ovary-active workers were larger; smaller groups led to higher worker longevity	Blacher et al. (2017)

Species	Level of Sociality	Focus	Results	Reference
Bombus terrestris	Intermediately eusocial	Queen longevity and fecundity	Reproductive costs for queens: decreased queen longevity with experimentally increased fecundity via egg removal	Collins et al. (2023)
Bombus terrestris	Intermediately eusocial	Longevity & Reproductive success	Colonies with late switch-point had more female-biased sex ratios; queens with larger workforce had greater reproductive success; queen longevity positively correlated with reproductive success	Lopez-Vaamonde et al. (2009)

1.3.2 Sociality, Ageing and Longevity: Proximate Causes

On a proximate (mechanistic) level, since within almost all species of eusocial Hymenoptera there are no genetic differences between the reproductive and worker castes, the disparity in longevities between them, like their other differences (Evans and Wheeler, 1999; Hoffman and Goodisman, 2007), must be caused by differential gene expression. Recent progress has been made in elucidating the network or networks of signalling pathways that could potentially be driving ageing in social insects (summarised in **Table 1.2**).

For most insects there is a positive association between Juvenile Hormone (JH) and Vitellogenin (Vg) (Raikhel and Dhadialla, 1992). However, in *A. mellifera*, the relationship between JH and Vg is inverted, with a negative ('double repressor') feedback loop existing in workers (Pinto et al., 2000; Amdam et al., 2009; Flatt et al., 2013; Rodrigues and Flatt, 2016). In nurse workers, in which stress levels are low, levels of Vg are high and levels of JH low, whereas in stress-exposed forager workers, levels of Vg are low and levels of JH high (Flatt et al., 2013; Rodrigues and Flatt, 2016; Rueppell et al., 2016). The transition of a worker from a nurse with a high

stress resistance to a forager with a low stress resistance is triggered by decreasing Vg titres and increasing JH titres (Amdam and Omholt, 2003). JH therefore has a 'pro-stress' and 'pro-ageing' effect, as in non-social insects. By comparison, contrary to its role in non-social insects, Vg seems to be involved in somatic maintenance in *A. mellifera* by acting as an anti-oxidant (**Section 1.2.2**; Seehuus et al. (2006); Nelson et al. (2007); Flatt et al. (2013); Rodrigues and Flatt (2016)).

Several more aspects in the JH-Vg/YP network are different in *A. mellifera* compared to other insects and might partly be the mechanistic basis of the extended lifespans of queens (**Table 1.2**; Corona et al. (2007); Rodrigues and Flatt (2016)). Additionally to its important functions as a pro-maintenance antioxidant and as a yolk protein precursor, Vg is proposed to be an endocrine signal between JH and IIS in *A. mellifera*, as part of a feedback loop that triggers insulin secretion resulting in further production of JH (Corona et al., 2007; Rodrigues and Flatt, 2016). However, the general relationship (by which IIS is triggered by nutrition) between JH and IIS as found in most other organisms also appears to be reversed in *A. mellifera*. A down-regulation of IIS and insulin-like peptides (ILPs) is still positively associated with longevity, though this is not achieved by low nutrition, but rather is associated with the high nutritional input of queens being fed by their workers (Corona et al., 2007; Rodrigues and Flatt, 2016). Lastly, the classical gonadotropic function of JH occurs in queens in the pharate stage (i.e. just before shedding the pupal cuticle), in which high JH titres induce the synthesis of Vg (Barchuk et al., 2002). In adult queens, JH is not needed for Vg production (Amdam et al., 2004a), which is even being suppressed by high JH concentrations (Corona et al., 2007; Rodrigues and Flatt, 2016). The apparent reversal of the fecundity-longevity trade-off in (advanced) eusocial insect queens is therefore proposed to be connected to a rewiring of the IIS-JH-Vg/YP circuit (Corona et al., 2007; Rodrigues and Flatt, 2016).

This unusual negative coupling between JH and Vg as found in *A. mellifera* has been documented for other advanced eusocial insects as well, including some termite and ant species (**Table 1.2**). By contrast, in *B. terrestris*, JH was found to play the typical role of a gonadotropin, with elevated titres of JH in workers being associated with longer oocytes (Bloch et al., 2000). In queens of this species, Vg titres are also elevated compared to those of workers and there is a connection between high Vg titres and productivity (comparing fertile to virgin queens) (Amsalem et al., 2014). Similarly, Lockett et al. (2016) found that ovary-active *B. terrestris* workers showed higher expression of *vitellogenin* in fat body than ovary-inactive ones. *B. terrestris* shows a consistent association between aggression and reproductivity in workers (e.g. (van der Blom, 1986; Amsalem et al., 2015; Almond et al., 2019). However, the

relationship between aggression, reproductivity and Vg level is complex, as Amsalem et al. (2014), along with confirming the link between aggression and reproductive activity, also demonstrated a connection between Vg and worker aggression before ovary maturation. Studies of the advanced eusocial fire ant *Solenopsis invicta* reveal a strong correlation between JH and the uptake of Vg by queen oocytes and therefore the maturation and laying of eggs (reviewed in Bloch et al. (2002)), so constituting a more typical JH-Vg relationship. Such differences between eusocial insect species suggest that there is some multifunctionality of JH and Vg within the group. It seems, though, that there is a common pattern in the involvement of the JH-Vg signalling network and other genes in the downstream part of the TI–J–LiFe network in insect life histories (Korb et al., 2021).

Though it is intriguing to assume that a queen's long lifespan is simply explained by an up-regulation of known antioxidant genes, no evidence for such a connection could be found in *Apis mellifera* (Corona et al., 2005) or the ant *Lasius niger* (Parker et al., 2004) (except for *SOD3*; Lucas and Keller (2018)). Surprisingly, in these systems, old queens showed lower expression of antioxidant genes than young queens and old workers; SOD activity or other antioxidants was not highest in the reproductive caste of those species (Parker et al., 2004; Corona et al., 2005). The free radical theory would predict that high activity of the TOR signalling pathway would lead to higher metabolic rates and therefore more free radical production, resulting in lower lifespans (Stanfel et al., 2009). Contrary to this prediction, TOR activity was found to be lower in small short-lived workers and higher in long-lived large queens in *Apis mellifera* (Parker, 2010).

In social insects some reproductive proteins seem to play an antioxidant function, most importantly *vitellogenin* (Vg) (Seehuus et al., 2006; Amdam et al., 2012; Salmela et al., 2016). Experimentally inducing oxidative stress revealed that Vg is the most preferentially oxidised haemolymph protein in honey bee workers (Seehuus et al., 2006), which is most likely linked to its zinc-binding capacity (Amdam et al., 2004b). This could explain increased Vg titers in long-lived individuals (queens and overwintering workers) compared to short-lived individuals (forager workers and males) in *A. mellifera* (Amdam et al., 2009). This is another example of how in social insects genes connected to reproduction and ageing have multiple functionalities, and potentially an example for the proposed rewiring of conserved networks causing extreme longevities (Corona et al., 2007; Rodrigues and Flatt, 2016).

Overall, there seems to be a high variance in antioxidant gene expression patterns and protein damage accumulation between different (even closely related) eusocial species (Kramer et al., 2021; Lucas and Keller, 2020). A study, comparing expression

patterns of different eusocial species and castes, although documenting a great lack of consistency between species, found that oxidation levels were never increased with age in the reproductive caste (Kramer et al., 2021). This study's authors therefore argue that oxidative stress is a 'significant factor' in ageing, though it is hard to make out a general pattern, since antioxidant defence mechanisms and the manifestation of oxidative stress differ substantially between species (Kramer et al., 2021).

Table 1.2. Summary of selected, key studies of possible proximate causes of ageing in eusocial insects and the influence of level of eusociality.

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Lasius niger	Advanced eusocial	CuZn Superoxide Dismutase 1 (SOD1)	Lower expression levels in long-lived queens than in workers and males	Parker et al. (2004)
Lasius niger	Advanced eusocial	CuZn Superoxide Dismutase 3 (SOD3)	Significantly higher expressed in queens (brains) than workers, independent of age	Lucas and Keller (2018)
Solenopsis invicta	Advanced eusocial	Vitellogenin (Vg) & Juvenile Hormone (JH)	Queen: Strong correlation between JH and the uptake of Vg by oocytes	Bloch et al. (2002)
Temnothorax rugatulus	Advanced eusocial	TOR pathway	Worker: Fertility induction altered several genes involved in mTOR; Queen-presence had a strong negative effect on gene expression	Negroni et al. (2021)

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Temnothorax rugatulus	Advanced eusocial	Toll signaling, TOR pathway, antioxidants	Young founding queens: high investment into immunity (high *Toll* activity)and resilience (*TOR* down-regulation); Older queens: high antioxidant production	Negroni et al. (2019)
Apis mellifera	Advanced eusocial	Antioxidant genes	Higher antioxidant gene expression in younger queens	Corona et al. (2005)
Apis mellifera	Advanced eusocial	CuZn Superoxide Dismutase 1 (SOD1)	Queen-biased expression in the brain	Grozinger et al. (2007)
Apis mellifera	Advanced eusocial	Oxidative stress	Worker: Overlap in genes up-regulated by reproductive activation and paraquat exposure; Reproductive activation increased survival of virus exposure and a oxidative stressor	Kennedy et al. (2021)
Apis mellifera	Advanced eusocial	Superoxide dismutase (SOD), Catalase (CAT), GPx, TR	Lower ROS levels and SOD, CAT and GPx activity in young queens vs. old queens; TR activity higher in young queens	Hsieh and Hsu (2013)

Species	**Level of Sociality**	**Focus**	**Results**	**Reference**
Apis mellifera	Advanced eusocial	Juvenile Hormone (JH)	Worker: JH titers increase initiates transition from nurse to forager; JH is pro-stress and pro-ageing	Amdam and Omholt (2003); Flatt et al. (2013)
Apis mellifera	Advanced eusocial	Vitellogenin (Vg)	Worker: VG promotes somatic maintenance as antioxidant	Seehuus et al. (2006); Nelson et al. (2007); Flatt et al. (2013)
Apis mellifera	Advanced eusocial	Vg & JH	Nurse workers (stress-resistant): high Vg, low JH; Foragers (stress-supsceptible): low Vg, high JH	Rodrigues and Flatt (2016)
Apis mellifera	Advanced eusocial	Vitellogenin (Vg)	Forager worker: Lower Vg lead to lower zinc levels and fewer hemocytes and immunocytes	Amdam et al. (2004b); Seehuus et al. (2006)
Apis mellifera	Advanced eusocial	Vg & JH	Adult queens: Vg production is independent of JH and surpressed by high JH titers; classic gonadotropic function only in pharate stage	Amdam et al. (2004a)

Species	Level of Sociality	Focus	Results	Reference
Apis mellifera	Advanced eusocial	Vitellogenin (Vg)	Worker: Increased Vg titers (due to reduced brood rearing) increased longevity and delayed foraging	Amdam et al. (2009)
Apis mellifera	Advanced eusocial	Vg & JH	Workers: Low JH results in Vg production; high JH suppresses Vg synthesis	Pinto et al. (2000)
Bombus terrestris	Intermediately eusocial	CoQ7, Dnmt3, for, vg	Queen: CoQ7, Dnmt3, for, vg expression increased with age; Worker: Dnmt3 increased in fat body with age	Lockett et al. (2016)
Bombus terrestris	Intermediately eusocial	Juvenile Hormone (JH)	Workers: Elevated JH titers associated with longer oocytes	Bloch et al. (2000)
Bombus terrestris	Intermediately eusocial	Juvenile Hormone (JH)	Worker: JH injection increased ovary development	van Doorn (1989)
Bombus terrestris	Intermediately eusocial	Vitellogenin (Vg) & JH	JH and Vg are uncoupled; Vg levels higher in fertile queens than in virgin queens and fertile workers;	Amsalem et al. (2014)

1.4 *Bombus terrestris*: a model for studies of the effects of sociality on ageing and longevity

Eusociality evolved independently multiple times within the order of Hymenoptera. Among the over 25,000 documented bee species, belonging to more than 4,000 gen-

era, most are found to be solitary living animals and only 1,000 species are considered to be eusocial with varying degrees of eusociality (Goulson, 2010; Hughes et al., 2008). Most of the eusocial bee species can be found in the Apidae family, from *Bombus* spp. that are representing an intermediate level of eusociality, with low worker-queen dimorphism (Collins et al., 2017) to *Apis* spp. that show advanced levels of eusociality with high worker-queen dimorphism and worker castes split into task-specific sub-castes (Seeley and Morse, 1976).

Bombus species are widely used in scientific studies, for several reasons. As they are commercially reared for pollination (Velthuis and van Doorn, 2006), they are available independent of seasonal limitations on wild colony availability. They have relatively small colonies and a short, annual colony life cycle, and can be readily reared in captivity, making them particularly suited for behaviour and life-history studies. Furthermore, the availability of the fully sequenced genome of *B. terrestris* (and *B. impatiens*) (Sadd et al., 2015) has made them a model system in the study of the genomic and transcriptomic basis of eusociality and associated traits. Due to the worldwide decline of bumblebees and other wild bees (Brown and Paxton, 2009; Potts et al., 2010; Brown, 2011), they have also been widely studied in the context of understanding and mitigating threats to wild pollinators to safeguard human food security.

As in other eusocial insects, ageing and longevity determination are key life-history traits of bumblebees as the lifespan of the queen and workers strongly influence the reproductive success of the colony. *B. terrestris*, the buff-tailed bumblebee, is very suitable to study the influence of sociality on ageing as it shares the small-scale colonies and annual colony cycle of the genus, as well as amenability to captive rearing, making it straightforward to measure queen and worker longevities at the individual level in relatively large samples. It also shows a large discrepancy in longevity between queens and workers (as adults, in nature, *Bombus* queens live approximately 12 months while workers typically live 1-2 months; Goulson (2010)). Although *B. terrestris* does not exhibit a high level of task specialisation among workers (Goulson, 2010), there is still large variability between workers with respect to reproductivity and longevity (e.g. Bloch and Hefetz (1999); Lopez-Vaamonde et al. (2004); Zanette et al. (2012); Livesey (2023)). This reproductive plasticity makes the worker caste in particular well suited to the study of the influence of different social factors and life-history traits (such as reproductivity) on longevity (e.g. Blacher et al. (2017); **Section 1.3.2**).

1.4.1 Colony Life Cycle

Bumblebee species can be found from South America to the Arctic but are most abundant in the temperate climates of the northern hemisphere. The 250 described species of bumblebees (Williams and Osborne, 2009) show different colony sizes, nesting preferences and brood rearing modes, but almost all share a similar annual colony life cycle. It begins in spring, when monandrous (singly mated) or weakly polyandrous (multiply mated) foundress queens emerge from hibernation. In *B. terrestris*, queens are monandrous (Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000; Lopez-Vaamonde et al., 2004), which is associated with a mating plug deposited by males in the female reproductive tract on mating (Sauter et al., 2001). Spring queens are those that were produced in the previous summer and that, after mating, dug themselves into the ground, where they then spent up to 6 months of overwintering diapause. During this first solitary, post-emergence phase of the colony cycle, the queen searches for a suitable nest site. In the case of *B. terrestris*, this is typically a pre-existing underground hole or cavity (Goulson, 2010). Into a small clump of wax and pollen, the queen then lays her first eggs (oviposition), which are worker-destined diploid eggs. She actively incubates the eggs to a temperature of 30−32 °C until the larvae hatch after approximately four days (Heinrich, 1972). During this time she stays with the brood, feeding herself with nectar she had collected externally and stored beforehand. In so-called 'pollen-storer' species, such as *B. terrestris*, the larvae are fed individually with a mixture of regurgitated pollen and nectar, which the queen then collects on further foraging flights, while still actively incubating the brood (Goulson, 2010). Once the larvae have pupated, the queen will resume laying eggs and collect more resources. After 16-25 days the first batch of workers ecloses (Heinrich, 1979). From this point onwards the workers take over foraging, brood care (also by individual feeding of the larvae) and nest maintenance, while the queen remains in the nest and continues to lay more diploid worker-destined eggs. These early-stage colonies are small, consisting of only the queen and the first few batches of workers (Heinrich, 1979). The colony then grows rapidly. as the queen continues to be the only egg layer, solely laying diploid worker-destined eggs. During this phase there is little to no between-individual aggression in the colony (Heinrich, 1979; Duchateau and Velthuis, 1988).

Once the colony has reached a sufficient size, the queen ceases to lay diploid eggs and instead, over a period of up to ten days, switches to laying only haploid eggs, from which males will eclose (Duchateau et al., 2004). This event is known as the 'switch point' (Duchateau and Velthuis, 1988). It is usually followed by the 'competition point', which is defined as the point at which workers start to

lay haploid eggs themselves (Duchateau and Velthuis, 1988), with these eggs being capable of developing into viable males, so affording workers a means of achieving direct fitness. In some colonies, queens lay queen-destined diploid eggs from their later batches of diploid eggs (Duchateau and Velthuis, 1988; Goulson, 2010; Lopez-Vaamonde et al., 2009). Workers are potentially able to sense differences in the queen's pheromones associated with the queen's change to producing new queens (gynes), which induces their own reproduction, i.e the competition point (Bourke and Ratnieks, 2001; Alaux et al., 2006; Amsalem et al., 2015). This phase is usually accompanied by increased aggression and policing (egg-eating) behaviour between the workers and between the queen and her workers (Duchateau and Velthuis, 1988; Duchateau, 1989; Zanette et al., 2012). However, within colonies, approximately 55-70% of workers never lay eggs (Bloch, 1999). As well as laying eggs in their natal colonies, workers are able to leave the nest as 'drifters' and, in a form of intraspecific social parasitism, successfully enter non-nestmate conspecific nests to produce sons inside them (Lopez-Vaamonde et al., 2004; Beekman and Oldroyd, 2008; Blacher et al., 2013). A recent study also showed that *B. terrestris* workers, and those of some other *Bombus* spp, are unexpectedly capable of mating and founding their own colonies independently (Zhuang et al., 2023); the prevalence of this in the field is unknown and deserves further study.

In nature, as summer comes to an end, so do the floral resources that the colony relies on and the colony stops growing. While the gynes and males leave the nests to mate, the queen and the remaining workers die and it is only the newly-mated queens that enter hibernation before starting a new colony cycle in the following spring (Heinrich, 1979; Goulson, 2010).

1.4.2 Intrinsic Quality Variation in Workers

From the foregoing sections, it is clear that, within colonies, *B. terrestris* queens and workers show a very large degree of variation. In workers, this variation occurs, for example, with respect to body size, reproductivity (as non-reproductive or reproductive workers, and, as reproductive workers, as natal, drifter or potentially mated ones), level of aggression, policing behaviour and longevity (**Sections 1.4.1**). Moreover, associations exist between these traits, e.g. between aggression and reproductivity, reproductivity and adult body size, and reproductivity and longevity (e.g. Blacher et al. (2017)). In non-social species, it has long been argued that, because individuals vary in resources and resource-holding potential, quality variation can lead to an attenuation of the negative fecundity – longevity relationship otherwise observed (van Noordwijk and de Jong, 1986; Reznick et al., 2000). That is, when

high-quality individuals (showing both high fecundity and longevity) are compared to lower quality individuals (showing low fecundity and low longevity), the result is a positive relationship between fecundity and longevity, even though costs of reproduction are still present and the fecundity – longevity trade-off still remains at the within-individual level (van Noordwijk and de Jong, 1986; Reznick et al., 2000). Following these concepts, Blacher et al. (2017) therefore hypothesised that, in *B. terrestris*, worker variation in the traits listed reflected within-colony variation in worker quality. Under this interpretation, there are high-quality workers able to bear the costs of reproduction to a certain extent, before experiencing costs of reproduction, whereas lower-quality workers can sense their own low quality and, if unmanipulated, refrain from becoming reproductively active in the first place, so accounting for the relationships observed (Blacher et al. (2017); **Section 1.3.2**). It may even be that those workers recently discovered to be capable of successfully mating (the interaction with males in mating proving lethal in some cases) (Zhuang et al., 2023) are particularly high-quality ones (and, correspondingly, especially via their queen-like ability to found colonies, would be expected to have particularly elevated longevities). Evidence for such quality differences can be found in the positive relationship between body size and reproductivity and reproductivity and longevity in *B. terrestris* workers (Blacher et al., 2017).

As mentioned, worker quality is likely to be a composite function of several traits, including adult body size, reproductive activity and longevity. The proximate basis of such variation is not fully known. But factors directly affecting worker development from egg to adult, such as egg quality, larval nutrition and level of brood care all seem likely to be influential. These could further be shaped by social factors of the colony, such as colony composition and the colony stage in the annual cycle.

1.5 Conclusion

The intersection of the study of social evolution and the study of the evolution of ageing is a potentially highly informative area of investigation. In particular, as the preceding sections have shown, eusocial insects can help provide new insights into the evolution of ageing and the ultimate and proximate drivers behind it. The evolution of sociality is correlated with long lifespans and the potential attenuation or abolition of certain physiological and life-history trade-offs, such as the fecundity-longevity trade-off, in reproductive individuals. Several ultimate causes connected to sociality and associated traits including reproductive division of labour have been hypothesised to drive this evolution towards elongated longevities of the reproductives

and reduced longevities of non-reproductives, such as: a shift in energy allocation towards somatic maintenance in reproductives; a reduction of extrinsic mortality risk; inclusive fitness benefits for both queens and workers, if the reproductive lives longer and produces more (sexual) offspring; and the age of first helping replacing (in workers) the age of first reproduction as the evolutionary trigger of ageing. However, despite substantial progress in understanding the full effects of sociality on ageing and longevity detailed in the previous sections, important gaps in our knowledge remain, some of which I aimed, in the current thesis, to address.

Studying variation in longevity among workers, particularly in groups of highly-related individuals such as those of eusocial insects including *B. terrestris*, can bear insights into the potential determinants of longevity under sociality, such as differences in worker quality and the effect of reproductivity on longevity. Yet relatively few studies exist measuring individual worker longevities in eusocial insects as a whole (**Table 1.1**), and no previous studies have tracked lifetime reproductivity in *B. terrestris* workers, both of which are needed to further understand the connection between sociality and ageing and to understand if longevity is influenced by characteristics of the individual or characteristics of the group/colony.

This thesis therefore set out to further investigate the connection between reproductivity and longevity under a social context using the study system provided by *B. terrestris* workers. **Chapter 2** experimentally tested the hypothesis that, in *B. terrestris* workers, a positive fecundity-longevity relationship is connected to high intrinsic quality in adults, and that this arises from better larval nutrition. It therefore investigated whether increased or decreased levels of nutrition during larval development will lead to workers with increased reproductivity and longevity and to workers with low reproductivity and longevity, respectively. **Chapter 3** aimed to discriminate experimentally between two alternative hypotheses for longevity determination in social organisms, namely the hypotheses that either individual or social factors take the leading role in this process (respectively, the Individual and Social Hypotheses). Utilising the finding that workers produced early in the colony cycle live longer than those produced late in the colony cycle, I conducted a reciprocal transfer experiment of workers between early and late colonies, predicting that either the stage of the donor colony (Individual Hypothesis) or that of the recipient colony (Social Hypothesis) would explain resulting patterns of worker longevity. In the same experiment, I also used gene expression data from mRNA-seq to investigate differences in the experimental workers in age-related gene expression and so test between the hypotheses at the transcriptomic level. **Chapter 4** followed up on a novel pattern uncovered in the current thesis, i.e. that the frequency distribution

of within-cohort worker longevity within *B. terrestris* colonies is often multimodal, aiming to use it to conduct a further experiment to discriminate between the Individual and Social Hypotheses. In particular, to test whether this pattern is robust and intrinsically anchored to worker quality, and whether it can be expressed by isolated individuals or requires a social environment, longevities of workers in isolation were compared to longevities of workers in a social colony setting. Lastly, **Chapter 5** used mRNA-seq data to investigate the transcriptomic basis of the connected traits of worker reproductivity and longevity, comparing age-related gene expression in ovary-active and ovary-inactive workers and characterising changes in the gene expression profiles of young workers during the process of ovary activation.

Overall, therefore, in this thesis, I aimed to generate new insights into the effect of sociality on ageing and longevity and in particular into its ultimate and proximate drivers.

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Chapter 2 | An Experimental Test of the Effect of Larval Nutrition on Reproductivity, Longevity and Body Size *Bombus terrestris* Workers

Abstract

The widespread trade-off between fecundity and longevity seems to be absent in reproductive females of eusocial insects. An example is the bumblebee *Bombus terrestris*, in which both queens and workers express a positive relationship between fecundity and longevity. However, experiments have shown the likely existence of condition-dependent costs of reproduction, with, in workers, quality varying within colonies such that low-quality workers are shorter-lived if forced to be reproductively active. This study therefore experimentally tested the hypothesis that, in *B. terrestris* workers, a positive fecundity-longevity relationship is connected to high intrinsic quality in adults, and that this arises from better larval nutrition. To test this, ten *B. terrestris* colonies were split into two halves, with worker-destined larvae receiving a high nutritional treatment in one half and a low nutritional treatment in the other. The reproductivity (egg-laying), longevity and body size of workers developing from these larvae were then individually measured. The results showed no direct effect of the nutritional treatments on worker reproductivity or longevity. However, workers that had received high levels of larval nutrition were significantly larger than those that received low levels of larval nutrition. In analyses considering all focal workers (irrespective of treatment), significant positive associations were found in workers between reproductivity and body size, reproductivity and longevity, and body size and longevity. Egg-laying had a significant positive effect on survival early in life but appeared to negatively affect survival later in life. In addition, bimodality was found both in the age at which egg-laying workers laid eggs and in the frequency distribution of longevity of all workers. Overall, the demonstration of significantly positive associations of reproductivity, longevity and body size was as predicted by the hypothesis. The findings regarding bimodality further suggested that at least some variation in worker quality might be discontinuous.

2.1 Introduction

A trade-off between the two major contributors to evolutionary fitness, fecundity and survival, is found widely throughout the animal kingdom (Stearns, 1989; Kirkwood et al., 1991). One possible explanation for this is within-organism competition for resources between somatic maintenance and reproduction (Kirkwood, 1977; Maklakov and Chapman, 2019). Where selection favours reproduction, and resources are allocated differentially, reproduction might come at a cost to somatic maintenance and ultimately longevity. For the organism, such a cost could therefore lead to somatic deterioration over time, decreasing the survival probability beyond a certain point in time, that is, to ageing (Williams, 1957; Hamilton, 1966; Kirkwood, 1977; Stearns, 1992; Edward and Chapman, 2011; Maklakov and Chapman, 2019).

Generally, social organisms show extended longevities compared to closely related solitary species (Kim et al., 2011). Additionally, the otherwise widespread fecundity-longevity trade-off is not always apparent or present at all. For the reproductive members of such social groups, from cooperatively breeding birds to eusocial insects, the trade-off seems to be abolished, and they often live longer than non-reproducing group members (Hammers et al., 2019; Korb and Heinze, 2021). In addition, within the reproductive caste, there can even be a positive fecundity-longevity relationship, with highly reproductive queens living longer than reproductively less active queens (e.g. *Cardiocondyla obscurior* ants; Heinze and Giehr (2021)). Queens of *C. obscurior* even increase their egg-laying-rate with age (Heinze and Schrempf, 2012) and only late in life (irrespective of the total lifespan) do they switch to producing sexual offspring (males and new queens) (Jaimes-Nino et al., 2022). This life-history strategy has been described as 'continuusparity' and consists of 'continuous reproduction with a fitness peak late in life' (Jaimes-Nino et al., 2022). In species with less complex eusocial structures, such as the bumblebee *Bombus terrestris*, the same positive relationship between fecundity and longevity can be found among queens, with high lifetime reproductive success (production of adult sexuals) being positively correlated with high longevity (Lopez-Vaamonde et al., 2009).

In many species of eusocial Hymenoptera, the workers are not completely sterile and are able to reproduce to a certain extent (Bourke, 1988). Workers may activate their ovaries and reproduce by laying unfertilized eggs that develop (through haplodiploiody) into haploid males. In such cases it has been found that the positive fecundity-longevity relationship that queens exhibit also occurs in reproductively active workers. For example, it has been reported that reproducing workers show higher longevities than their non-reproducing nestmates in honeybees (*Apis mellifera*, Dixon et al. (2014)), ants (*Temnothorax rugatulus*, Negroni et al. (2021)) and

bumblebees (*B. terrestris*, Blacher et al. (2017)).

In bumblebees, this positive relationship between fecundity and longevity in reproductives and workers has been hypothesised to be connected to differences in quality between individuals (Blacher et al., 2017). Blacher et al. (2017) argued that, just as in non-social organisms, in which an attenuation of the longevity-fecundity trade-off can occur in individuals with a high intrinsic quality or those that hold high quality resources (van Noordwijk and de Jong, 1986; Reznick et al., 2000), in eusocial insects too there may be high-quality individuals that reproduce more and live longer. In a comparison between individuals this then appears as a positive relationship between fecundity and longevity, but the costs of reproduction are still present, and the fecundity-longevity trade-off still remains at the within-individual level (van Noordwijk and de Jong, 1986; Reznick et al., 2000; Blacher et al., 2017).

Blacher et al. (2017) tested this hypothesis by allowing focal *B. terrestris* workers to freely activate their ovaries in unmanipulated colonies. In an experiment, they also placed focal workers either with two younger workers each, which induced the focal workers to activate their ovaries, or with two older workers each, which prevented focal workers from activating their ovaries. Workers in the unmanipulated colonies, which had voluntarily activated their ovaries (such that not all workers did so), exhibited increased longevity relative to non-reproductive workers (Blacher et al., 2017). By contrast, in the experiment, workers forced to be reproductively active exhibited reduced longevity relative to non-reproductive workers and hence, in each such worker, the common trade-off between fecundity and longevity seemed to be present (Blacher et al., 2017). The inference was that workers differ in intrinsic quality and that only high-quality workers can overcome the costs of reproduction. Hence, in unmanipulated colonies, it would be only the high-quality workers that choose to activate their ovaries, and a positive fecundity-longevity relationship is shown. But in the experiment, in which workers are forced to activate their ovaries regardless of their intrinsic quality, some workers will be of low-quality and unable to overcome the costs of reproduction, leading to a negative fecundity-longevity relationship. Collins et al. (2023) hypothesised that, similarly to the case in workers, costs of reproduction would be present but latent in *B. terrestris* queens, i.e. that these queens exhibit condition-dependent positive fecundity-longevity relationships. This was supported by an experiment in which the egg-laying-rate of queens was experimentally increased (by removing eggs, which causes queens to upregulate their rate of egg-laying). The result was that, as predicted by the hypothesis, the treatment queens expressed a cost of reproduction by showing shorter longevities than control queens whose egg-laying-rate remained unaltered (Collins et al., 2023).

Given that workers within eusocial insect colonies are (typically) highly related, the differences in quality hypothesised to occur between them are likely, at the proximate (mechanistic) level, to be partly caused by environmental differences. It has therefore been proposed that such quality differences could arise from unequal nutrition during larval development (Blacher et al., 2017). A connection between different levels in larval nutrition and fecundity in the adult workers was found to be present in *A. mellifera* workers, in which a high protein diet during either larval development or adult lifespan was connected to high levels of ovary development (Hoover et al., 2006). However, in ant workers (*T. rugatulus*), the same association could not be recorded. In this case, high protein diets led to shorter longevities, while not affecting the reproduction of fertile workers (Choppin et al., 2023). It may be, therefore, that a high protein diet does not necessarily represent a high quality diet across all taxa. The effect of nutrition on longevity and ageing has also been widely studied in the context of dietary restriction, which has been found to be associated with extended lifespans in nematodes, invertebrates and mammals (Mair and Dillin, 2008; Nakagawa et al., 2012; Cypser et al., 2013; Sultanova et al., 2021). Such findings seem to run counter to the hypothesis that a high quality diet might lead to greater fecundity and longevity. Yet, these studies usually focus on non-social organisms and on nutrition levels during adulthood rather than during development, suggesting that they are not necessarily incompatible with the hypothesis.

In eusocial insects with morphological adult castes (i.e. with queens differing morphologically from workers), an individual's caste is determined pre-imaginally, that is in the larval stage (Wheeler, 1986). In Vespidae (wasps), differences in nutritional levels during larval development were found to underpin pre-imaginal caste determination, as queen-destined larvae receive higher-quality nutrition (O'Donnell, 1998). In *Bombus*, within the worker caste, worker-destined larvae receive varying resource allocation and brood care depending on their physical position within the nest, with the more centrally-located larvae being fed more frequently, resulting in increased adult body sizes (Couvillon and Dornhaus, 2009). Blacher et al. (2017) found that, in *B. terrestris*, workers in unmanipulated colonies that had activated ovaries also had significantly larger body sizes (as well as higher longevities) than workers with inactive ovaries. Further, in *B. terrestris*, adult worker body size was found to be positively influenced by colony age and negatively correlated with the frequency of brood to queen contact, rather than caused by differences in egg quality (Shpigler et al., 2013). However, whether differences in larval nutrition affect adult body sizes in workers, with larger workers being of higher individual 'quality' in terms of reproduction and longevity, has not previously been experimentally tested

in either *B. terrestris* or other eusocial insects.

Using the *B. terrestris* study system, this study therefore aimed to test experimentally for the first time the hypothesis that differences in larval nutrition result in differences in body size among adult workers within colonies, with body size reflecting intrinsic quality such that larger (higher quality) individuals are, relative to smaller (lower quality) ones, able to exhibit a positive fecundity-longevity relationship. For this, we designed a split-colony approach, which allowed manipulation of larval nutrition to create different nutritional levels within single colonies. As a measure of reproductivity, the egg-laying rates of the workers that eclosed from these larvae were individually recorded, rather than their levels of ovary activation, because it is known that activated ovaries do not necessarily result in workers laying eggs (Alaux et al., 2004; Sibbald and Plowright, 2014). Longevity and body size of the adult workers were then also individually recorded. The two compartments of each colony received differentially manipulated quantities of pollen and nectar. The compartment under the 'high' nutrition-treatment received 130% of the daily per capita pollen and nectar requirement of larvae while the compartment under the 'low' nutrition-treatment received 70% of the daily per capita pollen and nectar requirement of larvae. According to the hypothesis, we predicted that workers that developed under the high nutritional-treatment would have larger body sizes, be more likely to be egg-layers and live longer than workers developing under the low nutritional-treatment.

2.2 Methods

2.2.1 Colony Rearing

Methods were as also described in Livesey (2023). Twelve queenright, pre-competition point *B. t. audax* colonies were received on 10 February 2022, having been supplied by Biobest (Westerlo, Belgium). (In *B. terrestris*, the competition point is defined as the point in the colony cycle at which workers first lay eggs; (Duchateau and Velthuis, 1988).) For the duration of the experiment, all colonies were kept in a controlled climate room (temperature: 28°C; humidity: 60%) in constant darkness. Upon arrival, the colonies each had a queen, a small amount of brood and a mean of 25.3 ± 5.1 SD workers. Each colony was randomly assigned a number between 1 and 12. On the day following arrival, the colonies were transferred into wooden colony nest-boxes (internal dimensions 17 cm x 27.5 cm x 16 cm high) with clear Perspex lids, in which they would remain for the duration of the experiment. These nest-boxes were equipped with two plastic frames glued closely to each other into the box along the middle line. This allowed for a split-colony design later on, i.e. for later splitting of each nest-box into two equal halves with a mesh barrier.

For transfer, all the workers were carefully removed from the supplier's box using forceps and temporarily placed into a 1 l conical flask. The queen was kept in a marking cage. Equal amounts of brood cells that were visually assessed to be containing eggs, along with equal amounts of all available pupae, were transferred into each half of the nest-boxes, directly onto the wooden floor. If damaged, the brood cells were re-sealed with wax of the same colony. These brood cells might have also contained 1st and 2nd instar larvae, as such larvae may occur in communal larval-cells that resemble egg-cells in size (Alford, 1975). Larvae of the 3rd instar (which also occur in communal larval-cells, but with these cells being recognisably larger) and 4th instar larvae (in individual brood-cells) were not transferred. This was because these larvae could have been influenced by external factors, such as nutrition availability and temperature, during transport, and would not have later experienced the experimental nutritional treatments (see below) for the majority of their development. Each queen was then carefully placed into the new nest-box for her colony before her workers were added. The brood left over in the supplier's box was discarded.

Any workers developing from the eggs transferred in this process were considered focal workers in this experiment. All the workers eclosing from the transferred pupae and the adult workers present on arrival were considered non-focal workers and were retained to provide a workforce to rear the focal brood (the brood developing under

the nutritional treatments described below). For the first 48 hours after transfer, the colonies were supplied with *ad libitum* access to sugar syrup (i.e. supplier's artificial nectar) and fresh pollen. The workers and queen could freely move around the whole nest-box, so ensuring recovery from transport and adjustment to the new environment and laboratory conditions.

2.2.2 Experimental Design

Split-colony Design

The experiment employed a split-colony design, which involved dividing each colony in its nest-box into two equal halves. For this purpose, on 13 February 2022, all the workers (non-focal workers) were removed from the nest-box and a thin sheet of metal mesh (17 cm x 16 cm) was then inserted between the two plastic frames. This created two equal-sized compartments divided by a mesh barrier (mesh size: 0.5 mm), with the mesh preventing workers and the queen from moving between the two compartments while still allowing a shared airflow within the colony . Each of the two compartments was then randomly assigned to one of two nutritional treatments: 1) the **H-treatment** with a high feeding regime that supplied larvae with 130% of the estimated mean per day per capita larval pollen and syrup intake; 2) the **L-treatment** with a low feeding regime that supplied larvae with 70% of the estimated mean per day per capita larval pollen and syrup intake. (The rationale for, and calculation of, these levels are described further below.) Before returning them to the nest-boxes, the non-focal workers within a given colony were randomly split into two equal groups and each group was then assigned to one of the treatments within the same nest-box. If the number of non-focal adult workers was not an even number, one random worker was removed and frozen, so allowing each nest-box half to receive the same number of workers. All non-focal workers were marked on the top of the thorax with non-toxic marking paint (Queen Marking Paint, Thorne Ltd., UK), which was purple if they were assigned to the H-treatment side, or blue if they were assigned to the L-treatment side. For twelve days following the transfer of the colonies to the nest-boxes, newly-eclosed workers in either compartment were considered to be non-focal workers and marked accordingly with purple or blue paint depending on the compartment they had eclosed in. This time period was chosen to include all the workers eclosing from the pupae present upon colony arrival as belonging to the group of non-focal workers (worker-destined pupal development time: 9.9 ± 0.9 days, Cnaani et al. (2000); Tian and Hines (2018)).

The absence of the queen in a *B. terrestris* colony can alter internal colony dy-

namics greatly. Adult workers are able to activate their ovaries and start laying eggs within 7-8 days if they cannot detect the queen (Duchateau and Velthuis, 1989; Alaux et al., 2007). For the workers to detect the queen and her pheromones, direct contact with her is necessary (Alaux et al., 2004). Diploid (female) larvae also respond to the queen's absence, which may lead to their development as gynes (newly-eclosed, unmated queens) rather than workers (Cnaani et al., 1997; Lopez-Vaamonde et al., 2007; Alaux et al., 2005). To maintain effectively queen-right conditions (i.e. conditions with queen present) within each compartment of a given colony, the queen was moved three times a day (every 6-12 hours) from one compartment to the other. This was done each day at 08:00, 14:00 and 20:00 by gently grasping the queen by one of her legs using long forceps and placing her in the other compartment on top of the brood. Within each colony, the timings of these transfers created a 6 h period of queen presence and a 6 h period of queen absence for each compartment during the day (08:00-20:00), while ensuring that the queen spent the longer night period (12 h, 20:00-08:00) in alternating compartments each night. In six of the colonies the queen's initial compartment was the H-treatment compartment and in the other six colonies it was the L-treatment one. The initial compartment was assigned randomly.

The experiment was divided into three phases. The first phase started with the splitting of the colonies and ended when the first focal workers eclosed; during this phase all focal brood underwent one of the two nutritional treatments as it developed. The second phase covered the period of eclosion of all the focal workers; during this phase the nutritional treatments continued and adult worker numbers of both compartments of a colony were regularly equalised. The third and final phase of the experiment started once the planned sample size for the focal workers was reached; at the start of this phase the mesh barrier was removed and the nutritional treatments were discontinued.

During daily inspections, all dead workers were removed from the colonies and their death and their individual identity recorded. In the third phase (after nutritional manipulation ended and all focal workers had eclosed) the colonies started producing reproductive offspring (gynes and males), and these were also removed (as such individuals disperse from colonies shortly after eclosion).

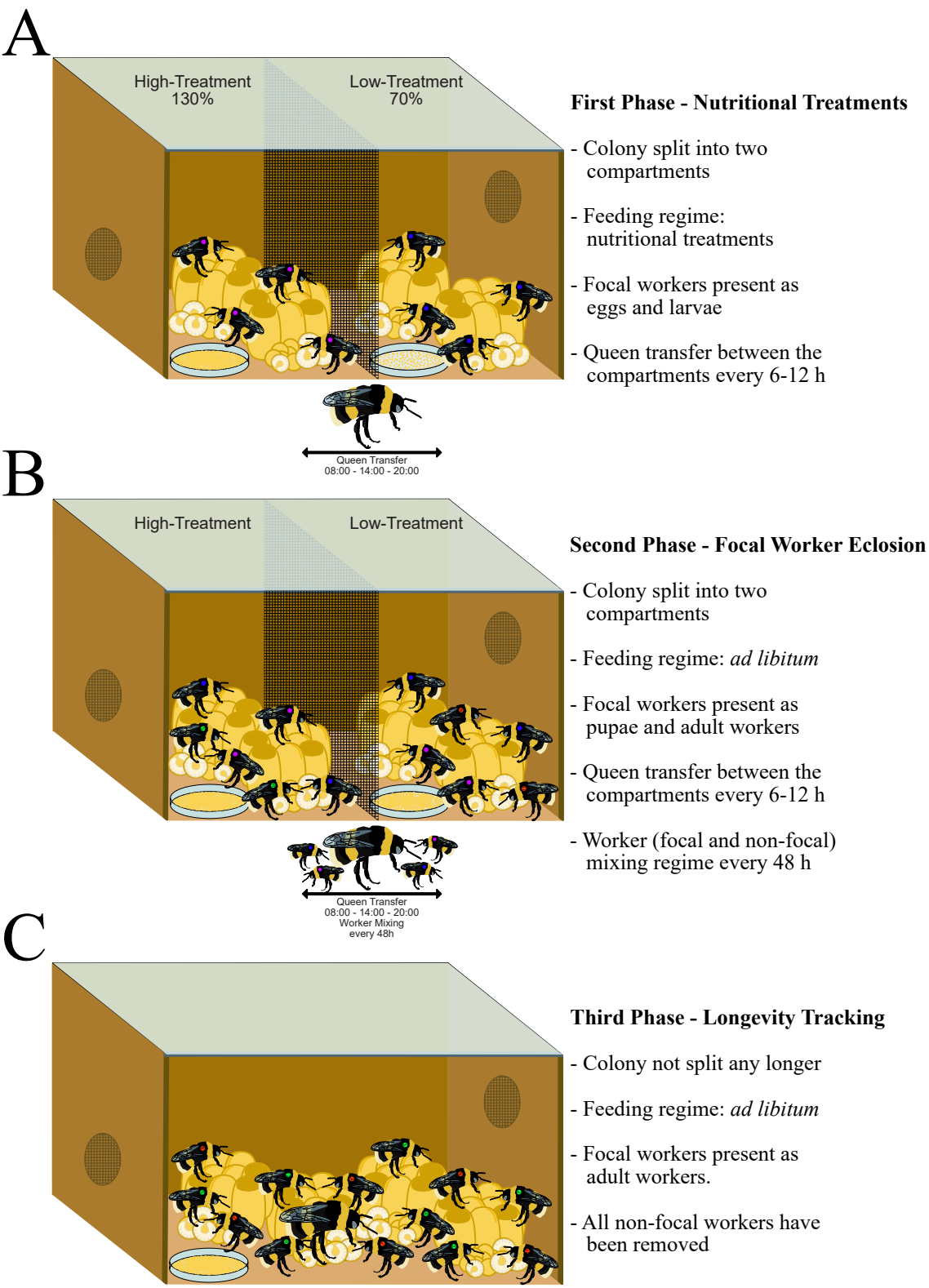

Figure 2.1. Schematic visualisation of the experimental setup throughout the three phases of the experiment using *Bombus terrestris audax* colonies. **A**: First Phase: Focal workers are developing as eggs and larvae under the two nutritional treatments in a split colony. The queen is moved between the compartment every 6-12 h. **B**: Second Phase: Focal workers have started eclosing or are in the pupal development stage. The nutritional treatments have been terminated and the colony is still split. The queen is moved between the compartment every 6-12 h. Every 48 h all the workers are randomly mixed between the two compartments. **C**: Third Phase: All focal workers have eclosed and all non-focal workers have been removed. The colony is no longer split. The longevities of the focal workers are being tracked and their behaviour observed.

First Phase - Nutritional Treatments

From 13 February 2022 until 4 March 2022, the feeding regime followed the nutritional treatments (described in detail below). In order to calculate the correct amount of pollen and sugar syrup given to each colony compartment according to the assigned feeding regime, workers and brood in each colony were censused every morning between 08:00 and 10:00. In *B. terrestris*, eggs and 1st to 3rd instar larvae can be found within communal wax cells containing 8-10 individuals of the same developmental stage (Alford, 1975; Duchateau and Velthuis, 1988; Zanette et al., 2012). It was therefore estimated that each egg or small larval cell contained 9 eggs or larvae respectively. By the time that larvae have reached their 4th instar, each larva is housed in an individual wax cell, and so such larvae were counted individually (**Equation S2.1**). In each compartment the nutritional treatments were ended once there was a minimum number of 15 focal pupae. Focal pupae were defined as those which were eggs or 1st instar larvae when the feeding regime started, such that all or nearly all their entire larval development was therefore under the assigned nutritional treatment.

For the nutritional treatments, the pollen and syrup requirements for the larvae were manipulated. Compartments assigned to the H-treatment received 130% of the larval requirements and compartments assigned to the L-treatment received 70% of the larval requirements, calculated as explained below. These levels were chosen to match observed levels of natural variation in adult worker body size within colonies (Owen, 1988; Goulson et al., 2002; Goulson, 2003). Nutritional requirements of adult workers were not manipulated, and this was to ensure that non-focal workers, which were needed to rear the focal brood, received a sufficient energy supply (Heinrich, 2004).

In the opening days of the first phase (between 13 February 2022 and 18 February 2022), nutritional requirements (100%) were calculated as the mid-point of the ranges estimated from the literature by Gradish et al. (2019) for the total amount (mg/day/bee) of nectar and pollen consumed per day for different life stages and castes of bumblebees. The assumed per day per capita requirements were therefore: 25.0 mg of pollen and 236.5 mg of nectar for adult workers and 25.0 mg of pollen and 42.0 mg of nectar for worker-destined larvae. These were then manipulated according to the treatments.

From 19 February 2022 onwards, pollen and nectar requirements were re-calculated to account for a surplus in pollen and a deficit of nectar observed in both treatments. For this, on each day, the mean mass of pollen and nectar consumed per larva during a 24 hour period two days previously was calculated. This was done by subtracting

the mass of pollen or nectar retrieved from a compartment from the mass put in the day before to obtain the mass that was consumed(**Equations S2.3, S2.2**). The mass that was expected to have been consumed by the adult non-focal workers (25 mg per capita of pollen and 236.5 mg (later 318.3 mg, see below) per capita of nectar, Gradish et al. (2019)) was subtracted from that mass. The resulting value for larval consumption was divided by the number of larvae that were present during that period of consumption. This resulted in a per capita larval consumption measure for each compartment. The mean between all compartments resulted in the daily consumption rate (dcr, **Equations S2.4, S2.5**) that was used to calculate the amount of pollen and nectar needed for each compartment according to the brood census of that day and the nutritional treatment assigned to the compartment, which remained at 130% of the (recalculated) daily requirement for the H-treatment and 70% of it for the L-treatment (**Equations S2.6, S2.7, S2.8, S2.9**). Because adult *Bombus* workers have a relatively high requirement for carbohydrates (found in the nectar) but a low requirement for proteins (found in the pollen) (Stabler et al., 2015), the daily nectar requirement for workers (=100%) was increased to 318.3 mg per worker (two thirds of the range in Gradish et al. (2019)) while the daily pollen requirement in the calculations was kept at the median level in Gradish et al. (2019).

Nutritional calculations and preparation of the required amounts of pollen and nectar were conducted every day between 10:00 and 14:00. To minimize disturbance of the colonies, feeders containing the calculated amounts of pollen and nectar were placed in each compartment of a given colony just after the moving of the queen at 14:00. At the same time, the previous day's feeders were retrieved and measurements for the calculation of consumption were taken. This meant that it was only possible to use the dcr values from two days prior for the daily calculations of pollen and nectar quantities.

Second Phase - Focal Worker Eclosion

The feeding regimes implementing the nutritional treatments (H and L) continued until there were 15 focal pupae present in a compartment. Brood in the two compartments of a given colony was likely to develop at different rates and in addition there was no guarantee that the queens would lay an equal number of eggs in each compartment. Therefore in some cases, the nutritional treatment in one compartment of a colony terminated earlier than in the other compartment (this was the case in 4 colonies). Once this point was reached the compartments were provided with *ad libitum* access to pollen and nectar.

Once focal workers had started eclosing, they were each marked individually

shortly after eclosion. A focal callow (newly-eclosed) worker was removed from the colony and placed in a marking cage, which was a cylindrical transparent tube open at one end and covered with a plastic mesh at the other end. The worker was then positioned by means of a foam plunger so that the area of the thorax between the wings was accessible through the mesh. With a toothpick applied through the mesh, a circular paper label bearing a number on a coloured background (Queen Marking Kit, Thorne Ltd., UK) was stuck to the worker's thorax by means of a small drop of shellac-based glue (Queen Marking Glue, Thorne Ltd., UK). Within a compartment, all marked workers received different numbers on the same colour of background, allowing each worker to be identified both individually and by compartment and colony.

While the focal workers were still eclosing, the mesh barrier was kept in place to ensure correct classification of newly-eclosed workers as having developed as larvae in either the H-treatment or L-treatment. Consequently, the procedure described previously of exchanging the queen between the two compartments of the colony was also continued.

Because focal worker eclosion spanned over several days and the mesh barrier was still dividing the colonies, to ensure that all focal workers had experienced similar social surroundings as adults during the days after eclosion such that any differences in adult longevity and behaviour resulting would have stemmed only from their nutritional treatment as larvae, a mixing regime between the two compartments was introduced. This regime was started for a colony once the first focal workers had eclosed and continued until all focal workers in that colony had eclosed. In this, within each colony, all workers (non-focal and focal) were removed every 48 hours, randomly split into two equal groups and returned to either compartment of their colony. Overall, this mixing regime took place between 2 March 2022 and 15 March 2022.

Third Phase - Longevity Tracking

This phase took place between 4 March 2022 and 22 August 2022. Once all focal workers in both compartments of a colony had eclosed and been marked the dividing mesh barrier was removed allowing the workers and the queen free movement throughout the whole nest-box.

At this point all non-focal workers were removed and frozen at $-20°C$. This measure was taken because for *Bombus* species it has been found that older workers tend to be more dominant than younger workers (van Doorn and Heringa, 1986; Duchateau and Velthuis, 1989). Dominant individuals are usually the ones that are

reproductively active and may suppress ovary activation in subordinate workers (van Doorn and Heringa, 1986; Duchateau, 1989; Bloch, 1999). Therefore, the removal of the non-focal workers, which from the experimental design were older than the focal workers, was carried out to ensure that the focal workers could express reproductive behaviour and egg laying without inhibition by older workers. In two colonies (7 and 8), only gynes or males eclosed, meaning that there were no worker-destined larvae present under the nutritional treatments and no focal workers were produced. These two colonies were therefore excluded from the final set of colonies in the experiment (leading to the final colony sample size, N = 10 colonies).

As previously stated, all colonies were checked daily for dead individuals, which were removed. If a dead focal worker was found, its death date was recorded and the worker was frozen at −20°C in a bag labelled with the worker's unique identity code (number + tag colour + colony) and the date of death. Any reproductives (gynes and males) that eclosed in the colonies were also removed. The last focal worker died on 22 August 2022, and this point marked the end of the third phase and the experiment as a whole.

2.2.3 Behavioural Observations

From the start of the first phase, the behaviour of the queens, focal and non-focal workers was recorded. This was done with direct in-person observation in bouts of 1 hour per colony conducted on five days per week. During the first phase (16-18 days), these observations resulted in 13-14 hours of behaviour observation data per colony. Once the mesh barrier and the non-focal workers had been removed (third phase), the behavioural observations were continued in the same manner, resulting in a further 52-54 hours of behaviour observation data per colony obtained between 4 March 2022 and 16 June 2022.

Various behaviours involving aggressive interactions between workers or between workers and the queen were recorded throughout the whole observation period (**Table 2.1**). Aggressive behaviours included: attack, butting, buzzing, darting and pumping (Duchateau, 1989). The identities of the individuals both performing and receiving aggression were recorded.

Reproduction was recorded by recording egg-laying in the observational bouts, both in the queen and any egg-laying workers. This behaviour was recognized by the individual placing its abdominal tip in an open egg-cell followed by visibly tapping its hind legs on the egg-cell wall (Bloch, 1999) (**Table 2.1**). The total number of egg-laying events performed by workers from each treatment group was recorded, as well the rate of egg-laying events per hour per colony and the number of egg-laying

individuals (egg-layers).

Table 2.1. Definitions of the behaviours recorded during observations according to Duchateau (1989); Bloch and Hefetz (1999) and Den Boer and Duchateau (2006) in *Bombus terrestris*, adapted from Livesey (2023).

Aggression	
Attack	Actor directly attacks recipient, by either grappling, stinging, or biting.
Butting	Actor makes an accelerated movement toward recipient, resulting in brief contact, before backing away.
Buzzing	Actor makes short wing vibrations directly at recipient. No physical contact between actor and recipient is made.
Darting	Actor makes sudden movement towards recipient but stops forward motion prior to making contact.
Pumping	Actor faces recipient and making pumping movements with abdomen, while audibly buzzing.
Reproduction	
Egg-laying	Worker places abdominal tip into an open egg-cell and visibly taps hind legs periodically on the egg-cell wall. Egg-layer typically waxes up opening of egg-cell after finishing egg-laying event.

2.2.4 Wing Measurements

In *B. terrestris* the length of the marginal wing cell can be used as a proxy for body size, because the two measures are highly correlated (Duchateau and Velthuis, 1989; Owen, 1988; Goulson et al., 2002). Using a *Zeiss SteREO Discovery.V12* dissection microscope, the length of the marginal cell of the left forewing of every focal worker was measured (the right forewing being measured if the left one was missing or deformed). To do this, a digital photograph of the forewing was taken with an AxioVision camera under a 15 x magnification and next to a 1 mm graticule. The length of the marginal cell was measured using the AxioVision software, with all such measurements being conducted by investigators blind to the longevity and nutritional treatment of the relevant focal worker (**Figure 2.2**).

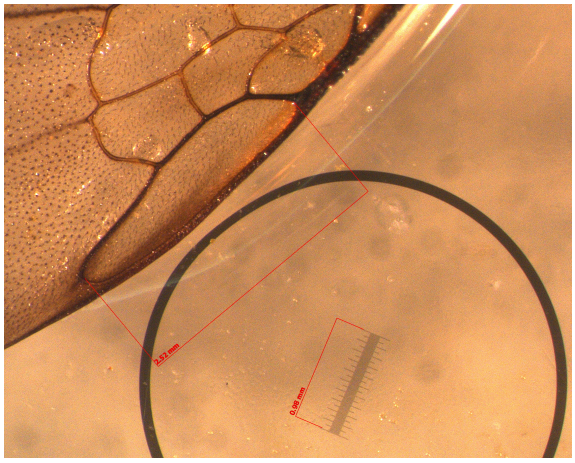

Figure 2.2. The length of the marginal wing cell is used as a proxy for body size in *Bombus terrestris* workers. Marginal wing cell of the left forewing of a *B. terrestris* worker under a *Zeiss SteREO Discovery.V12* dissection microscope at 15 x magnification with an AxioVision camera next to a 1 mm graticule (in black circle). The length was measured with the AxioVision software (red bars).

2.2.5 Statistical Analyses

All statistical analyses were performed in R-Studio (R Core Team, 2020; Posit team, 2024) version 4.4.1 (2024-06-14 ucrt). To test whether the feeding regimes under the two nutritional treatments actually resulted in different levels of larval pollen consumption, the mean daily per capita larval consumption rates per compartment were compared between the two treatment groups using a Mann-Whitney U test. The effect of treatments on adult focal worker body sizes (marginal wing cell length) was tested using a linear mixed model with the marginal wing cell length following a log-transformation as the response variable and the treatments ('Treatment') as a fixed effect. To account for differences between the ten colonies, the factor 'Colony' was included as a random effect. The model was constructed using the 'lme4'-package (Bates et al., 2015).

To compare different measures of reproductivity between the two treatment groups, a Mann-Whitney U test was conducted for the total occurrence of egg-laying events and the rate of egg-laying events per hour per colony. To compare the number of individual egg-layers between the two treatments, a generalized linear mixed Poisson model was used, with the count of egg-layers as the response variable, 'Treatment' as the fixed effect and 'Colony' as a random effect (Bates et al., 2015). The distribution of the age of the egg-layers at an egg-laying-event was tested for multimodality using the 'is.multimodal()'-function of the package 'LaplacesDemon' (Statisticat and LLC., 2021). This function uses a kernel density estimation and detects whether there are more than one 'true' peak (area under the curve $> 10\%$ of total density). If that is the case the distribution tests positive for multimodality, if not the distribution is considered unimodal. Afterwards, using the 'Modes()'-function of the same package, the number of distinct peaks and therefore the form of multimodality (bimodal, trimodal, etc.) was determined. (This analysis was conducted because later

experiments and analyses showed such a bimodality in worker longevity to be present within some colonies in *B. terrestris*; **Chapter 3**, **Chapter 4**). To test whether the treatments, body size or longevity predicted the likelihood of whether a focal worker was an egg-layer or not, a binomial generalized linear mixed model was implemented, with the binomial variable of being an egg-layer (yes/no) as the response variable and 'Treatment', 'Marginal Wing Cell' and 'Longevity' (the time difference in days between eclosion date and death date) as fixed effects and 'Colony' as a random effect (Bates et al., 2015). To achieve correct convergence of this model, the variables 'Marginal Wing Cell' and 'Longevity' had to be scaled because of largely different magnitudes.

For analyses including longevity and survival, only data from focal workers with a known death date could be included. Focal workers that were found dead under the comb material without a known date of death had to be excluded (N = 57) and those that did not die naturally (N = 10, e.g. drowned in the feeder) were censored to that date. The distributions of longevity in the treatment groups and per colony were tested for multimodality using the test described above. A survival analysis was conducted using a Cox proportional hazard regression model using functions from the 'survival'-package (Therneau, 2024). The model included a survival-object based on the longevity data as the response variable and 'Treatment' and 'Marginal Wing Cell' as fixed effects. Including whether a worker was an egg-layer or not did not fulfil the assumption of a proportional hazard ratio. This variable was therefore included in the model in two parts - as the binomial variable it self and as a time-dependent variable (Zhang et al., 2018). In order to analyse the change of the effect of egg-laying on survival, the 'adjusted hazard ratio' AHR in each day was calculated as AHR = exp(Coefficient(Egg-laying) + Coefficient(Egg-laying over time)*Day). To account for possible differences between the colonies while keeping the proportional hazard assumption, 'Colony' was included after stratification. Kaplan-Meier survival curves were created using the 'survminer'-package (Kassambara et al., 2020), including the results of a log-rank test. The same analysis was conducted for the subset of short-lived focal workers. This subset was defined as consisting of all workers that had longevities of the median longevity (92 days) or below. This analysis was conducted because a separate study (**Chapter 3**) suggested that relatively short-lived workers (i.e. the 50% dying before the median age of death) were more susceptible to factors affecting survival and longevity.

Workers' survival was further analysed using the Bayesian survival trajectory analysis of the BaSTA-package (Colchero et al., 2012). In this method, age-related mortality distributions are estimated using the Markov chain Monte Carlo approach.

Using the categories as the grouping factor, four simulations were run in parallel using a 'Gompertz' distribution with a 'simple' shape, 150,000 iterations, a burn-in of 15,001 chains and a thinning of 150. The distribution was chosen after running the 'multibasta'-function to determine which distribution best fit the data. To analyse differences between the categories, the Kullback Leibler discrepancy calibrations (KLDC) were compared, following the standard threshold by which a value higher than 0.85 suggests that the posterior distributions of the two categories are substantially different (Sultanova et al., 2021).

2.3 Results

A total of 603 focal workers were subject to the nutritional treatments during their larval development across all 10 colonies (300 focal workers in the H-treatment and 303 focal workers in the L-treatment). Per colony, the H-compartments produced a mean of 30.0 ± 18.2 (SD) focal workers and the L-compartments produced a mean of 30.3 ± 20.3 (SD) focal workers (t-test: t = -0.035, df = 17.773, p-value = 0.97). The estimated daily larval consumption rates of pollen were significantly lower (- 25%) in the compartments under the L-treatment (mean per day per capita: 4.83 mg ± 0.71 mg (SD)) than in those under the H-treatment (mean per day per capita: 6.45 mg ± 1.45 mg (SD); Wilcoxon test: W = 128, $p < 0.001$). This shows that the nutritional treatments delivered significantly different amounts of nutrition to larvae as intended (**Figure S2.1**). There was no difference in the total number of feeding events performed by adult non-focal workers recorded between the two treatments (H: mean 40.6 ± 13.17 (SD), L: mean 38.4 ± 13.27 (SD); Wilcoxon test: W = 61, p = 0.43).

2.3.1 Body Size

From the marginal wing cell length measurements, focal workers from the L-treatment (mean: 2.58 ± 0.23 (SD) mm) were significantly smaller than those from the H-treatment (mean: 2.64 ± 0.22 (SD) mm) (linear mixed model: $\chi^2 = 5.54$, p = 0.02, **Table 2.2**). This suggested that the nutritional treatments led to greater adult worker body size in the H-compartments and reduced adult worker body size in the L-compartments as predicted by the hypothesis. Note, however, that there was no significant difference in marginal wing cell length between L- and H-treatment focal workers in the subset of workers with longevities less than the overall median longevity of 92 days (**Table S2.1**). The analysis of adult worker body size excluded one worker from the H-treatment and one worker from the L-treatment, as

there were no wings left to measure on these individuals (H-treatment: N = 198; L-treatment: N = 208).

Table 2.2. Effect of the nutritional treatments on the length of the marginal wing cell (a proxy for body size) of adult *Bombus terrestris* workers. Summary of a linear mixed model: : log(Marginal wing cell) ~ Treatment + (1 | Colony). The low nutritional treatment (L-treatment, N = 208 from 10 experimental colonies) had a significant negative effect on the marginal wing cell length (p = 0.02), when compared to workers from the high nutritional treatment (H-treatment, N = 198 from 10 experimental colonies). Shown are the Estimate, the Standard Error, the degrees of freedom (df) and the t- and the p-value. For the random effect of Colony, the Standard Deviation and the Variance are displayed.

