Dominance and Policing in Social Insects: Testing the Hypotheses in Bumblebees

Photo taken by Jenny Livesey

Jennifer Sarah Livesey

A thesis submitted for the degree of Doctor of Philosophy

School of Biological Sciences

University of East Anglia, UK

July 2023

Thesis abstract

Animal societies can display remarkable levels of cooperation and altruism, but they are still susceptible to conflict and social cheats. Consequently, the maintenance of these societies, over evolutionary time, relies on the evolution of mechanisms that limit exploitation from both external and internal elements. Despite extensive research on conflict and conflict resolution in animal societies, uncertainties remain regarding the role coercive mechanisms, such as dominance and policing, play in enforcing cooperation and preventing group exploitation. Through a series of quantitative experiments involving colonies of the Buff-tailed bumblebee *Bombus terrestris,* I investigate uncertainties surrounding (1) the exact origins of worker policing (egg-eating) in eusocial societies, (2) the ultimate function of dominance behaviours in animal societies, and (3) the underlying factors behind variations in intrinsic quality, reproductive success, and longevity in workers of eusocial Hymenoptera. In an experiment aimed at discerning the origin of non-reproductive worker policing in eusocial Hymenoptera, partial support was found for both the public goods hypothesis and the selfish policing hypothesis, leaving the origin of non-reproductive worker policing unclear. However, additional findings of an association among reproductive workers between egg-laying, egg-eating and aggression provide new support for policing by reproductive workers originating as selfish policing. Behavioural analyses of queenless groups of full-sister *B. terrestris* workers also revealed that while (dominant) alpha workers were unable to completely suppress egg-laying in betas or subordinates, they effectively monopolised reproduction by exhibiting significantly higher rates of aggression, policing, and egg survivorship than other group members. These findings strongly support the hypothesis that dominance is positively correlated with reproductive success in *B. terrestris* workers and that increasing direct fitness is the ultimate function of dominance behaviour. Finally, an experiment that aimed to determine if the quantity of nutrition provided during larval development influences a worker's size and intrinsic quality did not generate distinct worker body sizes as intended. Nonetheless, associations between reproductive behaviours and longevity were still evident, as reproductive workers exhibited greater body size and longevity compared to non-reproductive workers. The data also revealed significant associations among workers between egg-laying, egg-eating, aggression, and body size, supporting the hypothesis that variations in intrinsic worker quality, reflected in body size, underlie positive associations between fecundity and longevity in *B. terrestris* workers. Overall, the findings of this thesis provide valuable insights into the complex dynamics of cooperation and conflict in animal societies, particularly in colonies of bumblebees and comparable social systems.

Access Condition and Agreement

Each deposit in UEA Digital Repository is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the Data Collections is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form. You must obtain permission from the copyright holder, usually the author, for any other use. Exceptions only apply where a deposit may be explicitly provided under a stated licence, such as a Creative Commons licence or Open Government licence.

Electronic or print copies may not be offered, whether for sale or otherwise to anyone, unless explicitly stated under a Creative Commons or Open Government license. Unauthorised reproduction, editing or reformatting for resale purposes is explicitly prohibited (except where approved by the copyright holder themselves) and UEA reserves the right to take immediate 'take down' action on behalf of the copyright and/or rights holder if this Access condition of the UEA Digital Repository is breached. Any material in this database has been supplied on the understanding that it is copyright material and that no quotation from the material may be published without proper acknowledgement.

Table of contents

Chapter 1 | General introduction: conflict and conflict resolution in animal societies **7**

Chapter 2 | Worker policing: testing the hypotheses with *Bombus terrestris* **41**

Chapter 3 | Testing the relationship between dominance behaviour and reproductive success with *Bombus terrestris* **100**

Acknowledgements

Undertaking a PhD amidst a global pandemic and a cost-of-living crisis has, at times, been an unusual experience. I owe the small shreds of sanity that remain to the many humans and animals that have supported me on this journey.

First and foremost, I would like to thank my supervisor, Professor Andrew Bourke, for his invaluable support, patience, and guidance. Andrew's profound knowledge of the principles of social evolution and his remarkable memory of decades' worth of research is truly awe-inspiring. I am grateful to have been able to learn and grow as a scientist under his mentorship. While it is bittersweet to finish this PhD, knowing that I will no longer be able to burst into his office on a daily basis to ask a multitude of questions, I am sure I will find new ways to pester him for snippets of wisdom. It has been a privilege, and I look forward to publishing chapters of this thesis with him.

I also wish to express my appreciation to past and present members of the Bourke lab group for their practical advice, constructive criticism, and friendship. In particular, I would like to thank Ryan Brock for teaching me molecular genetic techniques and the art of catching bumblebee queens. I would also like to thank David Collins for his positivity, good humour, and friendship throughout my PhD. In addition, I extend my thanks to Iain Barr, Phil Leftwich, Ellen Bell and Becky Lewis for providing me with fantastic teaching opportunities during my time at UEA. Teaching on the Ireland ecology field course was a particular highlight, and I thank all those who also taught on this course for making the experience so enjoyable and memorable.

I feel fortunate to have crossed paths with many wonderful people during my PhD, and I am incredibly grateful for all the joy and humour they have brought into my life. In particular, I owe my thanks to Tom, who has taught me to enjoy life, rather than just analyse it. I look forward to the many adventures ahead together. I am also grateful to all those who have made the Biological Sciences Department at UEA a supportive and social environment. A special mention goes to my amazing office buddies Mable Sydney, Alex Siddall and Harry Ewing for making me laugh and for providing much-needed motivation. In addition, I would like to thank my examiners David Richardson and Elli Leadbeater for an engaging and enjoyable viva experience. Thanks also go to my family, who have provided unconditional love and support, despite not truly understanding what it was I was doing these last 4 years. Thanks especially go to my mum and dad for making the long journey from Wales to Norwich several times a year to say hello.

Finally, I couldn't possibly write an acknowledgement section without a mention of thanks to the bumblebees for being such a fantastic study species. Despite spending countless hours observing them and enduring a few well-earned stings, I continue to find these creatures utterly fascinating. It has been a privilege and a joy to work with them and to help unravel the mysteries of their social behaviour.

Author contributions

Chapter 2 | Worker policing: testing the hypotheses with *Bombus terrestris*

Jenny Livesey designed the study along with Andrew Bourke. Jenny Livesey conducted all laboratory work and collected all data. Jenny Livesey performed all analyses and produced all figures, infographics, and tables. Jenny Livesey wrote the manuscript under the supervision of Andrew Bourke.

Chapter 3 | Testing the relationship between dominance behaviour and reproductive success with *Bombus terrestris*

Jenny Livesey designed the study along with Andrew Bourke. Jenny Livesey conducted all laboratory work and collected all data. Jenny Livesey performed all analyses and produced all figures and tables. Jenny Livesey wrote the manuscript under the supervision of Andrew Bourke.

Chapter 4 | Quality, reproductivity and longevity in *Bombus terrestris* workers

Jenny Livesey designed the study along with Andrew Bourke and Lilliana Fisher. Jenny Livesey and Liliana Fisher conducted the laboratory work and collected all data. Jenny Livesey performed all analyses and produced all figures, infographics, and tables. Jenny Livesey wrote the entire manuscript (abstract, introduction, methods results and discussion) under the supervision of Andrew Bourke.

1

Chapter

General introduction: conflict and conflict resolution in animal societies

Photo taken by Andrew Bourke

1.1 An overview

1.1.1 Social group formation and maintenance

Sociality has evolved independently in all domains of life. The key element of social groups is that separate entities cooperate with one another, disregarding their selfish interests, to an extent, in favour of the collective interests of the group (Maynard Smith and Szathmáry, 1995). Within the context of multicellular organisms, social groups (animal societies) are comprised of individuals who cooperate with other members of the same species, either those they are related to or those they are not (Hamilton, 1964; Trivers, 1971; Clutton-Brock, 2009a). They may vary in size or structure, but a fundamental aspect of these social groups is the ability of group members to distinguish one another from outsiders, whom they reject (Bourke, 2011).

The evolution and maintenance of group living has been researched and described in various studies (Hamilton, 1964; Wilson, 1971; Alexander, 1974; Maynard Smith and Szathmáry, 1995; Whitehouse and Lubin, 2005). Across taxa, in order for group living to be selected for in a species, the average fitness benefits to individuals living in a group must be greater than the fitness of those living solitarily (Hamilton, 1964; Alexander, 1974). For example, a cooperating pack of wolves *Canis lupus* is more likely to have a successful hunt than a lone wolf (McNulty et al., 2014). The benefits of cooperating (i.e., higher hunting success) outweigh the cost of sharing resources, such as food, with other individuals, and hence may increase an individual's chances of successful reproduction and survival. This allows individuals to thrive via group living in habitats that solitary individuals wouldn't survive in alone.

In many social groups, a reproductive skew exists in which reproduction is monopolised by one or a few individuals, while others forgo reproduction to care cooperatively for the young and take on the tasks of foraging and defence (Chase, 1974; Wilson, 1975; Clutton-Brock, 2009b; Strauss et al., 2022). In such a system, those that reproduce are considered 'dominant', while those that do not are considered 'subordinate' and are reproductively supressed to varying degrees (Keller and Reeve, 1994). In groups of non-kin or in groups where there is low relatedness, subordinates may be willing to supress their own reproduction in favour of another, for a time, in unfavourable environments where resources are scarce (Emlen, 1982) or there is a low frequency of nesting sites (Riehl, 2013). In harsh environments such as these, staying in a group may increase the likelihood of survival for a subordinate individual and the chance they may climb their way up the hierarchy and reproduce at a later date (Goodnight, 2005; Clutton-Brock, 2009a; Leadbeater et al., 2011; Riehl, 2013). However, groups that are made up of non-kin are only considered to have temporary stability, with dominant group members changing over time (Clutton-Brock, 2009a). For example, in a cooperatively breeding cichlid fish *Neolamprologus pulcher*, subordinate males rapidly change their behaviour and anatomy before aggressively attempting to gain the socially dominant position, if the original alpha is removed (Fitzpatrick, 2008).

As explained by inclusive fitness theory, the formation and maintenance of a stable social group, characterised by a permanent reproductive skew, where subordinates are not expected to reproduce in their lifetime, relies heavily on high within-group relatedness (Hamilton, 1964; Frank, 1998; Marshall, 2015). As, in groups consisting of closely related individuals, the initial costs for subordinates that refrain from reproduction are outweighed by the benefits of the altruistic behaviour, which ensures the successful reproduction of genetically related group members and, thus, the transmission of the subordinate's own genes to future generations. In essence, inclusive fitness theory encompasses both an individual's direct fitness and the indirect fitness they obtain from the reproduction of other genetically related group members. Overall, this theory highlights how subordinates can indirectly enhance their own inclusive fitness by assisting closely related relatives in reproduction. The evolution of such extreme forms of reproductive altruism are particularly evident in eusocial societies. These societies are characterised by a reproductive division of labour, cooperative brood care, and overlapping generations (Wilson, 1971). Eusocial societies have independently evolved more than 20 times across various taxa, with the most prevalent instances found in the Hymenoptera (ants, bees, and wasps) and termites (Bourke, 2011).

Yet, while living in a group can give an individual direct and indirect fitness benefits, reproductive conflict can still arise, even in eusocial societies (Wenseleers et al., 2021). Conflict can be costly, with those who partake in it potentially wasting energy via fighting or risking injury (Frank, 1995). However, conflict is not a rarity in social groups. One of the main reasons conflict can arise with in a group is because the relatedness of individuals in a group to group offspring is not equal (Bourke, 2011). This can cause the reproductive interests of group individuals to differ considerably, leading to conflict over how reproduction should be shared amongst group members. As such, individual known as ‘cheats’ are selected to maximise their personal reproductive output by exploiting the cooperative efforts of others in the group, with the effect being reduced fitness for those that cooperate (Ghoul et al., 2014; West and Ghoul, 2019). If a high number of group members were to selfishly reproduce, then the shared resources that sustain the group would be overexploited. This would lead to a ‘tragedy of the commons’ whereby the society either (1) collapses (collapsing tragedy) at a cost to both cooperative and selfish individuals, or (2) takes a large fitness cost (component tragedy) whereby all group members are forced to pay a fitness cost to avoid societal collapse (Rankin, 2007).

A great deal of research has been invested into understanding how social groups balance cooperation and conflict to prevent exploitation from social cheats, and thus the collapse of the society. Two principal mechanisms proposed to suppress cheats, and maintain social group stability, are dominance and policing (Ratnieks and Wenseleers, 2008; Bourke, 2011). These are coercive measures that aim to reduce cheating and enforce cooperation by ensuring that subordinates can only maximise their inclusive fitness by helping to maximise the fitness of the group (Frank, 2003).

Dominance is often defined as aggressive behaviour aimed at gaining direct fitness for the actor by imposing a within-group reproductive monopoly in which rank correlates with reproductive success (Wilson, 1975; Beekman et al., 2003; Beekman and Ratnieks, 2003; Bourke, 2011). In other words, it is considered that the aim of dominance is to aggressively deter individuals lower in rank from reproducing. Policing, on the other hand, is more a form of damage limitation that can occur after a group member has selfishly reproduced (Bourke, 1999; Wenseleers and Ratnieks, 2006a; Ratnieks and Wenseleers, 2008; Bonckaert et al., 2011b). Generally policing involves reproductive and non-reproductive group members searching for, and destroying, eggs or offspring produced by those that have selfishly reproduced. The main benefit to those that use energy to assert their dominance or police group members is that both these mechanisms can prevent those that cheat from successfully reproducing, and, in the case of dominance, can deter them from acting selfishly in the future (Heinze et al., 1994; Ratnieks, 1998; Reeve, 1998; Wenseleers and Ratnieks, 2006a). Overall, these measures are thought to promote social group stability and the production of future reproductives, thus ensuring the propagation of the social group's genes into the next generation.

1.1.2 Thesis aims

Despite the extensive research conducted on dominance and policing in social groups, significant uncertainties remain regarding the ultimate function of dominance behaviours in animal societies and the exact origins of worker policing. Furthermore, it is unclear why highly related individuals within eusocial societies differ so remarkably in the rate at which they perform reproductive behaviours (egg-laying, policing, and aggression). Without a comprehensive understanding of how conflicts originate, and are resolved, an in-depth understanding of social evolution cannot be achieved.

Therefore, the main aim of this thesis is to advance the understanding of how societies remain stable in the face of internal conflict. Specifically, through the use of quantitative experiments involving colonies of the Buff-tailed bumblebee *Bombus terrestris,* this thesis aims to contribute to the understanding of the role dominance hierarchies and policing play in maintaining social stability. Moreover, this thesis also seeks to determine the underlying factors behind reproductive dominance in workers in eusocial Hymenoptera, focusing on elucidating the reasons for within-colony variation in worker quality in eusocial societies that exhibit intermediate complexity.

In the current chapter, I aim to provide a general background on the themes of dominance and policing, firstly in animal societies as a whole and then with a specific focus on the model organism of this thesis, the bumblebee *B. terrestris*. This review of prior research also seeks to identify gaps or limitations in the existing knowledge of how dominance and policing maintain social group stability in animal societies. Overall, this chapter aims to establishes the research context and significance of the subsequent data chapters of this thesis.

1.2 Gaps in the literature

1.2.1 Dominance hierarchies and reproductive output

Agonistic interactions between individuals are commonplace in social groups (Huntingford et al., 1987). Individuals in groups participate in repeated agonistic interactions, many of which involve aggression, and these interactions lead to the formation of dominance hierarchies within a group. Those at the top of the hierarchy are considered the most ‘dominant’; defined as being the most aggressive, and so are expected to have the greatest access to resources and mates (Wilson, 1975). These hierarchies are often thought to be linear, or near linear, with the most dominant individual monopolising reproduction, while those below them in rank are increasingly less likely to achieve reproductive success (Chase, 1974; Strauss et al., 2022). Aggressive acts are thought to be exhibited by individuals in a group either to maintain their rank, or to help them challenge another and gain a higher rank (Ellis, 1995; Cant et al., 2006). Yet, while aggression has certainly been shown to play a role in determining an individual’s social status in a group, whether or not the level of aggression an individual displays, in comparison to others in the group, positively correlates with their overall reproductive success has yet to be confirmed as the general rule across species.

The generality of the correlation between social status and reproductive success was extensively reviewed by Ellis (1995). He found that, overall, the association between social status and reproductive output was positive in 75% of social groups covered in the review, most of these being groups of carnivores, ungulates and rodents. In terms of invertebrates, Ellis’s (1995) review indicated that female dominants had more offspring than subordinates. However, this conclusion was only based on five studies, one of which indicated that subordinates had more offspring. Since Ellis’s (1995) review, a significant positive correlation between social status and reproductive success has been found in additional cases, including a neotropical ponerine ant *Pachycondyla villosa* (Trunzer et al., 1999). However, studies have also found dominance and reproductive success do not correlate in other cases. For example, in colonies of the primitively eusocial paper wasps *Polistes japonicas* (Ishikawa et al., 2011) and *Ropalida marginata* (Gadagkar, 2001) the queen, despite monopolising reproduction, is not the most aggressive group member.

In addition, Ellis (1995) and numerous other researchers since have shown that factors such as group composition (Silk, 2007), group size (Bourke, 1999) and resource availability (Rodrigues, 2018) can affect a dominant individual’s ability to monopolise reproduction. Sneak copulation, whereby subordinate males opportunistically mate with females of their own group, or those of another social group, when a dominant is otherwise distracted, is another factor that affects a dominant's reproductive monopoly. Its occurrence means that dominant individuals are not always successful at monopolising access to the mates they guard. For example, molecular techniques have revealed high rates of extra group paternity in some species, with subordinate males from outside the social group, siring 25% of a group's offspring in meerkats (Young and Hammock, 2007). Sneak copulation is also responsible for relative fitness being found to be equal between dominant and subordinate collared lizards (York and Troy, 2019) and higher in subordinate fur seals *Arctocephalus australis* (Franco-Trecu et al., 2014). This suggests that having the highest dominance rank in a group is not always a guarantee of having the highest levels of direct fitness.

The issues with correlating reproductive success and dominance seem most apparent in invertebrate eusocial societies in which there are varying degrees of a queen-worker dimorphism and workers are often unable to produce one or both sexes of offspring (Beekman et al., 2003). With the exception of termites, these eusocial societies are restricted to those with a haplodiploid sex determination system (in which females arise from fertilised eggs and are diploid and males arise from unfertilised eggs and are haploid) in the order Hymenoptera. The original view of these societies being harmonious has long since dissipated and now they are seen more along the lines of ‘police states’ in which dominance, policing and punishment help enforce altruism amongst workers (Ratnieks and Wenseleers, 2008; Ratnieks and Helanterä, 2009).

In general, before Hymenopteran colonies have formed, mating has already occurred, and so these societies are founded by fertilised queens. Some colonies are monogynous (headed by a single queen) with a monandrous queen (one that has mated with a single male), with the result being that workers are highly related to their sisters, including the gynes (unmated, newly-produced queens) ($r = 0.75$) (Hamilton 1964). However, they are less related to their brothers ($r = 0.25$) and nephews ($r = 0.375$) than to their own sons ($r = 0.5$), and this creates potential queen-worker conflict over the sex ratio of brood and queen-worker and worker-worker conflict over male parentage (Trivers and Hare, 1976). As in other social groups, in some cases, aggression correlates with reproductive success. This is true in some colonies of queenless ant *Dinoponera quadriceps* in which ‘alpha females’ have been observed to dominate reproduction by performing the most aggressive behaviours (Monnin and Peeters, 1999). Orphaned workers of the ant species *Temnothorax nylanderi* with higher numbers of ovarioles tend also to achieve a higher dominance rank than workers with fewer ovarioles (Heinze et al., 1997). This supports research that suggests individuals with higher fecundity should display higher levels of aggression (Cant et al., 2006).

Yet, as mentioned previously, in some species of social wasp, though the queen is the most reproductively dominant, she may not be the most aggressive individual (Ishikawa., et al. 2011; Gadagkar, 2001). Worker aggression is expected to be at its highest when colony size is small, and there is little queen-worker dimorphism. In such a colony, workers have a high level of reproductive potential, which in turn leads to high-levels of reproductive conflict and aggression, directed at both other workers and the queen (Bourke, 2001). For example, in the yellow-jacket wasp *Dolichovespula arenia*, the conflict of interest over male parentage leads to direct confrontations between the queen and some workers. Despite the queen being reproductively dominant, in some colonies workers have been observed killing the queen (worker matricide) (Loope, 2015). While matricide is not common, incidents in which one or a few workers harass and kill the queen have been observed in multiple species (Bourke, 1994).

In one of these species, the bumblebee *Bombus terrestris*, it is possible that the queen has not evolved to be consistently the most aggressive group member because, for much of the colony cycle, she does not need to be. By being the sole producer of gynes (future queens) she provides fitness benefits to workers that they could not achieve by producing their own male offspring (Hamilton, 1964; Trivers and Hare, 1976). Therefore, it would be costly to workers to aggress or kill the queen during the early stages of the colony cycle. It is only in the second part of the colony cycle when the queen has started to produce mainly sexuals (gynes and males) that workers may gain reproductive benefits from killing the queen and reproducing themselves (Bourke, 1994). It has been shown that, at this point, there is a decline in the fecundity of the queen, increasing the incentive for workers to overthrow her and eventually making it in the queen's own fitness interest for workers to become reproductively dominant (Almond et al., 2019). Hence the correlation between dominance and reproductive output may be broken in cases in which changes in the social context alter the cost-to-benefit ratio of subordinate challenges to the dominant.

However, even if aggression provides a means for workers to form a hierarchy in which the most aggressive worker has the highest rank and is thus the most likely to reproduce, aggression and reproductivity are not inevitably linked and, if a colony becomes queenless, the most aggressive worker under queenright conditions (i.e., in the queen's presence) is not always the most aggressive or most likely to reproduce. For example, ovariectomised *B. terrestris* workers have been seen to perform the same aggressive behaviours as workers with intact, activated ovaries, including forming dominance hierarchies and aggressing workers with ovaries (Van Doorn, 1989). Gadagkar (2001) was able to show that, in the wasp *Ropalidia marginata,* dominance ranks between workers change once a queen is removed and that neither the original alpha or beta workers become reproductively dominant. Instead, a lower-ranked worker increases its rate of aggression drastically to gain the position of reproductive dominance. The reasons behind this are not fully understood but the result raises questions over why a group member would remain submissive to a higher-ranking member, if they had the means to overpower them. Potentially it is because the risk of being injured or killed in conflict, and lowering future fitness, is only a risk worth taking when the queen is absent and reproduction is conducted by workers alone (Cant and Field, 2001; Cant et al., 2006).

The results found by Gadagkar (2001) may not be universal. For example Friend and Bourke (2014) found that in the ant species *Leptothorax acervorum* future reproductive workers were more aggressive than their non-reproductive counterparts, both in the presence and absence of the queen. Still, the existence of variation in how an individual’s aggression is correlated with reproductive output in social groups suggests that the link between the two is not clear cut and raises questions over whether dominance ranks (based on aggression) can accurately explain the structure and dynamics of social groups, or be reliably used to predict an individual's reproductive success (Amsalem et al., 2015; Lewis, 2022).

1.2.2 Worker policing

Policing, like dominance, is aimed at supressing reproductive selfishness in social groups. However, it often occurs as a form of damage limitation, after a selfish individual has reproduced, rather than as a threat to deter individuals from acting selfishly in the first place (Ratnieks and Wenseleers, 2008; Riehl and Frederickson, 2016). Policing exists in varying degrees across the animal kingdom, examples being found in social groups of the banded mongoose *Mungos mungo* (Cant et al., 2014) and the honeybee *Apis mellifera* (Ratnieks, 1988). Generally, the more efficient the policing, the less likely a selfish individual is to continue to attempt to selfishly reproduce (Wenseleers and Ratnieks, 2006b). Also, unlike dominance, which aims to increase the direct fitness of the winner of conflict at the cost of the group, policing tends to act more as a ‘public good’, increasing average inclusive fitness of all group members by promoting group cohesion and productivity (Monnin and Ratnieks, 2001; Ratnieks and Wenseleers, 2005; Singh and Boomsma, 2015).

In general, group members police by monitoring other group members and destroying eggs (oophagy) (Ratnieks, 1988) or killing offspring (infanticide) (Young et al., 2007) of social cheats. In some species, policers also attack nest mates that lay eggs (Visscher and Dukas, 1995). It is likely policing evolved, in part, because it is of low cost to the actor. This is especially so in colonies of eusocial insects in which interactions with eggs form part of brood maintenance activities. Thus, the act of checking for worker-laid eggs and destroying them when found does not require large amounts of extra time or energy (Bourke, 2011). Policing systems usually require a mechanism whereby policing individuals can discriminate between brood to police and brood to be spared. In eusocial insects, this system appears to involve chemical cues that help workers differentiate between worker-laid eggs and eggs laid by the queen (Zanette et al., 2012; Oi et al., 2015).

Policing is most prevalent in eusocial Hymenoptera, where the behaviour has been recorded in colonies of ants, wasps and bees (Wenseleers and Ratnieks, 2006a). In many species workers retain ovaries but cannot mate, and so are capable (via haplodiploidy) of producing male offspring from unfertilised eggs (Bourke, 1988). In some species where there is a low level of queen-worker dimorphism, workers can even have the same number of ovarioles as the queen (Amsalem et al., 2015). Workers' ability to produce male offspring results in a high level of potential conflict over male parentage between the queen and workers (Trivers and Hare, 1976). In addition, on average, workers that lay eggs have been shown to perform fewer tasks for the colony than their non-laying counterparts (Roth et al., 2014). This means that the presence of reproductive workers risks causing a decline in colony productivity. Therefore, it has been hypothesised that, to mitigate this, coercion evolves in the form of policing (Wenseleers et al., 2004b). While policing does not generally injure policed individuals directly, or prevent them from laying eggs in the future, it does make egg laying costly and unprofitable. It therefore acts to dissuade individuals from selfishly reproducing (i.e., to evolve self-restraint), as the chances of successfully reproduction are too low to warrant the use of energy on egg laying (Wenseleers et al., 2004a).

1.2.3 Worker policing: maintenance

In some species, queens are polyandrous (mate with multiple males). For example, in the honeybee *Apis mellifera,* the queen mates with 10 drones on average, and this produces a worker caste of mixed paternity (Ratnieks, 1988). Polyandry is thought to be a major factor in the evolutionary maintenance of worker policing as it produces a relatedness skew in which workers are more related to their brothers (r = 0.25) than they are to their 'nephews', i.e., sons of nest-mate workers (r = c. 0.15). As workers are more related to their own male offspring (r = 0.5) there is still selection for some to lay eggs, but workers are also selected to police each other. This is because each worker receives higher indirect fitness benefits from ensuring the queen is reproductively successful than from ensuring that other workers are reproductively successful. Likewise, the queen is also selected to police workers as she is more related to her sons (r = 0.5) than she is to her grandsons (r = 0.25) (queen policing) (Ratnieks, 1988; Ernst et al., 2017).

Policing is an effective means of preventing workers from reproducing successfully and thus of helping maintain group stability. For example, in honeybee colonies *Apis mellifera* worker policing has been recorded as being so effective that workers only produce 0.1% of a colony's adult males. This is despite their laying approximately 7% of the colony's male-destined eggs (Visscher, 1996). In addition, worker policing has been shown to be effective because, as the threat of oophagy and aggression is so high, only 1 in 10,000 workers activate their ovaries in the presence of a highly reproductive queen (Ratnieks, 1993; Visscher and Dukas, 1995).

There is substantial comparative evidence for the relatedness differences outlined above having had a major effect on the maintenance of worker policing. Worker policing occurs more frequently in species where workers are more related to the queen's sons than in species where they are more, or equally related, to other workers' offspring (Wenseleers and Ratnieks, 2006a; Bonckaert et al., 2008). Moreover, an analysis of male parentage has shown that a significantly higher percentage of males are worker-derived in species where the workers are more related to other workers' sons (14%) than in species where they are more related to the queen's sons (0.14%) (Wenseleers and Ratnieks, 2006a). This suggests that, over evolutionary time, worker policing is more likely to be maintained in a species if relatedness between workers is lower than workers' relatedness to the queen. However, while these and the earlier-cited studies have uncovered the role policing plays in ensuring social stability, how policing originated in social groups remains less clear and there has been relatively little research in this area.

1.2.4 Worker policing: origin

It is unlikely that relatedness differences such as those associated with polyandry played a major role in the origin of worker policing. This follows because, while worker policing is widespread in colonies characterised by polyandry or polygyny, it has also been recorded in 20% of species characterised by monogyny and monandry

(Bonckaert et al., 2008; Zanette et al., 2012). In such a system, workers are more related to their sons (r = 0.5) and nephews (r = 0.375) than they are to the queen's sons, their brothers (r = 0.25), suggesting that workers should be selected to raise worker-produced over queen-produced males (Wenseleers and Ratnieks, 2006a; Brunner and Heinze, 2009). However, research has found that, despite these higher levels or relatedness in favour of worker-produced males, in some species workers selectively destroy other workers' viable eggs, while the queen’s male eggs remain mostly unpoliced (Zanette et al., 2012). In the eusocial Hymenoptera, polyandry is a derived trait, with monandry being the ancestral state for all eight independent origins of eusociality (Hughes et al., 2008). Hence it is possible that worker policing originated before polyandry in the eusocial Hymenoptera (Zanette et al., 2012). If so, a higher level of relatedness to brothers over 'nephews' (Starr, 1984; Ratnieks, 1988; Wenseleers and Ratnieks, 2006a) cannot be the reason worker policing originated.

It has also been hypothesised that colony efficiency benefits might favour the evolution of worker policing, within societies with monogyny and monandry (Ratnieks, 1988; Hammond and Keller, 2004). This hypothesis suggests that worker reproduction may result in a reduction in the number of sexuals produced overall, and thus have a high colony-level cost. This is because reproductive workers perform fewer tasks, on average, than their non-reproductive sisters, and the high number of eggs laid result in inefficiencies in brood rearing (Wenseleers et al., 2004a; Ratnieks et al., 2006). Therefore, worker policing could be selected for, even in monandrous conditions, as it maximises a worker's indirect fitness by increasing colony-level productivity (Ratnieks, 1988). However, in one of the few monandrous, monogynous species in which costs of worker reproduction were estimated, *Bombus terrestris*, research has suggested that costs of worker reproduction are low (Lopez-Vaamonde et al., 2003). This implies that saving costs to colony-level productivity is unlikely to have played an important role in the origin of worker policing.

Other research has suggested worker policing may have originated as a means to defend against intraspecific parasitism by drifter workers (Beekman and Oldroyd, 2008; O'Connor et al., 2013). Workers have been recorded ‘drifting’ from their natal colony to other colonies of the same species (Lopez-Vaamonde et al., 2004; O'Connor et al., 2013). Once these host colonies have been infiltrated, drifters have been observed laying twice as many eggs as natal workers. As drifters tend to have activated ovaries it has been suggested that this is an alternative worker reproductive strategy to staying in the natal colony to reproduce (Beekman and Oldroyd, 2008). Hence, it has been suggested that worker policing may have evolved as a defence against these parasitic drifters. However, the frequency of drifter workers has been estimated at 2-3 % in wild *Bombus terrestris* colonies (O'Connor et al., 2013; Zanette et al., 2014), and so does not seem high enough to have provided sufficient selection for policing to have evolved. While it is possible drifting may have been higher in the past, *B. terrestris* workers are unable to differentiate between queen-laid eggs and drifter worker-laid eggs (Holland et al. unpublished), consistent with drifter eggs evading policing (Lopez-Vaamonde et al., 2004). Had policing evolved to remove drifter eggs from a colony, it is likely workers would also have evolved to be able to differentiate between a natal queen's egg and a drifting worker's eggs. As this is not the case, it seems unlikely to be the reason policing originated.

A final mechanism that has been hypothesised to be responsible for the origin of worker policing is 'selfish worker policing'. This theory suggests that egg-laying workers police each other, not for the common good, but for personal gain, increasing their own direct fitness, by replacing consumed eggs with their own, and reducing competition. For example, in species in which brood are reared in cells, killing the eggs of a rival sister would make more cells available for a policer to oviposit in (Wenseleers et al., 2005). Therefore, policers would gain sufficient fitness benefits to warrant the cost of policing their sisters, even if the relatedness to their nephews is higher than that to their brothers. This hypothesis is supported by observations in colonies of ants (Stroeymeyt et al., 2007) wasps *Dolichovespula slyvestris* (Wenseleers et al., 2005)*; Dolichovespula norwegica* (Bonckaert et al., 2011a) and bumblebees *Bombus terrestris* (Zanette et al., 2012). In all these species, egg-laying workers were more likely to eat more eggs per capita, and to aggress other egg-laying workers, than non-laying workers. This directed aggression from egg-laying workers to other egg-laying workers suggests that worker policing may have originated as a means for reproductive workers to gain direct fitness benefits at the expense of rival reproductive worker nest mates. However, the selfish policing hypothesis does not fully account for the observations of non-reproductive workers engaging in egg-eating events, as will be discussed in more detail below (**Section 1.3.5**). This unresolved aspect indicates that there may be additional factors and complexities involved in the evolution of worker policing beyond the selfish motives of reproductive workers.