Fixed Effect	**Estimate**	**Standard Error**	**df**	**t-value**	**p**
Treatment (L)	-0.048	0.021	399.75	-2.33	0.02
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.040	0.113		

2.3.2 Reproductivity

Reproductivity and Treatment

A total of 540 h of behavioural observation data was collected. Each colony was observed for a mean of 54.0 ± 0.9 (SD) hours over 93-94 days, after the termination of the nutritional treatments (which means the data only included focal workers). Across all 10 colonies, a total of 304 egg-laying events were recorded. Overall, the rate of egg-laying events per hour per colony increased over the course of the experiment as the colonies matured (**Figure S2.2**). There was no difference in the total number of egg-laying events performed by focal workers of the H-treatment compared to the L-treatment (mean H: 16.4 ± 11.6 (SD) egg-laying events per colony, mean L: 14.0 ± 11.0 (SD) egg-laying events per colony, Wilcoxon-test: W = 57, p = 0.62), nor in the rate of egg-laying events per hour per colony (mean H: 0.2 ± 0.7 (SD) egg-laying events per hour per colony, mean L: 0.2 ± 0.6 (SD) egg-laying events per hour per colony, Wilcoxon test: W = 308473, p = 0.38). This means that the worker egg-laying activity in workers of both treatments was similar, because there was no significant difference in focal worker numbers between the treatments. Of all 603 focal workers, 151 individuals (25%) were observed to be active egg-layers

(i.e. observed to perform at least one egg-laying event). The proportion of active egg-layers did not differ between focal workers of the H-treatment (82 (27.3%) egg-laying individuals) and focal workers of the L-treatment (69 (22.9%) egg-laying individuals) (generalized linear mixed model, Poisson distribution: z = -1.27 p = 0.20, **Table 2.3**). Therefore, the nutritional treatments did not lead to significant differences in rates of egg-laying events per hour per colony or proportions of adult workers becoming egg-layers across the H- and L-compartments. The probability of being an egg-layer was not significantly affected by the nutritional treatments (**Table 2.4**).

Table 2.3. Effect of the nutritional treatments on the number of egg-laying workers in *Bombus terrestris*. Generalized linear mixed model (Poisson): count egg-layers ~ Treatment + (1 | Colony). Counts per compartment from the low nutritional treatment (L-treatment, N = 10) are compared to counts per compartment of the high nutritional treatment (H-treatment, N = 10). There was no significant effect of the nutritional treatments on the number of egg-laying workers per compartment. Shown are the Estimate, the Standard Error, and the z- and the p-value. For the random effect of Colony, the Standard Deviation and the Variance are displayed. The model explained 0.01 of the deviance (pseudo-R^2).

Fixed Effect	**Estimate**	**Standard Error**	**z-value**	**p**
Treatment (L)	-0.198	0.156	-1.270	0.204
Random effects	**Variable**	**Std Dev**	**Variance**	
Colony	Intercept	0.591	0.769	

Reproductivity and Body Size

For the focal workers as a whole (from both treatments), the probability of being an egg-layer was significantly positively associated with increased marginal wing cell length (body size) (binomial generalized linear mixed model: $p < 0.001$, **Figure 2.3A**, **Table 2.4**). Additionally, in focal workers as a whole, worker longevity was significantly positively associated with the probability of being an egg-layer (binomial generalized linear mixed model: $p < 0.001$, **Figure 2.3B**, **Table 2.4**).

Overall, therefore, although the nutritional treatments did not bring about the expected differences in worker reproductivity, the results demonstrated that, across all workers in both treatments , there were significantly positive associations in adult

workers between reproductivity and body size, and reproductivity and longevity, consistent with the predictions of the hypothesis.

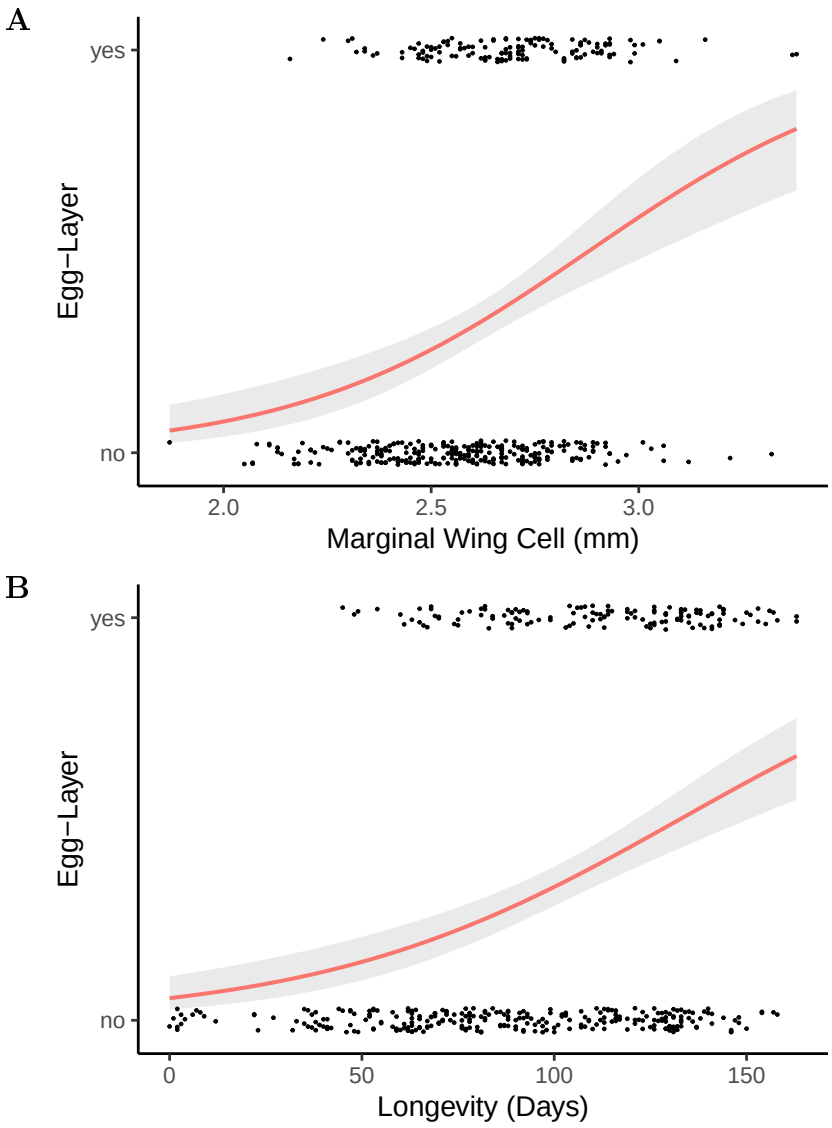

Figure 2.3. The effects of longevity and marginal wing cell length (proxy for body size) on the probability of being an egg-layer in adult *Bombus terrestris* workers. **A**:Marginal wing cell length was also a significant predictor for the binomial variable, 'Egg-Layer' (yes/no), i.e. larger focal workers were significantly more likely to be egg-layers. **B**: Longevity (days from eclosion to death) was a significant predictor for the binomial variable, 'Egg-Layer' (yes/no), i.e. longer-lived focal workers were significantly more likely to be egg-layers. N = 407 workers (irrespective of treatment, excluding workers with missing marginal wing cell length and missing longevity data). The model output summary can be found in Table 2.4.

Table 2.4. Effects of the nutritional treatments, marginal wing cell length (a proxy for body size) and longevity (days between eclosion and death) on the probability of being an egg-layer in *Bombus terrestris* workers. Generalized linear mixed model (binomial): egg-laying ~ Treatment + Marginal Wing Cell + Longevity + (1 | Colony). The variables Marginal Wing Cell and Longevity were scaled. Data from the low nutritional treatment (L-treatment, N = 209 from 10 colonies) were compared to data from the high nutritional treatment (H-treatment, N = 198 from 10 colonies). The nutritional treatments had no effect on the probability of being an egg-layer ($p = 0.33$). Marginal wing cell length and longevity each had a significantly positive effect ($p < 0.001$). The model explained 0.15 of the deviance (pseudo-R^2). Shown are the Estimate, the Standard Error, and the z- and the p-value. For the random effect of Colony, the Standard Deviation and the Variance are displayed.

Fixed Effect	**Estimate**	**Standard Error**	**z-value**	**p**
Treatment (L)	-0.234	0.244	-0.973	0.33
Marginal Wing Cell	0.591	0.135	4.377	< 0.001
Longevity	0.775	0.144	5.385	< 0.001
Random effects	**Variable**	**Std Dev**	**Variance**	
Colony	Intercept	0.035	0.187	

Reproductivity at different Ages

Analysing the age of all egg-laying workers (irrespective of treatment) at the point when they performed each egg-laying event revealed a significantly bimodal distribution with modes at 24 days of age and 88 days of age (**Figure 2.4**). Such a bimodal distribution of worker age at an egg-laying event was not a product of the nutritional treatments, as it was also found in workers of each nutritional treatment considered separately (**Figure S2.3**). Only 25 of the 151 focal workers (16.5%) that were observed egg-laying were recorded at ages within both modes (i.e. modal peak ± 15 days). A third of all egg-layers (33.8%) were observed laying eggs only at an age younger than the second mode ($\leq$ 74 days) and not thereafter. Within those, a quarter of all egg-layers (24.5%) were observed laying eggs only within the first mode (24 ± 15 days of age). Those egg-layers had a median longevity of 89 days. More than one third of all egg-layers (37.7%) were observed laying eggs only at an age greater than 39 days (24 + 15 days, representing the upper limit of the first mode). Within those, 31% of all egg-layers were observed laying eggs only within the second

mode (88 ± 15 days of age). Those egg-layers had a median longevity of 133 days. Overall, therefore, egg-laying workers appeared to fall into two groups, shorter-lived egg-layers, that (inevitably) only lay eggs at earlier ages, and longer-lived egg-layers, that start laying eggs only after the first group started dying.

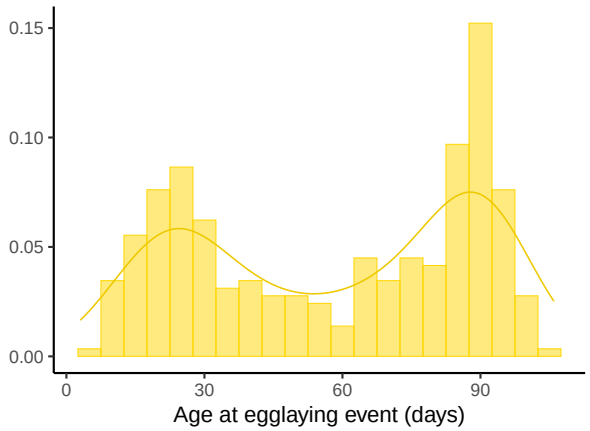

Figure 2.4. Worker age at egg-laying events in *Bombus terrestris* follows a bimodal distribution. Proportional histogram with a density curve of of the age of the workers on the date they were observed laying eggs (bin size = 5). This includes all recorded egg-laying events (N = 291, focal workers from both treatments), showing the proportion of egg-layers that laid eggs at a certain age (age in days from eclosion). The total area of all bins and under the curve respectively represents 100%. The distribution was significantly bimodal with modes at 24 days of age and 88 days of age.

2.3.3 Longevity

From the numbers of workers with both eclosion and certain natural death dates recorded, it was possible to obtain longevity data for 188 focal workers from the H-treatment and 181 focal workers from the L-treatment. The median longevity of all focal workers was 92 days (95% CI - upper: 98, lower: 88) (N = 369). The maximum longevity of any focal worker was 163 days.

Longevity and Treatment

The difference in median longevity between the two nutritional treatments was 3 days. For focal workers from the H-treatment, the median longevity was 94 days (95% CI - upper: 104, lower: 87) and the maximum longevity was 163 days. For focal workers from the L-treatment the median longevity was 91 days (95% CI - upper: 100, lower: 83) and the maximum longevity was 158 days.

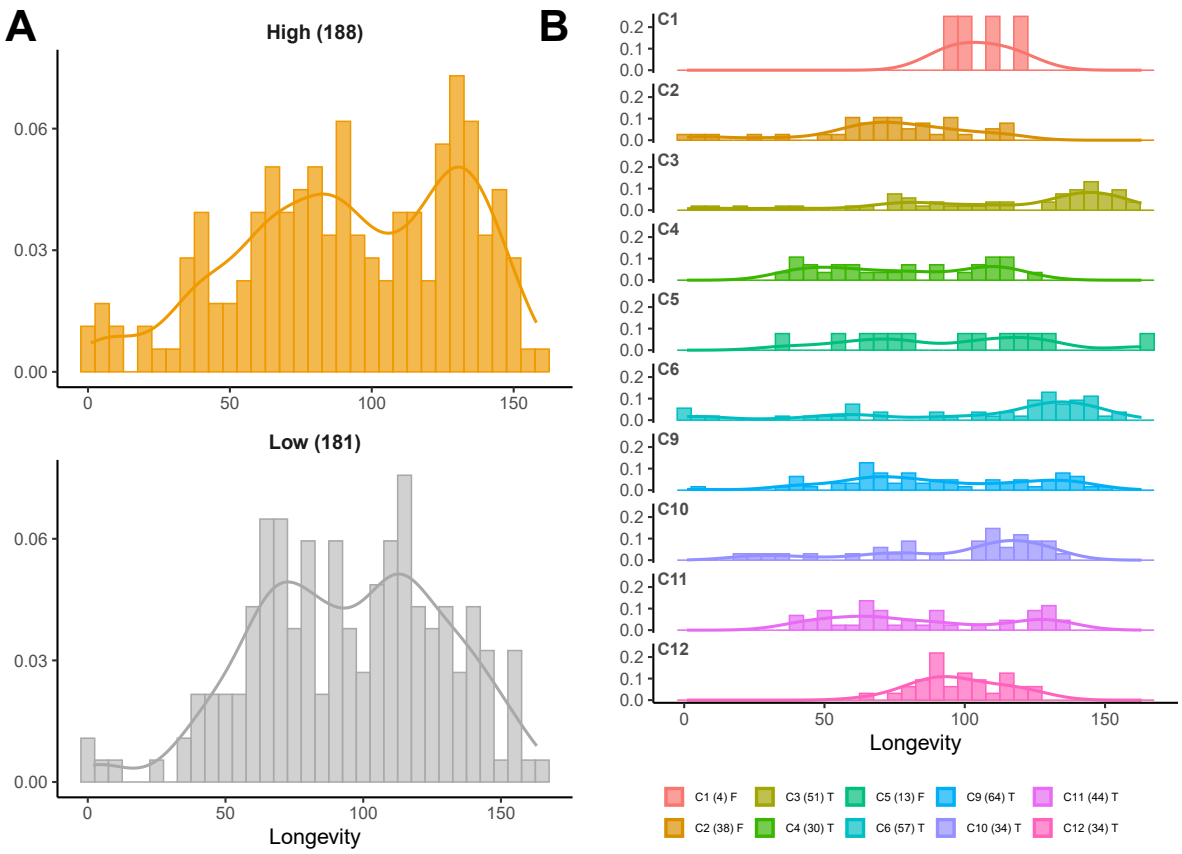

Figure 2.5. Worker longevity in *Bombus terrestris* follows a bimodal distribution. Distributions of focal worker longevity data. These data sets exclude censored workers. Proportional histograms with a density curve of worker longevities, showing the proportion of workers that died at a certain age (longevity in days from eclosion to death). The total area of all bins and under the curve respectively represents 100% of each sample. (Number in brackets representing the sample size; bin size 5 days.) **A**: Grouped by nutritional treatments (orange: high nutritional treatment, grey: low nutritional treatment) Both distributions tested significantly positive for bimodality with modes at 73 days and 113 days (H) and at 80 days and 130 days (L). **B**: Grouped by the 10 experimental colonies. The legend indicates which distributions tested significantly positive for multimodality (T=true) and which did not (F=false). C1: unimodal; C2: unimodal; C3: bimodal; C4: bimodal; C5: unimodal; C6: bimodal; C9: bimodal; C10: bimodal; C11: bimodal; C12: bimodal. Data for each colony includes both treatments.

The distribution of longevity of all focal workers was significantly bimodal, with modes at 73 days and 124 days. This bimodality was also found within each treatment group separately (H-treatment: modes of 73 days and 113 days, L-treatment: modes of 80 days and 131 days, **Figure 2.5A**). Within individual colonies, the distribution of longevities was significantly bimodal (including bimodal) in seven of the ten colonies (**Figure 2.5B**).

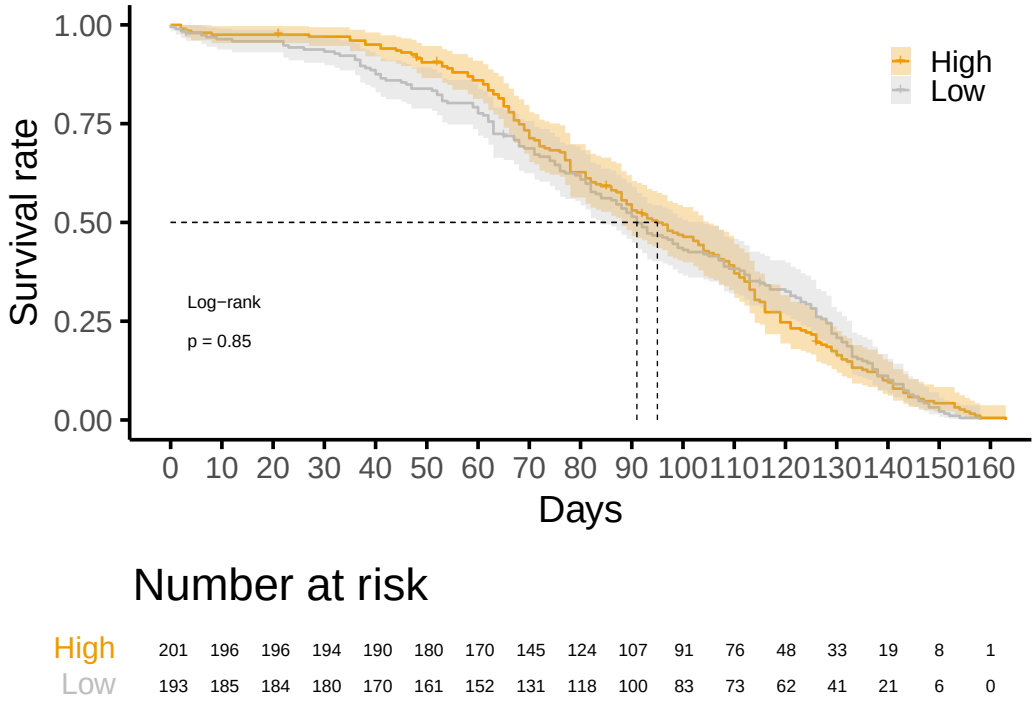

Figure 2.6. Effect of the nutritional treatment on the survival of the focal *Bombus terrestris* workers. Kaplan-Meier survival curves (including ± 95% confidence intervals) and log-rank test results comparing mean survival rates and (below) risk tables showing how many focal workers remained alive over the experimental days. High nutritional treatment (orange): N = 201; Low nutritional treatment (grey): N = 193. There was no significant difference in survival rates of focal workers in the two nutritional treatments, with the median longevities (dashed lines) of workers from the H-treatment workers from the L-treatment being, respectively, 94 days and 91 days (Cox proportional hazard analysis, **Table 2.5**).

Corresponding to the near-identical median longevities of workers in the two nutritional treatments, there was no significant effect of the nutritional treatments on the survival probability of focal workers (Cox proportional hazard model: hazard ratio = 0.91, 95% CI - upper: 1.15, lower: 0.72, p = 0.43, **Figure 2.6**, **Table 2.5**). Therefore, the differences in nutritional provisioning during larval development between the two treatments did not directly affect adult worker longevity. However, an analysis of a subset of the 50% of focal workers with longevities below the median (92 days) revealed a significant decrease in survival probability for workers from the L-treatment (Cox proportional hazard model: hazard ratio = 1.43, 95% CI - upper: 2.05, lower: 1.00, p = 0.045, **Table 2.5**, **Figure S2.4**). This analysis was conducted as data presented in Chapter 3 revealed, that those 50% of short-lived workers were more susceptible to factors affecting survival and longevity.

Table 2.5. Effect of the nutritional treatments, marginal wing cell length (a proxy for body size) and egg-laying (yes/no) on the longevity (risk of mortality) of the focal *Bombus terrestris* workers, analysed with a Cox proportional hazard model with mixed effects. Model: Surv(Longevity, censored) ~ Treatment + Marginal Wing Cell Length + Egg-laying + Change over time (Egg-laying). This model also includes a stratified variable for the colonies, to account for differences between colonies. Data from the low nutritional treatment (L-treatment) are compared to data from the high nutritional treatment (H-treatment). Workers with an uncertain death date were censored to the date they were last recorded alive. Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error and the Z- and the p-value. **A)** Analysis of the full data set. H-treatment: N = 201, L-treatment: N = 193. There was no significant difference in the risk of mortality between the two treatments (p = 0.422). Greater marginal wing cell length and being an egg-layer significantly reduced the risk of mortality (p < 0.001). However, the effect of being an egg-layer significantly changed over time such that there was a significantly increased risk of mortality associated for egg-layers at older ages (p < 0.001). **B)** Analysis of data from a subset of focal workers comprising the 50% of focal workers with longevities below the median (92 days). H-treatment: N = 110, L-treatment: N = 111. The risk of mortality was significantly increased in the L-treatment (p = 0.046). Marginal wing cell length did not affect risk of mortality (p = 0.512). being an egg-layer significantly reduced the risk of mortality (p < 0.001). However, as in the full data set, the effect of being an egg-layer significantly changed over time, becoming associated with an increased risk of mortality (p < 0.001).

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Std. Error**	**Z**	**p**
A) All focal workers					
Treatment (L)	-0.096	0.908	0.119	-0.803	0.422
Marginal Wing Cell Length	-1.078	0.340	0.295	-3.649	< 0.001
Egg-laying	-1.692	0.184	0.410	-4.128	< 0.001
Egg-laying over time	0.014	1.014	0.004	3.662	< 0.001
B) Shorter-lived focal workers					
Treatment (L)	0.361	1.434	0.181	1.992	0.046
Marginal Wing Cell Length	-0.328	0.720	0.500	-0.656	0.512
Egg-laying	-3.081	0.046	1.117	-2.757	0.006
Egg-laying over time	0.038	1.039	0.016	2.437	0.015

Analysing survival with the Bayesian survival trajectory analysis (BaSTA) showed slightly lower baseline mortality (b_0) for workers of the H-treatment than for those of the L-treatment, but this difference was not significant (Mean KLDC = 0.682, **Table 3.5**, **Figure 2.7A**). The change rate of mortality (b_1) was slightly higher for workers from the H-treatment than for those of the L-treatment and again this difference was not significant (Mean KLDC = 0.715, **Table 3.5**, **Figure 2.7B**).

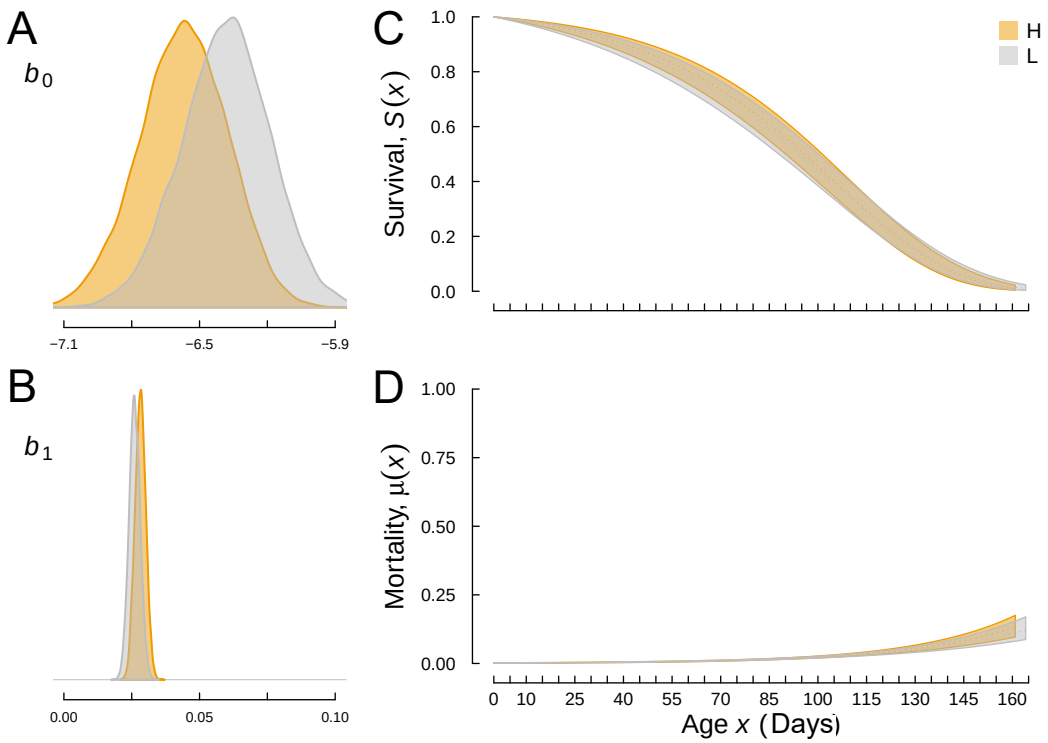

Figure 2.7. Effects of the nutritional treatments on age-specific survival and mortality of the focal *Bombus terrestris* workers, fitted with a simple Gompertz model (BaSTA analysis) across the full lifespan. High nutritional treatment (orange): N = 201 focal workers; low nutritional treatment (grey): N = 193 focal workers. The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) values are listed in **Table 3.5**. **A**: the baseline mortality rate b_0 was not substantially lower for either treatment. **B**: the Gompertz rate parameter change over time b_1 was not substantially lower for either treatment. **C**: Smoothed survival curves for the two nutritional treatments with the shaded areas representing 95% confidence intervals. There was no difference between the two treatments. **D**: the change in mortality over time (days) with the shaded areas representing 95% confidence intervals. This did not differ between the treatments.

In summary, the different survival analyses (Cox proportional hazard model and BaSTA analyses) each showed that the nutritional treatments had no direct effect on focal worker survival and longevity, except in the case of the 50% of focal workers with longevities below the median, in which the Cox proportional hazard analysis showed that the L-treatment significantly reduced worker survival.

Table 2.6. Effects of the nutritional treatments and egg-laying on age-specific survival and mortality of the focal *Bombus terrestris* workers. Results of a Gompertz model (BaSTA analysis) across the full lifespan: Mean Kullback-Leibler discrepancy calibration (KLDC) values for the analysed comparisons. b_0 represents the baseline mortality rate and b_1 the Gompertz rate parameter or the change rate in mortality. Group comparisons that result in KLDC values >0.85 are regarded as differing substantially from each other. **A**: Comparison between the two nutritional treatments (H: high nutritional treatment (N = 201); L: low nutritional treatment (N = 193). There was no difference in b_0 or b_1 between the treatments. **B**: Comparison between egg-layers and non-egg-layers. yes: focal workers that were observed laying eggs (N = 129); no: focal workers that were not observed laying eggs (N = 262). b_0 was substantially lower for egg-layers, they had higher chances of survival at the start. b_1 was substantially higher for egg-layers, meaning that with time egg-laying affected survival more negatively.

Mean KLDC	**b0**	**b1**
A Survival by Treatment		
L - H	0.682	0.715
B Survival by Reproductivity		
no - yes	1.0	1.0

Longevity and Body Size

Increased body size positively affected survival (Cox proportional hazard model: hazard ratio = 0.34, 95% CI - upper: 0.62 lower: 0.20, $p < 0.001$, **Table 2.5**, **Figure 2.8**), with larger workers having a 65% decrease in hazard (chance of death). The same effect was not found when the analysis was restricted to only the 50% of workers that died before the overall median longevity of 92 days (**Table 2.5**).

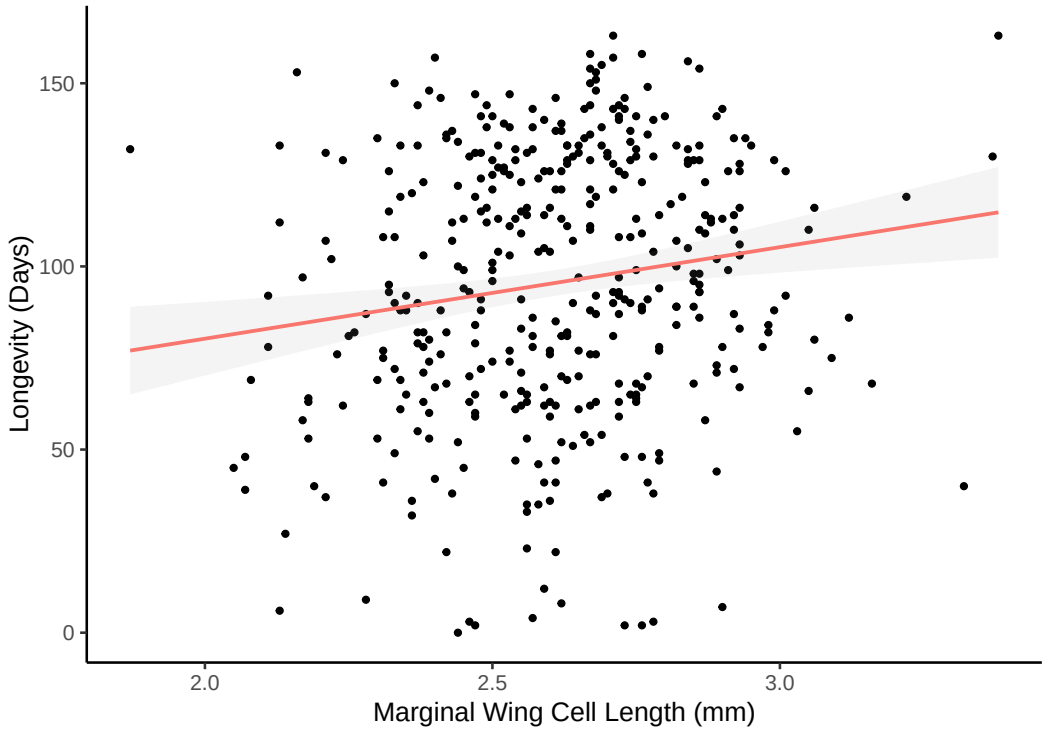

Figure 2.8. The effect of marginal wing cell length (a proxy for body size) on the longevity of the focal *Bombus terrestris* workers. Larger workers showed significantly greater longevities (Cox proportional hazard regression: hazard ratio = 0.35, $p < 0.001$). N = 407 workers. Regression line fitted in a simple linear model with 95% confidence intervals (shading).

Longevity and Reproductivity

Egg-laying focal workers (irrespective of treatment) had a significantly higher survival rate than focal workers that had not been observed egg-laying (Cox proportional hazard model: hazard ratio = 0.70, 95% CI - upper: 0.90 lower: 0.55, $p < 0.01$, **Figure 2.9**). Specifically, egg-laying workers had a 30% decrease in hazard. The hazard for egg-laying focal workers was significantly lower at shorter longevities than for non-egg-laying individuals. It increased with time, such that egg-laying seemed to have induced an increased risk of mortality for longevities over 122 days (**Figure S2.5**). The median longevity for egg-laying workers was 112 days (95% CI - upper: 119, lower: 104) and the median longevity for non-egg-laying workers was 88 days (95% CI - upper: 92, lower: 82).

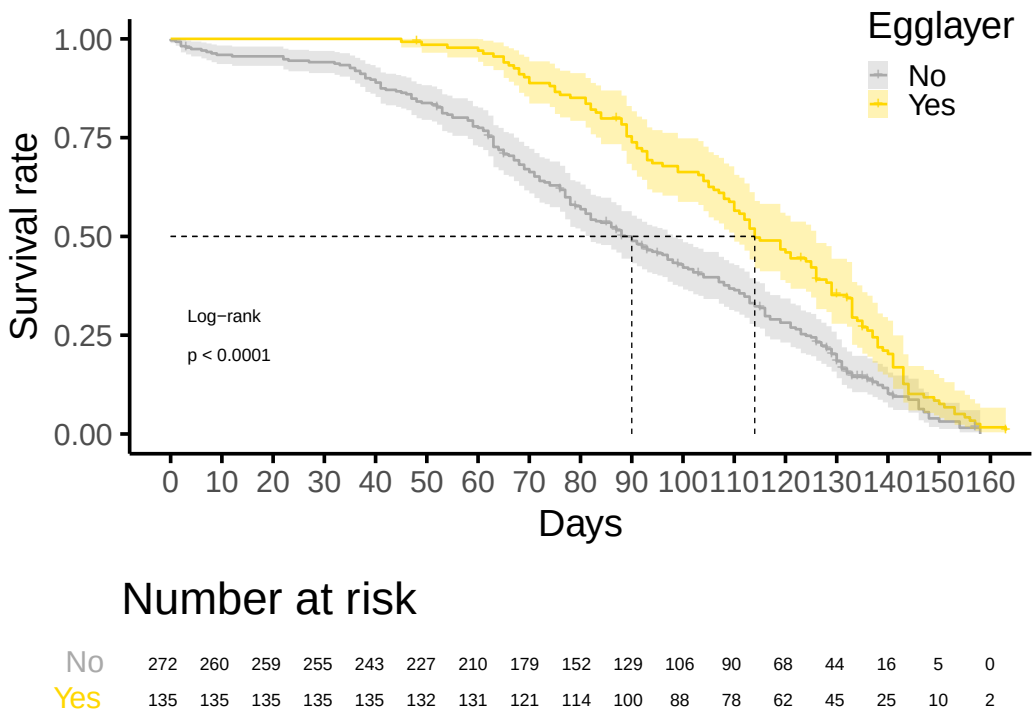

Figure 2.9. Effect of egg-laying on the survival trajectory of focal *Bombus terrestris* workers. Kaplan-Meier survival curves (including ± 95% confidence intervals) and log-rank test results comparing mean survival rates and (below) risk tables showing how many focal workers remained alive over the experimental days. Caption: yes (yellow): focal workers that were observed laying eggs (N = 135 focal workers); no (grey): focal workers that were not observed laying eggs (N = 272 focal workers). Egg-laying workers had significantly higher chances of survival than non-egg-laying workers, with the median longevities (dashed lines) of egg-laying workers and non-egg-laying workers being, respectively, 112 days and 88 days (Cox proportional hazard analysis, Table 2.5).

The Bayesian survival trajectory analysis (BaSTA) showed 'substantially' lower base line mortality (b_0) for workers that were observed to be egg-layers than for non-egg-laying workers (Mean KLDC = 1.0, **Table 3.5**, **Figure 2.10A**). This order was reversed for the change rate of mortality (b_1), which was 'substantially' higher for egg-laying workers than for non-egg-laying workers (Mean KLDC = 1.0, **Table 3.5**, **Figure 2.10B**). This supported the previous finding that egg-laying decreases the risk of mortality overall but increases it with increasing age (**Table 2.5**).

The egg-laying activity of focal workers therefore had an overall positive effect on survival (Cox proportional hazard model and BaSTA analyses). The magnitude of this effect decreased with age. At particularly great ages though (>122 days) egg-laying had a significantly negative effect on worker survival.

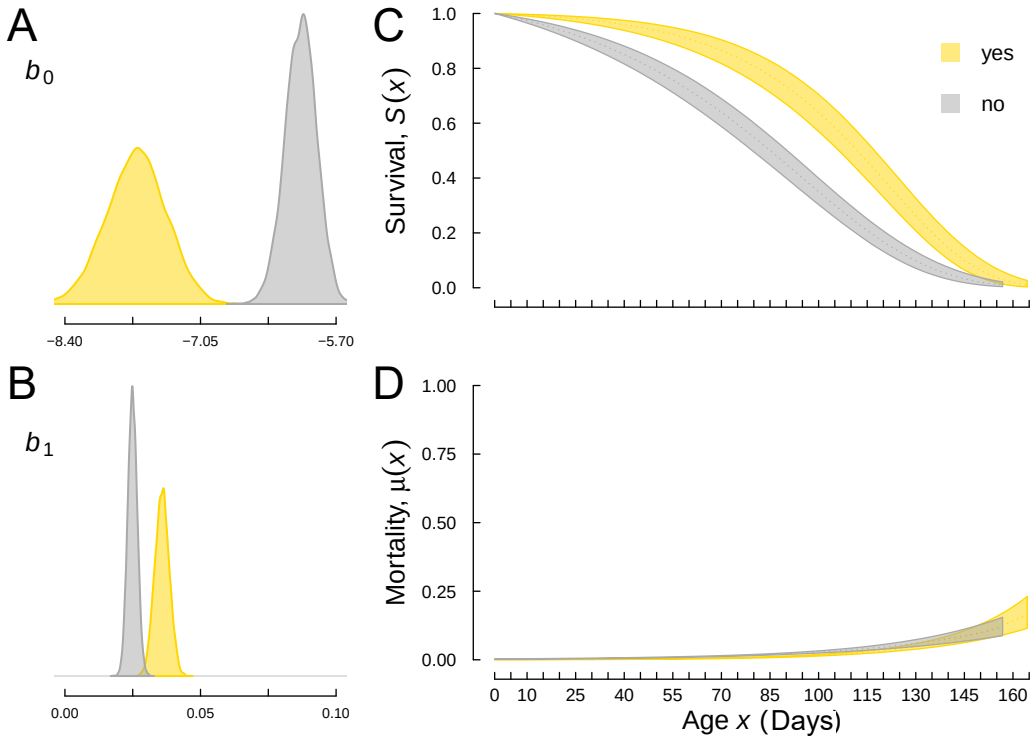

Figure 2.10. Effects of egg-laying on age-specific survival and mortality of the focal *Bombus terrestris* workers, fitted with a simple Gompertz model (BaSTA analysis) across the full lifespan. **yes** (yellow): focal workers that were observed laying eggs (N = 129); **no** (grey): focal workers that were not observed laying eggs (N = 262). The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) values are listed in Table 3.5. **A**: the baseline mortality rate b_0 was substantially lower for egg-layers. **B**: the Gompertz rate parameter change over time b_1 was substantially higher for egg-layers. **C**: Smoothed survival curves for egg-layers and non-egg-layers with the shaded areas representing 95% confidence intervals. Survival chances were higher for egg-layers. **D**: the change in mortality over time (days) with the shaded areas representing 95% confidence intervals. This did not differ between two groups.

2.4 Discussion

Using *Bombus terrestris*, this study sought to test experimentally the hypothesis that differences in adult worker quality are related to differences in larval nutrition (with better nourished larvae developing as higher-quality adult workers) and that it is high-quality adult workers that are able to express a positive fecundity-longevity relationship. Such workers were therefore predicted to have larger body sizes, be more likely to be egg-layers, and also have greater longevities. To test this, larvae within colonies were reared under two feeding regimes, a high nutritional treatment and a low nutritional treatment. As intended, this manipulation was associated with significantly lower estimated larval pollen consumption in the low treatment and significantly divergent body sizes in adult workers eclosing from the larvae (greater in the high treatment and reduced in the low treatment). However, the nutritional treatments did not lead to differences in the numbers of worker egg-laying events or the proportion of egg-laying workers per treatment group. There was also no significant effect of the nutritional treatments on worker longevity across all focal workers, but it did have a significant effect on longevity for the subset of focal workers with longevities below the median, with such short-lived workers from the low treatment showing significantly reduced longevities. In addition significant positive associations were found in workers between reproductivity and body size, reproductivity and longevity, and body size and longevity (irrespective of treatment). These results showed that adult worker body size, reproductivity and longevity were therefore positively associated as predicted by the hypothesis. If so, it is possible that the experimental results were as found because the experimental nutritional manipulation was sufficient to create a difference in body size, with increased body sizes in the H-treatment, though not sufficient to show an effect of the treatments on longevity and reproductivity. Unexpectedly, the frequency distribution of worker longevity also showed significant bimodality. These findings are now discussed in greater detail.

2.4.1 Effect of the Nutritional Treatments

The experimental manipulation delivered the intended difference between the two treatments in the amount of larval nutrition, since the daily per capita larval consumption of pollen was significantly lower in the L- compared with the H-treatment. Specifically, larvae developing under the L-treatment consumed significantly less pollen (ca. 2 mg or 25% less) per capita than those developing under the H-treatment (**Figure S2.1**).

As predicted, adult workers from the H-treatment had significantly larger body sizes than those of the L-treatment, although this difference was relatively small (**Table 2.2**). In *Bombus spp.*, the differences in body size between castes were previously shown to be related to larval nutrition, with high levels of food availability during larval development causing the larger adult body sizes of queens (Pereboom, 2000). The same mechanism has been suggested to cause the size variability among the workers (Sutcliffe and Plowright, 1988) and the findings presented here, that the focal workers of the L-treatment were smaller, evidence this further.

The nutritional treatments did not have an effect on the reproductive behaviour (likelihood of egg-laying) of the adult focal workers. Similarly, there was no difference in the proportion of egg-laying workers between the two treatment groups (**Table 2.3**), and nor did treatments predict whether an individual became an egg-layer or not (**Table 2.4**). The overall number of worker egg-laying events and the hourly rate of worker egg-laying events did also not differ between the two treatment groups. For many organisms, adult dietary restriction leads to reduced fecundity (Adler and Bonduriansky, 2014; Regan et al., 2020; Zajitschek et al., 2019). Yet, for *B. terrestris* workers no such connection could be confirmed between dietary restriction during larval development and the reproductivity of the adult workers in the current study.

There was no difference in longevity between workers that were reared under the H-treatment and workers that were reared under the L-treatment (**Figures 2.6, 2.7**). The median survival time only differed by 3 days between the two groups (**Figure 2.6**). Workers from the L-treatment seemed to experience a slightly increased baseline mortality (**Figure 2.7**, **Table 3.5**), represented by a steeper survival curve at the beginning of the experiment (**Figure 2.6**). Such early life differences in survival between the workers of the two treatments were supported by the results of an analysis (Cox proportional hazard analysis) including only the 50% of focal workers that died before the overall median longevity of 92 days, which did find a significant difference between the two treatments (**Figure S2.4**, **Table 2.5**). Specifically, consistent with the hypothesis, focal workers from the H-treatment had a significantly higher survival probability than those from the L-treatment (**Table 2.5**). It is therefore possible that the nutritional manipulation was not strong enough to have an effect on survival across the full range of worker longevities. The shorter-lived workers, though, seem to have been more susceptible to the effects of the nutritional treatments, with low nutrition negatively affecting longevity, as predicted by the hypothesis.

Although the hypothesis that differing nutritional levels during larval develop-

ment would directly affect adult worker longevities was not supported by the whole data set, the high nutritional treatment did produce larger adult workers, which might be a sign of increased quality, as increased body size was correlated with increased longevity (irrespective of treatment; **Figure 2.8**). Interestingly, for the 50% of focal workers that died before the overall median longevity between the two nutritional treatments, body sizes did not differ significantly (**Table S2.1**), although it was significantly higher for the H-treatment when analysing the full data set (**Table S2.1**). The reason behind this could again be that groups of different quality were present within the H-treatment, hence the higher-quality workers might be the ones of greater body size and higher longevity. A further potential indicator of the existence of different levels of worker quality not stemming from the experimental manipulation in larval nutrition was the bimodal distribution of focal worker longevities, which was present within each of the nutritional treatment groups (**Figure 2.5A**). The factors causing this pattern in worker longevities were therefore not connected to larval nutrition (**Section 2.4.2**).

Alternatively, the long-lived workers of the L-treatment could be a sign that within the L-treatment, the pollen provided was not equally distributed among the brood. The non-focal workers might have focussed their provisioning on a few larvae, which therefore received optimal nutrition and showed no reduction in quality as adult workers. Those adult workers might be the long-lived workers in the L-treatment, whereas the short-lived workers of the L-treatment might have developed from the larvae that received significantly lower levels of nutrition.

The feeding regimes in this study were designed to provide 100% of the syrup and pollen requirements for the non-focal adult workers that were rearing the focal brood, while either providing the brood with 130% of its requirement (H-Treatment) or restricting the brood's requirements to 70% (L-treatment) (Gradish et al., 2019). This was done to ensure that the non-focal workers in both treatment groups would still be able to perform all colony tasks without any restrictions, such as larval feeding and thermoregulation of the brood and the nest (Heinrich, 2004), as it is known that adult workers also require pollen for their own nutritional needs (Smeets and Duchateau, 2003). Hence the intention was to ensure that potential differences between the focal workers that developed under the two treatments would stem only from differences in larval nutrition. A potential drawback of this approach was that there was no experimental control over how the adult workers distributed the resources provided to each type of compartment. It cannot be fully ruled out that the non-focal workers in the L-treatment compartment focused their nutritional care on a subset of larvae, potentially at a central position of the nest (Couvillon and Dorn-

haus, 2009). Those larvae would therefore have not received nutritional restriction. Overall, though, the results were consistent with larvae in the L-treatment consuming significantly less pollen, which was consistent with the nutritional manipulation in this experiment having had the intended effect. Yet the relatively small difference in worker marginal wing cell length (hence body size) between the treatments might be an indication of the level of larval malnutrition in the L-compartments having not been quite low enough to generate larger differences in body size, reproductivity or longevity. While the manipulations applied lay within the natural ranges of pollen and nectar requirements of *Bombus* larvae (Gradish et al., 2019), it can be argued that the nutritional restriction in the L-treatment would have needed to be more severe to have created effects strong enough to fully return the predictions of the hypothesis.

2.4.2 Reproductivity, Longevity and Body Size

Reproductivity, longevity and body size can be seen as three different yet highly connected measures of worker quality. Because the nutritional treatments had no direct effect on reproductivity and longevity, the relationship between these two measures was analysed for all focal workers, irrespective of treatment. As hypothesised, in workers within each of the nutritional treatments, egg-laying workers had significantly greater marginal wing cell lengths, indicating larger body sizes, than workers that were not observed egg-laying, and, correspondingly, body size was a significant predictor of whether a worker would be an egg-layer or not (**Figure 2.3A**, **Table 2.4**). Longer-lived workers were also more likely to be egg-layers (**Figure 2.3B**). This was another sign of differing quality levels among the workers. Additionally, none of the egg-layers had longevities below 45 days (**Figure 2.3B**), with workers with lower longevities not becoming egg-layers even though egg-laying events of workers at ages from 3 days upwards were recorded (**Figure 2.4**). This suggested the existence of a threshold, and hence could have been a further indication of the presence of lower-quality individuals that are not reproductive and that are shorter-lived. Reproductivity further significantly positively affected longevity. Specifically, focal workers that were observed to be egg-layers had significantly greater longevities (median longevity: 114 days) than those workers that were never observed to be egg-layers (median longevity: 87 days, **Figure 2.9**, **Table 2.5**). Lastly, as predicted, larger body sizes were also significantly connected to greater longevities (**Figure 2.8**, **Table 2.5**). meaning that neither solely individual factors In sum, the large dataset generated in this study, which measured body size, reproductivity and longevity of workers on the individual level over the entire course of their adult

lives, strongly and positively linked reproductivity, longevity and body size in *B. terrestris* workers as predicted by the hypothesis.

Blacher et al. (2017) documented significantly larger body sizes for workers with activated ovaries in *B. terrestris* and a positive association of ovarian activation with increased longevity, as well as with increased body size. A positive relationship between body size and fecundity in females is widely found among insects (Honěk, 1993), but it was long unclear whether this was the case in bumblebees as well, as some previous studies failed to find such a relationship (Duchateau and Velthuis, 1989). The results presented here, coupled with those of Blacher et al. (2017), are strong evidence for such a positive relationship between body size and fecundity and could be an indicator of high quality individuals that have the resources to become reproductively active.

The fact that body size positively affects longevity was also reported in workers of the red imported fire ant (*Solenopsis invicta*) (Calabi and Porter, 1989), in which it was possibly connected to lower metabolic rates of the larger workers (Calabi and Porter, 1989). Similarly, a connection between low metabolic rates and greater longevities was documented for *Bombus impatiens* workers (Kelemen et al., 2019). For these workers the metabolic rate remained stable independent of age. It would therefore be interesting to further test to what extent the metabolic rate of an adult worker is connected to nutrition levels during larval development. A positive connection between reproductivity and longevity was also reported for *Apis mellifera* workers, in which the development level of worker ovaries (e.g. number of ovarioles) was positively correlated with increased longevity (Kuszewska et al., 2017). The findings of the current study represent even more robust evidence of the positive relationship between fecundity and longevity in workers of eusocial Hymenoptera than in previous studies, because the reproductivity of workers in the current study was recorded from observations of actual egg-laying, rather than from levels of ovarian activation, with ovarian activation not necessarily being a certain indicator for being an egg-layer (Alaux et al., 2004; Sibbald and Plowright, 2014).

Consistent with the effect of egg-laying on worker longevity, egg-laying correlated severely with a reduced the risk of mortality (hazard ratio) (**Figure 2.9**, **Table 2.5**). This positive effect was strongest early in the life of workers, as evidenced by the lower baseline mortality of egg-layers (**Figure 2.10**, **Table 3.5**). With increasing age this effect decreased, up to the age of 122 days (**Table 2.5**; higher Gompertz change rate of mortality for egg-layers, **Figure 2.10**, **Table 3.5**). After this age, the effect of egg-laying resulted in an increased risk of death (hazard ratio larger than 1, **Figure S2.5**). These findings support the hypothesis of reproductivity positively

affecting longevity, though add the new findings that this effect appears to decrease at particularly great ages. Some theories predict such increasing cost of reproduction with age, because of increased physical deterioration with age (Williams, 1966; Clutton-Brock, 1984). They argue that somatic maintenance is more energetically costly when more wear and tear accumulates with age. Investing into reproduction should therefore come at an higher cost for survival at greater age. This has also been described as 'Terminal Investment', whereby increased costs of reproduction are accepted to maximize reproductive success before death (Clutton-Brock, 1984).

It seems, though, that the workers experiencing the positive effects of egg-laying by laying eggs at younger ages were usually not the same individuals that experienced the negative effect of egg-laying by laying eggs at older ages. With respect to the age of focal workers at the time of a recorded egg-laying-event, the data showed a bimodal distribution, with modes at 24 days and 88 days, meaning that most egg-laying events were performed by workers around those ages (**Figure 2.4**). Only 16.5% of all individual egg-layers were recorded laying eggs at both of those ages ($\pm$ 15 days). Approximately one quarter of the egg-layers laid eggs only during the age-span defining the first mode, whereas another approximately one third of the egg-layers laid eggs only during the age-span of the second mode. The median longevities between those two groups differed by 44 days. As a corollary, only 7 workers were observed laying eggs throughout the whole time-frame of the experiment. Thus, there seemed to be two different strategies among the workers as regards when to start laying eggs. Some workers started laying eggs at young ages, potentially starting to activate their ovaries not long after eclosion. After a reproductive period they ceased to lay eggs and died at younger ages, as indicated by the lower median longevity of this group. Other workers only started laying eggs at older ages, around the age of median longevity of the first group. This group showed a higher median longevity.

The early group of egg-layers could consist of workers higher up in the social hierarchy, dominating other workers to determine which ones lay eggs and which ones do not, as egg-laying activity is often connected to higher levels of aggression (Bloch and Hefetz, 1999; Zanette et al., 2012). As a form of reproductive assurance, the late group of egg-layers potentially begins laying eggs only once the majority of those dominant workers have died or stopped laying eggs and the level of competition has reduced. Often it is older workers that occupy the top of the social hierarchy (van Doorn and Heringa, 1986; Duchateau and Velthuis, 1989; Duchateau, 1989; Bloch and Hefetz, 1999). In the current study, however, we had removed all non-focal workers after all the focal workers had eclosed and there was therefore no large

discrepancy in age between the focal workers. Increased competition experienced by younger individuals as an extra cost of reproduction can therefore be discounted in this case. The early group of egg-layers had possibly taken advantage of this 'gap' in the dominance hierarchy, created by the lack of older workers. Whether the same phenomenon would therefore be present in an unmanipulated colony is unclear. A second possible explanation could be that workers can sense cues of their own relative quality. If they sense they are of lower quality and destined to be shorter lived (potentially due to low nutrition during larval development), they might choose to lay eggs earlier on. Other workers might sense they are of higher quality and destined to be longer lived and so would lay eggs later. Such a direct connection to larval nutrition could not be confirmed in the current study, as egg-layers of both nutritional treatments appeared in both modes of the bimodal age distribution of egg-layers (**Figure S2.3**). For queens in *B. terrestris* such a connection between the nutritional/health status of the queen and her reproduction strategy has been hypothesized. Queens that hibernated longer and used up a lot of their metabolic resources, which will potentially reduce their lifespan, switched to producing males early in the colony cycle, perhaps as reproductive assurance, rather than continue to build colony strength and produce gynes later on (Duchateau et al., 2004). By contrast, queens that emerged earlier from hibernation, switched to male production later in the colony cycle and hence often showed a gyne-biased sex ratio. Duchateau et al. (2004) therefore suggested that queens could be sensing their metabolic status such that late-emerging, shorter-lived queens choose a different reproductive strategy focusing on the less-costly, yet of lesser reproductive success, production of males. Are the workers in the current study that lay eggs only early in life therefore sensing their own low metabolic status? The order of causation might also be the opposite. It might be that simply by not reproducing at a young age, the egg-layers of the late group save energy, which allows them to reach greater longevities. As those workers approach their life expectancy, they have those energy resources left to then be investing into reproduction.

The distribution of all focal workers' longevities tested positive for bimodality, and this was also the case within the majority of the individual colonies (**Figure 2.5**). This was not a pure artefact of the eclosion window of the focal workers, because the distinct modes in the worker longevity distributions were further apart than the length of the eclosion window ($<$ 2 weeks in all colonies). The finding was unexpected and suggests that the workers as a whole are split into shorter-lived and longer-lived workers. These worker groups of different longevities might be another indicator of groups of different intrinsic quality within the workers of one colony. As this pattern

was found for both of the nutritional treatment groups, driving factors independent of larval nutrition have to be assumed. Intrinsic quality differences in adult workers could be related to differences in egg-quality ('intentionally' or 'unintentionally' caused by the queen) or differences during the larval development, such as nest position and level of brood-care (Couvillon and Dornhaus, 2009; Holland and Bourke, 2015). The bimodality pattern in worker longevities could further be caused by the social environment of the colony. Peaks of shorter-lived workers (earlier deaths) might be an indicator for increased aggression and stress in the colony. Shifts in the social environment of a colony are related to the progression in the colony cycle and increased competition and aggression are related to increased egg-laying activity by the workers, once the queen has ceased to lay worker-destined eggs (Bourke and Ratnieks, 2001; Alaux et al., 2006). Further investigation is needed to disentangle the intrinsic or social factors underlying this pattern in worker longevities.

Recording live-time egg-laying activity and longevity records led to strong evidence in support of a positive relationship between reproductivity, longevity and body size in *B. terrestris* workers. Nevertheless, it needs to be mentioned that the fact that an individual worker was never observed laying eggs during observation bouts did not necessarily mean that it never laid eggs. Therefore, in the experiment, all egg-laying workers would have been correctly classified (i.e. any worker observed to perform at least one egg-laying event), but some workers classified as non-egg-layers might in fact have been egg-layers. However, given that the mean duration of observations was 54 h per colony and the mean rate of egg-laying events occurring was 0.2 events per hour per colony, in 54 h one would have expected to see a mean of 10.8 egg-laying events per colony; this suggests that it is unlikely that an event involving any one egg-laying worker was missed, in which case relatively few non-egg-laying workers would have been misclassified. Nonetheless, this factor could have meant that the long-lived egg-layers that were observed to lay eggs only early in life were perhaps in fact just not observed egg-laying late in life, although they did. Similarly, egg-layers that were observed to lay eggs only late in life might have just not been recorded laying eggs at younger ages although they did. Therefore, although there was a clear separation between young-age egg-layers and old-age egg-layers, this finding would need to be supported by behavioural observations conducted at a larger scale.

2.4.3 Conclusion

This study successfully demonstrates, in *B. t. audax* workers, that positive relationships between reproductivity, longevity and body size exist. Egg-laying workers are

longer lived and larger workers are more likely to become egg-layers while also being longer lived. The manipulation of larval nutrition in this experiment did not have a direct effect on reproductivity or longevity of the focal workers. Yet, adult body sizes (measured as marginal wing cell length) were significantly negatively affected by reduction in larval nutrition (L-treatment). The nutritional treatments therefore had a direct but weak effect (for methodological reasons discussed) on worker body size and, by extension, on reproductivity and longevity. Despite a lack of all the predicted effects of the treatments, including workers from both treatments showed that there were significant positive associations in workers between reproductivity and body size, reproductivity and longevity, and body size and longevity. These associations support the existence of varying levels of worker quality within colonies as predicted by the hypothesis. In addition, variation in quality became apparent in the bimodal distribution of worker longevities, splitting the workers into short-lived and long-lived workers. Analysis of a subset of the 50% of focal workers with longevities below the median (92 days) also revealed a direct effect of the nutritional treatments on worker longevity, as workers from the H-treatment showed higher survival. This again supported the conclusion that there are workers of intrinsically lower quality that are more susceptible to the effect of less favourable conditions, such as low larval nutrition, than workers of intrinsically higher quality that are able to buffer such effects. In addition, there seem to have been two different reproductive strategies among the egg-laying workers. A first group chose to lay eggs at younger ages only and also showed a lower median longevity, whereas a second group began laying eggs only at older ages and also showed a higher median longevity. This again might be a sign of underlying and discontinuous variation in intrinsic quality, even among egg-laying workers.

In conclusion, by providing an extensive dataset demonstrating positive associations of reproductivity, longevity and body size at the individual level in workers, this study strongly supports the existence of different levels of worker quality within colonies of *B. terrestris* and, by extension, potentially among workers within colonies of other species of eusocial Hymenopterans. In turn, this supports the concept of condition-dependence in the positive fecundity-longevity relationships observed in workers and queens of *B. terrestris* and (again by extension) some other eusocial species.

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Supplement

Equations for the Nutritional Treatments

$$n_{e+l_{d,i}} = (n_{\text{egg cells}_{d,i}} + n_{\text{larval cells}_{d,i}}) * 9 + n_{\text{ind. larvae}_{d,i}} \tag{S2.1}$$

$$m_{\text{pollen consumed}_{(d-2),i}} = m_{\text{pollen in}_{(d-2),i}} - m_{\text{pollen out}_{(d-1),i}} \tag{S2.2}$$

$$m_{\text{nectar consumed}_{(d-2),i}} = m_{\text{nectar in}_{(d-2),i}} - m_{\text{nectar out}_{(d-1),i}} \tag{S2.3}$$

$$dcr_{pollen_{d-1}} = \sum_{i=1}^{N} \frac{m_{\text{pollen consumed}_{(d-2),i}} - n_{worker_{(d-2),i}} \times 25mg}{n_{e+l_{(d-2),i}}} \div N \tag{S2.4}$$

$$dcr_{nectar_{d-1}} = \sum_{i=1}^{N} \frac{m_{\text{nectar consumed}_{(d-2),i}} - n_{worker_{(d-2),i}} \times 318.3mg}{n_{e+l_{(d-2),i}}} \div N \tag{S2.5}$$

$$H : m_{\text{pollen in}_{d,i}} = dcr_{pollen_{d-1}} \times n_{e+l_{d,i}} \times 1.3 + n_{worker_{d,i}} \times 25mg \tag{S2.6}$$

$$L : m_{\text{pollen in}_{d,i}} = dcr_{pollen_{d-1}} \times n_{e+l_{d,i}} \times 0.7 + n_{worker_{d,i}} \times 25mg \tag{S2.7}$$

$$H : m_{\text{nectar in}_{d,i}} = dcr_{nectar_{d-1}} \times n_{e+l_{d,i}} \times 1.3 + n_{worker_{d,i}} \times 318.3mg \tag{S2.8}$$

$$L : m_{\text{nectar in}_{d,i}} = dcr_{nectar_{d-1}} \times n_{e+l_{d,i}} \times 0.7 + n_{worker_{d,i}} \times 25mg \tag{S2.9}$$

$n_{\textbf{egg cells}_{d,i}}$	Number of egg cells on day d in compartment i	$n_{\textbf{larval cells}_{d,i}}$	Number of larval cells on day d in compartment i
$n_{\textbf{ind. larvae}_{d,i}}$	Number of individual larvae on day d in compartment i	$n_{e+l_{d,i}}$	Number of eggs and larvae on day d in compartment i
$m_{\textbf{X in}_{(d-2),i}}$	Weight of pollen or nectar put into compartment i on day $d-2$	$m_{\textbf{X out}_{(d-1),i}}$	Weight of left-over pollen/nectar from compartment i on day $d-1$
$m_{\textbf{X consumed}_{(d-2),i}}$	Total pollen or nectar consumed during day $d-2$ in compartment i	N	Number of compartments still undergoing the feeding regime
$dcr_{X_{d-1}}$	Daily consumption rate of pollen or nectar per larva for day $d-1$	H	High nutritional treatment
L	Low nutritional treatment	$n_{\textbf{worker}_{d,i}}$	Number of adult workers on day d in compartment i
$m_{\textbf{X in}}(d)$	Weight of pollen or nectar put into compartment i on day d		

2.4.1 Supplementary Figures

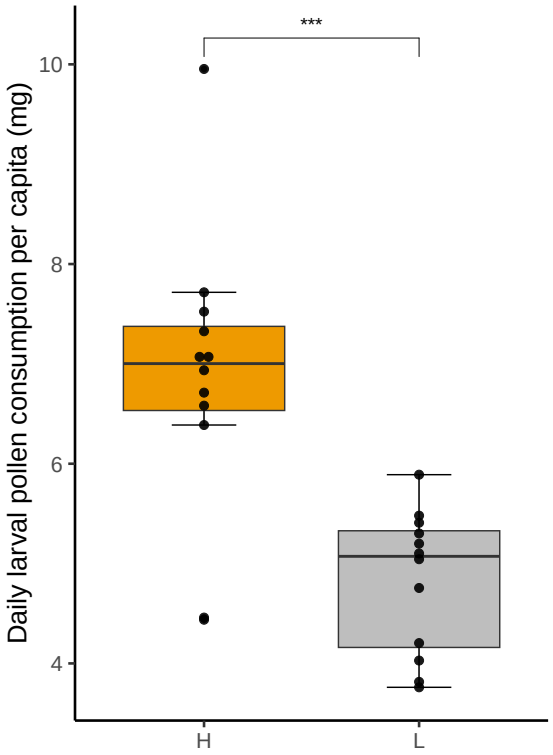

Figure S2.1. Differences in daily per capita larval pollen consumption between the two nutritional treatments of worker-destined *Bombus terrestris* larvae. **H**: high nutritional treatment (orange) - N colonies = 12; **L**: low nutritional treatment (grey) - N colonies = 12. Larvae in the H-treatment consumed significantly more pollen per larvae per day ($p < 0.001$). Each black dot represents data from one colony. For each boxplot the black line within each box represents the median consumption rate for that treatment, the upper and lower bounds of the box are the first quartile and the third quartile respectively and the whiskers extend to 1.5 × interquartile range after the box boundaries.

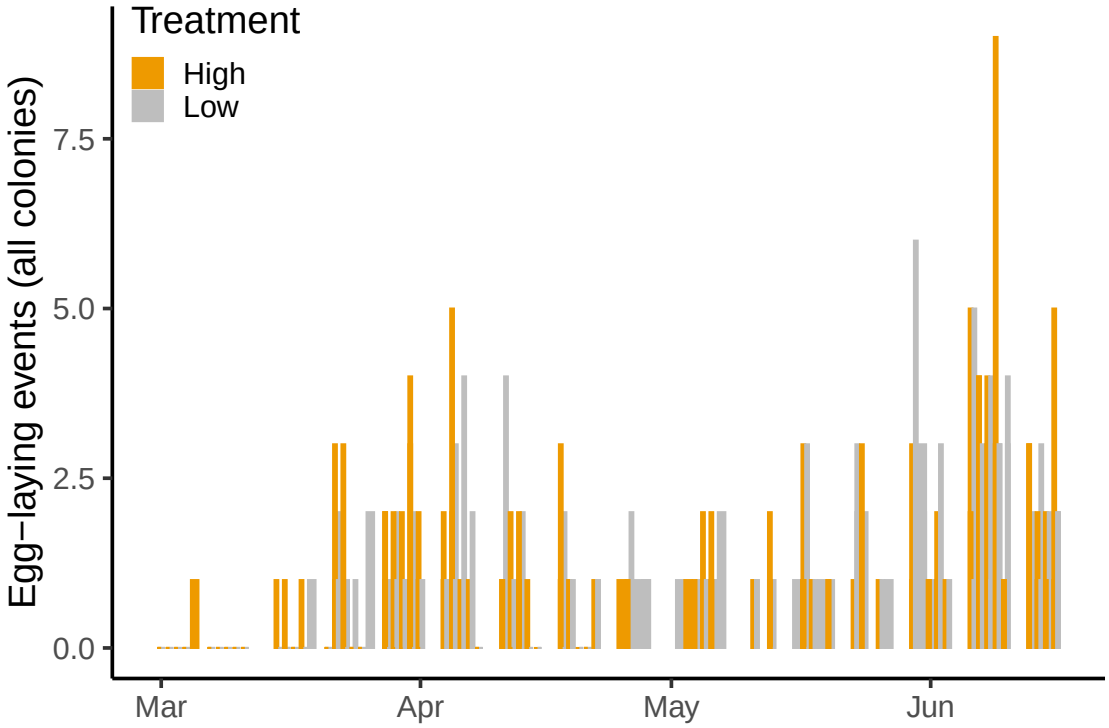

Figure S2.2. Number of worker egg-laying events per *Bombus terrestris* colony recorded per day per nutritional treatment after the non-focal workers had been removed. High nutritional treatment (orange): N colonies = 10; Low nutritional treatment (grey): N colonies = 10. Egg-laying activity increased over the first month. Then there was a reduction in egg-laying activity before it increased again towards the end of the experiment. The egg-laying activity did not differ between the two nutritional treatments.

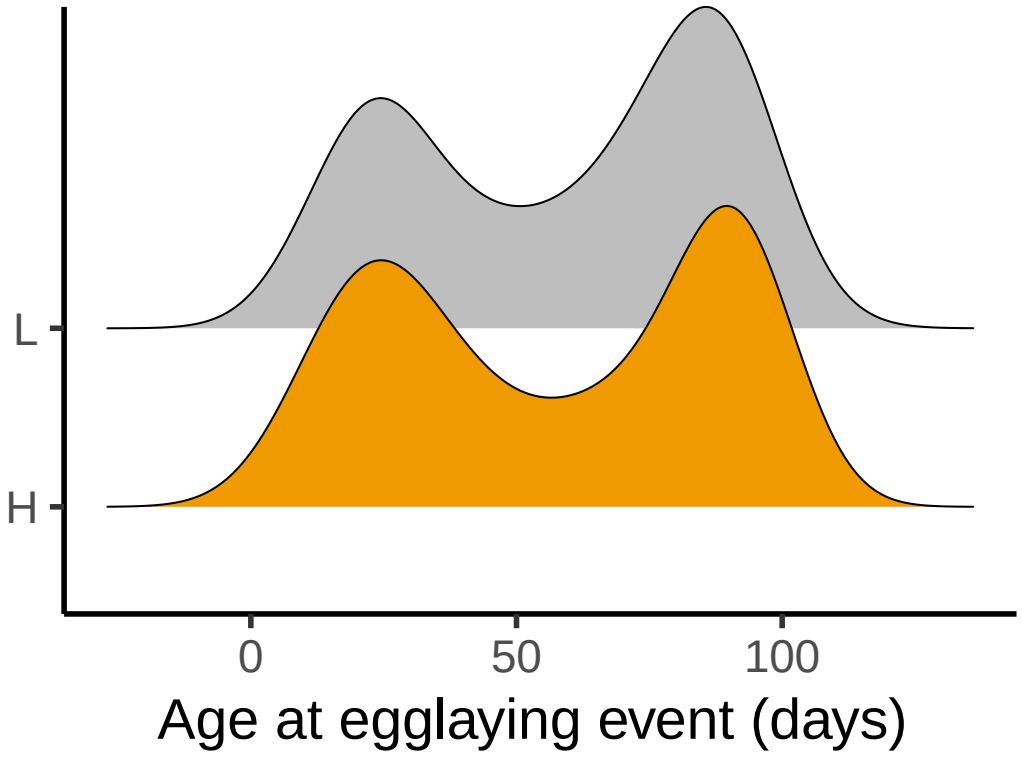

Figure S2.3. Worker age at egg-laying events in *Bombus terrestris* follows a bimodal distribution. Density plot of the age of the workers on the date they were observed laying eggs, split by the two nutritional treatments. H-treatment (orange): high nutritional treatment, N(egg-laying events) = 156; L-treatment (grey): low nutritional treatment, N(egg-laying events) = 135. The same worker might have been recorded multiple times. The y-axis shows the smoothed estimate of the data's distribution. The total area under the curve represents 100% (= 1). The distributions were significantly bimodal for both treatments.

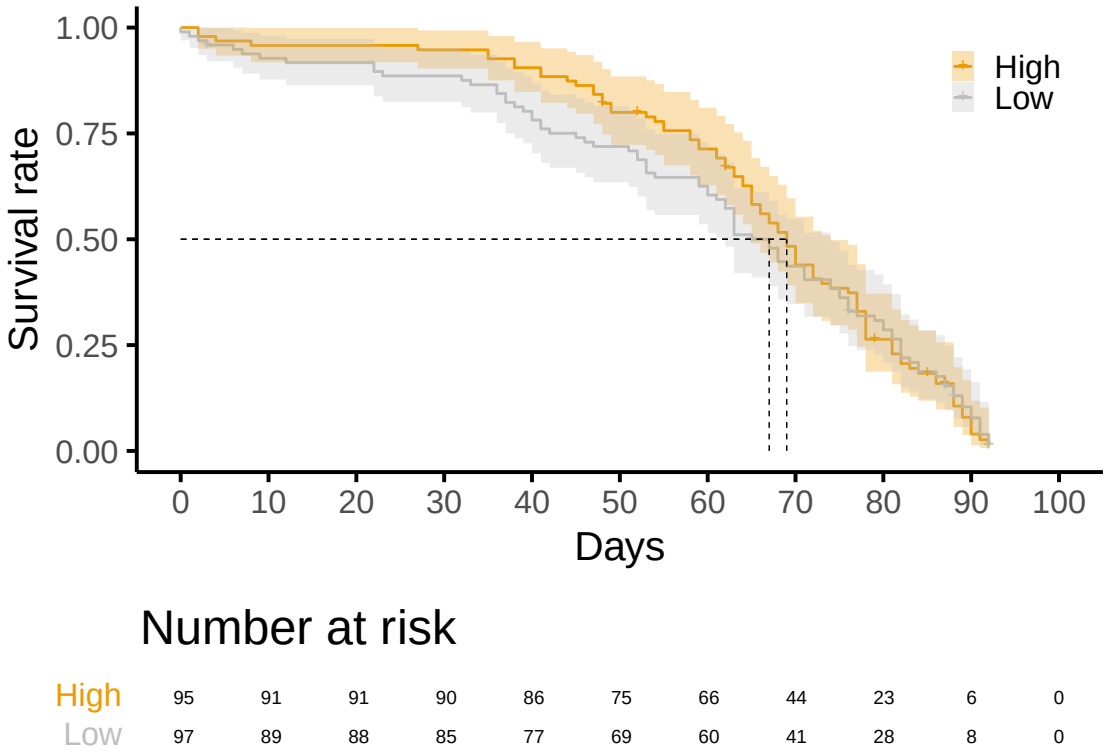

Figure S2.4. Effect of the nutritional treatments on the survival of the 50% of focal *Bombus terrestris* workers that had died until the total median longevity of 92 days. Kaplan-Meier survival curves (including ± 95% confidence intervals) and log-rank test results comparing mean survival rates and (below) risk tables showing how many focal workers remained alive over the experimental days. High nutritional treatment (orange): N = 95; Low nutritional treatment (grey): N = 97. There was a significant difference in survival rates of the two nutritional treatments for this subset of short-lived workers (Results of Cox proportional hazard analysis in Table 2.5).

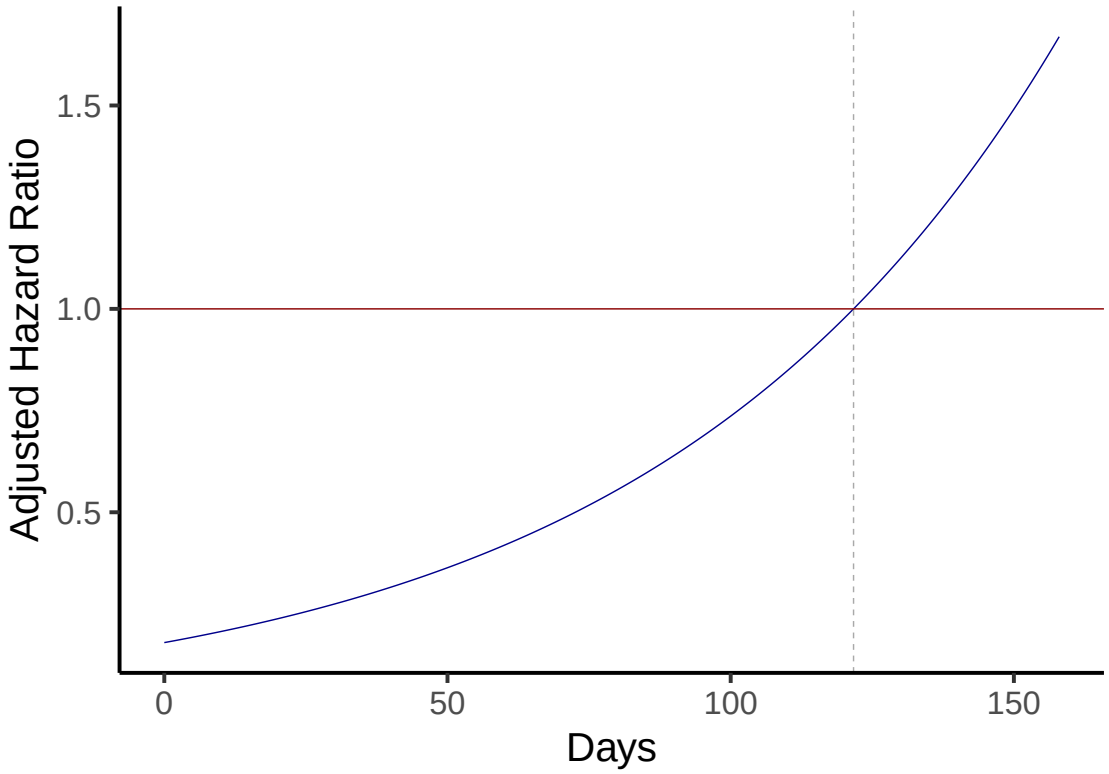

Figure S2.5. The changing effect of egg-laying activity on survival in *Bombus terrestris* workers according to the Cox proportional hazard model (Table 2.5). The Adjusted Hazard Ratio (AHR)is calculated as exp (coef(*Egg-laying*)+ coef(*Egg-laying over time*) * *Days*) (the coefficients are taken from the survival model in Table 2.5. The AHR is below 1 at the start of the experiment, resulting in a positive effect of egg-laying on survival. The AHR increases with the course of the experiment until it crosses 1.0 at Day 122 (grey dashed line). After this point egg-laying has a negative effect on survival. The red line marks AHR=1.0, the point were egg-laying does not affect survival positively or negatively.

2.4.2 Supplementary Tables

Table S2.1. Effect of the nutritional treatments on the length of the marginal wing cell (a proxy for body size) of the 50% of focal adult *Bombus terrestris* workers that had died until the total median longevity of 92 days. Summary of a linear mixed model: log(Marginal wing cell) ~ Treatment + (1 | Colony). The nutritional treatments (High: H-treatment, N = 95 from 10 experimental colonies; Low: L-treatment, N = 97 from 10 experimental colonies) had no effect on the marginal wing cell length (p = 0.55). Shown are the Estimate, the Standard Error, the degrees of freedom (df) and the t- and the p-value. For the random effect of Colony, the Standard Deviation and the Variance are displayed.

Fixed Effect	**Estimate**	**Standard Error**	**df**	**t-value**	**p**
Treatment (L)	-0.019	0.030	184.27	-0.593	0.55
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.019	0.137		

Chapter 3 | Individual versus Social Influences on Ageing in Social Organisms: A Phenotypic and Transcriptomic Test in Bumblebees

Abstract

In social organisms, an unsolved question concerns whether intrinsic properties of the individual or properties of the social environment are the major determinant of ageing and longevity. Using workers of the annual eusocial bumblebee, *Bombus terrestris*, I conducted an experiment aimed at discriminating between these two evolutionary hypotheses. A previous study suggested that workers produced early in the colony cycle live longer than those produced late, providing a context for this purpose. The Individual Hypothesis attributes this longevity difference to individual factors (intrinsic differences) affecting early- and late-produced workers and the Social Hypothesis attributes it to differences in the workers' social environments. Newly-eclosed workers were reciprocally transferred between early- and late-stage colonies and their longevities were compared with those of non-transferred workers. Control transfers into same-stage colonies showed that transfer itself did not affect worker longevity. To examine age-related gene expression in the experimental context, subsets of workers were also sampled for mRNA-seq of fat body tissue at worker ages of two and seven weeks. Longevity and survival analyses found an intrinsic difference between early-produced workers and late-produced workers, supporting the Individual Hypothesis. This difference was also shaped by the social environment, supporting the Social Hypothesis. The social effect on longevity seemed to act primarily on short-lived workers, as survival of late-produced workers in this subset was significantly higher following transfer to early colonies. Comparisons of age-related gene expression supported the occurrence of an intrinsic difference between early- and late-produced workers (Individual Hypothesis) with a social effect acting on late-produced workers only (Social Hypothesis), as transferred late- but not early-produced workers tended to exhibit the age-related gene expression profile of workers in the receiving social environment. Overall, this experiment suggests that a combination of individual and social factors determines longevity and its molecular underpinnings in social organisms.

3.1 Introduction

The evolutionary theory of ageing (ETA) states that selection favours reproduction over survival and that such selection becomes stronger with age (Williams, 1957; Hamilton, 1966; Kirkwood, 1977; Stearns, 1992; Wensink et al., 2017). In other words, because there is an ongoing trade-off between somatic maintenance and reproduction, and this trade-off increasingly favours reproduction with increasing age, somatic degradation becomes inevitable, resulting in ageing or senescence (i.e. a gradual deterioration of functional characteristics) (Edward and Chapman, 2011; Maklakov and Chapman, 2019). In addition, because the risk of extrinsic mortality accumulates with age, selection for genes that benefit fitness only at greater ages, which could potentially counteract or halt ageing effects, is weak, as not many individuals will reach such ages and the majority of reproduction will already have been achieved (Hamilton, 1966; Charlesworth, 1980, 2000).

The ETA in this classic form was largely based on a consideration of solitary (non-social) organisms, such that the within-organism trade-off between reproduction (fecundity) and somatic maintenance (longevity), as well as the risk and impact of extrinsic mortality, were considered to act on individuals in isolation from social effects (e.g., Hughes and Reynolds (2005); Rose (1994)). However, for social organisms, these underlying principles, and the ETA as a whole, need to be considered on the level of both the individual and its social partners or group. Not only do resource availability and the risk of extrinsic mortality change in a group and potentially differ for each group member (e.g. when social hierarchies occur), but also the life history and mortality schedule of each group member may affect those of the other group members (e.g. Alexander et al. (1991); Bourke (2007)). In cases where the group members are related, those factors would also affect the individual's inclusive fitness and would therefore be subject to kin selection (Lee, 2003; Travis, 2004; Bourke, 2007). Sociality and longevity are therefore inevitably strongly connected and influence one another's evolution (Lucas and Keller, 2020). Hence, in all organisms except strictly solitary ones, a full understanding of the evolution of ageing requires it to be considered in a social context.

Sociality has been proposed to affect ageing and longevity in several, related ways. First, in many cases, sociality increases longevity such that social organisms often show extended longevities compared to closely related solitary species (e.g. Kim et al. (2011)). In species with large social groups, there is often a positive association between social group size and longevity of the group members, and this occurs in cases both with and without cooperative breeding (Lucas and Keller, 2020). In extreme cases, the evolution of social life can lead to longevity increases of

a hundred-fold in the sole reproductive individuals of a eusocial group (e.g. queens of eusocial Hymenoptera), compared to their solitary relatives (Keller and Genoud, 1997). Several other examples can be found in which reproductive individuals of social species show significantly higher longevities than do individuals of closely related solitary species (Korb and Heinze, 2021), for example the eusocial vertebrate, the naked mole rat (*Heterocephalus glaber*) (Kim et al., 2011), or the cooperatively-breeding Seychelles warbler (*Acrocephalus sechellensis*) (Hammers et al., 2019).

Second, sociality may affect ageing and longevity by creating disruptive selection on longevity within social groups (Alexander et al., 1991; Lucas and Keller, 2020). Within eusocial societies, the reproductive individuals (queens or kings) are very long-lived compared to the less reproductive or sterile individuals (workers) (Keller and Genoud, 1997; Bourke, 2007; Lucas and Keller, 2020; Lopez-Vaamonde et al., 2009; Kramer et al., 2015; Southon et al., 2015; Rodrigues and Flatt, 2016). This phenomenon can be explained by reproductive individuals benefiting from the help of non-reproductive helpers or workers, so leading to selection on them to become longer-lived via the help received and buffering from sources of extrinsic mortality; and by simultaneous selection on the non-reproductive individuals to become shorter-lived. This latter effect occurs because, from the ETA, ageing begins with sexual maturation in reproductive individuals because this point marks the onset of fitness accrual (Hamilton, 1966). Fitness in this case is direct fitness (through offspring production). However, in societies with helpers or workers, non-breeding helpers/workers gain fitness via the indirect component of inclusive fitness, which they can start accruing as soon as they start helping, which might be earlier than the time at which they would reach sexual maturity themselves. This leads to an early onset of ageing and reduced longevities in such individuals, with the net result that, in societies with helpers or workers, selection favours longer-lived reproductive phenotypes and shorter-lived non-reproductive ones (Alexander et al., 1991; Bourke, 2007).

Third, sociality may affect longevity and ageing by leading to the evolution of positive fecundity-longevity relationships. In solitary organisms, the association of fecundity and longevity is typically negative, and this is explained in the ETA via the trade-off proposed to occur between reproduction and somatic maintenance. However, across queens within eusocial societies, there is evidence from multiple species for a positive fecundity-longevity relationship, such that the longest-lived queens are also the most fecund or productive (Hartmann and Heinze, 2003; Lopez-Vaamonde et al., 2009; Heinze and Schrempf, 2012; Tsuji et al., 2012; Heinze et al., 2013; Kramer et al., 2015; Rueppell et al., 2015; Monroy Kuhn and Korb, 2016;

Schrempf et al., 2017; Kennedy et al., 2021; Negroni et al., 2021; Jaimes-Nino et al., 2022; Collins et al., 2023). In some species of eusocial Hymenoptera, the workers are not completely sterile and may reproduce by laying unfertilized eggs that develop (through haplodiploiody) into males (Bourke, 1988). In several cases, these reproductive workers also exhibit a positive fecundity-longevity relationship (e.g. honeybees, Dixon et al. (2014); ants, Negroni et al. (2021); and bumblebees, *Bombus* spp., Blacher et al. (2017)).

In eusocial species that appear less advanced in social complexity, such as bumblebees, positive fecundity-longevity associations may occur in both queens and workers in a condition-dependent manner, such that only high-quality individuals are able to overcome costs of reproduction and exhibit such relationships (Blacher et al. (2017); Collins et al. (2023); **Chapter 2**, **Chapter 4**). Similarly, even in solitary species, it has been proposed that positive fecundity-longevity relationships can co-exist within populations if resources vary and well-resourced, high-quality individuals are able to invest strongly in both reproduction and survival, although a trade-off between fecundity and longevity still exists within individuals (van Noordwijk and de Jong, 1986). However, in advanced eusocial species, it has been proposed that positive fecundity-longevity relationships are not condition-dependent and that, at the proximate level, they have been achieved via a 'rewiring' of signalling pathways that regulate reproduction and ageing (Rodrigues and Flatt, 2016; von Wyschetzki et al., 2015; Lockett et al., 2016; Rueppell et al., 2016; Korb and Heinze, 2021). It has also been hypothesized that a major pathway or set of pathways of this kind involves the 'TI-J-LiFe network', i.e. the target of rapamycin (TOR)/ insulin/insulin-like growth factor 1 signalling (IIS)/juvenile hormone (JH) network (Korb et al., 2021). For example, in solitary insects, a down-regulation of the IIS-pathway leads to increased longevity but decreased fecundity (Russell and Kahn, 2007). By contrast, an increase in JH-levels causes increased fecundity while decreasing longevity (Flatt et al., 2005, 2008; Pamminger et al., 2016). Overall, this network triggers pro-reproduction and pro-ageing effects in non-social insects and also in vertebrates (Flatt et al., 2013; Rodrigues and Flatt, 2016). Correspondingly, a rewiring of the pathways within the network has been proposed to underpin the positive fecundity-longevity relationships found in eusocial insects (Rodrigues and Flatt, 2016; Korb et al., 2021). Likewise, Kramer et al. (2021) proposed that an enzymatic antioxidant gene set has played a parallel role in the molecular basis of ageing in eusocial insects.

Despite progress in these areas of the field's understanding of the possible effects of sociality on ageing and longevity, sociality also raises the general question as to whether, in social organisms, the main determinants of ageing and longevity

are individual or social factors, and this question remains unanswered. Examples of relevant individual factors are potentially those, described above, that underlie within-group variation in individual quality, leading to intrinsic differences between the workers. Examples of relevant social factors could occur when some individuals respond differentially to sociality relative to others, as seen most strongly in the contrast between reproductive breeders and non-reproductive helpers in eusocial societies. However, as their relative roles still need to be further understood, the aim of the current study was therefore to investigate experimentally the general question of whether ageing and longevity in social organisms are influenced more by individual or social factors.

The bumblebee *B. terrestris* provides a highly suitable study system in which to address this question, because colonies of different social environments have workers with systematically different longevities. Specifically, a study by Holland and Bourke (2015) reported that workers that eclosed early in the colony cycle lived significantly longer than workers eclosing later in the colony cycle. In their study, colonies were three weeks apart in the colony cycle and workers from the earlier colonies had a median longevity of 45 days compared to the median longevity of 30 days for the later colonies (Holland and Bourke, 2015). As it demonstrated longevity varying as a function of social environment, this study therefore provided a context for an experimental test of the roles of individual and social factors in longevity determination. Because bumblebees have an annual colony life cycle, early-stage colonies are small, consisting of only the queen and the first few batches of workers (Heinrich, 1979). In addition, behavioural interactions between the individuals of the colony are generally harmonious at this stage, with the workers being occupied with foraging and nest- and brood-care, while the queen lays worker-destined eggs (Goulson, 2010). Late-stage colonies are considerably larger, potentially containing several hundred workers alongside the queen. As they grow, colonies also reach the 'switch point', at which the queen no longer produces more workers but rather switches from laying diploid eggs to laying haploid eggs that develop into males (Duchateau et al., 2004; Duchateau and Velthuis, 1988; Goulson, 2010). The queen may also lay queen-destined eggs that develop into gynes (new queens) from her final batches of diploid eggs. Following this, the colony typically then reaches the 'competition-point', at which the workers start laying male-destined eggs themselves, competing with the queen and other workers over male production (Duchateau and Velthuis, 1988; Bourke and Ratnieks, 2001; Alaux et al., 2006). After this point aggression levels between queen and workers and among the workers increase sharply because of kin-selected conflict over the parentage of males (Duchateau and Velthuis, 1988;

Duchateau, 1989; Zanette et al., 2012). For these reasons, early- and late-stage *B. terrestris* colonies have very different social environments, which, as stated, provided a highly suitable context in which the relative influence of individual and social factors on ageing could be experimentally investigated.

In this study, I therefore performed a reciprocal transfer experiment in which I exchanged newly-eclosed adult workers (callow workers) between early- and late-stage colonies in *B. terrestris* and measured their longevities relative to those of controls, as well as sampling a subset of transferred and non-transferred workers to characterise their gene expression profiles using mRNA-seq. Hence, I sought to address the general question of how sociality affects ageing by discriminating experimentally between two hypotheses. The *Individual Hypothesis* proposes that the life-history schedule of individuals is determined by intrinsic properties of the individual. The *Social Hypothesis* proposes that the life-history schedule of individuals is determined by properties of the social environment. To discriminate these, workers of colonies early in the colony cycle (from here on referred to as 'early colonies') were compared to workers of colonies later in the colony cycle (from here on referred to as 'late colonies') in a transfer experiment, focussing on longevity, reproductivity, and gene expression. The predictions were that if, following transfers, the stage of the donor colony predicted worker longevity and gene expression changes, the Individual Hypothesis would be supported (as workers would have retained the traits they would have expressed in their colony of origin despite the change in social environment); but if the stage of the recipient colony predicted these metrics, the Social Hypothesis would be supported (as workers would have adopted the traits of the colony they were transferred into). Note that, in this study, longevity refers to adult longevity, and so, strictly speaking, individual and social factors refer to those operating from the start of adult life. The gene expression data were also used to investigate age-related changes in gene expression (defined as changes in expression of genes with age) in *B. terrestris* workers, as well as a possible role for genes in the TI-J-LiFe network of Korb et al. (2021) and the enzymatic antioxidant gene set of Kramer et al. (2021).

3.2 Methods

The design of the experiment involved the transfer of newly-eclosed workers to a colony of the other stage, for comparison (as regards longevity) with non-transferred workers that were retained in the colony they were produced in. Previous work has shown that newly-eclosed *B. terrestris* workers can be successfully introduced into non-nestmate colonies (e.g. Lopez-Vaamonde et al. (2003)). This design therefore created two types of transfer, i.e. transfers of workers from early to late colonies and from late to early colonies, or 'early to late' and 'late to early' transfers, respectively. Early and late colonies were three weeks apart in colony age, this difference having been chosen on the basis of previous research that found a significant difference in worker longevities when workers' eclosion dates were (on average) three weeks apart (Holland and Bourke, 2015). The experimental design also allowed, alongside comparisons between the transferred workers and nestmate workers (sisters) in the colony of origin, comparison with the other workers in the recipient colony, resulting in four experimental worker categories, i.e. TEW_{EL}, TEW_{LE}, $NTEW_E$ and $NTEW_L$ as defined in **Table 3.1**. According to the Individual Hypothesis, the longevities and gene expression changes of transferred workers should resemble those of nestmate workers that were retained in the donor colony. This predicted that, in early to late transfers, transferred workers would show longevities (high) and gene expression changes resembling those of non-transferred workers in the donor (early) colony. Similarly, in late to early transfers, transferred workers would show longevities (low) and gene expression changes resembling those of non-transferred workers in the donor (late) colony (**Table 3.2**). By contrast, according to the Social Hypothesis, the longevities and gene expression changes of transferred workers should resemble those of the other workers already present in the recipient colony. This predicted that, in early to late transfers, transferred workers would show longevities (low) and gene expression changes resembling those of the other workers in the recipient (late) colony. In late to early transfers, transferred workers would show longevities (high) and gene expression changes resembling those of the other workers in the recipient (early) colony (**Table 3.2**).

Table 3.1. Definitions of the categories of marked/focal *Bombus terrestris* workers. The sample size (N, number of workers) of each category per colony differed because colonies varied in the number of workers eclosing after the transfers had started. The total sample size represents all marked workers in a given category. The molecular sample sizes give numbers of workers (included in column for total N workers) flash-frozen for molecular analyses. Early colony, colony early in the colony cycle; late colony, colony later (by 3 weeks) in the colony cycle; *N/A*, not applicable.

	Definition	n per colony	n total	n molecular
$BTCW_E$	Before transfer control worker in an early colony (a colony that became a late control colony)	6 - 34	55	*N/A*
$BTEW_E$	Before transfer experimental worker in an early colony (a colony that became a late experimental colony)	14 - 22	63	*N/A*
$NTEW_E$	Non-transferred experimental worker in an early colony	19 - 24	140	16
$NTEW_L$	Non-transferred experimental worker in a late colony	4 - 16	156	13
TEW_{EL}	Transferred experimental worker transferred from an early colony to a late colony	18 - 24	130	15
TEW_{LE}	Transferred experimental worker transferred from a late colony to an early colony	4 - 16	63	13
$NTCW_E$	Non-transferred control worker in an early colony	18 - 20	110	*N/A*
$NTCW_L$	Non-transferred control worker in a late colony	1 - 18	98	*N/A*
TCW_{EE}	Transferred control worker transferred from an early colony to a (different) early colony	19 - 22	81	*N/A*
TCW_{LL}	Transferred control worker transferred from a late colony to a (different) late colony	1 - 18	31	*N/A*

Table 3.2. Predicted comparative *Bombus terrestris* worker longevities and gene expression changes under the Individual Hypothesis and the Social Hypothesis (**A**)) and the underlying assumptions for the experimental design as extracted from Holland and Bourke (2015) (**B**)). Worker categories are as defined in Table 3.1. Same, for the two worker categories, mean longevities are predicted to be equal, gene expression changes are predicted to resemble one another; Different, for the two worker categories, mean longevities are predicted to be unequal, gene expression changes are predicted not to resemble one another. In the longevity predictions, $<$, mean longevity of the first worker category is less than that of the second; $>$, mean longevity of the first worker category is greater than that of the second. The gene expression change predictions were tested in comparisons of TEWs and NTEWs alone.

Worker Categories	**Individual Hypothesis**	**Social Hypothesis**
A) Predictions		
TEW_{EL} vs. $NTEW_E$	Same	Different ($<$)
TEW_{EL} vs. $NTEW_L$	Different ($>$)	Same
TEW_{LE} vs. $NTEW_L$	Same	Different ($>$)
TEW_{LE} vs. $NTEW_E$	Different ($<$)	Same
B) Assumptions		
TCW_{EE} vs. $NTCW_E$	Same	Same
TCW_{LL} vs. $NTCW_L$	Same	Same
$BTCW_E$ vs. $NTCW_L$	Different ($>$)	Different ($>$)

3.2.1 Colony Rearing

Young, pre-competition point *B. terrestris audax* colonies were obtained from ©*Biobest Group NV, Belgium*. A total of 24 colonies were received in two batches of twelve, three weeks apart. The first batch arrived on 9 February 2023, with each colony including a queen, brood and a mean of 46.0 ± 13.1 (SD) workers. These twelve colonies were used to provide 'late colonies'. The second batch arrived three weeks later, on 3 March 2023, with each colony including a queen, brood and a mean of 27.8 ± 7.1 (SD) workers. These twelve colonies were used to provide 'early colonies'. The commercial supplier confirmed that the termination dates of the founding queens' hibernation and the colony initiation dates for the two batches of colonies provided were, respectively, approximately three weeks apart between the batches (Annette Van Oystaeyen, Biobest Group, in litt., February 2023). In the experiment, the focal workers were then defined as the workers that eclosed after the arrival of the colonies, were individually marked and had their eclosion date recorded (**Section 3.2.3**).

All colonies were kept in a controlled climate room (temperature: 28°C; humidity: 60%) in constant darkness. All handling of the colonies was carried out under red light, to minimize any stress they experienced. Of each batch of 12 colonies, 10

colonies were randomly selected to be used in the experiment. In ten early colonies, each colony was randomly assigned a number between 1 and 10, and in ten late colonies each colony was randomly assigned a number between 11 and 20. In each colony on the day following arrival, all workers were carefully removed from the supplier's box using forceps and temporarily placed in a 1 l conical flask. Next, by means of two spoons, the brood was carefully removed from the supplier's box and placed on the floor of a wooden colony nest-box near one of the shorter walls. The queen was then carefully transferred into the new nest-box and placed on the brood, before, lastly, her workers were taken from the flask and added. The wooden colony nest-boxes (internal dimensions 17 cm x 27.5 cm x 16 cm high), in which colonies were housed for the duration of the experiment (**Figure 3.1A**), were equipped with a circular ventilation hole (Ø3.5 cm) on either side covered with a metal mesh. A plastic container filled with BIOGLUC®('Biobest Group NV, Belgium), a ready-to use sugar solution (artificial nectar), was positioned underneath each nest-box. The bees could feed on this solution via a filter-tip forming a wick (passing through aligned holes in the floor of the nest-box and the roof of the container) from the container to the inside of the nest-box, ensuring *ad libitum* access. The bees were also fed with fresh honeybee-collected pollen on an *ad libitum* basis. A rectangular frame of acetate was glued onto the top rim of the box to act as a barrier against bees crawling out. Each box was then covered with a transparent Perspex lid. The floors of the boxes were covered with unscented, non-clumping cat litter ('Felight', Non-clumping cat litter, Bob Martin Petcare) to absorb faeces and moisture.

After arrival of the colonies, dead workers were removed every 1-2 days and the number of dead workers was recorded. In the case of a focal worker, the death date of the worker was also recorded. All dead workers were singly stored in a bag labelled with the experiment number, date and worker ID and frozen at −20°C.

Once a week all colonies were cleaned by removing faeces and replacing soiled litter with fresh cat litter. Males and gynes that eventually eclosed in the colonies were removed and frozen at −20°C and the numbers of all such removed sexuals were recorded. Each colony was terminated (with remaining individuals being frozen) once its last focal worker had died. The end date of the experiment was 26 July 2023. In one experimental and one control colony, focal workers remained alive on this date (1-2 per colony), and in these cases these focal workers were recorded and treated as data censored at that point (for longevity analyses).

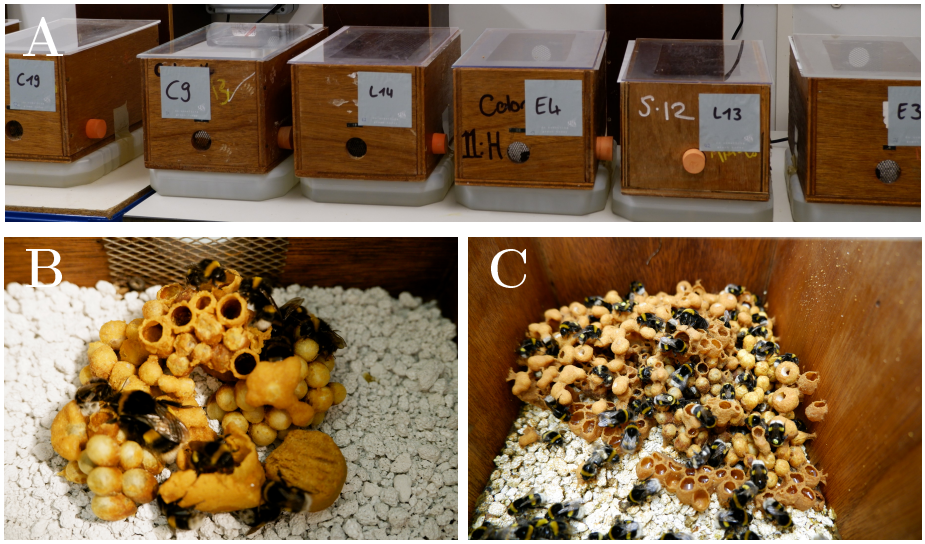

Figure 3.1. Rearing conditions of the experimental *Bombus terrestris* colonies. **A**: Wooden colony nest-boxes, with each nest-box housing one colony; underneath each nest-box is the plastic container of sugar solution (BioGluc); colonies paired for transfers were placed next to one another. **B**: Example of an 'early colony', with queen (large individual at lower left), workers and brood. **C**: Example of a 'late colony', i.e. a colony three weeks later in the colony cycle than an 'early colony', so having more workers and brood present.

3.2.2 Transfers

The early colonies that were assigned the numbers 1-6 were designated colonies E1 - E6, respectively, and the late colonies assigned the numbers 11-16 were designated L11 - L16, respectively. These twelve queenright colonies were used as the experimental colonies. Within the set of experimental colonies, each early colony was randomly paired with a late colony (e.g. E1 with L11) and transfers were carried out within a given pair only (**Table 3.3**, **Figure 3.2**). Early colonies 7-10 and late colonies 17-20 were used as control colonies (all queenright). Within the set of control colonies, each colony was randomly paired with a colony in the same stage (e.g. C8 with C9 and C18 with C19) and transfers were carried out within the pair only. This paired-design was implemented to minimize variation stemming from colony-specific factors within each pairwise set of transfers. Transfers between control colonies were conducted to test whether the experience of being transferred into a different colony as a newly-eclosed worker itself had an effect on worker longevity (**Table 3.3**, **Figure 3.2**).

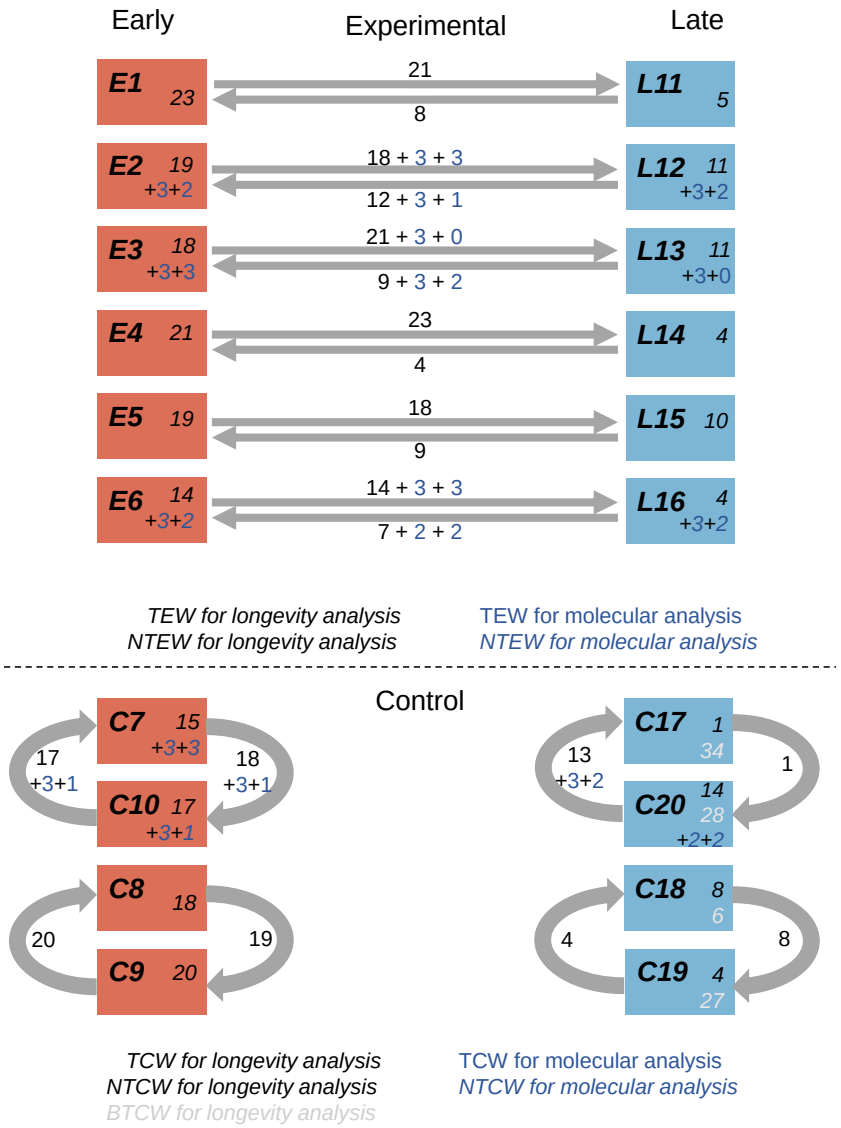

Figure 3.2. Design of the experiment. Red boxes represent 'early colonies' and blue boxes represent 'late colonies'. The bold number on the left within each box is the colony number. The numbers on the right within each box represent the sample sizes of marked focal workers that were not transferred (NTEW or NTCW) and were used for either longevity data (black) or molecular analysis (blue), with workers for molecular analysis being sampled at two different time points, i.e. worker ages of 2 weeks old (first number) and 7 weeks old (second number), these worker ages representing molecular sampling time point 1 (TP1) and time point 2 (TP2), respectively. The arrows illustrate the transfer of marked focal workers (TEW or TCW) and the sample sizes of such workers, shown as the number of workers for longevity data (black number) and the numbers of workers taken for molecular analysis over the two time points (first blue number and second blue number, respectively). (However , although control workers (NTCW and TCW) were sampled for molecular analyses as shown, molecular analyses on these two worker categories were not conducted, so as to allow such analyses to be prioritized in experimental workers.) Each row of the experimental colonies refers to one colony pair. Every experimental early colony was paired with one experimental late colony and transfers were conducted only within each pair. Control colonies were paired with one colony of the same stage, and transfers were also conducted only within each pair. In the late control colonies the grey numbers represent the sample size of marked workers from before the transfers started, when those colonies were still at an early stage (BTCW). Worker categories (TEW etc.) are as defined in Table 3.1.

This design led to the focal workers in the experiment being categorized into ten focal worker categories overall. Four experimental worker categories (TEW_{EL}, TEW_{LE}, $NTEW_E$ and $NTEW_L$) were used to test the hypotheses. Four control worker categories (TCW_{EE}, TCW_{LL}, $NTCW_E$ and $NTCW_L$) were used to monitor potential effects of being transferred itself. Lastly, two before-transfer worker categories ($BTEW_E$ and $BTCW_E$) were used to assess within-colony longevity differences between early-produced and late-produced workers that did not undergo transfers. Detailed descriptions of the worker categories are in **Table 3.1**.

Table 3.3. Colony pairs for the experimental transfer of workers. Each early experimental colony was paired with one late experimental colony. Each control colony was paired with another control colony of the same stage. Each row represents one pair of colonies (Colony A + Colony B). Sample size columns list, for each colony, the number (N) of adult non-focal workers present in the colony at the point of arrival.

Colony A	Stage	N at Arrival	Colony B	Stage	N at Arrival	Category
E1	early	16	L11	late	59	experimental
E2	early	26	L12	late	25	experimental
E3	early	26	L13	late	42	experimental
E4	early	27	L14	late	62	experimental
E5	early	27	L15	late	48	experimental
E6	early	20	L16	late	46	experimental
C7	early	28	C10	early	36	control
C8	early	41	C9	early	31	control
C17	late	44	C20	late	48	control
C18	late	24	C19	late	52	control

Marking of all newly-eclosed workers was conducted each morning for 5 consecutive days of each week and once every second morning for 2 days of each week. Each such worker was removed from the colony and placed in a marking cage (a cylindrical transparent tube, open at one end and covered with a plastic mesh at the other end). Using a foam plunger, the worker was then positioned so that the area of the thorax between the wings was accessible through the mesh. With a toothpick and a small drop of shellac-based glue (Queen Marking Glue, Thorne Ltd., UK), a circular paper label bearing a number on a coloured background (Queen Marking Kit, Thorne Ltd., UK) was attached to the worker's thorax. Within a colony all marked workers had different numbers on the same coloured background, allowing each worker to be identified both individually and by colony. All focal workers of

a colony were randomly assigned to be either a transferred or a non-transferred worker. This was done, during marking, by alternating the assigned class in successive workers. After marking, non-transferred workers were placed back into the colony they eclosed in, whereas transferred workers were transferred into the paired colony of the other stage. Depending on whether a worker eclosed in an early or a late colony and whether it was from one of the experimental or control colonies, the worker was then classified as one of eight worker categories (excluding $BTEW_E$ and $BTCW_E$, **Table 3.1**).

The target sample size was 18 workers per colony in each of the two classes of worker (transferred and non-transferred). This number was chosen because it was estimated to be less than one third of the total amount of workers already present in a small early colony, so, in the case of late to early transfers, was designed to avoid 'flooding' recipient colonies with too many transferred workers from donor colonies and thereby potentially altering the social environment. In addition, a power analysis suggested that a sample size of 18 individuals per worker treatment type would be sufficient to detect a significant difference (at the 0.05 level) between worker longevities with 0.80 power, assuming sample longevity means were 45 and 30 days, respectively, each with a standard deviation of 15 days (with these expected means and SD being taken from Holland and Bourke (2015),for purpose of the power analysis, medians treated as means).

Marking and transfer were conducted for three weeks after the early colonies had arrived. Additionally, newly-eclosed workers in the colonies that became the late colonies were marked in the three weeks before the early colonies arrived (at the same frequency as for the workers used for transfers). The longevity data from these workers ($BTEW_E$ and $BTCW_E$, as described above) were later used to compare the longevities of non-transferred early workers with those of non-transferred late workers of the same colony. This was to confirm that the longevity difference between early and late workers reported by Holland and Bourke (2015) was present in colonies used in the current experiment. Although the target sample size was reached and even exceeded for every early colony, it was not possible to reach it for workers (transferred or non-transferred) eclosing from any late colony except one (C20). This occurred because, soon after transfers started, no more workers eclosed in the late colonies (but instead males and gynes). Therefore, sample sizes for workers transferred from late colonies and remaining in late colonies were lower (**Table 3.1**, **Figure 3.2**).

3.2.3 Behavioural Observations

To check for effects of transfers on behaviour and to record any reproductive activity in workers, direct (in-person) observations were conducted in which a set of aggressive and reproductive behaviours was quantified. If the acting individual was a focal worker, its identity was recorded as its individual number (a combination of the number on the label and the first letter of the colour, e.g. 53R) and as the colony it originated in. If the acting individual was a non-focal worker, it was recorded as 'NF'. In the case of an aggressive behaviour (with an acting and a receiving individual), the receiving individual was recorded in the same way as for the acting one. Aggressive behaviours that were recorded included: darting (individual makes a sudden movement towards another individual but does not make contact); butting (individual makes an accelerated movement towards another individual, resulting in contact, then moves away); buzzing (individual faces another individual and makes fast short wing vibrations); pumping (individual faces another individual, its body is arched and the abdomen makes pumping movements); and attack (individual directly attacks another individual either by grappling, stinging, or biting) (Duchateau, 1989). Reproduction was recorded by noting the occurrence of egg-laying events (laying of an egg or eggs into a wax cell), both in the queen and any egg-laying workers. This behaviour was recognized by the individual placing its abdominal tip in an open egg-cell followed by visible tapping of the hind legs on the egg-cell wall (Bloch, 1999).

Observations were conducted daily for five days per week, alternating between colonies. In the three weeks after the start of transfers, each colony was watched for one hour at least every other day. Up to five colonies were watched by one person in parallel. Observations were undertaken such that the total observation time was similar for all colonies and the same observer did not watch the same colony twice in succession. In the following three weeks, colonies were watched for half an hour every other day. Overall, this resulted in total observation times of 8.5 - 10.0 hours per colony.

3.2.4 Wing Measurements

In *B. terrestris*, the length of the marginal wing cell of the forewing can be used as a proxy for body size, because the two measures are highly correlated (Duchateau and Velthuis, 1989; Owen, 1988; Goulson et al., 2002). Using a *Zeiss SteREO Discovery.V12* dissection microscope, the length of the marginal cell of the left forewing of every focal worker was measured (the right forewing being measured if the left

one was missing or deformed). To do this, a digital photograph of the forewing was taken with an AxioVision camera under a 15 x magnification and next to a 1 mm graticule. The length of the marginal cell was measured using the AxioVision software, with all such measurements being conducted by investigators blind to the longevity and category of the relevant focal worker.

3.2.5 Sampling for Molecular Analyses

To provide samples for RNA extraction for molecular analyses of gene expression changes in experimental and control workers, subsets of focal workers were sampled during the experiment and flash frozen. Samples were taken from each colony within three experimental colony pairs and within two control colony pairs (one early control, one late control), at two time points, i.e. corresponding to worker ages (for a given sampled worker) of two weeks and seven weeks after eclosion, these worker ages representing molecular sampling time point 1 (TP1) and time point 2 (TP2), respectively (**Figure 3.2**). Two weeks was picked as TP1 to ensure that the transferred workers had enough time to potentially be impacted by the social environment they were transferred into, while still remaining relatively young workers. Seven weeks was picked as TP2 in order to sample workers considerably older than those sampled at the first worker age, but before mortality prevented the collection of sufficient sample sizes (seven weeks being approximately the upper end of the interquartile range for worker longevities in Holland and Bourke (2015)). For each time point, if possible three focal workers per treatment type (transferred and non-transferred) per colony were taken (in total six per colony). This target sample size could not be reached in all cases, because some focal workers of the required age had died before the day of sampling (mainly the case for the second time point), with final sample sizes being a mean (range) of 2.9 (2-3) workers per colony per treatment type for time point one and 1.8 (0-3) workers per colony per treatment type for time point two (**Table 3.1**). For focal workers of the required age on the day of sampling, individuals were taken from the colony at random, placed in a 15 ml labelled falcon tube with ventilation holes in the lid and put on ice to induce torpor. Once all the samples for that day were collected on ice, each of the opened falcon tubes was flash frozen in liquid nitrogen. Frozen samples were kept on dry ice for the rest of the process before storage in a $-80°C$ freezer.

3.2.6 Dissections

For RNA extraction and sequencing, fat body tissue was dissected from each of the 57 experimental focal workers frozen for molecular analyses. The fat body was chosen as the tissue in which to profile gene expression because previous work in *B. terrestris* workers suggested that ageing-related gene networks were concentrated in fat body (Prince et al., 2024). Before a set of dissections commenced, all utensils used were cleaned with laboratory disinfectant (Distel High-level) and RNaseZap^{TM} (invitrogen) to ensure no contamination. Individuals were taken from the freezer and defrosted on ice. In each dissection, the abdomen was carefully separated from the rest of the body using a scalpel. Head and thorax were placed in a labelled tube and returned to the freezer. The abdomen was then dissected on ice under a dissection microscope in a Petri dish filled with cooled 1 x PBS. Using dissection forceps (Dumont No. 5), each segment of the cuticle on the ventral site of the abdomen was removed. Fat body tissue was collected from underneath the cuticle both ventrally and dorsally and from between the organs. Tissue was placed directly into a labelled 2 ml Eppendorf tube and excess PBS was removed with a 10 μl pipette. A mean of 29.35 mg $\pm$ 5.35 mg (SD) of tissue was collected per individual. The tube containing the tissue was placed in liquid nitrogen for flash freezing. The frozen tissue was then ground up (15 times) with a clean plastic pestle. Finally, TRI-reagent (Sigma-Aldrich, Gillingham, Dorset, UK) (500 μl) was added to the ground tissue before the sample was frozen and stored at -80°C. In addition, during dissections of the workers for the molecular samples, the ovaries were assessed as either activated, when mature oocytes/eggs were visible (Duchateau, 1989), or not activated when there was no sign of mature oocytes/eggs.

3.2.7 RNA Extraction and Sequencing

RNA was extracted from the collected fat body tissue of each individual separately using the Direct-zol$^{\text{TM}}$ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). For this, the samples on the TRI-reagent were defrosted and vortexed, incubated for 3 min, and then mixed with 100 μl of chloroform. After another 3 min, the samples were centrifuged for 15 min at 15,000 rpm and 4°C. A volume of 250 μl of the aqueous phase was removed and mixed with an equal volume of 100% ethanol. The samples were then each loaded onto a labelled Zymo-Spin$^{\text{TM}}$ IICR Column sitting in a collection tube before they were centrifuged at 13,000 rpm for 1 min and the flow through was discarded. The columns were then washed with 400 μl RNA-Wash buffer. To remove any DNA, 80 μl of DNase I reaction mix (DNase I : DNA digestion

buffer = 1:15) was added directly to the membranes and left to incubate for 15 min. Afterwards the samples were washed twice with 400 μl RNA-PreWash buffer before a last washing step with 700 μl RNA-Wash buffer. Following an extra spin in the centrifuge at 13,000 rpm for 2 min, the columns were placed into labelled Rnase-free 1.5 ml Eppendorf tubes, before the RNA was eluted with 21.5 μl RNase/DNase-free water at 13,000 rpm for 1 min.

To ensure that there was no DNA left in the RNA samples, an additional DNase treatment using the Turbo^{TM} DNA-free kit (Thermo Fisher Scientific, Loughborough, UK) was performed. For this, 3.5 μl DNase master mix (Turbo DNase : Turbo DNase buffer = 1:2.5) was added to each of the RNA samples, which were then left to incubate for 25 min at 37°C. Afterwards, 2.5 μl of inactivation reagent was added to each sample and left at 24°C for 5 min on a ThermoMixer for shaking at 300 rpm to mix the samples continuously. The RNA samples, including the inactivation reagent, were then transferred to 0.5 ml tubes containing 3.5 μl of DNase/RNase-free water, before being spun at 13,000 rpm for 1.5 min. A total of 23 μl of supernatant was removed carefully from each tube into labelled 2 ml Eppendorf tubes.

Using a Nanodrop 8000 spectrophotometer (ThermoFisher Scientific), the amount of RNA in each sample was quantified and the purity of the RNA was assessed to determine whether sample quality met standards for mRNA-sequencing (260/280 ratio $>$ 2.0). Twenty-seven samples were above this threshold and 30 samples were just below it (lowest detected: 1.82), but as the quantity of RNA was still very high in these 30 samples, all samples were retained. The 57 samples (each representing RNA from a single worker and hence a single biological replicate) were then sent to Novogene (Cambridge, UK) for sequencing as 150 bp paired-end reads on two lanes of an Illumina NovaSeqXPlus sequencer.

3.2.8 Statistical Analyses of Longevity and Behavioural Data

All statistical and graphical analyses were conducted using the software R version 4.4.0 (2024-04-24 ucrt)(R Core Team, 2020). For graphical visualisation of the data, the R package 'ggplot2' was used (Wickham, 2016).

As the behaviours were recorded at low frequencies and those observed were performed mainly by non-focal workers, the counts of behavioural actions were pooled by colony. Only one focal worker was recorded egg-laying. Therefore, reproductive behaviour in the focal workers could not be further analysed. To calculate the total egg-laying rate per colony, all recorded egg-laying events in a given colony were counted and divided by the total hours of observation of the colony. This yielded

a mean hourly rate of egg-laying (strictly, mean rate of performance of egg-laying events, since each event might involve laying more than one egg, from here on defined as 'egg-laying rate') per colony. As well as the total egg-laying rate, for each colony the mean hourly egg-laying rate of the queen and of the workers (as a group) were calculated separately. Within each of the colony stages (early and late), egg-laying rates were calculated using data from the first three weeks of observation only. This was to allow a comparison of egg-laying rates across the stages, as early colonies after three weeks would have entered the late stage of the colony cycle.

For each focal worker, worker longevity was calculated as the number of days between the worker's recorded eclosion date and its recorded death date. If there was no reliable death date for a given worker (as occurred if a worker's corpse was found hidden under the nest material), the data point was 'censored' to the date on which the worker was last seen alive (if this information was available). If this information was not available, the death date was treated as missing data and the given worker could not be included in the longevity analyses. The frequency distribution of the longevity data (excluding the censored workers) was investigated by testing for multimodality using the 'is.multimodal()'-function of the package 'LaplacesDemon' (Statisticat and LLC., 2021). This function uses a kernel density estimation and detects whether there are more than one 'true' peak (area under the curve $>$ 10% of total density). If that is the case the distribution tests positive for multimodality, if not the distribution is considered unimodal. Afterwards, using the 'Modes()'-function of the same package, the number of distinct peaks and therefore the form of multimodality (bimodal, trimodal, etc.) was determined.

Survival analyses using the worker longevity data were conducted with the R packages, 'survival' (Therneau, 2024) and 'survminer' (Kassambara et al., 2020). Survival analyses were conducted using a Cox proportional hazard regression mixed effect model with the four main worker categories ($NTEW_E$, TEW_{EL}, $NTEW_L$, TEW_{LE}) as a fixed effect and the colony transfer pairs as a random effect. The results were visualised using Kaplan-Meier curves. These analyses were repeated for a subgroup defined as 'short-lived workers'. For this, the median longevity of all experimental workers was calculated and only the 50% of workers with a longevity equal to or lower than that value were included in the subgroup. This was done because the form of the survival curves suggested a difference existed in survival between the categories in the first half of workers' adult life. Workers' survival was further analysed using the Bayesian survival trajectory analysis of the BaSTA-package (Colchero et al., 2012). In this method, age-related mortality distributions are estimated using the Markov chain Monte Carlo approach. Using the categories

as the grouping factor, four simulations were run in parallel using a 'Gompertz' distribution with a 'simple' shape, 150 000 iterations, a burn-in of 15001 chains and a thinning of 150. To analyse differences between the categories, the Kullback Leibler discrepancy calibrations (KLDC) were compared, following the standard threshold by which a value higher than 0.85 suggests that the posterior distributions of the two categories are substantially different (Sultanova et al., 2021).

To test whether the transfer itself affected worker longevity and survival, the same survival analyses (Cox proportional hazard and BaSTA) were conducted on the dataset from the control colonies, comparing transferred control workers (TCW_{EE} and TCW_{LL}) with non-transfer control workers of the same colony stage ($NTCW_E$ and $NTCW_L$). To test the underlying assumptions drawn from Holland and Bourke (2015), that early-produced workers have higher longevities than late-produced workers, further survival analyses (Cox proportional hazard and BaSTA) were conducted for three comparisons: 1) $BTEW_E$ and $BTCW_E$ vs. $NTEW_L$ and $NTCW_L$ (comparison within colonies); 2) $NTEW_E$ vs. $NTEW_L$ (comparison between colonies); 3) $NTCW_E$ and TCW_{EE} vs. $NTCW_L$ and TCW_{LL} (comparison between colonies).

3.2.9 Bioinformatic Analyses

The bioinformatic analyses were conducted using the pipeline in Collins et al. (2023), who customised standard pipelines for use on mRNA-seq data from *B. terrestris*. To assess the quality of the reads obtained from the mRNA-seq, FastQC v0.11.9 software (Andrews, 2015) was used to conduct quality tests such as base quality and potential adapter contamination in each sample and read, these tests then being combined into a quality report using the MultiQC v1.9 Python library (Ewels et al., 2016) with Python v3.7 (Python Core Team 2017). The reads were then aligned to the *B. terrestris* genome (BomTerr1.2_genomic.fna.gz; Crowley et al. (2023)) using HISAT2 v2.1.0 (Kim et al., 2015) and mapping statistics were documented. On the basis of the HISAT2 alignment files, the gene body coverage was calculated, testing for a 3' or 5' skew in the libraries, and the junction saturation was also calculated, testing whether all splice sites have been detected, using the RSeQC v3.0.1 Python library (Wang et al., 2012) with Python v3.7.

Kallisto v0.46.1 (Bray et al., 2016) was used to pseudoalign the reads to the *B. terrestris* transcriptome(BomTerr1.2_genomic.fna.gz; Crowley et al. (2023)). Only samples that had reached a threshold alignment of 20 million reads were used in further analyses. Estimated transcript counts for each gene were obtained using the tximport package v1.32.0 (Soneson et al., 2016) in R (v4.1.3) (R Core Team, 2020). These were then used for differential expression analysis with a model $\sim$ condition,

in which condition was the experimental category plus time point of the sample (DESeq2 package v1.44.0 (Love et al., 2014) in R (v4.1.3) (R Core Team, 2020). The FDR adjusted p-value threshold was set to 0.05.

The data set included data from ovary-active and ovary-inactive workers and age-related gene expression changes due to ovary activity are further analysed in **Chapter 5**. Pooling the data in the current chapter was justified by the fact that both ovary-active and ovary-inactive workers were present in all four worker categories in both time points (**Figure S5.1**). Additionally, it was previously found that the transfer of a worker into a non-native colony does not induce ovary activation in previously ovary-inactive workers (Yagound et al., 2012). Worker ovary-activity was therefore not expected to have been affected by the transfer. Ovary activity was therefore neglected in the current chapter, in which the focus laid on individual and social factors affecting longevity and age-related gene expression.

Principal component analysis (PCA) of the DESeq2 data was used to check for library clustering, and boxplots of the normalised count data were constructed to check the normalisation (Mohorianu et al., 2017). Overall, the analysis generated lists of differentially expressed genes (DEGs) containing genes that were significantly more highly expressed at the second time point (TP2) than at the first time point (TP1) (here defined as up-regulated DEGs, i.e. showing significantly increased expression with worker age), and genes that were significantly less highly expressed at the second time point (TP2) than at the first time point (TP1) (here defined as down-regulated DEGs, i.e. showing significantly decreased expression with worker age), within each of the four worker categories ($NTEW_E$, TEW_{EL}, $NTEW_L$, TEW_{LE}).

This analysis was repeated testing only for differential expression between the two time points (with all categories pooled), to analyse differential gene expression with worker age in general. The top 50 most differentially expressed genes from this analysis (combining up- and down-regulated), i.e. 'top-50 DEGs', were plotted in a heatmap showing the number of reads of these genes for each sample.

To analyse whether there were significant overlaps between the four worker categories in DEGs up- or down-regulated between the two time points, Fisher's exact tests were performed in R (R Core Team, 2020), including a Bonferroni correction to adjust for multiple testing.

Gene Ontology (GO) enrichment analysis and comparisons to other gene lists were conducted using OrthoFinder v2.5.2 (Emms and Kelly, 2019). This tool permitted gene orthologues between *B. terrestris* and *D. melanogaster* to be identified. Because GO annotations in *D. melanogaster* are considerably more detailed, only *D. melanogaster* single-copy orthologues for *B. terrestris* DEGs were used for

GO enrichment analysis. GO enrichment analysis was conducted in R (R Core Team, 2020)) using the clusterProfiler package (v4.12.0) (Yu et al., 2012) and the org.Dm.eg.db package (v3.19.1) (Carlson, 2024) for the biological processes GO annotations. Significantly overrepresented ($p < 0.05$ after adjustment for multiple testing with Benjamini-Hochberg) GO terms in a set of DEGs compared to all expressed genes were identified with an over-representation test (Boyle et al., 2004). Significantly overrepresented non-redundant GO terms are defined as 'enriched'.

Lastly, following the analysis in Collins et al. (2023), the DEG-lists were compared to two sets of *D. melanogaster* genes that have been hypothesised to be strongly associated with ageing in eusocial insects: the TI-J-LiFe network (Korb et al., 2021) and an enzymatic antioxidant gene set (Kramer et al., 2021). To identify significant overlaps with the TI-J-LiFe network and the enzymatic antioxidant gene set, the single-copy orthologues of the OrthoFinder results were used to compare the *B. terrestris* DEGs to the *D. melanogaster* orthologues. The up- and down-regulated DEGs in each worker category were pooled and then ranked by the log fold change in expression with time (Collins et al., 2023). For each worker category, the 50 genes with the most positive log fold change and the 50 genes with the most negative log fold change were selected ('top ± 50 genes'). If the number of DEGs in a given list were high enough, the lists for 'top ± 100 genes', 'top ± 200 genes', 'top ± 300 genes', 'top ± 500 genes', 'top ± 700 genes' and all genes were also created. For each worker category, these 'top ± gene'-lists were then compared for significant overlap with the gene lists from the TI-J-LiFe network and the enzymatic antioxidant gene set, using Fisher's exact tests with a Bonferroni correction to adjust for multiple testing.

3.3 Results

3.3.1 Behaviour

A total of 227.5 hours behaviour observation data were recorded. After the beginning of the transfers, 184 hours of behaviour observations were conducted and the mean observation time in this period was 9.2 ± 0.6 hours per colony.

Aggression in Workers as a Function of Colony Stage and Transfer

In total, 163 events of aggressive behaviour were recorded among all colonies from after the beginning of transfers. Most of these were buzzing events (61) or attack events (61). A significantly higher rate of hourly aggressive interactions was recorded in late colonies (mean: 1.42 ± 0.66 (SD), total of 130 interactions) compared to in early colonies (mean: 0.36 ± 0.18 (SD), total of 33 interactions; Mann-Whitney-U test: W = 0.5, p < 0.001). (Early colonies were observed for a total of 91.5 hours after transfers had started and late colonies were observed for a total of 92.5 hours after transfers had started.)

Aggression was largely displayed by non-focal workers and directed towards other non-focal workers. In 24 cases, a focal worker (experimental or control) showed aggressive behaviour. The recipient of aggression was a focal worker in 21 cases (12.9% of all aggressions), 5 of which were transferred individuals (3.1% of all aggressions). Hence, there was no evidence of increased aggression towards transferred workers (Chi-square test: expected counts of aggression directed towards non-transferred workers (N = 505): 13.09, expected counts of aggression directed towards transferred workers (N = 305): 7.91; χ^2 = 1.3, df = 1, p = 0.19). Therefore, longevity differences between worker categories were not caused by aggression incurred via transfers.