1.2.5 Intrinsic quality

While reproduction, dominance, and policing can provide an individual with fitness benefit, these behaviours can also come with significant physical and energetic costs. Reproduction especially is a resource-demanding process. In general, as organisms only have limited resources for growth, maintenance, and reproduction and, as natural selection prioritises reproductive success over survival, the allocation of resources towards reproduction comes at the expense of maintenance and repair mechanisms that promote longevity. The fecundity-longevity trade-off may, at the proximate level, result in molecular damage accumulating in an organism over time, resulting in a decreased probability of survival per unit time beyond a certain age, i.e., ageing (Williams, 1957; Hamilton, 1966; Kirkwood, 1977; Stearns, 1992; Edward and Chapman, 2011).

In multiple taxa, sociality can alter the relationship between reproduction and longevity, with reproductive individuals that live in cooperatively breeding groups, or eusocial societies, having significantly longer lifespans compared to their non-social relatives (Kim et al., 2011; Hammers et al., 2019; Korb and Heinze, 2021). Evidence of the absence of the fecundity-longevity trade-off is perhaps most apparent in eusocial

Hymenoptera (ants, bees, wasps) and termites, in which reproductive castes (queens and kings) have been shown to have significantly longer lifespans in comparison to non-reproductive, or less-reproductive, castes (workers) (Keller and Genoud, 1997; Bourke, 2007; Lopez-Vaamonde et al., 2009; Kramer et al., 2015; Southon et al., 2015; Rodrigues and Flatt, 2016).

However, the costs of reproduction have also been shown to vary significantly among individuals of a species, irrespective of their social organisation. In essence, both non-social individuals (Van Noordwijk and De Jong, 1986; Reznick et al., 2000) and social individuals (Keller and Genoud, 1997; Bourke, 2007; Lopez-Vaamonde et al., 2009; Kramer et al., 2015; Southon et al., 2015; Rodrigues and Flatt, 2016) differ in intrinsic quality (i.e., inherent characteristics or traits that contribute to their overall quality or fitness). Observations have shown high-quality individuals both reproduce more and live longer than poor-quality individuals. For example, research has shown that in species of ants (Negroni et al., 2021), honeybees (Dixon et al., 2014) and bumblebees (Blacher et al., 2017), workers that activate their ovaries and/or reproduce (via asexual reproduction of haploid sons) live longer than workers that do not reproduce during their lifetime.

Furthermore, recent research has suggested that in eusocial insects that exhibit an intermediate level of eusociality and an annual life-cycle, the reversal of the fecundity-longevity trade-off in queens and workers may be condition dependent, with only high quality, well-resourced individuals able to overcome the costs of reproduction (Blacher et al., 2017; Collins et al., 2023). Given the high relatedness observed among nest-mate workers in eusocial societies, the question arises as to how they might differ so substantially in intrinsic quality, to the extent that some are able to overcome the costs of reproduction while others cannot. It seems unlikely that genetic differences alone can explain the significant variation in intrinsic quality observed. Instead, it is possible that environmental factors play a crucial role in determining a worker's ability to overcome the costs of reproduction. However, this remains to be tested and further studies are required to investigate the role of genetic variation, environmental conditions, and social interactions in shaping individual traits and fitness. Understanding how these factors interact and contribute to the observed variations among individuals would provide valuable insights into the mechanisms underlying reproductive dominance in workers in eusocial Hymenoptera.

1.3 *Bombus terrestris*

1.3.1 Why study this genus & species?

Due to their high-levels of cooperation and conflict, insect societies have been used widely as a model system for investigating the causes of conflict and the mechanisms behind conflict resolution in social groups (Ratnieks et al., 2006). Within the Hymenoptera, eusociality has evolved independently multiple times, with different lineages showing varying degrees of eusociality (Hughes, 2008). Within the family Apidae*, Apis* species are considered to show an advanced level of eusociality in which there are strong queen-worker morphological differences, as well as specialised behavioural worker castes (Butler, 1954; Neumann et al., 2011; Dixon et al., 2014). In comparison, due to their weak queen-worker morphological differences, bumblebees (*Bombus* species) are considered to represent an intermediate level of eusociality (Collins et al., 2017). As both *Apis* and *Bombus* share a common ancestor (Cardinal and Danforth, 2011), bumblebees can be seen as representing a system that is likely to be both the closest extant model for this common ancestor and a potential forerunner of advanced eusociality. Thus, investigating the social biology of *Bombus* species not only offers an opportunity to explore the proximate mechanisms that regulate social behaviour, but also a chance to determine the ultimate mechanisms underlying the maintenance of intermediate eusociality and the origin of advanced eusociality from intermediate eusociality (Amsalem et al., 2015).

Though 250 species of bumblebee have been discovered, only five have been successfully reared commercially for the purpose of crop pollination (Velthuis and van Doorn, 2006). Two of these, *B. impatiens* and *B. terrestris*, are also used widely for laboratory and field studies. The availability of commercially reared colonies all year round in these species makes them advantageous in scientific studies as they ensure research is not limited by seasonal variation in the availability of wild colonies. In addition, *Bombus* species can be seen as ideal study species as their colonies are fairly small and have an annual colony cycle, meaning a full cycle can be observed within a few months. In recent years, both *B. impatiens* and *B. terrestris* have gained value as model organisms as their genomes have been sequenced (Sadd et al., 2015). Molecular tools have provided important insights into the evolution of *Bombus* species that previous observational methods had been unable to uncover. Continued advances in molecular and genomic methods, combined with a sequenced genome, mean *B. impatiens* and *B. terrestris* represent key study species for exploring the behavioural and genomic mechanisms behind the origin and evolution of social behaviour.

Among *Bombus* species, *B. terrestris* is a highly suitable one in which to research the origin of policing and the role of dominance in maintaining social stability. *B. terrestris* has already become a model species in the study of social evolution (Zanette et al., 2012), social and learning behaviour (Alem et al., 2016) and pollinator ecology (Becher et al., 2018), meaning there is a wealth of data on which to build future research. Aside from this, levels of worker aggression and ovary activation are high in both queenright and queenless *B. terrestris* colonies, with up to 64% of *B. terrestris* workers activating their ovaries by the end of the colony cycle (compared to 11% in colonies of *B. impatiens*) (Duchateau and Velthuis, 1989; Cnaani et al., 2002; Alaux et al., 2004; Jandt and Dornhaus, 2011). In addition, two recent studies in *Bombus terrestris* have suggested that, both queens and workers can experience costs of reproduction in this system, so do not exhibit a genuine reversal of the negative fecundity-longevity relationship (Blacher et al., 2017; Collins et al., 2023). Therefore. to determine how conflict originates and is resolved, as well as what mechanisms ensure social group stability and intrinsic quality among individuals of a group, *B. terrestris* makes an excellent model species.

1.3.2 Life Cycle

Of the 250 species of bumblebee that have been described (Williams and Osborne, 2009), almost all reside in the temperate regions of the northern hemisphere (Goulson, 2010), though some species occur in South America (i.e. *B. dahlbomi*) and others in the Arctic (i.e. *B. polaris*). Species may differ in their choice of nest site, and colony size, but the life cycle of most social bumblebees follows the same general pattern (**Figure 1.1**). These species are eusocial and typically have an annual colony life cycle in which monogynous colonies, headed by a monandrous or weakly polyandrous queen, rear one generation per year. The colony cycle begins in the spring with a solitary phase in which a lone queen emerges from 6 months of diapause and finds a suitable nest site, where she creates a brood clump and begins to oviposit, initially laying eggs that will produce workers (Goulson, 2003). This solitary phase is perhaps the most dangerous to the queen as she alone must perform all tasks required to maintain the colony, including foraging for pollen and nectar, nest maintenance and incubation of brood. If she dies during a foraging trip, then the colony will fail to develop. She is less at risk 16-25 days into the cycle, when the first cohort of workers ecloses and the eusocial phase of the cycle begins (Heinrich, 1979). Here workers take over nest maintenance, brood incubation, and foraging, allowing the queen to focus solely on laying eggs. Workers are considered to be flexible in terms of what task they perform, and division of labour does not seem to be strongly correlated with body size (Peat et al., 2005). This period is generally harmonious, and the colony undergoes a phase of accelerating growth (Heinrich, 1979; Lopez Vaamonde et al., 2009) as the queen continues to be the sole egg layer, laying exclusively diploid eggs that develop into workers.

Part-way through the colony cycle, the queen changes to laying eggs that develop into reproductives (diploid eggs developing into new queens and/or haploid eggs developing into males). The point at which the queen stops laying exclusively diploid eggs and begins to lay predominantly haploid eggs is known as the *switch point* (Duchateau and Velthuis, 1988). In *B. terrestris*, this is a well-defined event, with the queen switching from laying 0% to laying 100% haploid eggs over a period of approximately 10 days (Duchateau et al., 2004), although in other species this may not be the case. In some but not all populations of *B. terrestris*, colonies exhibit bimodal sex ratios, being either early-switching colonies that show protandry (early male production) and produce relatively many males or late-switching colonies that exhibit protogyny (late male production) and produce relatively many new queens (Duchateau and Velthuis, 1988; Bourke and Ratnieks, 2001; Duchateau et al., 2004; Lopez-Vaamonde et al., 2009). It is thought workers use changes in the pheromone output of the queen, caused by her switch to producing gynes, to determine when to begin laying haploid eggs (Bourke and Ratnieks, 2001; Alaux et al., 2006). This initiates the *competition point* (Duchateau and Velthuis, 1988) which is defined by the appearance of the first worker-laid eggs and is followed by intense competition over egg laying as cooperation between the queen and some workers breaks down. It is in this period that egg-policing by queens and workers occurs (Zanette et al., 2012). This competition, though intense, is short lived. This is in part due to bumblebees' reliance on floral resources as a source of forage. As these resources are only available during the summer months in temperate habitats, this limits the growing season of a colony and its overall size (Heinrich, 1979). Thus, between late summer and early autumn, colonies disintegrate, the old queens and her workers senesce, and the gynes and drones have left to mate. Newly inseminated queens then enter hibernation, allowing the cycle to begin again the following year (Heinrich, 1979, Goulson, 2003).

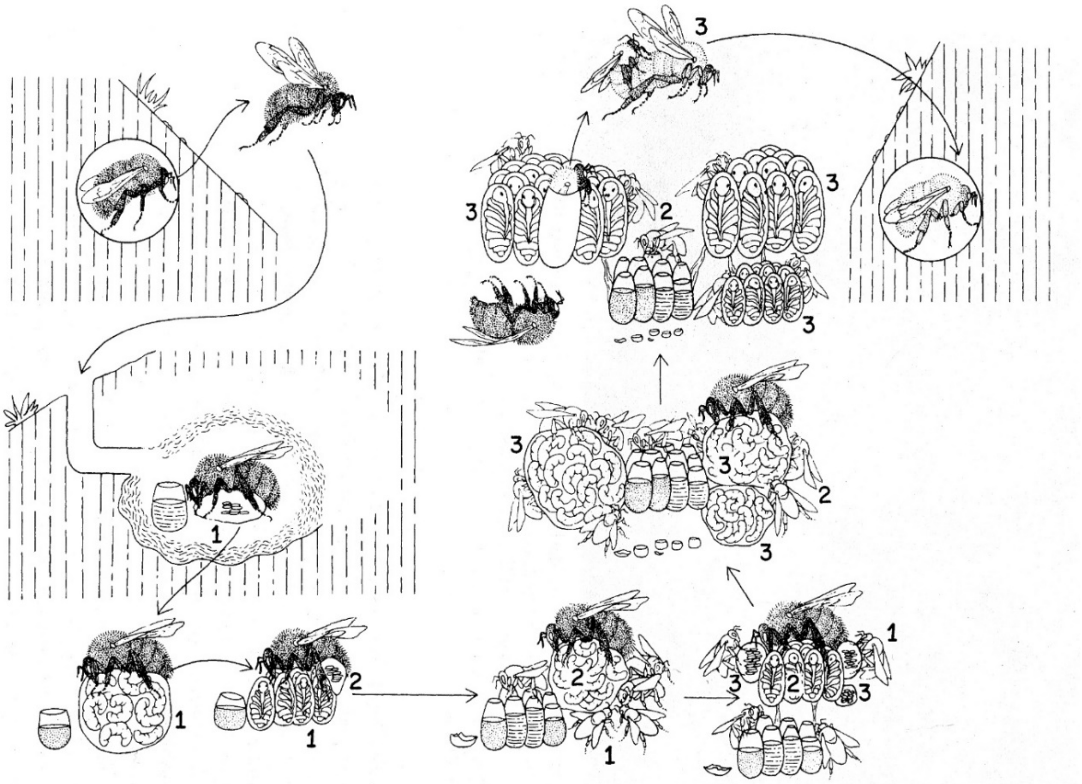


Figure 1.1. The colony cycle of a typical bumblebee species (top left to top right). Numbers 1 & 2 represent two worker broods, while number 3 represents gyne brood. All brood is seen to progress from eggs to larvae, pupae and adults (from Heinrich, 1979).

1.3.3 Queen-Worker Conflict

In *B. terrestris*, prior to the competition point, aggression appears to play only a minor role, if any, in skewing reproduction in the queen’s favour. Instead, it seems more likely that this reproductive monopoly is achieved simply because it is in the fitness interest of workers to forgo reproduction and altruistically aid the queen's reproduction during the growth stage of a colony. *B. terrestris* queens are obligatory monandrous (Estoup et al., 1995; Lopez-Vaamonde et al., 2004). Therefore, as described earlier, workers are more related to their sisters (r = 0.75) than to their own offspring (r = 0.5). Hence, other things equal, workers are selected to abstain from reproducing and instead work to ensure that, while the queen continues to lay eggs yielding workers and gynes, the reproductive success of the queen is as high as possible. However, potential conflict arises when, at the switch point, the queen begins to produce males. This is because workers are less related to their brothers (r = 0.25) than they are to their own sons (r = 0.5) or nephews (r = 0.375). For this reason, workers are selected to prefer to favour worker- over queen-produced males. This generates potential conflict with the queen as she is more related to her own sons (r = 0.5) than to her grandsons (r = 0.25) and with other workers who again who are selected to raise their own sons, rather than their nephews (r = 0.375) (**Figure 1.2**) (Hamilton, 1964; Trivers and Hare, 1976).

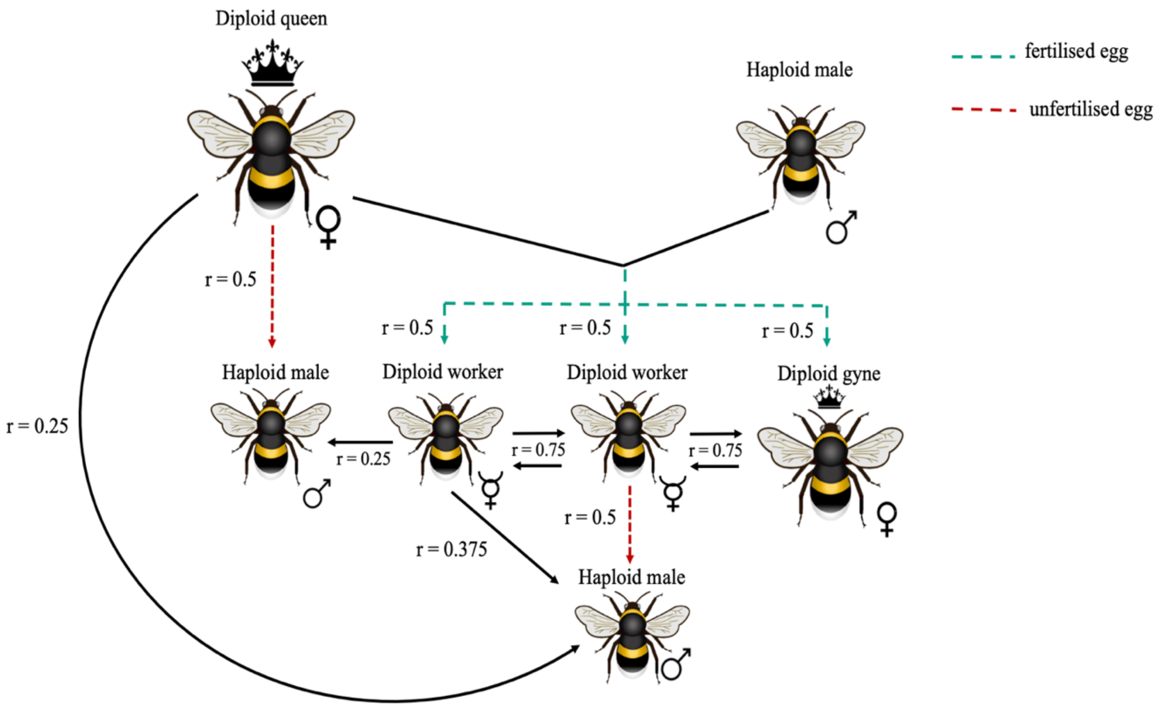


Figure 1.2. Within-colony relatedness values (r) between different castes and sexes in a colony of eusocial Hymenoptera headed by a single, monandrous queen (such as a colony of *Bombus terrestris*).

It is possible that, in eusocial Hymenoptera as a whole, aggression played a major role in producing a reproductive skew in the ancestral state, but pheromones have since evolved to become the main mechanism that regulates behaviour and reproduction in eusocial insect colonies (Van Oystaeyen et al., 2014). Bumblebees, like many social insect species, have a pheromone-mediated communication system that is believed to regulate queen-worker interactions (Van Oystaeyen et al., 2014; Holman, 2018). As in other species, it is thought the *B. terrestris* queen may produce a pheromone or pheromonal mix that honestly signals to workers her fecundity, i.e. that workers can use to determine the queen's level of fecundity and initiate egg laying in conditions where to do so would be most favourable to their inclusive fitness (Alaux et al., 2006; Lopez-Vaamonde et al., 2007). Research has suggested that pheromones produced by the queen are non-volatile and that workers must make direct contact with the queen to determine her levels of fecundity (Alaux et al., 2004; Lopez-Vaamonde et al., 2007). While research has yet to identify this queen pheromone, studies have suggested that the hydrocarbon pentacosane (C25) may serve as this pheromone (Holman, 2014; Van Oystaeyen et al., 2014). However, this has been disputed (Padilla et al., 2016) and more research is required to fully identify the pheromones responsible for regulating reproduction in bumblebee colonies.

Nonetheless, while the identity of the pheromone has yet to be discovered, research has shown that it is highly likely that workers use pheromones, produced by the queen, to determine whether or not it is in their interest to act as a helper or to selfishly oviposit (Zanette et al., 2012). Thus, while in *B. terrestris* colonies, the queen is the sole reproducer prior to the competition point, this seems less to do with her levels of aggression and more to do with her overall value to workers' fitness. This is emphasised by the fact that, even prior to the competition point, some workers have activated ovaries and yet refrain from laying eggs (Duchateau and Velthuis, 1989; Alaux et al., 2004). Aside from this, ovary-activated workers will revert to 'sterility' in the presences of a queen producing diploid eggs (Alaux et al., 2007). These findings all suggest that workers choose to defer reproduction to maximise their inclusive fitness (Bourke and Ratnieks, 2001; Almond et al., 2019).

Queen-worker conflict only becomes apparent following the competition point, which is characterised by aggression, worker oviposition and oophagy (Duchateau and Velthuis, 1988, 1989; Bourke and Ratnieks, 2001). As previously stated, conflict arises because the relative relatedness to queen-derived males differs between a queen and her workers (Trivers and Hare, 1976). Recent research has shown that a queen lays significantly fewer eggs per cell as the time since the switch point increases (Almond et al., 2019). In addition to this, the workers' share of male parentage decreased significantly with queen longevity (Almond et al., 2019). Thus, in some circumstances, i.e. when they sense that queens are returning a decreasing level of indirect fitness to them, workers may kill the queen in order to gain nest inheritance and lay eggs unhindered by queen policing (Bourke, 1994). It has been shown that workers that eventually lay eggs are more likely to aggress the queen than workers that do not (Almond et al., 2019). This suggests that there is a link between aggression and reproductive output and that, in some cases, the queen may lose her top rank as the result of harassment from workers. However, it is not yet clear if the most reproductively active worker is the most likely to aggress, and even kill, the queen. In most cases, despite the high rates of egg laying by workers (69% of all male eggs; Zanette et al., 2012), only 5%-6.9% of adult males are produced by workers in *B. terrestris* colonies (Alaux et al., 2004b; Zanette et al., 2012). In part, this is due to the high rates of oophagy performed by the queen, who is responsible for around 50% of all policing of workers' eggs (Zanette et al., 2012). Therefore, it is not fully understood why matricide, in colonies characterised by one singly mated queen, is not a common occurrence after the switch point, when egg-laying workers would benefit from high direct fitness by disposing of their queen (Almond et al., 2019).

1.3.4 Worker-Worker Conflict

In *B. terrestris*, aggression seems to play an important role in establishing social and reproductive dominance ranks between workers. Previous research has categorised aggression into three main behaviours. The first, and most aggressive, of these behaviours is *Attack*. This involves one worker making physical contact with another bee in an aggressive manner, resulting in biting, pushing, struggling or attempted stinging. The second behaviour, *Humming*, is characterised by short bursts of wing vibrations directed at another bee. The third behaviour is *Darting* and involves a worker making a sudden and directed movement towards another bee, without making actual contact (Duchateau and Velthuis, 1989; Bloch and Hefetz, 1999; Amsalem and Hefetz, 2010; 2011; Sibbald and Plowright, 2013; 2014; Amsalem et al., 2015).

It has been suggested that, in queenright colonies the formation of dominance ranks, amongst workers, begins even before the competition stage. Various studies have suggested that an ‘elite worker group’ forms prior to the competition point, with members interacting predominantly with one another and the queen (Van Honk and Hogeweg, 1981; Van Doorn and Heringa, 1986). The high level of interaction with the queen may represent a means of determining her fecundity via her pheromonal signals as described earlier. This elite group is thought to be made up of older workers, from the first and second cohorts, some of which have transitioned from foragers to in-house workers (Van Doorn and Heringa, 1985; Duchateau and Velthius, 1989; Duchateau, 1989; Bloch and Hefetz, 1999). While workers are able to join or leave this group prior to the competition point, only those who are members of the elite group, once the competition phase has commenced, are thought to lay eggs (Van Doorn and Heringa, 1985; Duchateau, 1989). Aggression, expressed predominantly by ovary-active workers towards other ovary-active workers, has been observed to begin during the competition phase (Van Doorn and Heringa, 1985; Duchateau, 1989). However, workers with active ovaries have also been shown to be significantly more likely to aggress the queen, after the competition point, than workers with non-active ovaries (Almond et al., 2019) and to inhibit the ovary development of younger workers (Van Doorn and Heringa, 1985; Duchateau and Velthius, 1989; Bloch and Hefetz, 1999). By contrast, those that forage, once the competition point has been passed, are unlikely to have activated ovaries (Duchateau and Velthius, 1989) and may even emit a ‘sterility signal’, secreted from their Dufour’s gland, to signal that they are not reproductively active to dominant workers, and so render them safe from attack (Amsalem et al., 2013).

The above studies show that only a subset of workers activate their ovaries and those with activated ovaries are more aggressive than their ovary-inactive sisters. They also show that subordinates may emit a sterility signal that reduces costly fighting between reproductive workers and subordinates, allowing subordinates to forage for the colony and to boost productivity (Amsalem et al., 2013). This suggests that a dominance hierarchy forms between workers, with ovary-active workers showing higher ranks than their ovary-inactive counterparts. However, as these studies measured level of reproductivity only as the degree of ovary activation, it is unclear if these dominant workers would have been able to use aggression to monopolise egg-laying in colonies or if another worker would have laid more eggs and, overall, produced more male offspring. For example, previous research has shown that up to 64% of workers may have active ovaries capable of egg-laying within a colony, but only 38% may lay eggs (Alaux et al., 2004). In addition, a study found that ovariectomised workers perform the same aggressive behaviours as workers with intact, activated ovaries (Van Doorn, 1989). This suggests that ovarian activation may not necessarily lead to oviposition and that aggressive behaviours are not limited to ovary-active workers. Indeed, research that has focused on measuring an individual's dominance rank against their egg-laying rate has found no relationship between the levels of aggression a worker exhibited and the number of egg-laying events they performed, both in the presence of the queen (Duchateau, 1989) and in queenless conditions (Sibbald and Plowright, 2013; 2015). Thus, while it is clear there is a link between aggression and ovary activation, it is not clear how strong this link is, and what role other factors play in regulating worker reproduction and in making one worker reproductively dominant over another.

1.3.5 Worker Policing

Zanette et al. (2012) showed that it is highly likely that worker policing originated in *B. terrestris* in the form of selfish worker policing. In this study, queens were the most prolific eaters of worker-laid eggs (eating 49.3% of all worker-laid eggs eaten), followed by reproductive workers (27.6%) and non-reproductive workers (23.1%). The study found that egg-eating workers were significantly more likely to have activated ovaries, than their non-egg eating sisters, suggesting that though policing is not exclusively performed by reproductive workers, there is still a positive link between reproduction and egg-eating behaviours. Most worker eggs were eaten soon after they had been laid, so accounting for low proportion of adult males found to be worker-produced (Alaux et al., 2004; Lopez-Vaamonde et al., 2004). Zanette et al. (2012) also showed that queen- and worker-laid male eggs had equal viability and that workers can discriminate between queen- and worker-laid eggs using cues (most probably chemical cues) on eggs and egg-cells. Therefore, it is unlikely worker-laid eggs are eaten for hygienic reasons in this system.

Though the theory that worker policing has a selfish origin is highly plausible, it fails to explain the occurrence of two policing-related phenomena observed in some species, including *B. terrestris.* The first is workers' avoidance of policing queen eggs. Despite the fact that, in species with monogyny and monandry, workers are less related to their brothers than their sons or nephews, workers in two such species rarely ate their queen's eggs when observed (*Dolichovespula sylvestris*: Wenseleers et al., 2005; *B. terrestris*; Zanette et al., 2012). This was despite the queen actively policing workers' eggs (Wenseleers et al., 2005; Zanette et al., 2012). Why workers tend to avoid eating queen laid eggs is not known. Research suggests that, while workers are able to use surface cues on eggs to differentiate between queen-laid and worker-laid male eggs, they may be selected to avoid eating both queen-laid male and female eggs, so as to avoid eating eggs developing into gynes (Zanette et al., 2012). However, further research is required to determine if haploid and diploid eggs laid by a queen have the same surface chemical profile or not.

The second behaviour that selfish policing fails to explain in *B. terrestris* is policing of worker-laid eggs by non-laying workers (Zanette et al., 2012). At first glance this behaviour seems maladaptive as, by policing their sisters’ eggs, non-reproductive workers are not only decreasing their sisters' direct fitness benefits, but their own indirect benefits as well. Though various theories have been put forward to explain this phenomenon including the public good hypothesis and selfish policing hypothesis (**Section 1.2.4**), currently, mainly through lack of investigation, there is little evidence for any hypothesis. For example, empirical research that has attempted to determine if worker reproduction is costly at the colony level within monandrous monogynous systems has provided mixed results (Lopez-Vaamonde et al., 2003; Tsuji et al., 2012). It is therefore unclear if worker policing improves the overall productivity of a colony, a key component of the public goods hypothesis. In terms of the selfish policing hypothesis, no empirical studies exist that test whether non-laying workers observed policing in these systems later become reproductive. Hence, though policing by non-reproductive workers is acknowledged as a major mechanism of conflict resolution within eusocial societies (see above), how it originated remains unresolved.

1.3.6 Variation in intrinsic quality among workers

A study by Blacher et al. (2017) found evidence that in the bumblebee *Bombus terrestris,* a positive relationship between ovary activation and longevity exists among workers in unmanipulated colonies, indicating that workers who naturally activate their ovaries tend to live longer. However, when workers were experimentally manipulated to induce ovary activation, the positive relationship was reversed. This result suggests that within unmanipulated colonies, there exists a variation in intrinsic quality among workers. Specifically, those workers who freely choose to reproduce are of higher quality, enabling them to overcome the costs associated with reproduction (Blacher et al., 2017). Expanding upon these findings, a study by Collins et al. (2023) found that *B. terrestris* queens that were experimentally manipulated to increase their egg-laying rate had significantly lower longevities than control queens whose egg-laying rates were not manipulated. In this case, the positive fecundity-longevity relationship is thought to arise because the costs of reproduction for queens are latent, meaning that in unmanipulated colonies, queens perform egg-laying events at a rate that does not induce a reproductive cost, thus allowing them to exhibit both high fecundity and high longevity (Collins et al., 2023).

Overall, these studies suggest that in social systems, such as those found in *Bombus* species, both queens and workers may experience costs of reproduction, indicating that there is no genuine reversal of the negative fecundity-longevity relationship. However, inherent differences in intrinsic quality allow some individuals to overcome these costs. This interpretation aligns with general arguments that fecundity-longevity relationships can become positive when there is variation in individual quality within populations, enabling high-quality individuals to be both highly fecund and long-lived (Van Noordwijk and De Jong, 1986). The cause of these inherent differences in quality, have yet to be determined, however Blacher et al. (2017) put forward a compelling hypothesis that the observed variation in workers' intrinsic quality could be linked to differences in their rearing conditions during development. In other words, workers that receive a greater quantity of larval nutrition during their development eclose as adults with larger body sizes. In turn, a larger body size provides these workers with superior physical conditions and resource-holding abilities, contributing to their enhanced reproductive success.

In the competitive social environment of a bumblebee colony, having a larger body size could confer reproductive advantages for a worker, especially during aggressive interactions. However, while prior research has established that a positive relationship between worker body size and ovary activation does exist in colonies of *Bombus terrestris* (Blacher et al., 2017), other studies have indicated that ovarian activation does not necessarily result in a worker performing egg-laying events during its lifetime (Alaux et al., 2004; Sibbald and Plowright, 2014). Indeed, a study that investigated the egg-laying rate and body size of *B. terrestris* workers found no correlation (Duchateau and Velthuis, 1989), challenging the notion that body size plays a crucial role in determining a worker's likelihood of becoming an egg-layer (Duchateau and Velthuis, 1989).

Therefore, further investigations are required to establish a direct link between larval nutrition, adult body size, reproductive behaviours, and longevity. Such studies would provide definitive evidence for the role of rearing conditions in shaping worker intrinsic quality in *B. terrestris* colonies, and potentially other eusocial species.

1.4 Summary

As shown above, the bumblebee *Bombus terrestri*s represents a model organism for investigating the origins of policing, the ultimate function of dominance hierarchies in animal societies, and the underlying factors influencing variations in worker intrinsic quality within eusocial societies.

Using colonies of *B. terrestris*, in **Chapter 2** of this thesis, I aim to discriminate between the two primary hypotheses - the public goods hypothesis and selfish policing hypothesis - proposed for the origin of non-reproductive worker policing in eusocial Hymenoptera. Further to this, in **Chapter 3** of the thesis, I utilise queenless groups of *B. terrestris* workers to test for a within-group positive association of dominance and reproductive success. Lastly, in **Chapter 4** of this thesis, I aim to test the hypothesis that positive associations between fecundity and longevity among adult *B. terrestris* workers are a consequence of within-colony variation in intrinsic worker quality, resulting from rearing conditions during larval development and specifically from the level of larval nutrition provided.

1.5 References

Alaux, C., Boutot, M., Jaisson, P., Hefetz, A., 2007. Reproductive plasticity in bumblebee workers (*Bombus terrestris*) - reversion from fertility to sterility under queen influence. Behavioral Ecology and Sociobiology 62, 213-222.

Alaux, C., Jaisson, P., Hefetz, A., 2004. Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies. Insectes Sociaux 51, 287-293.

Alaux, C., Jaisson, P., Hefetz, A., 2006. Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal. Behavioral Ecology and Sociobiology 60, 439-446.

Alem, S., Perry, C.J., Zhu, X., Loukola, O., Ingraham, T., Sovik, E., Chittka, L., 2016. Associative Mechanisms Allow for Social Learning and Cultural Transmission of String Pulling in an Insect. PLoS Biology 14, e1002589.

Alexander, R.D., 1974. The evolution of social behavior. Annual Review Ecology Systematics 5, 325-383.

Almond, E.J., Huggins, T.J., Crowther, L.P., Parker, J.D., Bourke, A.F.G., 2019. Queen longevity and fecundity affect conflict with workers over resource inheritance in a social insect. American Naturalist 193, 256-266.

Amsalem, E., Grozinger, C.M., Padilla, M., Hefetz, A., 2015. The physiological and genomic bases of bumble bee social behaviour. Advances in Insect Pysiology 48, 37-93.

Amsalem, E., Hefetz, A., 2010. The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers. Behavioral Ecology and Sociobiology 64, 1685-1694.

Amsalem, E., Hefetz, A., 2011. The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*. PLoS One 6, e18238.

Amsalem, E., Shamia, D., Hefetz, A., 2013. Aggression or ovarian development as determinants of reproductive dominance in *Bombus terrestris*: interpretation using a simulation model. Insectes Sociaux 60, 213-222.

Becher, M.A., Twiston-Davies, G., Penny, T.D., Goulson, D., Rotheray, E.L., Osborne, J.L., 2018. Bumble-BEEHAVE: A systems model for exploring multifactorial causes of bumblebee decline at individual, colony, population and community level. Journal of Applied Ecology 55, 2790-2801.

Beekman, M., Komdeur, J., Ratnieks, F.L.W., 2003. Reproductive conflicts in social animals: who has power? Trends in Ecology and Evolution 18, 277-282.

Beekman, M., Oldroyd, B.P., 2008. When workers disunite: intraspecific parasitism by eusocial bees. Annual Review of Entomology 53, 19-37.

Beekman, M., Ratnieks, F.L.W., 2003. Power over reproduction in social Hymenoptera. Philosophical Transactions of the Royal Society of London Series B 358, 1741-1753.

Blacher, P., Huggins, T.J., Bourke, A.F.G., 2017. Evolution of ageing, costs of reproduction and the fecundity-longevity trade-off in eusocial insects. Proceedings of the Royal Society B - Biological Sciences 284, 20170380.

Bloch, G., Hefetz, A., 1999. Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. Behavioral Ecology and Sociobiology 45, 125-135.

Bonckaert, W., Tofilski, A., Nascimento, F.S., Billen, J., Ratnieks, F.L.W., Wenseleers, T., 2011a. Co-occurrence of three types of egg policing in the Norwegian wasp *Dolichovespula norwegica*. Behavioral Ecology and Sociobiology 65, 633-640.

Bonckaert, W., Van Zweden, J.S., D'Ettorre, P., Billen, J., Wenseleers, T., 2011b. Colony stage and not facultative policing explains pattern of worker reproduction in the Saxon wasp. Molecular Ecology 20, 3455-3468.

Bonckaert, W., Vuerinckx, K., Billen, J., Hammond, R.L., Keller, L., Wenseleers, T., 2008. Worker policing in the German wasp *Vespula germanica*. Behavioral Ecology 19, 272-278.

Bourke, A.F.G., 1988. Worker reproduction in the higher eusocial Hymenoptera. Quarterly Review of Biology 63, 291-311.

Bourke, A.F.G., 1994. Worker matricide in social bees and wasps. Journal of Theoretical Biology 167, 283-292.

Bourke, A.F.G., 1999. Colony size, social complexity and reproductive conflict in social insects. Journal of Evolutionary Biology 12, 245-257.

Bourke, A.F.G., 2001. Social insects and selfish genes. Biologist 48, 205-208.

Bourke, A.F.G., 2007. Kin selection and the evolutionary theory of aging. Annual Review of Ecology, Evolution and Systematics 38, 103-128.

Bourke, A.F.G., 2011. Principles of Social Evolution. Oxford University Press, Oxford.

Bourke, A.F.G., Ratnieks, F.L.W., 2001. Kin-selected conflict in the bumble-bee *Bombus terrestris* (Hymenoptera: Apidae). Proceedings of the Royal Society of London Series B 268, 347-355.

Brunner, E., Heinze, J., 2009. Worker dominance and policing in the ant Temnothorax unifasciatus. Insectes Sociaux 56, 397-404.

Butler, C.G., 1954. The World of the Honeybee. Collins, London.

Cant, M.A., Field, J., 2001. Helping effort and future fitness in cooperative animal societies. Proceedings of the Royal Society of London Series B 268, 1959-1964.

Cant, M.A., Llop, J.B., Field, J., 2006. Individual variation in social aggression and the probability of inheritance: theory and a field test. American Naturalist 167, 837-852.

Cant, M.A., Nichols, H.J., Johnstone, R.A., Hodge, S.J., 2014. Policing of reproduction by hidden threats in a cooperative mammal. Proceedings of the National Academy of Sciences, U.S.A. 111, 326-330.

Cardinal, S., Danforth, B.N., 2011. The antiquity and evolutionary history of social behavior in bees. PLoS One 6, e21086.

Chase, I.D., 1974. Models of hierarchy formation in animal societies. Behavioral Science 19, 374-382.

Clutton-Brock, T., 2009a. Cooperation between non-kin in animal societies. Nature 462, 51-57.

Clutton-Brock, T., 2009b. Structure and function in mammalian societies. Philosophical Transactions of the Royal Society B 364, 3229-3242.

Cnaani, J., Schmid-Hempel, R., Schmidt, J.O., 2002. Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson. Insectes Sociaux 49, 164-170.

Collins, D.H., Mohorianu, I., Beckers, M., Moulton, V., Dalmay, T., Bourke, A.F.G., 2017. MicroRNAs associated with caste determination and differentiation in a primitively eusocial insect. Scientific Reports 7, 45674.

Collins, D.H., Prince, D.C., Donelan, J.L., Chapman, T., Bourke, A.F.G., 2023. Costs of reproduction are present but latent in eusocial bumblebee queens. BMC Biol. 21, 153.

Dixon, L., Kuster, R., Rueppell, O., 2014. Reproduction, social behavior, and aging trajectories in honeybee workers. Age 36, 89-101.

Duchateau, M.J., 1989. Agonistic behaviors in colonies of the bumblebee *Bombus terrestris*. J. Ethol. 7, 141-151.

Duchateau, M.J., Velthuis, H.H.W., 1988. Development and reproductive strategies in *Bombus terrestris* colonies. Behaviour 107, 186-207.

Duchateau, M.J., Velthuis, H.H.W., 1989. Ovarian development and egg laying in workers of *Bombus terrestris*. Entomologia Experimentalis et Applicata 51, 199-213.

Duchateau, M.J., Velthuis, H.H.W., Boomsma, J.J., 2004. Sex ratio variation in the bumblebee *Bombus terrestris*. Behavioral Ecology 15, 71-82.

Edward, D.A., Chapman, T., 2011. Mechanisms underlying reproductive trade-offs: costs of reproduction, in: Flatt, T., Heyland, A. (Eds.), Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs, Oxford University Press, Oxford, pp. 137-152.

Ellis, L., 1995. Dominance and reproductive success among nonhuman animals: a cross-species comparison. Ethology and Sociobiology 16, 257-333.

Emlen, S.T., 1982. The evolution of helping. I. An ecological constraints model. American Naturalist 119, 29-39.

Ernst, U.R., Cardoen, D., Cornette, V., Ratnieks, F.L.W., De Graaf, D.C., Schoofs, L., Verleyen, P., Wenseleers, T., 2017. Individual and genetic task specialization in policing behaviour in the European honeybee. Animal Behaviour 128, 95-102.

Estoup, A., Scholl, A., Pouvreau, A., Solignac, M., 1995. Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites. Molecular Ecology 4, 89-93.

Fitzpatrick, J.L., 2008. Female-mediated causes and consequences of status change in a social fish. Proc. R. Soc. B 275, 929–936.

Franco-Trecu, V., Costa, P., Schraam, Y., Tassino, B., Inchausti, P., 2014. Sex on the rocks: reproductive tactics and breeding success of South American fur seal males. Behavioral Ecology 25, 1513–1523.

Frank, S.A., 1995. Mutual policing and repression of competition in the evolution of cooperative groups. Nature 377, 520-522.

Frank, S.A., 1998. Foundations of Social Evolution. Princeton University Press, Princeton.

Frank, S.A., 2003. Repression of competition and the evolution of cooperation. Evolution 57, 693-705.

Friend, L.A., Bourke, A.F.G., 2014. Workers respond to unequal likelihood of future reproductive opportunities in an ant. Animal Behaviour 97, 165-176.

Gadagkar, R., 2001. Division of labour and organization of work in the primitively eusocial wasp *Ropalidia marginata*. Proceedings of the Indian National Science Academy B67, 397-422.

Ghoul, M., Griffin, A.S., West, S.A., 2014. Toward an evolutionary definition of cheating. Evolution 68, 318-331.

Goodnight, C.J., 2005. Multilevel selection: the evolution of cooperation in non-kin groups. Population Ecology 47, 3-12.

Goulson, D., 2003. Bumblebees: their Behaviour and Ecology. Oxford University Press, Oxford.

Goulson, D., 2010. Bumblebees: Behaviour, Ecology, and Conservation. 2nd, ed. Oxford University Press, Oxford.

Hamilton, W.D., 1964. The genetical evolution of social behaviour I, II. Journal of Theoretical Biology 7, 1-52.

Hamilton, W.D., 1966. The moulding of senescence by natural selection. Journal of Theoretical Biology 12, 12-45.

Hammers, M., Kingma, S.A., Spurgin, L.G., Bebbington, K., Dugdale, H.L., Burke, T., Komdeur, J., Richardson, D.S., 2019. Breeders that receive help age more slowly in a cooperatively breeding bird. Nature Communications 10, 1301.

Hammond, R.L., Keller, L., 2004. Conflict over male parentage in social insects. PLoS Biology 2, e248.

Heinrich, B., 1979. Bumblebee Economics. Harvard University Press, Cambridge, Mass.

Heinze, J., Hölldobler, B., Peeters, C., 1994. Conflict and cooperation in ant societies. Naturwissenschaften 81, 489-497.

Heinze, J., Puchinger, W., Hölldobler, B., 1997. Worker reproduction and social hierarchies in *Leptothorax* ants. Animal Behaviour 54, 849-864.

Holman, L., 2014. Bumblebee size polymorphism and worker response to queen pheromone. PeerJ 2, e604.

Holman, L., 2018. Queen pheromones and reproductive division of labor: a meta-analysis. Behavioral Ecology, doi:10.1093/beheco/ary1023.

Hughes, W.O.H., Oldroyd, B.P., Beekman, M., Ratnieks, F.L.W., 2008. Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320, 1213-1216.

Huntingford, F.A., Turner, A.K., Downie, L.M., 1987. Animal conflict. Chapman & Hall/CRC.

Ishikawa, Y., Yamada, Y.Y., Matsuura, M., Tsukada, M., Tsuchida, K., 2011. *Polistes japonicus* (Hymenoptera, *Vespidae*) queens monopolize ovipositing but are not the most active aggressor in dominant-subordinate interactions. Insectes Sociaux 58, 519-529.

Jandt, J.M., Dornhaus, A., 2011. Competition and cooperation: bumblebee spatial organization and division of labor may affect worker reproduction late in life. Behavioral Ecology and Sociobiology 65, 2341-2349.

Keller, L., Genoud, M., 1997. Extraordinary lifespans in ants: a test of evolutionary theories of ageing. Nature 389, 958-960.

Keller, L., Reeve, H.K., 1994. Partitioning of reproduction in animal societies. Trends in Ecology and Evolution 9, 98-102.

Kim, E.B., Fang, X.D., Fushan, A.A., Huang, Z.Y., Lobanov, A.V., Han, L.J., Marino, S.M., Sun, X.Q., Turanov, A.A., Yang, P.C., Yim, S.H., Zhao, X., Kasaikina, M.V., Stoletzki, N., Peng, C.F., Polak, P., Xiong, Z.Q., Kiezun, A., Zhu, Y.B., Chen, Y.X., Kryukov, G.V., Zhang, Q., Peshkin, L., Yang, L., Bronson, R.T., Buffenstein, R., Wang, B., Han, C.L., Li, Q.Y., Chen, L., Zhao, W., Sunyaev, S.R., Park, T.J., Zhang, G.J., Wang, J., Gladyshev, V.N., 2011. Genome sequencing reveals insights into physiology and longevity of the naked mole rat. Nature 479, 223-227.

Kirkwood, T.B.L., 1977. Evolution of ageing. Nature 270, 301-304.

Korb, J., Heinze, J., 2021. Ageing and sociality: why, when and how does sociality change ageing patterns? Philosophical Transactions of the Royal Society B 376, 20190727.

Kramer, B.H., Schrempf, A., Scheuerlein, A., Heinze, J., 2015. Ant colonies do not trade-off reproduction against maintenance. PLoS One 10, e0137969.

Leadbeater, E., Carruthers, J.M., Green, J.P., Rosser, N.S., Field, J., 2011. Nest inheritance is the missing source of direct fitness in a primitively eusocial insect. Science 333, 874-876.

Lewis, R.J., 2022. Aggression, rank and power: why hens (and other animals) do not always peck according to their strength. Philosophical Transactions of the Royal Society B.

Loope, K.J., 2015. Queen killing is linked to high worker-worker relatedness in a social wasp. Current Biology 25, 2976-2979.

Lopez-Vaamonde, C., Brown, R.M., Lucas, E.R., Pereboom, J.J.M., Jordan, W.C., Bourke, A.F.G., 2007. Effect of the queen on worker reproduction and new queen production in the bumble bee *Bombus terrestris*. Apidologie 38, 171-180.

Lopez-Vaamonde, C., Koning, J.W., Brown, R.M., Jordan, W.C., Bourke, A.F.G., 2004. Social parasitism by male-producing reproductive workers in a eusocial insect. Nature 430, 557-560.

Lopez-Vaamonde, C., Koning, J.W., Jordan, W.C., Bourke, A.F.G., 2003. No evidence that reproductive bumblebee workers reduce the production of new queens. Animal Behaviour 66, 577-584.

Lopez-Vaamonde, C., Raine, N.E., Koning, J.W., Brown, R.M., Pereboom, J.J.M., Ings, T.C., Ramos-Rodriguez, O., Jordan, W.C., Bourke, A.F.G., 2009. Lifetime reproductive success and longevity of queens in an annual social insect. Journal of Evolutionary Biology 22, 983-996.

Marshall, J.A.R., 2015. Social Evolution and Inclusive Fitness Theory: An Introduction. Princeton University Press, Princeton, NJ.

Maynard Smith, J., Szathmáry, E., 1995. The Major Transitions in Evolution. W.H. Freeman, Oxford.

McNulty, D.R., Tallian, A., Stahler, D.R., Smith, D.W., 2014. Influence of Group Size on the Success of Wolves Hunting Bison. PLoS One 9, e112884.

Monnin, T., Peeters, C., 1999. Dominance hierarchy and reproductive conflicts among subordinates in a monogynous queenless ant. Behavioral Ecology 10, 323-332.

Monnin, T., Ratnieks, F.L.W., 2001. Policing in queenless ponerine ants. Behavioral Ecology and Sociobiology 50, 97-108.

Negroni, M.A., Macit, M.N., Stoldt, M., Feldmeyer, B., Foitzik, S., 2021. Molecular regulation of lifespan extension in fertile ant workers. Philosophical Transactions of the Royal Society B 376.

Neumann, P., Härtel, S., Kryger, P., Crewe, R.M., Moritz, R.F.A., 2011. Reproductive division of labour and thelytoky result in sympatric barriers to gene flow in honeybees (*Apis mellifera* L.). Journal of Evolutionary Biology 24, 286-294.

O'Connor, S., Park, K.J., Goulson, D., 2013. Worker drift and egg dumping by queens in wild *Bombus terrestris* colonies. Behavioral Ecology and Sociobiology 67, 621-627.

Oi, C.A., van Zweden, J.S., Oliveira, R.C., Van Oystaeyen, A., Nascimento, F.S., Wenseleers, T., 2015. The origin and evolution of social insect queen pheromones: novel hypotheses and outstanding problems. Bioessays 37, 808-821.

Padilla, M., Amsalem, E., Altman, N., Hefetz, A., Grozinger, C., 2016. Chemical communication is not sufficient to explain reproductive inhibition in the bumblebee *Bombus impatiens*. Royal Society Open Science 3, 160576.

Peat, J., Tucker, J., Goulson, D., 2005. Does intraspecific size variation in bumblebees allow colonies to efficiently exploit different flowers? Ecological Entomology 30, 176-181.

Rankin, D.J., 2007. Resolving the tragedy of the commons: the feedback between intraspecific conflict and population density. Journal of Evolutionary Biology 20, 173-180.

Ratnieks, F.L.W., 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. American Naturalist 132, 217-236.

Ratnieks, F.L.W., 1993. Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. Behavioral Ecology and Sociobiology 32, 191-198.

Ratnieks, F.L.W., 1998. Conflict and cooperation in insect societies, in: Schwarz, M.P., Hogendoorn, K. (Eds.), Social Insects at the Turn of the Millennium, XIII International Congress of IUSSI, Adelaide, pp. 14-17.

Ratnieks, F.L.W., Foster, K.R., Wenseleers, T., 2006. Conflict resolution in insect societies. Annual Review of Entomology 51, 581-608.

Ratnieks, F.L.W., Helanterä, H., 2009. The evolution of extreme altruism and inequality in insect societies. Philosophical Transactions of the Royal Society B 364, 3169-3179.

Ratnieks, F.L.W., Wenseleers, T., 2005. Policing insect societies. Science 307, 54-56.

Ratnieks, F.L.W., Wenseleers, T., 2008. Altruism in insect societies and beyond: voluntary or enforced? Trends in Ecology and Evolution 23, 45-52.

Reeve, H.K., 1998. Acting for the good of others: kinship and reciprocity with some new twists, in: Crawford, C., Krebs, D. (Eds.), Handbook of Evolutionary Psychology, Lawrence Erlbaum, Hillsdale NJ, pp. 43-85.

Reznick, D., Nunney, L., Tessier, A., 2000. Big houses, big cars, superfleas and the costs of reproduction. Trends in Ecology and Evolution 15, 421-425.

Riehl, C., 2013. Evolutionary routes to non-kin cooperative breeding in birds. Proc. R. Soc. B 280, 20132245.

Riehl, C., Frederickson, M.E., 2016. Cheating and punishment in cooperative animal societies. Philosophical Transactions of the Royal Society B 371.

Rodrigues, A.M., 2018. Resource availability and adjustment of social behaviour influence patterns of inequality and productivity across societies. PeerJ, 6:e5488.

Rodrigues, M.A., Flatt, T., 2016. Endocrine uncoupling of the trade-off between reproduction and somatic maintenance in eusocial insects. Current Opinion in Insect Science 16, 1-8.

Roth, K.M., Beekman, M., Allsopp, M.H., Goudie, F., Wossler, T.C., Oldroyd, B.P., 2014. Cheating workers with large activated ovaries avoid risky foraging. Behavioral Ecology 25, 668-674.

Sadd, B.M., Barribeau, S.M., Bloch, G., de Graaf, D.C., Dearden, P., Elsik, C.G., Gadau, J., Grimmelikhuijzen, C.J.P., Hasselmann, M., Lozier, J.D., Robertson, H.M., Smagghe, G., Stolle, E., Van Vaerenbergh, M., Waterhouse, R.M., al., e., 2015. The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol. 16, 76.

Sibbald, E.D., Plowright, C.M.S., 2013. On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees (*Bombus impatiens*). Insectes Sociaux 60, 23-30.

Sibbald, E.D., Plowright, C.M.S., 2014. Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (Bombus impatiens). Behavioral processes 103, 150-155.

Sibbald, E.D., Plowright, C.M.S., 2015. Reproductive potential and its behavioural consequences in orphaned bumblebee workers (*Bombus impatiens*). Apidologie 46, 618-627.

Silk, J.B., 2007. The adaptive value of sociality in mammalian groups. Philosophical Transactions of the Royal Society B-Biological Sciences 362, 539-559.

Singh, M., Boomsma, J.J., 2015. Policing and punishment across the domains of social evolution. Oikos 124, 971-982.

Southon, R.J., Bell, E.F., Graystock, P., Sumner, S., 2015. Long live the wasp: adult longevity in captive colonies of the eusocial paper wasp *Polistes canadensis* (L.). PeerJ 3, e848.

Starr, C.K., 1984. Sperm competition, kinship, and sociality in the Aculeate Hymenoptera, in: Smith, R.L. (Ed.), Sperm Competition and the Evolution of Animal Mating Systems, Academic Press, Orlando, pp. 427-464.

Stearns, S.C., 1992. The Evolution of Life Histories. Oxford University Press, Oxford.

Strauss, E.D., Curley, J.P., Shizuka, D., Hobson, E.A., 2022. The centennial of the pecking order: current state and future prospects for the study of dominance hierarchies. . Philosophical Transactions of the Royal Society B 377, 20200432.

Stroeymeyt, N., Brunner, E., Heinze, J., 2007. "Selfish worker policing" controls reproduction in a Temnothorax ant. Behavioral Ecology and Sociobiology 61, 1449-1457.

Trivers, R.L., 1971. The evolution of reciprocal altruism. Quarterly Review of Biology 46, 35-57.

Trivers, R.L., Hare, H., 1976. Haplodiploidy and the evolution of the social insects. Science 191, 249-263.

Trunzer, B., Heinze, J., Hölldobler, B., 1999. Social status and reproductive success in queenless ant colonies. Behaviour 136, 1093-1105.

Tsuji, K., Kikuta, N., Kikuchi, T., 2012. Determination of the cost of worker reproduction via diminished life span in the ant *Diacamma* sp. Evolution 66, 1322-1331.

Van Doorn, A., 1989. Factors influencing dominance behaviour in queenless bumblebee workers (*Bombus terrestris*). Physiological Entomology 14, 211-221.

Van Doorn, A., Heringa, J., 1986. The ontogeny of a dominance hierarchy in colonies of the bumblebee *Bombus terrestris* (Hymenoptera, Apidae). Insectes Sociaux 33, 3-25.

Van Honk, C., Hogeweg, P., 1981. The ontogeny of the social structure in a captive *Bombus terrestris* colony. Behavioral Ecology and Sociobiology 9, 111-119.

Van Noordwijk, A.J., De Jong, G., 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. American Naturalist 128, 137-142.

Van Oystaeyen, A., Oliveira, R.C., Holman, L., Van Zweden, J.S., Romero, C., Oi, C.A., D'Ettorre, P., Khalesi, M., Billen, J., Wäckers, F., Millar, J.C., Wenseleers, T., 2014. Conserved class of queen pheromones stops social insect workers from reproducing. Science 343, 287-290.

Velthuis, H.H.W., van Doorn, A., 2006. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37, 421-451.

Visscher, P.K., 1996. Reproductive conflict in honey bees: a stalemate of worker egg-laying and policing. Behavioral Ecology and Sociobiology 39, 237-244.

Visscher, P.K., Dukas, R., 1995. Honey bees recognize development of nestmates' ovaries. Animal Behaviour 49, 542-544.

Wenseleers, T., Hart, A.G., Ratnieks, F.L.W., 2004a. When resistance is useless: policing and the evolution of reproductive acquiescence in insect societies. American Naturalist 164, E154-E167.

Wenseleers, T., Helanterä, H., Hart, A., Ratnieks, F.L.W., 2004b. Worker reproduction and policing in insect societies: an ESS analysis. Journal of Evolutionary Biology 17, 1035-1047.

Wenseleers, T., Princen, S., Oliveria, R., Oi, C.A., 2021. Conflicts of Interest Within Colonies. Encyclopedia of Social Insects 28, 279-293.

Wenseleers, T., Ratnieks, F.L.W., 2006a. Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. American Naturalist 168, E163-E179.

Wenseleers, T., Ratnieks, F.L.W., 2006b. Enforced altruism in insect societies. Nature 444, 50.

Wenseleers, T., Tofilski, A., Ratnieks, F.L.W., 2005. Queen and worker policing in the tree wasp *Dolichovespula sylvestris*. Behavioral Ecology and Sociobiology 58, 80-86.

West, S.A., Ghoul, M., 2019. Conflict within cooperation. Current Biology 29, R425-R426.

Whitehouse, M.E.A., Lubin, Y., 2005. The functions of societies and the evolution of group living: spider societies as a test case. Biological Reviews 80, 347-361.

Williams, G.C., 1957. Pleiotropy, natural selection, and the evolution of senescence. Evolution 11, 398-411.

Williams, P.H., Osborne, J.L., 2009. Bumblebee vulnerability and conservation world-wide. Apidologie 40, 367-387.

Wilson, D.S., 1975. A theory of group selection. Proceedings National Academy of Sciences, U.S.A. 72, 143-146.

Wilson, E.O., 1971. The Insect Societies. Belknap Press, Cambridge, MA.

York, J.R., Troy, A.B., 2019. Sexual selection on female collared lizards favours offspring production with multiple males. Animal Behaviour 147, 17-23.

Young, L.J., Hammock, E.A.D., 2007. On switches and knobs, microsatellites and monogamy. Trends Genet. 23, 209-212.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Almond, E.J., Huggins, T.J., Jordan, W.C., Bourke, A.F.G., 2012. Reproductive conflict in bumblebees and the evolution of worker policing. Evolution 66, 3765-3777.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Lopez-Vaamonde, C., Bourke, A., 2014. Bumble bee workers drift to conspecific nests at field scales. Ecological Entomology 39, 347-354.

2
Chapter

Worker policing: testing the hypotheses with *Bombus terrestris*

Photo taken by Jenny Livesey

2.1 Abstract

In many eusocial Hymenopteran societies workers suppress the reproduction of nest-mate workers by eating their male-destined eggs (worker policing). Though this coercive behaviour helps to enforce cooperation and prevent colony exploitation, the evolutionary origins of worker policing are not fully understood. In monogynous monandrous *Bombus terrestris* colonies, reproductive workers are known to selfishly police nest-mate workers for direct fitness benefits. However, it remains unclear why non-reproductive workers have also been observed policing nest-mate workers in these colonies, in the absence of clear relatedness benefits. By quantifying workers' response to the removal of the colony queen, this study aimed to discriminate between the two main hypotheses (the public goods hypothesis and selfish policing hypothesis) proposed for the origin of non-reproductive worker policing in the eusocial Hymenoptera. The results showed that, of the non-laying but egg-eating workers observed pre-queen removal, in queenless conditions 42% remained as non-layers but ceased egg-eating (as predicted by the public goods hypothesis) and 58% started egg-laying (as predicted by the selfish policing hypothesis). The results therefore provided partial support for both hypotheses. Additional findings, for example multiple lines of evidence for an association in reproductive workers between egg-laying, egg-eating and aggression towards other egg-laying workers, provided new support for policing by reproductive workers originating as selfish policing. Hence, indirectly, these additional findings added to support for the selfish policing hypothesis for policing by non-reproductive workers. Finally, the results confirm the co-occurrence of three forms of policing in *B. terrestris* colonies, namely, queen policing, worker policing by reproductive workers and worker policing by non-reproductive workers and help elucidate the high-levels of reproductive conflict in eusocial species with ancestrally low mating frequencies.

2.2 Introduction

Sociality has evolved independently in all domains of life, with natural selection favouring group formation where the (fitness) benefits of group living outweigh the costs of living solitarily (Hamilton, 1964; Alexander, 1974). While social groups vary in complexity, all social groups are vulnerable to conflicts of interest and exploitative elements that would use group resources for their own reproduction, at the expense of the group (Wade and Breden, 1980; Clutton-Brock and Parker, 1995). Consequently, the maintenance of any social group, over evolutionary time, relies on mechanisms being selected that allow group members to limit exploitation from both external and internal elements (Bourke, 2011).

This is true even for the most advanced societies (Maynard Smith and Szathmáry, 1995). Though advanced eusocial societies consist of groups of highly related individuals with a permanent reproductive skew, high-levels of altruism and conflict co-exist (Wilson, 1971; Ghoul et al., 2014; Wenseleers et al., 2021). Within colonies of eusocial Hymenoptera (ants, bees and wasps), conflict varies in intensity and form (Ratnieks et al., 2006; Wenseleers et al., 2021) and can be expressed over sex allocation (Bourke and Franks, 1995; Crozier and Pamilo, 1996), caste determination (Bourke and Ratnieks, 1999; Ratnieks and Wenseleers, 2008) and male parentage (Wenseleers and Ratnieks, 2006a). Conflict over male parentage is possible in many species of eusocial Hymenoptera as, despite having lost the ability to mate, workers have retained ovaries and so are capable (via haplodiploidy) of producing male offspring from unfertilised eggs (Bourke, 1988). Egg-laying by workers is a common behaviour observed in multiple societies of eusocial Hymenoptera (Ratnieks and Wenseleers, 2005). However, worker reproduction can be costly at the colony level for several reasons, including if it leads to inefficiency in brood-rearing and, in effect, reduces colony productivity (Ratnieks, 1988; Ratnieks and Wenseleers, 2005; Roth et al., 2014). If it occurs in colonies where workers are more closely related to queen-produced males than worker-produced males then inclusive fitness costs to non-reproductive workers are also high (Ratnieks, 1988; Bourke and Franks, 1995). Hence, how such societies reduce internal conflict over worker reproduction and prevent societal collapse is a question of major evolutionary interest (Frank, 2003; Wenseleers and Ratnieks, 2006b; Bourke, 2011).

Policing is one of the chief mechanisms that have evolved within eusocial Hymenoptera to reduce colony exploitation from selfishly reproducing workers (Ratnieks and Wenseleers, 2008; Bourke, 2011). For the most part, policing involves either the colony queen (queen policing) and/or workers (worker policing) monitoring group members and reducing the level of successful worker reproduction by eating worker-laid eggs (oophagy) (Ratnieks, 1988). Though policing does not generally injure egg-laying individuals directly, by lowering the chances of successful reproduction it makes egg-laying costly and unprofitable (Wenseleers et al., 2004). Hence, policing acts as a coercive measure that aims to enforce cooperation by ensuring that workers can only maximise their inclusive fitness by helping to maximise the fitness of the group (Frank, 2003). Worker policing mechanisms vary between species, and can be performed by either, or both, non-reproductive workers (Ratnieks 1998; D'Ettore et al., 2004) and reproductive workers (Stroeymeyt et al., 2007; Bonckaert et al., 2011; Zanette et al., 2012) within a colony. Worker policing was first observed being performed by non-reproductive workers in the honeybee *Apis mellifera* (Ratnieks 1998), in which colonies are headed by one, polyandrous (multiply-mated) queen, but this behaviour has since been documented in colonies of eusocial Hymenoptera with varying relatedness asymmetries, including polygynous colonies (with multiple queens) such as the ponerine ant *Pachycondyla inversa* (D'Ettore et al., 2004) and monandrous monogynous colonies (i.e. headed by one, singly-mated queen) such as those of the Buff-tailed bumblebee *Bombus* *terrestris* (Zanette et al., 2012). However, though well documented, it has yet to be fully understood how this coercive mechanism originated in eusocial Hymenoptera lineages.

Following the discovery of worker policing in *A. mellifera,* it was first hypothesised that worker policing occurs within eusocial Hymenoptera due to the effects of queen mating frequency on worker-male relatedness (Ratnieks, 1988). For example, in *A. mellifera,* the queen is polyandrous and mates with approximately 10 males on average, and this produces workers of mixed paternity and a relatedness asymmetry in which workers are more related to their brothers (r = 0.25) than they are to their 'nephews', i.e., sons of nest-mate workers (r = c. 0.15) (Ratnieks, 1988). In this system, worker policing is performed by non-reproductive workers, as though workers are more related to their own male offspring (r = 0.5) and so there is selection for some to lay eggs, the average worker receives higher indirect fitness from rearing queen-produced males than from rearing worker-produced males. Hence, in this system, few workers attempt to lay their own male eggs because workers, the majority of which are non-reproductive, police the eggs of other workers so effectively that egg-laying is costly and unprofitable for all colonies members aside from the queen (Ratnieks, 1988; Pamilo, 1991; Ernst et al., 2017). However, though worker policing may be maintained by the relatedness asymmetries caused by polyandry, it is unlikely that it originated due to them. This is because polyandry is a derived trait in the eusocial Hymenoptera, with monandry reflecting the ancestral mating system for all eight independent origins of Hymenopteran eusociality (Hughes et al., 2008). As worker policing has been observed in extant monandrous, monogynous colonies, including in the ant *Temnothorax unifasciatus* (Stroeymeyt et al., 2007), wasps *Dolichovespula sylvestris* (Wenseleers et al., 2005b)*, Dolichovespula norwegica* (Bonckaert et al., 2011) and bumblebee *B. terrestri*s (Zanette et al., 2012), it follows that worker policing originated prior to polyandry (Zanette et al., 2012).

Monandry and monogyny, combined with a haplodiploid genetic system, results in colonies with relatedness asymmetries among colony members by which workers are more related to their sons (r = 0.5) and nephews (r = 0.375) than they are to the queen's sons, their brothers (r = 0.25) (**Figure 2.1**). Therefore, in monandrous monogynous colonies, workers gain higher indirect fitness benefits from favouring male production by workers over male production by the queen. Hence, other factors, aside from workers being more highly related to queen- than to worker-produced males, must have contributed to the origin of worker policing in eusocial Hymenoptera (Pirk et al., 2007; Beekman and Oldroyd, 2008). Previous research suggests that worker policing by reproductive workers has evolved from selfish origins, i.e., as 'selfish policing' (Wenseleers et al., 2005a; Bonckaert et al., 2011; Zanette et al., 2012). Specifically, worker policing by reproductive workers is likely to be selected to maximise their direct fitness gains (i.e., through permitting reproductive workers to gain a greater share of resources for their own offspring by reducing competition from offspring of rival egg-laying workers). Evidence that supports this theory includes data that show egg-laying workers are more likely to eat more eggs per capita, and to aggress other egg-laying workers, than non-laying workers in monandrous monogynous colonies of ants, wasps and bees (Wenseleers et al., 2005a; Stroeymeyt et al., 2007; Bonckaert et al., 2011; Zanette et al., 2012). Polyandry may have instead been more important in modulating and maintaining worker policing over evolutionary time (Pamilo, 1991; Ohtsuki and Tsuji, 2009; Helanterä and Ratnieks, 2019). Evidence that polyandry has a major effect on the maintenance of worker policing includes data showing worker policing occurs more frequently in species in which workers are more related to the queen's sons than in species where they are more, or equally related, to other workers' offspring (Wenseleers and Ratnieks, 2006a; Bonckaert et al., 2008).