Egg-laying

Egg-laying Rate as a Function of Colony Stage

After the beginning of transfers, a total of 160 egg-laying events were recorded over 184 hours of observation. The mean hourly total rate of performance of egg-laying events in early colonies (0.17 ± 0.17 egg-laying events per hour per colony) was significantly lower than in late colonies (1.10 ± 1.36 egg-laying events per hour per colony) (Mann-Whitney-U test: W = 11.5, p = 0.004; **Figure 3.3A**).

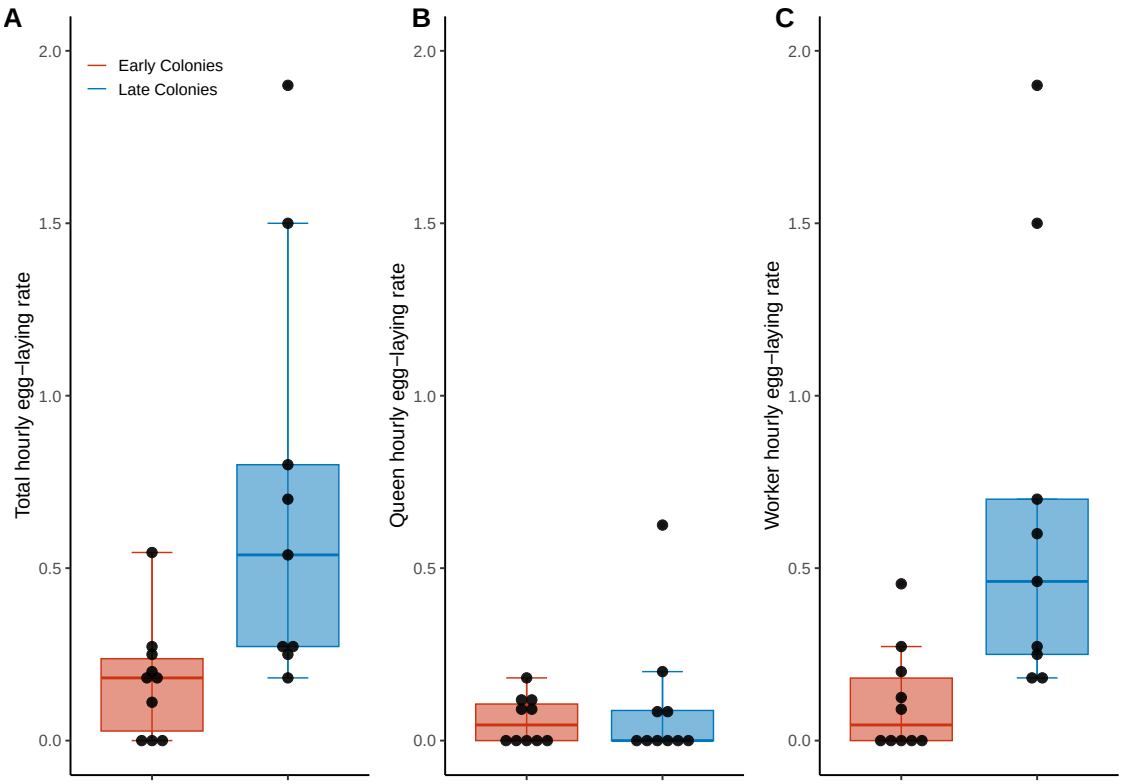

Figure 3.3. Rates of performance of egg-laying events ('egg-laying rate') per *Bombus terrestris* colony per hour of observation as a function of colony stage. 'Early colonies' are shown in red on the left side of each plot and 'late colonies' are shown in blue on the right side. Each black dot represents data from one colony (N = 10 colonies per stage). For each box the black line is median egg-laying rate, the upper and lower bounds of the box are the first quartile and the third quartile respectively and the whiskers extend to 1.5 × interquartile range after the box boundaries. **A**: The total mean hourly rate of performance of egg-laying events per colony, i.e. including queen and worker egg-laying. This rate was significantly higher in late colonies (p = 0.004). **B**: The mean hourly rate of performance of egg-laying events by the queen per colony. There was no significant difference in this rate between early and late colonies (p = 0.772). **C**: The mean hourly rate of performance of egg-laying events by workers per colony. This rate was significantly lower in early colonies (p = 0.002). The colonies were observed for 184 hours and a total of 160 egg-laying events were recorded.

The mean hourly rate of performance of egg-laying events by queens did not differ significantly between early colonies (0.06 ± 0.07 egg-laying events per hour per colony) and late colonies (0.10 ± 0.19 egg-laying events per hour per colony) (per colony) (Mann-Whitney-U test: W = 54, p = 0.772; **Figure 3.3B**). The difference in total rate of performance of egg-laying events between early and late colonies was driven by a difference in worker egg-laying activity, which was significantly lower in

early colonies (mean of 0.11 ± 0.15 egg-laying events per hour per colony) than in late colonies (mean of 1.01 ± 1.20 egg-laying events per hour per colony) (Mann-Whitney-U test: W = 9.5, p = 0.002; **Figure 3.3C**).

3.3.2 Worker Body Size as a Function of Colony Stage and Worker Category

The length of the marginal wing cell of the left forewing (a proxy for body size) was measured in 367 focal workers. There was no difference in marginal wing cell length between early-produced workers and late-produced workers (Mann-Whitney U test: W = 16417, p = 0.83). There was also no difference in marginal wing cell length between the four experimental categories (Kruskal-Wallis test: χ^2 = 1.96, df = 3, p = 0.58).

3.3.3 Worker Longevity and Survival

Overview of samples and overall longevity

Of 810 marked focal workers, longevity data were available for 488 (238 experimental workers, 127 control workers and 123 before-transfer workers). Those workers with missing longevity data could not be included in the longevity or survival analyses. Of the 488 focal workers included, 105 workers (71 experimental workers, 30 control workers and 4 before-transfer workers) had their longevity data included in comparisons of longevities as censored data. In these cases, censoring was conducted either because the focal workers were still alive when the colony was terminated (N = 2 experimental workers and 1 control worker), or because the workers were found dead underneath the brood and last recorded alive at an earlier date (N = 12 experimental workers, 2 control worker and 4 before-transfer workers), or because the workers were removed as samples for the molecular analyses (N = 57 experimental workers and 27 control workers). Censored longevities could not be included in analyses of the frequency distributions of longevity. The median longevity of all un-censored workers was 34 days (range: 1 day to 150 days; N = 383 workers).

Worker Longevity Distributions

The worker longevity distributions of the experimental focal workers (excluding censored workers) were not normally distributed (Kolmogorov-Smirnov test: D = 0.117, p = 0.003), and instead were significantly multimodal (bimodal) with a mode at 24 days and 56 days.

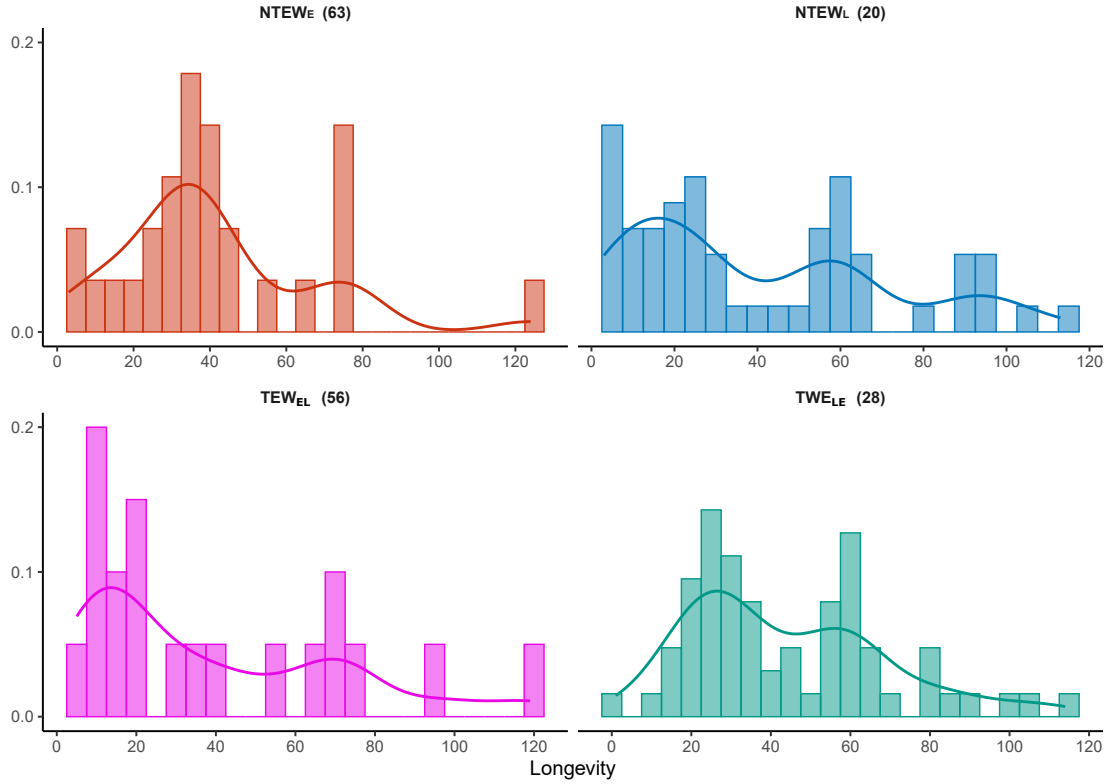

Figure 3.4. Worker longevity in *Bombus terrestris* follows a bimodal distribution. Proportional histograms with a density curve of worker longevities, showing the proportion of workers that died at a certain age (longevity in days from eclosion to death). These data sets exclude censored workers. The total area of all bins and under the curve respectively represents 100% of each sample. (Number in brackets representing the sample size; bin size 5 days.) Focal worker categories: $NTEW_E$ (red), $NTEW_L$ (blue), TEW_{EL} (purple), TEW_{LE} (green). Definitions of the experimental worker categories are in Table 3.1. The distributions for $NTEW_E$, TEW_{EL} and TEW_{LE} showed significant multimodality.

Three of the experimental worker categories had significantly multimodal longevity distributions, i.e. $NTEW_E$ (bimodal, modes at 26 days and 56 days), TEW_{EL} (bimodal, modes at 35 days and 75 days) and TEW_{LE} (trimodal, modes at 17 days, 55 days and 90 days, **Figure 3.4**). No multimodality was detected in the category $NTEW_L$. When non-transfer workers were grouped by colony of origin, 11/20 colonies had significantly multimodal worker longevity distributions (5/10 early colonies, 6/10 late colonies, **Figure S3.2**). To ensure this was not an artefact of the period of almost six weeks (three weeks before the transfers started and three weeks for the transfers) over which the focal workers had eclosed, the distributions were also tested with workers grouped by eclosion-week (non-transfer workers from all colonies pooled). Worker longevity distributions were significantly multimodal (bimodal) for 4/6 eclosion weeks (**Figure S3.1**). Therefore, overall, the data showed

within-colony and within-cohort multimodality in the frequency distribution of adult worker longevity.

Worker Longevity and Survival: tests of the hypotheses

Of the four experimental categories, $NTEW_E$ workers showed the highest median longevity of 37 days (range: 1-130 days; N = 63 workers) (Figure 3.5A) and (in fact the single individual whose longevity was 130 days might have lived even longer, as it was censored at the termination point of the experiment). $NTEW_L$ workers had a median longevity of 27 days (range: 5-119 days; N = 20 workers), TEW_{EL} workers a median of 28 days (range: 3-113 days; N = 56 workers), and TEW_{LE} workers a median of 35 days (range: 3-124 days; N = 28 workers) (**Figure 3.5A**). (Median longevities did not include data from censored workers.) A Cox proportional hazard model with mixed effects (colony-pair as a random effect) showed no significant difference in survival between the four categories of experimental workers, i.e., in longevity, $TEW_{EL} = NTEW_E$, $TEW_{EL} = NTEW_L$, $TEW_{LE} = NTEW_L$ and $TEW_{LE} = NTEW_E$ (**Table 3.4A)**, **Figures 3.5 B-E**). (Reflecting the previous analysis of the effects of size (**Section 3.3.2**), marginal wing cell length was not a significant predictor of survival in this model and the model was a better fit without it.) The absence of any pairwise differences between the longevities of the four main experimental worker categories did not support the predictions of either the Individual or the Social Hypothesis (**Table 3.2**).

As described in the Methods (**Section 3.2.8**), the analysis was repeated on the subset of the data consisting of only the 50% of focal adult *B. terrestris* workers that died before the total median longevity of 34 days (short-lived focal workers). In these workers, a Cox proportional hazard model with mixed effects showed significantly greater survival of TEW_{LE} and $NTEW_E$ workers compared to TEW_{EL} and $NTEW_L$ workers (**Figure 3.6A**), reflected in increased hazard ratios (risk of mortality) for TEW_{EL} and $NTEW_L$ workers (**Figure 3.6B**, **Table 3.4B)**). There was no significant difference in survival between TEW_{LE} and $NTEW_E$ workers nor between TEW_{EL} and $NTEW_L$ workers (**Figure 3.6B**, **Table 3.4B)**). (As in the corresponding survival analysis of the full data set (**Table 3.4A)**), marginal wing cell length did not influence longevity in this subgroup of workers and was therefore not a predictor in the final model.) In sum, in longevity of short-lived workers, $TEW_{EL} < NTEW_E$, $TEW_{EL} = NTEW_L$, $TEW_{LE} > NTEW_L$ and $TEW_{LE} = NTEW_E$. This pattern of pairwise differences and similarities in the longevities of short-lived workers in the four experimental worker categories matched exactly the predictions of the Social Hypothesis (**Table 3.2**).

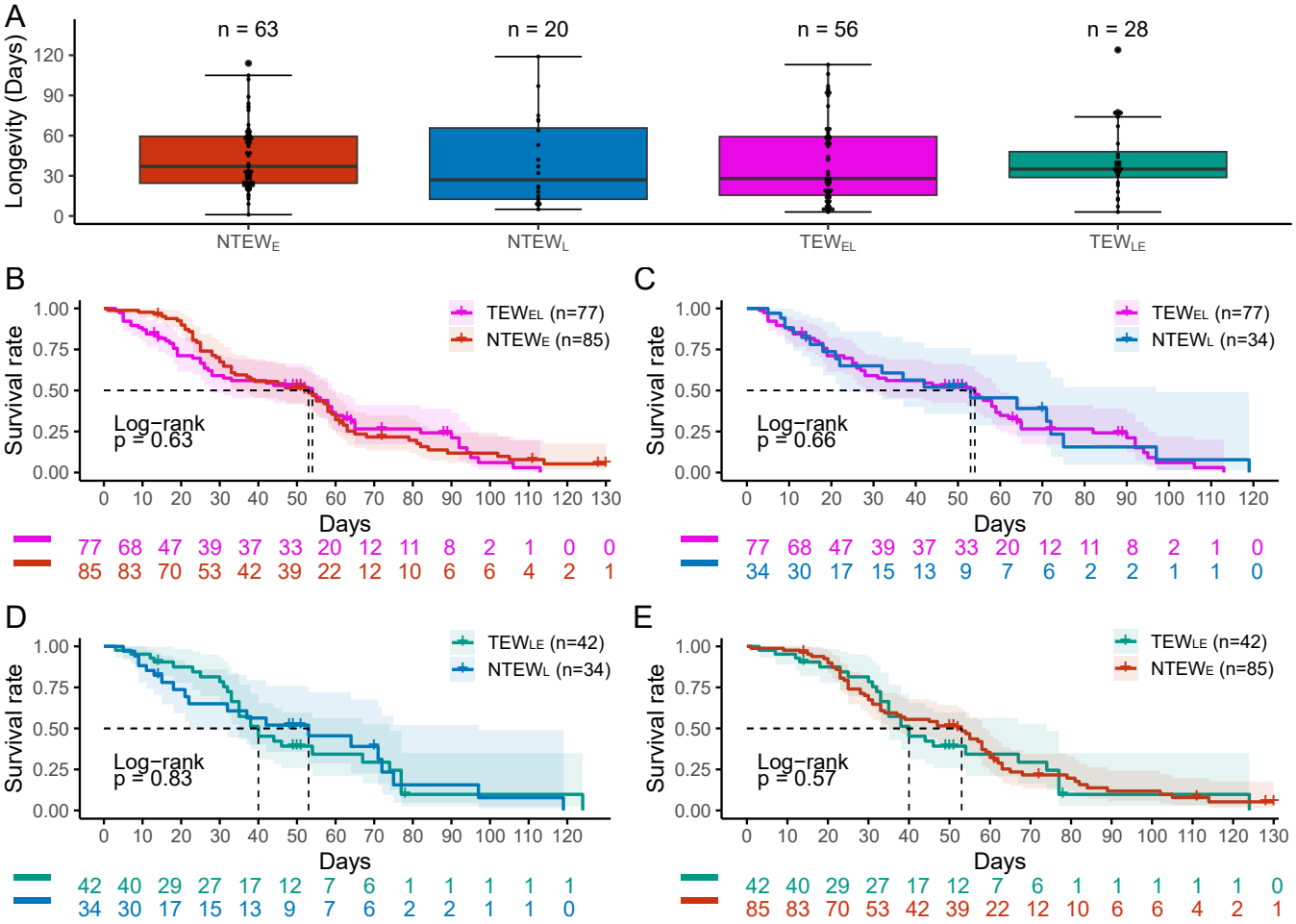

Figure 3.5. Longevity and survivorship of *Bombus terrestris* workers in the four main experimental categories. Focal worker categories: $NTEW_E$ (red), $NTEW_L$ (blue), TEW_{EL} (purple), TEW_{LE} (green). Definitions of the experimental worker categories are in Table 3.1. **A**: Box-plots of worker longevity within each category (censored workers are excluded, accounting for sample sizes lower than in panels B-E). For each box-plot, sample size is shown on top, the black line is median longevity, the upper and lower bounds of the box are the first quartile and the third quartile respectively and the whiskers extend to 1.5 × interquartile range after the box boundaries. **B - E**: Kaplan-Meier survival curves for compared pairs of experimental worker categories (in accordance to Table 3.2), with each curve including +- 95% confidence intervals (shading), and log-rank test results comparing mean survival rates; plus, below each pair of curves, risk tables showing how many workers of the given category remained alive at 10-day intervals throughout the experiment. Dotted lines indicate median longevity for each worker category. **B**: TEW_{EL} compared with $NTEW_E$ (same colony of origin). **C**: TEW_{EL} compared with $NTEW_L$ (same colony stage) **D**: TEW_{LE} compared with $NTEW_L$ (same colony of origin). **E**: TEW_{LE} compared with $NTEW_E$ (same colony stage). In all cases (B-E), there was no significant difference in survival rates between worker categories in the pair (pairwise comparisons (log-rank test), p = 0.57-0.83; Cox regression mixed model, Table 3.4A).

Table 3.4. Effect of the worker category on the longevity of focal adult *Bombus terrestris* workers, analysed with a Cox proportional hazard model with mixed effects. Model: Surv(Longevity, censored) ~ Category + (1 | Colony Pair). Workers with an uncertain death date were censored to the date they were last recorded alive. Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error and the Z- and the p-value. The three categories shown were compared to $NTEW_E$. **A)**: Analysis on the full data set (All focal workers). $NTEW_E$: N = 85; $NTEW_L$: N = 34; TEW_{EL}: N = 77; TEW_{LE}: N = 42. There was no significant difference between the four categories. **B)**: Analysis on the 50% of focal adult *B. terrestris* workers that had died before the total median longevity of 34 days (Short-lived focal workers). $NTEW_E$: N = 40; $NTEW_L$: N = 20; TEW_{EL}: N = 40; TEW_{LE}: N = 20. The hazard ratio (risk of mortality) was significantly increased for $NTEW_L$ and TEW_{EL}. Definitions of the experimental worker categories are in Table 3.1.

A) All focal workers

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
$NTEW_L$	0.167	1.182	0.266	0.63	0.53
TEW_{EL}	0.161	1.174	0.195	0.83	0.41
TEW_{LE}	0.142	1.152	0.230	0.62	0.54
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony Pair	Intercept	0.428	0.183		

B) Short-lived focal workers

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
$NTEW_L$	0.754	2.126	0.367	2.05	0.040
TEW_{EL}	0.717	2.049	0.267	2.68	0.007
TEW_{LE}	-0.039	0.962	0.345	-0.11	0.910
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony Pair	Intercept	0.009	0.00008		

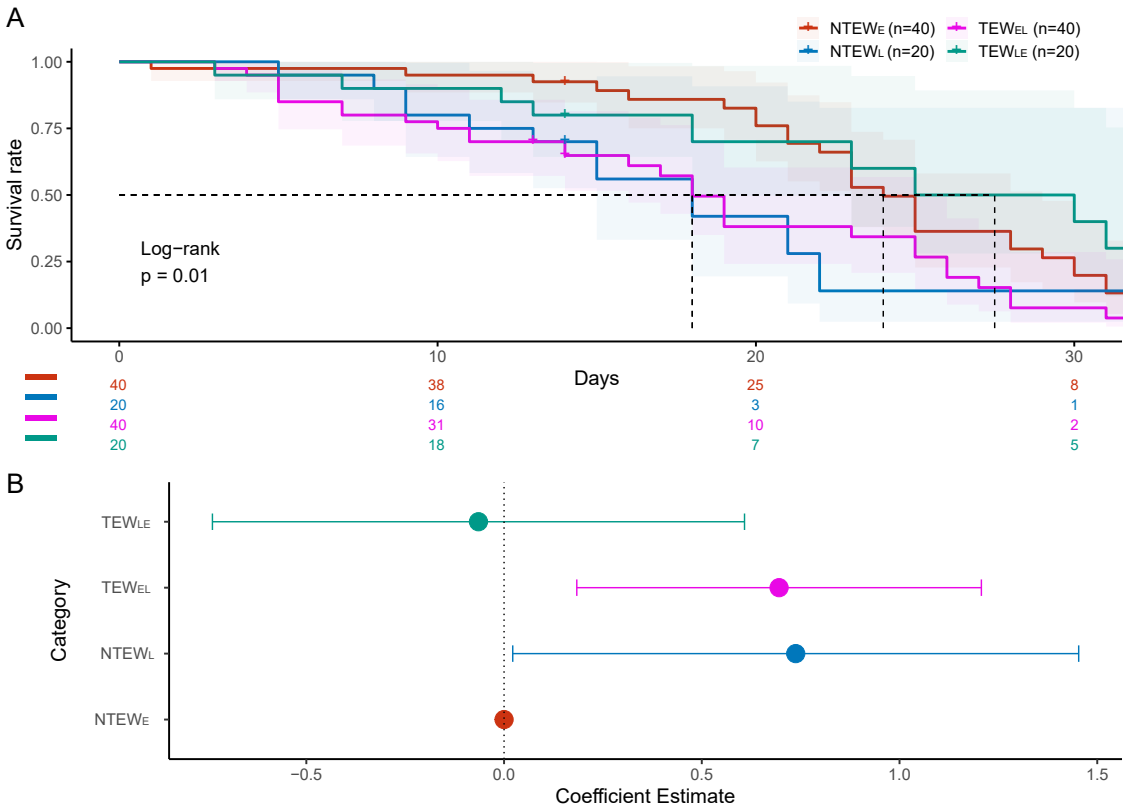

Figure 3.6. Survival analysis for the 50% of focal adult *Bombus terrestris* workers that died before the total median longevity of 34 days (short-lived focal workers). **A**: Kaplan-Meier survival curves for compared pairs of experimental worker categories, with each curve including ± 95% confidence intervals (shading), and log-rank test results comparing mean survival rates; plus, below, a risk table showing how many workers of the given category remained alive at 10-day intervals throughout the experiment. Dotted lines indicate median longevity for each worker category. TEW_{LE} and $NTEW_E$ showed lower survival probabilities than TEW_{EL} and $NTEW_L$ (log-rank test: p = 0.011). **B**: Forest plot for mixed Cox proportional hazard model. Workers of the category $NTEW_E$ serve as the reference. To the left of the dotted line: lower hazard (risk of mortality); to the right of the dotted line: higher hazard (risk of mortality). Bars represent the confidence intervals (-1.96 SE to +1.96 SE). The categories TEW_{EL} and $NTEW_L$ show a significantly increased hazard compared to $NTEW_E$ (Table 3.4B)). Definitions of the experimental worker categories are in Table 3.1.

The worker longevity data (full data set) were further analysed using Bayesian survival trajectory analysis. The baseline mortality rate (b_0, with lower baseline mortality rates translating to higher chances of survival for workers early in their lives) was substantially lower for the TEW_{EL} category relative to the $NTEW_L$ category but not relative to the $NTEW_E$ category (**Figure 3.7A**, **Table 3.5**). In addition, the baseline mortality rate was substantially higher for the TEW_{LE}

category relative to the $NTEW_E$ category but not relative to the $NTEW_L$ category (**Figure 3.7A**, **Table 3.5**). In sum, as regards chances of survival of workers early in life (inverse of baseline mortality rate) in the full data set, $TEW_{EL} = NTEW_E$, $TEW_{EL} > NTEW_L$, $TEW_{LE} = NTEW_L$ and $TEW_{LE} < NTEW_E$. Therefore, unlike the results of the Cox regression analysis for short-lived workers (**Figure 3.6**, **Table 3.4B)**), this pattern of pairwise similarities and differences in the four experimental worker categories matched exactly the predictions of the Individual Hypothesis and did not match the predictions of the Social Hypothesis (**Table 3.2**).

The change rate of mortality (b_1, Gompertz rate parameter, with higher values translating to steeper increases in mortality, hence lower survival) of the TEW_{EL} and TEW_{LE} categories did not differ substantially from those of the $NTEW_E$ and $NTEW_L$ categories or from one another (**Figure 3.7B**, **Table 3.5**). Therefore, overall, for this parameter, $TEW_{EL} = NTEW_E$, $TEW_{EL} = NTEW_L$, $TEW_{LE} = NTEW_L$ and $TEW_{LE} = NTEW_E$. The b_1 values for TEW_{EL} and TEW_{LE} fell between the b_1 values for $NTEW_E$ and $NTEW_L$ (which differed substantially from each other, **Table 3.5**). This result did not clearly support either the Individual Hypothesis or the Social Hypothesis (**Table 3.2**).

Table 3.5. Age-specific survival and mortality of the focal *Bombus terrestris* workers of the four experimental categories. Results of a simple Gompertz model (BaSTA analysis) across the full dataset: Mean Kullback-Leibler discrepancy calibration (KLDC) values for the analysed comparisons. b_0 represents the baseline mortality rate and b_1 the Gompertz rate parameter or the change rate in mortality. Each row represents the comparison between two categories. Group comparisons that result in KLDC values >0.85 are regarded as differing 'substantially' from each other. $NTEW_E$: N = 85; $NTEW_L$: N = 34; TEW_{EL}: N = 77; TEW_{LE}: N = 42. b_0 was substantially lower in $NTEW_E$ and TEW_{EL} than in $NTEW_L$ and TEW_{LE}. b_1 was substantially higher in $NTEW_E$ than in $NTEW_L$. Definitions of the experimental worker categories are in Table 3.1.

Comparisons	**Mean KLDC**	
	b0	**b1**
TEW_{EL} - $NTEW_E$	0.781	0.649
TEW_{EL} - $NTEW_L$	0.874	0.704
TEW_{LE} - $NTEW_L$	0.744	0.764
TEW_{LE} - $NTEW_E$	0.868	0.602
$NTEW_L$ - $NTEW_E$	0.990	0.913
TEW_{LE} - TEW_{EL}	0.550	0.543

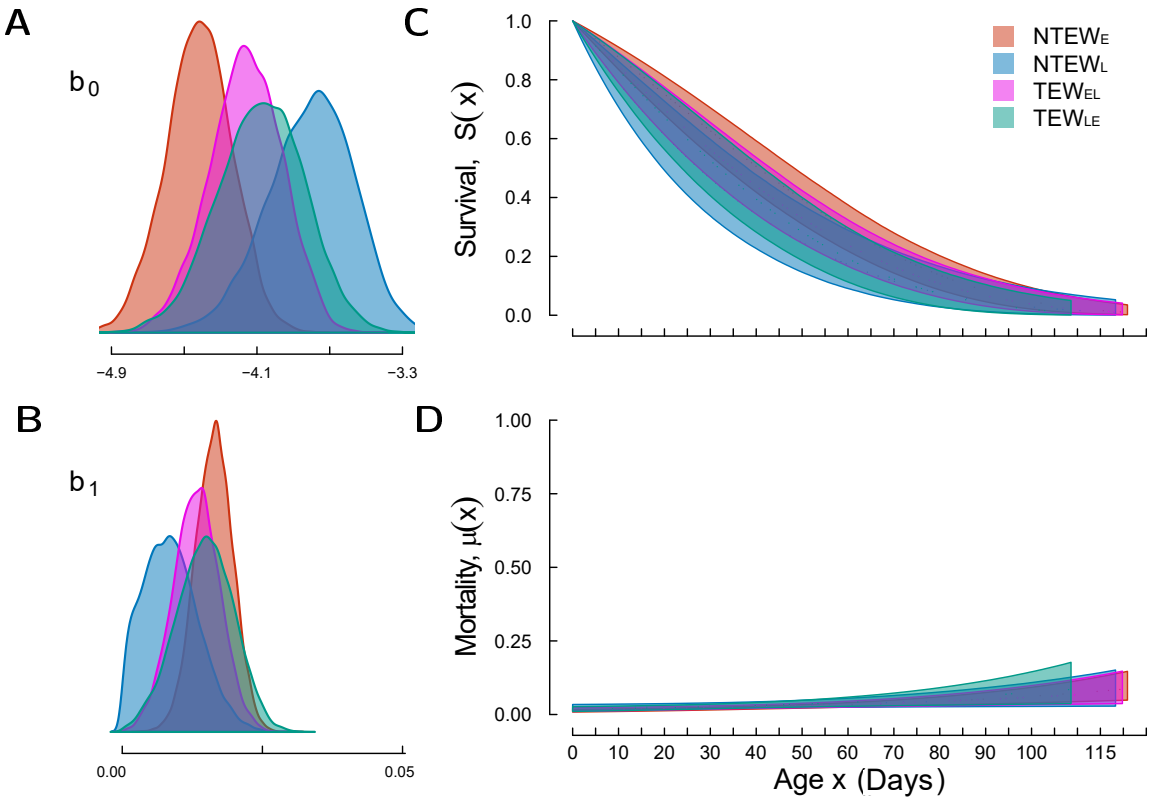

Figure 3.7. Age-specific survival and mortality of the focal *Bombus terrestris* workers of the four experimental categories, fitted with a simple Gompertz model (BaSTA analysis) across the full dataset. $NTEW_E$ (red): N = 85; $NTEW_L$ (blue): N = 34; TEW_{EL} (pink): N = 74; TEW_{LE} (green): N = 42. The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) values are listed in Table 3.5. **A**: Baseline mortality rate (b_0) was substantially lower in TEW_{EL} and $NTEW_E$ than in TEW_{LE} and $NTEW_L$. **B**: Gompertz rate parameter (mortality change over time, b_1) was substantially higher in $NTEW_E$ than in $NTEW_L$. **C**: Smoothed survival curves for the four experimental categories with the shaded areas representing 95% confidence intervals. There was no difference between the four categories. **D**: Change in mortality over time (days) with the shaded areas representing 95% confidence intervals. This did not differ between the categories. Definitions of the experimental worker categories are in Table 3.1.

The results of the longevity and survival analyses with respect to the predictions of the Individual Hypothesis and the Social Hypothesis are summarised in **Table 3.6**.

Effect of Transfers

Transferred control workers (TCW_{EE}: median longevity = 34 days (range, 1-130 days), N = 40 workers; TCW_{LL}; median longevity = 47 days (range, 4-105 days), N = 15 workers) did not differ significantly in longevity from non-transfer control

workers in the colony of origin nor the receiving colony ($NTCW_E$: median longevity = 21 days (range, 1-91 days), N = 35 workers; $NTCW_L$: median longevity = 47 days (range, 4-95 days), N = 7 workers) (mixed effects Cox proportional hazard model for transferred control workers compared to non-transfer control workers: early comparison: p = 0.47, late comparison: p = 0.81, **Table S3.2**).

The same conclusion was supported by the fact that there was no difference found in the baseline mortality (b_0) between the transferred control workers (TCW_{EE} and TCW_{LL}) and the non-transfer control nestmate workers of the same colony stage ($NTCW_E$ and $NTCW_L$) (**Figure S3.4A**, **Table S3.3A**). Similarly, the change rate in mortality (b_1) did not differ between the TCW_{LL} workers and the $NTCW_L$ workers, though it was substantially lower in the TCW_{EE} workers than in the $NTCW_E$ workers (**Figure S3.4B**, **Table S3.3A**).

Nevertheless, overall, as predicted, the transfer of newly-eclosed workers to non-nestmate colonies had, of itself, no effect on workers' adult longevity in the current experiment (**Table 3.2B**).

Longevity and Survival of Workers eclosing in Early versus Late Colonies

An underlying assumption of this experiment was that, in unmanipulated conditions, early-produced workers (i.e. workers eclosing in early colonies) have higher longevities than late-produced workers (i.e. workers eclosing in late colonies), as found by Holland and Bourke (2015) (**Table 3.2B**). To test this assumption with data from the current study, longevity comparisons were conducted between early-produced, non-transferred focal workers (i.e. workers eclosing in colonies before the transfers started, $BTEW_E$ and $BTCW_E$ pooled) and late-produced, non-transferred workers of the same colonies ($NTEW_L$ and $NTCW_L$ pooled). These within-colony comparisons were only possible in the experimental and control colonies that became the late colonies in the experiment. Early-produced workers in these colonies were those that eclosed in the three weeks prior to the start of transfers. These early-produced workers had a median longevity of 38 days (range, 2-138 days; N = 119 workers) and the late-produced workers had a median longevity of 32 days (range: 4-119 days: N = 27 workers), similar to the difference reported in Holland and Bourke (2015), though there was no difference in survival between the two groups (mixed effects Cox proportional hazard model: p = 0.72, **Table S3.1**).

The assumption was also tested using Bayesian survival trajectory analysis. Baseline mortality (b_0) was substantially lower for the early-produced workers (within-colony comparison, **Figure S3.3A**, **Table S3.3B**, mean KLDC = 0.95), meaning

that, as assumed, the early-produced workers had higher chances of survival in early life than the late-produced workers. However, the mortality change over time (b_1, Gompertz rate parameter) was substantially higher for the early-produced workers (**Figure S3.3A**, **Table S3.3B**, mean KDLC = 0.85), suggesting that the chances of mortality increased more steeply for the early-produced workers than for the late-produced workers. This could potentially explain why overall survival did not differ significantly between the two groups (**Table S3.1**).

Similarly, in the full data set of the experimental worker categories the assumption was not confirmed, as survival did not differ between the $NTEW_E$ category and the $NTEW_L$ category (between-colony comparison, Cox proportional hazard model with mixed effects, **Table S3.1**). Nevertheless, the median longevity was the highest in the $NTEW_E$ category with 37 days and the lowest in the $NTEW_L$ category with 27 days (**Figure 3.5A**). This difference in median longevity of 11 days, between the early-produced workers and the late-produced workers, was similar to the difference found by Holland and Bourke (2015) (approximately 15 days). Furthermore, the baseline mortality (b_0, Bayesian survival trajectory analysis) was again substantially lower for the $NTEW_E$ category than for the $NTEW_L$ category (**Figure 3.7A**, **Table 3.5**), meaning that the underlying chances of survival in early life were higher for $NTEW_E$ category. The change rate of mortality (Gompertz rate parameter b_1) on the other hand was substantially higher for the $NTEW_E$ category than the $NTEW_L$ category (**Figure 3.7B**, **Table 3.5**), suggesting that the chances of mortality increased more steeply for the early-produced workers than for the late-produced workers. Yet, the combination of lower baseline mortality (b_0), but higher change rates in mortality (b_1), for the early-produced workers in both of the comparisons (and the opposite for the late-produced workers) may explain, why the overall survival of the workers did not differ between early-produced and late-produced workers (**Table 3.4A**, **Table S3.1**).

Additionally, in the control colonies (comparison between colonies), early workers ($NTCW_E$ and TCW_{EE}) showed a substantially higher baseline mortality rate (b_0) than workers from late colonies ($NTCW_L$ and TCW_{LL}) (**Figure S3.4A**, **Table S3.3A**), contradicting the initial assumption (Holland and Bourke, 2015). The change rate of mortality though, was substantially lower in TCW_{EE} than in $NTCW_L$ and in TCW_{LL}, meaning that the mortality rate increased slower in TCW_{EE} than in $NTCW_L$ and in TCW_{LL} which again was in alignment with the assumption (Holland and Bourke, 2015).

Overall, there seemed to have been a trend following the underlying assumption extracted from Holland and Bourke (2015), that early-produced workers had higher

survival and longevities than late-produced workers. Different longevity and survival analyses found some statistical support for this, but also no difference between early-produced workers and late-produced workers in many instances. The assumption could therefore not be fully confirmed, yet there was evidence for a trend in the assumed direction.

3.3.4 Gene Expression Analysis

The sequencing provider created 57 libraries from the 57 *B. terrestris* worker fat body samples sent for mRNA-seq (one library per worker). Four of the samples did not reach the set threshold of an alignment of 20 million reads and were not used in the following analyses. In these four samples, Slow Bee Paralysis Virus was among the overrepresented sequences. Across the remaining 53 libraries, the mean read pairs per library was 30.31 million base-pairs. These libraries aligned to the *B. terrestris* transcriptome with a mean (range) percentage pseudoalignement of 82.02% (76.1% - 87.4%). There was evidence for normalisation in the normalised count data of the remaining samples (**Figure S3.5**).

Differential Gene Expression: as a Function of Worker Age

Gene expression changed strongly between the two time points at which the samples were taken (TP1 and TP2, at worker ages of 2 and 7 weeks, respectively). This was represented by the samples clustering strongly together by time point in the principal component analysis (**Figure 3.8**).

In the 50 genes most highly differentially expressed between the two time points, i.e. with worker age (top-50 DEGs), the gene with the highest read counts across all the samples was *venom serine protease 34*, which enables serine-type endopeptidase activity and is involved in proteolysis (Burge et al., 2012; Tang et al., 2019) **Figure S3.6**). The top-50 DEGs also included genes associated with defence or stress responses, development, cell division and organisation, and metabolism. The biggest difference in read counts between the two time points was in genes associated with immune responses (e.g. *phenoloxidase 1*, Giacomini et al. (2023)), and germ cell migration (e.g. *protein trapped in endoderm-1*, Ishimoto et al. (2000), both of which were up-regulated with worker age (**Figure S3.6**).

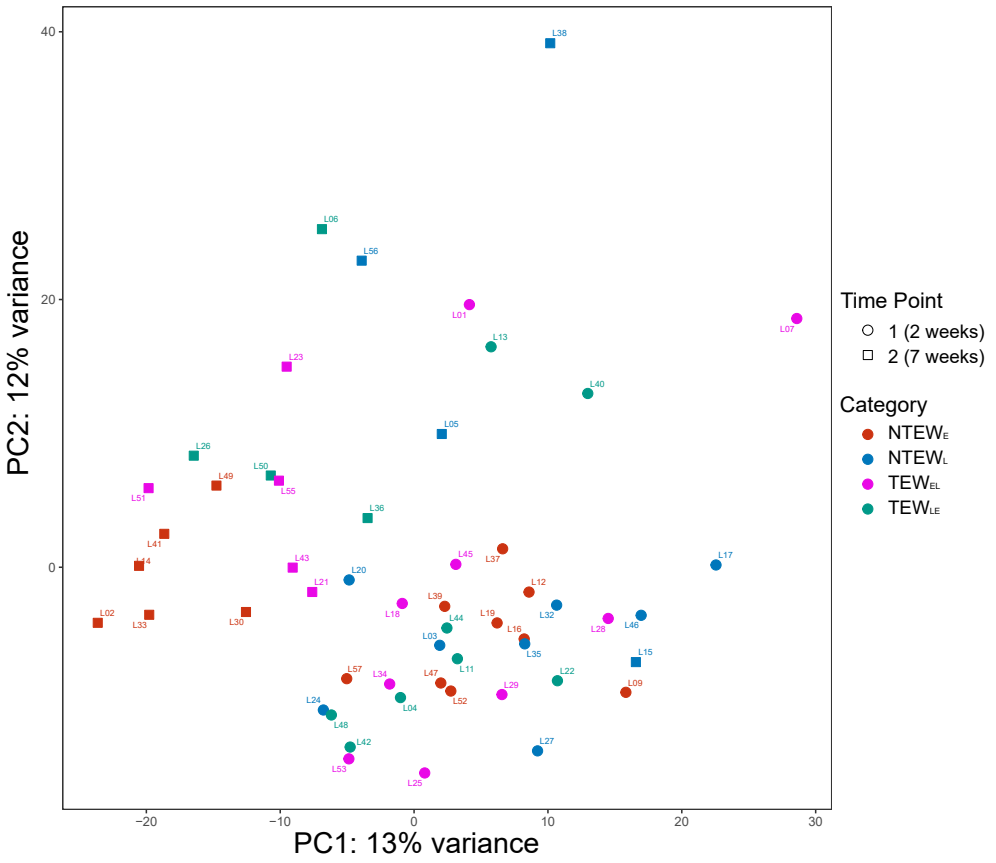

Figure 3.8. Principal component analysis (PCA) plot of mRNA-seq libraries from fat body of *Bombus terrestris* workers in four experimental worker categories over two time points (TP1 and TP2, worker ages of 2 and 7 weeks, respectively). Axes represent principal components; individual points represent individual fat body samples (one per worker), with colour denoting experimental category and shape denoting time point. Only samples above a threshold of an alignment of 20 million reads are included. Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 7; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 5; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 6; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 5. Definitions of the experimental worker categories are in Table 3.1.

Differential Gene Expression: Tests of the Hypotheses

The change with age was largest in the $NTEW_E$ category (888 genes up-regulated with age and 819 genes down-regulated with age) (**Figure 3.9**). Within each worker category, considered across all four categories, there were 304-888 DEGs up-regulated with worker age and 254-819 DEGs down-regulated with worker age (**Figure 3.9**). Comparisons within time points of DEGs between worker categories showed that TEW_{EL} workers resembled $NTEW_E$ workers (2-3 DEGs within TP1, 2-20 DEGs within TP2) but differed from $NTEW_L$ workers (25-57 DEGs within

TP1, 234-375 DEGs within TP2) (**Figure 3.9**). Transferred early workers therefore consistently resembled workers from the donor but not the recipient colony stage, matching the predictions of the Individual Hypothesis. By contrast, TEW_{LE} workers resembled $NTEW_L$ workers at time point 1 (2-8 DEGs within TP1) but not at time point 2 (145-196 DEGs within TP2), and resembled $NTEW_E$ workers at time point 1 (8-25 DEGs within TP1) but less so at time point 2 (47 up- and down-regulated DEGs within TP2) (**Figure 3.9**). Therefore, transferred late workers exhibited greater relative change in gene expression profile when transferred into a new social environment (early colonies), but not so as to become more similar to older workers of that environment, suggesting partial support for the Social Hypothesis.

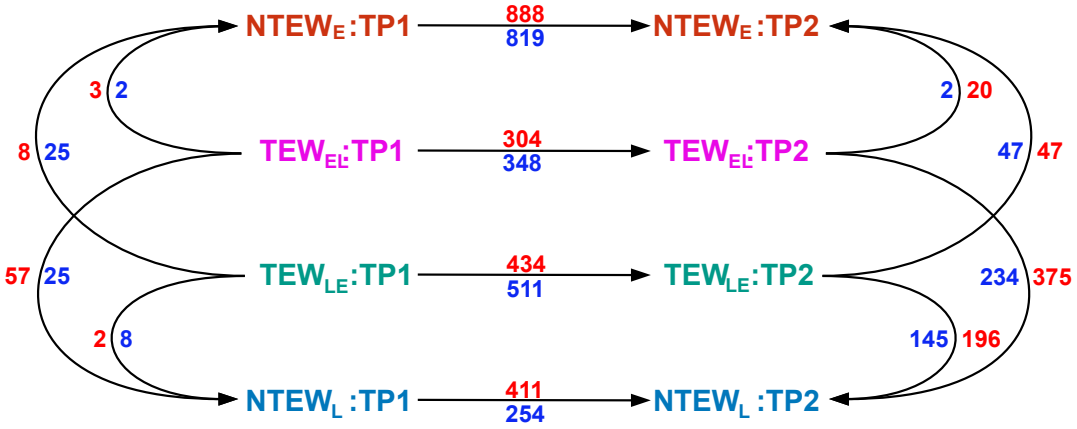

Figure 3.9. Comparison of mRNA-seq gene expression profiles in *Bombus terrestris* workers on the basis of differentially expressed genes (DEGs) over two worker ages. Colours (of abbreviations) represent the experimental worker categories ($NTEW_E$ – red; $NTEW_L$ – blue; TEW_{EL} – pink; TEW_{LE} – green). TP1: Time Point 1 (worker age of 2 weeks); TP2: Time Point 2 (worker age of 7 weeks). Arrows indicate the direction of the comparison between two conditions. Numbers in red: up-regulated DEGs (increased expression in the second condition of the comparison); numbers in blue: down-regulated DEGs (decreased expression in the second condition of the comparison). (For comparisons between time points, up-regulated DEGs therefore increased expression with age and down-regulated DEGs decreased expression with age.) Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 5; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 4. Definitions of the experimental worker categories are in Table 3.1.

The predictions of the Individual and Social Hypotheses as regards gene expression (**Table 3.2**) were also tested by checking for significant overlaps in DEGs changing expression with worker age in pairwise comparisons of the four experimental worker

categories.

In the comparison of TEW_{EL} and $NTEW_E$, there was significant overlap, with 44.7% of TEW_{EL} up-regulated DEGs shared with $NTEW_E$ (**Table S3.4**, **Figure 3.10A**) and with 40.5% of TEW_{EL} down-regulated DEGs shared with $NTEW_E$ (**Table S3.4**, **Figure 3.10B**). In the comparison of TEW_{EL} and $NTEW_L$, there was significant overlap for up-regulated DEGs, with 11.8% of TEW_{EL} up-regulated DEGs shared with $NTEW_L$ (**Table S3.4**, **Figure 3.10A**). The overlap of down-regulated DEGs, with 2.0% of TEW_{EL} down-regulated DEGs shared with $NTEW_L$, was not significant (**Table S3.4**, **Figure 3.10B**). In the comparison of TEW_{LE} and $NTEW_L$, there was significant overlap, with 10.1% of TEW_{LE} up-regulated DEGs shared with $NTEW_L$ (**Table S3.5**, **Figure 3.10C**) and with 6.3% of TEW_{LE} down-regulated DEGs shared with $NTEW_L$ (**Table S3.5**, **Figure 3.10D**). In the comparison of TEW_{LE} and $NTEW_E$, there was significant overlap, with 33.4% of TEW_{LE} up-regulated DEGs shared with $NTEW_E$ (**Table S3.5**, **Figure 3.10C**) and with 44.8% of TEW_{EL} down-regulated DEGs shared with $NTEW_E$ (**Table S3.5**, **Figure 3.10D**).

Among the DEGs exclusively shared between TEW_{EL} and $NTEW_E$, was *allatotropin*, which was up-regulated. *Allatotropin* is listed as part of the TI-J-LiFe network (Korb et al., 2021) and acts as a stimulator of juvenile hormone production (Bede et al., 2007). Among the DEGs exclusively shared between TEW_{LE} and $NTEW_E$, was *copper chaperone for superoxide dismutase*, which was down-regulated and is part of an ageing-related enzymatic antioxidant gene-set (Kramer et al., 2021). Full lists of DEGs that were exclusively shared between TEW_{EL} and $NTEW_E$, as well as those exclusively shared between TEW_{LE} and $NTEW_E$, are shown in **Table S3.6**.

In sum, in comparisons of their up- and down-regulated age-related DEGs, TEW_{EL} and $NTEW_E$ workers showed a strong resemblance (significant overlaps in 2/2 comparisons, of 40.5-44.7% DEGs), especially compared to the relative lack of resemblance shown by TEW_{EL} and $NTEW_L$ workers (significant overlap in 1/2 comparisons, but of 2.9-11.8% DEGs only). This pattern matched the predictions of the Individual Hypothesis (**Table 3.2A**). However, TEW_{LE} and $NTEW_L$ workers showed a relative lack of resemblance (significant overlaps in 2/2 comparisons, but of 6.3-10.1% DEGs only) compared to the resemblance shown by TEW_{LE} and $NTEW_E$ (significant overlaps in 2/2 comparisons, of 33.4-44.8% DEGs). This pattern matched the predictions of the Social Hypothesis (**Table 3.2A**). Therefore, these analyses of age-related gene expression, like the earlier comparisons within time points of DEGs between worker categories, suggested the existence of an in-

teraction of individual and social factors with colony stage. In particular, they both suggested that, with respect to age-related gene expression, workers from early colonies are more influenced by individual factors and workers from late colonies are more influenced by social factors.

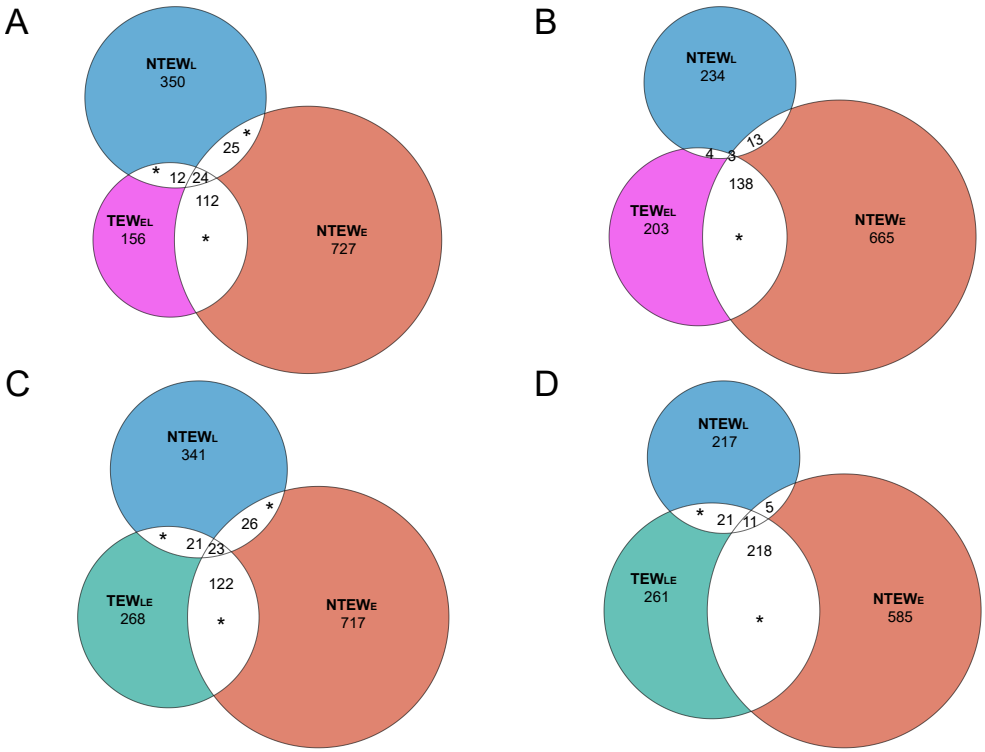

Figure 3.10. Euler diagrams comparing changes in mRNA-seq gene expression profiles with age in the fatbody of *Bombus terrestris* workers. Plotted are the number of differentially expressed genes (DEGs) that changed expression between time point 1 (TP1, worker age of 2 weeks) and time point 2 (TP2, worker age of 7 weeks) and the extent of the overlap between the experimental worker categories (white area). Asterisks (*), significant overlap in DEGs (Fisher's exact test, $p < 0.05$ after Bonferroni correction). The corresponding DEG numbers and Fisher's exact test results can be found in Tables S3.4 and S3.5. Up-regulated DEGs: DEGs significantly more expressed in TP2 than TP1, i.e. that increased expression with age; down-regulated DEGs: DEGs significantly more expressed in TP1 than TP2, i.e. that decreased expression with age. Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 5; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 4. Definitions of the experimental worker categories are in Table 3.1.

Strikingly, there was very little overlap in up- and down-regulated age-related DEGs between $NTEW_E$ and $NTEW_L$ workers. Only 5.5% of up-regulated $NTEW_E$ DEGs and 11.9% of up-regulated $NTEW_L$ DEGs were shared between the two cat-

egories (significant overlap, **Table S3.4**, **Figure 3.10A,C**). Similarly, only 2.9% of down-regulated $NTEW_E$ DEGs and 6.3% of down-regulated $NTEW_L$ DEGs were shared between the two categories (non-significant overlap, **Table S3.4**, **Figure 3.10B,D**). This finding supports the assumption that ageing in early-produced workers differs from ageing in late-produced workers.

Gene Ontology

Gene Ontology (GO) analysis was conducted using OrthoFinder to investigate the biological functions of the DEGs and compare them between the four experimental worker categories. A total of 5,932 single orthologues (between *B. terrestris* and *D. melanogaster*) were found (47.4% of the 12,514 genes expressed in the analysed *B. terrestris* mRNA-seq libraries). Using these, 221 non-redundant enriched GO terms were isolated for the DEGs. The TEW_{EL} category shared 7 GO terms (all derived from down-regulated DEGs) with the $NTEW_E$ category, which were mainly associated with the mitochondrial respiratory chain (5/7) (**Table S3.6**). There were no shared GO terms between the TEW_{EL} category and the $NTEW_L$ category. There were also no shared GO terms between the TEW_{LE} category and the $NTEW_L$ category. The TEW_{LE} category shared 29 GO terms (all derived from down-regulated DEGs) with the $NTEW_E$ category, which were largely associated with metabolic and catabolic processes (13/29), vesicular transport (7/29), symbiont-host interactions (3/29) and viral life cycles (2/29) (**Table S3.6**). Between the TEW_{EL} category, the TEW_{LE} category and the $NTEW_E$ category 10 enriched GO terms were shared (all derived from down-regulated DEGs), 6/10 were directly involved in cellular respiration (**Table S3.6**). In addition, there were no enriched GO terms shared between $NTEW_L$ category and any of the other categories. This is in line with the finding of little overlap in age-related DEGs between the $NTEW_L$ category and the other three categories, further supporting the assumption that late-produced workers in a late colony show different patterns of age-related gene expression than early-produced workers and/or workers in an early colony.

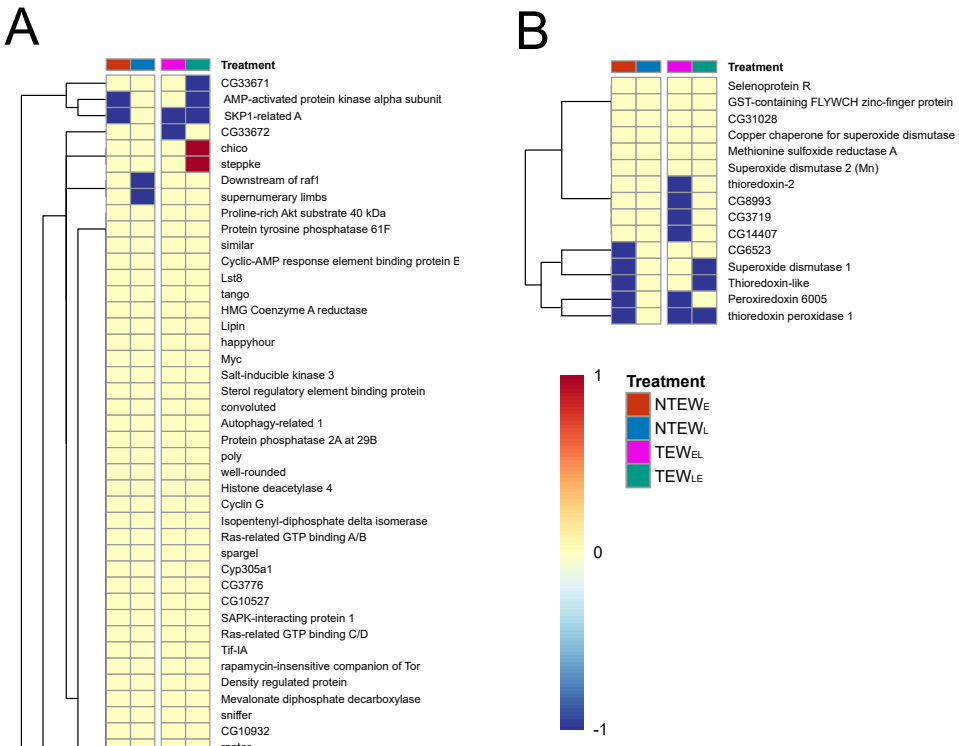

Figure 3.11. A comparison of changes in mRNA-seq gene expression profiles with age in *Bombus terrestris* workers to two sets of genes hypothesised to be ageing-related in eusocial insects. Each column corresponds to one of the experimental worker categories, pooling the data for all samples (single workers) in this category, with the colours coded accordingly ($NTEW_E$ – red; $NTEW_L$ – blue; TEW_{EL} – pink; TEW_{LE} – green). Definitions of the experimental worker categories are in Table 3.1. Gene expression was compared between two time points (TP1 and TP2, representing worker ages of 2 weeks and 7 weeks, respectively). A cell for a specific gene is colour coded to denote whether it was up-regulated between the two time points (red), or down-regulated (blue) or not differently expressed (beige). **A**: Comparison with TI-J-LiFe network; each row represents a gene from *D. melanogaster* found in the TI-J-LiFe network of Korb et al. (2021) and having a single-copy orthologue in *B. terrestris*. **B**: Comparison with enzymatic antioxidant gene set; each row represents a gene from *D. melanogaster* found in the enzymatic antioxidant gene set of Kramer et al. (2021) and having a single-copy orthologue in *B. terrestris*. The dendogram at the left groups genes that cluster according to their gene expression patterns. Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 5; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 4.

Differential Gene Expression: comparison with published gene sets

Some of the age-related DEGs isolated in the current study were also found in the set of ageing-related genes in the TI-J-LiFe network proposed by Korb et al. (2021). For the $NTEW_E$ category, 9 up-regulated DEGs and 5 down-regulated DEGs occurred in the TI-J-LiFe network (**Figure 3.11A**). For the $NTEW_L$ category, 0 up-regulated DEGs and 2 down-regulated DEGs occurred in the TI-J-LiFe network (**Figure 3.11A**). For the TEW_{EL} category, 3 up-regulated DEGs and 2 down-regulated DEGs occurred in the TI-J-LiFe network (**Figure 3.11A**). Lastly, for the TEW_{LE} category, 3 up-regulated DEGs and 5 down-regulated DEGs occurred in the TI-J-LiFe network (**Figure 3.11A**). However, in none of these cases was there significant overlap of age-related DEGs in focal workers and TI-J-LiFe network genes (Fisher's exact tests; p = 0.24–1.00, **Table S5.2A)**).

In line with the previous results, this analysis also revealed the $NTEW_L$ category to differ from the other three categories.

The DEGs of the current study were also compared to the set of ageing-related enzymatic antioxidant genes proposed by Kramer et al. (2021). For the $NTEW_E$ category, 0 up-regulated DEGs and 5 down-regulated DEGs occurred in the antioxidant gene set (**Figure 3.11B**, **Table S5.2B)**). For the $NTEW_L$ category, 0 up-regulated and 0 down-regulated DEGs occurred in the antioxidant gene set (**Figure 3.11B**, **Table S5.2B)**). For the TEW_{EL} category, 0 up-regulated DEGs and 6 down-regulated DEGs occurred in the antioxidant gene set (only significant overlap; **Figure 3.11B**, **Table S5.2B)**). Lastly, for the TEW_{LE} category, 0 up-regulated DEGs and 3 down-regulated DEGs occurred in the antioxidant gene set (**Figure 3.11B**, **Table S5.2B)**). This showed the $NTEW_L$ category again to differ from the other three categories.

The main gene expression results relevant to the hypotheses are summarised in **Table 3.6**.

Overall, the results show support for the Individual Hypothesis in that the baseline mortality was lower for early-produced workers than for late-produced workers and there was support for the Social Hypothesis in the longevity data of the 50% of focal workers that had died until the total median longevity of 35 days, in which workers in the late colonies showed lower longevities than the workers in early colonies (**Table 3.6**). The gene expression comparisons showed support for the Individual Hypothesis in the TEW_{EL} category, though also support for the Social Hypothesis in the TEW_{LE} category (**Table 3.6**).

Table 3.6. Overview of comparative *Bombus terrestris* worker longevities and gene expression changes between the experimental worker categories in relation to the predictions according to the Individual Hypothesis and the Social Hypothesis (columns 2 and 3, Table 3.2A). Worker categories are as defined in Table 3.1. Results of the current study are listed in columns 4-8 with references to the Figures and Tables showing the respective results. Cox PH: Cox-proportional hazard analysis; Full dataset: the four experimental focal worker categories: Short-lived: 50% of workers dying before median longevity; Baseline Mortality and Mortality Change: BaSTA-analysis. **Same**: for the two worker categories, mean longevities are predicted to be equal, age-related gene expression changes are predicted to resemble one another; **Different**: for the two worker categories, mean longevities are predicted to be unequal, age-related gene expression changes are predicted not to resemble one another. In the longevity predictions, $<$, mean longevity of the first worker category is less than that of the second; $>$, mean longevity of the first worker category is greater than that of the second. For results regarding b_0 and b_1, $<$ or $>$ are implications for survival (inverse of mortality).

	Predictions		**Results**				
Worker Categories	**Individual Hypothesis**	**Social Hypothesis**	**Cox PH Full dataset**	**Cox PH Short-lived**	**Baseline Mortality (b_0)**	**Mortality Change (b_1)**	**Age-related Gene Expression**
TEW_{EL} vs. $NTEW_E$	**Same**	**Different (<)**	**Same** Figure 3.5B Table 3.4A)	**Different (<)** Figure 3.6 Table 3.4B)	**Same** Figure 3.7A Table 3.5	**Same** Figure 3.7B Table 3.5	**Same** Figure 3.9 Figure 3.10A,B
TEW_{EL} vs. $NTEW_L$	**Different (>)**	**Same**	**Same** Figure 3.5C Table 3.4A)	**Same** Figure 3.6 Table 3.4B)	**Different** Figure 3.7A Table 3.5	**Same** Figure 3.7B Table 3.5	**Different** Figure 3.9 Figure 3.10A,B
TEW_{LE} vs. $NTEW_L$	**Same**	**Different (>)**	**Same** Figure 3.5D Table 3.4A)	**Different (>)** Figure 3.6 Table 3.4B)	**Same** Figure 3.7A Table 3.5	**Same** Figure 3.7B Table 3.5	**Different** Figure 3.9 Figure 3.10C,D
TEW_{LE} vs. $NTEW_E$	**Different (<)**	**Same**	**Same** Figure 3.5E Table 3.4A)	**Same** Figure 3.6 Table 3.4B)	**Different** Figure 3.7A Table 3.5	**Same** Figure 3.7B Table 3.5	**Same** Figure 3.9 Figure 3.10C,D

3.4 Discussion

The general question addressed by this study was whether ageing and longevity in social organisms are influenced primarily by individual (intrinsic) or social factors. The experiment tested this using the bumblebee *B. terrestris*, exploiting differences in longevity previously found between *B. terrestris* workers eclosing in colonies early and late in the colony cycle along with known differences in the social environments of early and late colonies. Two hypotheses were formulated. The Individual Hypothesis attributes longevity differences between workers in early and late colonies to intrinsic differences between individuals (individual factors), whereas the Social Hypothesis attributes them to differences between the social environments of colonies (social factors). To discriminate between these hypotheses, a reciprocal transfer experiment was performed in which newly-eclosed workers produced by early colonies were transferred into late colonies (3 weeks further along in the colony cycle) and newly-eclosed workers produced by those late colonies were transferred into the early colonies. This resulted in four experimental categories of workers: transferred experimental workers from an early to a late colony (TEW_{EL}), transferred experimental workers from a late to an early colony (TEW_{LE}), non-transferred experimental workers from an early colony ($NTEW_E$), and non-transferred experimental workers from a late colony ($NTEW_L$). These categories were compared for longevity and survival and (using mRNA-seq) age-related changes in levels of gene expression. Transfers occurred successfully, as there was no evidence of increased aggression towards transferred workers (**Section 3.3.1**) and longevities of the transferred control workers did not differ significantly from those of the non-transfer workers from colonies of the same colony stage (**Section 3.3.3**, **Figure S3.4**, **Tables S3.2** and **S3.3A**).

As now discussed in detail, the comparison of focal worker longevities and gene expression changes between the categories did not offer uniform support for either hypothesis, but instead suggested the existence of interactions between individual and social factors driving the longevity patterns.

3.4.1 Longevity and Survival of Workers eclosing in Early versus Late Colonies

Based on the findings of Holland and Bourke (2015), an underlying assumption of the current study was that workers produced early in the colony cycle would show higher longevities and higher survival than workers produced late in the colony cycle. This assumption could not be fully confirmed in the current study, yet there was a trend following these assumptions with higher median longevities of early-produced work-

ers than late-produced workers. As in Holland and Bourke (2015), early-produced workers (non-transferred) in the current study showed a median longevity that was 11 days greater than that of late-produced workers (non-transferred). This trend also showed in lower baseline mortality (b_0, BaSTA analysis) for early-produced workers than late-produced workers, suggesting higher chances of survival in early life (**Section 3.3.3**).

Unexpectedly, the mortality change rate (b_1, BaSTA analysis) was higher for the early produced workers than for the late-produced workers, which suggested steeper increases in mortality, which could explain that overall there was no difference in survival between early-produced and late-produced workers, despite the lower baseline mortality in early-produced workers.

The reason for the unexpected increased $b1$-rates in the early-produced workers is potentially explained by the fact that early colonies eventually progress to have a 'late' social environment as well. This could mean that, for early-produced workers surviving into the late colony stage, intrinsically higher survival is, later in the workers' lives, negatively affected by the late-colony social environment; by contrast, late-produced workers, starting with intrinsically lower survival, would show an overall lower change rate (b_1), as observed. This interpretation is also supported by the fact that, for the 50% of focal workers of the experimental worker categories that died before the total median longevity of 34 days, the chances of survival were significantly higher for the $NTEW_E$ category compared to the $NTEW_L$ category (**Figure 3.6**), again aligning with the assumptions drawn from the findings in Holland and Bourke (2015).

Overall, the longevities of early-produced workers and late-produced workers differed as expected from Holland and Bourke (2015), at least as a trend. The current study also confirmed that late colonies (as well as containing more workers) have social environments strongly differing from those of early colonies (see Introduction), e.g. in having greater levels of aggression (**Section 3.3.1**), which might be expected to constitute a social factor negatively affecting workers' longevity. Therefore, although the longevity difference assumed between unmanipulated early- and late-produced workers was not as clear-cut as was found in the study of Holland and Bourke (2015), the current experiment remains a test of the effects on workers' longevity of changes in social environment that, a priori, might be expected to strongly affect longevity in the same direction as that assumed by the hypotheses.

3.4.2 Worker Survival and Longevity: Tests of the Hypotheses

The data on workers' longevity and survival provided a mixture of support for both the Individual and Social Hypothesis (**Table 3.6**). In the full data set, overall worker survival did not differ between the four experimental worker categories (Cox proportional hazard model, **Table 3.6**). This pattern did not match predictions of either hypothesis and nor did it show a consistent trend with respect to either hypothesis (**Figure 3.5B-E**), meaning that neither solely individual factors nor solely social factors influenced the survival of the experimental workers. This suggests that there are likely additional factors influencing worker longevity as well as the possibility that individual and social factors could be interacting (see below). However, in the equivalent Cox proportional hazard model analysis including only the short-lived workers (50% of workers dying before median longevity), the pattern of similarities and differences in the pairwise comparisons of survival between the experimental worker categories matched all the predictions of the Social Hypothesis (**Table 3.6**). For example, short-lived TEW_{EL} workers (those transferred from early to late colonies) showed significantly lower survival than short-lived $NTEW_E$ workers (those remaining in early colonies) (**Figure 3.6**; **Table 3.4B)**) i.e. the transferred workers expressed the reduced longevity characteristic of the late-colony social environment. This suggests that social factors do indeed influence workers' longevity as the Social Hypothesis predicted, but that their influence is stronger in short-lived workers than in longer-lived workers.

In the Bayesian survival trajectory analyses (BaSTA), results for baseline mortality (b_0) yielded a pattern of pairwise similarities and differences between the experimental worker categories matching the predictions of the Individual Hypothesis (**Table 3.6**). This finding appears at odds with results from the Cox proportional hazard model analysis of the short-lived workers, especially since lower baseline mortality rates translate to higher chances of survival for workers early in their lives. However, although in these results TEW_{EL} workers did not differ significantly from $NTEW_E$ workers, and did show significantly lower baseline mortality than $NTEW_L$ workers, the data show that their baseline mortalities shifted away from those of $NTEW_E$ workers towards those of $NTEW_L$ workers (shift of pink curve from red curve towards blue curve in **Figure 3.7A**). Similarly, although TEW_{LE} workers did not differ significantly in baseline mortality from $NTEW_L$ workers, and did show significantly higher baseline mortality than $NTEW_E$ workers, their baseline mortalities shifted from those of $NTEW_L$ workers towards those of $NTEW_E$ workers (shift of green curve from blue curve towards red curve in **Figure 3.7A**).

In short, for baseline mortality, the consistent trend was for transferred workers to move closer to the pattern shown by workers of recipient colonies, as expected from the Social Hypothesis, allowing the conclusion that there are intrinsic differences between early-produced and late-produced workers that are additionally shaped by the social environment. In this sense, the baseline mortality results, though providing some support for the Individual Hypothesis, did show consistency with the results of the Cox proportional hazard model analysis of the short-lived workers. Results for mortality change rate (b_1) showed no overall difference between the experimental worker categories (**Table 3.6**), which, as for the results of the Cox proportional hazard model analysis of the full data set, did not match predictions of either hypothesis meaning that neither solely individual factors nor solely social factors influenced the change rate of worker mortality over time, again suggesting additional factors to be playing a role and/or an interplay of individual and social factors. However, for mortality change rate, the data showed the same consistent trend as was found for baseline mortality (TEW_{EL} workers shifted towards $NTEW_L$ workers, TEW_{LE} workers shifted towards $NTEW_E$ workers), that is, for transferred workers to move closer to the pattern shown by workers of recipient colonies (**Figure 3.7B**). This again suggested support for the Social Hypothesis.

Overall, these results, in providing support for elements of both hypotheses, suggest that an interaction of individual and social factors influences worker longevity in this system. The baseline mortality results provide evidence for intrinsic differences caused by individual factors affecting survival chances in early life, such that early-produced workers (TEW_{EL} and $NTEW_E$) had higher chances of survival than late-produced workers (TEW_{LE} and $NTEW_L$) in early life irrespective of the social environment, in line with the Individual Hypothesis (**Table 3.6**). However, these intrinsic differences also seemed to be shaped to some extent by effects of the social environment, such that short-lived workers showed longevity patterns matching the predictions of the Social Hypothesis and the baseline mortality and mortality change rate of all transferred workers tended to grow closer to those of workers of the recipient colonies. These countervailing influences of individual and social factors, as well as the trend for negative associations between b_0 and b_1 within worker categories, could have helped account for the lack of difference shown across worker categories between median longevities in the full data set. In sum, the conclusion from the longevity and survival results of this experiment is that individual and social factors interact to influence ageing and longevity in this system and, by extension, in other social organisms. In a sense this was expected given that sociality has evolved from a non-social state (e.g. **Chapter 1**), such that the necessarily exclusively individual

factors influencing ageing and longevity of non-social organisms acquire, in social evolution, an overlay of social influences.

3.4.3 Differential Gene Expression: Tests of the Hypotheses

In agreement with the longevity and survival results, comparisons of patterns of resemblance and non-resemblance in age-related differential gene expression between the experimental worker categories also provided a mixture of support for both the Individual and Social Hypothesis (**Table 3.6**; **Section 3.3.4**). In particular, comparisons within time points of DEGs between worker categories (**Figure 3.9**) suggested that early workers' gene expression matched more closely the predictions of the Individual Hypothesis but late workers' gene expression matched more closely the predictions of the Social Hypothesis. Similarly, in the analysis of age-related DEGs (**Figure 3.10**), the pairwise comparisons of TEW_{EL} vs. $NTEW_{E}$ workers (relatively similar) and of TEW_{EL} vs. $NTEW_{L}$ workers (relatively different) matched predictions of the Individual Hypothesis, whereas the pairwise comparisons of TEW_{LE} vs. $NTEW_{L}$ workers (relatively different) and of TEW_{LE} vs. $NTEW_{E}$ workers (relatively similar) matched predictions of the Social Hypothesis (**Figure 3.10**). In other words, transferred early-produced workers tended to retain their particular profile of age-related gene expression despite introduction into a new social environment (late colonies), but transferred late-produced workers tended to adopt the profile of age-related gene expression closer to that of workers from their new social environment (early colonies). As a result, both sets of analyses suggested that, with respect to age-related gene expression, workers from early colonies are more influenced by individual factors and workers from late colonies are more influenced by social factors. In turn, this leads to the conclusion that intrinsic differences are present and are too strong to be shaped by transfer to the late social environment but can be overcome by transfer to a (more benign) early social environment. This is further suggested by the fact that age-related gene expression in the late-produced workers that remained in the late colonies differed greatly from that of the other three worker categories.

3.4.4 Behavioural Differences between Early and Late Colonies

Consistent with previous literature on *B. terrestris* (**Chapter 1**) and with an assumption of the current experiment, the behavioural observations provided evidence for very different social environments in early and late colonies. Both worker egg-

laying activity and aggression increased in late colonies (**Figure 3.3**). The queens' egg-laying rates were similar in early and late colonies (**Figure 3.3B**), and the overall significant increase in egg-laying activity in late colonies was therefore driven by workers becoming reproductively active (**Figure 3.3C**). Such increased worker egg-laying activity in late colonies relative to early colonies was expected and showed that late colonies had passed their competition point, at which workers start laying haploid eggs and aggression between workers increases (Duchateau and Velthuis, 1988; Bourke and Ratnieks, 2001; Alaux et al., 2006; Zanette et al., 2012), whereas the early colonies had not yet reached this point. Along with the significant differences in worker egg-laying activity and aggressive behaviour between early colonies and late colonies, this change underscored the strong differences that exist between the social environments of early and late colonies. In late colonies, the increasingly hostile interactions between workers could potentially be a driver of reduced worker longevity in such colonies. On average, it is the more aggressive workers that become reproductively active (Alaux et al., 2004; Bloch et al., 1996; Duchateau, 1989; van Doorn, 1989; Foster et al., 2004), and those also seem to have greater longevities (**Chapter 2**). Hence increased levels of stress and energy expenditure by workers receiving the aggression could be causing lower survival rates. Increased stress levels might also explain why this social effect was found only in the subset of the shorter-lived workers, i.e. the 50% of focal workers that had died before the total median longevity of 34 days. These workers might have been weaker/lower-quality workers that were more susceptible to stress, such that, when these levels were increased in a late social environment, the consequences in terms of reduced longevity were expressed. In addition, the fact that this social effect appeared to be present only in short-lived workers was in a sense unsurprising. As mentioned above (**Section 3.4.1**), the early colonies of this experiment continued to progress in the colony cycle, and therefore three weeks (21 days) into the experiment, by the definition followed in the current study, these colonies had entered a 'late' social environment. Therefore, it seems that the advantages of the early social environment may not have left lasting effects on longevity and survival that could still be detected when the colonies had progressed to a later colony stage.

The exact nature of the underlying intrinsic differences caused by individual factors in the young workers that affect longevity and survival is unknown, but such differences could stem from various factors during development. For example, there could initially be differences in egg quality of the worker-destined eggs laid by the queen. The so-called 'Lansing Effect' has been reported for many animal species (vertebrates and invertebrates) and describes a direct negative correlation between

the age of the mother and the longevity of the adult offspring (Ivimey-Cook et al., 2023). Such an effect would be consistent with *B. terrestris* queens in early colonies (and therefore relatively young queens) producing longer-lived workers, as found by Holland and Bourke (2015). It could also mean that queens in early colonies lay higher-quality eggs, potentially containing more resources, than queens of late colonies (older in age), that might have fewer such resources with which to provision eggs. Intrinsic differences in the adult workers could also stem or be further enhanced by differences during larval development. Early colonies usually have less brood and no male or queen brood yet. The attendance frequency of the brood-caring workers to the worker larvae might therefore be higher than in late colonies, as it has been shown that male-destined larvae are fed at a higher frequency than worker-destined larvae and queen-destined larvae are fed at a higher frequency than male-destined larvae (Ribeiro et al., 1999). This should result in higher feeding frequencies and optimal thermal development conditions for worker-destined larvae in early colonies, whereas in late colonies, single larvae may be competing with more brood for food and attendance, potentially leading to less optimal development conditions. Brood attendance might also become less when the competition among workers to lay eggs increases. Hence, such differences in rearing conditions causing intrinsic differences in the adult workers, may also be shaped by the changes in the social environment of a colony.

3.4.5 Worker Longevity Distributions

An unanticipated finding of this study was multimodality in the frequency distribution of worker longevity. For example, the frequency distribution of the longevity of all focal experimental workers was significantly bimodal (**Figure 3.4**). Splitting longevities by experimental worker category also revealed significant multimodality in worker longevity within three of the categories ($NTEW_E$, TEW_{EL}, and TEW_{LE}) and for non-transfer workers within 11 of the 20 colonies (5/10 early colonies and 6/10 late colonies) (**Figure S3.2**). This multimodality was not an artefact of the fact that the eclosion window of the focal workers stretched over six weeks (three weeks for experimental workers). When all non-transfer workers were grouped by the week of eclosion, multimodality was still detected in the longevities of the focal workers in 4/6 of the eclosion weeks (**Figure S3.1**). Similarly, such a bimodal distribution was detected in the longevity data analysed in **Chapter 2**. Because both these experiments were conducted in the laboratory with each colony being a closed system, one can theoretically assume equal chances of mortality from external sources for all focal workers, if they were intrinsically similar, which would also

be consistent with their high relatedness (Hamilton, 1964; Trivers and Hare, 1976). The fact that worker longevities do not follow a normal distribution might therefore be evidence for intrinsic differences in worker quality within the groups investigated. As considered elsewhere in this thesis (**Chapter 2**, **Chapter 4**), individual quality, while complex to define, in *B. terrestris* workers appears to be a function of positively associated traits of body size, reproductivity and longevity. The occurrence of multimodal longevity distributions suggests that, among workers of the same colony, there are some that live long lives while others under the same conditions do not, consistent with longevity being one element of individual quality. The fact that multimodality in the longevity distributions appeared within three of the experimental worker categories (and within some of the colonies), suggests that even though the four experimental worker categories might still overall differ (due to individual and social factors), there were still underlying quality differences within each category. Such differences could be intrinsic and connected to egg-quality or could arise from differences during larval development, such as position in the nest and nutrition (Couvillon and Dornhaus (2009); Holland and Bourke (2015); **Chapter 2**).

3.4.6 Adult Body Size and Longevity

Because larger body sizes in *B. terrestris* workers have previously been found to be associated with increased longevities and better rearing conditions (Blacher et al. (2017); **Chapter 2**), it was assumed that intrinsic differences of the adult workers would potentially manifest in differences of adult body size. However, body size (measured as marginal wing cell length) had no effect on survival either in the full data set or in the short-lived workers (**Figure 3.6**, **Table 3.4B)**). Increased body size was therefore not connected to increased longevity in this experiment. This could be because the strength of the relationship between longevity and body size is relatively low, accounting for its variable occurrence across studies.

3.4.7 Other Aspects of the Gene Expression Analysis

Four libraries (corresponding to four samples and four workers) were excluded from the analyses due to low alignment (one library per category, all time point 2). In these four samples, sequences associated with the Slow Bee Paralysis Virus (SBPV, *Iflavirus apistardum*) were among the over-represented sequences. It is therefore likely that in these libraries the low alignment to the *B. terrestris* genome was caused by the large number of SBPV reads. The same virus has been previously reported in RNA-seq data from *B. terrestris* queens in commercial colonies (Collins

et al., 2023). The virus is also known to be present in wild *B. terrestris* (McMahon et al., 2015). However, because it did not affect the longevity of experimentally infected workers (Manley et al., 2017) and Collins et al. (2023) reported that the presence of SBPV was not likely to have altered gene expression in their sample, it is assumed that it had no effect on gene expression of the retained samples in the current experiment.

Among the 50 genes that changed the most with age, the '*venom serine protease 34*' had the largest expression change (**Figure S3.6**). This gene enables serine-type endopeptidase activity and is involved in proteolysis (Burge et al., 2012; Tang et al., 2019). Proteolysis helps maintain protein homeostasis by degrading damaged or misfolded proteins and it has been suggested that a decline in efficiency in the molecular networks involved in this is one of the proximate causes of ageing (Koga et al., 2011; Maklakov and Immler, 2016). An increase in the "*venom serine protease 34*" activity could therefore be such a sign of ageing. Genes associated with immune response reactions and germ cell migration had higher reads for the second time point than for the first. Opposing to these results, immune activity in *B. terrestris* workers has previously been found to decline with age (Moret and Schmid-Hempel, 2009). Increased germ cell migration could be a sign of increased reproductive activity in the older workers.

The shared GO terms between the three experimental categories $NTEW_E$, TEW_{EL} and TEW_{LE} were mainly connected to cellular respiration, all of which were down-regulated. A reduction in the activity of the respiratory chain in *D. melanogaster*, has been found to increase longevity without negatively affecting fecundity (Fridell et al., 2005). Whether a similar effect is behind the current finding remains inconclusive, because longevity data for the sampled workers is could not be collected. The GO terms shared between $NTEW_E$ and TEW_{LE} though (mainly associated with metabolic and catabolic processes, all-down regulated) and the GO terms shared between $NTEW_E$ and TEW_{EL} (mainly associated with the mitochondrial respiratory chain, all down-regulated), further suggests a slowing of the metabolism with age in these categories. Metabolism and cell respiration are the main contributors to oxidative stress (Finkel and Holbrook, 2000). Oxidative stress can cause damages such as DNA mutations, protein oxidation and the peroxidation of membrane lipids (Li-Byarlay and Cleare, 2020) and the accumulation of such forms of damage has been proposed to be one of the main proximate drivers of ageing (Harman, 1956; Kirkwood and Holliday, 1979). Examples for this have been found in other eusocial Hymenopterans, including honey bees (Seehuus et al., 2006; Corona et al., 2007) and ants (Schneider et al., 2011; Majoe et al., 2021).

There was no significant overlap between age-related genes isolated in the current study with ageing-related genes in the TI-J-LiFe network (Korb et al., 2021) (**Figure 3.11A**). A recent study also found very little significant overlap of age-related genes in *B. terrestris* queens with genes in this network and could not define any conclusive ageing-related patterns (Collins et al., 2023). Despite the lack of significant overlap, some of the genes in the TI-J-LiFe network were found to be up- or down-regulated with age in the current study, though which genes these were differed greatly between all four experimental worker categories and was therefore not conclusive for either hypothesis (**Figure 3.11A**). There was some significant overlap of age-related genes in the current study with genes in the ageing-related enzymatic antioxidant gene set of (Kramer et al., 2021), though not for the $NTEW_L$ category ((**Figure 3.11B**)). The network genes that did overlap in the other three categories were all down-regulated with age in the current study. In both the TEW_{EL} category and the TEW_{LE} category, some of the down-regulated genes were the same as those down-regulated in the $NTEW_E$ category, but none of the patterns were fully the same ((**Figure 3.11B**)).

3.4.8 Conclusion

The main conclusion of this study with respect to the hypotheses is that both the longevity and survival data, and the gene expression data, point to workers' longevity in the study system being affected by an interaction of individual and social factors. Specifically, from the longevity and survival data, there seems to be evidence for individual differences between early and late workers, possibly associated with intrinsic worker quality differences, by which early-produced workers have an increased chance of survival at the start of life, as suggested by the Individual Hypothesis. Additionally, as suggested by the Social Hypothesis, there seem to be social factors affecting workers' longevity as well, possibly associated with the social environment in late colonies being more stressful and so resulting in higher mortality. This social effect seems to act especially strongly on short-lived workers, i.e. those making up the 50% of workers that died before the median longevity of all workers, which showed longevity patterns matching the predictions of the Social Hypothesis. Similarly, the gene expression data revealed that, with respect to age-related gene expression, the intrinsic difference between early and late workers remained for early workers even when these workers were transferred into the late social environment, whereas late workers transferred into the early social environment seemed to adopt profiles of age-related gene expression more like those of the workers of the recipient colonies. Worker longevity in this study could therefore be

explained by an interaction of individual and social factors, in a way whereby the intrinsic differences caused by individual factors determined how susceptible workers were to social factors affecting longevity.

Additionally, there seem to be underlying differences in worker quality that were not readily explained by the Individual or Social Hypothesis. The general pattern of longevity distributions seemed to provide evidence that, even within the experimental worker categories, there are underlying differences among the workers, resulting in multi-modal longevity patterns irrespective of whether workers were early or late workers (**Chapter 4**). A possible reason for such patterns is within-group variation in worker quality, i.e. within each group of workers (early or late) there were individuals of potentially lower quality that were therefore more susceptible to effects of the social environment, so expressing relatively reduced longevity, and individuals of potentially higher quality that were less susceptible to the effects of the social environment, so expressing relatively greater longevity.

In sum, in the study system (*B. terrestris* workers), longevity differences could be explained with a combination of individual factors underpinning the intrinsic longevity advantage of early-produced workers and social factors that manifest as the adverse effect of the social environment on worker longevity in late colonies, to which shorter-lived workers seemed more susceptible. Therefore, the overall conclusion of this experiment is that both individual and social factors may interact to influence longevity, along with its molecular underpinnings, in social organisms.

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Supplement

Additional supplementary material is available at: `https://github.com/lilianafischer/PhD-Thesis-Longevity-Ageing-and-Reproductivity-in-a-Social-Insect`.

Supplementary Figures

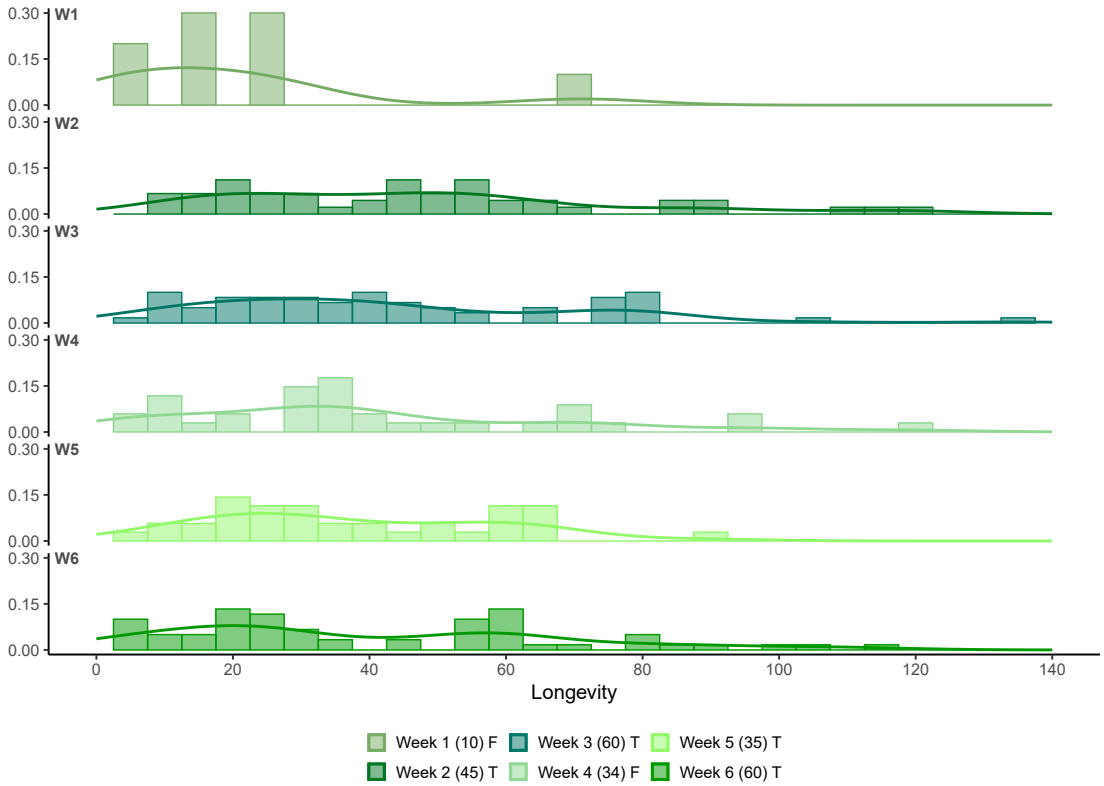

Figure S3.1. Worker longevity distributions of *Bombus terrestris* in days (all non-transfer workers, excluding censored workers), grouped by the week of eclosion (pooled from all colonies). Proportional histograms with a density curve of worker longevities, showing the proportion of workers that died at a certain age (longevity in days from eclosion to death). The total area of all bins and under the curve respectively represents 100% of each sample. (Number in brackets representing the sample size; bin size 5 days.) Week 1: N = 10; Week 2: N = 45; Week 3: N = 60; Week 4: N = 34; Week 5: N = 35; Week 6: N = 60. The distribution of worker longevities was significantly bimodal for 4/6 eclosion weeks.

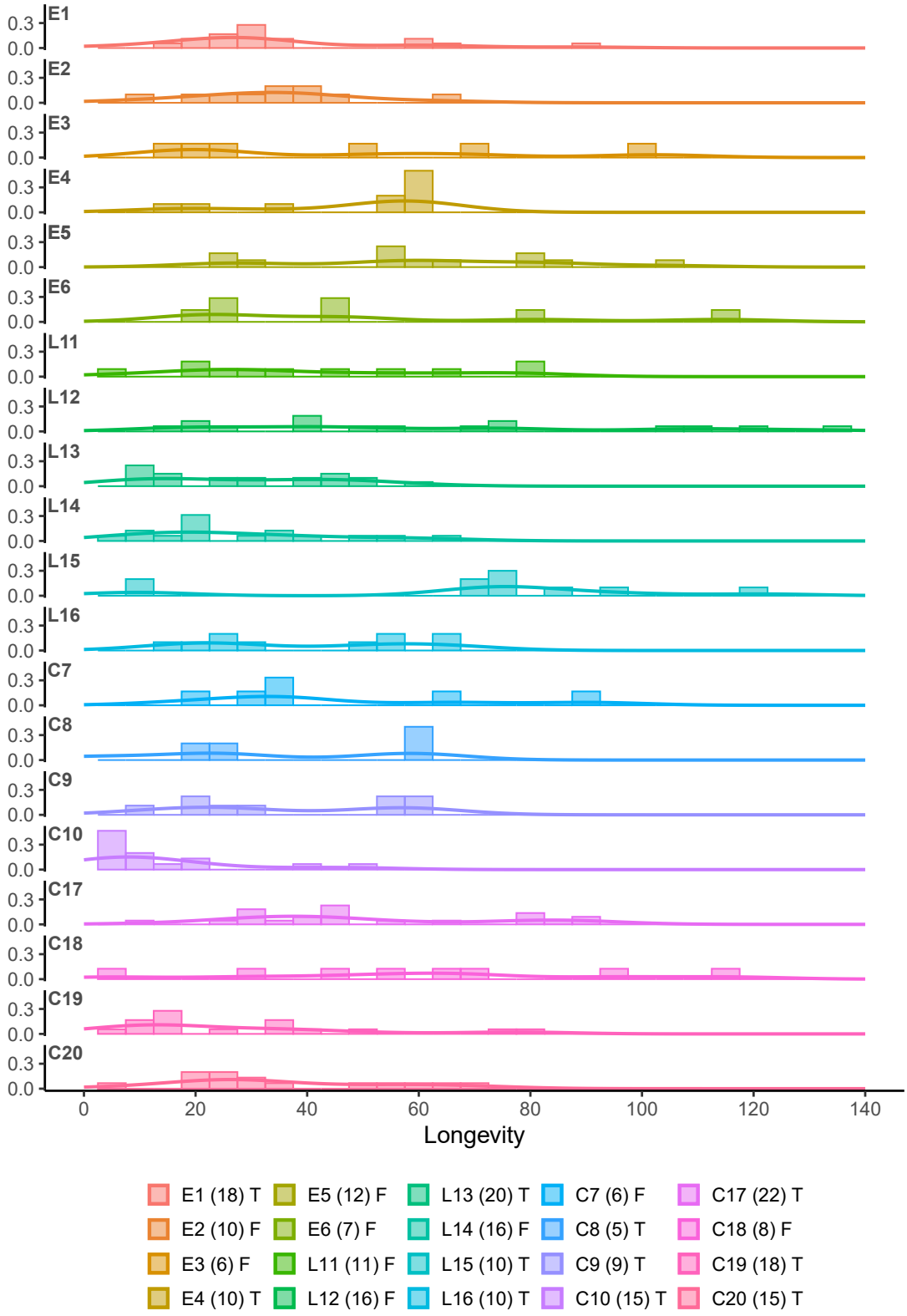

Figure S3.2. Worker longevity distributions of *Bombus terrestris* in days (all non-transfer workers, excluding censored workers), grouped by colony. Proportional histograms with a density curve of worker longevities, showing the proportion of workers that died at a certain age (longevity in days from eclosion to death). The total area of all bins and under the curve respectively represents 100% of each sample. (Number in brackets representing the sample size; bin size 5 days.) The distribution of worker longevities was bimodal (T) for 11/20 colonies and unimodal (F) for 9/20 colonies. E1 - E6: early experimental colonies; L11 - L16: late experimental colonies; C7 - C10: early control colonies; C17 - C20: late control colonies.

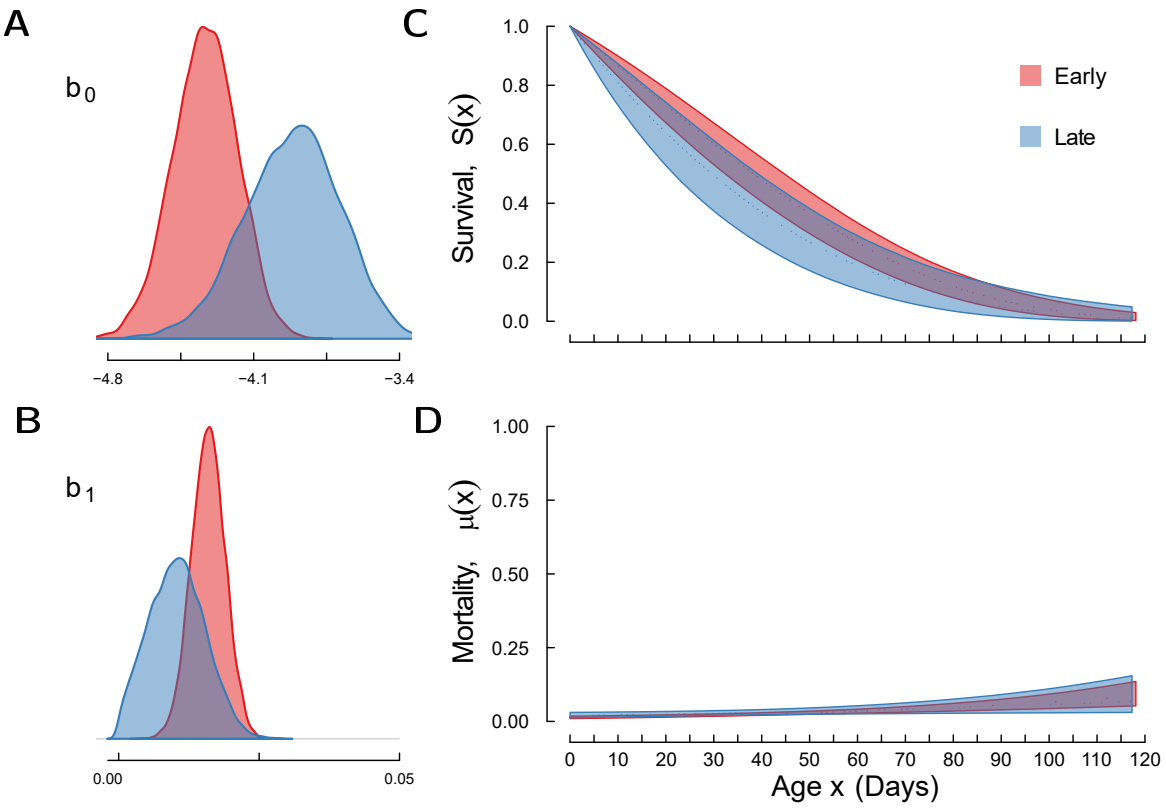

Figure S3.3. Age-specific survival and mortality of the focal *Bombus terrestris* workers produced at an early stage and at a late stage within the same colonies, fitted with a simple Gompertz model (BaSTA analysis) across the full lifespans. Early (red): N = 123 ($BTEW_E$ and $BTCW_E$ pooled); Late (blue): N = 45 ($NTEW_L$ and $NTCW_L$ pooled). Definitions for the worker categories are in Table 3.1. The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) values are listed in Table 3.5. **A**: the baseline mortality (b_0) was substantially lower in the early-produced workers than in the late-produced workers. **B**: the Gompertz rate parameter (b_1, change rate in mortality over time) was slightly higher in the earl-produced workers than in the late-produced workers. **C**: Smoothed survival curves for the two nutritional treatments with the shaded areas representing 95% confidence intervals. There was no difference between the two groups. **D**: the change in mortality over time (days) with the shaded areas representing 95% confidence intervals. This did not differ between the two groups.

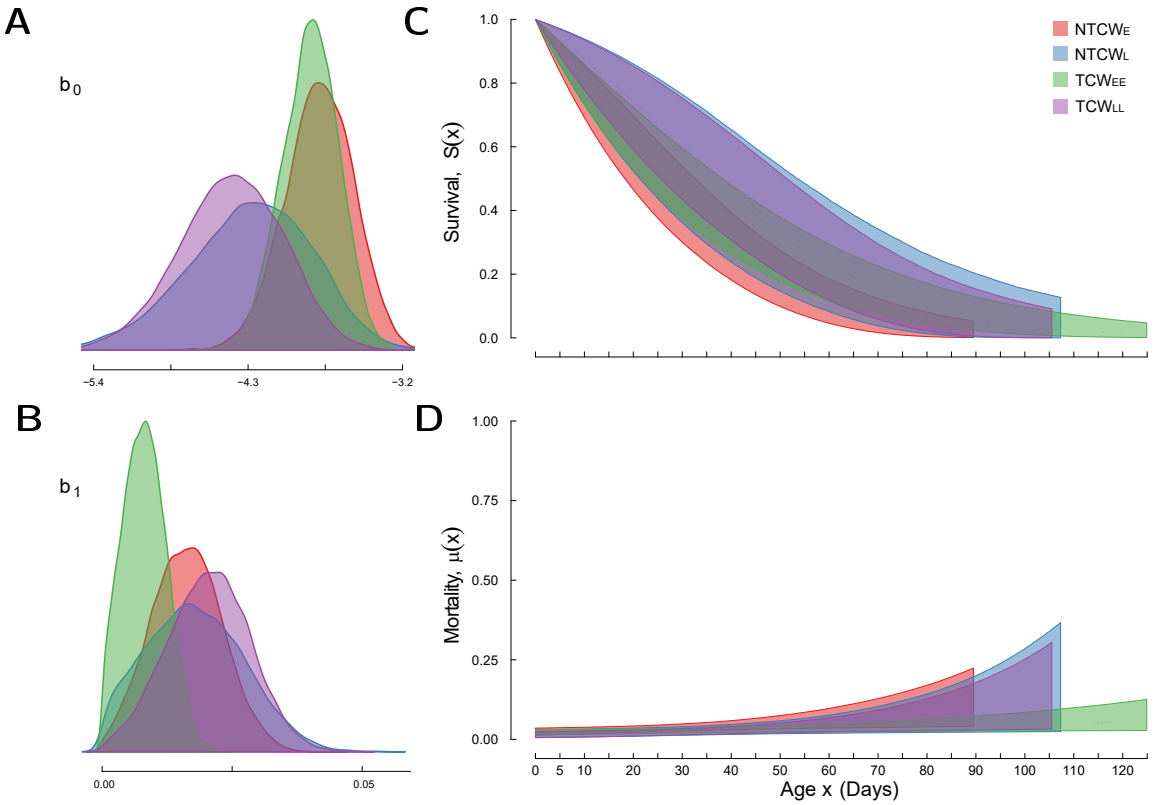

Figure S3.4. Age-specific survival and mortality of the focal *Bombus terrestris* workers of the control categories (as defined in Table 3.1), fitted with a simple Gompertz model (BaSTA analysis) across the full lifespans. $NTCW_E$ (red): N = 46; $NTCW_L$ (blue): N = 11; TCW_{EE} (green): N = 50; TCW_{LL} (purple): N = 20. Definitions of the control worker categories are in Table 3.1. The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) values are listed in Table 3.5. **A**: the baseline mortality (b_0) was substantially lower in $NTCW_L$, TCW_{LL} than in $NTCW_E$ and TCW_{EE}. It did not differ between transferred (TCW_{EE} and TCW_{LL}) and non-transferred ($NTCW_E$ and $NTCW_L$) workers. **B**: the Gompertz rate parameter (b_1, change rate in mortality over time) was substantially lower TCW_{EE} than in the other categories. **C**: Smoothed survival curves for the two nutritional treatments with the shaded areas representing 95% confidence intervals. There was no difference between the four categories. **D**: the change in mortality over time (days) with the shaded areas representing 95% confidence intervals. This did not differ between the categories.

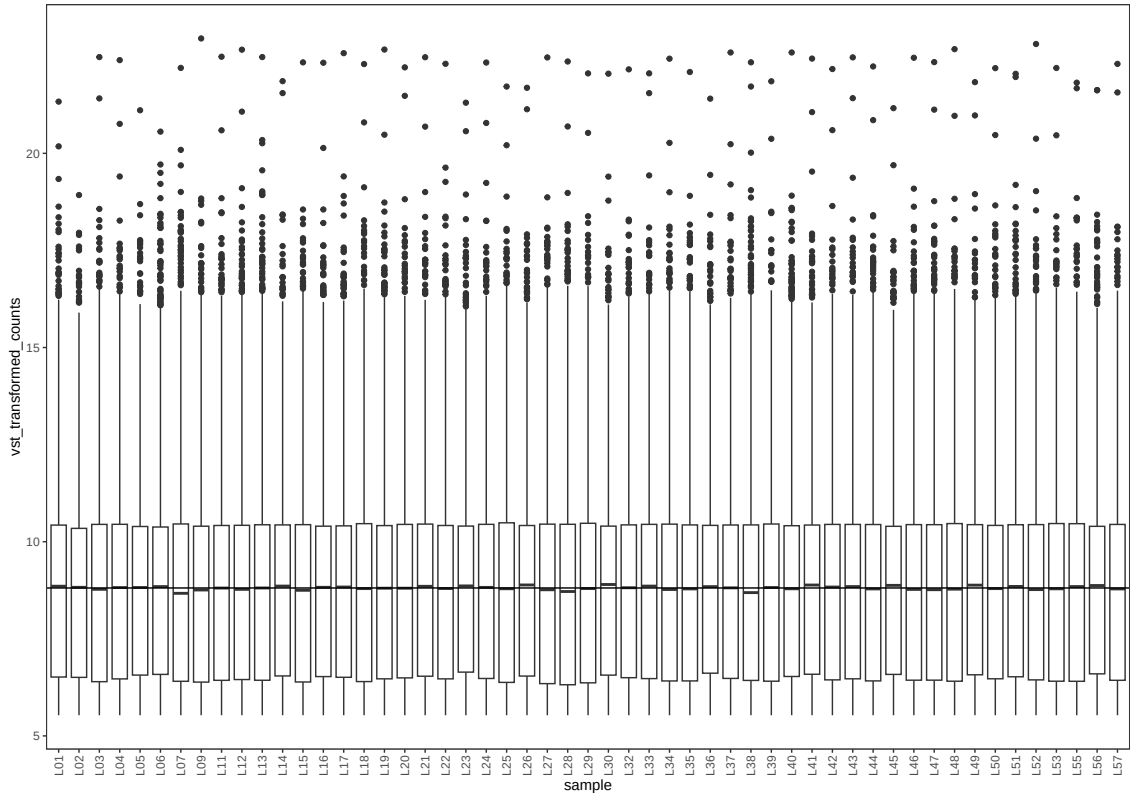

Figure S3.5. Exploratory plots from the differential gene expression analysis in fat bodies of *Bombus terrestris* workers. Normalisation boxplots of the regularised log_2-(rlog) transformed value of mRNA-seq expression for genes in all libraries. Black horizontal bars: medians; boxes: interquartile ranges; whiskers: 10th to 90th percentile ranges. Each boxplot corresponds to an individual sample (individual worker). Only samples that were over the threshold of an alignment of 20 million reads are included.

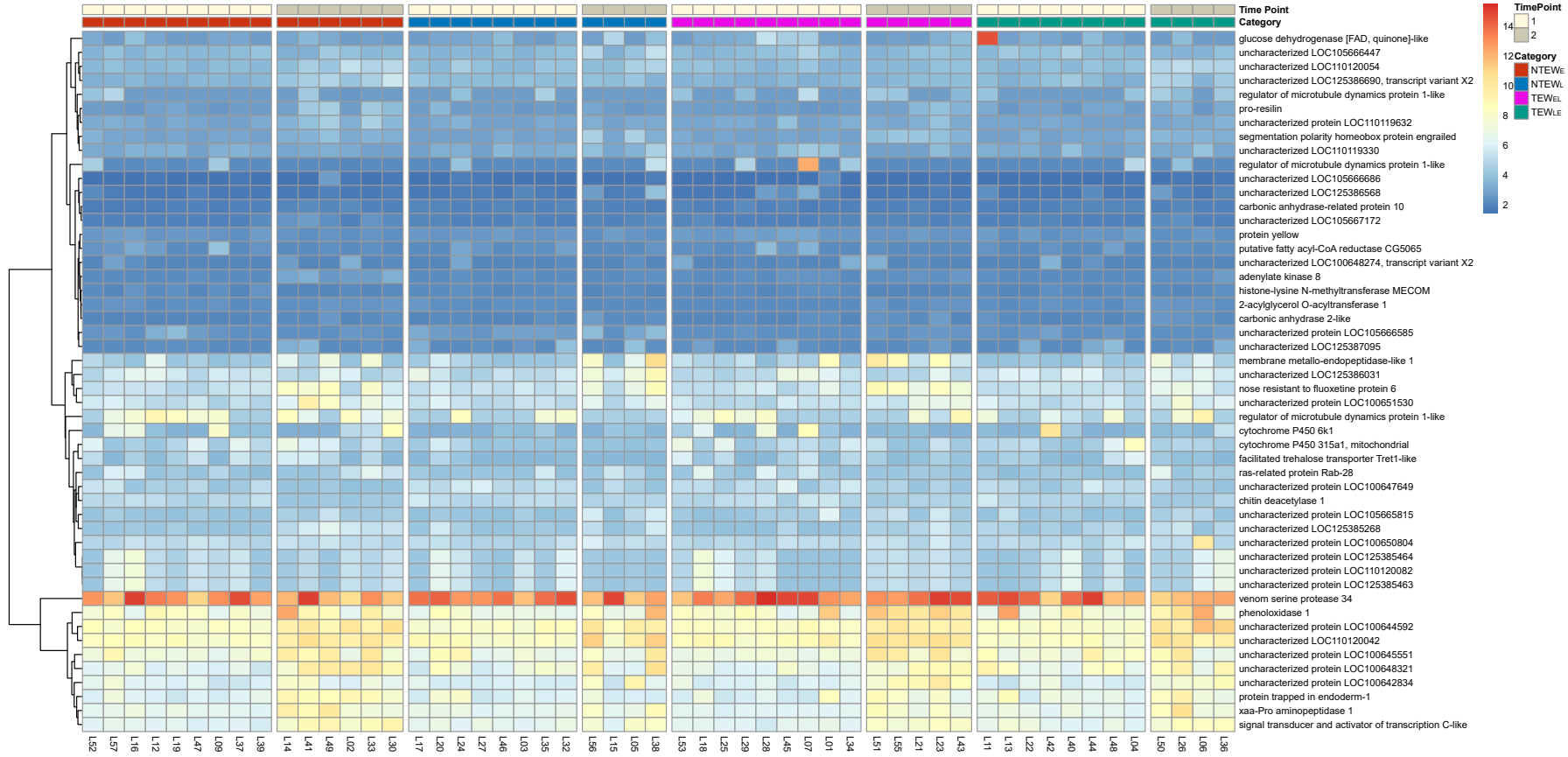

Figure S3.6. Gene expression patterns (read counts) of the top 50 DEGs between the two time points. Each row represents an individual gene from the *Bombus terrestris* genome. Each column represents a single sample (single worker). The top two rows define the phenotypes, with the colours coded according to time point (1 = 2 weeks (beige), 2 = 7 weeks (grey)) and worker category ($NTEW_E$ – red; $NTEW_L$ – blue; TEW_{EL} – pink; TEW_{LE} – green). Vertical breaks separate the eight combinations of category and time point. The dendogram at the left groups genes that cluster according to their gene expression patterns. Numbers at the bottom correspond to sample IDs. Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 5; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 4. Definitions of the experimental worker categories are in Table 3.1.

Supplementary Tables

Table S3.1. Effect of the colony stage on the longevity of focal adult *Bombus terrestris* workers within a colony, analysed with a Cox proportional hazard model with mixed effects. A comparison of workers produced while the colony was at an early colony stage ($BTEW_E$ (N = 66) and $BTCW_E$ (N = 57)) with workers produced while the colony was at a late colony stage ($NTEW_L$ (N = 34) and $NTCW_L$ (N = 11)). Model: Surv(Longevity, Colony Stage) ~ Colony Stage + (1 | Colony). Workers with an uncertain death date were censored to the date they were last recorded alive. Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error and the Z- and the p-value. The late-produced workers were compared to the early-produced workers as the baseline. There was no significant difference between the two groups. Definitions of the worker categories are in Table 3.1.

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
Late	0.081	1.084	0.229	0.35	0.724
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.671	0.450		

Table S3.2. Effect of transfer on the longevity of focal adult *Bombus terrestris* workers, analysed with Cox proportional hazard models with mixed effects. Model: Surv(Longevity, censored) ~ Transfer + (1 | Colony). Workers with an uncertain death date were censored to the date they were last recorded alive. Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error and the Z- and the p-value. **A)**: Comparison of early transferred control workers (TCW_{EE} (N = 50)) to early non-transfer control workers ($NTCW_E$ (N = 46)). **B)**: Comparison of late transferred control workers (TCW_{LL} (N = 20)) to late non-transfer control workers and with non-transfer control workers ($NTCW_L$ (N = 11)). The transferred workers were compared to the non-transfer workers as the baseline. There was no significant difference between the two groups. Definitions of the worker categories are in Table 3.1.

A) Early Control Workers

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
Transferred	-0.170	0.843	0.236	-0.72	0.471
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.078	0.006		

B) Late Control Workers

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
Transferred	0.111	1.117	0.470	0.24	0.813
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.10	0.010		

Table S3.3. Age-specific survival and mortality of the focal *Bombus terrestris* workers of the control categories. Results of two simple Gompertz models (BaSTA analysis) across the full lifespan: Mean Kullback-Leibler discrepancy calibration (KLDC) values for the analysed comparisons. b_0 represents the baseline mortality rate and b_1 the Gompertz rate parameter or the change rate in mortality. Group comparisons that result in KLDC values >0.85 are regarded as differing 'substantially' from each other. **A**: Comparisons between the control worker categories. $NTCW_E$: N = 46; $NTCW_L$: N = 11; TCW_{EE}: N = 50; TCW_{LL}: N = 20 (b_0) was substantially lower in $NTCW_L$, TCW_{LL} and $BTCW_E$ than in $NTCW_E$ and TCW_{EE}. It did not differ between transferred (TCW_{EE} and TCW_{LL}) and non-transferred ($NTCW_E$ and $NTCW_L$) workers. b_1 was substantially lower in TCW_{EE} than in the other categories. **B**: Comparison between early-produced non-transferred workers ($BTEW_E$ and $BTCW_E$) and late produced non-transferred workers of the same colonies ($NTEW_L$ and $NTCW_L$). BTW_E: N = 123; NTW_L: N = 45. b_0 was substantially lower in BTW_E than in NTW_L and b_1 was substantially higher in BTW_E than in NTW_L. Definitions of the worker categories are in Table 3.1.

Comparisons		**Mean KLDC**	
		b0	**b1**
A	$NTCW_L$ - $NTCW_E$	0.879	0.556
	TCW_{EE} - $NTCW_E$	0.533	0.876
	TCW_{EE} - $NTCW_L$	0.887	0.903
	TCW_{LL} - $NTCW_E$	0.941	0.593
	TCW_{LL} - $NTEW_L$	0.539	0.525
	TCW_{LL} - TCW_{EE}	0.937	0.945
B	BTW_E - NTW_L	0.952	0.848

Table S3.4. The overlap of differentially expressed genes with age in fat body between *Bombus terrestris* workers of the TEW_{EL} category and the $NTEW_E$ and the $NTEW_L$ category. DEGs: Count of genes differently expressed between the two time points (2 weeks and 7 weeks). The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167). Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 5. Definitions of the experimental worker categories are in Table 3.1.

	Up-regulated	Down-regulated
TEW_{EL} DEGs	304	348
$NTEW_E$ DEGs	888	819
$NTEW_L$ DEGs	411	254
TEW_{EL} - $NTEW_E$ overlapping DEGs	136	141
TEW_{EL} - $NTEW_L$ overlapping DEGs	36	7
$NTEW_E$ - $NTEW_L$ overlapping DEGs	49	16
TEW_{EL} - $NTEW_E$ - $NTEW_L$ overlapping DEGs	24	3
DEGs only in TEW_{EL}	156	203
DEGs only in $NTEW_E$	727	665
DEGs only in $NTEW_L$	350	234
DEGs in TEW_{EL} and $NTEW_E$ not in $NTEW_L$	112	138
DEGs in TEW_{EL} and $NTEW_L$ not in $NTEW_E$	12	4
DEGs in $NTEW_E$ and $NTEW_L$ not in TEW_{EL}	25	13
DEGs in neither	11246	11398
p-value TEW_{EL} - $NTEW_E$	5.93e-80	1.29e-78
p-value TEW_{EL} - $NTEW_L$	8.74e-18	0.27
p-value $NTEW_E$ - $NTEW_L$	7.28e-06	0.49
Odds-ratio TEW_{EL} - $NTEW_E$	13.48	11.90
Odds-ratio TEW_{EL} - $NTEW_L$	7.41	1.68
Odds-ratio $NTEW_E$ - $NTEW_L$	2.16	1.17
$\%TEW_{EL}$ - $NTEW_E$ DEGs overlap	44.7	40.5
$\%TEW_{EL}$ - $NTEW_L$ DEGs overlap	11.8	2.0
$\%NTEW_E$ - $NTEW_L$ DEGs overlap	5.5	2.0

Table S3.5. The overlap of differentially expressed genes with age in fat body between *Bombus terrestris* workers of the TEW_{LE} category and the $NTEW_E$ and the $NTEW_L$ category. DEGs: Count of genes differently expressed between the two time points (2 weeks and 7 weeks). The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167). Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 4. Definitions of the experimental worker categories are in Table 3.1.

	Up-regulated	Down-regulated
TEW_{LE} DEGs	434	511
$NTEW_E$ DEGs	888	819
$NTEW_L$ DEGs	411	254
TEW_{LE} - $NTEW_E$ overlapping DEGs	145	229
TEW_{LE} - $NTEW_L$ overlapping DEGs	44	32
$NTEW_E$ - $NTEW_L$ overlapping DEGs	49	16
TEW_{LE} - $NTEW_E$ - $NTEW_L$ overlapping DEGs	23	11
DEGs only in TEW_{LE}	268	261
DEGs only in $NTEW_E$	717	585
DEGs only in $NTEW_L$	341	217
DEGs in TEW_{LE} and $NTEW_E$ not in $NTEW_L$	122	218
DEGs in TEW_{LE} and $NTEW_L$ not in $NTEW_E$	21	21
DEGs in $NTEW_E$ and $NTEW_L$ not in TEW_{LE}	26	5
DEGs in neither	11154	11429
p-value TEW_{LE} - $NTEW_E$	1.71e-65	6.41e-147
p-value TEW_{LE} - $NTEW_L$	1.17e-16	1.12e-14
p-value $NTEW_E$ - $NTEW_L$	3.13e-06	0.17
Odds-ratio TEW_{LE} - $NTEW_E$	8.41	17.12
Odds-ratio TEW_{LE} - $NTEW_L$	5.37	6.45
Odds-ratio $NTEW_E$ - $NTEW_L$	2.23	1.44
$\%TEW_{LE}$ - $NTEW_E$ DEGs overlap	33.4	44.8
$\%TEW_{LE}$ - $NTEW_L$ DEGs overlap	10.1	6.3
$\%NTEW_E$ - $NTEW_L$ DEGs overlap	5.5	2.0

Table S3.6. Results of a gene ontology (GO) enrichment analysis, listing gene orthologues between *B. terrestris* and *D. melanogaster* present in the mRNA-seq libraries of B. terrestris fat body and which experimental worker categories they were found in. The expression with age corresponds to a comparison in expression between camples from time point 1 (2 weeks) to samples from time point 2 (7 weeks). Up-regulated: more highly expressed in time point 2; Down-regulated: more highly expressed in time point 1. Definitions of the experimental worker categories are in Table 3.1.

GO term ID	GO term description	Expression with age	Categories
GO:0006120	mitochondrial electron transport NADH to ubiquinone	down-regulated	NTEWE TEWEL
GO:0010257	NADH dehydrogenase complex assembly	down-regulated	NTEWE TEWEL
GO:0015980	energy derivation by oxidation of organic compounds	down-regulated	NTEWE TEWEL
GO:0022900	electron transport chain	down-regulated	NTEWE TEWEL
GO:0032981	mitochondrial respiratory chain complex I assembly	down-regulated	NTEWE TEWEL
GO:0033108	mitochondrial respiratory chain complex assembly	down-regulated	NTEWE TEWEL
GO:0051205	protein insertion into membrane	down-regulated	NTEWE TEWEL
GO:0006091	generation of precursor metabolites and energy	down-regulated	NTEWE TEWEL TEWLE
GO:0006119	oxidative phosphorylation	down-regulated	NTEWE TEWEL TEWLE
GO:0009060	aerobic respiration	down-regulated	NTEWE TEWEL TEWLE
GO:0019646	aerobic electron transport chain	down-regulated	NTEWE TEWEL TEWLE
GO:0022904	respiratory electron transport chain	down-regulated	NTEWE TEWEL TEWLE
GO:0042773	ATP synthesis coupled electron transport	down-regulated	NTEWE TEWEL TEWLE
GO:0042775	mitochondrial ATP synthesis coupled electron transport	down-regulated	NTEWE TEWEL TEWLE
GO:0045048	protein insertion into ER membrane	down-regulated	NTEWE TEWEL TEWLE
GO:0045333	cellular respiration	down-regulated	NTEWE TEWEL TEWLE
GO:0071816	tail-anchored membrane protein insertion into ER membrane	down-regulated	NTEWE TEWEL TEWLE
GO:0006163	purine nucleotide metabolic process	down-regulated	NTEWE TEWLE
GO:0006508	proteolysis	down-regulated	NTEWE TEWLE
GO:0006511	ubiquitin-dependent protein catabolic process	down-regulated	NTEWE TEWLE
GO:0006888	endoplasmic reticulum to Golgi vesicle-mediated transport	down-regulated	NTEWE TEWLE
GO:0006900	vesicle budding from membrane	down-regulated	NTEWE TEWLE
GO:0007029	endoplasmic reticulum organization	down-regulated	NTEWE TEWLE
GO:0009057	macromolecule catabolic process	down-regulated	NTEWE TEWLE
GO:0010498	proteasomal protein catabolic process	down-regulated	NTEWE TEWLE
GO:0016032	viral process	down-regulated	NTEWE TEWLE
GO:0016050	vesicle organization	down-regulated	NTEWE TEWLE
GO:0016192	vesicle-mediated transport	down-regulated	NTEWE TEWLE
GO:0019058	viral life cycle	down-regulated	NTEWE TEWLE
GO:0019362	pyridine nucleotide metabolic process	down-regulated	NTEWE TEWLE
GO:0019941	modification-dependent protein catabolic process	down-regulated	NTEWE TEWLE
GO:0030163	protein catabolic process	down-regulated	NTEWE TEWLE
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	down-regulated	NTEWE TEWLE
GO:0043632	modification-dependent macromolecule catabolic process	down-regulated	NTEWE TEWLE
GO:0044403	biological process involved in symbiotic interaction	down-regulated	NTEWE TEWLE
GO:0044409	symbiont entry into host	down-regulated	NTEWE TEWLE
GO:0045184	establishment of protein localization	down-regulated	NTEWE TEWLE
GO:0046496	nicotinamide nucleotide metabolic process	down-regulated	NTEWE TEWLE
GO:0046718	symbiont entry into host cell	down-regulated	NTEWE TEWLE
GO:0046907	intracellular transport	down-regulated	NTEWE TEWLE
GO:0048193	Golgi vesicle transport	down-regulated	NTEWE TEWLE
GO:0051603	proteolysis involved in protein catabolic process	down-regulated	NTEWE TEWLE
GO:0051701	biological process involved in interaction with host	down-regulated	NTEWE TEWLE
GO:0061024	membrane organization	down-regulated	NTEWE TEWLE
GO:0072524	pyridine-containing compound metabolic process	down-regulated	NTEWE TEWLE
GO:1901565	organonitrogen compound catabolic process	down-regulated	NTEWE TEWLE
GO:0006412	translation	up-regulated	NTEWL
GO:0006518	peptide metabolic process	up-regulated	NTEWL
GO:0043043	peptide biosynthetic process	up-regulated	NTEWL
GO:0006412	translation	down-regulated	TEWEL
GO:0006518	peptide metabolic process	down-regulated	TEWEL
GO:0043043	peptide biosynthetic process	down-regulated	TEWEL

GO:0030239	myofibril assembly	up-regulated	TEWEL TEWLE
GO:0031032	actomyosin structure organization	up-regulated	TEWEL TEWLE
GO:0032989	cellular anatomical entity morphogenesis	up-regulated	TEWEL TEWLE
GO:0035107	appendage morphogenesis	up-regulated	TEWEL TEWLE
GO:0035114	imaginal disc-derived appendage morphogenesis	up-regulated	TEWEL TEWLE
GO:0035120	post-embryonic appendage morphogenesis	up-regulated	TEWEL TEWLE
GO:0035220	wing disc development	up-regulated	TEWEL TEWLE
GO:0042692	muscle cell differentiation	up-regulated	TEWEL TEWLE
GO:0045214	sarcomere organization	up-regulated	TEWEL TEWLE
GO:0048168	regulation of neuronal synaptic plasticity	up-regulated	TEWEL TEWLE
GO:0048736	appendage development	up-regulated	TEWEL TEWLE
GO:0048737	imaginal disc-derived appendage development	up-regulated	TEWEL TEWLE
GO:0051146	striated muscle cell differentiation	up-regulated	TEWEL TEWLE
GO:0055001	muscle cell development	up-regulated	TEWEL TEWLE
GO:0055002	striated muscle cell development	up-regulated	TEWEL TEWLE

Table S3.7. A comparison of changes in fat body mRNA-seq gene expression profiles with age in *Bombus terrestris* workers to two sets of genes hypothesised to be ageing-related in eusocial insects. DEGs: Count of genes differently expressed between the two time points (2 weeks and 7 weeks). The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167) **A**: Comparison with TI-J-LiFe network; each row represents a gene from *D. melanogaster* found in the TI-J-LiFe network of Korb et al. (2021) and having a single-copy orthologue in *B. terrestris*. **B**: Comparison with enzymatic antioxidant gene set; each row represents a gene from *D. melanogaster* found in the enzymatic antioxidant gene set of Kramer et al. (2021) and having a single-copy orthologue in *B. terrestris*. The dendogram at the left groups genes that cluster according to their gene expression patterns. Sample sizes: $NTEW_E$:TP1 = 9; $NTEW_E$:TP2 = 6; $NTEW_L$:TP1 = 8; $NTEW_L$:TP2 = 4; TEW_{EL}:TP1 = 9; TEW_{EL}:TP2 = 5; TEW_{LE}:TP1 = 8; TEW_{LE}:TP2 = 4. Definitions of the experimental worker categories are in Table 3.1.

	$NTEW_E$	$NTEW_L$	TEW_{EL}	TEW_{LE}
A) TI-J-LiFe Network				
B. terrestris DEGs	977	355	373	568
D. melanogaster genes	81	81	81	81
Overlapping genes	14	2	5	8
Genes only in *B. terrestris*	963	353	368	560
Genes only in *D. melanogaster*	67	79	76	73
Genes in neither	4850	5460	5445	5253
p-value	0.88	0.24	1.00	0.85
Odds-ratio	1.05	0.39	0.97	1.03
Percentage of gene-set present	17.3	2.5	6.2	9.9
B) Enzymatic Antioxidant Gene-set				
B. terrestris DEGs	977	355	373	568
D. melanogaster genes	15	15	15	15
Overlapping genes	5	0	6	3
Genes only in *B. terrestris*	972	355	367	565
Genes only in *D. melanogaster*	10	15	9	12
Genes in neither	4907	5524	5512	5314
p-value	0.09	1.00	<0.001	0.17
Odds-ratio	2.52	0.00	10.00	2.35
Percentage of gene-set present	33.3	0.0	40.0	20.0

Chapter 4 | An Experimental Test of the Effect of social Isolation and Reproductivity on Longevity in *Bombus terrestris* Workers

Abstract

Studies of longevity in individual *Bombus terrestris* workers in the current thesis have shown the frequency distribution of within-cohort worker longevity within colonies to be multimodal. This appears to be a novel pattern, but it is unclear what causes it and whether it stems from individual (intrinsic) or social factors affecting workers. This study therefore firstly aimed to test the hypothesis (H1) that multimodality in worker longevity distributions is a common and replicable phenomenon in *B. terrestris* colonies. It then sought to discriminate between individual and social factors as drivers of multimodality, by testing a second hypothesis that either the pattern stems from individual properties of workers (H2a), so predicting it would therefore be exhibited by workers kept in isolation, or, alternatively, that it stems from social factors (H2b), predicting it would therefore be exhibited only by workers within colonies. Longevities were recorded of newly-eclosed workers from the same set of colonies that were either kept singly in isolation boxes, which were provided with *ad libitum* access to food, or remained in their natal colonies. Longevities were also recorded of newly-eclosed workers in additional, unmanipulated control colonies. Worker longevity distributions in both social groups were significantly bimodal, whereas the longevity distribution of the isolated workers was unimodal. These findings therefore supported H1 and H2b, showing that the multimodality of worker longevity is a robust phenomenon and stems from social factors. An additional finding was that, surprisingly, isolated workers showed significantly increased longevities, compared to the social workers. Moreover, among egg-laying isolated workers, there was a significant positive relationship between reproductive output (number of adult males produced) and longevity. These results therefore both elucidate the causes of multimodality in worker longevity distributions and provide further evidence for varying levels of worker quality within colonies.