Though previous research has gone some way in furthering understanding of how worker policing is likely to have originated in reproductive workers and be maintained in eusocial lineages, a complete understanding of the origins of worker policing is lacking. Specifically, the origin of worker policing by non-reproductive workers remains unclear and there has been little theoretical or empirical research of this conundrum. As non-reproductive workers have been observed policing workers in monandrous monogynous colonies of wasps and bumblebees (Bonckaert et al., 2011a; Zanette et al., 2012), it is highly likely that, like selfish policing by reproductive workers, policing by non-reproductive workers originated prior to polyandry in eusocial Hymenoptera (Zanette et al., 2012). If so, then non-reproductive worker policing also originated in a system where workers are more related to worker-produced males than queen-produced males (**Figure 2.1**). However, unlike selfishly-policing workers, non-reproductive workers cannot be selected to police their sisters to replace less closely related male eggs with more closely related ones, as, by policing their sisters' eggs, non-reproductive workers are, other things equal, not only decreasing their sisters' direct fitness but their own indirect benefits (Wenseleers et al., 2005b).Yet, for worker policing of worker-produced males by non-reproductive workers to undergo selection in these systems, policing non-reproductive workers must be benefiting indirectly (Helanterä and Ratnieks, 2019).

There are two main hypotheses for the origin of worker policing by non-reproductive workers in monandrous monogynous colonies. The first hypothesis is the *public goods hypothesis* (or efficiency hypothesis) (Ratnieks, 1988; Hammond and Keller, 2004). This suggests that worker reproduction has a high colony-level cost, as laying workers perform less colony-beneficial work, on average, than their non-reproductive sisters. This, combined with the high number of eggs laid by queens and reproductive workers, results in inefficiencies in the rearing of reproductives and causes colony productivity to decrease (Wenseleers et al., 2004; Wenseleers and Ratnieks, 2006a). Therefore, the hypothesis suggests that policing by non-reproductive workers acts as a 'public good' to maximise colony efficiency. More specifically, non-reproductive workers benefit from selectively destroying male-laid eggs as by doing so they are preventing an indirect fitness cost caused by inefficiencies in brood rearing and are instead maximising their indirect fitness by enhancing the production of queen-produced males (Ratnieks, 1988; Hammond and Keller, 2004).

The second hypothesis is the *selfish policing hypothesis* (Wenseleers et al. 2005b; Stroeymeyt et al., 2007; Zanette et al. 2012,), which proposes that non-laying workers performing worker policing behaviours are preparing to activate their ovaries and lay eggs at a later date, and so are 'hopeful reproductives' that consume other workers' eggs for selfish reasons. Hence, these workers are themselves potential egg-layers and under selection to destroy competing workers' eggs to reduce competition and increase their own direct fitness (Wenseleers et al., 2005b; Zanette et al. 2012).

Currently, mainly through lack of investigation, there is little evidence for either hypothesis. For example, empirical research that has attempted to determine if worker reproduction is costly at the colony level within monandrous monogynous systems has provided mixed results (Lopez-Vaamonde et al., 2003; Tsuji et al., 2012). It is therefore unclear if worker policing improves the overall productivity of a colony, a key component of the public goods hypothesis. In terms of the selfish policing hypothesis, no empirical studies exist that test whether non-laying workers observed policing in these systems later become reproductive. Hence, though policing by non-reproductive workers is acknowledged as a major mechanism of conflict resolution within eusocial societies (see above), how it originated remains unresolved.

The eusocial bumblebee *B. terrestris* provides a study system well placed to investigate the origin of worker policing. Like nearly all bumblebees (*Bombus* spp.), *B. terrestris* exhibits an annual cycle in which colonies, each headed by one, monandrous queen, rear one generation per year. Part-way though the colony cycle, queens change from producing workers to reproductives (males and new queens or gynes). The queen's males are produced when she ceases laying diploid eggs (yielding workers or gynes) and starts laying unfertilised, haploid eggs, an event termed the *switch point* (Duchateau and Velthuis 1988). New queens are reared from the queen's final batches of diploid eggs, and it is thought that workers use changes in the pheromone output of the queen, associated with her starting to lay queen-destined eggs, to determine when to begin laying their own male (haploid) eggs (Bourke and Ratnieks, 2001; Alaux et al., 2006a; Alaux et al., 2006b). The appearance of the first worker-laid eggs is termed the *competition point* (Duchateau and Velthuis 1988) and typically follows the switch point (Lopez-Vaamonde et al., 2009). The competition point is associated with the onset of physical aggression between workers and between workers and the queen over egg-laying, and, since both workers and queens are then laying male eggs, this represents conflict over male parentage. The high level of conflict over male parentage makes *B. terrestris* an ideal system for studying the role policing plays in maintaining colony stability. *B. terrestris* also currently represents one of the few monandrous, monogynous eusocial systems in which all three forms of policing (queen policing, worker policing by reproductive workers and worker policing by non-reproductive workers) have been shown to occur (Zanette et al., 2012). Hence, this species provides an excellent system not only for elucidating the conditions selecting for the origin of worker policing in non-reproductive workers but also for investigating policing by queens and reproductive workers in order to enhance the understanding of the relationship between all three forms of policing.

Therefore, the primary aim of this study was to perform an experiment using the *B. terrestris* study system to discriminate between the public goods and selfish policing hypotheses. The secondary aim was to collect data on queen and worker egg-laying and egg-eating rates in different social contexts so as to better understand the factors affecting these rates and how they are associated. The experiment involved observing colonies of individually marked workers with the queen present (pre-queen removal phase), removing queens, and then observing the same colonies following queen removal (post-queen removal phase). *B. terrestris* colonies in the field undergo natural loss of the queen through queen death, including by worker matricide (Bourke 1994), and natural populations contain a proportion of queenless colonies, with these colonies producing the large majority of worker-produced males (Lopez-Vaamonde et al. 2004, 2009), most likely because the absence of queen policing and/or gynes to rear creates conditions maximally conducive to worker male production. Therefore, queenless conditions towards the end of the colony cycle represent a natural state in *Bombus* species and are associated with enhanced worker reproduction.

The public goods hypothesis predicted that, if non-reproductive workers police their sisters' eggs to improve colony efficiency (and their indirect fitness) by maximising the queen’s productivity, then workers that were non-reproductive egg-eating workers in the pre-queen removal phase should cease policing following queen removal. This is because, in queenless colonies, only worker-laid eggs would be present and a non-reproductive worker is equally related to all laying workers' sons, i.e., nephews ($r = 0.375$), so policing worker-laid eggs after queen removal would serve no purpose in maximising non-reproductive workers' indirect fitness. By contrast, the selfish policing hypothesis predicted that, if they are prospective reproductive workers, then workers that were non-reproductive, policing workers in the pre-queen removal phase should, following queen removal, start to lay their own eggs (whether or not they continued policing). In other words, if non-reproductive policing workers are would-be reproductive workers, then they should exhibit reproductive behaviour in the conditions most conducive to it.

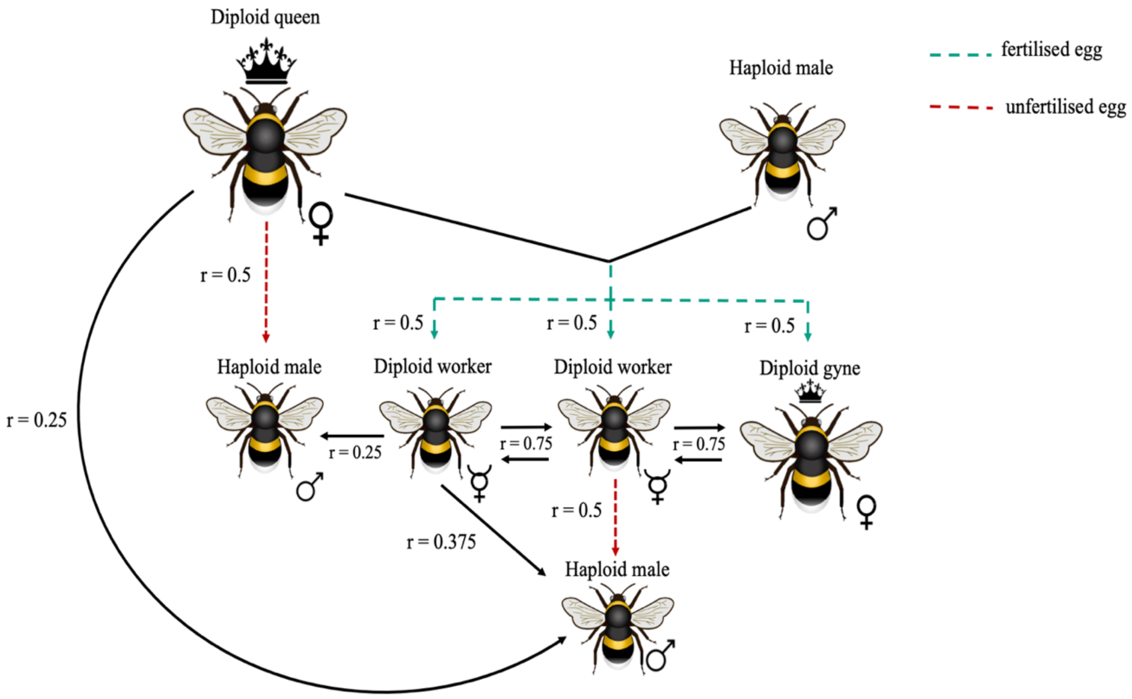


Figure 2.1. Within-colony relatedness values (r) between different castes and sexes in a colony of eusocial Hymenoptera headed by a single, monandrous queen (such as a colony of *Bombus terrestris*).

2.3 Materials & Methods

2.3.1 Rearing and development of *Bombus terrestris* colonies

Fifteen pre-competition point *Bombus terrestris audax* colonies were obtained from a commercial supplier (Biobest, Westerlos, Belgium). On delivery (29 March 2019), each colony contained 1 queen, a mean (± SD) of 7 ± 3 workers and a small quantity of brood at varying stages of development (e.g., 12-15 clumps of eggs, larvae or pupae). Colonies were transferred into wooden colony nest boxes (internal dimensions 17 cm × 27.5 cm × 16 cm high) with clear, colourless Perspex lids, kept under standard conditions (28°C, 60% relative humidity, constant darkness), and fed *ad libitum* on sugar syrup and fresh pollen (Attracker; Koppert Biological Systems, Haverhill, UK) for the duration of the experiment.

Between 30 March 2019 and 14 June 2019, colony development was monitored every 1-2 days in order to (1) census and individually mark newly-eclosed workers, (2) census and remove newly-eclosed reproductives (gynes and males), and (3) determine the onset of the competition point. Between 30 March 2019 and 26 April 2019, all newly-eclosed workers were individually marked daily with a unique numbered disc glued to the thorax (Queen Marking Kit, Thorne Ltd., UK), and any adult sexuals were removed on the day of their eclosion. To reduce levels of disturbance to colony queens, these queens were not marked. However, as newly-eclosed gynes differ in colour and behaviour from colony queens, it was possible to differentiate between gynes and colony queens throughout the experiment. Because of a sharp increase in numbers of workers eclosing daily across colonies, from 27 April 2019 to 12 May 2019 (the date on which the last worker eclosed in any colony), newly-eclosed workers were individually marked every two days, rather than daily. As the mean date on which the last worker eclosed in each colony was 4 May 2019, the mean number of days that newly-eclosed workers were not marked in colonies was four. Overall, at the end of the experiment, the mean per colony percentage of marked workers was 67.5% (range, 52.1% - 94.7%; **Table A2.1**). For reasons similar to those stated above, adult sexuals in each colony were removed every 2-3 days from 27 April 2019.

The date of the competition point, in individual colonies, was determined as the date of first observation of either (1) worker egg-laying or (2) oophagy (egg-eating) by workers (following the procedures adopted in Zanette et al. 2012). The worker behaviours associated with worker egg-eating and laying are distinct and therefore easy to recognise. The former involves opening of an egg-cell and visible consumption of eggs, and the latter involves placing of the abdominal tip in an open egg-cell for a period of at least 2 minutes and visibly tapping the hind legs on the egg-cell wall (Bloch & Hefetz 1999; **Table 2.1**, **Figure 2.2**). To ensure the date of the competition point could be accurately recorded, each colony was directly observed in 30-minute bouts daily, following the date of arrival, i.e., from 30 March 2019 to 13 April 2019. Once the competition point had been detected in at least 2 colonies (14 April 2019), each colony was directly and continuously observed in a 45-minute bout (direct observations) and digitally filmed in an additional 2-hour bout (digital observations) each day for 5-6 days per week until 14 June 2019. All observations (direct and digital) occurred between 08:00 and 20:00 each day.

2.3.2 Behavioural observations in the pre-queen removal phase

In the pre-queen removal phase of the experiment (queenright conditions), the queen of each colony remained present. Colonies were both observed directly in the 45-minute direct observations (total of 19.5–21.0 h per colony over a period of 26-28 days) and filmed digitally in the 2-h digital observations (50.0-56.0 h per colony over a period of 25-28 days).

During direct and digital observations, for all egg-eating and egg-laying events observed, the individual identities of all egg-layers and egg-eaters were recorded (**Table 2.1**). As it was not possible to attribute these behaviours to individual unmarked workers, records of all behaviours performed by unmarked workers were pooled within each colony. Within an observation bout, if an egg-eating event occurred from an egg-cell containing eggs for which the identity of the egg-layer was known, then the origin of the eggs eaten was recorded.

In some cases, workers were observed laying into an egg-cell that (1) already visibly contained eggs or (2) could not be determined to be empty at the start of the observed egg-laying event. In these cases, if egg-eating from the cell then occurred, eaten eggs could have included eggs of both known and unknown parentage. Therefore, where required, later analyses distinguished between eggs of known and unknown origin (parentage). Queens were never observed laying into an egg-cell that already contained eggs in any direct or digital observation bout, and so the need to classify eaten eggs into eggs of known and unknown origin applied only to workers' eggs. For queens and marked workers, rates of both egg-laying and egg-eating were quantified as the rate of events observed per individual per hour, where 'event' referred to either an individual being observed to lay eggs into an egg-cell (egg-laying) or to an individual being observed to eat eggs from an egg-cell (egg-eating). The rates of behaviours for unmarked workers were quantified in the same way, with the rates of events carried out by unmarked workers being divided by the total number of unmarked workers within the colony. The actual numbers of eggs laid, and eggs eaten per hour could not be accurately quantified, because egg-cells typically contain multiple eggs (Zanette *et al.* 2012; Almond *et al.* 2019; **Figure 3.1**), with more than one egg being laid or eaten per event, and during most egg-laying and egg-eating events the depth of the egg-cell and the position of the queen or worker's body prevented the number of eggs laid or eaten being accurately counted. As *B. terrestris* queens tend to lay more eggs per egg-laying event and eat more eggs per egg-eating event than workers (Zanette et al. 2012), this meant that comparisons of rates between queens and workers need to be treated as minimal comparisons, i.e., they underestimate the comparatively greater rates of egg-laying and egg-eating by queens (see Discussion).

In addition, in the direct observations only, any occurrences of directed agonistic behaviours were recorded, along with the identities of the actors and the recipients of each behaviour. Potential agonistic behaviours recorded included those described by Duchateau (1989) and Bloch and Hefetz (1999a) and consisted of 'attack', 'butting' 'buzzing', 'darting' and 'pumping' (**Table 2.1**).

Digital filming of each colony was conducted using Sony HDR CX240E digital camcorders mounted on tripods to film the nest comb from above through the Perspex lid of the nest-box. Workers' numbered discs could only be clearly observed on the digital films under white light and preliminary observations determined that the behaviour of colony members under red light did not differ from that under dim white light. Therefore, to ensure that all workers could be individually identified in the digital observations, filming was conducted under dim white light (3W LED lights). To ensure sufficient resolution in films, only part of the total comb area (around 30%) of each nest, centred on a focal egg-cell, was filmed at any one time. For each digital film observation, the focal egg-cell was selected for filming according to one of the following criteria: (1) if it was being laid into by an identified egg-layer; or (2) in instances where no egg-laying events were observed, if it had a high level of worker and/or queen activity near it.

Directed Aggression	
Behaviour	Description
Attack	Actor directly attacks recipient, either by grappling, stinging or biting.
Butting	Actor makes an accelerated movement towards recipient, resulting in brief contact, before backing away.
Buzzing	Actor makes brief wing vibrations while facing recipient, without making physical contact
Darting	Actor makes sudden acclerated movement towards recipient, but stops forward motion prior to making contact.
Displacing egg-layer*	Actor grabs hold of a recipient engaged in an egg-laying event and forcefully removes them from an egg-cell, bringing the egg-laying event to an abrupt end. This behaviour may proceed egg-eating.
Eating egg-cell wall*	Actor eats away at the wall of an egg cell in which recipient is simultaneously laying with-in. This behaviour may expose newly-laid eggs and proceed egg-eating.
Pumping	Actor faces recipient while standing on tarsi, arching body and making pumping movements with abdomen.
Oviposition	
Egg-laying	Queen/worker places abdominal tip into an open egg-cell for a period of at least 2-minutes and visibly taps hind legs on the egg-cell wall. Egg-layer typically waxes up opening of egg cell after finishing egg-laying event.
Policing (oophagy)	
Egg-eating	Queen/worker opens egg-cell and eats some or all eggs present.

Table 2.1. Behaviours in *Bombus terrestris* queens and/or workers recorded during the pre-queen and post-queen removal phase of the experiment. *, behaviour described for the first time in the current study; all other behaviours are as described in Duchateau (1989) or Bloch and Hefetz (1999a).

2.3.3 Behavioural observations in the post-queen removal phase

To create queenless conditions, the queen of each colony was removed on the evening of 15 May 2019, which was 32 days after the first two colonies had reached their competition points, and immediately frozen at -20°C. Both direct and digital observations of the queenless colonies were then conducted following methods identical to those used in the pre-queen removal phase described in the previous section. During the post-queen removal phase, colonies were directly observed for a total of 17.5-18.75 h per colony over 23-25 days. Colonies were also digitally filmed for a total of 42-44 h per colony over 21-22 days. On 14 June 2019, once the post-queen removal phase had run for 30 days, i.e., approximately as long as the pre-queen removal phase, the experiment was terminated, and all colonies were frozen within 3 days at -20°C.

Figure 2.2. Behaviours involved in conflict over the parentage of males in *Bombus terrestris* colonies. (A) Worker performing an egg-laying event with the abdominal tip placed into an egg-cell. (B) Worker #36 white eating male-destined eggs during an egg-eating event. (C) Colony queen and worker #53 white simultaneously eating worker-laid male-destined eggs from an egg-cell. All photos taken by Jenny Livesey with a Canon DSLR camera and EF-S 18-55 mm lens.

2.3.4 Data extraction from digital films

Following the end of the experiment, 10 digital observations (20 h of film) from the pre-queen removal phase and 6 digital observations (12 h of film) from the post-queen removal phase from each colony were chosen at random and viewed for data extraction. Any films recorded prior to the competition point, or switch point + 8 days (see below), were excluded from analysis.

The data types extracted from each digital observation viewed included egg-laying and egg-eating by the queen and both marked and unmarked workers, as recorded in the direct observations. The acts of aggression 'attack', 'butting', 'buzzing', 'darting' and 'pumping' (**Table 2.1**) were rarely seen during the direct observations. Therefore, these behaviours were not recorded in digital observations.

During direct observations, both queens and workers were observed aggressing other colony members (queen or worker) performing egg-laying events. Such behaviour has been previously observed in *B. lapidarius* (Hoffer *et al.* 1802, Sladen 1912, Free *et al.* 1969, Alford 1975) and *B. terrestris* (Sladen 1912, Alford 1975). However, these studies did not distinguish the different forms that they take. I observed that these behaviours could be classified as either: (1) 'displacing egg-layer': actor physically grasps (with mandibles and legs) an egg-layer engaged in an egg-laying event and forcibly removes it from the egg-cell, bringing egg-laying by the recipient to an abrupt end; this behaviour may precede egg-eating; and (2) 'eating egg-cell wall': actor eats into egg-cell wall while an egg-layer is engaged in an egg-laying event; this behaviour may expose the newly-laid eggs and precede egg-eating. (**Table 2.1**). As these behaviours were observed and recognised as distinct only after direct observations had started, they were quantified from the digital observations only. For all occurrences of these behaviours, the identity of the individuals involved (i.e., the actor and the recipient) recorded.

All digital observations were viewed for data extraction blind to the results produced by direct observations. Therefore, no individual workers were targeted for data collection and instead the behaviour of all workers in the field of view were recorded. Data for egg-laying and egg-eating events recorded from digital observations were combined with the data for these events collected from direct observations, with rates of each type of event being calculated over the total duration of the direct and digital observations. As the behaviours 'displacing egg-layer' and 'eating egg-cell wall' were recorded only from digital recordings, the rates of these behaviours were calculated over the total duration of digital observations for each phase.

2.3.5 Worker dissections

After the freezing of all colonies, a sample of workers from each class was dissected to determine degree of ovarian activation. All workers identified as non-laying egg-eating workers in the pre-queen removal phase (NLEEs$_{pre}$) were dissected, along with a sample of workers observed performed egg-laying events in the post-queen removal phase, and a sample of workers that were not observed performing either egg-laying events or egg-eating events in either the direct or digital observations. Dissections were performed under a dissection microscope in double distilled water. For each worker, ovaries were photographed at 15× magnification, next to an ocular scale. This procedure was repeated with a forewing of each worker. Measurements were then taken from images using the software 'Image J'. For each worker, the largest terminal oocyte on three different ovarioles was measured. The mean length of these three oocytes was then used as an index of ovarian activation (Duchateau and Velthuis, 1989). For each forewing, the marginal cell length was measured and used as a proxy for worker size (Duchateau and Velthuis, 1989; Owen, 1989; Goulson et al., 2002). Worker ovaries were also classified into one of seven stages, depending on the degree of ovarian activation (Duchateau and Velthuis, 1989; **Figure A2.1**) .

2.3.6 Data analyses

In 3/15 colonies (Colonies 1-3) the colony queen died prior to the planned date of removal (15 May 2019). In a fourth colony (Colony 6) the colony queen was aggressed frequently, and any eggs laid by the queen were promptly eaten by workers. From the data collected, it was predicted that the queen in Colony 6 was 'usurped' 16 days prior to the date of queen removal. This suggests the queen of Colony 6 lost her reproductive monopoly early in the colony cycle and so a large portion of the pre-queen removal phase involved a high rate of worker egg-laying and low rate of queen egg-laying. As the experimental design relied on the queen of each colony being present and maintaining a reproductive monopoly throughout the pre-queen removal phase, these four colonies (1-3, 6) were excluded from further analysis.

In *B. terrestris*, the switch point (first queen-laid male egg) usually precedes the competition point (first worker egg-laying), but occasionally it occurs after it (Duchateau and Velthuis 1988; Duchateau *et al.* 2004; Lopez-Vaamonde *et al*. 2009). In the current study, the date of the switch point was estimated as the date of eclosion of the first adult male, produced by the colony, minus 26 days, since 26 is the egg-to-adult development time for male *B. terrestris* (Bourke and Ratnieks 2001). This showed that the current colonies followed the general pattern from previous studies, with the switch point preceding the competition point in 9/11 colonies and following it in only 2/11 colonies (colonies 12 and 14; **Table A2.2**). Previous studies also suggest that, at the switch point, queens switch from laying 100% diploid eggs to laying 100%

haploid eggs over a period of c. 8 days (Duchateau *et al.* 2004). In the current colonies, the date equal to the switch point plus 8 days occurred after the competition point in 4 of 11 colonies (colonies 8, 10, 12 and 14; **Tables A2.1, A2.2**). (In all 4 of these colonies, gyne eclosion proceeded male eclosion by 5-12 days, and this may have affected the timing of the competition point.) In these 4 colonies, data used in analyses, whether from direct observations or digital observations, were taken only from the period starting after the date equal to the switch point plus 8 days (**Table A2.2**). In the remaining seven colonies (in which the switch point preceded the competition point by more than 8 days), data used in analyses were taken from the date of the competition point onwards (**Table A2.2**). These procedures were followed to ensure that, in all colonies, comparisons of rates of egg-laying and eating events in queens versus workers involved exclusively or almost exclusively male eggs.

All egg-laying and egg-eating events recorded in the digital observations were added to the data collected from direct observations, with rates of each type of event being calculated over the total duration of direct and digital observations (starting post competition point or switch point + 8 days) for each colony. As the camera's field of view did not cover the entire brood mass, it is possible some egg-laying and egg-eating events were performed off-screen while filming of a focal egg-cell was underway. However, as the hourly rate of egg-laying and egg-eating per individual within each class was fairly low (**Table 2.3**), it is unlikely that many (if any) events were being performed simultaneously off-screen, if egg-laying or egg-eating events were being recorded on-screen.

Across the 11 colonies, rates of directed aggression towards individuals, outside of an egg-laying event, were low. Across all colonies, only 14 acts of directed aggression towards individuals not engaged in an egg-laying event were observed in the pre-queen removal phase and only 15 such acts were observed in the post-queen removal phase. As these rates were so low, directed aggression that did not involve an egg-laying event was excluded from further analysis and all analyses involving acts of aggression involved only aggression directed at egg-laying individuals engaged in an egg-laying event, i.e., 'displacing egg-layer' or 'eating egg-cell wall' (**Table 2.1**) (with these data being collected from digital observations only).

All marked workers in each of the 11 colonies were classified into one of four classes, based on the egg-laying and egg-eating behaviours they were observed performing in both direct and digital observations (**Table 2.2**). As the hypotheses being tested concerned the eating of worker-laid eggs, unless stated otherwise only worker-laid eggs and eggs of unknown origin (assumed worker-laid eggs) were included in analyses (in both pre- and post-queen removal phases). (In the pre-queen removal phase, eaten eggs of unknown origin were treated in analyses as worker-laid because the results showed that, in this phase, workers accounted for the great majority (88%) of all egg-laying events (Results 2.4.2).) Therefore, although some workers were observed eating known queen-laid eggs in the pre-queen removal phase, these egg-eating events were not used to classify workers. Workers were only classed as egg-eaters, and placed in the appropriate category (i.e., LEE or NLEE as defined below), if they were seen eating either known worker-laid eggs or assumed worker-laid eggs. Each marked worker was classified independently in each phase, according to its behaviour in that phase, i.e., once at the end of the pre-queen removal phase and once at the end of the post-queen removal phase. Classes of marked worker in the pre-queen removal and post-queen removal phases are distinguished by the subscripts 'pre' and 'post', respectively (e.g., LEEs$_{\text{pre}}$ and LEEs$_{\text{post}}$). Within phases, the four classes of workers were as follows: **LEEs** (laying egg-eating workers); **LNEEs** (laying non-egg-eating workers); **NLEEs** (non-laying egg-eating workers); and **NLNEEs** (non-laying, non-egg-eating workers) (**Table 2.2**). Analyses examined the transitions that workers made, if any, from one class to another from the pre- to the post-queen removal phase, and how numbers of individuals and rates of behaviour within each class differed between the two phases. In these classifications, the terms 'laying' and 'non-laying' were used instead of 'reproductive' and 'non-reproductive' because the phase(s) in which workers laid eggs varied. For example, some workers laid eggs only following the removal of the queen. As these workers were reproductive when their entire lifetime was considered, they were classed as non-laying, rather than non-reproductive, in the pre-queen removal phase. Similarly, some workers laid eggs only in the pre-queen removal phase. Therefore, these workers were classed as 'laying' in the pre-queen removal phase and 'non-laying', rather than non-reproductive, in the post-queen removal phase.

Classes of Worker	Observed performing the following behaviours	
	Egg-laying	Egg-eating
LEE (**L**aying **E**gg **E**ating Worker)	YES	YES
LNEE (**L**aying **N**on-**E**gg **E**ating Worker)	YES	NO
NLEE (**N**on-**L**aying **E**gg-**E**ating Worker)	NO	YES
NLNEE (**N**on-**L**aying **N**on-**E**gg **E**ating Worker)	NO	NO

Table 2.2. Summary of the classes in which individual marked workers were placed, based on egg-laying and egg-eating behaviours they were observed performing in either pre- or post-queen removal phases. For these classifications, egg-eating refers to the eating of known or assumed worker-laid-eggs. Each marked worker was classified independently in each phase, according to its behaviour in that phase, i.e., once at the end of the pre-queen removal phase and once at the end of the post-queen removal phase. The classification in each phase is indicated in the text by the suffixes 'pre' and 'post' and transitions from one class to another are indicated by combining terms; for example, a worker observed both egg-laying and egg-eating in the pre-queen removal phase and observed only egg-laying in the post-queen removal phase would transition from a LEE$_{\text{pre}}$ to a LNEE$_{\text{post}}$, denoted LEE$_{\text{pre}}$/LNEE$_{\text{post}}$.

2.3.7 Statistical analyses

All statistical analyses were performed using R v 4.2.1 (R Core Team 2022).

A generalised linear mixed effects model (GLMM) with a Poisson distribution was constructed to analyse numbers of individuals within each class during the pre-queen removal and post-queen removal phases, as well as the relationship between colony phase and numbers of individuals in each class. The GLMM was implemented using the package *lme4* (Bates *et al.* 2015). Number of workers was used as a fixed effect, with class and phase used as covariates and colony as a random effect. An ANOVA was performed before pairwise comparisons were obtained using the *contrast* and *lsmeans* functions in the *lsmeans* package (Lenth, 2016). A Bonferroni correction was applied to pairwise comparisons to adjust the threshold of significance to correct for multiple comparisons.

To analyse rates of egg-laying, egg-eating and aggression directed at individuals engaged in an egg-laying event, count data taken from both direct and digital observations were pooled together for each individual in each class for the pre- and post-queen removal phases and converted into rates of 'events/h' per individual for egg-laying events and egg-eating events and 'events/24 h' per individual for aggression directed at individuals engaged in an egg-laying event. As the rates of behaviour comprised of zero-inflated continuous data, the relationship of rates between individuals in different classes was tested using the Kruskal-Wallis rank sum test in the *native stats* package (R Core Team, 2022). Multiple pairwise comparisons were obtained using Dunn's test in the *FSA* package (Ogle *et al.* 2022) and a Bonferroni correction was applied to adjust the threshold of significance to correct for multiple comparisons.

'In order to determine the response of NLEEs$_{pre}$ to queenless conditions, paired-sample Wilcoxon tests were used to test differences in rates of behaviour of NLEEs$_{pre}$ between the pre- and post-queen removal phases. In addition, paired Wilcoxon tests were also used to determine the difference in rates of behaviour between the pre- and post-queen removal phases of all other classes of worker. The relationship between the rates at which different classes of worker performed egg-laying, egg-eating and aggressive events in the pre-queen removal phase and the rates at which they performed egg-laying, egg-eating and aggressive events in the post-queen removal phase was assessed using Spearman rank correlation. Spearman rank correlation was also used to assess the relationship between the rates at which LEEs$_{pre/post}$ performed egg-laying and egg-eating events. In addition, Fisher's exacts tests were used to compare the activation stage of workers ovaries in different classes.

To test if there was an effect of origin of eggs (queen-laid or worker-laid) on the survivorship of eggs laid, a survival analysis was performed and visualised with Kaplan-Meir plots using the *survival* package (Therneau, 2022). For this analysis, only eggs of known origin (either known queen-laid eggs or known marked worker-laid eggs [i.e., eggs known to have been laid by marked workers]) recorded in digital observations were used. No assumed worker-laid eggs were used in this analysis. For all eggs of known origin laid, either a time to event recording (the length of time between an egg-laying event and egg-eating event) or a censored recording (the length of time between an egg-laying event and end of recording) was taken. In all censored recordings the survivorship of eggs beyond the point of the recording were unknown. Often queens and workers did not eat all eggs present within an egg-cell during a single egg-eating event. This meant that multiple egg-eating events could occur for the same egg-cell during a single observation bout, with each egg-eating event being performed by a different individual. In these instances, a time to event recording was made for each egg-eating event from a single egg-cell, resulting in multiple time to event recordings being taken for single egg-laying events. In addition, some workers were observed egg-laying into egg-cells that already contained worker-laid eggs. If an egg-eating event from an egg-cell containing eggs from more than one worker was observed, a time to event recording was taken for each worker. In these instances, for each worker the length of time between the egg-laying event by each worker and the egg-eating event was recorded as a time to event. If eggs remained in a shared egg-cell at the end of a digital recording, then a censored recording was made for each worker that had laid within that egg-cell during the observation. Queens were not observed sharing egg-cells for egg-laying with workers in any observation, so each egg-eating event involving queen-laid eggs was classed as one event and therefore reflects the number of egg-eating events observed in the digital observations. It was not possible to obtain a median survival time for queen-laid eggs as queen-laid eggs had not dropped to 50% survival at the end of the 120-minute period. However, two Cox Proportional- Hazard models were constructed with the *survival* package. The first model tested the effect of origin of eggs, and colony, on the overall survivorship of eggs. The second model tested the effect of origin of eggs on the survivorship of eggs when only egg-eating events by workers were considered. Initially an inspection of Schoenfeld residuals revealed the categorical variable 'Colony' did not meet the proportional hazards assumption; however, the stratification of this variable allowed for the assumptions of the proportional hazards model to be met.

A GLMM with a binomial distribution was also implemented, using the *lsmeans* package and functions stated above, to determine if the proportion of egg-laying events followed by an egg-eating event (in which eggs from the egg-laying event were eaten by either queens or workers) differed as a function of the origin of eggs. The number of eggs eaten and surviving were used as fixed effects with origin of eggs (queen or worker) used as a covariate and hours of observation and colony as random effects. A 'type II' (sum of squares) ANOVA was performed, followed by pairwise comparisons.