4.1 Introduction

Sociality and longevity are known to be strongly positively connected, and both may affect one another's evolution (Lucas and Keller, 2020). In extreme cases, the evolution of social life is associated with longevity increases of a hundred-fold in reproductive phenotypes (queens of some ants), compared to their solitary relatives (Keller and Genoud, 1997). Several other examples can be found in which reproductive individuals of social species, in either cooperatively breeding groups or eusocial societies, show significantly higher longevities than individuals of closely related solitary species (Korb and Heinze, 2021), with cases including the naked mole rat *Heterocephalus glaber* (Kim et al., 2011) and the Seychelles warbler *Acrocephalus sechellensis* (Hammers et al., 2019).

Different factors play a role in the increased longevities associated with sociality. The reduction in extrinsic mortality risk due to selfish herd effects is an obvious benefit from sociality that can arise even if group members are not cooperating in any way (Hamilton, 1971). The benefit arises from individuals reducing their own chances of being attacked and predated simply by clustering together (Hamilton, 1971; Kokko et al., 2001). There can also be factors in social living that have adverse effects on longevity, such as costs arising from increased levels of competition and increased risk of disease transmission (Alexander, 1974). If there is cooperation among the members of a group, these costs can be offset and benefits of sociality on longevity can be enhanced due to sharing of tasks like resource acquisition and defence (Alexander, 1974; **?**; Kokko et al., 2001). Lastly, if the cooperation within the group extends to cooperative breeding, dominant breeders can benefit from sociality, often to the greatest extent, as they can share the costs of reproduction with non-reproducing helpers (Crick, 1992).

Many social structures involve kin groups living together (Ruxton, 2002; Lukas and Clutton-Brock, 2018; Pereira et al., 2023). In kin groups, all members may gain inclusive fitness benefits, on top of the individual benefits of group living (Hamilton, 1964, 1971; Bourke, 2007). The inclusive fitness gain leads to interdependence among the members of the group, meaning that the fitness of one individual is also dependent on the fitness (reproductive success and survival) of another. This can lead to the evolution of extreme longevities of some group members, if the inclusive fitness of all kin depends on their survival (Keller and Genoud, 1997; Bourke, 2007). Examples for this effect can be found in eusocial insect queens, in which the workers gain inclusive fitness benefits by the queen's survival and reproduction (Keller and Genoud, 1997; Bourke, 2007). Kin selection can therefore lead to the evolution of extreme longevities in the reproductive group members. These indi-

viduals (e.g. eusocial insect queens and kings) receive most of the social benefits, such as reduced extrinsic mortality and shared costs of reproduction. At the same time, the non-reproducing helpers (e.g. eusocial insect workers) might even experience shortened longevities as a result of sociality (Toth et al., 2016; Lucas and Keller, 2020). For all group members, the process of ageing is proposed to start with the onset of reproduction, hence with the accrual of fitness (Hamilton, 1966). Helpers in cooperatively breeding groups or workers in eusocial insect societies, by helping to raise brood of kin, may gain fitness via inclusive fitness before they have sexually matured themselves. This would lead to a premature onset of ageing that results in a decrease of longevity in those non-reproductive group members (Alexander et al., 1991; Bourke, 2007; Lucas and Keller, 2020). Such a disruptive selection on longevity in highly social species can lead to different phenotypes within a social group. Eusocial insect species show this strongly, often having an extremely long-lived reproductive phenotype (queens and kings) and a short-lived less reproductive or fully sterile helper-phenotype (workers).

In less complex social structures, such as those of cooperatively breeding species, it is, as mentioned, also the dominant breeders that show increased longevities in the presence of helpers in the nest (Berg et al., 2018; Crick, 1992; Paquet et al., 2015). In such cases it seems that the positive effect of sociality on the breeder's longevity is largely attributable to the position at the top of the social hierarchy rather than to sociality itself (Lucas and Keller, 2020).

In bumblebees such as *Bombus terrestris*, there are, as in other eusocial Hymenoptera, two distinct phenotypes among the females. The queen is the longest-lived member of the colony, while also being the most reproductive (van Doorn and Heringa, 1986). The other phenotype consists of the shorter-lived workers. These are less reproductive or non-reproductive yet have inclusive fitness gain by helping to rear the queen-laid eggs and maintaining colony functions (Heinrich, 1979). Among the workers, there are also strong hierarchical structures headed by a few dominant and often aggressive workers, which are the ones most likely to become reproductively active (laying haploid eggs) (laying haploid eggs) (Bourke, 1988; Bloch, 1999; Zanette et al., 2012; Almond et al., 2019). For such reproductive bumblebee workers there is a significantly positive association between the level of ovarian activation and longevity (Blacher et al., 2017). They are seemingly able to overcome the costs of reproduction, similar to queens (Blacher et al., 2017). Such a connection between social dominance, reproduction and longevity, might suggest that these workers are intrinsically higher quality individuals, relative to the subordinate workers that do not reproduce and experience lower longevities (Blacher et al., 2017).

Variation in worker quality within a bumblebee colony is likely to be high, as reflected by the large variation in adult body size among workers within colonies (Goulson, 2010), adult body size being positively associated with longevity and reproductivity (Ayasse et al., 1995; Blacher et al., 2017). Findings reported elsewhere in this thesis provide support for this theory as well. Specifically, there is a small but significant influence of larval nutrition on worker body size, and worker body size was found to be positively associated with reproductivity and longevity (**Chapter 2**). Further, such quality differences were found to be partly due to intrinsic differences (caused by factors acting before the eclosion of the adult worker) and partly due to changes in the social environment of a colony, with workers of lower intrinsic quality being more susceptible to the influence of the social environment (**Chapter 3**).

The datasets analysed in those two chapters both showed further large variation in worker longevities, a potential indication of different levels of worker quality. In an unanticipated finding, the distributions of worker longevities (within treatment groups and within cohorts within colonies) also tested positively for multimodality (in most cases bimodality) rather than showing a unimodal (including normal) distribution. These longevity distributions were found in workers within cohorts eclosing in a relatively narrow eclosion window, and so their form of bi- or multimodality is distinct from that shown by worker longevities pooled across the entire colony cycle, by which workers from early-stage colonies are longer-lived than those from late-stage colonies (Holland and Bourke 2015; **Chapter 3**). In the new form of multimodality, worker longevities showed two or more distinct peaks around modes, splitting the workers in shorter-lived and longer-lived workers. A similar pattern was found in a reanalysis of the dataset of Blacher et al. (2017) (**Figure S4.1A**). Here such bimodality in longevity was found in both workers with activated ovaries and workers with inactive ovaries (**Figure S4.1B**), and within most of the colonies (**Figure S4.1C**). It therefore seems that this pattern occurs irrespective of larval nutrition levels of those workers (**Chapter 2**), the point in the colony cycle when workers are produced (**Chapter 3**) and the workers' ovary activation status (Blacher et al., 2017). Moreover, as the relevant datasets all stemmed from entirely captive colonies, differential survival of workers arising from external foraging cannot have been a contributing factor.

Multimodality of longevities of workers within eusocial insect colonies seems to be a novel or little-reported phenomenon and, as such, merits further investigation. There have been relatively few previous studies measuring individual longevities of eusocial insect workers (**Chapter 1**, but see Hartmann and Heinze (2003); Tsuji et al. (2012); Dixon et al. (2014)), and they do not usually record whether their

longevity data showed multimodal distributions or not. One of the few other previous instances of this phenomenon (in addition to the one provided by the current thesis's reanalysis of Blacher et al. (2017)) was found in *Apis mellifera* workers (Dixon et al., 2014), in which there seem to have been two distinct peaks in worker mortality rates.

The question therefore arises as to what might cause this striking pattern. In principle, the observed multimodality could be linked to different levels of worker quality, with quality, as outlined above, reflecting differences in reproductivity and longevity. Alternatively, but not mutually exclusively, the phenomenon could be linked to processes connected to the social nature of a colony. In either case, the occurrence of such multimodality allows a further test of the relative roles of individual versus social influences on worker longevity, as in **Chapter 3**.

The objective of the current study was therefore to test a set of hypotheses arising from the occurrence of multimodality in the distribution of worker longevities within cohorts. The first hypothesis postulated that the frequency distribution of longevity of workers eclosing within the same cohort within *B. terrestris* colonies is multimodal, the aim being to test this in a sample of colonies independent from those previously analysed (**Chapter 3**) and so establish whether or not multimodality represents a robust pattern (Hypothesis 1, H1). Hence H1 predicted that cohorts of workers within intact colonies should exhibit multimodality. The study also sought to discriminate between individual and social factors as drivers of multimodality. Therefore the second hypothesis was that multimodality is either due to individual (intrinsic) differences between workers, so predicting that it would be exhibited by workers that are kept in isolation (i.e. singly), without any social interaction (Hypothesis 2a, H2a), or due to social factors, so predicting that, while present in intact colonies, it would not be exhibited by workers kept in isolation (Hypothesis 2b, H2b).

4.2 Methods

4.2.1 Colony Rearing

Nine queenright (including a queen), pre-competition point *B. t. audax* colonies were received on 17 April 2024, having been supplied by Biobest (Westerlo, Belgium). For the duration of the experiment, all colonies were kept in a controlled climate room (temperature: 28°C; humidity: 60%) in constant darkness. Each colony had a queen, brood and a mean of 38.3 ± 10.4 (SD) workers upon arrival. The two colonies with the highest number of workers were chosen to be 'spare' colonies, because larger worker numbers indicate that a colony is further advanced in the colony cycle and will therefore soon stop producing more workers. The other seven colonies were ranked by worker numbers and assigned a number between 1 and 7. Colonies 1-4 were then allocated as experimental colonies and colonies 5-7 were allocated as control colonies (**Table 4.1**). The colonies were ranked and assigned in this way to ensure that enough new workers would eclose in the experimental colonies to reach the intended sample sizes (larger colonies are further along in the colony cycle and more likely to start producing males and gynes (newly-produced queens) soon). In each experimental colony, half of the newly-eclosed workers were put into isolation boxes (see below) and the other half were retained in their colony. These workers that were retained in their colony served as the social control to the isolated workers of the same colony. In each control colony, all newly-eclosed workers were retained in their colony. These control colonies served therefore to measure worker longevity in an additional set of unmanipulated colonies from which isolation workers were not drawn.

A day after arrival, all colonies were moved into wooden colony nest-boxes (internal dimensions, 17 cm x 27.5 cm x 16 cm high) with clear Perspex lids. The colonies were kept in those boxes for the duration of the experiment (except for the workers that were put into isolation). All nest-boxes had circular ventilation holes (ø 3.5 cm) on either side, covered with a metal mesh. Underneath each nest-box there was a container filled with BIOGLUC®('Biobest Group NV, Belgium), a ready-to use sugar solution (artificial nectar), that was connected to the inside of the nest-box via a filter-tip wick ensuring *ad libitum* access for the colony. Additionally, the bees were fed with fresh honeybee-collected pollen on an *ad libitum* basis. To prevent the bees from crawling out of the nest-box, a rectangular frame of acetate was glued onto the top rim of the box. The floor in the corners of the boxes was covered with unscented non-clumping cat litter (Felight, Non-clumping cat litter, Bob Martin Petcare) to absorb faeces and moisture.

To transfer a colony into the nest-box, all workers were carefully removed from the supplier's box using forceps and temporarily placed in a 1 l conical flask. The brood was then also carefully removed from the supplier's box and placed into the nest-box towards the back wall using two table spoons. Last, the queen was carefully transferred into the new nest-box and placed on the brood, following which the workers were added.

On a daily basis (except once per weekend), dead workers were removed from the colonies and the number of deaths was recorded. For focal workers (see below), the death date was recorded on the corresponding data sheet. Dead workers were kept in a labelled (experiment number; date; worker ID) bag and frozen at −20°C. Males and gynes that eventually eclosed in the colonies were recorded and removed. Each colony was terminated (with remaining individuals being frozen, which was only the case in one control colony) once its last focal worker had died or, in cases in which this had not yet occurred, on 25 July 2024. The end date of the experiment as a whole was 21 October 2024, when the last isolated focal worker died.

4.2.2 Experimental Design

All newly-eclosing workers in each colony were individually marked with a unique numbered disk glued to the thorax, within 48 hours after eclosion. For this, a focal callow worker was removed from the colony and placed in a marking cage, which was a cylindrical transparent tube open at one end and covered with a plastic mesh at the other end. Using a foam plunger, the worker was then positioned so that the area of the thorax between the wings was accessible through the mesh. With a toothpick, a circular paper label bearing a number on a coloured background (Queen Marking Kit, Thorne Ltd., UK) was stuck to the worker's thorax by means of a small drop of shellac-based glue (Queen Marking Glue, Thorne Ltd., UK). Within each colony, all marked workers had different numbers on the same colour of background, allowing each worker to be identified both individually and by colony. The marked workers were defined as the "focal workers" of this experiment.

Marked workers from the control colonies (C5-C7) were placed back into their colonies. They were categorised as Social Focal Control Workers (SFCWs). Marked workers from the experimental colonies (E1-E4) were either placed back into their colony and categorised as Social Focal Experimental Workers (SFEWs) or placed singly into isolation boxes and categorised as Isolated Focal Workers (IFWs). The assignment to either category was done at by alternation as they were marked. Final sample sizes were as listed in **Table 4.1**. The isolation boxes were plastic boxes (13.0 cm x 7.0 cm x 4.5 cm) with ventilation from the bottom and a removable lid

(**Figure 4.1A**). A cylindrical, up-right syrup feeder was attached from the outside to ensure that each IFW received *ad libitum* access to artificial nectar. Pollen was provided *ad libitum* in a small Petri dish placed on the floor of the box. IFWs were also provided with a ball of 'pollen bread' (made from ground pollen mixed with artificial nectar) to serve as a substrate on which each IFW was potentially able to construct brood cells (**Figure 4.1B**). Such brood cells are the wax cells that eggs are laid into in intact, unmanipulated colonies. In such colonies, brood cells are constantly being built by the workers, mainly for the queen to lay eggs into. Brood cells are usually constructed on existing structures such as pupal cocoons. Such a structure was therefore provided so that the isolated worker would have opportunities for egg-laying as they would have in a colony.

Table 4.1. Properties of the four experimental colonies and the three control *Bombus terrestris* colonies. Listed are the colony name, the category, the number of alive adult workers that were present in the colony upon arrival, the number of isolated focal workers (IFWs) that originated in the colony, the number of social focal experimental workers (SFEWs) that originated in the colony and the number of social focal control workers (SFCWs) that originated in the colony. *N/A*: this category did not originate in the colony.

Colony	Category	n workers at start	n IFWs	n SFEWs	n SFCWs
E1	Experimental	26	15	15	*N/A*
E2	Experimental	29	17	17	*N/A*
E3	Experimental	31	15	15	*N/A*
E4	Experimental	35	21	21	*N/A*
C5	Control	36	*N/A*	*N/A*	30
C6	Control	36	*N/A*	*N/A*	50
C7	Control	46	*N/A*	*N/A*	32

The duration of marking new focal workers was kept to a maximum 12 consecutive days (8-12 days, depending on how productive a colony was), simultaneously for all colonies. This length of time was selected to ensure that any within-colony variation in worker longevity did not stem from eclosion dates varying widely within a given colony's colony cycle, while still permitting sufficient numbers of focal workers to be obtained. (Target sample sizes for focal workers were selected on the basis of a power analysis informed by the data on multimodality in the previous analyses (**Chapters 2**, **3**), i.e. such that multimodality would be detectable at alpha = 0.05 with power = 0.8.) The longevity of all focal workers was measured by recording

their death dates. For each IFW, in cases in which the isolated worker laid eggs developing to adulthood, the date of the first appearance of male pupae (i.e. developing from haploid eggs laid by the IFW) and the first eclosion of adult males was also recorded. All adult males eclosing in the isolation boxes were then censused and removed.

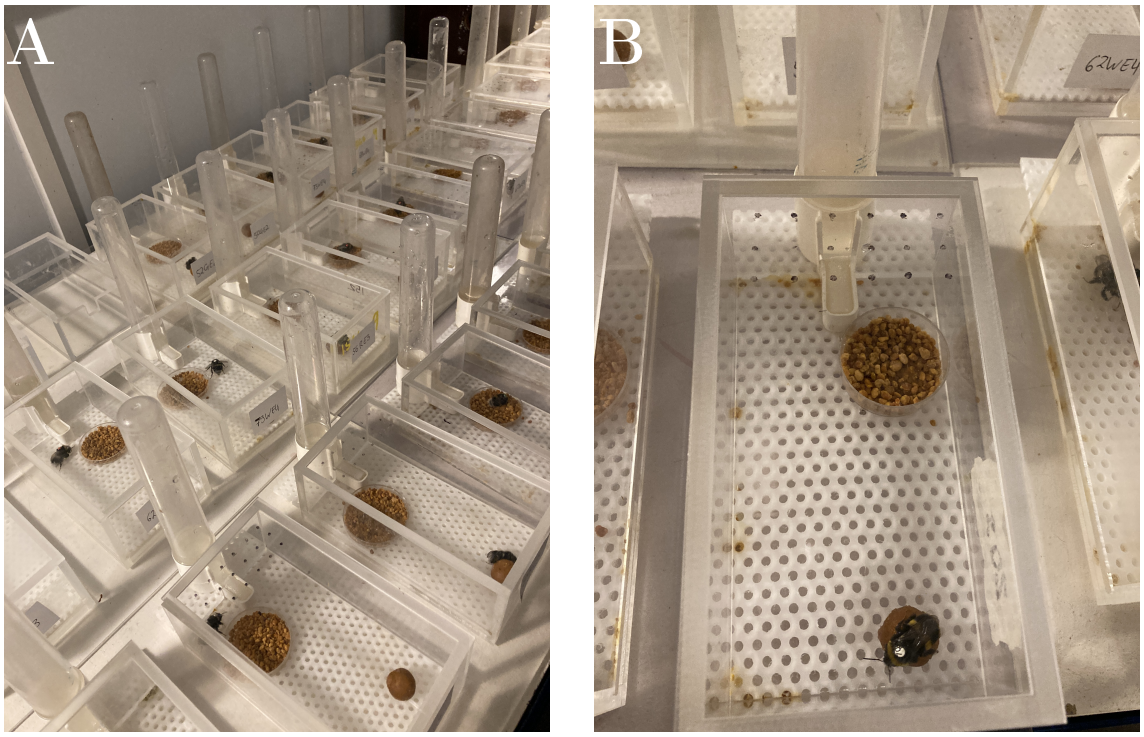

Figure 4.1. Isolation boxes used in the experiment. Focal *Bombus terrestris* workers from colonies E1–4 selected for isolation (IFWs) were placed in plastic isolation boxes after marking. **A**: Set of boxes. Each box had inner dimensions of 13.0 cm × 7.0 cm × 4.5 cm, and ventilation was provided through the perforated floor, which was inserted 0.7 cm above the table surface. **B**: Close-up of a single isolation box. Each box was fitted with an upright feeder (upper centre of image) providing *ad libitum* access to artificial nectar. A filter tip in the opening of the feeder prevented smaller workers from drowning in the artificial nectar. Pollen was provided *ad libitum* in a small Petri dish (centre right). A ball of 'pollen bread' (lower right) made from a mixture of ground pollen and artificial nectar was provided to serve as a substrate on which each IFW was potentially able to construct brood cells.

4.2.3 Behaviour Observations

Because one potential measure of worker quality is reproductive output or reproductive activity in general, individual worker behaviours associated with reproductivity were recorded in the both the experimental and control colonies. The behaviour of SFCW and SFEW workers was observed using direct (in-person) observations. Observations were conducted on all seven colonies (containing SFCWs and SFEWs)

in parallel for one hour daily for five days per week. Behaviour observations were conducted from 07 May 2024 to 14 July 2024.

The acting individual was recorded as either a focal worker (with its identity then being noted), a non-focal worker ("NF") or the queen. Reproductive activity was recorded as "egg-laying", in which the individual (queen or worker) places its abdominal tip in an open egg-cell followed by visible tapping of the hind legs on the egg-cell wall (Bloch, 1999). Based on these observation data, a focal worker was classified as either an "egg-layer" if it had been observed egg-laying at least once, or a "non-egg-layer", if it had never been observed egg-laying. This meant that there was the potential for some workers classified as "non-egg-layers" to have been misclassified, i.e. if they laid eggs outside of observation bouts.

To control for effects of activity on longevity, the activity of the IFWs was recorded twice daily (before the colony observations and afterwards). For this the IFW was either recorded as active, if it was seen in scan-sampling to be moving around the isolation box or the brood, or as inactive if it was seen resting motionless. These observations were conducted for 35 days until 14 July 2024.

4.2.4 Wing Measurements

In *B. terrestris*, the length of the marginal wing cell can be used as a proxy for body size, because the two measures are highly correlated (Duchateau and Velthuis, 1989; Owen, 1988; Goulson et al., 2002). Using a *Zeiss SteREO Discovery.V12* dissection microscope, the length of the marginal cell of the left forewing of every focal worker (in all three categories) was measured (the right forewing being measured if the left one was missing or deformed). To do this, a digital photograph of the forewing was taken with an AxioVision camera under a 15 x magnification and next to a 1 mm graticule. The length of the marginal cell was measured using the AxioVision software, with all such measurements being conducted by investigators blind to the longevity and worker category of the relevant focal worker.

4.2.5 Statistical Analyses

All statistical analyses were performed in R-Studio (R Core Team, 2020; Posit team, 2024) version 4.4.1 (2024-06-14 ucrt). Worker longevity ("Longevity") was calculated as the time difference in days between eclosion date and death date. The frequency distributions of the longevity data (excluding the censored workers) for all three categories (IFW, SEFW, SCFW) was then investigated by testing for multimodality using the 'is.multimodal()'-function of the package 'LaplacesDemon' (Sta-

tisticat and LLC., 2021). This function uses a kernel density estimation and detects whether there are more than one 'true' peak (area under the curve > 10% of total density). If that is the case the distribution tests positive for multimodality, if not the distribution is considered unimodal. Afterwards, using the 'Modes()'-function of the same package, the number of distinct peaks and therefore the form of multimodality (bimodal, trimodal, etc.) was determined. The distribution of IFW longevities was further tested for normality using the Shapiro-Wilk test. For longevity and survival analyses, only data from focal workers with a known death date could be included. Focal workers that were found dead under the comb material without a known date of death had to be excluded (N = 48). Those workers that were still alive when the colonies were terminated were censored to that date (N = 20). A survival analysis was conducted on the two experimental categories of focal workers (IFWs and SFEWs) using a Cox proportional hazard regression model using functions from the "survival"-package (Therneau, 2024). The model included a survival-object based on the longevity data as the response variable and Category and Marginal Wing Cell Length as fixed effects. To account for possible differences between colonies, Colony was included as a random effect. Kaplan-Meier survival curves were created using the "survminer"-package (Kassambara et al., 2020), including the results of a log-rank test.

Workers' survival was further analysed using the Bayesian survival trajectory analysis of the BaSTA-package (Colchero et al., 2012). In this method, age-related mortality distributions are estimated using the Markov chain Monte Carlo approach. Using the categories as the grouping factor, four simulations were run in parallel using a "Gompertz" distribution with a "simple" shape, 150,000 iterations, a burn-in of 15,001 chains and a thinning of 150. The distribution was chosen after running the "multibasta"-function to determine which distribution best fitted the data. To analyse differences between the categories, the Kullback Leibler discrepancy calibrations (KLDC) were compared, following the standard threshold by which a value higher than 0.85 suggests that the posterior distributions of the two categories are substantially different (Sultanova et al., 2021).

The distributions of social focal worker body sizes (measured as marginal wing cell length), within each of the colonies were tested for multimodality, using the methodology described above. Where multimodality was detected, the dataset was grouped by the modes of the body size distribution. A mode was defined around the peak marginal wing cell length detected by the test and expanded 0.11 mm to either side. This value corresponded to 0.5 x SD of all focal worker marginal wing cell lengths and was picked to ensure no overlap between the modes. The median

longevity for workers in each marginal wing cell length mode was calculated and longevities between workers of each mode compared using a Mann-Whitney U test.

Reproductivity was analysed only among the IFWs, as there were only 7 events of egg-laying recorded by focal workers in the social colonies (i.e. colonies E1-4 following removal of workers for isolation and colonies C5-7). Activity levels of IFWs were included in analyses as, for a given worker, the proportion of recordings in which the worker was scored as active. In addition, to test whether body size, activity or longevity predicted the likelihood of whether a IFW was an egg-layer or not, a binomial generalized linear mixed model was implemented, with the binomial variable of being an egg-layer (yes/no) as the response variable, Marginal Wing Cell Length, Activity and Longevity as fixed effects and Colony as a random effect (Bates et al., 2015). Due to large differences in the magnitudes of the fixed effects (large eigenvalue ratio), all fixed effects were scaled before including them in the final model. Model assumptions were tested by plotting binned deviance residuals against the fitted values. Assumptions were met if the majority of the fitted values fell within the binned deviance residuals. Reproductive output of the IFWs was analysed as number of adult males produced per egg-laying IFW (non-egg-laying IFWs excluded) with a mixed linear model, including the Number of Adult Males Produced as the response variable, Longevity and Male Eclosion Time (the time between marking of the IFW and the eclosion of the first male in the IFW's isolation box) as fixed effects and Colony as a random effect (Bates et al., 2015).

A further Cox proportional hazard analysis was conducted on the longevities of the IFWs as described above. The fixed effects in the model were the binomial Egg-Layer (yes/no), Marginal Wing Cell Length, Number of Adult Males Produced by the IFW and Activity. Additionally, the same Cox proportional hazard model was run on only the egg-laying IFWs, including Male Eclosion Time as a further fixed effect. Lastly, the Bayesian survival trajectory analysis described above (Colchero et al., 2012) was conducted for egg-laying IFWs versus non-egg-laying IFWs to investigate the effect of egg-laying on survival.

4.3 Results

The total number of marked focal *B. terrestris* workers was 248. Of those, 68 were Isolated Focal Workers (IFWs), i.e. transferred after eclosion and marking into isolation boxes. The remaining workers (180) were, after eclosion and marking, retained in the social environment of their natal colonies, either as Social Focal Experimental Workers (SFEWs, N = 71) if they originated from the same colonies as the IFWs, or as Social Focal Control Workers (SFCWs, N = 109) if they originated in the control colonies.

4.3.1 Worker Longevity Distributions

The numbers of focal workers with a known death date were 42, 70 and 68 for SFEWs, SFCWs and IFWs , respectively (**Figure 4.2A**). In each of the SFEW and SFCW category of workers, the longevity distributions were significantly bimodal (**Figure 4.2A**). The longevity distribution of SFEWs showed modes at 6.6 days and 38.8 days, and the longevity distribution of SFCWs showed modes at 45.2 days and 86.1 days. When the longevity data of all focal workers in social colonies were split by colony, all 7 colonies, except for one (E1), displayed significantly bimodal distributions (**Figure 4.2B**). (It needs to be mentioned that, although the longevity distribution of colony E1 seemed to have two peaks, the later peak consisted of only one data point, explaining why the distribution was not bimodal.) The presence of multimodality in the worker longevity distribution in SFEW workers as a whole, the SFCW workers as a whole and within 6/7 individual social colonies supported H1 , i.e. that multimodality in worker longevity distributions within cohorts is a common and replicable phenomenon in *B. terrestris* colonies.

Within the IFW workers, the longevity distribution was unimodal, with a single mode at 132 days (**Figure 4.2A**). When the two left-most outliers (two workers that lived substantially lower than the rest, 5 days and 28 days) were removed, the longevity distribution of the IFW workers followed a normal distribution (Shapiro-Wilk test: p = 0.11). The fact that multimodality of the worker longevity distributions was not detected in the IFWs, i.e. workers kept in isolation, whereas it was detected in the SFEW workers in the social colonies from which the IFWs were drawn , failed to support H2a and instead supported H2b, i.e. that multimodality in worker longevity distributions stems from social factors present within colonies.

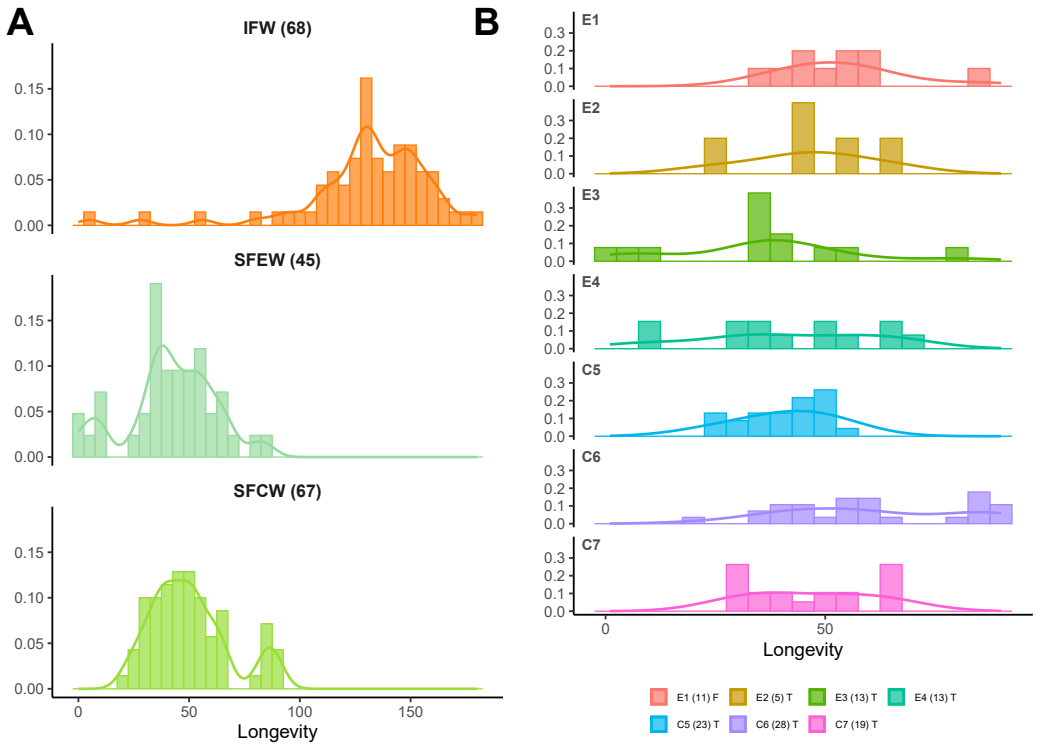

Figure 4.2. Distributions of focal *Bombus terrestris* worker longevity in days. Proportional histograms with a density curve of worker longevities, showing the proportion of workers that died at a certain age (longevity in days from eclosion to death). These data sets exclude censored workers. The total area of all bins and under the curve respectively represents 100% of each sample. (Number in brackets representing the sample size; bin size 5 days.) These data sets exclude censored workers. **A**: Worker longevities grouped by focal worker categories: IFW (orange) = Isolated Focal Worker; SFEW (dark green) = Social Focal Experimental Worker; SFCW (light green) = Social Focal Control Worker. The curves for SFEW, and SFCW showed significant multi-modality (T=true), the curve for IFW did not (F=false). **B**: Worker longevities of social focal workers (SFEW and SFCW)grouped by colony. The legend indicates which curves were truly multimodal (T=true, in all cases bimodal) and which did not (F=false). E1-E4, experimental colonies 1-4; C5-C7, control colonies 5-7.

Adult worker body sizes (measured as marginal wing cell length) of social focal workers (SFEWs and SFCWs) were significantly bimodally distributed for workers originating from colony E1 (modes at 2.70 mm and 3.06 mm), from colony E2 (modes at 2.25 mm and 3.05 mm), from colony C6 (modes at 2.52 mm and 2.91 mm) and from colony C7 (modes at 2.73 mm and 3.07 mm). In all cases, the median longevity of workers belonging to the first size mode was lower than the median longevity of workers belonging to the second size mode (E1: 42 days vs. 57.5 days; E2: 44 days vs. 65 days; C6: 55 days and 66 days; C7: 44.5 days vs. 47 days), but in none of the cases was this difference significant (Mann-Whitney U tests: $p > 0.05$).

4.3.2 Effect of Isolation on Longevity

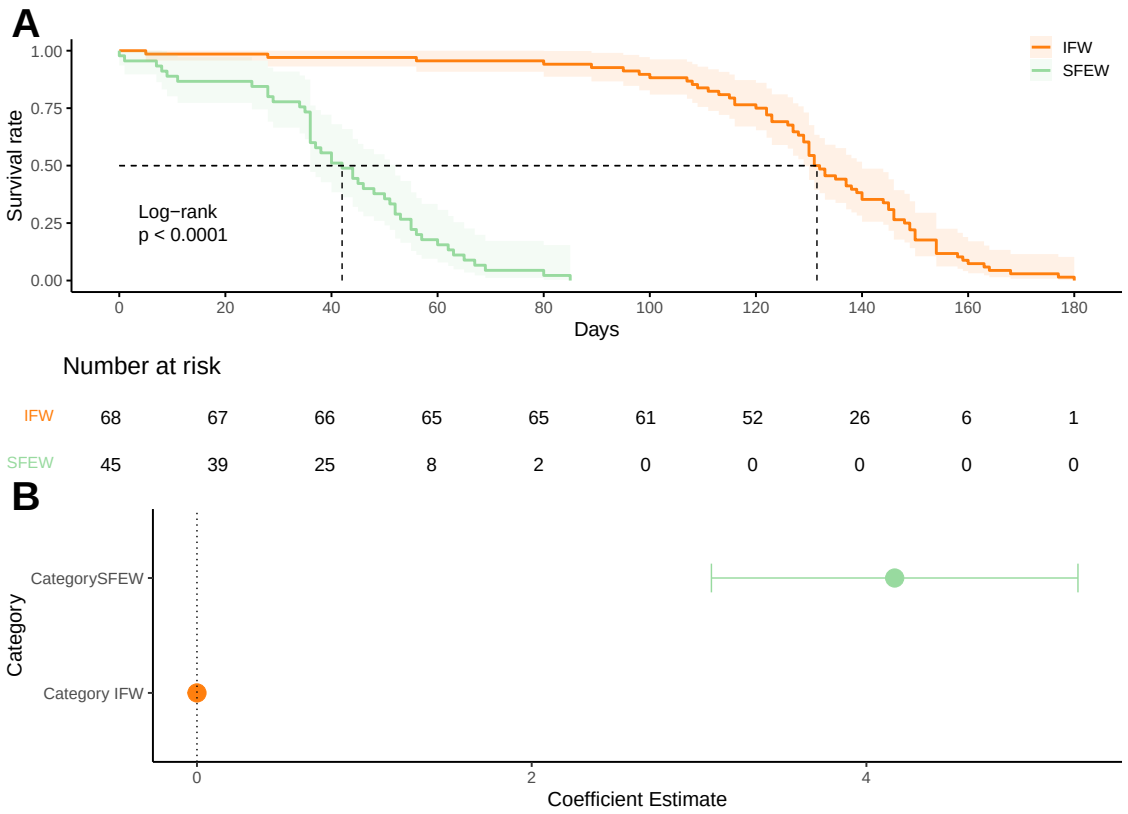

Figure 4.3. Effect of social isolation on the survival of the focal *Bombus terrestris* workers. **A**: Kaplan-Meier survival curves including 95% confidence intervals (shading), and log-rank test results comparing mean survival rates and (below) risk tables showing how many workers of the given category remained alive at 20-day intervals throughout the experiment. Dotted lines indicate median longevity for each worker category. IFW (orange): Isolated Focal Workers, N = 68; SFEW (green): Social Focal Experimental Workers, N = 45. Median longevity was significantly higher for the IFW category (log-rank test: $p < 0.001$). Median longevities of IFWs and SFEWs were 131.5 days and 42.0 days, respectively. **B**: Forest plot for mixed Cox proportional hazard model (Table 4.2). IFW workers serve as the reference. To the left of the dotted line: lower hazard (risk of mortality); to the right of the dotted line: higher hazard (risk of mortality) (i.e. coefficient estimate > 0). The confidence intervals stretch from -1.96 x the standard error to +1.96 x the standard error. SFEWs showed a significantly increased hazard (chance of mortality) compared to IFWs.

The median longevity (range) of all focal workers with a known death date was 60.5 (1-180) days (N = 180 workers). The median (range) longevities of IFWs, SFEWs and SFCWs were 131.5 (5-180) days (N = 68), 42.0 (1-85) days (N = 42) and 49.0 (21-90) days (N = 70) respectively.

Isolation had a large and significant positive effect on worker survival (**Figure 4.3**). Median longevity of IFWs (131.5 days) was significantly greater than that of

SFEWs (42.0 days), i.e. workers in the social colonies from which IFWs originated (**Figure 4.3**). Correspondingly, SFEWs had a 57.2 times higher risk of mortality (hazard ratio) than the IFWs (Cox proportional hazard model: 95% CI - upper: 172.26, lower: 19.02, $p < 0.001$, **Table 4.2, Figure 4.3**). Worker longevity was not affected by adult body size (Marginal Wing Cell Length, Cox proportional hazard model: 95% CI - upper: 1.52, lower: 0.26, $p = 0.303$, **Table 4.2**).

Table 4.2. Effect of worker isolation and marginal wing cell length (a proxy for body size) on the longevity of the focal *Bombus terrestris* workers, analysed with a Cox proportional hazard model with mixed effects. Colony is included as a random effect. Model: Surv(Longevity, censored) ~ Category + Marginal Wing Cell Length + (1|Colony). Workers with an uncertain death date were censored to the date they were last recorded alive. Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error and the Z- and p-value. Data from the social workers (SFEW: Social Focal Experimental Worker, N = 45) are compared to those from isolated workers originating from the same colonies (IFW: Isolated Focal Workers, N = 68). IFWs had significantly higher survival than SFEWs ($p < 0.001$). Marginal wing cell length did not affect survival ($p = 0.303$).

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
SFEW	4.047	57.242	0.562	7.20	< 0.001
Marginal Wing Cell Length	-0.463	0.629	0.450	-1.03	0.303
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.0090	8.14×10^{-05}		

The Bayesian Survival Trajectory Analysis also showed that there were significant differences in survival between IFWs and SFEWs (**Figure 4.4**). The baseline mortality (b_0) was remarkably lower for IFWs (mean KDLC = 1.0, **Table 4.3, Figure 4.4A**) and so was the Gompertz rate parameter (mortality change over time, b_1) (mean KDLC = 0.92, **Table 4.3, Figure 4.4B**). This difference was also shown by the differences in the survival trajectories, with IFWs showing higher survival (**Figure 4.4C**), and in the mortality trajectories, with IFWs showing a later increase in mortalities (**Figure 4.4D**). Overall, therefore, isolation had a strongly positive effect on worker longevity.

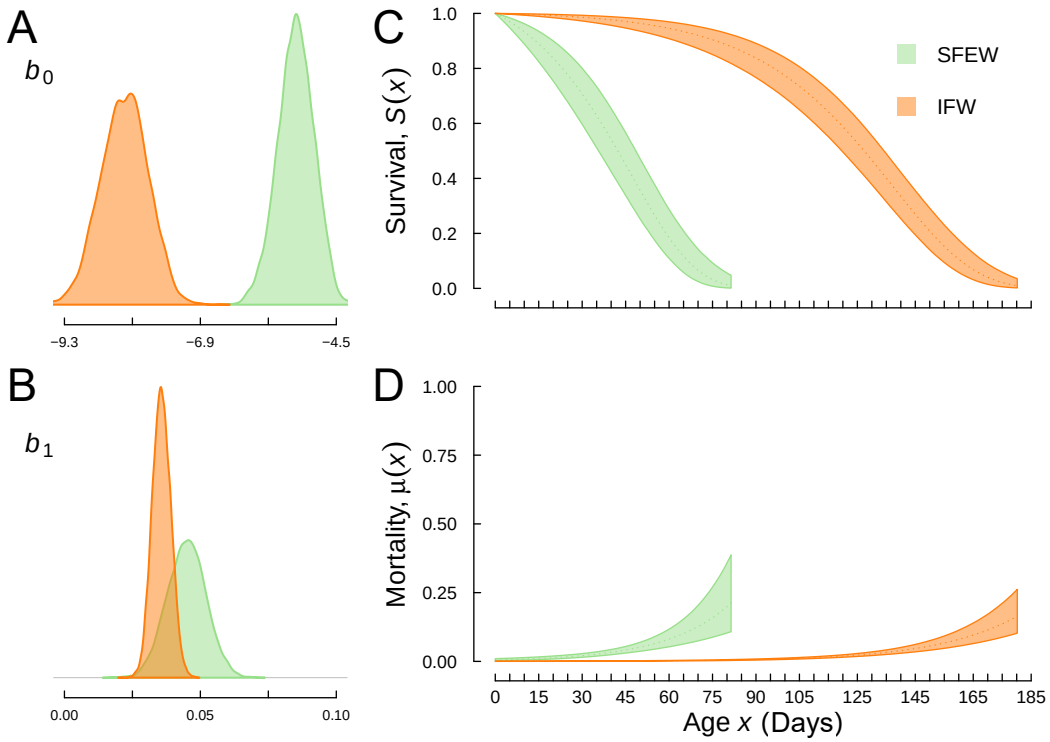

Figure 4.4. Effects of isolation on age-specific survival and mortality on experimental focal *Bombus terrestris* workers, fitted with a simple Gompertz model (BaSTA-analysis) across the full lifespan. IFW (orange): Isolated Focal Workers (N = 68); SFEW (green): Social Focal Experimental Workers (N = 45). The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) values are listed in Table 4.3. **A**: Baseline mortality rate (b_0) for IFWs and SFEWs; this rate was substantially lower for IFWs, which means they had higher chances of survival early in life. **B**: Gompertz rate parameter (mortality change over time, b_1) for IFWs and SFEWs; this parameter was substantially lower for IFWs, which means they had a slower increase in mortality rate with time. **C**: Smoothed survival curves for IFWs and SFEWs (with shaded areas representing 95% confidence intervals); survival chances were higher for IFWs. **D**: Change in mortality over time (days) for IFWs and SFEWs (with shaded areas representing 95% confidence intervals); SFEWs had a steeper increase in mortality than IFWs, resulting in earlier death of all SFEWs.

Table 4.3. Effects of worker category (isolated vs. social) and of egg-laying status on age-specific survival and mortality in the focal *Bombus terrestris* workers. Results of a Gompertz model across the full lifespan: mean Kullback-Leibler discrepancy calibration (KLDC) values for the analysed comparisons. b_0 represents the baseline mortality rate and b_1 the Gompertz rate parameter or the mortality change over time. Group comparisons that result in KLDC values >0.85 are regarded as differing substantially from each other. **A**: Comparison between the isolation category and the social category (IFW: Isolated Focal Workers (N = 68); SFEW: Social Focal Experimental Worker (N = 45)). b_0 was considerably lower for IFWs, i.e. they had higher chances of survival early in life. b_1 was considerably lower for IFWs, i.e. they had a slower increase in mortality rate with time. **B**: Within IFWs, comparison between egg-layers and non-egg-layers: *yes*: egg-laying IFWs (N = 48); *no*: non-egg-laying IFWs (N = 20). b_0 was considerably lower for egg-layers, i.e. they had higher chances of survival early in life. b_1 was considerably higher for egg-layers, i.e., over time, egg-laying affected survival more negatively.

Mean KLDC	**b0**	**b1**
A Survival by Category		
SFEW - IFW	1.0	0.92
B Survival by Reproductivity		
no - yes	0.99	0.99

4.3.3 Effect of Reproductivity on Longevity

Among the 28 recorded egg-laying events in the social colonies performed by workers, only 9 were performed by marked focal workers (SFEW and SFCW combined, by 7 different focal workers). Because, therefore, data on reproductivity were available for only 7 of 180 social focal workers, reproductivity data from the social colonies were not included in further analyses.

Of the 68 IFWs, 48 workers laid at least one egg (and were therefore classified as 'egg-layers'). Of these, 45 workers successfully produced adult males (mean 3.0 ± 3.2 (SD), range 1-12, adult males per worker). More active IFWs were significantly less likely to become egg-layers ($p = 0.005$, **Table 4.4**). Worker longevity and worker body size (measured as marginal wing cell length) did not affect whether an IFW was an egg-layer or not. The number of adult males produced by individual egg-laying IFWs was significantly associated with longevity ($p = 0.002$), with longer-lived workers producing more adult males (**Table S4.1**) and significantly negatively associated with first male eclosion times ($p < 0.001$) (**Table S4.1**), so that longer time spans from marking of the IFW to the eclosion of the first male produced by that IFW, resulted in lower numbers of males produced by that IFW. These results

imply that longer-lived workers were quicker to produce their first adult male.

Within the IFWs as a whole, marginal wing cell length (body size) had a significantly positive effect on survival , meaning that larger workers had higher longevities (Cox proportional hazard model: 95% CI - upper: 0.639, lower: 0.029, p = 0.011, **Table 4.5**). The probability of being an egg-layer, number of adult males produced and worker activity did not affect survival (**Table 4.5**). When parallel analyses were conducted for egg-laying IFW alone, it was found that marginal wing cell length (body size) had a positive effect of survival (non-significant, p = 0.066) (**Table 4.6**). In addition, worker activity, male eclosion time and number of adult males produced each had a significantly positive effect on survival (**Table 4.6**).

Table 4.4. Effects of marginal wing cell length (a proxy for body size), longevity (days between eclosion and death) and worker activity on the probability of isolated *Bombus terrestris* workers (IFW, N = 68) being egg-layers or not. Generalized linear mixed model (binomial): Egg-layer ~ Marginal Wing Cell Length + Longevity + Activity + (1 | Colony). All fixed effects were scaled. Marginal wing cell length and longevity had no significant effect on the probability of an IFW being an egg-layer, whereas worker activity was associated with a significantly lower probability of being an egg-layer (p = 0.006). Shown are the Estimate, the Standard Error, the z-value and the p-value, and, for the random effect of Colony, the Standard Deviation and the Variance.

Fixed Effect	**Estimate**	**Standard Error**	**Z**	**p**
Marginal Wing Cell Length	0.539	0.364	1.481	0.139
Longevity	0.429	0.474	1.481	0.366
Activity	-1.145	0.411	-2.789	0.005
Random effects	**Variable**	**Std Dev**	**Variance**	
Colony	Intercept	0.440	0.663	

The Bayesian survival trajectory analysis (BaSTA) showed that egg-laying IFWs had a substantially lower base line mortality (b_0,) than non-egg-laying IFWs (Mean KLDC = 0.99, **Table 4.3**, **Figure 4.5A**), meaning egg-layers initially had better chances of survival in early life. By contrast, egg-laying IFWs had a substantially higher change rate of mortality (b_1) than non-egg-laying IFWs (Mean KLDC = 0.99, **Table 4.3**, **Figure 4.5B,D**), meaning that egg-laying had an increasingly negative effect on survival with age. This could help explain why there was overall no effect of egg-laying, in IFWs, on worker longevity in the Cox proportional hazard analysis (**Table 4.5**). The survival curves though, suggest a general trend of egg-layers living longer than non-egg-layers (**Figure 4.5C**). with relatively broad confidence intervals suggesting high variation in survival of the non-egg-layers, potentially leading to reduced statistical power.

Table 4.5. Effect of egg-laying, marginal wing cell length (a proxy for body size), the number of adult males produced and worker activity on the longevity of the isolated *Bombus terrestris* workers (IFW, N = 68), analysed with a Cox proportional hazard model with mixed effects. Colony is included as a random effect. Model: Surv(Longevity, censored) ~ Egg-layer + Marginal Wing Cell Length + Number of Adult Males + Activity + (1|Colony). Workers with an uncertain death date were censored to the date they were last recorded alive. Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error, the Z-value and the p-value. Larger IFWs had significantly higher survival (p = 0.011).

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
Egg-layer	-0.193	0.824	0.388	-0.50	0.618
Marginal Wing Cell Length	-2.010	0.134	0.790	-2.54	0.011
Number of Adult Males	0.026	1.026	0.049	0.54	0.592
Activity	-3.510	0.705	1.209	-0.29	0.773
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.430	0.185		

Table 4.6. Effect of marginal wing cell length (a proxy for body size), worker activity, the number of adult males produced and the time until the first adult male eclosed on the longevity of the egg-laying isolated *Bombus terrestris* workers (egg-laying IFW, N = 48), analysed with a Cox proportional hazard model with mixed effects. Colony is included as a random effect to account for the grouping. Model: Surv(Longevity, censored) ~ Marginal Wing Cell Length + Activity + Number of Adult Males + Male Eclosion Time + (1|Colony). Shown are the Coefficient, the Hazard Ratio (exp(coef)), the Standard Error and the Z- and the p-value.

Fixed Effect	**Coefficient**	**Hazard Ratio**	**Standard Error**	**Z**	**p**
Marginal Wing Cell Length	-2.103	0.122	1.146	-1.83	0.066
Activity	-3.930	0.020	1.845	-2.13	0.033
Number of Adult Males	-0.182	0.833	0.068	-2.96	0.007
Male Eclosion Time	-0.042	0.959	0.013	-3.15	0.002
Random effects	**Variable**	**Std Dev**	**Variance**		
Colony	Intercept	0.020	0.0003		

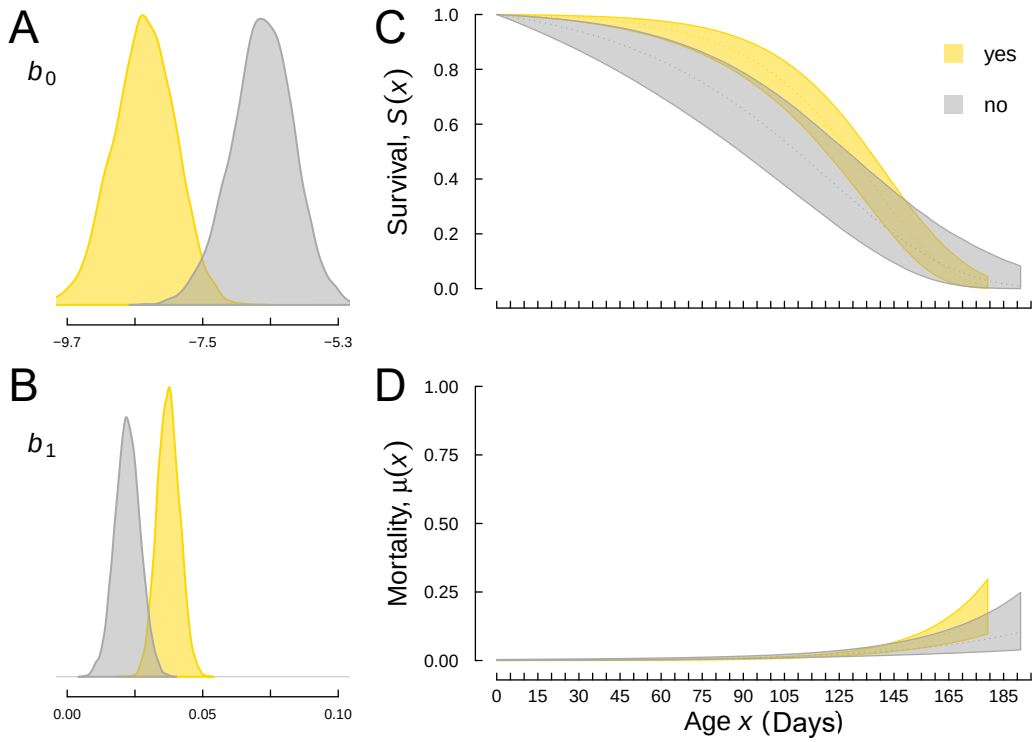

Figure 4.5. Effects egg-laying activity on age-specific survival and mortality on isolated focal *Bombus terrestris* workers (IFWs), fitted with a simple Gompertz model across the full lifespan. *yes* (yellow): egg-laying IFWs (N = 48); *no* (grey): non-egg-laying IFWs (N = 20). The corresponding mean Kullback-Leibler discrepancy calibration (KLDC) are listed in Table 4.3. **A**: Baseline mortality rate (b_0) for egg-laying IFWs and non-egg-laying IFWs; this rate was substantially lower for egg-layers. **B**: Gompertz rate parameter (mortality change over time, b_1) egg-laying IFWs and non-egg-laying IFWs; this parameter was substantially higher for egg-layers. **C**: Smoothed survival curves for egg-laying IFWs and non-egg-laying IFWs (with shaded areas representing 95% confidence intervals); survival chances did not differ between egg-layers and non-egg-layers. **D**: Change in mortality over time (days) for egg-laying IFWs and non-egg-laying IFWs (with shaded areas representing 95% confidence intervals); egg-laying IFWs had a steeper increase in mortality than non-egg-laying IFWs.

Overall, larger IFWs had significantly higher longevities. While body size had no direct effect on the probability of being an egg-layer and the number of males produced by egg-layers, longer-lived egg-layers produced significantly more males. Additionally, egg-layers had higher chances of survival early in life, yet that effect faded over time, resulting in no overall effect of egg-laying on the survival of IFWs. Furthermore, the number of males produced by an egg-laying IFW (reproductive output) had a significant positive effect on survival of the egg-layers. This means there was a positive relationship between body size, longevity and reproductive output in (egg-laying) IFWs.

4.4 Discussion

This study set out to provide further understanding of the underlying drivers of multimodality found in worker longevity distributions within worker cohorts in *B. terrestris* colonies. The first hypothesis tested was that the longevity distribution of workers within the same cohort and colony is multimodal (H1). The second hypothesis attributed this either to intrinsic differences between workers and therefore predicted the same pattern to be exhibited by workers kept in isolation (H2a), or to social factors acting on worker longevity and therefore predicted the pattern would not be exhibited by workers kept in isolation, but only by workers in a social colony setting (H2b). These hypotheses were tested by comparing the longevities of focal workers that were kept in isolation with those of focal workers that were kept in the social environment of a queenright colony. The results supported H1 and H2b, and also showed a strikingly large positive effect of isolation on worker longevity, as now discussed in detail.

4.4.1 Worker Longevity Distributions

In both categories of social workers (SFEWs and SFCWs), the worker longevity distribution was significantly bimodal (**Figure 4.2B**). Further, a bimodal distribution of social focal worker longevity could be detected in 6 of the 7 individual colonies (**Figure 4.2B**). These findings provided evidence for H1, that indeed multimodality in worker longevity distributions is a common and replicable pattern in *B. terrestris* colonies.

The fact that bimodality was present in worker longevity distributions in the current study, as well as in the datasets presented in **Chapter 2** and **Chapter 3**, and the dataset reanalysed from Blacher et al. (2017), is evidence for this being a robust phenomenon that is likely to occur in most *B. terrestris* colonies (**Table S4.2**).

For the focal workers kept in isolation (IFWs), the longevity distribution was not multimodal. Instead, the distribution of IFWs longevities followed more closely a normal distribution, with a tail to the left caused by three shorter lived IFWs (5 days, 28 days and 56 days) (**Figure 4.2**). Such a normal distribution in longevities of a population is the more common pattern found across taxa (Kannisto, 2001; Moser et al., 2015; Montoya et al., 2023).

The fact that multimodality in the worker longevity distributions of this study was only found in the social categories (SFEW and SFCW) and not among the workers kept in isolation (IFWs) provided clear support for H2b, stating that the pattern is driven by the social environment of a colony rather than by purely individual

differences between the workers.

There are several potential reasons for the existence of the observed form of bimodality in worker longevity within *B. terrestris* colonies. One set of reasons invokes colony-level benefits. Potentially, a strong full worker force is only needed until the majority of the sexual larvae have pupated and therefore no longer need feeding. The foraging needs of the colony decrease at that point and this could be timed to match a seasonal decline in resources in the natural habitat. If worker numbers decline then, but a smaller subset of long-lived workers survives, it could be because such workers are required to maintain the nest and carry out the thermoregulation of the sexual pupae until they eclose, meaning that colony requirements could be accounting for and reenforcing bimodality.

In *Bombus* colonies, several parallel forms of non-uniformity among the workers can be found and the regulation of major colony and nest tasks has been hypothesised to be achieved through such non-uniformity. For example, it has been suggested that the large within-colony variation in adult worker body sizes has evolved to facilitate the performance of different tasks (Goulson, 2003), although there is no clear empirical evidence for this (Jandt and Dornhaus, 2014). In social insects in general, colony tasks are likely to be regulated via variation in response thresholds of workers (Beshers and Fewell, 2001). Examples include the nest and brood thermoregulation behaviour performed by *Bombus* workers (Weidenmüller et al., 2002; Weidenmüller, 2004), in which some workers react to changing temperatures more quickly than others in changing their behaviour (fanning or incubation) according to need. Similar mechanisms may regulate foraging behaviour, whereby some workers start switching from collecting pollen to collecting nectar at higher brood densities than others (Russell et al., 2017). Therefore, the variation in, and multimodal distribution of, worker longevities within colonies in *B. terrestris* is conceivably another example of such a regulative mechanism. A caveat is that all the evidence presented in this thesis for within-cohort multimodality in the worker longevity distributions of *B. terrestris* has come from commercially-supplied bees reared in captive colonies (**Table S4.2**). Therefore, the prevalence of this phenomenon in wild colonies in nature (and across *Bombus* species in general) is strictly speaking unknown, and determining it remains a key goal for future investigation. In the current context, this point also highlights that, in wild colonies, the added extrinsic mortality of foraging workers may already lead to varying longevities among the workers, and this could providing a means of achieving the regulatory benefits proposed.

A second set of potential reasons for the observed multimodality in worker longevity invokes individual-level factors. In particular, such multimodality arguably provides

further evidence for within-colony variation in individual worker quality. This is because other indicators of worker quality, such as body size and reproductivity, have been found to be positively associated with longevity (**Chapter 2**). Accordingly, a positive association of body size and longevity was found in the IFWs (**Table 4.5**, further discussed below in **Section 4.4.3**). In addition, larger IFWs produced significantly more adult male offspring, and, among the egg-laying IFWs, there was a significantly positive association of the number of adult male offspring produced and worker longevity (**Table 4.6**, further discussed below in **Section 4.4.3**). From the positive associations between reproductivity, body size and longevity, one would expect, given multimodality in longevity, a similar pattern of multimodality in worker body size distributions. In the current study, such a pattern occurred in 3 of the 7 colonies and in the SFEWs. In addition, in all these cases the median longevity of workers that fell within the first body size mode (smaller) had a lower median longevity than those that fell into the second body size mode (larger), although there was no significant difference in longevity between the two modes. This is consistent with, but does not prove, an association between bimodality in worker longevity and body size distributions, and so in turn potentially reflects a connection between longevity bimodality and individual worker quality.

Bimodality in the worker longevity distribution might specifically reflect the existence of a subset of particularly high-quality workers. Moreover, other known aspects of *B. terrestris* social biology suggest that the existence of such workers could be maintained by selection for workers to gain direct fitness (fitness from offspring production) by producing sons. In *B. terrestris*, it is well known that workers may start laying haploid eggs (male-destined) within their natal colony (Duchateau and Velthuis, 1988; Lopez-Vaamonde et al., 2009), though only a low proportion (about 5%) of the adult males produced by a colony stem from worker-laid eggs (Alaux et al., 2004; Lopez-Vaamonde et al., 2004). The relatively small amount of realised adult male production by workers is largely due to the fact that the workers practice policing behaviour, which includes detecting worker-laid eggs and eating them (van Doorn and Heringa, 1986; Duchateau, 1989; Zanette et al., 2012), and also aggressive behaviour towards egg-laying workers (Amsalem and Hefetz, 2011). Dominant workers are also able to inhibit ovarian development of other workers (Bloch and Hefetz, 1999). Therefore, success in dominance interactions and in the production of adult sons could also be aspects of high quality. Furthermore, *B. terrestris* workers may sometimes 'drift' to other non-natal colonies and may successfully produce male offspring in the new, non-natal colony (Lopez-Vaamonde et al., 2004; Zanette et al., 2014). (see also review of Beekman and Oldroyd (2008)). In accordance with

the concept of the existence of a subset of high-quality workers, workers with the greatest levels of ovarian activation are the ones most likely to become drifters and also reproduce in the new colony (Blacher et al., 2013). Therefore, a subset of high-quality workers could be maintained by selection for individual-level direct fitness gains in both the natal colony and (via drifting) in non-natal colonies.

Lastly, the fact that the worker longevity distribution of the IFWs was not bimodal (**Figure 4.2**) suggests that, even if bimodality reflects individual-level differences in quality, such quality differences require a social context in which to be expressed. Alternatively, it is possible that the far greater overall longevity of IFWs (further discussed below in **Section 4.4.2**) to some extent masked any bimodality that might otherwise have been exhibited. Notwithstanding this possibility, the idea that individual-level differences require a social context is consistent with the earlier findings of this thesis that both individual and social factors influence longevity in *B. terrestris* (**Chapter 3**).

4.4.2 Effect of Isolation on Longevity

The experiment reported in the chapter also revealed an unanticipated but very strong positive effect of isolation on worker longevity. Focal workers' longevities differed greatly between the isolated workers (IFWs) and the social workers (SFEWs and SFCWs), with the median (range) longevities of IFWs, SFEWs and SFCWs being 131.5 (5-180) days (N = 68), 42 (1-85) days (N = 42) and 49 (21-90) days (N = 70), respectively, (**Figure 4.3A**). The difference between the IFWs and SFEWs is especially striking, as those workers originated in the same set of colonies. Correspondingly, the Cox proportional hazard analysis revealed an increase in the mortality chance (hazard ratio) for the SFEWs compared to the IFWs by a factor of 57 (**Figure 4.3B**, **Table 4.2**), meaning the focal workers under social conditions were 57 times more likely to die than the isolated workers. This was also reflected in the Bayesian survival trajectory analysis that showed, in the IFWs, significantly lower baseline mortality as well as a lower change rate in mortality over time, leading to better survival (**Figure 4.4**, **Table 4.3**).

A study on *B. impatiens* found that, if callow workers were isolated (for 9 consecutive days), gene expression and brain development were disrupted (Wang et al., 2022). Workers reared under isolation also showed increased social behaviour when introduced to another worker (Wang et al., 2022). By contrast, workers reared in small social groups of four nest mates and workers reared in their natal colonies did not show these effects (Wang et al., 2022). Similar results regarding brain development were reported for workers of the ant *Camponotus floridanus* (Seid and

Junge, 2016). In the current study, the brains of the IFWs were not analysed, but the extended longevities recorded suggest that, even if brain disruption occurred, it was not severe and/or did not impair longevity.

The extreme positive effect of isolation on longevity in the current study has not previously been reported in *Bombus* and to my knowledge there are no other examples in the literature that track longevity of isolated bee workers. This novel finding was unexpected because it was the reverse of patterns typically reported from other eusocial insects and from non-eusocial insects. Social isolation has typically been found to be associated with reductions in longevity, with examples including the fruit-flies *Drosophila melanogaster* (Ruan and Wu, 2008) and *Bactrocera dorsalis* (Wang et al., 2016). In ants, a reduction in longevity due to isolation of workers was found in *Temnothorax nylanderi* and the red imported fire ant *Solenopsis invicta* (Wang et al., 2016). In a *Forelius* sp. ant, workers had a high chance of dying after a single night of isolation from the nest and colony in the field (Tofilski et al., 2008). In the carpenter ant *Camponotus fellah*, social isolation of workers also led to greatly reduced longevities, compared to those of workers kept with one other nestmate worker or in small social groups of workers (Boulay, 1999; Koto et al., 2015, 2023). At the proximate level, social isolation in these ants led to hyperactivity and the reduction in longevity seemed to be caused by increases in oxidative stress, unrelated to the increase in activity (Koto et al., 2023). Further, the reduction in longevity due to social isolation was suggested to be connected to reduced energy income in the isolated workers, even under constant food supply (Koto et al., 2015). In this species, workers share food resources via trophallaxis, i.e. liquid food exchange (Hölldobler and Wilson, 1990), and isolated workers have been found to retain food in their crop for future transfer to other workers rather than consuming it to provide energy for themselves (Koto et al., 2015). Trophallactic feeding is not found in bumblebees, which could be a proximate reason why isolation did not have a negative effect on survival in the IFWs of the current study.

Instead, as mentioned, isolation of *B. terrestris* workers in the current study had a strongly positive effect on longevity. At the ultimate level, there could be several reasons for this. First, as in other large-bodied bee species, *Bombus* workers are known to forage for long distances externally, e.g. maxima of 0.8 to 2.9 km in five *Bombus* species, including *B. terrestris*, in the field study of Redhead et al. (2016). On such occasions, it seems likely that foraging workers might frequently become temporarily lost and may be away from the colony, and hence isolated, for a considerable time. This is also consistent with the strong homing ability of displaced workers over long distances and several days in the field (Goulson and Stout, 2001).

This factor might therefore select for the ability of workers to survive isolation. Alternatively, but not mutually exclusively, as workers of an intermediately eusocial species with relatively high frequencies of worker-produced males (in the natal colony or as reproductive drifters), including in wild colonies (Zanette et al. (2014); **Section 4.4.1**), *Bombus* workers might be selected to survive social isolation better in order to realise direct fitness opportunities. This could occur with respect to male production by unmated, drifter workers as discussed above (**Section 4.4.1**), consistent with the positive association of longevity and number of adult males produced in egg-laying IFWs (**Table 4.6**). In addition, it has recently been shown that *Bombus* workers are able to mate with males and produce diploid female offspring and found a colony in a queen-like manner (Zhuang et al., 2023). (Likewise, in the current study, IFWs, which were unmated, were able to raise up to 12 adult males each; **Section 4.3.2.**) Voluntary mating behaviour of workers was found by Zhuang et al. (2023) to be directly positively correlated with isolation of the worker in the seven days after eclosion. Workers that were placed in the social environment of their natal colony after eclosion did not mate when presented with a male (Zhuang et al., 2023). Therefore, not only are *Bombus* workers able to mate and found their own colonies and thereby gain direct fitness, but also they appear better able to do so in the context of social isolation. Zhuang et al. (2023) argue that workers' mating ability might have evolved to buffer early queen loss in a colony. Longevity increases with isolation in *B. terrestris* workers could therefore have evolved to ensure survival of potentially highly-reproductive and/or colony-founding, queen-like individuals.

4.4.3 Effect of Body Size and Reproductivity on Longevity

Among the IFWs as a whole , survival was significantly positively influenced by body size (marginal wing cell length) (**Table 4.5**). This was in accordance with earlier results (**Chapter 2**), in which larger workers also showed greater longevities.

As regards fecundity-longevity associations, among IFWs as a whole, there was no significant association between longevity and the probability of being an egg-layer (**Table 4.5**). However, in egg-laying IFWs, there was a significant positive association between longevity and the number of adult males produced (**Table 4.6**). This means there was a positive fecundity-longevity association in this sample of egg-laying *B. terrestris* workers. Consistent with this, in egg-laying IFWs, baseline mortality (BaSTA analysis) was substantially lower than for non-egg-layers (**Figure 4.5A**, **Table 4.3**), translating to lower chances of mortality in early life for egg-layers. By contrast, the change rate of the mortality risk (BaSTA analysis) was increased for egg-layers, meaning that with age the effect of egg-laying on sur-

vival became increasingly negative (**Figure 4.5B**, **Table 4.3**). Similar results were reported in **Chapter 2** , in which this effect was even stronger, eventually leading to a negative influence of egg-laying on survival. The finding of an overall positive fecundity-longevity association in the egg-laying IFWs independently confirms the existence of such an association in *B. terrestris* workers that freely choose to become reproductive, as described by Blacher et al. (2017) and earlier in this thesis (**Chapter 2**). Moreover, fecundity in the current case was measured by adult male production, which arguably provides the measure of fecundity most closely aligned to direct fitness.

In IFWs as a whole, the activity of the IFW did also not affect longevity (**Table 4.5**), but in egg-laying IFWs, activity and longevity were significantly associated (**Table 4.6**). Therefore, the overall longevity contrast between isolated and social workers (**Section 4.4.2**) seems unlikely to have been mediated by activity effects, but, in egg-laying IFWs, activity and longevity may have been associated because longer-lived egg-laying IFWs also produced more adult males, and hence may have shown more brood care.

In egg-laying IFWs, interestingly, there was a significantly positive association of worker longevity and male eclosion time (**Table 4.6**), which could be a sign that activation of the ovaries too early and/or laying eggs too early was not beneficial for survival. This effect was relatively small (Hazard Ratio: 0.959, meaning a reduction in mortality risk of only 0.041). However, it aligned with results reported in **Chapter 2**, in which some workers started laying eggs at younger ages but showed lower average longevities, whereas other workers started laying eggs only at older ages and showed higher longevities.

It was particularly noteworthy to find that, of the total sample of 68 IFWs, 20 workers, representing a substantial proportion of the sample (29%) (**Section 4.3.2**), never laid any eggs. Therefore, even when provided with seemingly optimal conditions in which to become egg-layers (e.g. lack of social interference, *ad libitum* food, prolonged lifespan), in conditions in which the majority of workers did lay eggs, some workers failed to become reproductive throughout their entire adult lives. This finding adds further to the evidence that there are different levels of worker quality, and in particular that workers of low quality exist for which the costs of reproduction are sufficiently deterrent to effectively prevent reproduction from occurring when workers have a choice of whether to reproduce or not, as argued by Blacher et al. (2017). Similarly, in whole colonies, not all workers (up to 55%) lay eggs (Bloch and Hefetz, 1999), and this could be for the same reason.

Finally, a positive relationship between lifetime reproductive success (adult sexual

production) and longevity, mirroring the one found between adult male production and longevity in IFWs of the current study, has previously been described for *B. terrestris* queens (Lopez-Vaamonde et al., 2009). This could be further support for some high-quality workers indeed having queen-like properties, as suggested by Zhuang et al. (2023), and so reinforcing the conclusion than increased longevities in isolated workers have most likely evolved for direct fitness gains.

4.4.4 Conclusion

Overall, this experiment has confirmed that multimodality in worker longevity distributions is a robust phenomenon in *B. terrestris*. In addition, it clearly shows that a social environment is required for this pattern to be expressed. When the social environment of a colony was absent, the distribution of worker longevities followed a unimodal, approximately normal distribution. These findings therefore attest to the existence of a novel phenomenon in the study of the relationship between sociality and longevity, which potentially exists in eusocial Hymenoptera more widely.

An unanticipated finding of this experiment was that the isolation had a strong positive effect on worker longevity, whereas the opposite effect can be found in many other (eusocial) insects. Coupled with evidence of positive fecundity-longevity associations in the workers of this study, this effect seemed likely to stem from workers in *B. terrestris* being able to gain direct fitness. By presenting further evidence for positive relationships between body size, reproductivity and longevity in *B. terrestris* workers, this study also supported the concept of within-colony variation in worker quality affecting longevity in this species, and by extension in other eusocial species as well.

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Supplement

4.4.1 Supplementary Figures

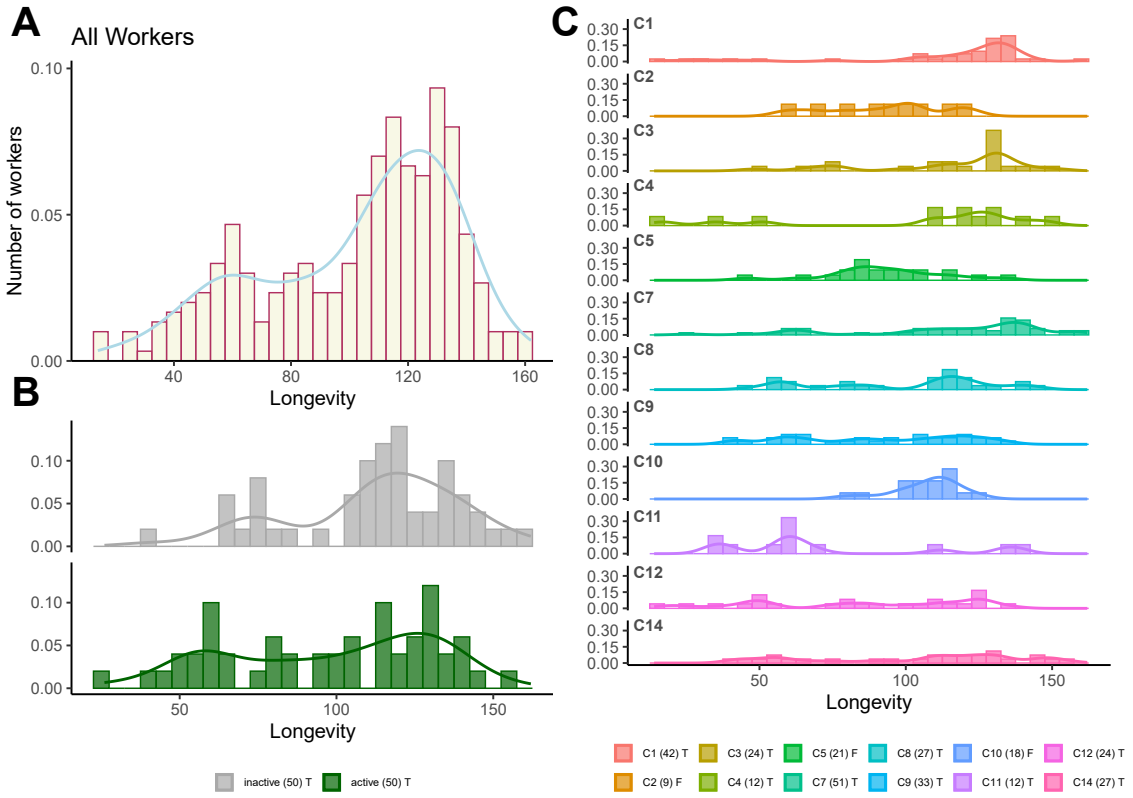

Figure S4.1. Distributions of *Bombus terrestris audax* worker longevity in the data set of Blacher et al. (2017) as reanalysed in the current study. Proportional histograms with a density curve of worker longevities, showing the proportion of workers that died at a certain age (longevity in days from eclosion to death). These data sets exclude censored workers. The total area of all bins and under the curve respectively represents 100% of each sample. (Number in brackets representing the sample size; bin size 5 days.) **A**: All focal workers' longevities (N = 300), showing how many workers died at a certain age (longevity in days). The distribution tested positive for multimodality (bimodal). **B**: Worker longevities grouped by treatments (green: active ovaries (N = 50); grey: inactive ovaries (N = 50)). Both curves tested positive for multimodality (bimodal). **C**: Worker longevities grouped by colony. Out of 12 colonies, 9 showed significant multimodality (bimodal). The legend indicates which curves tested positive for multimodality (T=true) and which did not (F=false).

4.4.2 Supplementary Tables

Table S4.1. Effects of worker longevity and male eclosion time (a proxy for body size), on the number of adult males produced by an isolated egg-laying *Bombus terrestris* worker (IFW, N = 68). Linear mixed model: Males ~ Longevity + Male Eclosion Time + (1 | Colony). Longer-lived workers produced more adult males ($p = 0.002$), and longer male eclosion times resulted in less males produced ($p < 0.001$). Shown are the Estimate, the Standard Error, and the t- and the p-value. For the random effect of Colony, the Standard Deviation and the Variance are displayed.

Fixed Effect	**Estimate**	**Standard Error**	**t**	**p**
Longevity	0.0611	0.018	3.366	0.002
Male Eclosion Time	-0.108	0.020	-5.404	<0.001
Random effects	**Variable**	**Std Dev**	**Variance**	
Colony	Intercept	1.05	1.02	

Table S4.2. Summary list of the *Bombus terrestris* worker longevity datasets (re)analysed in this thesis. All colonies were obtained from the commercial supplier Biobest, Belgium. Reference: where this dataset was initially analysed; Dataset: description of what the dataset includes; Year: the year were the data was collected; Median Longevity: the median worker longevity in days from eclosion to death; Longevity Range: the minimum to maximum range in worker longevities in days, Sample Size: number of of adult workers included in the dataset; Figure Reference: thesis figure regarding this dataset; Experimental Treatment: experimental manipulation if any; Rearing Conditions: conditions under which the workers were reared; Living Conditions: the conditions in which the workers were kept; Modality: Classification of the worker longevity distribution; Eclosion Window: time frame in days in which the workers had eclosed. Censored workers were excluded from all datasets.

Reference	Dataset	Year	Median Longevity (days)	Longevity Range (days)	Sample Size	Figure Reference	Experimental Treatment	Rearing Condition	Living Condition	Modality	Eclosion Window (Days)
Chapter 2 current thesis	Full dataset	2022	93	1 - 158	369	Figure 2.5	N/A	Queenright colony	Queenright colony	Bimodal	17
Chapter 2 current thesis	Subset H-treatment	2022	95	2 - 158	187	Figure 2.5	High larval nutrition	Queenright colony	Queenright colony	Bimodal	16
Chapter 2 current thesis	Subset L-treatment	2022	92	1 - 158	180	Figure 2.5	Low larval nutrition	Queenright colony	Queenright colony	Bimodal	23
Chapter 3 current thesis	All experimental workers	2023	33	1 - 124	167	Figure 3.4	N/A	Queenright colony	Queenright colony	Bimodal	20
Chapter 3 current thesis	Non-transfer experimental workers early	2023	37	1 - 114	63	Figure 3.4	Non-transfer	Queenright early colony	Queenright early colony	Bimodal	19
Chapter 3 current thesis	Non-transfer experimental workers late	2023	27	5 - 119	20	Figure 3.4	Non-transfer	Queenright late colony	Queenright late colony	Unimodal	11
Chapter 3 current thesis	Transferred experimental workers early to late	2023	28	3 - 113	56	Figure 3.4	Transferred	Queenright early colony	Queenright late colony	Trimodal	20
Chapter 3 current thesis	Transferred experimental workers late to early	2023	35	3 - 124	28	Figure 3.4	Transferred	Queenright late colony	Queenright early colony	Bimodal	12
Chapter 3 current thesis	Non-transfer control workers early	2023	21	Jan-91	35	N/A	Non-transfer	Queenright early colony	Queenright early colony	Bimodal	18
Chapter 3 current thesis	Non-transfer control workers late	2023	47	May-95	7	N/A	Non-transfer	Queenright late colony	Queenright late colony	Unimodal	6
Chapter 3 current thesis	Transferred control workers early to early	2023	34	1 - 126	40	N/A	Transferred	Queenright early colony	Queenright early colony	Unimodal	20
Chapter 3 current thesis	Transferred control workers late to late	2023	47	4 - 102	15	N/A	Transferred	Queenright late colony	Queenright late colony	Unimodal	10
Chapter 4 current thesis	Full dataset	2024	60.5	1 - 180	180	Figure 4.2	N/A	Queenright colony	N/A	Bimodal	12
Chapter 4 current thesis	Social experimental workers	2024	42	Jan-85	42	Figure 4.2	N/A	Queenright colony	Queenright colony	Bimodal	12
Chapter 4 current thesis	Social control workers	2024	49	21 - 90	70	Figure 4.2	N/A	Queenright colony	Queenright colony	Bimodal	11
Chapter 4 current thesis	Isolated workers	2024	131.5	5 - 180	68	Figure 4.2	N/A	Queenright colony	Isolated	Unimodal	12
Blacher et al., 2017	Full dataset	2014	112	14 - 162	300	Figure S4.1	N/A	Queenright colony	Groups of 3 workers	Bimodal	12
Blacher et al., 2017	Focal workers only	2014	114.5	26 - 162	100	Figure S4.1	N/A	Queenright colony	Groups of 3 workers	Bimodal	6
Blacher et al., 2017	Ovary-active focal workers	2014	104	26 - 155	50	Figure S4.1	Kept with younger workers	Queenright colony	Groups of 3 workers	Bimodal	4
Blacher et al., 2017	Ovary-inactive focal workers	2014	121	63 - 162	43	Figure S4.1	Kept with older workers	Queenright colony	Groups of 3 workers	Bimodal	6

Chapter 5 | Gene Expression associated with Ovary Activation in *Bombus terrestris* Workers

Abstract

In eusocial insects, female reproductivity is known to have a positive effect on longevity. However, little is known about the molecular processes that drive this positive relationship. Reproductively active workers of the eusocial bumblebee *Bombus terrestris* represent a system exhibiting a condition-dependent positive-fecundity relationship. Therefore, to elucidate the molecular basis of such relationships, two transcriptomic (mRNA-seq) sequencing experiments were conducted using selected tissues of worker *B. terrestris*. In Experiment A, age-related gene expression changes in fat body were compared between ovary-active workers and ovary-inactive workers, each of two different ages (2- and 7-week-old adults). In Experiment B, mRNA-seq data from *B. terrestris* brain, fat body and ovary from focal workers activating their ovaries in queenless microcolonies were compared between three time points (24 h and 96 h after becoming queenless and at first egg-laying). In Experiment A, large differences in gene expression occurred between ovary-active and ovary-inactive workers. These differences increased with age and a majority of age-related differentially expressed genes, some associated with known ageing-related gene networks or sets, were found exclusively in either phenotype. These findings suggested that consistent with the occurrence of condition-dependent positive-fecundity relationships, the molecular basis of ageing may differ between reproductive and non-reproductive workers. They also revealed potential candidates for genes underlying positive fecundity-longevity relationships. In Experiment B, the largest changes in gene expression occurred in all three tissues between the 24 h and 96 h time points, i.e. within the first four days of ovarian activation. Therefore, genes changing expression in this period, especially those shared across tissues, again represent candidates for genes underlying positive longevity-fecundity relationships in eusocial insects. Combined, these results support hypothesized links between reproductivity and ageing in eusocial insects and provide a basis for further investigation of specific genes and pathways potentially involved in them.

5.1 Introduction

The evolution of a reproductive division of labour in eusocial insects, whereby individuals are split into reproductive phenotypes (queens and kings) and less reproductive or fully sterile phenotypes (workers), represents one of the major transitions in evolution (Smith and Szathmary, 1997; Bourke, 2011; Boomsma, 2022). In obligate eusocial societies, the morphological separation between queens and workers extends to the point that workers are unable to mate and therefore found colonies independently (Boomsma, 2022). Evolutionary, this has been explained by inclusive fitness (kin selection) theory, which in this case attributes morphological worker evolution to inclusive fitness gains of workers from rearing related brood (Hamilton, 1964; West et al., 2007; Bourke, 2011). Yet, within the obligate eusocial Hymenoptera (ants, wasps and bees), there are also many examples of unmated workers being capable of direct reproduction through producing haploid male offspring from unfertilized eggs (Bourke, 1988). Eusocial complexity in eusocial species can vary greatly. In 'advanced' eusociality, species can show very strong morphological dimorphism between queens and workers and worker reproductivity is usually low, with workers in some cases being completely sterile. In 'primitive' or 'intermediate' eusociality, queen-worker morphological dimorphism is low and the level of worker reproductivity is typically higher (Bourke, 2011). In some cases, workers in intermediate eusocial societies are even able to mate and initiate (smaller) colonies (*Bombus terrestris*, Zhuang et al. (2023)). Overall, therefore, the level of worker reproductivity decreases with increasing social complexity in eusocial evolution (Bourke, 2011).

The study of the molecular basis of worker reproductivity in eusocial Hymenoptera is relevant to researchers' understanding of not only social evolution, but also the influence of sociality on ageing and longevity. This is because, as previous chapters of the current thesis have shown (**Chapters 2-4**), worker reproductivity provides a set of phenomena demonstrating such an influence and permitting it to be experimentally investigated. Specifically, reproductively active Hymenopteran workers have been found to exhibit a positive relationship between fecundity and longevity, and to have higher longevity than non-reproductively active worker nestmates (honeybees Dixon et al. (2014), ants Negroni et al. (2019) and bumblebees Blacher et al. (2017)), and evidence from *B. terrestris* suggests that this could be a function of worker quality varying within colonies such that high-quality workers are able to overcome costs of reproduction (Blacher et al. (2017); **Chapters 2-4**). Eusocial insect queens also exhibit positive fecundity-longevity relationships, and it has been proposed that a rewiring of signalling pathways and networks that regulate reproduction and ageing is the proximate mechanism behind this apparent abolition of

the fecundity-longevity trade-off typically found in non-social organisms (Rodrigues and Flatt, 2016; von Wyschetzki et al., 2015; Lockett et al., 2016; Korb and Heinze, 2021). One network hypothesized to have this role is the 'TI-J-LiFe network', a signalling network between the target of rapamycin (TOR), insulin/insulin-like growth factor 1 signalling (IIS) and juvenile hormone (JH) (Korb et al., 2021), as this network is associated with pro-reproduction and pro-ageing effects in non-social insects and vertebrates (Flatt et al., 2013; Rodrigues and Flatt, 2016). Another hypothesized network of ageing-related genes that could potentially be rewired over eusocial evolution is a set of enzymatic antioxidant genes identified by Kramer et al. (2021)). A recent study in queens of *B. terrestris* reported the possibility that a partial remodelling of gene networks underpinning ageing and longevity, including genes in the TI-J-LiFe network and the enzymatic antioxidant gene set, has indeed occurred in this species (Collins et al., 2023). By extension, in cases in which reproductive workers exhibit positive fecundity-longevity relationships, similar remodelling of underpinning gene networks may have occurred.

For these reasons, it is of interest to investigate the molecular basis of worker reproductivity in the eusocial Hymenoptera. Several previous studies have addressed this topic by comparing gene expression in ovary-active versus ovary-inactive workers, e.g. in the bees *Bombus* and *Apis* (e.g. *Bombus*: Pereboom et al. (2005); Harrison et al. (2015); Prince et al. (2024); *Apis*: Grozinger et al. (2007); Cardoen et al. (2011); Galbraith et al. (2016); Duncan et al. (2020).) In particular, Prince et al. (2024) used transciptomics (mRNA-seq) to isolate genes differentially expressed between ovary-active and ovary-inactive workers aged 3-5 weeks in *B. terrestris*. However, although exceptions exist (e.g. Lockett et al. (2016)), few studies in *Bombus*, *Apis* or other groups have investigated differential gene expression with respect to ovarian activation over time, or had a particular focus on relevance to the evolution of ageing and longevity.

The study presented in the current chapter therefore aimed to profile, using mRNA-seq, gene expression differences associated with ovary activation in *B. terrestris* workers with a particular focus on changes over time. This was achieved in two experiments. In the first, Experiment A, the mRNA-seq data from *B. terrestris* worker fat body presented in **Chapter 3** were further analysed, but instead of a focus on comparisons across experimental categories, analysis was focused on differential gene expression between ovary-active and ovary-inactive workers and, within these phenotypes, between the two time points (2 weeks and 7 weeks of worker age). In the second, Experiment B, mRNA-seq data from *B. terrestris* worker brain, fat body and ovary were analysed from a separate experiment in which, in queenless

microcolonies, ovary-activated or egg-laying workers were sampled at three time points (worker ages) post eclosion, i.e. 4, 7 and (if egg-layers) 7-12 days. Across both experiments, the three tissues investigated were selected because previous studies suggest that gene pathways influencing ageing and reproduction are localized in them (Grozinger et al., 2007; Page et al., 2012; Duncan et al., 2016; Lockett et al., 2016; Duncan et al., 2020; Prince et al., 2024). Differentially expressed genes isolated between ovary-active and ovary-inactive workers and over time were compared with those highlighted from previous studies and with genes in networks or gene sets hypothesized to underpin relationships of ageing, longevity, reproductivity and sociality.

5.2 Methods

5.2.1 Experiment A: Ovary-active vs. Ovary-inactive Workers

As stated, the mRNA-sequencing data from *B. terrestris audax* worker fat body sampled from queenright colonies (i.e. with a queen present) presented in **Chapter 3** were further analysed, with samples grouped into the two worker phenotypes, ovary-active and ovary-inactive workers (and with all experimental categories pooled). Upon dissection of the workers to obtain the fat body tissue from which the mRNA was extracted, the ovaries were assessed as either activated, defined in this experiment as with mature oocytes/eggs visible (Duchateau, 1989), or not activated, defined in this experiment as showing no sign of mature oocytes/eggs. Workers were sampled for RNA extraction at two time points, 2 weeks after eclosion (TP1) and 7 weeks after eclosion (TP2). From the 57 samples sent for mRNA-sequencing, four could not be used in further analyses, because they had not reached the threshold of an alignment of 20 million reads (2 libraries of ovary-active workers in TP2 and 2 libraries of ovary-inactive workers in TP2). This resulted in 53 mRNA-libaries, i.e. 38 libraries from 38 ovary-active workers and 15 libraries from 15 ovary-inactive workers (ovary-active workers: TP1, N = 24 workers; TP2, N = 14 workers; ovary-inactive workers: TP1,N = 10 workers; TP2, N = 5 workers). The dataset included transferred and non-transferred workers as well as early-produced workers and late-produced workers. Ovary-active and ovary-inactive workers were equally present among the transferred and the non-transferred workers, as well as among the early-produced and the late-produced workers (**Figure S5.1**). Therefore effects due to age-related changes in gene expression due to these groupings should be equally

present in both phenotypes and not affect the analysis of age-related differential gene expression due to ovary activity. It also needs to be mentioned that workers that were dissected at the 2 week time point and found to be ovary-inactive, could have potentially, later in life activated their ovaries, as results in **Chapter 2** showed one reproductive strategy to be laying eggs at older ages only. All further details on colony rearing, dissections and mRNA-extraction can be found in **Section 3.2**.

5.2.2 Experiment B: Stages of Ovary Activation in Workers

In Experiment B, newly-eclosed *B. terrestris audax* workers were reared in queenless microcolonies and sampled for RNA in brain, fat body and ovary at time points 24 h, 96 h and (in egg-laying workers) 96-216 h post microcolony initiation, corresponding to worker ages (post eclosion) of 4, 7 and 7-12 days, respectively; with the time of microcolony initiation representing the point at which 3-day old workers were transferred from queenright to queenless ones, so inducing ovary activation.

Colony Rearing

Twelve *B. terrestris audax* colonies were obtained from ©*Biobest Group NV, Belgium* on 12th March 2015 for use as source colonies. For the duration of the experiment, the colonies were maintained in a controlled climate room (temperature: 28°C; relative humidity: 60%) in constant darkness. Upon arrival the colonies were left to acclimatise for 24 hours. After this time, the colonies were moved from the commercial boxes into individual wooden colony nest-boxes (internal dimensions 17 cm x 27.5 cm x 16 cm high). Each colony was randomly assigned a number from 1 to 12. The number of workers at this stage (mean ± SD) was 26.6 ± 12.3 workers per colony. Because these colonies reached the competition point (first worker egg-laying, representing a marker of the change in the colony cycle from worker production to the production of sexuals, i.e. new queens and/or males) before sufficient workers had been obtained to complete the experiment, a set of additional source colonies was then obtained. This set consisted of fifteen *B. terrestris audax* colonies obtained from 'Biobest Group NV, Belgium on 2 April 2015. These colonies were acclimatised and transferred to wooden colony nest-boxes as described above. The number of workers in the second set of source colonies at this stage (mean ± SD) was 15.7 ± 5.1 workers per colony). Each colony was randomly assigned a number from 13 to 27. The nest-boxes in which all colonies were housed for the duration of the experiment were equipped with a circular ventilation hole (Ø3.5 cm) on either side covered with a metal mesh. A plastic container filled with BIOGLUC®('Biobest

Group NV, Belgium), a ready to use sugar solution (artificial nectar), was positioned underneath each nest-box. The bees could feed on this solution via a filter-tip forming a wick (passing through aligned holes in the floor of the nest-box and the roof of the container) from the container to the inside of the nest-box, ensuring *ad libitum* access. The bees were also fed with fresh honeybee-collected pollen on an *ad libitum* basis. A rectangular frame of acetate was glued onto the top rim of the nest-box to act as a barrier against bees crawling out.

Worker Marking

Individual marking of workers was conducted each morning on all newly-eclosed workers (callow workers). Each such worker was removed from the colony and placed in a marking cage (a cylindrical transparent tube, open at one end and covered with a plastic mesh at the other end). Using a foam plunger, the worker was then positioned so that the area of the thorax between the wings was accessible through the mesh. With a toothpick and a small drop of shellac-based glue (Queen Marking Glue, Thorne Ltd., UK), a circular paper label bearing a number on a coloured background (Queen Marking Kit, Thorne Ltd., UK) was attached to the worker's thorax. The date, colony number and disc number were all recorded in order to monitor the age and identity of individual bees. The workers were then returned to their colony and retained there until potential use in the microcolonies.

Newly-eclosed workers from a colony were marked and/or used for microcolony construction until the source colony had reached its competition point or males were consistently eclosing. The competition point for each source colony was identified by observing the colony every 1-2 days with two three minutes scans. Following Duchateau et al. (2004), a colony was said to have passed the competition point when at least one of the following criteria was observed: 1) multiple open egg cells; 2) egg-eating by queen or workers; 3) aggression between queen and workers; or 4) egg-laying by workers.

Microcolony Construction

Microcolonies were set up following the methods of Alaux et al. (2007), who showed that egg-laying was induced in focal *B. terrestris* workers when they were placed in queenless conditions with two newly-eclosed workers three days younger in age. In the current experiment (Experiment B), workers individually marked as described were used as as focal workers. Focal workers were removed from their natal colony 3 days after marking, and formed into queenless groups of three workers each consisting of a focal worker and two, newly-eclosed workers from different colonies (i.e. all

three workers were from a different source colony). Following Alaux et al. (2007), it was anticipated that the focal worker, being older, would activate its ovaries and potentially then lay eggs. Each microcolony was established in a clear plastic box (7.5 x 14 x 5 cm high) with a sugar solution feeding tube, a dish for pollen and a dish of wax. Wax for cell construction was provided to microcolonies from source colonies other than those from which workers in the microcolony were drawn. Microcolonies were supplied with sugar solution (Attracker, Koppert) and pollen (Koppert) *ad libitum*. A total of 161 microcolonies were set up over the course of the experiment.

Worker Dissections

All microcolonies were scanned daily for egg laying. If blister-like cells of wax (egg-containing cells) were observed to have been formed on the surface of the box, or at the base of the 'honeypot', i.e. the cell constructed from the wax provided in which sugar solution was stored, they were carefully investigated for the presence of eggs. The microcolonies were also randomly assigned to one of three groups. In group 1, all workers in a microcolony were dissected 24 hours post microcolony initiation; in group 2, all workers in a microcolony were dissected 96 hours (4 days) post microcolony initiation; and in group 3, all workers were dissected after first eggs were observed, which occurred 4-9 days post microcolony initiation. Removed workers were anaesthetised on ice for 10 minutes before dissection. The brain, fat body and ovaries of each focal worker were immediately dissected on ice in insect ringer solution in order to minimise RNA degradation. A Leica MZ6 microscope with a Leica CLS 150X light source were used for dissection. Dissections were carried out using a scalpel (Swann Morton No10) and fine tweezers (cleaned with RNAse Zap (Sigma-Aldrich)). The level of ovary activation was scored based on Duchateau and Velthuis (1989), with ovaries with scores of 0 and 1 being designated inactive ovaries, those with scores of 2 and 3 being designated intermediate ovaries, and those with scores of 4 and above being designated active ovaries. Dissected brains and ovaries were stored in 1.5 ml microcentrifuge tubes containing RNAlater (Sigma-Aldrich) and dissected fat bodies were stored in 1.5 ml microcentrifuge tubes containing AllProtect (Qiagen). Tubes were kept at 5°C overnight to allow the reagent to permeate the tissue, before being stored at −20°C. In each microcolony, the other two workers were dissected to score ovary activation but their tissues were not sampled for RNA. Tissue was not kept from workers in any microcolony in which one of the workers died before any worker from the microcolony was dissected (in the case of 15 microcolonies). Because workers and males can be hard to distinguish as callows, some microcolonies were inadvertently set up with a male or males present;

therefore, pre-dissection, all individuals were checked for sex, and all workers from microcolonies in which any male was found were also discarded (in the case of 10 microcolonies). Further, if the egg-laying worker was not the focal worker of the microcolony, the microcolony was also discarded (in the case of 7 microcolonies).

The tissues collected from the dissections of the focal workers were allocated to nine pools (three for workers sampled at 24 hours, three for workers sampled at 4 days (96 hours) and three for workers sampled after first egg-laying) of ten individuals each. All workers collected at 24 hours had an ovary activation score of 0 or 1. At 96 hours (4 days), workers that had an ovary activation score of 3 (which were in the majority, 30/47) were used for the RNA extractions. Laying of eggs was first observed between 4 and 9 days post microcolony initiation, with the majority (in 32/40 microcolonies) happening between 6 and 8 days. Focal workers from this sampling period (6-8 days) were used for the RNA extractions. In total, these procedures resulted in 27 samples, consisting of 3 tissues (brain, ovary and fat body) × 3 time points (24 hours, 96 hours, egg-laying [at 144-216 hours]) each × 3 biological replicates each (of 10 pooled workers each). As workers were transferred into microcolonies 3 days after being marked as newly-eclosed adults, in full the worker phenotypes sampled at the three time points in Experiment B were as follows: '24 h': workers 1 day after transfer to queenless conditions and aged 4 days post eclosion (with ovary activation scores of 0 or 1); '96 h': workers 4 days after transfer to queenless conditions and aged 7 days post eclosion (with ovary activation scores of 3); and 'egg-laying': workers 6-8 days after transfer to queenless conditions and aged 9-11 days post eclosion (and that were also egg-layers).

RNA Extractions and Sequencing

Samples were ground in liquid nitrogen in 2 ml tubes using plastic 2 ml pestles (Eppendorf), and the powder was then suspended in Tri-reagent (Ambion) (1000 μl for each tissue type). Next, samples were vortexed and then centrifuged for 1.5 minutes. Supernatants were transferred to a fresh tube and an equal volume of 100% ethanol was added. Samples were mixed by vortexing and then bound to the columns in the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). The kit protocol was followed, including a 15 minute on-column DNase treatment, with the column finally being eluted twice with the same 26 μl volume of dH_2O.

An additional DNase treatment was carried out for all fat body and ovary samples (as they contained more RNA than the brain samples). Ambion Turbo™ DNA-free kit (Thermo Fisher Scientific, Loughborough, UK) DNase treatment was used. For this, 3.5 μl DNase master mix (Turbo DNase : Turbo DNase buffer = 1:2.5) was

added to each of the RNA samples (21.5 μl per sample), which were then left to incubate for 25 min at 37°C. Afterwards, 2.5 μl of inactivation reagent was added to each sample and left at 24°C for 5 min, with the tube being flicked two or three times to mix the contents. The reaction was then transferred to a 1.5 ml tube and centrifuged for 1.5 minutes at 13,000 rpm. The supernatant (20 μl) was carefully removed to a fresh tube containing 1.5 μl dH_2O and was then flash frozen and stored at −80°C. The samples were then quantified on a Nanodrop (Thermo). The 27 samples (each representing a single biological replicate of RNA from 10 pooled workers) were then sent to Edinburgh Genomics (Edinburgh, UK) for sequencing as 50 bp single-end reads on four lanes of an HiSeq 2500 sequencer.

5.2.3 Bioinformatic Analyses

For both Experiments A and B, bioinformatic analyses were conducted using the pipeline in Collins et al. (2023), who customised standard pipelines for use on mRNA-seq data from *B. terrestris*. To assess the quality of the readings obtained from the mRNA-seq, FastQC v0.11.9 software (Andrews, 2015) was used to conduct quality tests such as checks for base quality and potential adapter contamination in each sample and read, these tests then being combined into a quality report using the MultiQC v1.9 Python library (Ewels et al., 2016) with Python v3.7 (Python Core Team 2017). The reads were then aligned to the *Bombus terrestris* genome (BomTerr1.2_genomic.fna.gz; Crowley et al. (2023)) using HISAT2 v2.1.0 (Kim et al., 2015) and mapping statistics were documented. On the basis of the HISAT2 alignment files, the gene body coverage was calculated, testing for a 3' or 5' skew in the libraries, and the junction saturation was also calculated, testing whether all splice sites have been detected, using the RSeQC v3.0.1 Python library (Wang et al., 2012) with Python v3.7.

Kallisto v0.46.1 (Bray et al., 2016) was used to pseudoalign the reads to the *Bombus terrestris* transcriptome (BomTerr1.2_genomic.fna.gz; Crowley et al. (2023)). Estimated transcript counts for each gene were obtained using the tximport package (Soneson et al., 2016) in R (v4.1.3) (R Core Team, 2020) and these were then used for differential expression analysis. For Experiment A this was done with a model $\sim$ time point + ovary status, and for Experiment B his was done with a model $\sim$ time point (DESeq2 package v1.44.0 Love et al. (2014) in R (v4.1.3) (R Core Team, 2020). The FDR adjusted p-value threshold was set to 0.05. Principal component analysis (PCA) of the DESeq2 data was used to check for library clustering, and boxplots of the normalised count data were constructed to check the normalisation (Mohorianu et al., 2017). Overall, the analysis generated lists of differentially ex-

pressed genes (DEGs) between the time points (Experiment A: between TP1 and TP2, separately for ovary-active and ovary-inactive workers, these are being called age-related DEGs; Experiment B: between 24 h and 95 h time point, between 96 h time point and egg-laying time point, between 24 h and egg-laying time point, separately for each tissue). Genes that were more highly expressed in the second time point of the comparison were termed 'up-regulated' and genes that were more highly expressed in the first time point of the comparison were termed 'down-regulated'. For Experiment B, the 50 most differentially expressed genes in these three time point comparisons (top-50 DEGs) were plotted in a separate heatmap per tissue showing the number of reads of these genes for each sample (split by time point).

To analyse whether there were significant overlaps between the ovary-active and ovary-inactive workers (Experiment A) or the three tissues (Experiment B) in DEGs up- or down-regulated between two time points, Fisher's exact tests were performed in R (R Core Team, 2020), including a Bonferroni correction to adjust for multiple testing. Following the analysis of Prince et al. (2024), in Experiment B for comparisons with the egg-laying time point, the full ovary DEG-lists were considered, as well as DEG-lists with a list of egg-expressed genes (EEGs, Prince et al. (2024)) removed, in order to address differential gene expression in the ovarian tissue only.

Gene ontology (GO) enrichment analysis and comparisons to other gene lists were conducted using OrthoFinder v2.5.2 (Emms and Kelly, 2019). This tool permitted gene orthologues between *B. terrestris* and *D. melanogaster* to be identified. Because GO annotations in *D. melanogaster* are considerably more detailed, only *D. melanogaster* single-copy orthologues for *B. terrestris* DEGs were used for GO enrichment analysis. GO enrichment analysis was conducted in R (R Core Team, 2020) using the clusterProler package (v4.12.0) (Yu et al., 2012) and the org.Dm.eg.db package (v3.19.1) (Carlson, 2024) for the biological processes GO annotations. Significantly overrepresented ($p < 0.05$ after adjustment for multiple testing with Benjamini-Hochberg) GO-terms in a set of DEGs compared to all expressed genes were identified with an over-representation test (Boyle et al., 2004). Significantly overrepresented non-redundant GO terms were defined as 'enriched'.

Lastly, following the analysis in Collins et al. (2023), the DEG-lists of Experiment A were compared to two sets of *D. melanogaster* genes that have been hypothesised to be strongly associated with ageing in eusocial insects: the TI-J-LiFe network (Korb et al., 2021) and an enzymatic antioxidant gene set (Kramer et al., 2021). To identify significant overlaps with the TI-J-LiFe network (123 genes) and the enzymatic antioxidant gene set (58 genes), the single-copy orthologues of the OrthoFinder results were used to compare the *B. terrestris* DEGs to the *D. melanogaster* ortho-

logues. The up- and down-regulated DEGs in each worker category were pooled and then ranked by the log fold change in expression with time (Collins et al., 2023). For each worker category, the 50 genes with the most positive log fold change and the 50 genes with the most negative log fold change were selected ('top ± 50 genes'). If the number of DEGs in a given list were high enough, the lists for 'top ± 100 genes', 'top ± 200 genes', 'top ± 300 genes', 'top ± 500 genes', 'top ± 700 genes' and all genes were also created. For each worker category, these 'top ± gene'-lists were then compared for significant overlap with the gene lists from the TI-J-LiFe network and the enzymatic antioxidant gene set, using Fisher's exact tests with a Bonferroni correction to adjust for multiple testing.

5.3 Results

5.3.1 Experiment A: Ovary-active vs. Ovary-inactive Workers

Across these 53 libraries, the mean read pairs per library was 30.31 million base-pairs. These libraries aligned to the *B. terrestris* transcriptome with a mean percentage pseudoalignement of 82.02% (76.1% - 81.50%). There was evidence for normalisation in the normalized count data of the remaining samples (**Figure S3.6**).

Differential Gene Expression

Gene expression in fat body changed strongly between the two time points at which the samples were taken (2 weeks and 7 weeks) and therefore with the age of the workers (**Figure 5.1**). Specifically, in workers as a whole, 655-898 DEGs were up-regulated and 681-783 DEGs were down-regulated with worker age (**Figure 5.1**). Gene expression between ovary-active and ovary-inactive workers differed more at time point 2 than at time point 1, with total numbers of DEGs in fat body between the worker phenotypes (91 versus 229) increasing more than 2.5 times with worker age (**Figure 5.1**).

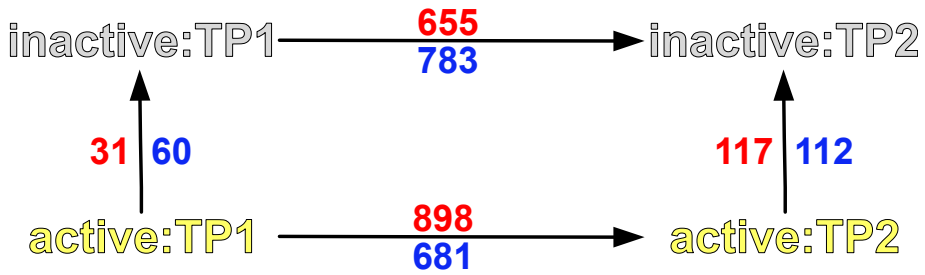

Figure 5.1. Comparison of mRNA-seq gene expression profiles in fat body of ovary-active and ovary-inactive *Bombus terrestris* workers on the basis of differentially expressed genes (DEGs) (Experiment A). Active (yellow): ovary-active workers (N = 38). Inactive (grey): ovary-inactive workers (N = 15), TP1: Time Point 1 (worker age of 2 weeks); TP2: Time Point 2 (worker age of 7 weeks). Arrows indicate the direction of the comparison. Number on top of the arrows (red): up-regulated DEGs (more expressed in the direction of the arrow); number below the arrow (blue): down-regulated DEGs (less expressed in the direction of the arrow). Sample sizes: active:TP1 (N = 24 workers), active:TP2 (N = 14 workers), inactive:TP1 (N = 10), inactive:TP2 (N = 5).

In the comparison between ovary-active and ovary-inactive workers with increasing worker age (DEGs in fat body between time point 1 and time point 2), there was

a significant overlap of 28.6% of ovary-active worker up-regulated DEGs and 39.3% of ovary-inactive up-regulated DEGs (**Figure 5.2A**, **Table S5.1**). There was also a significant overlap of 49.8% of ovary-active down-regulated DEGs and 43.3% of ovary-inactive down-regulated DEGs (**Figure 5.2B**, **Table S5.1**). In ovary-active workers, 71.4% of up-regulated DEGs and 50.2% of down-regulated DEGs were exclusively found in this phenotype. In ovary-inactive workers, 60.8% of up-regulated DEGs and 56.7% of down-regulated DEGs were exclusively found in this phenotype (**Figure 5.2**). Therefore, overall, both worker phenotypes exhibited age-related DEGs in fat body, and significant overlaps showed that some of these DEGs were shared, but the proportion of age-related DEGs exclusive to each phenotype (50.2-71.4%) exceeded the proportion shared (28.6-49.8%).

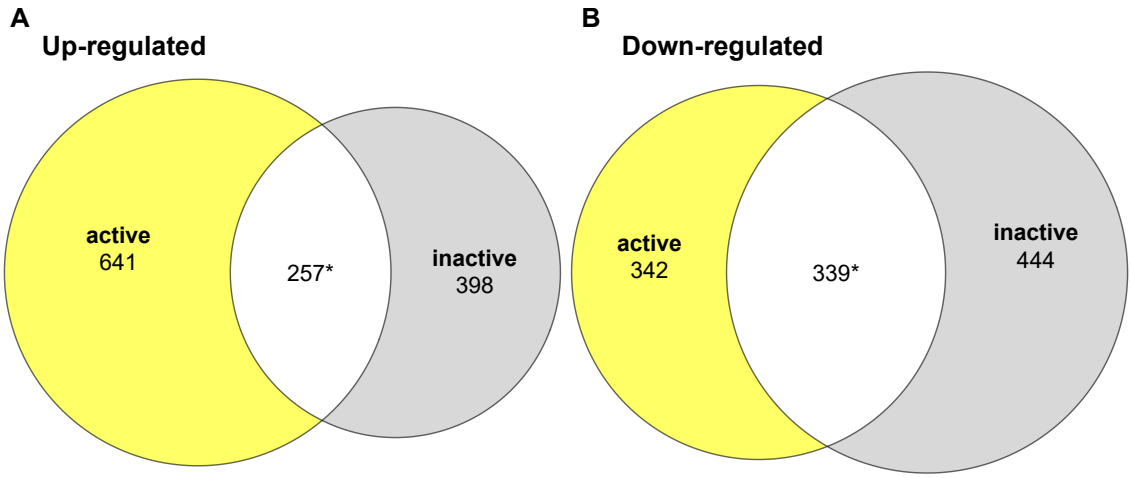

Figure 5.2. Euler diagrams comparing changes in mRNA-seq gene expression profiles in fat body with age in *Bombus terrestris* workers (Experiment A). Ovary-active workers (yellow, N = 38) were compared to ovary-inactive workers (grey, N = 15). Plotted are the number of DEGs that changed expression between time point 1 (2 weeks in age) and time point 2 (7 weeks in age) and the extent of the overlap between the two worker phenotypes (white area). Asterisks (*), significant overlap in DEGs (Fisher's exact test, $p < 0.05$ after Bonferroni correction). Up-regulated DEGs: DEGs significantly more expressed in TP2 than TP1, i.e. that increased expression with age; down-regulated DEGs: DEGs significantly more expressed in TP1 than TP2, i.e. that decreased expression with age. Sample sizes: active: TP1 (N = 24 workers), active: TP2 (N = 14 workers), inactive: TP1 (N = 10), inactive: TP2 (N = 5).

Gene Ontology

Age-related DEGs were matched to orthologues in the *D. melanogaster* genome. GO terms derived from up-regulated DEGs that were found only in ovary-active workers were associated with nucleotide metabolic processes, muscle cell differentiation, and nervous system processes. GO-terms that were derived from down-regulated DEGs that were found only in ovary-active workers were associated (among others) with catabolic processes (11/53), metabolic processes (11/53), cellular respiration (11/53), vesicular transport (9/53), symbiont-cell interactions (4/53), viral processes (2/53), and cell redox homeostasis (1/53). Up-regulated GO terms that were found only in ovary-inactive workers were associated (among others) with ion transport (9/37), metabolic processes (5/37), muscle cell differentiation (5/37), DNA-complex organisation (4/37), (epigenetic) regulation of gene expression (4/37), negative regulation of biosynthetic process (3/37), and behaviour (2/37). Down-regulated GO terms that were found only in ovary-inactive workers were associated (among others) with metabolic processes (23/115), cellular respiration (16/115),vesicular transport (15/115), catabolic processes (10/115), protein localization (8/115), responses to mis- or unfolded proteins (6/115), protein maturation and modification (5/115), symbiont-cell interactions (4/115), and viral processes (2/115).

Overlap with ageing-related gene networks and gene sets

Some of the age-related genes in worker fat body in Experiment A overlapped with ageing-related genes in the TI-J-LiFe network (Korb et al., 2021). In the ovary-active workers, 8 of the TI-J-LiFe network genes were up-regulated with age and 3 of these genes were down-regulated with age (**Figure 5.3A**). In ovary-inactive workers, 4 of the TI-J-LiFe network genes were up-regulated with age and 7 of these genes were down-regulated with age (**Figure 5.3A**). Three of the up-regulated genes and 2 of the down-regulated genes were found in both worker phenotypes. However, none of these overlaps were statistically significant (Fisher's exact test; **Table S5.2A)**).

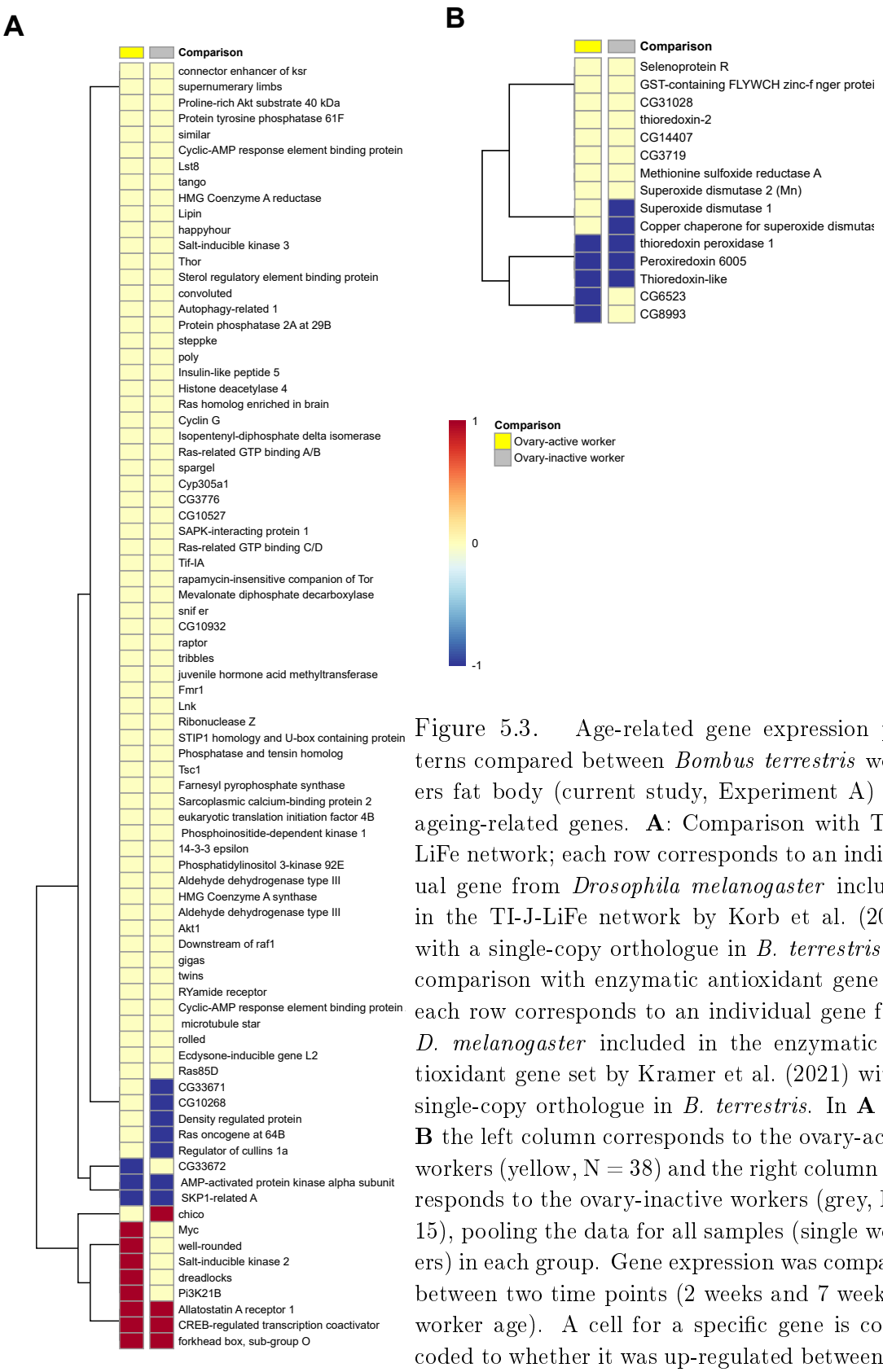

Figure 5.3. Age-related gene expression patterns compared between *Bombus terrestris* workers fat body (current study, Experiment A) and ageing-related genes. **A**: Comparison with TI-J-LiFe network; each row corresponds to an individual gene from *Drosophila melanogaster* included in the TI-J-LiFe network by Korb et al. (2021) with a single-copy orthologue in *B. terrestris*; **B**: comparison with enzymatic antioxidant gene set; each row corresponds to an individual gene from *D. melanogaster* included in the enzymatic antioxidant gene set by Kramer et al. (2021) with a single-copy orthologue in *B. terrestris*. In **A** and **B** the left column corresponds to the ovary-active workers (yellow, N = 38) and the right column corresponds to the ovary-inactive workers (grey, N = 15), pooling the data for all samples (single workers) in each group. Gene expression was compared between two time points (2 weeks and 7 weeks in worker age). A cell for a specific gene is colour coded to whether it was up-regulated between the two time points (red), or down-regulated (blue) or not differently expressed (beige). The dendogram at the left groups genes that cluster according to their gene expression patterns.

Similarly, some of the age-related genes in worker fat body in Experiment A overlapped with ageing-related genes in the enzymatic antioxidant gene set (Kramer et al., 2021). In the ovary-active workers, 0 of these antioxidant genes were up-regulated and 5 of these antioxidant genes were down-regulated, with the overlap for down-regulated genes being significant (Fisher's exact test; **Figure 5.3B**; **Table S5.2B)**). In ovary-inactive workers, 0 of these antioxidant genes were up-regulated and 5 of these antioxidant genes were down-regulated, with the overlap for down-regulated genes being significant (Fisher's exact test; **Figure 5.3B**; **Table S5.2B)**). Of these down-regulated genes, 3 were the same for ovary-active and ovary-inactive workers (associated to peroxidase activity).

Some overlap with both networks/ genes sets occurred for both phenotypes of workers. There was some similarity in these overlaps between the phenotypes, but also some genes that were only found differently expressed in one of the phenotypes.

5.3.2 Experiment B: Stages of Ovary Activation in Workers

Differential Gene Expression between the Three Time Points

In all three tissues (brain, fat body, ovary), gene expression differed strongly between the three time points at which the samples were taken (24 hours, 96 hours and egg-laying). This was evidenced by most samples clustering according to time point in different regions of the PCA plot in the PCA for each tissue (**Figure 5.4**). In brain, there was high variance between the biological replicates of the 24 h time point, whereas replicates within each of the other two time points clustered more closely together (excepting one replicate of the egg-laying time point) (**Figure 5.4A**). In fat body and ovary, replicates clustered more closely in each time point (excepting one replicate of the 96 h time point in each case) (**Figure 5.4B,C**). In sum, in all three tissues, PCA both showed consistency of replicates and evidenced strong changes in gene expression with worker age and the progression of ovary activation.

PCA also showed that samples from the two later time points, 96 h and egg-laying, tended to cluster together, and separately from the 24 h time point, especially for fat body and ovary (**Figure 5.4**) and this was reflected in the numbers of DEGs between time points in each tissue (**Figure 5.5**). Specifically, in brain there were 690-1311 DEGs between the 24 h and 96 h time points, but only 1-4 DEGs between the 96 h and egg-laying time points (**Figure 5.5**). Similarly, in fat body, there were 1064-1205 DEGs between the 24h and 96h time points, and 124-186 DEGs between the 96 h time point and the egg-laying time point (**Figure 5.5**). Finally, in ovary, there were 3277-3585 DEGs between the 24 h and 96 h time points, and 862-1146

(182-356 with EEGs excluded) DEGs between the 96 h and egg-laying time points (**Figure 5.5**). These data showed that, in addition, across all three tissue, total numbers of age-related DEGs were least in brain, higher in fat body and highest in ovary, showing that the greatest change in age-related gene expression over the entire time span sampled occurred in this tissue (**Figure 5.5**). Overall, therefore, within tissues, the greatest change in age-related gene expression occurred in the earlier stages of ovarian activation (between 24 h and 96 h after microcolony initiation).

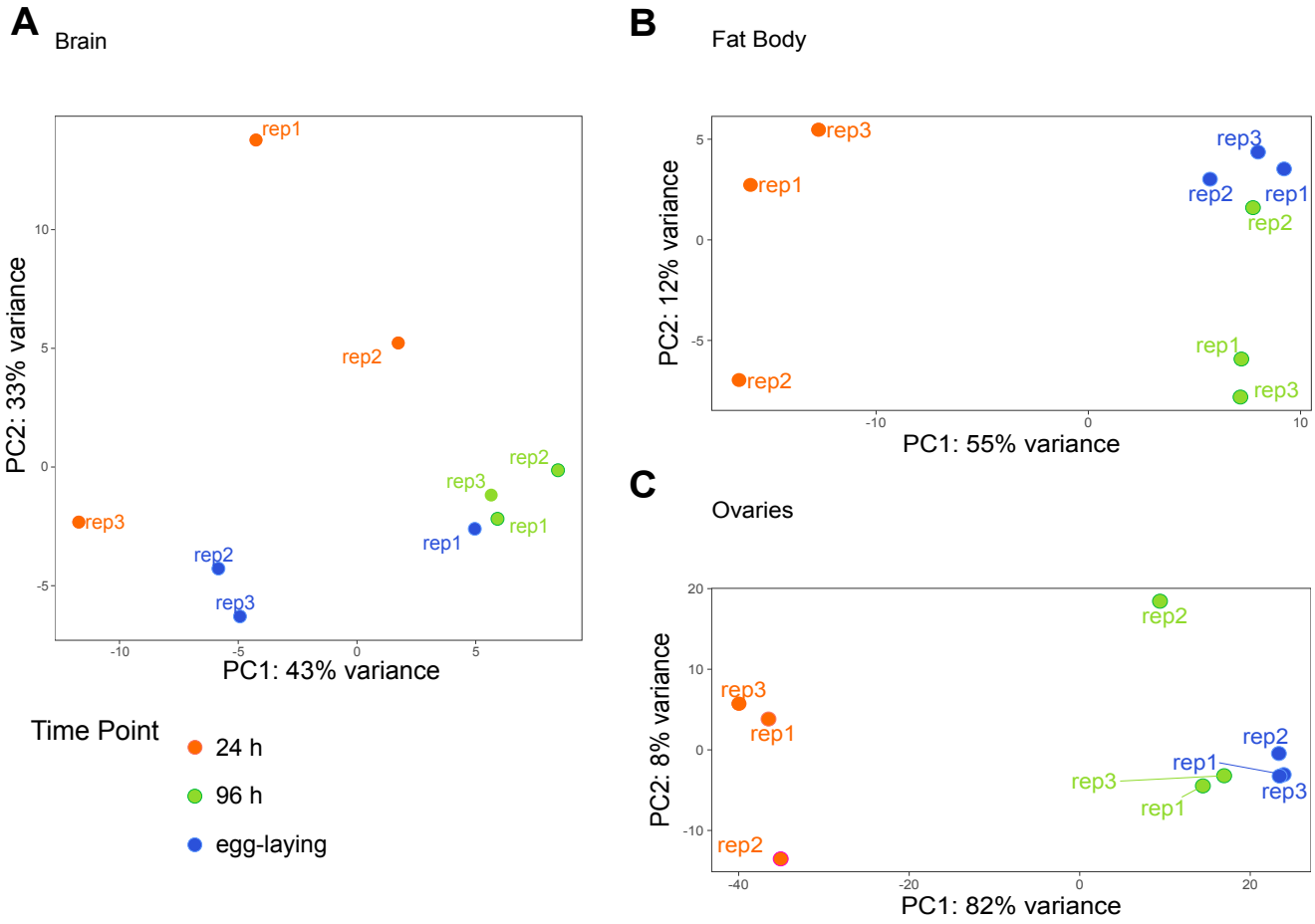

Figure 5.4. Principal component analysis (PCA) plot of mRNA-seq libraries from three tissues of *Bombus terrestris* workers activating their ovaries in queenless microcolonies, sampled at three different time points (Experiment B). Axes represent principal components describing gene expression levels; individual points represent biological replicates (pooled tissue from 10 workers), coloured by time point. Time points: 24h (orange): 24 hours (1 day) after microcolony initiation; 96h (green): 96 hours (4 days) after being placed in the microcolony; lay (blue): 7 days after being placed in the microcolony and laying eggs. **A**: Brain. **B**: Fat body. **C**: Ovary.

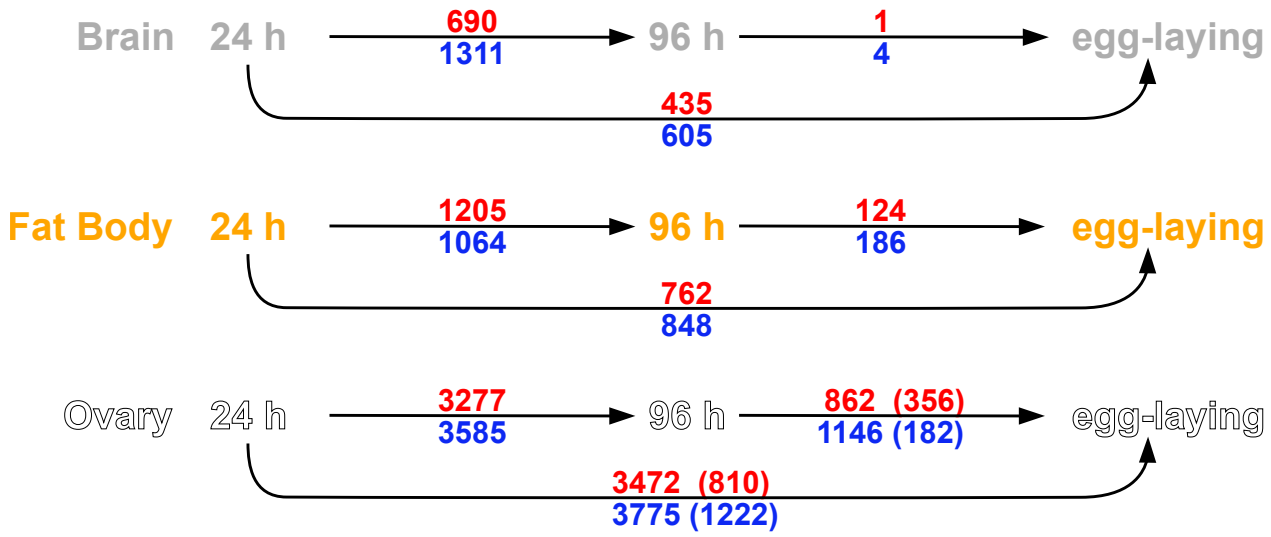

Figure 5.5. Comparison of mRNA-seq gene expression proles in brain, fat body and ovary of *Bombus terrestris* workers in queenless microcolonies along the time line of ovary activation on the basis of differentially expressed genes (DEGs) (Experiment B). Time points: 24 h: 24 hours (1 day) after microcolony initiation; 96 h: 96 hours (4 days) after being placed in the microcolony; egg-laying: 7 days after being placed in the microcolony and laying eggs. In each time point data from three biological replicates was pooled, each replicate consisting of pooled tissue from 10 workers. Arrows indicate the direction of the comparison. Number on top of the arrows (red): up-regulated DEGs (more expressed in the direction of the arrow); number below the arrow (blue): down-regulated DEGs (less expressed in the direction of the arrow). Numbers in parentheses are numbers of DEGs with the egg-expressed genes (EGGs) removed.