To analyse levels of ovary activation between classes of worker, a GLMM was constructed using the package *lme4* (Bates *et al.* 2015). A Shapiro-Wilk test was first used to test the normality of data distribution. As the dependent variable (mean oocyte length) was found to have a right-skewed distribution, the *bestnormalize* package (Peterson, 2021) was then used to transform the data using the most suitable transformation method (Ordered Quantile technique). Transformed mean oocyte length was used as a fixed effect, with the class of worker and marginal cell length (as an index of body size) being used as covariates and colony as a random effect. A 'type II' (sum of squares) ANOVA was performed followed by pairwise comparisons using the *contrast* and *lsmeans* functions from the *lsmeans* package (Lenth, 2016). A 'Tukey's HSD' correction was applied for pairwise comparisons to adjust the threshold of significance to correct for multiple comparisons.

2.4 Results

2.4.1 Pre-queen removal phase: numbers of egg-laying and egg-eating workers

The pre-queen removal phase of experiment took place in the period 14 April 2019 – 15 May 2019 with a total of 369.2 h of observation ($n_{\textit{direct observations}}$ = 152.25 h, $n_{\textit{digital observations}}$ = 220.00 h) across 11 colonies (nos. 4-5, 7-15) used in analyses. In these 11 colonies, the onset of the switch point and competition point varied considerably, with a mean ± SD of 8.4 ± 19.1 days between colony arrival and the switch point and a mean ± SD of 14.3 ± 19.9 days between the switch point and the competition point (**Table A2.2**). In total 956 workers were marked upon eclosion during the experiment. Colonies produced a mean ± SD of 125.2 ± 26.7 workers, with a mean of 86.9 ± 24.5 marked upon eclosion and 38.3 ± 22.3 remaining unmarked (**Table A2.1**). If workers died earlier than 10 days prior to the end of the pre-queen removal phase, they were excluded from analyses ($n_{\text{worker mortalities earlier than 10 days prior to the end of the pre-queen removal phase}}$ = 19) as these workers were absent in over 1/3 of observations and so could not be confidently placed in a worker class. Any workers that died later than 10 days prior to the end of the pre-queen removal phase were included in analyses as they were present in over 2/3 of observations and they were unlikely to have changed their behaviours and class during the remaining observations of the pre-queen removal phase ($n_{\text{worker mortalities later than 10 days prior to the end of the pre-queen removal phase}}$ = 10). In total 937 marked workers were included in the analyses of the pre-queen removal phase. During this phase, 31.7 % of these 937 marked workers were observed performing either both egg-laying and egg-eating events (involving worker-laid eggs and assumed worker-laid eggs) or one of these two behaviours. Within individual colonies, numbers of workers in each class varied significantly (GLMM: $\chi^2 = 334.75$, df = 2, $p < 0.001$; **Table 2.3, Table A2.3**), with 2-tailed pairwise comparisons showing that within colonies there were significantly more $\text{LNEEs}_{\text{pre}}$ than LEEs_{pre} or $\text{NLEEs}_{\text{pre}}$. ($\text{LNEEs}_{\text{pre}}$ vs LEEs_{pre}, Z

=12.85, $p < 0.001$; LNEEs$_{\text{pre}}$ vs NLEEs$_{\text{pre}}$, Z = 14.48, $p < 0.001$). Numbers of LEEs$_{\text{pre}}$ were also significantly higher than numbers of NLEEs$_{\text{pre}}$ (Z = 6.54, $p < 0.001$) (**Table 2.3, Table A2.3**). Overall, in each colony, LEEs$_{\text{pre}}$, LNEEs$_{\text{pre}}$, and NLEEs$_{\text{pre}}$ made up 5.7%, 23.3% and 2.7% of all marked workers, respectively. The remaining 68.3% were classed as NLNEEs$_{\text{pre}}$ (**Table 2.3, Table A2.3**). This suggests that, following the competition point, only a subset of workers lay eggs. In addition, many laying and non-laying workers do not perform egg-eating events and workers that do not lay eggs yet eat other workers' eggs (NLEEs) occur in queenright colonies, albeit not at high frequency.

2.4.2 Pre-queen removal phase: rates of egg-laying and egg-eating events

In total, 638 egg-laying events ($n_{\textit{Queen egg-laying events}} = 82$, $n_{\textit{LEEspre egg-laying events}} = 139$, $n_{\textit{LNEEspre egg-laying events}} = 354$, $n_{\textit{unmarked workerspre egg-laying events}} = 63$) were recorded in the pre-queen removal phase. The rate (mean ± SE) at which individuals in different classes performed egg-laying events varied significantly (Kruskal-Wallis test: $\chi^2 = 64.642$, df = 3, $p < 0.001$), being 0.22 ± 0.04, 0.08 ± 0.01, 0.05 ± 0.00 and 0.01 ± 0.00 events per individual per hour for queens, LEEs$_{\text{pre}}$, LNEEs$_{\text{pre}}$ and unmarked workers$_{\text{pre}}$, respectively (**Figure 2.3A, Table 2.3**). Pairwise comparisons showed that, at the individual level, queens performed egg-laying events at a significantly higher rate than each worker class (Dunn's test: queen vs LEEs$_{\text{pre}}$, Z = 2.83, $p < 0.001$; queen vs LNEEs$_{\text{pre}}$, Z = 4.63, $p < 0.001$; queen vs unmarked workers$_{\text{pre}}$, Z = 7.04, $p < 0.001$). Among classes of worker, LEEs$_{\text{pre}}$ performed egg-laying events at a significantly higher rate than each other worker class (Dunn's test: LEEs$_{\text{pre}}$ vs LNEEs$_{\text{pre}}$, Z = 3.26, $p < 0.001$; LEEs$_{\text{pre}}$ vs unmarked workers$_{\text{pre}}$, Z = 6.25, $p < 0.001$). Overall queens, LEEs$_{\text{pre}}$, LNEEs$_{\text{pre}}$ and unmarked workers$_{\text{pre}}$ were responsible for 12.03%, 21.70%, 52.64%, 13.64% of egg-laying events within colonies, respectively, over the course of the pre-queen removal phase (**Table 2.3**). Therefore, workers collectively accounted for 88.00% of all egg-laying events in this phase.

Queens, laying workers and non-laying workers were all recorded eating eggs in the pre-queen removal phase. In total, in this phase, 315 egg-eating events involving known worker-laid eggs and eggs of unknown origin (assumed worker-laid eggs – see below) were recorded ($n_{\textit{egg-eating events by queens}} = 181$, $n_{\textit{egg-eating events by LEEspre}} = 99$, $n_{\textit{egg-eating events by NLEEspre}} = 27$, $n_{\text{egg-eating events by unmarked workerspre}} = 8$). As with egg-laying events, the rate (mean ± SE) at which individuals within different classes performed egg-eating events (of worker-laid eggs and assumed worker-laid eggs) varied significantly (Kruskal-Wallis test: $\chi^2 = 59.38$, df = 3, $p < 0.001$), being 0.50 ± 0.07, 0.06 ± 0.01, 0.03 ± 0.01 and 0.00 ± 0.00 events per individual per hour for queens, LEEs$_{\text{pre}}$, NLEEs$_{\text{pre}}$ and unmarked workers, respectively (**Figure 2.3B, Table 2.3**). Again, pairwise comparisons showed that queens performed egg-eating events at a significantly higher rate than each worker class (Dunn's test: queen vs LEEs_{pre}, Z = 4.29, $p < 0.001$; queen vs $\text{NLEEs}_{\text{pre}}$ Z = 5.31, $p < 0.001$; queen vs unmarked $\text{workers}_{\text{pre}}$, Z= 7.42, $p < 0.001$). LEEs_{pre} also performed egg-eating events at a significantly higher rate than each other worker class (Dunn's test: LEEs_{pre} vs $\text{NLEEs}_{\text{pre}}$, Z = 2.05, $p < 0.001$; LEEs_{pre} vs unmarked $\text{workers}_{\text{pre}}$, Z = 5.28, $p < 0.001$). Overall, queens, LEEs_{pre}, $\text{NLEEs}_{\text{pre}}$ and unmarked $\text{workers}_{\text{pre}}$ were responsible for 53.64%, 33.55%, 7.86%, and 4.95% of egg-eating events within colonies, respectively (**Table 2.3**).

	Pre-Queen Removal Phase								Post-Queen Removal Phase					
	Rate of egg-eating events per individual per hour				Rate of egg-laying events per individual per hour				Rate of egg-eating events per individual per hour			Rate of egg-laying events per individual per hour		
Colony I.D	By Queen	By LEEs$_{Pre}$	By NLEE$_{Pre}$	By unmarked workers$_{pre}$	By Queen	By LEEs$_{Pre}$	By LNEE$_{Pre}$	By unmarked workers$_{pre}$	By LEEs$_{Post}$	By NLNEE$_{pre}$ /NLEEs$_{Post}$	By unmarked workers$_{post}$	By LEE$_{Post}$	By LNEE$_{Post}$	By unmarked workers$_{post}$
4	0.26	0.03	0.03	0.000	0.18	0.07	0.04	0.001	0.16	0.00	0.001	0.09	0.05	0.009
5	0.64	0.03	0.03	0.000	0.18	0.04	0.06	0.008	0.10	0.03	0.004	0.08	0.05	0.013
7	0.75	0.10	0.07	0.000	0.23	0.07	0.04	0.002	0.16	0.03	0.005	0.11	0.05	0.010
8	0.74	0.04	0.03	0.002	0.29	0.10	0.05	0.002	0.08	0.03	0.004	0.09	0.05	0.010
9	0.25	0.05	0.02	0.000	0.30	0.06	0.04	0.010	0.09	0.00	0.004	0.10	0.05	0.033
10	0.49	0.10	0.04	0.004	0.17	0.19	0.05	0.013	0.14	0.00	0.004	0.13	0.04	0.010
11	0.35	0.05	0.03	0.001	0.58	0.06	0.05	0.007	0.12	0.03	0.002	0.12	0.05	0.011
12	0.42	0.04	0.04	0.002	0.13	0.11	0.07	0.002	0.13	0.03	0.004	0.09	0.04	0.019
13	0.37	0.07	0.03	0.001	0.18	0.07	0.03	0.009	0.36	0.03	0.003	0.13	0.04	0.012
14	0.97	0.03	0.03	0.000	0.21	0.07	0.06	0.007	0.10	0.00	0.002	0.05	0.04	0.007
15	0.25	0.15	0.00	0.003	0.00	0.09	0.05	0.012	0.08	0.00	0.000	0.08	0.04	0.028
Mean	0.50	0.06	0.03	0.001	0.22	0.08	0.05	0.007	0.14	0.02	0.003	0.10	0.05	0.015
± SE	0.07	0.01	0.01	0.000	0.04	0.01	0.00	0.001	0.02	0.00	0.000	0.01	0.00	0.003
Mean no. of individuals per class per colony	1.0	4.9	2.3	38.3	1.0	4.9	19.8	38.3	5.9	0.7	38.3	5.9	20.6	38.3
Mean no. of events per class per colony in 24 h period	11.98	7.49	1.76	1.11	5.35	9.64	23.39	6.06	19.57	0.27	2.89	13.77	22.52	13.40

Table 2.3. Data on mean (± SE) rates of egg-eating (of worker-laid eggs and eggs of unknown origin, which are assumed to be worker-laid) and egg-laying events per individual per hour in 11 *Bombus terrestris* colonies (nos. 4-5, 7-15). For each colony, rates of behaviour are calculated from a combination of daily 45-minute long direct observation recordings and 2-hour long digital film recordings. All data were collected after the onset of the competition phase (or switch point + 8 days) (14 April 2019 - 9 May 2019). In the pre-queen removal phase, data were collected under queenright conditions (mean ± SD of 13.84 ± 4.81 h of direct observation and 20.00 ± 0.00 h of digital observations per colony). In the post-queen removal phase, data were collected in queenless conditions (mean ± SD of 18.20 ± 0.56 h of direct observations and 12.00 ± 0.00 h of digital observations per colony). See Table 2.2 for full definitions of classes of worker.

Overall, the data suggest that queens both laid eggs and ate worker-laid eggs at the highest individual rates. In addition, among workers, LEEs_{pre} both laid eggs and ate worker-laid eggs at the highest individual rates.

Of the 315 egg-eating events, 59% involved colony queens or workers eating known worker-laid eggs that could be assigned to a single marked egg-laying worker as its parent. The remaining 41% of egg-eating events involved queens or workers eating eggs of unknown origin, i.e., egg-eating events in which the eggs that were eaten were laid prior to a direct or digital observation and so could not be assigned to a single individual ($n_{\text{egg-eating events of known worker origin}} = 186$, $n_{\text{egg-eating events of eggs of unknown origin}} = 129$). Colony queens were responsible for 85 egg-eating events in which eggs of unknown origin were eaten. As colony queens were not observed eating known queen-laid eggs in any direct or digital observations of the 11 colonies throughout the queen-removal phase ($n_{\text{total hours of observation in pre-queen removal phase}} = 369.2$), it was assumed that any egg-eating events performed by a colony queen involved the consumption of worker-laid eggs. Workers were responsible for 44 egg-eating events involving eggs of unknown origin. Eggs eaten in these events were also assumed to be worker-laid because (1) workers were responsible for 88.0% of all egg-laying events during the pre-queen removal phase **(Table 2.3)**, so there was a high likelihood that the eggs were laid by a worker, rather than the colony queen, and (2) both LEEs_{pre} and $\text{NLEEs}_{\text{pre}}$ performed egg-eating events in which known worker-laid eggs were eaten at a significantly higher (mean ± SE) rate per hour than they performed egg-eating events in which known queen-laid eggs were eaten (Wilcoxon rank sum test: LEE_{pre} egg-eating events of known worker-laid eggs vs LEE_{pre} egg-eating event of known queen-laid eggs, $W = 2.5$, $p < 0.001$; NLEE_{pre} egg-eating events of known worker-laid eggs vs NLEE_{pre} egg-eating events of known queen-laid eggs, $W = 10$, $p < 0.001$; **Table A2.4**). This suggests that workers ate eggs laid by other workers at a higher rate than they ate eggs laid by the colony queen. For these reasons, eggs of unknown origin are termed 'assumed worker-laid eggs'.

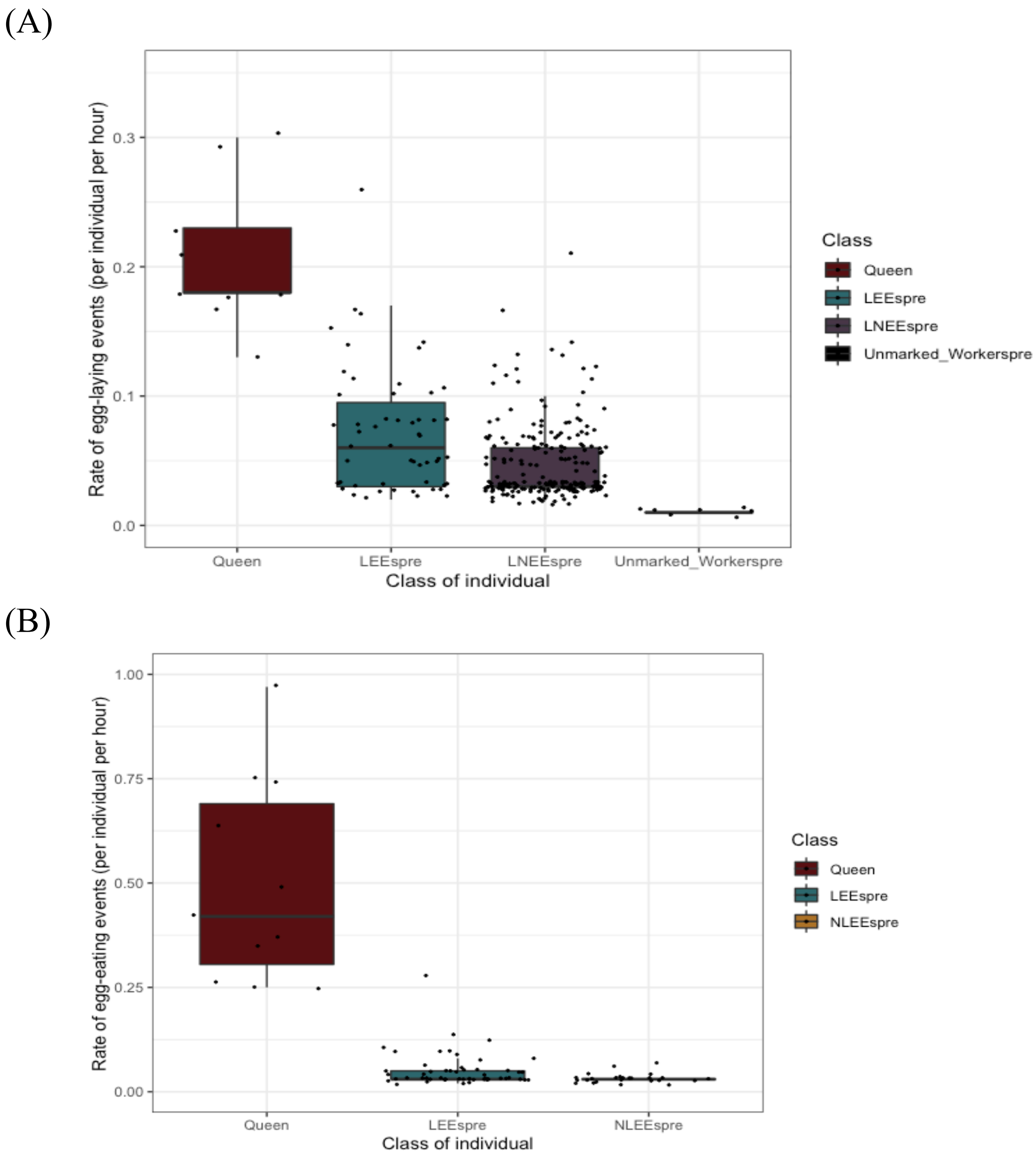


Figure 2.3. Rates of (**A**) egg-laying and (**B**) egg-eating events by different classes in 11 *Bombus terrestris* colonies (n *queen* = 11, n $LEEs_{pre}$ = 54, n $LNEEs_{pre}$ = 217, n *unmarked* $workers_{pre}$ = 11 and n $NLEEs_{pre}$ = 25) (see Table 2.2 for full definitions). Rates of behaviours (per individual per hour) are calculated from a combination of daily 45-minute-long direct observations and multiple 2-hour long digital observations (mean ± SD of 13.84 ± 4.81 h of direct observation and 20.00 ± 0.00 h of digital observations per colony). All data were collected after the competition point, under queenright conditions (or switch point + 8 days) in the pre-queen removal phase (from 14/04/2019 to 09/05/2019). All egg-eating events included involved worker-laid eggs (or assumed worker-laid eggs). Thick horizontal bars, medians; boxes, interquartile range (IQR); filled black circles, 'jittered' raw data, with each point representing a single individual within each class.

2.4.3 Pre-queen removal phase: egg-eating events involving queen-laid eggs and comparison with egg-eating events involving worker-laid eggs

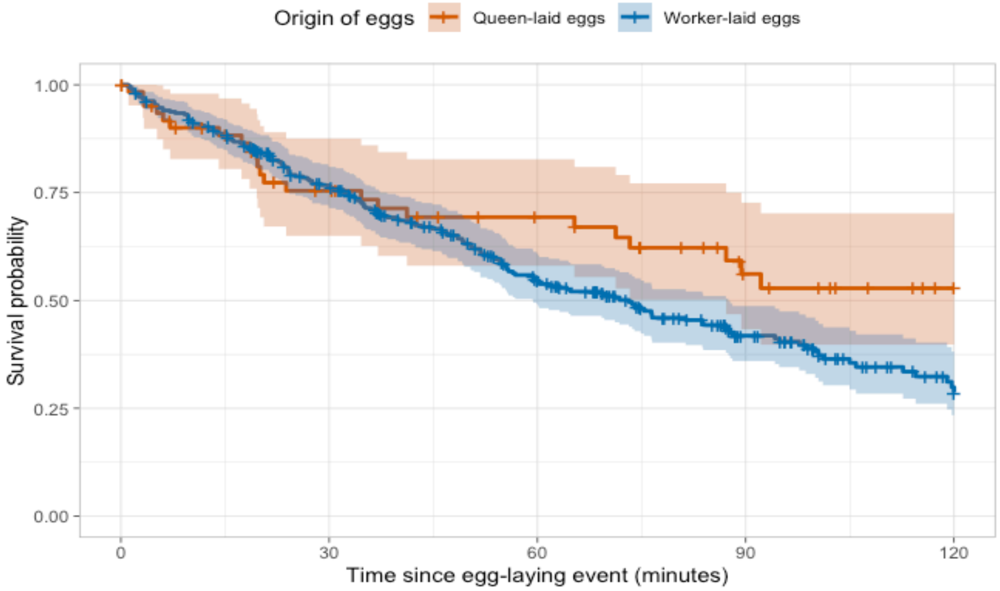
In addition to the 314 egg-eating events recorded in which worker-laid eggs (and assumed worker-laid eggs) were eaten, there were 28 egg-eating events recorded in which known queen-laid eggs were eaten. Queen-laid eggs were eaten in 6/11 colonies, and in total egg-eating events in which queen-laid eggs were eaten made up 17.4 % of all egg-eating events performed by workers across 11 colonies in the pre-queen removal phase ($n_{\textit{queen-laid egg} \text{ eating events performed by workers}} = 28$, $n_{\textit{worker-laid egg} \text{ eating events performed by workers}} = 133$). LEEs_{pre}, $\text{NLEEs}_{\text{pre}}$ and unmarked $\text{workers}_{\text{pre}}$ were all recorded eating queen-laid eggs ($n_{\textit{queen-laid egg-eating events performed by LEEspre}} = 20$, $n_{\textit{queen-laid egg-eating events performed by NLEEspre}} = 7$, $n_{\textit{queen-laid egg-eating events performed by unmarked workerspre}} = 1$), performing 71.4%, 25.0% and 3.6% of the 28 events, respectively. Of the seven queen-laid egg-eating events performed by $\text{NLEEs}_{\text{pre}}$, six of these events were performed by $\text{NLEEs}_{\text{pre}}$ that would become laying workers in the post-queen removal phase (either $\text{LEEs}_{\text{post}}$ or $\text{LNEEs}_{\text{post}}$) and only one event was performed by a $\text{NLEEs}_{\text{pre}}$ that would go on to become a $\text{NLNEEs}_{\text{post}}$ in the post-queen removal phase. This suggests workers that lay eggs within their lifetime are more likely to eat queen-laid eggs than worker that do not lay eggs within their lifetime. All workers that were observed eating queen-laid eggs were also observed eating known worker-laid eggs and/or assumed worker-laid eggs.

In the digital observations recorded during the pre-queen removal phase, it was possible to continuously monitor the survival of eggs laid in 311 egg-laying events for up to 120-minutes ($n_{\textit{egg-laying events performed by queen}} = 45$, $n_{\textit{egg-laying events performed by marked workers}} = 266$). This accounted for 45/82 (54.9%) of egg-laying events performed by queens and 266/357 (74.5%) of egg-laying events performed by marked workers, across the 11 colonies, in this phase. Of these egg-laying events, 24 egg-eating events were observed in which queen-laid eggs were eaten and 189 egg-eating events were observed in which worker-laid eggs were eaten ($n_{\textit{queen-laid egg-eating events performed by workers}} = 24$, $_{\textit{worker-laid egg-eating events performed by queens}} = 101$, $n_{\textit{worker-laid egg-eating events performed by workers}} = 88$). It was found worker-laid eggs had significantly reduced survivability relative to queen-laid eggs (Cox's proportional hazards analysis: hazard ratio = 1.63, 95% Cl [1.04, 2.54], $p = 0.033$; **Figure 2.4A**). However, when only egg-eating events (of queen and worker-laid eggs) performed by workers were used in analyses ($n_{\textit{queen-laid eggs (eaten by workers)}} = 40$, $n_{\textit{worker-laid eggs (eaten by workers)}} = 76$), the survivability of worker-laid eggs relative to queen-laid eggs did not differ significantly (hazard ratio = 1.08, 95% Cl [0.66, 1.75], $p = 0.8$; **Figure 2.4B**). This suggests workers did not preferentially eat worker-laid eggs over queen-laid eggs.

In order to further analyse the survivability of queen- and worker-laid eggs, all data (i.e., from both direct and digital observations), were considered in a generalised linear model. As it was not possible to obtain egg survival times in the direct observations, data were instead used to determine if the proportions of egg-laying events that were followed by an egg-eating event (in which eggs from the laying event were eaten within a direct or digital observation bout) differed depending on whether the eggs were queen-laid or known worker-laid. An analysis that included all egg-laying events and egg-eating events performed by workers and queens from both direct and digital observations suggested that there was a significant difference in the proportion of queen and worker egg-laying events that were followed by an egg-eating event (GLMM: χ^2= 14.06, df = 1, $p < 0.001$; **Table A2.5**). Pairwise comparisons showed queen-laid eggs were significantly less likely to be eaten following an egg-laying event than worker-laid eggs (Z = -3.79, $p < 0.001$). However, when only egg-eating events (of queen and worker-laid eggs) performed by workers were considered, there was no significant difference between the proportion of queen and worker egg-laying events that were followed by an egg-eating event (GLMM: χ^2 = 0.06, df = 1, $p = 0.80$; **Table A2.6**).

Overall, these two analyses suggest that queen-laid eggs have higher survivorship than worker-laid eggs and that workers do not preferentially eat worker-laid eggs over queen-laid eggs or vice versa. These findings are likely to stem from the fact that queen-laid eggs are eaten only by a small number of workers whereas worker-laid eggs are eaten by both queens and workers.

(A)



(B)

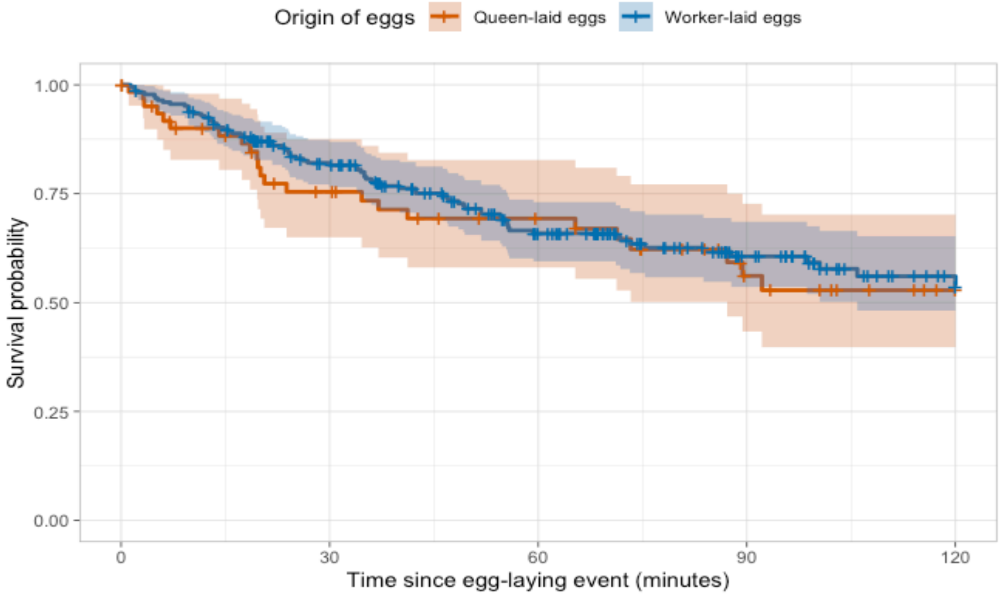


Figure 2.4. The Kaplan-Meyer curves (±95 CI) for the cumulative probability of survival for queen-laid (red line) and worker-laid (blue line) eggs, for up to 120 minutes of observation in the pre-queen removal phase. (**A**) survival probability of queen- and worker-laid eggs with egg-eating events by queens and workers included (censored recordings: n *queen-laid eggs* = 40, n *worker-laid eggs* = 150; time to event recordings: n *queen-laid eggs* = 24*,* n *worker-laid eggs* = 170). (**B**) survival probability of queen- and worker-laid eggs with only egg-eating events performed by workers included (censored recordings: n *queen-laid eggs* = 40, n *worker-laid eggs* = 150; time to event recordings n *queen-laid eggs* = 24*,* n *worker-laid eggs* = 76*).* Data were obtained from 11 *Bombus terrestris* colonies. Data include censored data and were collected from multiple 2-hour long digital film recordings (pre-queen removal phase: 20.00 ± 0.00 h of digital observations per colony) after the onset of the competition point (or switch point + 8 days) (between 14/04/2019 and 09/05/2019). All eggs (laid and/or eaten) were highly likely to have been male eggs (**Section 2.3.6**).

2.4.4 Pre-queen removal phase: acts of aggression directed at individuals engaged in egg-laying events

During the pre-queen removal phase, acts of aggression directed at individuals engaged in egg-laying events were relatively infrequent ($n_{\textit{acts of aggression performed by queens}} = 33$, $n_{\textit{acts of aggression performed by workers}} = 106$), and so the two component behaviours ('displacing egg-layers' and 'eating egg-cell wall'; **Table 2.1**) were pooled to provide one total rate for the overall behaviour for each individual within a colony. The mean rates at which queens, $LEEs_{pre}$, $LNEEs_{pre}$ and $NLEEs_{pre}$ performed acts of aggression directed at individuals engaged in an egg-laying event differed significantly (Kruskal-Wallis rank sum test: $\chi^2 = 17.90$, df = 3, $p < 0.001$; Figure 2.5, Table A2.7). Pairwise comparisons showed queens performed these acts at a significantly higher rate than $LNEEs_{pre}$ and $NLEEs_{pre}$ (Dunn's test: queen vs $LNEEs_{pre}$, Z = 3.48, $p < 0.003$; queen vs $NLEEs_{pre}$, Z = 3.41, $p = 0.004$). However, the rate at which queens and $LEEs_{pre}$ performed these acts did not differ significantly (Dunn's test: queen vs $LEEs_{pre}$, Z = 1.60, $p = 1.00$). The rate at which different classes of workers performed these acts also did not differ significantly (Dunn's test: $LEEs_{pre}$ vs $LNEEs_{pre}$, Z = 1.97, $p = 0.49$; $LEEs_{pre}$ vs $NLEEs_{pre}$, Z = 1.88, p = 0.59; $LNEEs_{pre}$ vs $NLEEs_{pre}$, Z = -0.80, $p = 1.00$). Overall, queens, $LEEs_{pre}$, $LNEEs_{pre}$ and $NLEEs_{pre}$ performed these acts at a mean (± SE) rate of 3.27 ± 0.76, 0.64 ± 0.20, 0.05 ± 0.03 and 0.04 ± 0.02 acts per individual per 24 h, respectively (**Figure 2.5, Table A2.7**). In summary, queens were significantly more likely to perform acts of aggression directed at individuals engaged in egg-laying events than were any of the other classes. LEEs may also have shown elevated rates of this form of aggression, but this could not be supported statistically from the current data. Hence, the results for queens, and possibly for LEEs, suggest an association of egg-laying and egg-eating with this form of aggression

Queens, $LEEs_{pre}$, $LNEEs_{pre}$ and unmarked workers were all aggressed while engaged in egg-laying events. The rate at which these classes were aggressed differed significantly (Kruskal-Wallis rank sum test: $\chi^2 = 26.75$, df = 3, $p < 0.001$; Figure 2.5, Table A2.7). Pairwise comparisons showed queens were aggressed while engaged in egg-laying events at a significantly higher rate than all classes of worker (Dunn's test: queen vs $LEEs_{pre}$, Z = 2.26, $p < 0.001$; queen vs $LNEEs_{pre}$, Z = 2.95, $p < 0.001$; queen vs unmarked $workers_{pre}$, Z = 5.13, $p < 0.001$). While the rate at which $LEEs_{pre}$ and $LNEEs_{pre}$ were aggressed while engaged in egg-laying events did not differ significantly, both classes of worker were aggressed while engaged in egg-laying events at a higher rate than unmarked workers (Dunn's test: $LEEs_{pre}$ vs unmarked $worker_{pre}$, Z = 2.87, $p < 0.001$; $LNEEs_{pre}$ vs unmarked $workers_{pre}$, Z = 2.17, $p < 0.001$). Overall, queens, $LEEs_{pre}$, $LNEEs_{pre}$, and unmarked $workers_{pre}$ were aggressed while engaged in egg-laying events at a mean (±SE) rate of 3.60 ± 0.90, 0.44 ± 0.09, 0.28 ± 0.05 and 0.02 ± 0.02 aggressive acts received per individual per 24 h respectively (**Figure 2.5, Table A2.7**).These results suggest that, at the individual level, queens were aggressed while engaged in egg-laying events (involving male-destined eggs) at a higher rate than any other egg-laying individual. This may be a result of queens having significantly higher rates of egg-laying per 24 h than individuals in any other class.

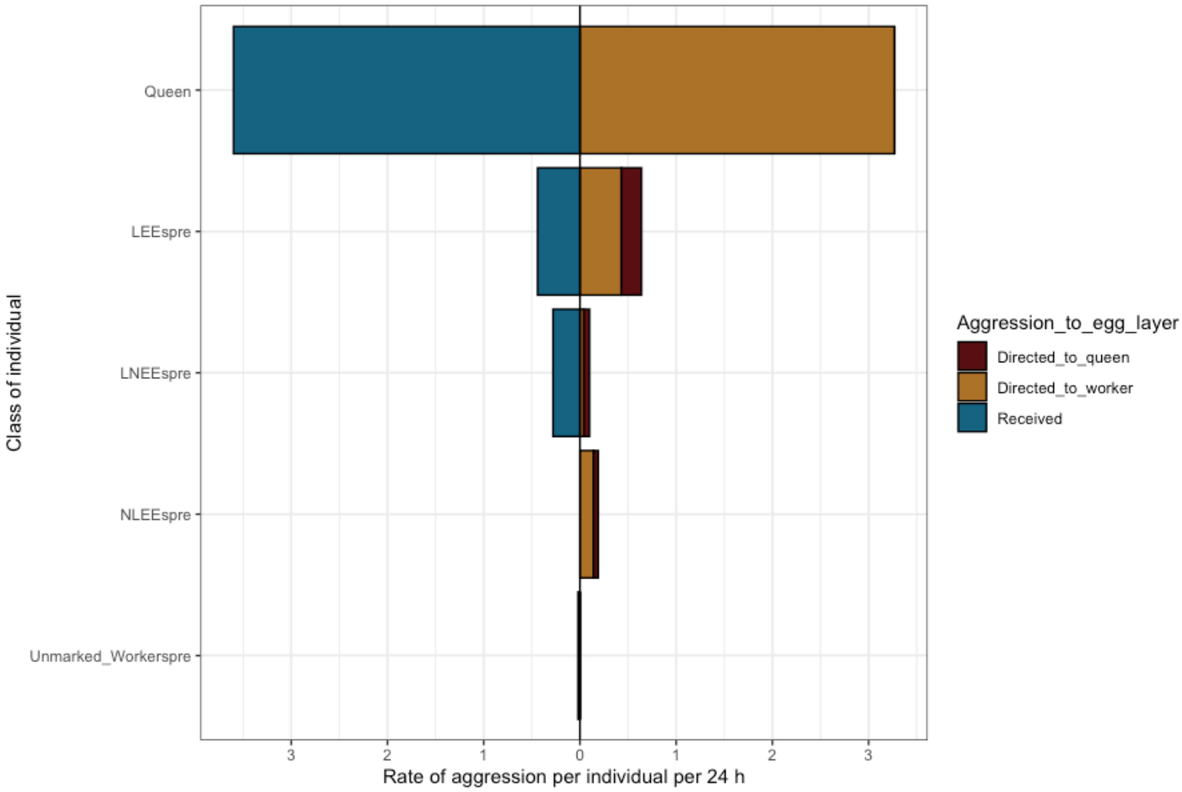


Figure 2.5. Rates of received (at left) and exhibited (at right) aggression towards individuals in different classes engaged in egg-laying events, in 11 *Bombus terrestris* colonies in the pre-queen removal phase. Rates of behaviour (mean per individual per 24 h) are calculated from multiple 2-hour long digital observations (20.00 ± 0.00 h of digital observations per colony) taken under post-competition point (or switch point + 8 days) queenright conditions (from 14/04/2019 to 09/05/2019). A total of 139 acts of aggression ($n_{\textit{acts of aggression performed by queens}} = 33$, $n_{\textit{acts of aggression performed by workers}} = 106$) were recorded towards individuals engaged in egg-laying over 220 hours of observations. Blue bars, mean rate of aggression received per individual per 24 h; gold bars, mean rate of aggressive acts directed at workers per individual per 24 h; maroon bars, mean rate of aggressive acts directed at queens per individual per 24 h.

2.4.5 Post-queen removal phase: numbers of egg-laying and egg-eating workers

The post-queen removal phase took place over the period 16 May 2019 – 14 June 2019 with a total of 332.75 hours ($n_{\textit{direct observations}} = 200.75$ h, $n_{\text{digital observations}} = 132.00$ h) of observations being recorded across 11 colonies. All workers that died during the pre-queen removal phase, as well as any worker that died earlier than 10 days prior to the end of the post queen-removal phase, were excluded from analyses. Workers that died later than 10 days prior to the end of the post-queen removal phase were retained in analyses as these workers were present in over 2/3 of observations and they were unlikely to have changed their behaviours or class during the remaining observations of the post-queen removal phase ($n_{\text{number of worker mortalities during the pre-queen removal phase}} = 29$, $n_{\text{number of worker mortalities earlier than 10 days prior to the end of the post-queen removal phase}} = 19$, $n_{\text{number of worker mortalities later than 10 days prior to the end of the post-queen removal phase}} = 7$). Of the 19 workers more than 10 days before the end of the post-queen removal phase, six had been observed performing egg-laying and/or egg-eating events in the pre-queen removal phase ($n_{\text{number of LEEpre mortalities}} = 3$, $n_{\text{number of LNEEspre mortalities}} = 2$, $n_{\text{number of NLEEspre mortalities}} = 1$, $n_{\text{number of NLNEEspre mortalities}} = 13$). These workers were excluded from all analyses that compared the behavioural response of individuals in each class of worker in the pre- and post- queen removal phases.

Overall, 908 marked workers were included in analyses for the post-queen removal phase. During this phase, 34.6% of the 908 marked workers were observed performing egg-laying and/or egg-eating events. In each colony, $\text{LEEs}_{\text{post}}$ $\text{LNEEs}_{\text{post}}$, and $\text{NLEEs}_{\text{post}}$ made up 7.2%, 25.0% and 2.4% of all marked workers, respectively. The remaining 65.4% of workers were classed as $\text{NLNEEs}_{\text{post}}$ (**Table 2.3**). Overall, the number of workers within each class did not differ significantly from the number of workers within each class during the pre-queen removal phase (GLMM: $\chi^2 = 0.0067$, df = 1, $p = 0.94$; **Table A2.3**). This suggests that, within the timeframe studied, the removal of the queen did not have significant impact on the numbers of egg-laying and egg-eating workers present within a colony.

2.4.6 Post-queen removal phase: discriminating between the public goods and selfish policing hypotheses by determining the response of $\text{NLEEs}_{\text{pre}}$ to queenless conditions

The behavioural response of $\text{NLEEs}_{\text{pre}}$ to queenless conditions was variable (**Figure 2.6**). Overall, $\text{NLEEs}_{\text{pre}}$ were recorded in 10/11 colonies during the pre-queen removal phase and there was a mean ± SD of 2.27 ± 0.41 $\text{NLEEs}_{\text{pre}}$ per colony ($n_{\textit{total numbers of NLEEspre in pre-queen removal phase}} = 25$; **Figure 2.6**). Following the removal of the colony queens, no NLEE_{pre} was classed as a $\text{NLEEs}_{\text{post}}$ in the post-queen removal phase (**Figure 2.6**). One NLEE_{pre} died more than 10 days prior to the end of the post-queen removal phase and so was excluded from any analysis in the post-queen removal phase (Section 2.4.5).

Of the remaining 24 $\text{NLEEs}_{\text{pre}}$, 14 (58%) performed egg-laying events (in direct or digital observations) in the post-queen removal phase. Of these 14 $\text{NLEEs}_{\text{pre}}$, five performed both egg-laying and egg-eating events, and so were classed as $\text{LEEs}_{\text{post}}$. The other nine performed egg-laying events only and were classed as $\text{LNEEs}_{\text{post}}$ (**Figure 2.6**). The remaining 10 $\text{NLEEs}_{\text{pre}}$ (42%) did not perform either egg-laying or egg-eating events in the post-queen removal phase, so were classed as $\text{NLNEEs}_{\text{post}}$ (**Figure 2.6**). Overall, the percentages of $\text{NLEEs}_{\text{pre}}$ that became $\text{LEEs}_{\text{post}}$, $\text{LNEEs}_{\text{post}}$, $\text{NLEEs}_{\text{post}}$ and $\text{NLNEEs}_{\text{post}}$ were 21%, 37%, 0% and 42%, respectively.

The public goods hypothesis predicted that all $\text{NLEEs}_{\text{pre}}$ workers would cease egg-eating in queenless conditions, and so become $\text{NLNEEs}_{\text{post}}$ workers in the post-queen removal phase. By contrast, the selfish policing hypothesis predicted that all $\text{NLEEs}_{\text{pre}}$ workers would start laying eggs and so become $\text{LEEs}_{\text{post}}$ or $\text{LNEEs}_{\text{post}}$ workers in the post-queen removal phase. The results (**Figure 2.6**) showed that all (24/24) $\text{NLEEs}_{\text{pre}}$ changed their behaviour in queenless conditions, but also that they exhibited a mixture of responses that did not fully match the predictions of either hypothesis, with 10/24 $\text{NLEEs}_{\text{pre}}$ ceasing egg-eating while remaining non-layers and so conforming to the public goods hypothesis prediction, and 14/24 $\text{NLEEs}_{\text{pre}}$ starting egg-laying and so conforming to the selfish policing hypothesis prediction.

The rate at which egg-eating was performed in the pre-queen removal phase by $\text{NLEEs}_{\text{pre}}$ that became $\text{LEEs}_{\text{post}}$ or $\text{LNEEs}_{\text{post}}$ did not differ significantly from the rate at which egg-eating was performed in the pre-queen removal phase by $\text{NLEEs}_{\text{pre}}$ that became $\text{NLNEEs}_{\text{post}}$ (Wilcoxon rank sum test: W =76, n = 24, $p = 0.975$). Therefore, the differential response of $\text{NLEEs}_{\text{pre}}$ to queenless conditions was not explained by their egg-eating rates in queenright conditions. There was also no relationship between the rate at which $\text{NLEEs}_{\text{pre}}$ workers aggressed workers engaged in an egg-laying event during the pre-queen removal phase and their subsequent rate of egg-laying (Spearman rank correlation: $\rho = 0.136$, n = 24, $p = 0.517$) or egg-eating (Spearman rank correlation: $\rho = 0.08$, n = 24, $p = 0.719$) in the post-queen removal phase.

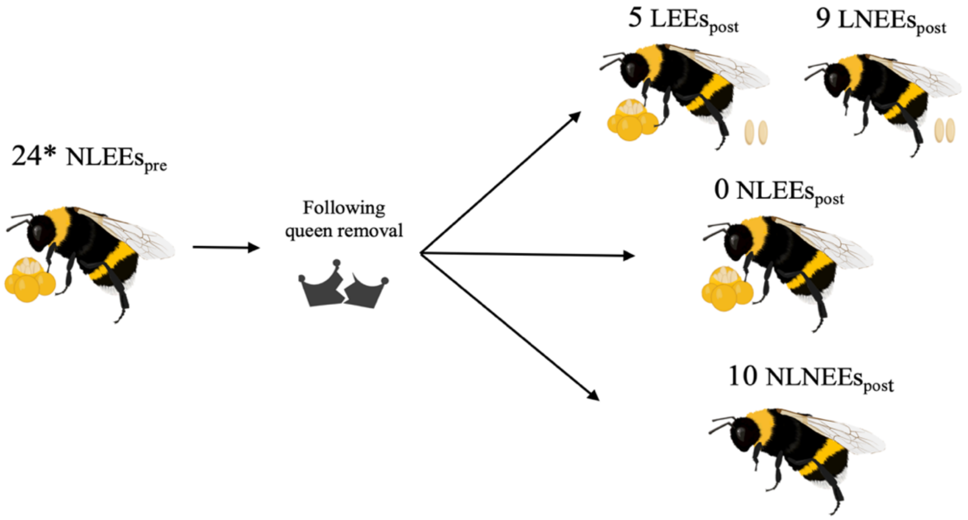


Figure 2.6. Behavioural response of non-laying, egg-eating workers in the pre-queen removal phase ($\text{NLEEs}_{\text{pre}}$) to queenless conditions in the post-queen removal phase (n = 24). Data collected across 11 *Bombus terrestris* colonies over 705 h of observations ($n_{\textit{pre-queen removal phase observations}}$ = 372.25 h, $n_{\textit{post-queen removal phase observations}}$ = 332.75 h). $\text{LEEs}_{\text{post}}$: worker observed egg-laying and egg-eating in the post-queen removal phase; $\text{LNEEs}_{\text{post}}$: worker observed egg-laying but not egg-eating in the post-queen removal phase; $\text{NLEEs}_{\text{post}}$: worker not observed egg-laying but observed egg-eating in the post-queen removal phase; $\text{NLNEEs}_{\text{post}}$: worker observed neither egg-laying nor egg-eating in the post-queen removal phase. Numbers above bee images are numbers of workers in each class. * Twenty-five NLEE_{pre} workers were originally present during the pre-queen removal phase but one was excluded from analyses as it died earlier than 10 days prior to the end of the post-queen removal phase.

2.4.7 Post-queen removal phase: response of other worker classes to queenless conditions

Like NLEEs$_{\text{pre}}$, individual workers in the classes LEEs$_{\text{pre}}$, LNEEs$_{\text{pre}}$, and NLNEEs$_{\text{pre}}$ also varied in their behavioural response to queenless conditions following the removal of the colony queens.

Of the 51 LEEs$_{\text{pre}}$ used in analyses, 33.3%, 43.1%, 5.9% and 17.7% became LEEs$_{\text{post}}$, LNEEs$_{\text{post}}$, NLEEs$_{\text{post}}$ and NLNEEs$_{\text{post}}$, respectively, in the post-queen removal phase (**Figure 2.7A**). Therefore, of the 51 LEEs$_{\text{pre}}$, the majority (76.5%) remained egg-layers but only 39.2% remained as egg-eaters. Of the 216 LNEEs$_{\text{pre}}$ used in analyses, 12.1%, 37.0%, 5.1% and 45.8% became LEEs$_{\text{post}}$, LNEEs$_{\text{post}}$, NLEEs$_{\text{post}}$ and NLNEEs$_{\text{post}}$, respectively, in the post-queen removal phase (**Figure 2.7B**). Therefore, of the 216 LNEEs$_{\text{pre}}$, almost half (49.1%) remained as egg-layers but 17.1% were observed performing egg-eating events for the first time. Of the 617 NLNEEs$_{\text{pre}}$ used in analyses, 2.8%, 18.8%, 1.3% and 77.2% were classed as LEEs$_{\text{pre}}$, LNEEs$_{\text{pre}}$, NLEEs$_{\text{pre}}$ and NLNEEs$_{\text{post}}$, respectively, in the post-queen removal phase (**Figure 2.7C**). Therefore, of the 617 NLNEEs$_{\text{pre}}$, the majority (77.2%) remained in the same class of worker, although 21.6% were observed performing egg-laying events for the first time and 4.1% were observed performing egg-eating events for the first time.

Although 22 workers were classed as NLEEs$_{\text{post}}$ (**Figures 2.6, 2.7**), 14 of these workers had been observed egg-laying in the pre-queen removal phase, as either LEEs$_{\text{pre}}$ or LNEEs$_{\text{pre}}$ (n = $_{\text{LEEspre/NLEEspost}}$ = 3, n = $_{\text{LNEEspre/NLEEspost}}$ = 11). Only eight NLEEs$_{\text{post}}$ workers had not been observed egg-laying at any point during direct or digital observations in the pre-queen removal phase, i.e., all eight had been classed as NLNEEs$_{\text{pre}}$ (**Figure 2.7C**). To allow for a direct comparison with NLEEs$_{\text{pre}}$, which were not observed egg-laying throughout the pre-queen removal phase, only NLEEs$_{\text{post}}$ that had not been observed egg-laying at any point in any observations pre- or post-queen removal were used in further analyses. This meant the 14 workers that had been LEEs$_{\text{pre}}$ or LNEEs$_{\text{pre}}$ were excluded from analyses. All analyses of NLEEs$_{\text{post}}$ therefore include only the eight workers that were NLNEEs$_{\text{pre}}$, i.e., NLNEEs$_{\text{pre}}$/NLEEs$_{\text{post}}$ workers **(Table 2.3, Figure 2.8D).**

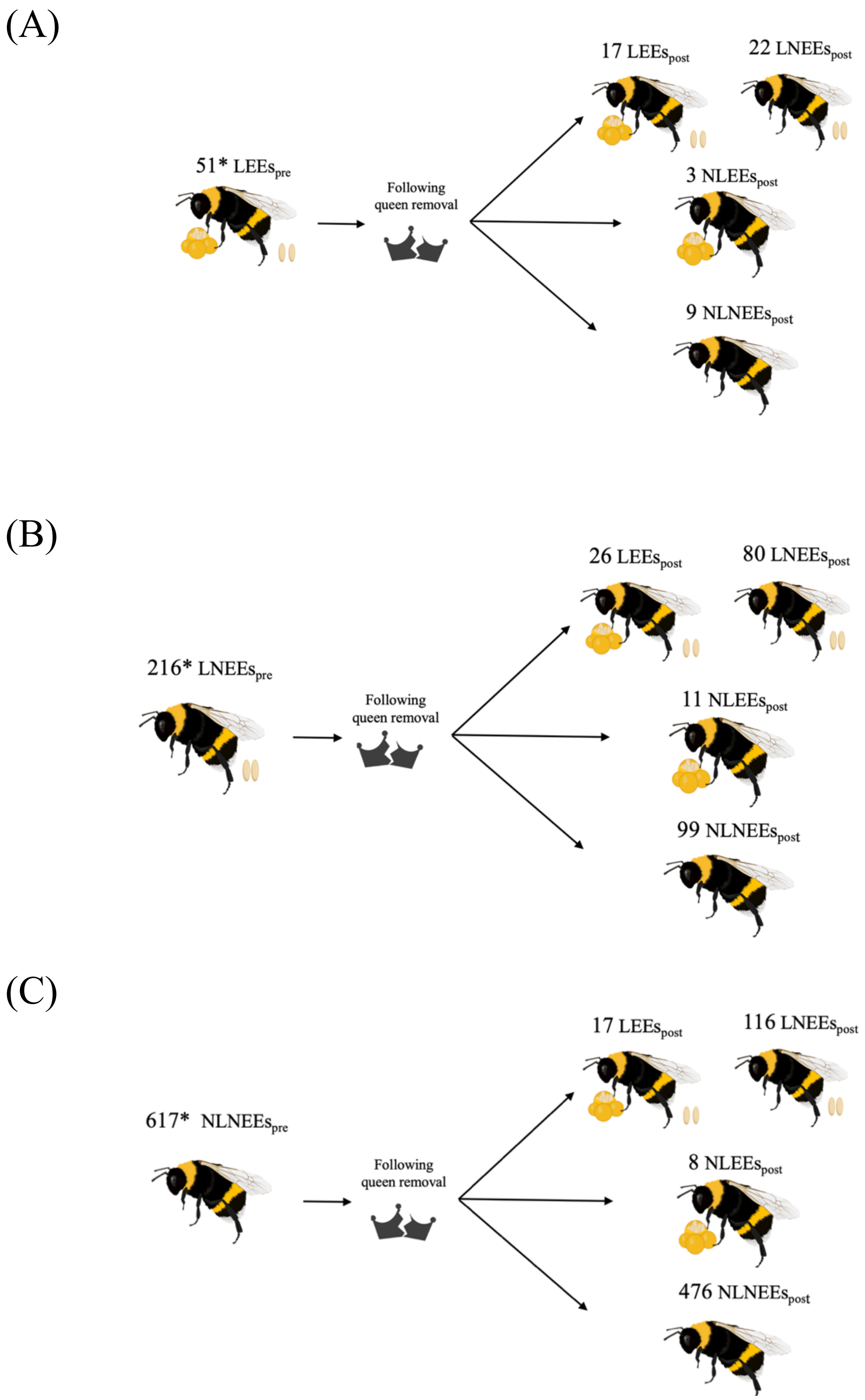


Figure 2.7. Behavioural responses of (A) LEEs_{pre} (n = 51), (B) $\text{LNEEs}_{\text{pre}}$ (n = 216) and (C) $\text{NLNEEs}_{\text{pre}}$ (n = 617) to queenless conditions in the post-queen removal phase. All data collected across 11 colonies over 705 h of observations ($n_{\textit{pre-queen removal phase observations}}$ = 372.25 h, $n_{\textit{post-queen removal phase observations}}$ = 332.75 h). $\text{LEEs}_{\text{post}}$: worker observed egg-laying and egg-eating in the post-queen removal phase; $\text{LNEEs}_{\text{post}}$: worker observed egg-laying but not egg-eating in the post-queen removal phase; $\text{NLEEs}_{\text{post}}$: worker not observed egg-laying but observed egg-eating in the post-queen removal phase; $\text{NLNEEs}_{\text{post}}$: worker observed neither egg-laying nor egg-eating in the post-queen removal phase. Numbers above bee images are numbers of workers in each class. *Workers present during the pre-queen removal phase were excluded from analyses if they died earlier than 10 days prior to the end of the post-queen removal phase.

2.4.8 Post-queen removal phase: rates of egg-laying and egg-eating events and comparison with pre-queen removal phase

A total of 650 egg-laying events were observed being performed by workers, across the 11 colonies, during the post-queen removal phase (n $_{\textit{LEEspost egg-laying events}}$ = 182, n $_{\textit{LNEEspost egg-laying events}}$ = 315, n $_{\textit{unmarked workerspost egg-laying events}}$ = 153). The mean rate (± SE) per individual per hour at which LEEs$_{\text{post}}$ performed egg-laying events (0.10 ± 0.01) was 1.25 times higher than the rate at which LEEs$_{\text{pre}}$ (0.08 ± 0.01) performed egg-laying events, while the rate at which LNEEs$_{\text{post}}$ performed egg-laying events (0.05 ± 0.01) did not differ to the rate at which LNEEs$_{\text{pre}}$ performed egg-laying events (0.05 ± 0.01) (**Table 2.3**). Overall, the rate at which LEEs$_{\text{post}}$ and LNEEs$_{\text{post}}$ performed egg-laying events did not differ significantly from the rate at which LEEs$_{\text{pre}}$ and LNEEs$_{\text{pre}}$ performed egg-laying events (paired Wilcoxon rank sum test: LEEs$_{\text{post}}$ vs LEEs$_{\text{pre}}$ V = 46, *p* = 0.265; **Figure 2.8A**, **Table 2.3**; LNEEs$_{\text{post}}$ vs LNEEs$_{\text{pre},}$, V = 27, *p* = 0.63; **Figure 2.8B**, **Table 2.3**). In contrast, the rate at which unmarked workers$_{\text{post}}$ performed egg-laying events (0.015 ± 0.003) was 2.1 times higher than the rate at which unmarked workers$_{\text{pre}}$ performed egg-laying events (0.007 ± 0.001) and this difference was statistically significant (paired Wilcoxon rank sum test: unmarked workers$_{\text{post}}$ vs unmarked workers$_{\text{pre}}$,V = 21, *p* = 0.03; **Table 2.3**). These findings suggest that queenless conditions had no effect on the rates at which individuals in the classes LEEs$_{\text{post}}$ and LNEEs$_{\text{post}}$ performed egg-laying events but did have the effect of increasing the per capita rate of egg-laying in unmarked workers.

A total of 295 egg-eating events were observed being performed by workers across the 11 colonies during the post-queen removal phase (n $_{\textit{LEEspost egg-eating events}}$ = 244, n $_{\textit{NLEEspost egg-eating events}}$ = 10, n $_{\text{unmarked workerspost egg-eating events}}$ = 41). The mean rate (± SE) per individual per hour at which LEEs$_{\text{post}}$ performed egg-eating events (0.14 ± 0.02) was 2.3 times higher than the rate at which LEEs$_{\text{pre}}$ (0.06 ± 0.01) performed egg-laying events, while the rate at which NLNEEs$_{\text{pre}}$/NLEEs$_{\text{post}}$ performed egg-eating events (0.02 ± 0.00) was 0.6 times lower than that of NLEEs$_{\text{pre}}$ (0.03 ± 0.01) (**Table 2.3**). Overall, the rate at which LEEs$_{\text{post}}$ performed egg-eating events was significantly higher than the rate at which LEEs$_{\text{pre}}$ performed egg-eating events and, in contrast, the rate at which NLNEEs$_{\text{pre}}$/NLEEs$_{\text{post}}$ performed egg-eating events was significantly lower than that rate at which NLEEs$_{\text{pre}}$ performed egg-eating events (paired Wilcoxon rank sum test: LEEs$_{\text{post}}$ vs LEEs$_{\text{pre}}$ V = 61, *p* = 0.02; **Figure 2.8C**, **Table 2.3**; NLEEs$_{\text{post}}$ vs NLNEEs$_{\text{pre}}$/NLEEs$_{\text{post}}$ V = 0, *p* = 0.04; **Figure 2.8D**, **Table 2.3**). In addition, the rates at which unmarked workers performed egg-eating events were low in both phases of the experiment (unmarked workers$_{\text{post}}$: 0.003 ± 0.000; unmarked workers$_{\text{pre}}$: 0.001 ± 0.000) and did not differ significantly between the phases (paired Wilcoxon rank sum test: unmarked workers$_{\text{post}}$ vs unmarked workers$_{\text{pre}}$ V = 1, *p* = 1.00). These findings show that workers continue to eat worker-laid eggs in queenless conditions and that, following queen removal, egg-laying workers show increased rates of egg-eating whereas non-egg-laying workers show decreased rates of egg-eating.

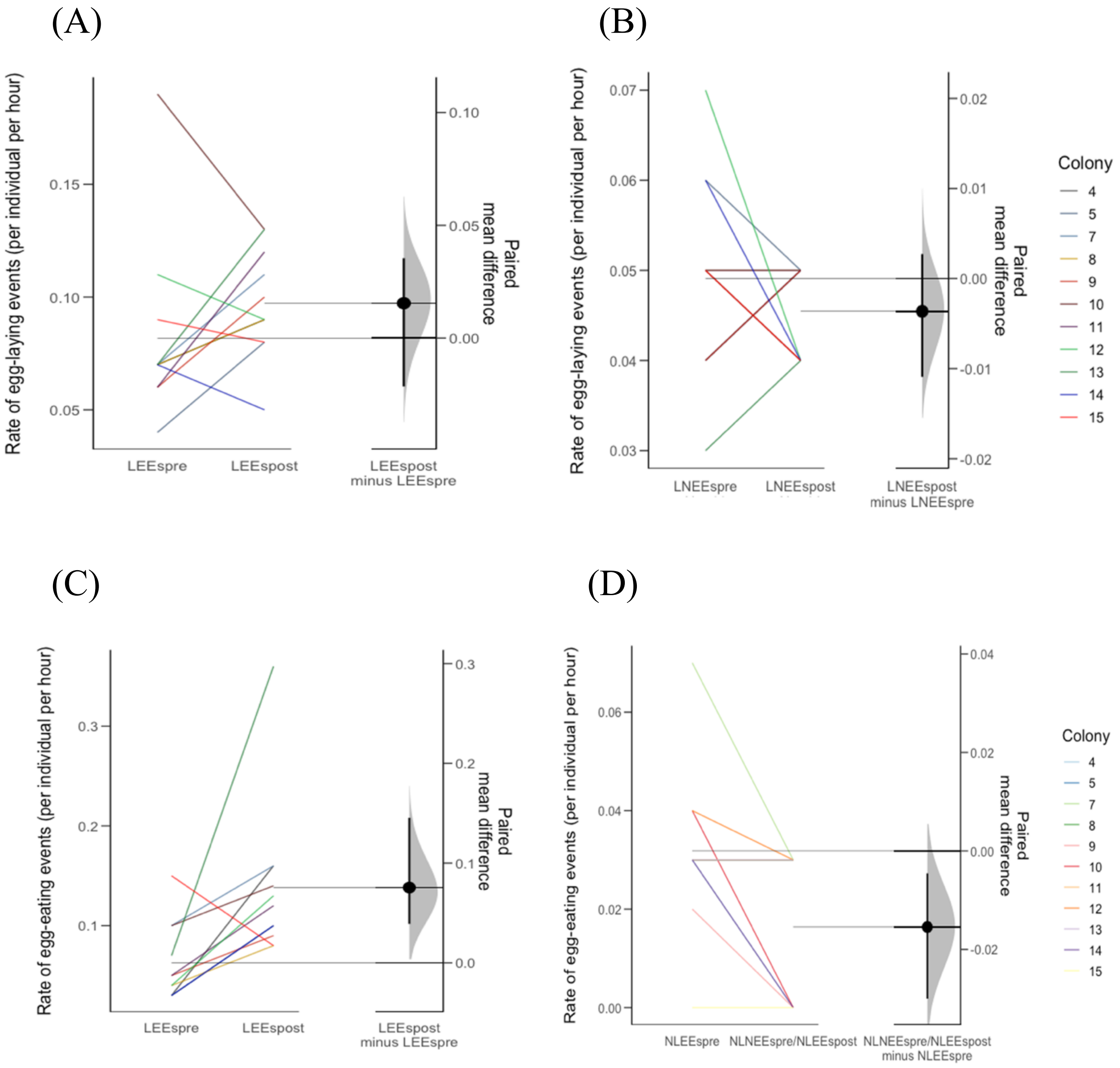


Figure 2.8. Comparison of rates of egg-laying or egg-eating in queenless versus queenright conditions for different worker classes. The paired mean differences in the per individual per hour mean rates of **(A)** LEEs$_{pre}$ and LEEs$_{post}$ performance of egg-laying events, **(B)** LNEEs$_{pre}$ and LNEEs$_{post}$ performance of egg-laying events, **(C)** LEEs$_{pre}$ and LEEs$_{post}$ performance of egg-eating events; and **(D)** NLEEs$_{pre}$ and NLNEEs$_{pre}$/NLEEs$_{post}$ performance of egg-eating events, between the pre- and post-queen removal phases. All data were obtained from 11 *Bombus terrestris* colonies. Rates of behaviours are calculated from a combination of daily 45-minute-long direct observations and multiple 2-hour long digital observations (pre-queen removal phase: mean ± SD of 13.84 ± 4.81 h of direct observations and 20.00 ± 0.00 h of digital observations per colony; post-queen removal phase: mean ± SD of 18.29 ± 0.56 h of direct observations and 12.00 ± 0.00 h of digital observations per colony). All data were collected after the onset of the competition point (or switch point + 8 days) (14/04/2019 – 09/05/2019). On each panel, raw data are plotted at left, with a coloured line (one per colony) connecting each paired data set; paired mean difference is plotted next to the right axis as a bootstrap sampling distribution; the difference in means is depicted as a filled black circle and the vertical black line represents the 95% confidence interval; sideways grey Gaussian curve represents the probability distribution of the difference between the two means.

2.4.9 Post-queen removal phase: acts of aggression directed at individuals engaged in egg-laying events

Acts of aggression directed at workers engaged in egg-laying events continued to be observed across colonies in the post-queen removal phase ($n_{\textit{LEEspost acts of aggression}} = 16$, $n_{\textit{LNEEspost acts of aggression}} = 17$, $n_{\text{NLNEEspre/NLEEspost acts } \textit{of aggression}} = 0$, $n_{\textit{unmarked workerspost acts of aggression}} = 1$). The mean (± SE) rate per individual per 24 h at which LEEs$_{post}$ performed acts of aggression directed at workers engaged in egg-laying events (0.72 ± 0.25) was 1.7 times greater than the rate at which LEEs$_{pre}$ (0.43 ± 0.21) performed acts of aggression directed at workers engaged in egg-laying events, while the rate at which LNEEs$_{post}$ performed acts of aggression directed at workers engaged in egg-laying events (0.15 ± 0.07) was 3.8 times greater than the rate at which LNEEs$_{pre}$ performed acts of aggression directed at workers engaged in egg-laying events (0.04 ± 0.02) (**Figure 2.9**, **Table A2.8, A2.9**). Overall, LEEs$_{post}$ and LNEEs$_{post}$ performed acts of aggression directed at workers engaged in egg-laying events at a significantly higher rate than LEEs$_{pre}$ and LNEEs$_{pre}$, respectively (paired Wilcoxon rank sum test: LEEs$_{post}$ vs LEEs$_{pre}$, V = 45, $p = 0.009$; LNEEs$_{post}$ vs LNEEs$_{pre}$, V = 36, $p = 0.014$ **Table A2.8, A2.9**). In contrast, NLNEEs$_{pre}$/NLEEs$_{post}$ were not observed performing acts of aggression directed at workers engaged in egg-laying events (0.00 ± 0.00) (**Figure 2.9**, **Table A2.8, A2.9**). In contrast, unmarked workers$_{post}$ performed acts of aggression directed at workers engaged in egg-laying events at a mean rate (0.07 ± 0.03) that was significantly higher than that of unmarked workers$_{pre}$ (0.00 ± 0.00) (paired Wilcoxon rank sum test: unmarked workers$_{post}$ vs unmarked workers$_{pre}$, V = 0, $p = 0.17$; **Table A2.8, A2.9**). Therefore, overall these results suggested that egg-laying workers upregulated their rates of this form of aggression in queenless conditions while non-laying workers down-regulated their rates of this form of aggression in queenless conditions.

LEEs$_{post}$, LNEEs$_{post}$ and unmarked workers$_{post}$ were all the recipients of aggression while engaged in egg-laying events, being aggressed at a mean (± SE) rate of 0.36 ± 0.11, 0.17 ± 0.03 and 0.09 ± 0.05 aggressive acts received per individual per 24 h, respectively (**Figure 2.9, Table A2.8**). These rates were lower than those shown by the corresponding worker classes in the pre-queen removal phase, although these differences were not statistically significant (paired Wilcoxon rank sum test: LEEs$_{post}$ vs LEEs$_{pre}$, V = 28.5, $p = 0.722$; LNEEs$_{post}$ vs LNEEs$_{pre}$, V = 18.5, $p = 0.213$; unmarked workers$_{post}$ vs unmarked workers$_{pre}$, V = 22.5, $p = 0.176$; **Table A2.8, A2.9**).