Top-50 DEGs

In brain, among the top-50 DEGs, genes associated with phospholipase A2 (venom), pupal cuticle protein, lysine metabolism, keratin, chitin hydrolysis, actin and myosin, troponin, glucose metabolism, and synthesis of fatty alcohols (Teerawanichpan et al., 2010) were more highly expressed at the 24 h time point than at the other two time points (**Figure S5.2A**). Genes associated with *vitellogenin*, *odorant receptor 13a*, and *hexamerin* were least expressed at the 24 h time point, more expressed at the 96 h time point and most highly expressed at the egg-laying time point (**Figure S5.2A**). Genes associated with tissue morphogenesis, venom, the glycine decarboxylase complex, glucocerebrosidase, and the homeotic protein Sex combs reduced, were most expressed in the egg-laying time point and least expressed in the 96 h time point (**Figure S5.2A**). In fat body, among the top-50 DEGs, genes associated with chitin hydrolysis, glucose metabolism, and proteasome as-

sembly were more highly expressed in the 24h-time point than in the other two time points (**Figure S5.2B**). Genes associated with the mitotic spindle, cholesterol metabolism, cuticle proteins immune response (González-Santoyo and Córdoba-Aguilar, 2012), protein catabolism, neuroparsin, DNA- binding, juvenile hormone synthesis (Cardoso-Júnior et al., 2017), and histones, were little expressed in the 24 h time point and highly expressed in the other two time points (**Figure S5.2B**). Lastly, in ovary, among the top-50 DEGs, a gene associated with the 40S ribosomal protein was highly expressed in the egg-laying time point but not in the other two time points (**Figure S5.2C**). Genes associated with cuticle proteins, chitin hydrolysis, vesicle tracking, proteostasis, aminopeptidase activity (EEGs), neuroparsin (EEG), and trehalose transport, showed little expression in the 24 h time point and more highly expressed in the other two time points (**Figure S5.2C**). Genes associated with a transmembrane protease, a phospholipase, the venom bombolitin 1 and the 40S ribosomal protein were more strongly expressed in the 24 h time point than in the other two time points (**Figure S5.2C**).

Differential Gene Expression between Tissues

Age-related DEGs were compared between the three tissues. In the DEGs between the 24 h time point and the 96 h time point, 21 DEGs were up-regulated and 17 DEGs were down-regulated in all three tissues. Between brain and fat body, 49 up-regulated DEGs and 61 down-regulated DEGs were shared and this level of overlap was significant (**Table S5.3**) with 2.1% of up-regulated and 4.5% of down-regulated brain DEGs and 2.6% of up-regulated and 3.6% of down-regulated fat body DEGs being exclusively shared between these two tissues (**Figure 5.6A,D**). Between brain and ovary, 468 up-regulated DEGs and 361 down-regulated DEGs were shared and this level of overlap was significant (**Table S5.3**), with 34.1% of up-regulated and 35.5% of down-regulated brain DEGs and 12.5% of up-regulated and 10.5% of down-regulated ovary DEGs being exclusively shared between these two tissues (**Figure 5.6A,D**). Between fat body and ovary 405 up-regulated DEGs and 359 down-regulated DEGs were shared and this level of overlap was significant (**Table S5.3**), with 36.0% of up-regulated and 28.4% of down-regulated fat body DEGs and 10.7% of up-regulated and 10.4% of down-regulated ovary DEGs being exclusively shared between these two tissues (**Figure 5.6A,D**). The percentage overlap was therefore highest between brain and ovary.

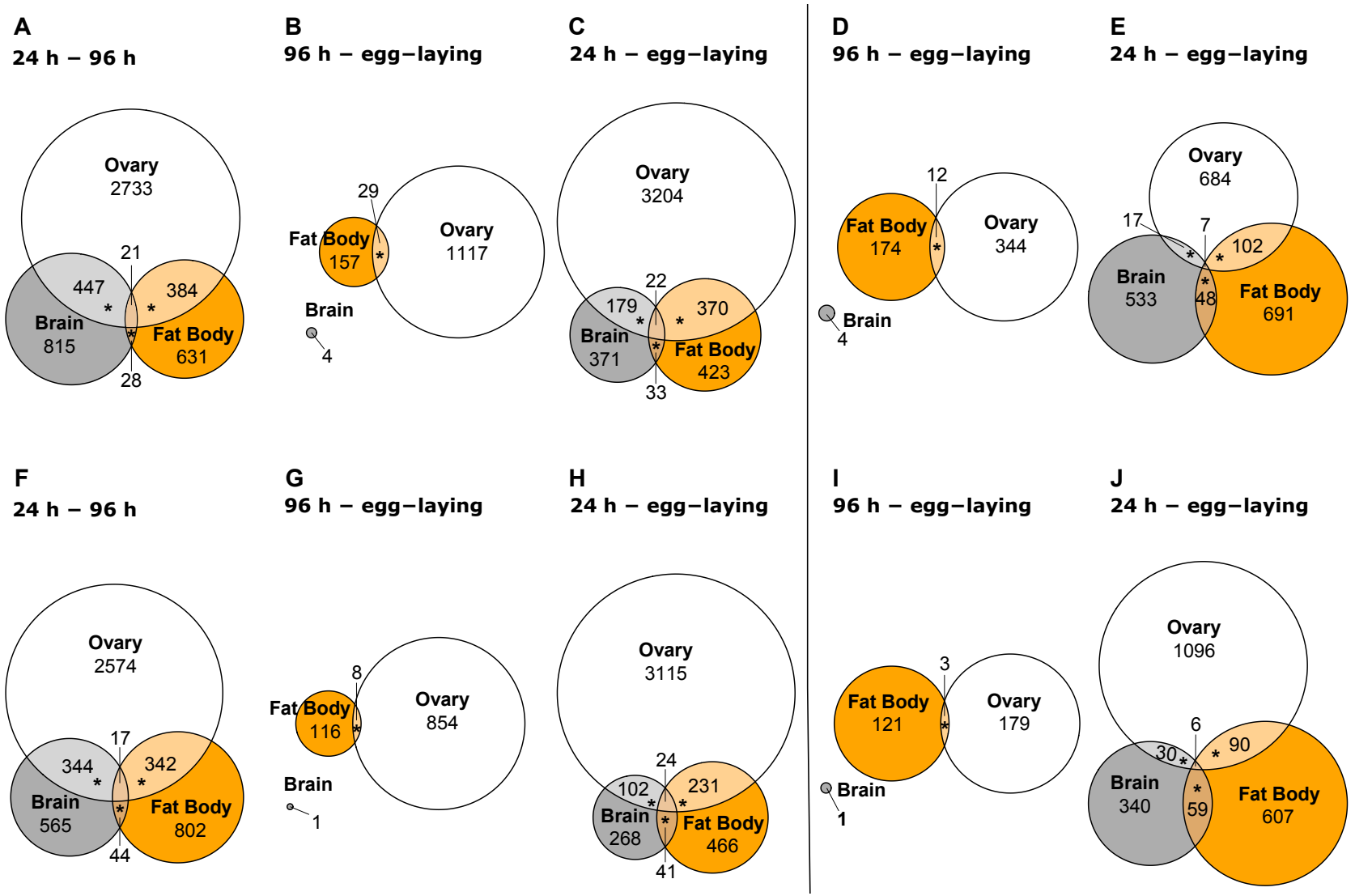

Figure 5.6. Euler diagrams comparing changes in mRNA-seq gene expression profiles between three time points in three tissues of *Bombus terrestris* workers activating their ovaries in queenless microcolonies (Experiment B). Plotted are the number of DEGs that changed expression in pairwise comparisons of the three time points (24 h, 96 h after microcolony initiation and at first egg-laying) and the extent of the overlap between the tissues in each comparison. Asterisks (*), significant overlap in DEGs (Fisher's exact test, $p < 0.05$ after Bonferroni correction, applied only to two-way comparisons between tissues). **A-E**: Up-regulated DEGs. DEGs significantly more expressed in the second time point of the comparison, i.e. that increased expression with age. **F-J**: Down-regulated DEGs. DEGs significantly more expressed in the first time point of the comparison, i.e. that decreased expression with age. **A-C** & **F-H**: Comparisons with the egg-laying time point include egg expressed genes (EEGs) in the ovary DEG-lists. **D-E** & **I-J**: EEGs are excluded from the ovary DEG-lists. Sample sizes: 3 biological replicates (tissue pooled form 10 workers) for each tissue in each time point.

In the DEGs between the 96 h time point and the egg-laying time point, no DEGs were shared between all three tissues. Between fat body and ovary, 15.6% (6.4% with EEGs excluded) of up-regulated and 6.4% (2.4%) of down-regulated fat body DEGs and 2.5% (3.4%) of up-regulated and 0.9% (1.6%) of down-regulated ovary DEGs, were shared and this level of overlap was significant (**Table S5.4**, **Figure 5.6B,E**). There was therefore less overlap between fat body and ovary between the 96 h time point and the egg-laying time point than there was between the 24 h time point and the 96 h time point.

In the DEGs between the 24 h time point and the egg-laying time point, 22 DEGs (7 with EEGs excluded) were up-regulated and 24 DEGs (6 with EEGs excluded) were down-regulated in all three tissues. Between brain and fat body, 55 up-regulated DEGs and 65 down-regulated DEGs were shared and this level of overlap was significant (**Table S5.5**), with 5.4% (7.9% with EEGs excluded) of up-regulated and 9.4% (13.6%) of down-regulated brain DEGs and 3.9% (7.0%) of up-regulated and 5.4% (7.7%) of down-regulated fat body DEGs being exclusively shared between these two tissues (**Figure 5.6C,F**). Between brain and ovary, 201 up-regulated DEGs (24) and 126 down-regulated DEGs (36) were shared and this level of overlap was significant (**Table S5.5**), with 33.2% (3.9%) of up-regulated and 28.9% (8.3%) of down-regulated brain DEGs and 5.3% (3.0%) of up-regulated and 3.6% (2.9%) of down-regulated ovary DEGs being exclusively shared between these two tissues (**Figure 5.6C,F**). Between fat body and ovary, 392 up-regulated DEGs (109) and 255 down-regulated DEGs (96) were shared and this level of overlap was significant (**Table S5.5**), with 43.6% (12.0%) of up-regulated and 30.3% (11.8%) of down-regulated fat body DEGs and 9.8% (12.6%) of up-regulated and 6.6% (7.4%) of down-regulated ovary DEGs being exclusively shared between these two tissues (**Figure 5.6C,F**). In the comparison of the 24 h time point (the start of ovary activation) and the egg-laying time point (the final time point in oocyte maturation), i.e. the comparison representing the entire time span of ovary activation, the largest percentage overlap was again found in the fat body shared with the ovary, even with EEGs excluded.

Generally, therefore, there were very few DEGs that were shared between all three tissues in all three time point comparisons, yet there were larger overlaps between any two of the tissues (except in the 96 h to egg-laying comparison).

Gene Ontology

Gene Ontology (GO) analysis was conducted using OrthoFinder to focus on the biological functions of the DEGs and compare them between the three time points.

Between *B. terrestris* and *D. melanogaster*, OrthoFinder detected in 5,928 single orthologues (49.28% of the 12,028 genes expressed across the analysed *B. terrestris* mRNA-seq libraries). Using these, 851 non-redundant enriched GO terms were isolated from the DEG-lists generated from the comparisons between the time points in each tissue.

In the 24 h time point vs. 96 h time point comparison, 0 up-regulated GO terms (i.e. terms derived from up-regulated DEGs) and 21 down-regulated GO terms (i.e. terms derived from down-regulated DEGs) associated with metabolism were shared between brain and fat body. Brain and ovary shared 79 up-regulated GO terms notably development, cell cycle, and oocyte development and 17 down-regulated GO terms (mainly biosynthesis and metabolism). Fat body and ovary shared 0 up-regulated GO terms and 4 down-regulated GO terms related to cell and neuron recognition. Brain-specific GO terms (all up-regulated, 253 total) were primarily associated with development, signalling, cytoskeleton, and neurogenesis. Fat body-specific GO terms (all down-regulated, 307 total) were mostly associated with development, signalling, and motility. Ovary-specific GO terms (all up-regulated, 8 total) were associated with cytoskeleton, morphogenesis, oocyte development, metabolism, and immunity, with one down-regulated GO term associated with neural development (**Table OS5.1**).

In the 96 h time point vs. egg-laying time point comparison, three up-regulated GO terms associated with morphogenesis and neural development were shared between fat body and ovary. Additionally, two up-regulated GO terms (RNA splicing and metabolism) were unique to the fat body, while two down-regulated terms with similar functions were unique to ovary (**Table OS5.1**).

In the 24 h time point vs. egg-laying time point comparison, 0 up-regulated GO terms and 5 down-regulated GO terms (associated with metabolism and cellular respiration) were shared between brain and fat body. Between fat body and ovary, 0 up-regulated GO terms and two down-regulated GO terms (associated with nucleotide biosynthesis) were shared. Brain-specific GO terms (all up-regulated, 14 total) were mainly associated with eye development, other developmental processes, and cytoskeleton/motility. Fat body-specific GO terms included 20 down-regulated (development, cytoskeleton, metabolism, transport) and 6 up-regulated (metabolism, peptide biosynthesis, translation). Ovary-specific GO terms (all up-regulated, 6 total) were associated with metabolism, catabolism, transport, and endocytosis (**Table OS5.1**).

Overall, the up-regulated GO terms in all three tissues were mainly connected to different developmental processes, meaning that genes underpinning these processes

increased their expression with time. There were also GO terms connected to a down-regulation of gene expression found in all three tissues in comparisons to the 24 h time point, which was potentially a sign of metabolic activity for the relevant processes slowing with time.

5.4 Discussion

Using mRNA-sequencing, gene expression differences associated with ovary activation in *B. terrestris* workers were analysed in two experiments. Experiment A focused on the molecular basis of the distinction between ovary-active and ovary-inactive workers, characterizing differential gene expression in fat body between these two phenotypes, and, within them, age-related gene expression differences (as workers were sampled at worker ages of 2 weeks and 7 weeks). Experiment B focused on the molecular basis of the process of ovary activation in egg-laying workers, investigating changes in gene expression in brain, fat body and ovary of workers in queenless microcolonies during the process of ovary activation (as focal workers were sampled at three time points, i.e. 1, 4 and (in egg-laying workers) 4-9 days after microcolony initiation, corresponding to worker ages of 4, 7 and 7-12 days post eclosion, respectively).

Experiment A revealed differences in gene expression in the fat body between ovary-active and ovary-inactive workers, which increased with age. Such an increasing difference in gene expression patterns with age is evidence for different processes being activated and inactivated with age depending on ovarian activity, which are very likely to be connected to the positive fecundity-longevity relationship in reproductive workers. Furthermore, the majority of age-related DEGs was exclusively found in either phenotype, making these DEGs interesting target genes for future studies aiming to investigate the molecular basis of ageing differences between reproductive and non-reproductive workers. In addition, the results of Experiment B suggest that differences in gene expression due to ovarian activation arise in the early stages of that process. Further, it was revealed that there were some genes that were equally affected in more than one tissue, making these sets of genes additional valuable target genes for further investigations into the positive fecundity-longevity relationship of reproductive *B. terrestris* workers and other female eusocial Hymenoptera.

5.4.1 Experiment A: Ovary-active vs. Ovary-inactive Workers

Differential Gene Expression between Ovary-Active and Ovary-Inactive Workers

Previous studies using mRNA-seq to profile gene expression differences between ovary-active and ovary-inactive workers at the tissue level in *B. terrestris* include those of Marshall et al. (2019) (who sampled head tissue from workers aged 6 days) and Prince et al. (2024) (who sampled brain, fat body and ovary from workers aged c. 4 weeks). *Vitellogenin* is a key gene in the reproduction of insects as vitellogenin is an egg-yolk precursor protein which is secreted to the haemolymph and then taken up by developing oocytes (Robinson and Vargo (1997); Bloch and Grozinger (2011), **Section 1.2.1**). Its production is regulated by juvenile hormone, also making it part of the TI-J-LiFe network (Korb et al., 2021). Marshall et al. (2019) found that *vitellogenin* (gene ID: LOC100650436) was among the most up-regulated genes in head of reproductive *B. terrestris* workers, but, in Experiment A of the current study, *vitellogenin* did not appear in any of the (fat body) DEG lists (**Table OS5.2**) and *vitellogenin-like precursor* appeared to be down-regulated with age in ovary-active workers (**Table OS5.2**). However, Prince et al. (2024) found *vitellogenin* to be up-regulated in both brain and fat body of ovary-active workers. Using qRT-PCR of single genes, Lockett et al. (2016), who sampled workers aged c. 2-6 weeks, also found *vitellogenin* to be up-regulated in fat body of ovary-active workers. Moreover, Lockett et al. (2016) found that, in brain, *vitellogenin* was up-regulated in ovary-active workers but only in younger workers, with the difference between *vitellogenin* expression between ovary-active and ovary-inactive workers falling with worker age (from c. 2 to 6 weeks of age). Therefore, with respect to *vitellogenin*, the results of Marshall et al. (2019) were consistent with those of Lockett et al. (2016) and, to some extent, those of Prince et al. (2024). In addition, in the current study, the absence of up-regulation of *vitellogenin* in ovary-active workers could have stemmed from age effects similar to those detected by Lockett et al. (2016), at least among workers aged 7 weeks, which would also be consistent with the study's finding that *vitellogenin-like precursor* was down-regulated with age in ovary-active workers.

Marshall et al. (2019) also found two genes coding for *serine-protease inhibitors* to be up-regulated in ovary-active *B. terrestris* workers compared to ovary-inactive workers and hence argued that this might be linked to reproduction (Bao et al., 2014). By contrast, in the current study, the gene *serine protease inhibitor 3/4-like* was found to be up-regulated with age only in ovary-inactive workers and accord-

ingly the gene *serine protease snake* was down-regulated with age in ovary-inactive workers. Furthermore, the gene *serine protease nudel* was up-regulated with age in ovary-active workers. Given the age differences between the sampled workers across the studies, it is possible that these differences between the findings of **?** and the current study also resulted from worker age effects, although differences between the sampled tissues cannot be ruled out.

Age-related Differential Gene Expression between the Worker Phenotypes

Within each of the ovary-active and ovary-inactive worker phenotypes there was a large difference in gene expression in fat body with age (655-898 DEGs, **Figure 5.1**). In addition, differences in gene expression between ovary-active and ovary-inactive workers were greater at seven weeks (TP2) than at two weeks (TP1), showing that the difference in gene expression in workers due to ovarian activity and egg-laying increased with age.

Both ovary-active and ovary-inactive workers exhibited age-related DEGs, and significant overlaps between their gene sets showed that some of these age-related DEGs were shared, but the proportion of age-related DEGs exclusive to each phenotype (50.2-71.4%) exceeded the proportion shared (28.6-49.8%) (**Figure 5.2**). The shared age-related DEGs in this context presumably include those underpinning ageing in workers independently of the workers' reproductive status. These genes therefore represent good candidates for key ageing-related genes (i.e. genes that directly affect ageing) in *B. terrestris* and, by extension, other eusocial Hymenoptera (**Section 3.4.3**). However, the fact that the age-related DEGs exclusive to one or the other worker phenotype formed the majority of the age-related DEGs suggests that the molecular basis of ageing may differ to some extent between ovary-active and ovary-inactive workers. This suggestion was also supported by the finding of enriched GO terms derived from the age-related DEGs exclusive to one or the other worker phenotype, indicating that some of the biological processes underlying ageing differed between ovary-active and ovary-inactive workers. Combined, these results were consistent with other findings of the thesis that ovary-active workers represent, on average, workers of higher quality whose longevity determination differs from that of workers as a whole (**Chapter 2**). Moreover, the age-related DEGs that exclusively occurred in ovary-active workers (342-641 DEGs; **Figure 5.2**) represent potential candidates for genes underpinning the positive fecundity-longevity relationship reported for reproductively active workers in *B. terrestris* (Blacher et al. (2017); **Chapter 2**) and, again by extension, in other reproductive females in euso-

cial Hymenoptera. Relevant to this, the fact that some of the GO terms exclusively found in ovary-inactive workers were connected to down-regulated genes associated with responses to mis- or unfolded proteins and protein maturation hints at protein control becoming less effective with age in ovary-inactive workers being a contributor to their reduced longevity.

Comparison with Ageing-related Gene Networks and Gene Sets

Although there was no statistically significant overlap of the age-related DEGs in Experiment A of the current study with the ageing-related genes in the TI-J-LiFe network (Korb et al., 2021), some of the network genes were found to be up-regulated or down-regulated with age in the *B. terrestris* workers (**Figure 5.3A**). They included genes that were age-related exclusively in one or the other worker phenotypes, which again suggested that the molecular basis of ageing may differ to some extent between ovary-active and ovary-inactive workers. In detail, there were 5 TI-J-LiFe network genes that were present only in the up-regulated DEGs of ovary-active workers, and these have functions as growth-promoting transcription factors (*Myc*) (Johnston et al., 1999), in enabling protein phosphatase activator activity (*well-rounded*) (Viquez et al., 2006), in enabling protein serine/threonine kinase activity (*Salt-inducible kinase 2*) (Choi et al., 2011), or as genes involved in the insulin signalling pathway (*dreadlocks* (Song et al., 2003) and *Pi3K21B* (Britton et al., 2002)). The gene dreadlocks has also been found to be up-regulated with age in *B. terrestris* queens with age (Collins et al., 2023). The same study found *Pi3K21B* to be down-regulated with age in queens (Collins et al., 2023), suggesting that the role of this gene differs between ovary-active workers and queens. There was only one TI-J-LiFe network gene (*CG33672*, uncharacterised) that was down-regulated with age only in ovary-active workers. In ovary-inactive workers, there was one TI-J-LiFe network gene (*chico*, uncharacterised) that was up-regulated with age only in these workers. There were five TI-J-LiFe network genes that were down-regulated only in ovary-inactive workers, and these have functions in enabling mevalonate kinase activity (*CG33671* and *CG10268*) (Faust et al., 2012), in enabling translation initiation factor activity (*Density regulated protein*) (Schleich et al., 2014), as genes involved in the Ras GTPase signalling pathways (*Ras oncogene at 64B*) (Mirey et al., 2003) and as genes involved in cell population proliferation (*Regulator of cullins 1a*) (Noureddine et al., 2002). These 12 genes of the TI-J-LiFe network (Korb et al., 2021), which were differentially expressed with age in one or other phenotype only, are therefore of particular interest as potential candidates for genes associated with the differences observed in ageing and longevity between ovary-active and ovary-inactive

workers (**Chapter 2**).

The comparison of the age-related DEGs in the current study with the ageing-related enzymatic antioxidant gene set (Kramer et al., 2021) resulted in a significant overlap between genes in this gene set and the age-related DEGs. This comprised 5 down-regulated genes in the ovary-active workers and 5 down-regulated genes in the ovary-inactive workers, with three of these genes being found in both phenotypes (**Figure 5.3B**). These three genes may therefore have a general involvement with ageing irrespective of workers' reproductive status. Correspondingly, the occurrence of genes in the ageing-related enzymatic antioxidant gene set exclusive to one or other of the worker phenotypes was again consistent with the molecular basis of ageing differing between the phenotypes. The two genes of the ageing-related enzymatic antioxidant gene set that were down-regulated with age in ovary-active workers only and were not differentially expressed in the ovary-inactive workers (*CG6523* and *CG8993*) have roles in enabling the activity of the protein-glutathione oxidoreductase (Mondal and Singh, 2022) and potentially the protein-disulfide reductase (Svensson and Larsson, 2007). These two genes were also found to be down-regulated with age in *B. terrestris* queens in ovary although not in fat body (Collins et al., 2023). This finding hints at these two genes being involved in the positive fecundity-longevity relationship found in queens and reproductive workers in *B. terrestris* (Blacher et al. (2017); Collins et al. (2023); **Chapter 2**) and possibly other eusocial Hymenoptera. The two genes of the ageing-related enzymatic antioxidant gene set that were down-regulated with age in ovary-inactive workers only and were not differentially expressed in the ovary-active workers were *Superoxide dismutase 1* and *Copper chaperone for superoxide dismutase. Superoxide dismutase 1* was found to be down-regulated with age in fat body of *B. terrestris* queens manipulated to increase their rate of egg-laying (Collins et al., 2023). Unlike unmanipulated queens, these manipulated queens also expressed costs of reproduction (Collins et al., 2023), suggesting that *Superoxide dismutase 1* is involved in condition-dependence in fecundity-longevity relationships.

Overall, therefore, these comparisons identified some ageing-related genes of interest that seem to be differentially expressed with age in relation to workers' reproductivity status. A potential rewiring of gene pathways underpinning ageing has been suggested to be the potential mechanism behind the positive relationship between fecundity and longevity in eusocial insect queens and reproductive workers (Korb et al., 2021). The genes of interest identified by the current study represent potential players in this process and hence candidates for further investigation in this respect.

5.4.2 Experiment B: Stages of Ovary Activation in Workers

Differential Gene Expression between the three time points

The results showed that, in the process of ovary activation in recently queenless, young *B. terrestris* workers induced to activate their ovaries and lay eggs, the greatest change in gene expression occurred in the first 4 days (96 h) of ovary activation from an ovary-inactive state. This conclusion was based on clustering patterns in the PCA and counts of DEGs between the three time points sampled up to and including the first egg-laying, with gene expression at the 24 h time point differing most from gene expression at the other two time points (at 96 h and at first egg-laying at 4-9 days after microcolony initiation, which were more similar to one another) (**Figures 5.4**, **5.5**). This overall pattern was found in all three tissues sampled (brain, fat body and ovary) and was most extreme in brain (**Figure 5.5**). and was reflected in the top-50 most differently expressed genes for each tissue (**Figure S5.2**). In the top-50 list of the ovary, the replicates from the 24 h time point showed similar expression patterns (e.g. in *neuroparsin A*) as was found in 20-35 day old ovary-inactive *B. terrestris* workers (Prince et al., 2024). This allows the assumption that the changes in gene expression between the three time points in the current study (Experiment B) was largely related to the process of ovary activation, rather than a change in worker age.

The high numbers of up- and down-regulated DEGs between workers at the first two time points (24 h and 96 h) (**Figure 5.5**) were likely to have reflected the fact that, in these relatively young, recently queenless workers, maturation was still ongoing. Accordingly, enriched GO terms found in the 24 h time point to 96 h time point comparison were largely connected to morphogenesis, development and cytoskeleton organisation. The strongest change in gene expression between the 24 h and 96 h time points was found in the ovary, indicating maturation and activation of that tissue (**Figure 5.5**). The ovaries of all workers included in the 96 h time point had an ovary activation score of 3 (Duchateau, 1989), which was defined as representing intermediate ovary activation (with ovaries scoring 4 or more being defined as representing active ovaries). Workers at the 96 h time point had therefore almost completed the process of ovary activation, which is likely to have been the reason why gene expression changed relatively less from this time point onwards, i.e. between this time point and the egg-laying time point. Nonetheless, in ovary specifically, there were relatively high DEG counts between the 96 h time point and the egg-laying time point (**Figure 5.5**), which was almost certainly connected to the maturation of the oocytes. This conclusion was supported by the fact that DEG

counts between the 96 h time point and the egg-laying time point in ovary were greatly reduced when the egg-expressed genes (EEGs) were removed from the DEG lists (**Figure 5.5**).

In the comparison of the 24 h time point and the egg-laying time point there were some GO terms derived from down-regulated DEGs connected to metabolic and catabolic functions, in line with the results of Experiment A, that suggested a slowing of the metabolism with age. Yet, there were also GO terms derived from up-regulated DEGs connected to metabolic and catabolic processes. The oldest workers in Experiment B were only around 11-12 days in age, whereas the oldest workers in Experiment A were 7 weeks old, comparing age-related changes in gene expression is therefore inconclusive.

The study of Prince et al. (2024) analysing gene expression of 20-35 day old *B. terrestris* workers found relatively little difference in gene expression in the brain between ovary-active and ovary-inactive workers, consistent with the small amount of gene expression change in brain as workers proceeded from the state (at 96 h) just prior to ovary activation to egg-laying (**Figure 5.5**). Combined, these findings could be evidence that becoming an egg-layer has very little effect on gene expression in brain in worker *B. terrestris*. This is perhaps surprising as egg-laying is associated with the appearance of other behaviours in egg-laying workers, namely aggression to the queen and other workers and egg-eating (e.g. (Duchateau and Velthuis, 1989; Zanette et al., 2012; Almond et al., 2019). However, it matches studies that have suggested that the physiological pathways affecting aggression, ovary activation and egg laying in *B. terrestris* workers may act partly independently of one another (van Doorn, 1987; Duchateau and Velthuis, 1989; Amarasinghe et al., 2014; Amsalem et al., 2014).

The fact that the highest change in gene expression occurred between the 24 h time point and the 96 h time point makes these early stages of ovary activation, which are therefore also the first steps towards reproductive activity, of particular interest. Interestingly, the gene ontology analysis found enriched GO terms in the 24 h time point to 96 h time point comparison in the brain and the ovary associated with oocyte development (up-regulated). Although ovary activation in workers does not necessarily mean that they will become egg-layers (Duchateau and Velthuis, 1989), the genes that were differentially expressed between the 24 h and the 96 h time points might therefore be further candidates for genes involved in driving the positive fecundity-longevity relationship found in reproductively active workers (Blacher et al. (2017); **Chapter 2**).

Differential Gene Expression between Tissues

The number of DEGs shared between all three tissues was relatively low for all of the three comparisons between the time points (**Figure 5.6**). Yet in most comparisons, there were relatively large overlaps of DEGs between any two tissues, though such overlaps were greater between the 24 h and 96 h time points than between the 96 h and egg-laying time points (with this remaining the case when egg-expressed genes were excluded from ovary data of egg-laying workers) (**Figure 5.6**). Correspondingly, pairwise comparisons of the tissues also showed the sharing of GO terms derived from up- or down-regulated DEGs shared between them.

The overall pattern suggests that the physiological changes associated with ovary activation involve gene expression changes at a more systemic level in their first stages, before becoming more tissue-specific in the later stages. There was relatively little overlap in DEGs between brain and fat body and similar findings in *B. terrestris* workers were reported by Prince et al. (2024) in their comparison of ovary-active and ovary-inactive workers. The genes changing expression during ovary activation in all three tissues are likely to be genes of particular importance to this process; the relevant gene lists can be found in **Tables OS5.3**, **OS5.4** and **OS5.5**.

A clear sign of ovary activation followed by oocyte activation was the high expression of *vitellogenin* in the brain samples at the 96 h time point and the egg-laying time point, while it a low expression at the 24 h time point (**Figure S5.2A**). This is concurrent with previous findings of Marshall et al. (2019) and Prince et al. (2024) that found *vitellogenin* to be up-regulated in brain of ovary-active workers. *Vitellogenin* has previously also been found to be highly expressed in the fat body of ovary-active workers (Prince et al., 2024; Lockett et al., 2016), and in the comparison between the 24 h time point and the 96 h time point and between the 24 h time point and the egg-laying time point in the fat body, *vitellogenin* (gene ID: LOC100650436) was up-regulated, though this change in expression was relatively low (**Table OS5.4**). The gene *serine protease nudel* was up-regulated in the ovary replicates of the 96 h time point and the egg-laying time point, but down-regulated at the 24 h time point (**Figrue S5.2**), this is in line with Experiment A, in which the *serine protease nudel* was up-regulated with age in ovary-active workers, making it a gene of interest for ageing in reproductively-active workers. Yet, previously serine-protease inhibitors were found to be up-regulated in the head of ovary-active workers (Marshall et al., 2019), which indicates that the interaction between serine proteases and their inhibitors could be of interest in understanding the positive relationship of reproductivity and longevity in reproductive workers.

5.4.3 Conclusion

Previous studies suggest that female eusocial Hymenoptera (queens and workers) exhibit positive fecundity-longevity relationships (e.g. Korb and Heinze (2021)) and that, in *B. terrestris*, these relationships are expressed only by high-quality individuals, i.e. are condition dependent (Blacher et al. (2017); Collins et al. (2023); **Section 5.1**; **Chapter 2**). Hence the two experiments of this chapter, in providing new data on the molecular basis of worker reproductivity as a function of time in *B. terrestris*, help elucidate the molecular underpinnings of such relationships and the phenomenon of condition-dependence in both the study species and, since similar phenomena are likely to occur widely, especially in species at a similar level of eusocial complexity (Collins et al., 2023), other eusocial Hymenoptera.

In Experiment A, the comparison of gene expression in fat body of ovary-active and ovary-inactive *B. terrestris* workers at two time points (worker ages of 2 and 7 weeks) revealed that gene expression differences between the two worker phenotypes increased with age and that the majority of age-related DEGs, including some genes within the ageing-related TI-J-LiFe network and enzymatic antioxidant gene set, occurred exclusively within one or other of the phenotypes. This suggests that the molecular basis of ageing may differ between ovary-active and ovary-inactive workers, which is consistent with the concept (**Chapter 2**) that ovary-active workers represent high-quality individuals able to express a positive fecundity-longevity relationship. As a result, investigating these DEGs further could help elucidate the basis of the apparent differences in worker quality that lead to this phenomenon. In particular, the age-related DEGs that exclusively occurred in ovary-active workers represent potential candidates for genes associated with these workers' ability to overcome the costs of reproduction while other workers cannot.

In Experiment B, the analysis of gene expression (in brain, fat body and ovary) of young queenless workers as they followed a trajectory from an ovary-inactive state to becoming egg-laying workers (aged 7-12 days) revealed that the largest gene expression changes occurred between the 24 h time point (i.e. 24 h after becoming queenless) and the 96 h time point. This general pattern occurred in all three tissues sampled. After the 96 h time point differential gene expression seems to have been driven mainly by oocyte maturation. Therefore the DEGs found between the 24 h and 96 h time points, especially those shared by more than one of the tissues, are again likely to be genes of particular interest for understanding the positive fecundity-longevity relationship of reproductively active *B. terrestris* workers and other female eusocial Hymenoptera. In sum, the current study helps support the interpretation of the phenotypic ageing and longevity traits associated with sociality

reported in previous studies and further elucidated in this thesis, and provides a basis for further investigation of specific genes and pathways potentially involved in them.

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Supplement

Additional supplementary material (including Tables OS5.1, OS5.2, OS5.3, OS5.4 and OS5.5) is available at: `https://github.com/lilianafischer/PhD-Thesis-Longevity-Ageing-and-Reproductivity-in-a-Social-Insect`.

Supplementary Figures

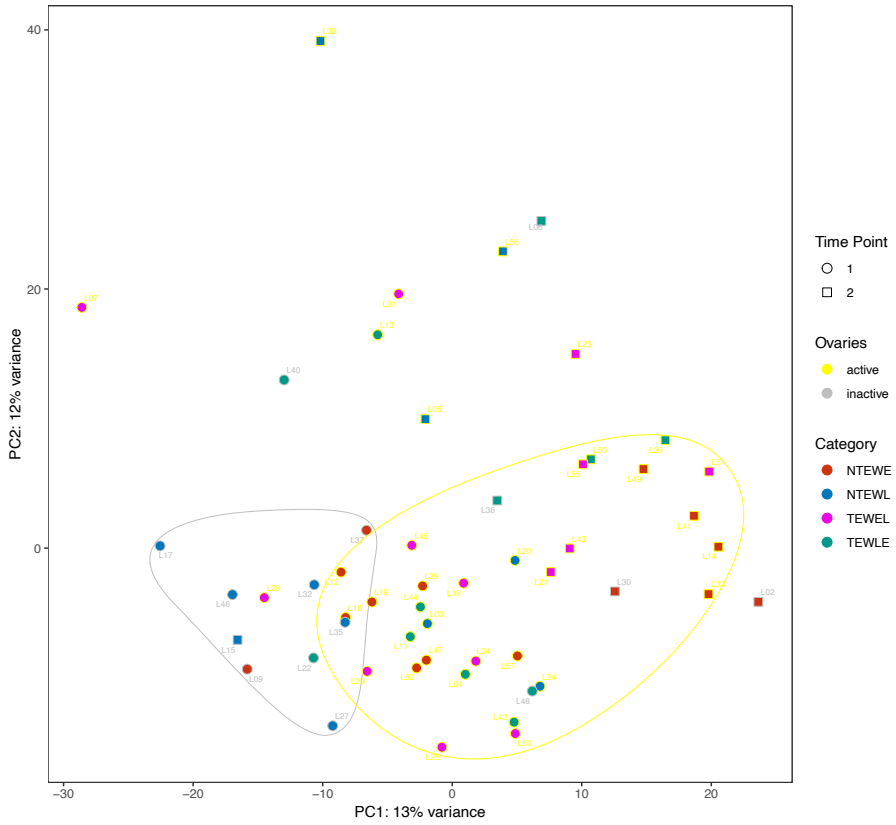

Figure S5.1. Principal component analysis (PCA) plot of mRNA-seq libraries from fat body of *Bombus terrestris* workers in four experimental worker categories (Experiment A; as described in Chapter 3) over two time points (TP1 and TP2, worker ages of 2 and 7 weeks, respectively) of ovary-active and ovary-inactive workers (active: yellow edge; inactive: grey edge). Axes represent principal components; individual points represent individual fat body samples (one per worker), with colour denoting experimental category and shape denoting time point. Only samples above a threshold of an alignment of 20 million reads are included. Sample sizes: $NTEW_E$:TP1:active = 7; $NTEW_E$:TP1:inactive = 2; $NTEW_E$:TP2:active = 4; $NTEW_E$:TP2:inactive = 3; $NTEW_L$:TP1:active = 3; $NTEW_L$:TP1:inactive = 5; $NTEW_L$:TP2:active = 6; $NTEW_L$:TP2:inactive = 6; $NTEW_L$:TP2:inactive = 1; TEW_{EL}:TP1:active = 9; TEW_{EL}:TP1:inactive = 0; TEW_{EL}:TP2:active = 6; TEW_{EL}:TP2:inactive = 0; TEW_{LE}:TP1:active = 5; TEW_{LE}:TP1:inactive = 3; TEW_{LE}:TP2:active = 2; TEW_{LE}:TP2:active = 3. Definitions of the experimental worker categories are in Table 3.1.

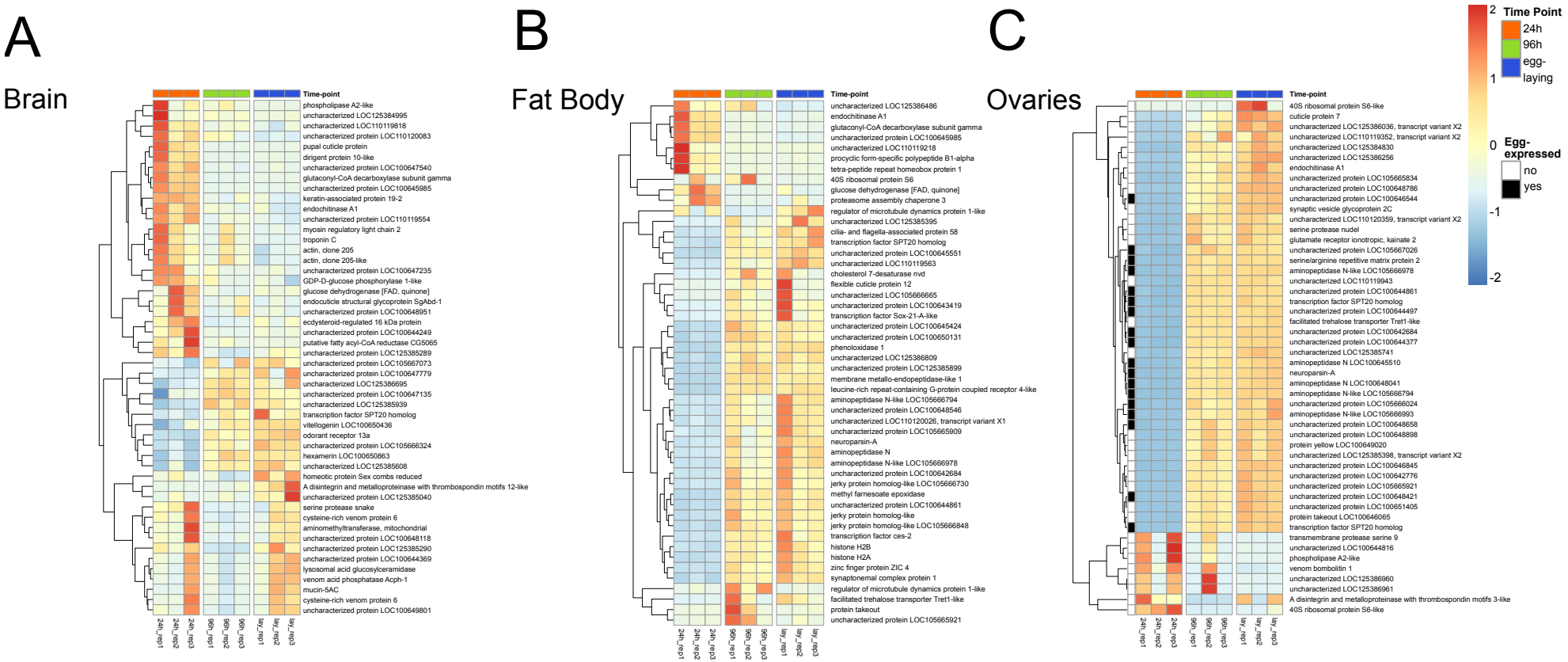

Figure S5.2. Gene expression patterns (read counts) of the top 50 DEGs between three stages of ovary activation (Experiment B). Each row represents an individual gene from the *Bombus terrestris* genome. Each column represents a single biological replicate (ten workers pooled). The top row defines the time point after becoming queenless (stage of ovary activation; 24 h: orange, 96 h: green,, egg-laying: blue). The dendogram at the left groups genes that cluster according to their gene expression patterns. Numbers at the bottom correspond to replicate IDs. **A**: Brain; **B**: Fat Body; **C**: Ovary

Supplementary Tables

Table S5.1. The overlap of differentially expressed genes with age in fat body between ovary-active and ovary-inactive *Bombus terrestris* workers (Experiment A). DEGs: Count of genes differently expressed between the two time points (2 weeks and 7 weeks). The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167).

	Up-regulated	Down-regulated
Ovary-active worker DEGs	898	681
Ovary-inactive worker DEGs	655	783
Overlapping DEGs	257	339
DEGs only in ovary-active workers	641	342
DEGs only in ovary-inactive workers	398	444
DEGs in neither	11207	11378
p-value	1.41e-131	1.96e-246
Odds-ratio	11.28	25.38
% Ovary-active worker DEGs overlap	28.6	49.8
% Ovary-inactive worker DEGs overlap	39.3	43.3

Table S5.2. A comparison of changes mRNA-seq gene expression profiles in fat body with age in ovary-active and ovary-inactive *Bombus terrestris* workers to two sets of genes hypothesised to be ageing-related in eusocial insects (Experiment A). DEGs: Count of genes differently expressed between the two time points (2 weeks and 7 weeks). The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167) **A**: Comparison with TI-J-LiFe network; each row represents a gene from *D. melanogaster* found in the TI-J-LiFe network of Korb et al. (2021) and having a single-copy orthologue in *B. terrestris*. **B**: Comparison with enzymatic antioxidant gene set; each row represents a gene from *D. melanogaster* found in the enzymatic antioxidant gene set of Kramer et al. (2021) and having a single-copy orthologue in *B. terrestris*. The dendogram at the left groups genes that cluster according to their gene expression patterns. Sample sizes: active:TP1 = 24; active:TP2 = 14; inactive:TP1 = 10; inactive:TP2 = 5

	Ovary-Active Workers	**Ovary-Inactive Workers**
A) TI-J-LiFe Network		
B. terrestris DEGs	797	851
D. melanogaster genes	81	81
Overlapping genes	11	11
Genes only in *B. terrestris*	786	840
Genes only in *D. melanogaster*	70	70
Genes in neither	5027	4973
p-value	1.00	1.00
Odds-ratio	1.00	0.93
Percentage of gene-set present	13.6	13.6
B) Enzymatic Antioxidant Gene-set		
B. terrestris DEGs	797	851
D. melanogaster genes	15	15
Overlapping genes	5	5
Genes only in *B. terrestris*	792	846
Genes only in *D. melanogaster*	10	10
Genes in neither	5087	5033
p-value	0.04	0.05
Odds-ratio	3.21	2.97
Percentage of gene-set present	33.3	33.3

Table S5.3. The overlap of differentially expressed genes in the comparison between the 24 h time point and the 96 h time point, between brain, fat body and ovary of *Bombus terrestris* workers (Experiment B). DEGs: Count of genes differently expressed between the two time points. The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167).

	Up-regulated	Down-regulated
Brain DEGs	1311	970
Fat Body DEGs	1064	1205
Ovary DEGs	3585	3277
Brain - Fat Body overlapping DEGs	49	61
Brain - Ovary overlapping DEGs	468	361
Fat Body - Ovary overlapping DEGs	405	359
Brain - Fat Body - Ovary overlapping DEGs	21	17
DEGs only in Brain	815	565
DEGs only in Fat Body	631	802
DEGs only in Ovary	2733	2574
DEGs in Brain and Fat Body not in Ovary	28	44
DEGs in Brain and Ovary not in Fat Body	447	344
DEGs in Fat Body and Ovary not in Brain	384	342
DEGs in neither	1760	1494
p-value Brain - Fat Body	3.39e-45	7.50e-40
p-value Brain - Ovary	5.96e-54	3.84e-41
p-value Fat Body - Ovary	8.81e-37	8.65e-86
Odds-ratio Brain - Fat Body	0.17	0.20
Odds-ratio Brain - Ovary	1.60	1.19
Odds-ratio Fat Body - Ovary	1.79	0.83
% Brain DEGs overlap only with Fat Body	2.1	4.5
% Fat Body DEGs overlap only with Brain	2.6	3.6
% Brain DEGs overlap only with Ovary	34.1	35.5
% Ovary DEGs overlap only with Brain	12.5	10.5
% Fat Body DEGs overlap only with Ovary	36.0	28.4
% Ovary DEGs overlap only with Fat Body	10.7	10.4

Table S5.4. The overlap of differentially expressed genes in the comparison between the 96 h time point and the egg-laying time point, between brain, fat body and ovary of *Bombus terrestris* workers (Experiment B). DEGs: Count of genes differently expressed between the two time points. The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167). Numbers in parentheses correspond to the analysis when egg-expressed genes (EEGs) were excluded from the ovary DEG-lists.

	Up-regulated	Down-regulated
Brain DEGs	4	1
Fat Body DEGs	186	124
Ovary DEGs	1146 (356)	862 (182)
Brain - Fat Body overlapping DEGs	0	0
Brain - Ovary overlapping DEGs	0 (0)	0 (0)
Fat Body - Ovary overlapping DEGs	29 (12)	8 (3)
Brain - Fat Body - Ovary overlapping DEGs	0 (0)	0 (0)
DEGs only in Brain	4 (4)	1 (1)
DEGs only in Fat Body	157 (174)	116 (121)
DEGs only in Ovary	1117 (344)	854 (179)
DEGs in Brain and Fat Body not in Ovary	0 (0)	0 (0)
DEGs in Brain and Ovary not in Fat Body	0 (0)	0 (0)
DEGs in Fat Body and Ovary not in Brain	29 (12)	8 (3)
DEGs in neither	58 (24)	16 (6)
p-value Brain - Fat Body	0.006 (0.30e-03)	0.128 (0.05)
p-value Brain - Ovary	6.96e-06 (2.61e-05)	0.02 (2.61e-05)
p-value Fat Body - Ovary	3.89e-124 (9.99e-66)	1.10e-122 (9.52e-60)
Odds-ratio Brain - Fat Body	0.0	0.0
Odds-ratio Brain - Ovary	0.0 (0.0)	0.0 (0.0)
Odds-ratio Fat Body - Ovary	0.07 (0.01)	0.01 (0.001)
% Brain DEGs overlap only with Fat Body	0.0	0.0
% Fat Body DEGs overlap only with Brain	0.0	0.0
% Brain DEGs overlap only with Ovary	0.0 (0.0)	0.0 (0.0)
% Ovary DEGs overlap only with Brain	0.0 (0.0)	0.0 (0.0)
% Fat Body DEGs overlap only with Ovary	15.6 (6.4)	6.4 (2.4)
% Ovary DEGs overlap only with Fat Body	2.5 (3.4)	0.9 (1.6)

Table S5.5. The overlap of differentially expressed genes in the comparison between the 24 h time point and the egg-laying time point, between brain, fat body and ovary of *Bombus terrestris* workers (Experiment B). DEGs: Count of genes differently expressed between the two time points. The significance of the overlap was tested with Fisher's exact tests (Bonferroni correction: alpha-value = 0.0167). Numbers in parentheses correspond to the analysis when egg-expressed genes (EEGs) were excluded from the ovary DEG-lists.

	Up-regulated	Down-regulated
Brain DEGs	605	435
Fat Body DEGs	848	762
Ovary DEGs	3775 (810)	3472 (1222)
Brain - Fat Body overlapping DEGs	55	65
Brain - Ovary overlapping DEGs	201 (24)	126 (36)
Fat Body - Ovary overlapping DEGs	392 (109)	255 (96)
Brain - Fat Body - Ovary overlapping DEGs	22 (7)	24 (6)
DEGs only in Brain	371 (533)	268 (340)
DEGs only in Fat Body	423 (691)	466 (607)
DEGs only in Ovary	3204 (684)	3115 (1096)
DEGs in Brain and Fat Body not in Ovary	33 (48)	41 (59)
DEGs in Brain and Ovary not in Fat Body	179 (17)	102 (30)
DEGs in Fat Body and Ovary not in Brain	370 (102)	231 (90)
DEGs in neither	1208 (348)	796 (370)
p-value Brain - Fat Body	3.60e-09 (7.05e-122)	6.46e-10 (1.27e-58)
p-value Brain - Ovary	8.08e-68(1.41e-147)	4.90e-82 (3.37e-75)
p-value Fat Body - Ovary	4.01e-41 (1.07e-122)	1.81e-118 (2.10e-94)
Odds-ratio Brain - Fat Body	0.42 (0.05)	0.41 (0.12)
Odds-ratio Brain - Ovary	0.20 (0.02)	0.12 (0.06)
Odds-ratio Fat Body - Ovary	0.35 (0.08)	0.14 (0.10)
% Brain DEGs overlap only with Fat Body	5.4 (7.9)	9.4 (13.6)
% Fat Body DEGs overlap only with Brain	3.9 (7.0)	5.4 (7.7)
% Brain DEGs overlap only with Ovary	33.2 (3.9)	28.9 (8.3)
% Ovary DEGs overlap only with Brain	5.3 (3.0)	3.6 (2.9)
% Fat Body DEGs overlap only with Ovary	43.6 (12.0)	30.3 (11.8)
% Ovary DEGs overlap only with Fat Body	9.8 (12.6)	6.6 (7.4)

Chapter 6 | Thesis Conclusion

6.1 Thesis Overview

Study systems in which the evolution of sociality and the evolution of ageing intersect, as in eusocial insect societies, are highly informative for providing further understanding of the ultimate and proximate drivers of ageing and its evolution. Eusociality is associated with extreme longevities and the absence of life-history tradeoffs in reproductive individuals, and with reduced longevities in non-reproductive individuals. Focussing on the workers of the bumblebee *Bombus terrestris*, which show a high variability in reproductivity and longevity, this thesis set out to provide new insights into the effect of sociality on ageing and longevity by further investigation of the connection between these two fitness-related life-history traits. In detail, this thesis aimed to investigate experimentally the origin of differences between workers linked to worker quality, following the idea that it is workers of high quality that become reproductively active and experience a positive fecundity-longevity relationship. The thesis then investigated experimentally, at both the phenotypic and transcriptomic level, whether individual or social factors take the leading role in influencing ageing and longevity in social organisms. Next, the thesis experimentally explored a novel pattern in the frequency distribution of worker longevity uncovered by the work (within-cohort multimodality). Lastly, the thesis characterised the transcriptomic basis behind these life-history traits by analysing (age-related) changes in gene expression as a function of workers' reproductive status and the gene expression changes connected to the process of ovarian activation.

Methodologically, to achieve these aims, the thesis formulated relevant hypotheses and then tested them employing a variety of techniques. Primarily, these involved controlled, manipulative experiments in the laboratory using captive colonies (commercially-supplied *Bombus terrestris audax* colonies), with these experiments involving individually-marked workers whose longevities were measured over the workers' entire lifespans of up to several months. Laboratory techniques also included, in particular to characterise the reproductive status and associated behaviours of individual workers, direct observations of the behaviour of individually-marked workers and ovarian dissections of them using stereomicroscopy, as well as measurements of wing cell length (a proxy for body size) using an imaging software measuring tool. In addition, RNA extraction of specific tissues dissected from workers followed by next-generation sequencing (mRNA-seq) and bioinformatic analyses were conducted to profile gene expression changes associated with the experimental manipulations and/or workers' age and reproductive status. The rest of this chapter summarises the key findings and conclusions of the thesis.

6.2 Positive Association of Adult Body Size, Reproductivity and Longevity

The Fecundity-Longevity Relationship in Workers

In the experimental *B. terrestris* workers in this thesis, there was a positive relationship between adult body size, reproductivity and longevity. In **Chapter 2**, I found that larger workers were significantly more likely to be egg-layers (irrespective of larval nutrition treatment), and that larger workers were significantly longer-lived. Longer-lived workers were significantly more likely to be egg-layers and consequently egg-layers were significantly longer-lived. The positive effect of egg-laying on worker survival was strongest early in life and decreased with worker age, to a point when, at an advanced worker age, egg-laying had a negative effect on the chances of worker survival. In **Chapter 4**, I reported similar results for isolated *B. terrestris* workers. It was again the larger workers that were significantly longer-lived. However, in this case neither adult body size nor longevity was a predictor of whether a worker became an egg-layer or not. Nevertheless, among the egg-layers in this experiment, there was a significantly positive relationship between lifetime reproductive output (number of adult males produced by an isolated worker) and worker longevity. Additionally, egg-laying again had a positive effect on worker survival early in life, though this became increasingly negative (higher increase in mortality rate) with age.

In **Chapter 2**, this effect was potentially reflected in the novel observation from the work that workers showed bimodality in the timing of their egg-laying, suggesting that there are two different reproductive strategies among the egg-layers with respect to the age at which they laid eggs. The majority of egg-layers were either part of a subset of workers that laid eggs only at young ages, and which had low median longevity, or part of a subset of workers that laid eggs only at older ages, and which had a high median longevity. Alongside evidence that high worker longevities are connected to high worker quality, this finding supported the concept that this second subset of workers consists of high-quality workers that are indeed able to bear the costs of reproduction late in life.

Overall, these results are evidence for strong variation in worker quality within *B. terrestris* colonies, which could therefore apply to other species of eusocial Hymenopterans. The findings confirm and extend the evidence that high-quality workers tend to be larger, more reproductive and longer-lived and it is such high-quality workers that can bear the costs of reproduction, whereas lower-quality workers tend to be smaller, less reproductive and shorter-lived and less able to bear these costs.

The Effect of Larval Nutrition on Worker Quality

Variation in adult worker quality within *B. terrestris* colonies has been hypothesized to stem from differences during larval development (Blacher et al., 2017). In **Chapter 2**, I experimentally tested the hypothesis that one factor contributing to such differences is the quantity of larval nutrition. I therefore investigated the effects of elevated levels of larval nutrition versus restrictive levels of larval nutrition on adult worker body size, reproductivity and longevity. As predicted, adult body sizes were significantly larger in workers developing under the high nutritional treatment. The experimental manipulation of larval nutrition had no effect on the probability of adult workers becoming egg-layers and on the longevity of the adult workers, yet both of these measures were positively correlated with increased body sizes. Therefore, although there was no direct effect of larval nutrition on reproductivity and longevity (two important components of worker quality), they were indirectly positively affected via the effects of larval nutrition on adult body size. For the shorter-lived workers, the nutritional treatments affected worker longevity as expected in that workers that developed under the high larval nutrition treatment had significantly higher chances of survival. Overall, these results confirm the assumption of varying levels of worker quality within a colony and provide evidence that variation in larval nutrition is indeed a likely driver. However, there also seem to be additional factors determining worker quality, as workers of lower quality (shorter-lived) workers seemed to be more susceptible to the effect of less favourable conditions, such as low larval nutrition.

Consequently, it is possible that the way larval nutrition was manipulated in this experiment, which was calibrated to reflect the level of natural variation in worker body size within colonies, was too weak to yield the expected effects on reproductivity and longevity, while being strong enough to cause the observed differences in adult body size. Therefore, future work building on these results might benefit by increasing the difference between the high and the low nutritional treatment (while remaining within the bounds of natural variation). Other aspects of larval development, such as nutritional quality (e.g., protein content) and temperature, along with egg-quality and the effects of all these factors on adult worker quality, would be worth studying to further understand the origin of worker quality variation in the study system and similar species.

6.3 The Effect of Individual versus Social Factors on Worker Longevity

In social organisms, an unsolved question concerns whether intrinsic properties of the individual or properties of the social environment are the major determinant of ageing and longevity. In **Chapter 3**, I therefore made use of the finding by Holland and Bourke (2015) that, in *B. terrestris*, workers eclosing in colonies early in the colony cycle are longer-lived than workers eclosing late in the colony cycle to experimentally discriminate between these two possibilities. To this end, in a transfer experiment between early colonies and late colonies, I tested two hypotheses: the Individual Hypothesis attributes the workers' longevity differences to intrinsic differences between early-produced and late-produced workers, predicting transferred workers to resemble in their longevity patterns the workers remaining in their colony of origin; the Social Hypothesis attributes the longevity differences to differences in the social environment of early and late colonies, predicting transferred workers to resemble in their longevity patterns the workers in the receiving colony. Analyses of the survival and longevity analyses of this experiment revealed that there was an interaction between individual and social factors. Specifically, there were intrinsic differences favourable for survival in early-produced workers and unfavourable for survival in late-produced workers. These intrinsic differences were further shaped by the social environment, which was more favourable to workers' survival in the early colonies than in the late colonies. This social effect was strongest for the short-lived workers, which showed significantly higher chances of survival in an early social environment than in a late social environment. The fact that the short-lived workers were most affected by this social effect was further evidence for varying levels of worker quality within a colony.

The hypotheses were simultaneously tested using mRNA-seq to profile gene expression changes in a sample of transferred and non-transferred workers. Based on these data, analysis of age-related differential gene expression confirmed the existence of an interaction between individual factors and social factors. On the one hand, intrinsic differences between early-produced workers and late-produced workers in age-related gene expression remained for early-produced workers even when they were transferred into a late social environment. On the other hand, late-produced workers that were transferred into early social environments adopted an age-related gene expression profile more like those of the workers of the early recipient colonies.

Therefore, the work reported in this chapter found worker longevities in *B. ter-*

restris to be determined by a combination of individual and social factors. In particular, the results suggested that individual factors lead to intrinsic differences, and these determined the workers' susceptibility to social factors affecting longevity. Hence, the overall conclusion of this experiment is that both individual and social factors may interact to influence longevity, along with its molecular underpinnings, in social organisms.

6.4 Effects of Isolation and Sociality on Worker Longevities

Multimodality in Worker Longevity Distributions

An unanticipated finding of **Chapter 2** and **Chapter 3** was that the distributions of *B. terrestris* worker longevities were multimodal (**Table S4.2**). This multimodality was found irrespective of treatment or colony stage and also within most of the colonies and within narrow eclosion windows. In **Chapter 4**, I therefore set out to replicate the finding of this pattern and to use it in a further experiment to discriminate between individual and social factors affecting longevity in social organisms. For this experiment, I compared the longevity distributions of workers kept singly in isolation and workers kept in the social environment of the colony. I predicted that, if longevity is intrinsically anchored to worker quality, a multimodal frequency distribution of longevity should appear in the isolated workers as well as in the social context, and if it is a phenomenon caused solely by the social environment of a colony, it would not appear in isolated workers. Multimodality in worker longevity distribution proved to be a common and robust phenomenon and, in experiment, was detected in the social worker treatment (and within most of the colonies), but not in the set of isolated workers. These findings showed that the multimodality in worker longevity distributions is an effect of the social environment of a colony, thereby supporting the role of social factors in longevity determination in this context. Overall, these findings attest to the existence of a novel phenomenon in the study of the relationship between sociality and longevity, which potentially exists in eusocial Hymenoptera more widely.

In addition, the several instances of multimodality detected in the worker longevity distributions in this experiment, other experiments presented in this thesis and also in the dataset of Blacher et al. (2017) as reanalysed in this thesis, provide further evidence that there are varying levels of worker quality within *B. terrestris* colonies, with this being the case even in controlled laboratory settings with *ad libitum* access

to food and without extrinsic mortality caused by workers externally foraging. Although this pattern appeared so frequently throughout the studies reported and/or analysed in this thesis, it was not a uniform pattern. There were different shapes of multimodality present. In some cases the majority of worker longevities fell in the peak around the first mode, while in other cases the majority of worker longevities fell in the peak around the second mode (**Table S4.2**). Additionally, in some cases the multimodality was due to a peak of very short lived workers, with longevities considerably lower than the median longevity, whereas in other cases the multimodality was due to a peak of very long liver workers, with longevities considerably higher than the median (**Table S4.2**). These different distributions were especially marked in the experiment presented in **Chapter 4**, between the two social categories of colonies and between the colonies. Such different forms of multimodality might suggest differences in the causation of multimodality in worker longevities and further raise the question as to which factor of the social context of a colony drive these patterns.

In further research, it would therefore be informative (a) to determine if these patterns also occur in wild colonies and (b) to conduct experiments testing if they are adaptive, for example by selective removal of workers to manipulate workers' longevity distributions followed by measurement of the effects on sexual production.

Effect of Isolation on Worker Longevity

In **Chapter 4**, I also unexpectedly found that the isolated *B. terrestris* workers lived significantly longer than workers retained in colonies, with this difference being extremely large (difference in median longevity > 75 days). For workers of a eusocial insect species, a positive effect of isolation on longevity has not been documented before and isolation of workers has usually been found to have negative effects on survival. Although not all the isolated workers became reproductively active, the egg-laying workers showed a positive fecundity-longevity relationship in that the longer-lived ones produced more adult sons in isolation; from this it is likely that workers' prolonged longevity under isolation can be explained by greater direct fitness gains for long-living workers. Moreover, in general, whether workers' longevity is increased or decreased by isolation could, like condition-dependent positive fecundity-longevity relationships, reflect their level of eusocial complexity, which could in future be tested through comparative studies.

6.5 Changes in Gene Expression in Connection with Ovary Activation

In **Chapter 5**, I provided new data on the molecular basis of worker reproductivity as a function of time in *B. terrestris*, which helps elucidate the molecular underpinnings of such relationships and the phenomenon of condition-dependence in both the study species and, since similar phenomena are likely to occur widely, especially in species at a similar level of eusocial complexity (Collins et al., 2023), other eusocial Hymenoptera.

Gene expression differences in fat body of ovary-active to ovary-inactive workers increased with age and the majority of age-related DEGs was exclusively found in either phenotype (Experiment A). This suggests that the molecular basis of ageing may differ between ovary-active and ovary-inactive workers, which is consistent with the concept that ovary-active workers represent high-quality individuals able to express a positive fecundity-longevity relationship. Genes that were exclusively found to change expression with age in the ovary-active workers will therefore be of particular interest in future studies to further understand the positive fecundity-longevity relationship found in reproductive *B. terrestris* workers and other female eusocial Hymenoptera. Further, the analysis of gene expression (in brain, fat body and ovary) of young queenless workers as they followed a trajectory from an ovary-inactive state to becoming egg-laying workers (Experiment B) revealed that the largest gene expression changes occurred within the first 96 h after becoming queenless. Therefore, the DEGs found between the 24 h and 96 h time points, especially those shared by more than one of the tissues, are again likely to be genes of particular interest for understanding the positive fecundity-longevity relationship of reproductively active *B. terrestris* workers and other female eusocial Hymenoptera.

6.6 Concluding Remarks

This thesis provides valuable novel insights into the nature of the influence that social evolution has on the evolution of ageing and longevity. It has confirmed previous hypotheses, tested new ones with novel results and uncovered new phenomena at the intersection of sociality and ageing. Although these results have been obtained through experiments using the study system provided by *B. terrestris*, because the hypotheses tested are general ones, and eusociality has evolved through stages from non-social to complex independently in multiple lineages, and other forms of sociality have evolved in many more, the results should also be generalisable to numerous other species and contexts.

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