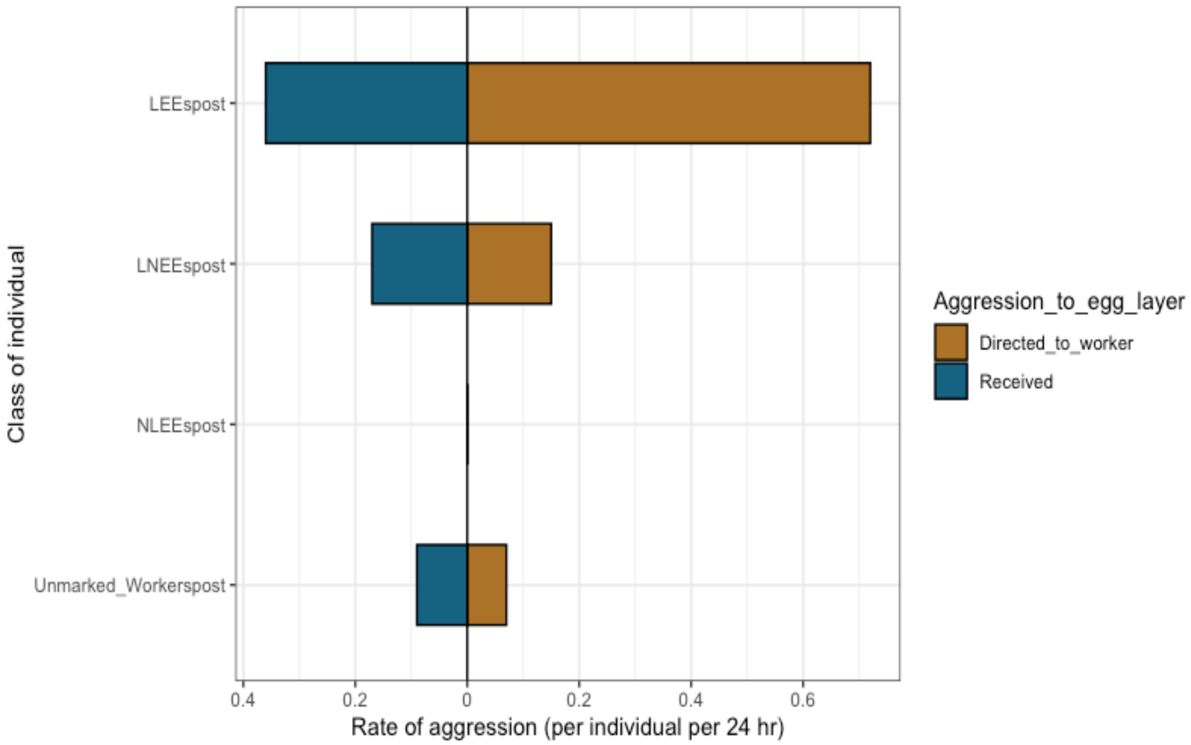


Figure 2.9. Rates of aggression received from (at left) or directed to (at right) workers within different classes engaged in egg-laying events in 11 *Bombus terrestris* colonies in the post-queen removal phase. Rates of behaviour (mean per individual per 24 h) are calculated from multiple 2-hour long digital film observations (12.00 ± 0.00 h of digital observations per colony) taken under post-competition point (or switch point + 8 days) queenless conditions (from 14/05/2019 to 09/05/2019). A total of 34 acts of aggression were recorded towards individuals engaged in egg-laying over 132 hours of observations. Blue bars, mean rate of aggression received per individual per 24 h; gold bars, mean rate of aggressive acts directed to workers per individual per 24 h.

2.4.10 Pre- and post-queen removal phases: relationship between egg-laying and egg-eating

Though only 33.3% of individual LEEs$_{pre}$ continued to perform both egg-laying and egg-eating events in the post-queen removal phase (**Figure 2.7A**), LEEs$_{pre}$ that became LEEs$_{post}$ had a significantly higher rate of egg-laying in the post-queen removal phase than LEEs$_{pre}$ that became LNEEs$_{post}$ (Wilcoxon rank sum test: LEEs$_{pre}$/LEEs$_{post}$ vs LEEs$_{pre}$/LNEEs$_{post}$, W = 302.5, $p < 0.001$). Likewise, LNEEs$_{pre}$ that became LEEs$_{post}$ had a significantly higher rate of egg-laying in the post-queen removal phase than LNEEs$_{pre}$ that became LNEEs$_{post}$ (Wilcoxon rank sum test: LNEEs$_{pre}$/LEEs$_{post}$ vs LNEEs$_{pre}$/LNEEs$_{post}$, W = 1365, p = 0.01) and NLNEEs$_{pre}$ that became LEEs$_{post}$ had a significantly higher rate of egg-laying, in the post-queen removal phase, than NLNEEs$_{pre}$ that became LNEEs$_{post}$ (NLNEEs$_{pre}$/LEEs$_{post}$ vs NLNEEs$_{pre}$/LNEEs$_{post}$, W = 1546, $p < 0.001$). These results suggested that, in the transitions occurring across the phases, egg-eating was associated with increased egg-laying in workers.

While the individual workers that made up the classes LEEs$_{post}$ and LNEEs$_{post}$ consisted of a mixture of LEEs$_{pre}$, LNEEs$_{pre}$, NLEEs$_{pre}$ and NLNEEs$_{pre}$ (**Figures 2.6, 2.7**), LEEs$_{post}$ performed egg-laying events at a significantly higher rate than LNEEs$_{post}$ (Welch t-test: $t_{(72.3)} = 6.00, p < 0.001$). In the pre-queen removal phase, there was a weak but significant, linear correlation between the rates at which individual LEEs$_{pre}$ performed egg-laying and egg-eating events (Spearman rank correlation: ρ = 0.284, n = 54, $p = 0.04$). In the post-queen removal phase, the rates at which individual LEEs$_{post}$ workers performed egg-laying and egg-eating events were not significantly correlated (Spearman rank correlation: $\rho = 0.22$, n = 65, $p = 0.08$). As the individual-level relationships from both phases showed the same directionality, the data from both pre- and post-queen removal phases were combined for greater power; analysis of the combined data again returned a weak but significantly positive correlation between the rates at which individual LEEs (i.e., LEEs$_{pre}$ or LEEs$_{post}$) performed egg-laying and egg-eating events (Spearman rank correlation: $\rho = 0.3$, n = 119, $p < 0.001$; **Figure 2.10**). Therefore, the individual-level data also suggested that egg-eating was associated with increased egg-laying rates in workers.

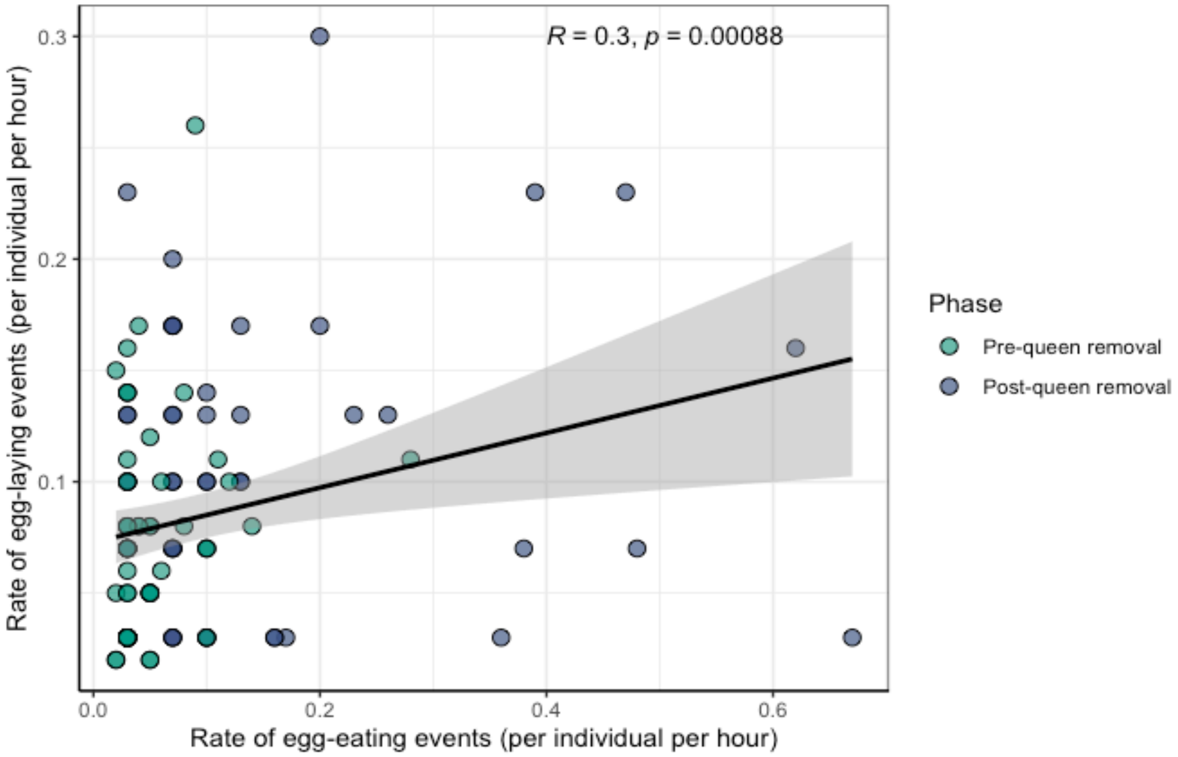


Figure 2.10. Relationship between the rate (per individual per hour) at which LEEs$_{pre}$ and LEEs$_{post}$ performed egg-laying events and egg-eating events. All data were obtained from 11 *Bombus terrestris* colonies. Rates of behaviours are calculated from a combination of daily 45-minute-long direct observations and multiple 2-hour long digital film observations (pre-queen removal phase: mean ± SD of 13.84 ± 4.81 h of direct observations and 20.00 ± 0.00 h of digital observations per colony; post-queen removal phase: mean ± SD of 18.29 ± 0.56 h of direct observations and 12.00 ± 0.00 h of digital observations per colony). All data were collected after the onset of the competition point (or switch point + 8 days) (14/04/2019 – 09/05/2019). Spearman rank correlation: $\rho = 0.3$, n = 119, $p < 0.001$. Each data point represents a single worker ($n_{LEEspre} = 54$ and $n_{LEEspost} = 65$). Colours represent phase (pre- or post-queen removal). Line is plotted from the correlation analysis, with grey shading representing 95% confidence intervals.

2.4.11 Relationship between ovarian activation, body size and class of worker.

In total, 123 workers were dissected across 11 colonies to determine the relationship between level of ovarian activation, body size and class of worker ($n_{\text{NLEEspre/LEEspost\_\&\_NLEEspre/LNEEspost}} = 14$, $n_{\text{LEEspost or LNEEspost}} = 50$, $n_{\text{NLNEEspre/NLNEEspost}} = 50$, $n_{\text{NLEEspre/NLNEEspost}} = 9$). All available workers that had been classed as $\text{NLEEs}_{\text{pre}}$ were dissected to determine if there was a difference in degree of ovary activation and body size between (1) $\text{NLEEs}_{\text{pre}}$ that were observed egg-laying in the post-queen removal phase (i.e., either $\text{NLEEs}_{\text{pre}}/\text{LEEs}_{\text{post}}$ or $\text{NLEEs}_{\text{pre}}/\text{LNEEs}_{\text{post}}$) and (2) $\text{NLEEs}_{\text{pre}}$ that had stopped egg-eating, and had not been observed performing egg-laying events, in the post-queen removal phase (i.e., $\text{NLEEs}_{\text{pre}}/\text{NLNEEs}_{\text{post}}$). In addition, to increase sample sizes and allow the degree of ovary activation of individual $\text{NLEEs}_{\text{pre}}$ to be compared to those of known egg-laying workers and workers that had never been observed egg laying, 50 workers that were observed egg-laying in the post-queen removal phase (either $\text{LEEs}_{\text{post}}$ or $\text{LNEEs}_{\text{post}}$) and 50 workers that were not observed egg-laying or egg-eating throughout the pre- and post-queen removal phases ($\text{NLNEEs}_{\text{pre}}/\text{NLNEEs}_{\text{post}}$) were also dissected.

Based on the above definitions, dissected workers were pooled to create two distinct groups with the following criteria: (1) 'known laying workers': workers observed performing an egg-laying event at least once in the post-queen removal phase; with this group consisting of $\text{NLEEs}_{\text{pre}}/\text{LEEs}_{\text{post}}$, $\text{NLEEs}_{\text{pre}}/\text{LNEEs}_{\text{post}}$ and $\text{LEEs}_{\text{post}}$ or $\text{LNEEs}_{\text{post}}$ ($n_{\text{known laying workers}} = 64$); and (2) 'suspected non-laying workers': workers not observed egg-laying in either the pre-queen removal phase or post-queen removal phase; with this group consisting of $\text{NLEEs}_{\text{pre}}/\text{NLNEEs}_{\text{post}}$ and $\text{NLNEEs}_{\text{pre}}/\text{NLNEEs}_{\text{post}}$ ($n_{\text{suspected non-laying workers}} = 59$). A generalised linear model showed that the mean length of the terminal oocytes was significantly larger in known laying workers than in suspected non-laying workers ($\chi^2 = 5.74$, df = 1, $p = 0.017$; **Figure 2.11**; **Table A2.9**). However, the length of the marginal cell on the forewing did not differ significantly between the two groups ($\chi^2 = 0.92$, df = 1, $p = 0.338$; **Table A2.9**). This suggests that, as might be expected, the degree of ovarian activation in known egg-laying workers was significantly greater than in suspected non-laying workers; however, this difference in degree of ovarian activation was not associated with a difference in body size.

In addition, the stage of oogenesis differed between known egg-laying workers and suspected non-laying workers (stages 1-7; **Figure A2.1**), with a significantly higher number of workers having active ovaries in the group of known egg-laying worker than in the group of suspected non-laying workers (Fisher's exact test: $p = 0.009$). Of the 64 known egg-laying workers, 49 (76.6%) had well-developed, active ovaries capable of producing mature oocytes (stage 5-6), while the remaining 15 (23.4%) had ovaries in a state of active regression (stage 7). In comparison, of the 59 suspected non-laying workers, 24 (40.7%) had well-developed, active ovaries capable of producing mature oocytes (stage 5-6), while 21 (35.6%) had ovaries in a state of active regression (stage 7) and the remaining 14 workers (23.7%) had inactive ovaries (stage 1-4). Of the 24 suspected non-laying workers that had well-developed active ovaries (stages 5-6), 2 (8.3%) were workers classed as NLEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ and 22 (91.7%) were workers classed as NLNEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$. Similarly, of the 21 suspected non-laying workers that had ovaries in a state of active regression (stage 7), 4 (19.0%) were workers classed as NLEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ and 17 (81.0%) were workers classed as NLNEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$. In the group of suspected non-laying workers, there was no difference in the number of workers in each class of worker (i.e., NLEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ and NLNEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$) with active ovaries (Fisher's exact test: $p = 0.34$). In addition, there was no difference in the number of workers in each class with ovaries in a state of active regression (Fisher's exact test: $p = 0.40$). In contrast, the subset of NLEEs$_{\mathrm{pre}}$/LEEs$_{\mathrm{post}}$ and NLEEs$_{\mathrm{pre}}$/LNEEs$_{\mathrm{post}}$ in the group of known egg-laying workers that had active ovaries was significantly higher than the number of NLEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ with active ovaries in the group of suspected non-laying workers (Fisher's exact test: $p = 0.02$).

Overall, the ovaries of NLEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ and NLNEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ were significantly smaller than the ovaries of NLEEs$_{\mathrm{pre}}$/LEEs$_{\mathrm{post}}$ and NLEEs$_{\mathrm{pre}}$/LNEEs$_{\mathrm{post}}$. Additionally, an analyses of levels of ovary activation between classes showed NLEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ had ovaries with a degree of activation similar to that of NLNEEs$_{\mathrm{pre}}$/NLNEEs$_{\mathrm{post}}$ which were not observed egg-laying or egg-eating throughout the experiment and were suspected to be non-reproductive throughout their lifetime. In turn, this suggests that, although observations in the experiment were not continuous (such that some worker egg-laying would have occurred between direct or digital observation bouts and so been unrecorded), NLEEs$_{\mathrm{pre}}$ workers were classified as non-layers with a relatively high degree of accuracy.

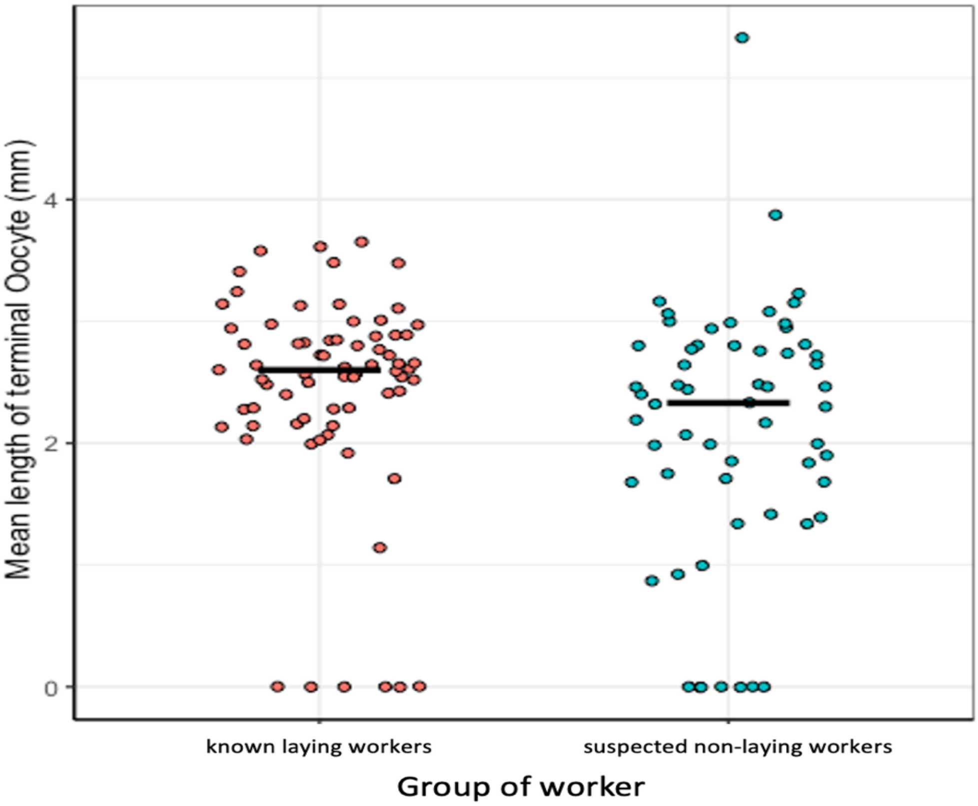


Figure 2.11. Terminal oocyte length for known egg-laying workers and suspected non-laying workers across 11 colonies *Bombus terrestris*. Mean length of terminal oocyte (mm) for each individual was determined as the mean of the largest terminal oocyte on three different ovarioles. Sample sizes: $n_{\text{known laying workers}} = 64$, $n_{\text{suspected non-laying workers}} = 59$. Filled circles, values for single individuals; black horizontal bars, mean value for each group of workers.

2.5 Discussion

2.5.1 Overview

This study set out to discriminate between two hypotheses (public goods and selfish policing hypotheses) for the origin of policing of other workers' eggs by non-reproductive workers in eusocial Hymenoptera. This was done in an experiment using *B. terrestris* colonies to quantify the behavioural response of non-laying, egg-eating workers (NLEEs$_{pre}$) to queenless conditions experimentally created by the removal of the colony queen. The results confirmed the existence of such workers in queenright *B. terrestris* colonies (Zanette et al. 2012), albeit at low frequency (2.7%). They therefore confirmed the co-occurrence in this species of the three forms of policing of worker-laid eggs, namely queen policing, policing by laying (reproductive) workers and policing by non-laying (non-reproductive) workers (Zanette et al. 2012). However, with respect to the hypotheses, the results found that, following removal of the queen, 10/24 NLEEs$_{pre}$ ceased egg-eating (policing) while remaining non-layers and so conformed to the prediction of the public goods hypothesis and 14/24 NLEEs$_{pre}$ started egg-laying and so conformed to the prediction of the selfish policing hypothesis (**Figure 2.6**). Hence the results found partial support for both hypotheses. In addition, I provide empirical data that advances the understanding of queen-worker and worker-worker conflict over reproduction and of policing mechanisms within eusocial Hymenoptera by (1) showing an association in reproductive workers between egg-laying, egg-eating and aggression towards other egg-laying workers and (2) quantifying the rate at which workers eat queen-laid male-destined eggs.

2.5.2 The public goods hypothesis

The finding that 42.0% (10/24) of NLEEs$_{pre}$ neither ate nor laid eggs in queenless conditions (**Figure 2.6**) provides some support for the public goods hypothesis. In essence, non-reproductive workers that consumed the eggs of other workers in queenright conditions in order to enhance the production of queen-derived sexuals (as predicted by the public goods hypothesis) would not be selected to destroy worker-laid eggs in the absence of the queen, as each worker is equally related to any potential nephew ($r = 0.375$), and so policing worker-laid eggs after the queen's removal would serve no purpose in maximising their inclusive fitness. The fact that the subset of NLEEs$_{pre}$ that became non-laying non-egg eating workers (NLNEEs$_{post}$) conformed to this prediction provides evidence to support the public goods hypothesis by suggesting that, in queenright conditions, these NLEEs$_{pre}$/NLNEEs$_{post}$ were maximising their inclusive fitness through favouring male production by the colony queen over male production by workers.

As 20% of the $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ had active ovaries and a further 40% had ovaries in a state of reabsorption (**Section 2.4.11**), it is possible these workers either performed egg-laying events (i.e., outside of all observations) or were 'hopeful reproductives' (see below) that intended to lay eggs within their lifetime and were thus selfishly policing rival workers' eggs. Though possible, these interpretations remain unlikely for several reasons: (1) colonies were observed extensively (~ 702 hours over 2 months), and so, if a worker that was classed as non-laying did perform egg-laying events, these events would have to have been performed at very low rates throughout the experiment; (2) the median longevity of *B. terrestris* workers in whole colonies is between 46-54 days (Smeets and Duchateau, 2003; Blacher et al., 2017), and so any workers that were 'hopeful reproductives' might have been expected to perform egg-laying events within the 2 month period of observations. In addition, previous research has shown that up to 64% of *B. terrestris* workers may have active ovaries capable of egg-laying within a colony, but only 38% may lay eggs (Alaux et al., 2004a). Therefore, as $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ workers in the current study were not observed performing egg-laying events in any observation over a 2-month period, it is still likely that they were non-reproductive despite the presence (in some) of active ovaries. Furthermore, if $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ had performed egg-laying events, it would be expected that their ovaries would have been in a similar stage of activation to those of known egg-laying workers. However, I found the ovaries of $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ were significantly smaller and less active than those of $\text{NLEEs}_{\text{pre}}$ that became laying egg-eating workers ($\text{LEEs}_{\text{post}}$) or laying non-egg eating workers ($\text{LNEEs}_{\text{post}}$). Instead, the ovaries of $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ were of a similar size and stage of activation to that of workers that were not observed egg-laying throughout their lifetime ($\text{NLNEEs}_{\text{pre}}$/$\text{NLNEE}_{\text{post}}$) (**Figure 2.11**). Hence, it is highly likely that $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ workers were indeed non-reproductive over their entire lifetimes.

While the public goods hypothesis could explain why $\text{NLEEs}_{\text{pre}}$/$\text{NLNEEs}_{\text{post}}$ were observed policing worker-laid eggs in queenright conditions, other predictions of the public goods hypothesis still require formal testing and validation. In particular, the relationship between worker policing by egg-eating and colony efficiency remains unclear (Helanterä and Ratnieks, 2019). The current study found that worker policing did not induce egg-laying workers to cease egg-laying activities, with LEEs_{pre} and $\text{LNEEs}_{\text{pre}}$ performing 74.0% of egg-laying events in the pre-queen removal phase, despite low egg survivorship (**Figures 2.3, 2.4**). As a result, it is unlikely that the presence of queen and/or worker policing behaviours led to an increase in the rate at which LEEs_{pre} and $\text{LNEEs}_{\text{pre}}$ performed other colony tasks, such as brood care or foraging, which would potentially enhance the overall efficiency of the colony. It is possible that worker policing by non-reproductive workers enhances colony productivity by ensuring the quantity of eggs laid does not exceed the numbers that can be successfully reared to adults by workers present (Wenseleers et al., 2004; Wenseleers and Ratnieks, 2006a). However, previous research has shown that worker reproduction does not reduce gyne production within *B. terrestris* colonies (Lopez-

Vaamonde et al., 2003). As worker-laid eggs have also been shown to be as viable as queen-laid male-destined eggs (Zanette et al., 2012) and worker-derived adult males shown to have the same reproductive ability as queen-derived adult males (Zhao et al., 2021), the rearing of worker-derived males is also unlikely to negatively impact the inclusive fitness of non-reproductive workers.

Therefore, while it appears a small number of non-reproductive workers do police reproductive workers in queenright conditions, as first reported in *B. terrestris* by Zanette et al. (2012), it remains unclear why non-reproductive workers would be selected to favour the rearing of queen-derived males ($r = 0.25$) over worker-derived males ($r = 0.375$) in colonies of *B. terrestris.* For the public goods hypothesis to be receive stronger support, empirical data are required to demonstrate the predicted positive relationship between worker policing and colony efficiency (Ratnieks, 1988).

2.5.3 The selfish policing hypothesis

In the study colonies, there was stronger evidence for selfish policing by reproductive workers than for policing by non-reproductive workers. It is likely the remaining 58% of NLEEs$_{\text{pre,}}$ that were observed egg-laying in queenless conditions initially engaged in policing behaviours as 'hopeful reproductives', i.e., were, in the pre-queen removal phase, preparing to lay eggs on a later occasion (**Figure 2.6**). The results therefore suggest that egg-laying behaviours do not necessarily have to precede egg-eating behaviours for selfish policing to be selected for in workers that are reproductive during their lifetime. Selfish policing by reproductive workers has been observed in colonies of ants (Stroeymeyt et al., 2007; Barth et al., 2010) and wasps (Wenseleers et al., 2005b), although, unlike the current study, none of these studies explicitly sought to discriminate the public goods and selfish policing hypotheses.

Moreover, several additional findings of the current study provide further evidence to suggest that worker policing by reproductive workers in monandrous monogynous colonies has evolved from selfish origins (Stroeymeyt et al., 2007; Bonckaert et al., 2011; Zanette et al., 2012). Firstly, the number of LEEs, and the rate at which LEEs performed egg-eating events, were each significantly higher than corresponding values for NLEEs in both phases of the experiment (**Sections 2.4.1, 2.4.5**), confirming that worker policing is performed primarily by reproductive workers in *B. terrestris* colonies (Zanette et al., 2012). Secondly, reproductive workers continued to aggress and police each other following the removal of the queen (**Section 2.4.5**). In addition, both LEEs$_{\text{post}}$ and LNEEs$_{\text{post}}$ performed acts of aggression towards workers engaged in egg-laying events at a significantly higher rate than LEEs$_{\text{pre}}$ and LNEEs$_{\text{pre}}$ (**Figure 2.8**). The current study is the first to fully describe and quantify such acts of aggression (which, for the remainder of the Discussion, are termed simply 'acts of aggression' for brevity). Likewise, LEEs$_{\text{post}}$ were observed performing egg-eating events at a significantly higher rate than LEEs$_{\text{pre}}$ (**Figure 2.8**). The finding that policing events continued following queen removal provides additional support for the selfish origins of worker policing. In essence, reproductive workers would still be selected to consume the eggs of rival workers in the absence of the queen, as doing so would still reduce competition and provide direct fitness benefits, as each policing reproductive worker in queenless conditions is effectively replacing nephews ($r = 0.375$) with sons ($r = 0.5$) . Moreover, the finding that LEEs increased their rate of policing following queen removal suggests that, in queenless conditions, reproductive workers are able to modify their policing behaviour in response to the absence of queen policing. Therefore, these results further suggest workers are under selection to selfishly both egg-lay and police other workers' eggs throughout the colony cycle in monogynous eusocial Hymenoptera, but particularly in the queenless phase when most worker-derived males are produced (Lopez-Vaamonde et al., 2009; Friend and Bourke, 2014).

2.5.4 Association between egg-laying, egg-eating and aggression

Prior to their removal, colony queens performed both egg-laying and egg-eating events, as well as acts of aggression, at significantly higher rates than all workers, irrespective of the workers' class (**Figures 2.3, 2.5**). In addition, when egg-eating events by both queens and workers were considered, the survivorship of queen-laid eggs was significantly higher than that of workers' eggs (**Figure 2.4**). These findings support those of previous research (Duchateau, 1989; Zanette et al., 2012) and provide further empirical evidence that explains why, on average, >95% of adult males are queen-produced in queenright *B. terrestris* colonies, despite intense reproductive conflict (Alaux et al., 2004b; Lopez-Vaamonde et al., 2004).

One surprising result of this study was that 19.8% – 22.3% of reproductive workers (LEEs) policed the eggs of rival workers (**Table 2.3**; **Sections 2.4.1, 2.4.5**). According to the selfish policing hypothesis, it is expected that all reproductive workers would benefit from consuming eggs laid by rival workers. Hence, it is unclear why the number of reproductive workers not observed performing policing behaviours (LNEEs) was significantly higher than those that were (LEEs). However, though egg-eating events were performed by relatively few laying workers, a strong association was found between the rates of egg-laying, egg-eating and aggression performed by individuals in both phases of the experiment, as I now discuss.

As stated above, queens had the highest rates of all three behaviours. However, this association was also found among worker classes. Specifically, in both phases, LEEs performed egg-laying, egg-eating and acts of aggression at significantly higher rates than LNEEs and all other classes of worker (**Figures 2.3, 2.5, 2.8, 2.9**). Among LEEs, the rate of egg-laying was also positively correlated with the rate of egg-eating at the individual level (**Figure 2.10**). Furthermore, $LEEs_{post}$ performed egg-eating events at a higher rate than $LEEs_{pre}$, whereas $NLNEEs_{pre}/NLEEs_{post}$ performed egg-eating events at a lower rate than $\text{NLEEs}_{\text{pre}}$ (**Figures 2.8C, 2.8D**), suggesting that queenless conditions cause egg-eating to be upregulated only among egg-laying workers. Overall, therefore, it is clear that there is a positive association between egg-laying, egg-eating and acts of aggression among workers in queenright and queenless *B. terrestris* colonies. These findings provide additional support for worker policing being performed by reproductive workers for selfish reasons in this system and, hence, indirectly, for the selfish policing hypothesis for policing by non-reproductive workers.

As a link between worker policing and aggression has previously been found in a *Temnothorax* ant (Stroeymeyt et al., 2007), it is possible that, among competing reproductive nest-mate workers, policing events can be viewed an extension of aggression, as both are used by reproductive workers to inhibit reproduction of other workers for direct fitness gains. Therefore, as dominance hierarchies (based on aggression) are known to exist among workers in *Bombus* species (Van Doorn and Heringa, 1986; Duchateau, 1989), and previous research has theorised only strong resource-rich individuals should perform policing events (Frank, 1996; 2003), it is possible that LEEs are high-ranking workers that use policing, along with aggression, to maintain a reproductive monopoly. Egg-policing could also provide a nutritional benefit to reproductive workers, so adding a proximate driver to this behaviour aligned to its ultimate direct fitness benefits. As it is known that protein (in the form of pollen) is essential in order to activate adult workers ovaries (Duchateau and Velthuis, 1989), it is possible that the protein content of eggs eaten provides policing workers (LEEs) with a reproductive advantage over reproductive workers that do not eat eggs (LNEEs). Overall, in order to elucidate the relationship between these three behaviours, further behavioural and molecular investigations are required to discovering the underlying mechanisms that determine why some reproductive workers are egg-eaters and others are not.

2.5.5 Policing of queen-laid male-destined eggs

Though previous studies have mentioned anecdotal observations of worker-to-queen aggression and the destruction of queen-laid eggs in *Bombus* species (Sladen, 1912; Alford, 1975; Van der Blom, 1986), the rate of worker-to-queen aggression has not previously been quantified. The rate at which queen-laid eggs are eaten by workers was previously quantified in one other study (Zanette et al., 2012), which found it to be very low (approximating zero). In the current study, I found that queens were aggressed while engaged in an egg-laying event at a significantly higher rate than any worker (**Figure 2.5**) and, in contrast to the findings of Zanette et al. (2012), that workers regularly consumed queen-laid eggs following the competition point. Specifically, during the pre-queen removal phase, 17.4% of egg-eating events performed by workers involved the consumption of queen-laid eggs (**Section 2.3.4**). The contrast with the results of Zanette et al. (2012) conceivably arose because of natural variation and/or the use in that study of colonies of the subspecies *B. terrestris*

terrestris, unlike the current study, which used colonies of the subspecies *B. terrestris audax*.

The high-levels of queen-worker conflict over male parentage observed in the current study are predicted by inclusive fitness theory, since, in eusocial systems where effective paternity is less than two, workers are more closely related to worker-laid male eggs than to queen-laid ones (Ratnieks, 1988; Bourke, 1994; Loope, 2015). Specifically, under monandry, the destruction of queen-laid male eggs by both reproductive and non-reproductive workers is predicted because workers are more related to sons ($r = 0.5$) and nephews ($r = 0.375$) than to brothers ($r = 0.25$) (Hamilton, 1964; Trivers and Hare, 1976). However, in the current study, all marked workers observed eating queen-laid eggs were also observed eating worker-laid eggs. Moreover, 96.4% of these workers were egg-layers during their lifetime. It is therefore possible that the observed worker policing of queen-laid eggs is an extension of selfish worker policing. In addition, though worker-laid eggs survived less well overall, the probability of survival of queen-laid and worker-laid eggs did not differ significantly when only egg-eating events performed by workers were considered (**Figure 2.4**). As workers that were reproductive during their lifetime were responsible for all but one egg-eating event involving queen-laid eggs, and LEEs_{pre} ate worker-laid eggs at a significantly higher rate than any other worker class, it is conceivable these reproductive workers destroyed both worker- and queen-laid eggs indiscriminately for selfish gains, as in both instances reproductive workers would receive direct fitness benefits from replacing the consumed eggs with their own.

In the case of the one observation of a non-reproductive worker destroying queen-laid eggs, it is possible that this worker ate queen-laid eggs for the indirect fitness benefits received from favouring the rearing of worker-derived males (nephews) over queen-derived males. However, if non-reproductive workers generally favoured the rearing of worker-derived males over queen-derived males, it would be expected that, in the pre-queen removal phase, instances of NLEE_{pre} eating queen-laid eggs would have been higher. As the single $\text{NLEE}_{\text{pre}}/\text{NLNEE}_{\text{post}}$ observed consuming queen-laid eggs was also observed eating worker-laid eggs in the pre-queen removal phase, it is possible that, in this case, queen-laid eggs were eaten following a discrimination error, as reported in colonies of *A. mellifera* (Karcher and Ratnieks, 2014).

2.5.6 Other aspects of study

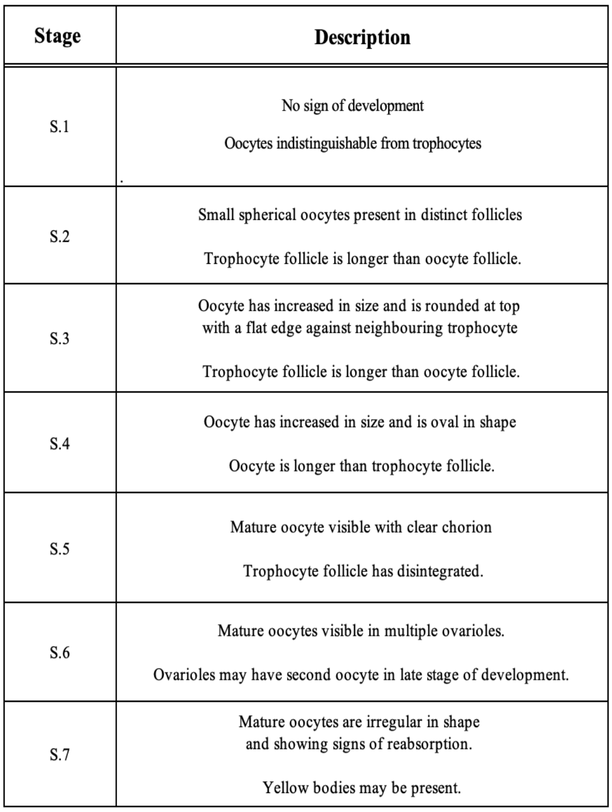
As colonies were not observed continuously over the course of the current experiment, it is likely that some behavioural events (i.e., egg-laying and egg-eating) were not observed and that as a result some individual workers were placed in the incorrect worker class. However, observations were extensive, with colonies observed for a total of ~702 hours over a period of 2 months. Thus, in either phase, the rate at which a worker performed a missed behaviour would have had to be very low for the behaviour to have remained undetected in all observation bouts. It therefore seems likely that the behaviours observed in queens and workers were representative of the behaviours and interactions that occurred in each colony. Additionally, as rates at which egg-laying and egg-eating events occurred were quantified at the event level, the rates of these behaviours could not be expressed in terms of number of eggs laid or eaten per unit time. Zanette et al. (2012) found that queens laid a mean (+SD) of 5.7 ± 2.5 eggs per egg-laying event and consumed a mean of 2.7 ± 1.5 eggs per egg-eating (policing) event. In comparison, they found that workers laid a mean 2.7 ± 1.5 eggs per egg-laying event and consumed 1.7 ± 1.0 eggs per egg-eating event. These results are consistent with the findings of the current study by confirming that queens are the most prolific egg-layers and egg-eaters. They also imply that, as mentioned, queen rates of egg-eating and egg-laying were underestimated in the current study. This should not have affected the conclusions, as the key comparisons of the current study are among workers alone. However, in the current study, it is unknown whether the number of eggs laid per event differed between LEEs and LNEEs, or similarly, if the number of eggs eaten per event differed between LEEs and NLEEs. Despite this, as LEEs performed egg-laying events and egg-eating events at a significantly higher rate than both LNEEs and NLEEs in both phases, and as in general egg-eating and egg-laying were positively associated, it appears highly unlikely that any systematic differences in the number of eggs laid or eaten per event among worker classes, if present, would affect the conclusion that LEEs were the most prolific egg-layers and egg-eaters among worker classes in both phases of the experiment.

2.5.7 Conclusions

In summary, these findings confirm that, in *B. terrestris* colonies, a small number of non-reproductive workers police the eggs of nest-mate workers, although these events occur at low frequencies. Though the predictions of the public goods hypothesis were met by a subset of NLEEs$_{pre}$ following the queen removals, most NLEEs$_{pre}$ started egg-laying in queenless conditions and so conformed with the prediction of the selfish policing hypothesis. Hence, overall, the origin of policing by non-reproductive workers remains unclear, and further investigation are required to determine why these workers are selected to police the eggs of other workers in monandrous monogynous colonies. In contrast, as most NLEEs$_{pre}$ became reproductive during their lifetime (i.e., in queenless conditions), the evidence from the current study suggests that the majority of worker policing events performed in *B. terrestris* colonies are selfish in origin, with reproductive workers consuming both queens and worker-laid eggs to increase their own direct fitness. Further to this, these results suggest that worker egg-laying and selfish policing are intricately linked by showing that (1) only a subset of reproductive workers perform policing events and (2) there was an association between egg-laying, egg-eating and aggression. Overall, these results provide valuable insights into the evolutionary basis of complex social interactions within eusocial insect colonies and enhance the understanding of conflict resolution in social systems in general.

2.6 Appendix

(A)



Stage	Description
S.1	No sign of development Oocytes indistinguishable from trophocytes .
S.2	Small spherical oocytes present in distinct follicles Trophocyte follicle is longer than oocyte follicle.
S.3	Oocyte has increased in size and is rounded at top with a flat edge against neighbouring trophocyte Trophocyte follicle is longer than oocyte follicle.
S.4	Oocyte has increased in size and is oval in shape Oocyte is longer than trophocyte follicle.
S.5	Mature oocyte visible with clear chorion Trophocyte follicle has disintegrated.
S.6	Mature oocytes visible in multiple ovarioles. Ovarioles may have second oocyte in late stage of development.
S.7	Mature oocytes are irregular in shape and showing signs of reabsorption. Yellow bodies may be present.

(B)

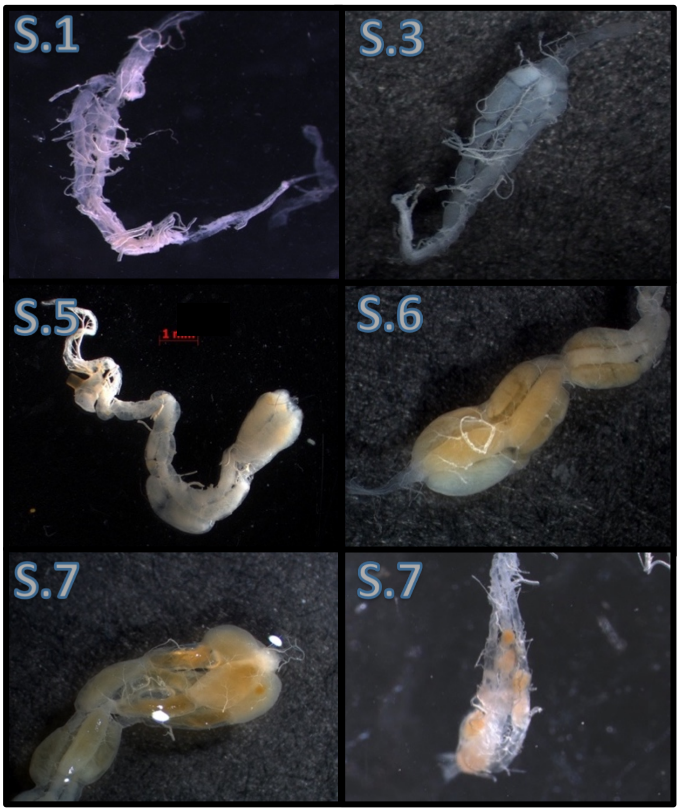


Figure A2.1. Stages of ovary activation in *Bombus terrestris* workers. (**A**) Description of the seven stages of ovarian activation in *B. terrestris* workers based on Ducheatau and Velthuis (1989). Ovaries in stages S.1- S.4 and S.7 are considered inactive and ovaries in stages S.5-S.6 are considered active. (**B**) *B. terrestris. audax* worker ovaries from the current study photographed in different stages of activation. No ovaries were found in stages S.2 or S.4 and so these stages are not illustrated. Level S.7 is shown in two versions to illustrate variation in the appearance of ovaries in a state of reabsorption. All photographs were taken by Jenny Livesey using an AxioVision camera attached to a dissection microscope.

Colony Number	No. of workers on arrival	No. of marked workers at end of experiment	No. of unmarked workers at end of experiment	Total no. of workers at end of experiment	Total no. of gynes produced per colony	Total no. of males produced per colony	Date of switch point	Date of competition point
4	8	61	56	117	13	502	05/03/2019	18/04/2019
5	2	96	24	120	175	483	12/04/2019	25/04/2019
7	9	73	65	138	78	364	20/04/2019	30/04/2019
8	6	126	48	174	120	546	12/04/2019	14/04/2019
9	9	102	7	109	2	530	02/04/2019	14/04/2019
10	4	125	7	132	97	438	16/04/2019	17/04/2019
11	5	63	46	109	188	259	15/03/019	29/04/2019
12	5	70	62	132	169	318	01/05/2019	23/04/2019
13	11	92	36	128	142	460	18/04/2019	16/04/2019
14	10	92	58	150	169	507	24/04/2019	22/04/2019
15	4	56	12	68	0	589	11/03/2019	22/04/2019
Mean	6.6	86.9	38.3	125.2	104.8	454.2	08/04/2019	20/04/2019
SD	2.9	24.5	22.3	26.7	72.4	101.7		

Table A2.1. Demographic, productivity and colony cycle data from 11 *Bombus terrestris* colonies used for data analyses. Colonies were received in queenright condition on 30/03/2019 and demographic data were collected from this date until the termination of the experiment (14/06/2019), i.e., over both the pre-queen removal and post-queen removal phases. Switch point is defined as the date of first male eclosion minus 26 days. Competition point is defined as the first date at which either worker egg-laying or eating was first observed. Dates are given in the format dd/mm/yyyy.

Colony	Date of Switch point	Date of Competition point	Days between switch and competition points	Date from which data were used for analysis
4	05/03/2019	18/04/2019	44	18/04/2019
5	12/04/2019	25/04/2019	13	25/04/2019
7	20/04/2019	30/04/2019	10	30/04/2019
8	12/04/2019	14/04/2019	2	22/04/2019
9	02/04/2019	14/04/2019	12	14/04/2019
10	16/04/2019	16/04/2019	0	24/04/2019
11	15/03/2019	29/04/2019	45	29/04/2019
12	01/05/2019	23/04/2019	-8	09/05/2019
13	02/04/2019	16/04/2019	14	16/04/2019
14	24/04/2019	22/04/2019	-2	02/05/2019
15	11/03/2019	22/04/2019	42	22/04/2019

Table A2.2. Timings of colony cycle events in 11 *Bombus terrestris* colonies, for calculation of date from which data were used for analysis (see text for further details). Dates are in the format dd/mm/yyyy.

A) Random effects

Groups	Name	Variance	Std.Dev
Colony	Intercept	0.1042	0.3228

Number of obs: 66, groups: Colony, 11

B) Fixed effects	Estimate Std. Error	Error	Z-value	Pr(>|z|)
Intercept	1.649594	0.119121	13.848	<2e-16 ***
Phase	0.003344	0.040761	0.082	0.935
LNEE	-0.012864	0.08546	-0.151	0.88
NLEE	1.308335	0.072546	18.035	<2e-16 ***

C) Anova	Chisq	df	Pr(>Chisq)
Intercept	191.7671	1	<2e-16 ***
Phase	0.0067	1	0.9346
Class	334.7572	2	<2e-16 ***

D) Pairwise Comparisons

contrast	ratio	SE	df	null	z.ratio	*p.* value
POST LEE / PRE LEE	1.007	0.0821	Inf	1	0.082	1
POST LEE / POST LNEE	0.267	0.0274	Inf	1	-12.847	<.0001
POST LEE / PRE LNEE	0.269	0.0353	Inf	1	-10.017	<.0001
POST LEE / POST NLEE	3.606	0.7071	Inf	1	6.541	<.0001
POST LEE / PRE NLEE	3.63	0.771	Inf	1	6.071	<.0001
PRE LEE / POST LNEE	0.265	0.0348	Inf	1	-10.118	<.0001
PRE LEE / PRE LNEE	0.267	0.0274	Inf	1	-12.847	<.0001
PRE LEE / POST NLEE	3.582	0.7607	Inf	1	6.008	<.0001
PRE LEE / PRE NLEE	3.606	0.7071	Inf	1	6.541	<.0001
POST LNEE / PRE LNEE	1.007	0.0821	Inf	1	0.082	1
POST LNEE / POST NLEE	13.515	2.4303	Inf	1	14.48	<.0001
POST LNEE / PRE NLEE	13.606	2.6862	Inf	1	13.222	<.0001
PRE LNEE / POST NLEE	13.425	2.6506	Inf	1	13.154	<.0001
PRE LNEE / PRE NLEE	13.515	2.4303	Inf	1	14.48	<.0001
POST NLEE / PRE NLEE	1.007	0.0821	Inf	1	0.082	1

Table A2.3. GLMM summary for analyses of numbers of individuals within 11 *B.terrestris* colonies in each class during the pre-queen removal and post-queen removal phases and the relationship between colony phase and numbers of individuals in each class *** $p < 0.001$. For pairwise comparisons (D), p value adjustment was conducted using the Bonferroni method (for 15 tests).

Rate of egg-eating by workers during the pre-queen removal phase (per individual per hour)

Colony	LEEspre		NLEEspre		Unmarkedworkerspre	
	Known queen-laid eggs	Known worker-laid egg	Known queen-laid eggs	Known worker-laid egg	Known queen-laid eggs	Known worker-laid egg
4	0.02	0.04	0.02	0.03	0.00	0.00
5	0.00	0.03	0.00	0.03	0.00	0.00
7	0.00	0.07	0.00	0.07	0.00	0.00
8	0.03	0.04	0.00	0.03	0.00	0.00
9	0.01	0.04	0.02	0.02	0.00	0.00
10	0.02	0.05	0.00	0.02	0.00	0.00
11	0.02	0.03	0.00	0.03	0.00	0.00
12	0.00	0.03	0.00	0.04	0.00	0.00
13	0.01	0.03	0.01	0.02	0.00	0.00
14	0.00	0.03	0.00	0.03	0.00	0.00
15	0.00	0.12	0.00	0.00	0.00	0.00
Mean	0.01	0.05	0.00	0.03	0.00	0.00
± SE	0.00	0.01	0.00	0.01	0.00	0.00

Table A2.4. Data from the pre-queen removal phase on mean (± SE) rates of egg-eating per individual per hour of known queen-laid eggs and known worker-laid eggs in 11 colonies of *Bombus terrestris*. For each colony and class of worker, rates of behaviour are calculated from a combination of daily 45-minute long direct observation recordings and 2-hour long digital film observations. All data were collected after the onset of the competition phase (or switch point + 8 days) (14/04/2019 – 09/05/2019). In the 'pre-queen removal phase', data were collected under queenright conditions (mean ± SD of 13.84 ± 4.81 h of direct observation and 20.00 ± 0.00 h of digital observations per colony).

A) Random effects

Groups	Name	Variance	Std.Dev
Hours	Intercept	1.29E-08	0.0001136
Colony	Intercept	8.85E-02	0.2975115

Number of obs: 21, groups: Hours, 9; Colony, 11

B) Fixed effects	Estimate Std. Error	Error	Z-value	Pr(>|z|)
Intercept	-0.6626	0.2545	-2.604	0.009215 **
Worker	0.9521	0.2539	3.749	0.000177***

C) Anova	Chisq	df	Pr(>Chisq)
Intercept	6.7807	1	0.0092149 **
Class	14.0568	1	0.001774 ***

D) Pairwise Comparisons

contrast	ratio	SE	df	null	z.ratio	*p.* value
Queen/Worker	0.386	0.098	Inf	1	-3.749	0.0002

Table A2.5. GLMM model summary for analyses of the proportion of egg-laying events (queen or worker) that were followed by an egg-eating event, performed by either queens or workers, in 11 colonies of *Bombus terrestris*. Analysis included all egg-laying events and egg-eating events performed by both queens and workers from both direct and digital observations *** $p < 0.001$ ** $p < 0.01$. For pairwise comparison (D) test was performed on the log odds ratio scale.

A) Random effects

Groups	Name	Variance	Std.Dev
Hours	Intercept	1.53E-08	0.0001237
Colony	Intercept	2.74E-01	0.5232257

Number of obs: 21, groups: Hours, 9; Colony, 11

B) Fixed effects	Estimate Std. Error	Error	Z-value	Pr(>|z|)
Intercept	-0.70954	0.29143	-2.435	0.0149 *
Worker	0.06661	0.2687	0.248	0.8042

C) Anova	Chisq	df	Pr(>Chisq)
Intercept	5.9278	1	0.0149 *
Class	0.0615	1	0.8042

D) Pairwise Comparisons

contrast	ratio	SE	df	null	z.ratio	*p.* value
Queen/Worker	0.936	0.251	Inf	1	-0.248	0.8042

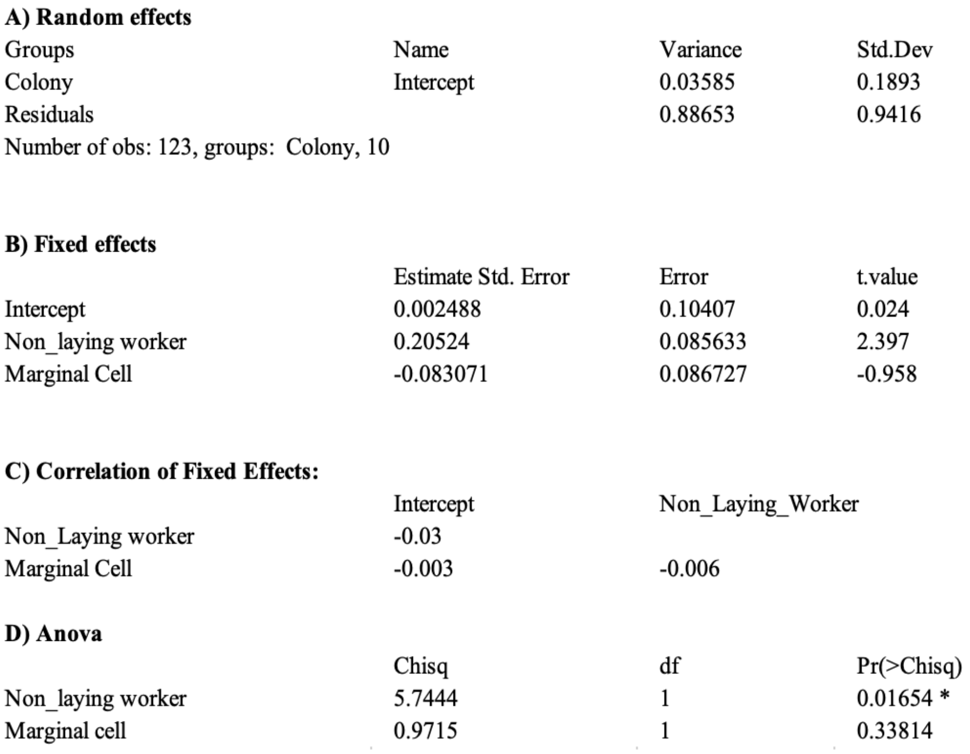
Table A2.6. GLMM model summary for analyses of the proportion of egg-laying events (queen or worker) that were followed by an egg-eating event, performed by workers only, in 11 colonies of *Bombus terrestris*. Analysis included egg-laying events performed by queens and workers and only egg-eating events performed by workers. Any egg-laying events performed by workers, which were followed by an egg-eating event by a queen were excluded from analyses. * $p < 0.05$. For pairwise comparison (D), test was performed on the log odds ratio scale.

	Rate of aggression to egg layer (per individual per 24 h)							Rate of aggression received while egg laying (per individual per 24h)			
	Actor Queen	Actor LEEpre		Actor LNEEpre		Actor NLEEpre					
Colony	Recipient Worker	Recipient Queen	Recipient Worker	Recipient Queen	Recipient Worker	Recipient Queen	Recipient Worker	Recipient Queen	Recipient LEEpre	Recipient LNEEpre	Recipient Unmarked Workers
4	1.20	0.00	0.48	0.00	0.00	0.00	0.60	3.60	0.48	0.55	0.00
5	0.00	0.00	0.48	0.04	0.00	0.00	0.00	1.20	0.24	0.13	0.00
7	4.80	1.20	0.00	0.10	0.10	0.00	0.00	4.80	0.00	0.00	0.00
8	7.20	0.72	0.00	0.17	0.06	0.00	0.00	9.60	0.40	0.32	0.00
9	3.60	0.00	0.36	0.00	0.04	0.00	0.00	1.20	0.44	0.16	0.00
10	6.00	0.00	0.00	0.07	0.00	0.00	0.60	2.40	0.60	0.21	0.17
11	3.60	0.00	0.48	0.00	0.00	0.60	0.00	3.60	0.00	0.40	0.03
12	6.00	0.40	0.00	0.11	0.00	0.00	0.00	2.40	0.40	0.55	0.00
13	0.00	0.00	0.48	0.00	0.00	0.00	0.30	2.40	0.48	0.20	0.03
14	1.20	0.00	0.00	0.17	0.21	0.00	0.00	8.40	0.96	0.21	0.00
15	2.40	0.00	2.40	0.00	0.00	0.00	0.00	0.00	0.80	0.35	0.00
Mean	3.27	0.21	0.43	0.06	0.04	0.05	0.14	3.60	0.44	0.28	0.02
± SE	0.76	0.12	0.21	0.02	0.02	0.05	0.07	0.90	0.09	0.05	0.02
Mean no. of individuals per class per colony	1.00	4.90		20.10		2.20		1.00	4.90	20.10	38.30

Table A2.7. Data from the pre-queen removal phase on rates (mean ± SE) of aggression directed at an individual performing an egg-laying event per individual per hour (left) and rates of aggression received while egg-laying per hour (right) in 11 *Bombus terrestris* colonies. Rates of behaviour were calculated from multiple 2-hour long digital film observations. All data were collected after the onset of the competition phase (or switch point + 8 days) (14/04/2019 – 09/05/2019) under queenright conditions (20.00 ± 0.00 h of digital observations per colony).

	Rate of aggression to egg-layer (per individual per 24 h)			Rate of aggression received while egg-laying (per individual per 24 h)		
	Actor LEEspost	Actor LNEEspost	Actor Unmarked workerspost			
Colony	Recipient Worker	Recipient Worker	Recipient Worker	Recipeint LEEspost	Recipient LNEEspost	Recipient Unmarked Workerspost
4	0.50	0.11	0.00	1.00	0.00	0.00
5	0.50	0.12	0.08	0.50	0.12	0.17
7	1.00	0.13	0.03	0.33	0.26	0.03
8	0.20	0.00	0.00	0.20	0.00	0.00
9	0.29	0.12	0.00	0.43	0.08	0.00
10	0.67	0.00	0.29	0.67	0.33	0.00
11	0.80	0.00	0.17	0.80	0.18	0.09
12	0.00	0.06	0.03	0.00	0.13	0.00
13	3.00	0.07	0.11	0.00	0.22	0.17
14	0.00	0.16	0.10	0.00	0.22	0.07
15	1.00	0.86	0.00	0.00	0.29	0.50
Mean	0.72	0.15	0.07	0.36	0.17	0.09
± SE	0.25	0.07	0.03	0.11	0.03	0.05
Mean no. of individuals per class per colony	6.4	20.6	38.3	6.4	20.6	38.3

Table A2.8. Data from the post-queen removal phase on rates (mean ± SE) of aggression directed at an individual performing an egg-laying event per individual per hour (left) and rates of aggression received while egg-laying per hour (right) in 11 *Bombus terrestris* colonies. Rates of behaviour were calculated from multiple 2-hour long digital film observations. All data were collected after the onset of the onset of the competition phase (or switch point + 8 days) (14/04/2019 – 09/05/2019) under queenless conditions (mean 12.00 ± 0.00 h of digital observations per colony).



A) Random effects

Groups	Name	Variance	Std.Dev
Colony	Intercept	0.03585	0.1893
Residuals		0.88653	0.9416

Number of obs: 123, groups: Colony, 10

B) Fixed effects

	Estimate Std. Error	Error	t.value
Intercept	0.002488	0.10407	0.024
Non_laying worker	0.20524	0.085633	2.397
Marginal Cell	-0.083071	0.086727	-0.958

C) Correlation of Fixed Effects:

	Intercept	Non_Laying_Worker
Non_Laying worker	-0.03	
Marginal Cell	-0.003	-0.006

D) Anova

	Chisq	df	Pr(>Chisq)
Non_laying worker	5.7444	1	0.01654 *
Marginal cell	0.9715	1	0.33814

Table A2.9. GLMM model summary for analyses of levels of ovary activation and marginal cell length, between two groups of workers. Egg-laying workers (reference category): workers observed performing an egg-laying event at least once in the post-queen removal phase; and non-laying workers : workers not observed egg-laying in either the pre-queen removal phase or post-queen removal phase. * $p < 0.05$.

2.7 References

Alaux, C., Hefetz, A., Jaisson, P., 2006a. Plasticity of worker reproductive strategies in *Bombus terrestris*: lessons from artificial mixed-species colonies. Animal Behaviour 72, 1417-1425.

Alaux, C., Jaisson, P., Hefetz, A., 2004a. Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies. Insectes Sociaux 51, 287-293.

Alaux, C., Jaisson, P., Hefetz, A., 2006b. Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal. Behavioral Ecology and Sociobiology 60, 439-446.

Alaux, C., Savarit, F., Jaisson, P., Hefetz, A., 2004b. Does the queen win it all? Queen-worker conflict over male production in the bumblebee, *Bombus terrestris*. Naturwissenschaften 91, 400-403.

Alexander, R.D., 1974. The evolution of social behavior. Annual Review Ecology Systematics 5, 325-383.

Alford, D.V., 1975. Bumblebees. Davis-Poynter, London.

Barth, M.B., Kellner, K., Heinze, J., 2010. The police are not the army: context-dependent aggressiveness in a clonal ant. Biology Letters 6, 329-332.

Beekman, M., Oldroyd, B.P., 2008. When workers disunite: intraspecific parasitism by eusocial bees. Annual Review of Entomology 53, 19-37.

Blacher, P., Huggins, T.J., Bourke, A.F.G., 2017. Evolution of ageing, costs of reproduction and the fecundity-longevity trade-off in eusocial insects. Proceedings of the Royal Society B - Biological Sciences 284, 20170380.

Bonckaert, W., Tofilski, A., Nascimento, F.S., Billen, J., Ratnieks, F.L.W., Wenseleers, T., 2011. Co-occurrence of three types of egg policing in the Norwegian wasp *Dolichovespula norwegica*. Behavioral Ecology and Sociobiology 65, 633-640.

Bonckaert, W., Vuerinckx, K., Billen, J., Hammond, R.L., Keller, L., Wenseleers, T., 2008. Worker policing in the German wasp *Vespula germanica*. Behavioral Ecology 19, 272-278.

Bourke, A.F.G., 1988. Worker reproduction in the higher eusocial Hymenoptera. Quarterly Review of Biology 63, 291-311.

Bourke, A.F.G., 1994. Worker matricide in social bees and wasps. Journal of Theoretical Biology 167, 283-292.

Bourke, A.F.G., 2011. Principles of Social Evolution. Oxford University Press, Oxford.

Bourke, A.F.G., Franks, N.R., 1995. Social Evolution in Ants. Princeton University Press, Princeton.

Bourke, A.F.G., Ratnieks, F.L.W., 1999. Kin conflict over caste determination in social Hymenoptera. Behavioral Ecology and Sociobiology 46, 287-297.

Bourke, A.F.G., Ratnieks, F.L.W., 2001. Kin-selected conflict in the bumble-bee *Bombus terrestris* (Hymenoptera: Apidae). Proceedings of the Royal Society of London Series B 268, 347-355.

Clutton-Brock, T.H., Parker, G.A., 1995. Punishment in animal societies. Nature 373, 209-216.

Crozier, R.H., Pamilo, P., 1996. Evolution of Social Insect Colonies: Sex Allocation and Kin Selection. Oxford University Press, Oxford.

D'Ettore, P., Heinze, J., Ratnieks, F.L.W., 2004. Worker policing by egg eating in the ponerine ant *Pachycondyla inversa*. Proceedings of the Royal Society of London Series B 271, 1427-1434.

Duchateau, M.J., 1989. Agonistic behaviors in colonies of the bumblebee *Bombus terrestris*. J. Ethol. 7, 141-151.

Duchateau, M.J., Velthuis, H.H.W., 1989. Ovarian development and egg laying in workers of *Bombus terrestris*. Entomologia Experimentalis et Applicata 51, 199-213.

Ernst, U.R., Cardoen, D., Cornette, V., Ratnieks, F.L.W., De Graaf, D.C., Schoofs, L., Verleyen, P., Wenseleers, T., 2017. Individual and genetic task specialization in policing behaviour in the European honeybee. Animal Behaviour 128, 95-102.

Frank, S.A., 1996. Policing and group cohesion when resources vary. Animal Behaviour 52, 1163-1169.

Frank, S.A., 2003. Repression of competition and the evolution of cooperation. Evolution 57, 693-705.

Friend, L.A., Bourke, A.F.G., 2014. Workers respond to unequal likelihood of future reproductive opportunities in an ant. Animal Behaviour 97, 165-176.

Ghoul, M., Griffin, A.S., West, S.A., 2014. Toward an evolutionary definition of cheating. Evolution 68, 318-331.

Goulson, D., Peat, J., Stout, J.C., Tucker, J., Darvill, B., Derwent, L.C., Hughes, W.O.H., 2002. Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? Animal Behaviour 64, 123-130.

Hamilton, W.D., 1964. The genetical evolution of social behaviour I, II. Journal of Theoretical Biology 7, 1-52.

Hammond, R.L., Keller, L., 2004. Conflict over male parentage in social insects. PLoS Biology 2, e248.

Helanterä, H., Ratnieks, F.L.W., 2019. Worker Conflict and Worker Policing. Encyclopedia of Animal Behavior (Second Edition), Pages 743-753,.

Hughes, W.O.H., Oldroyd, B.P., Beekman, M., Ratnieks, F.L.W., 2008. Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320, 1213-1216.

Karcher, M.H., Ratnieks, F.L.W., 2014. Killing and replacing queen-laid eggs: low cost of worker policing in the honeybee. American Naturalist 184, 110-118.

Loope, K.J., 2015. Queen killing is linked to high worker-worker relatedness in a social wasp. Current Biology 25, 2976-2979.

Lopez-Vaamonde, C., Koning, J.W., Jordan, W.C., Bourke, A.F.G., 2003. No evidence that reproductive bumblebee workers reduce the production of new queens. Animal Behaviour 66, 577-584.

Lopez-Vaamonde, C., Koning, J.W., Jordan, W.C., Bourke, A.F.G., 2004. A test of information use by reproductive bumblebee workers. Animal Behaviour 68, 811-818.

Lopez-Vaamonde, C., Raine, N.E., Koning, J.W., Brown, R.M., Pereboom, J.J.M., Ings, T.C., Ramos-Rodriguez, O., Jordan, W.C., Bourke, A.F.G., 2009. Lifetime reproductive success and longevity of queens in an annual social insect. Journal of Evolutionary Biology 22, 983-996.

Maynard Smith, J., Szathmáry, E., 1995. The Major Transitions in Evolution. W.H. Freeman, Oxford.

Ohtsuki, H., Tsuji, K., 2009. Adaptive reproduction schedule as a cause of worker policing in social Hymenoptera: a dynamic game analysis. American Naturalist 173, 747-758.

Owen, R.E., 1989. Differential size variation of male and female bumblebees. Journal of Heredity 80, 39-43.

Pamilo, P., 1991. Evolution of colony characteristics in social insects. II. Number of reproductive individuals. American Naturalist 138, 412-433.

Pirk, C.W.W., Neumann, P., Hepburn, H.R., 2007. Nestmate recognition for eggs in the honeybee (*Apis mellifera* L.). Behavioral Ecology and Sociobiology 61, 1685-1693.

Ratnieks, F.L.W., 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. American Naturalist 132, 217-236.

Ratnieks, F.L.W., Foster, K.R., Wenseleers, T., 2006. Conflict resolution in insect societies. Annual Review of Entomology 51, 581-608.

Ratnieks, F.L.W., Wenseleers, T., 2005. Policing insect societies. Science 307, 54-56.

Ratnieks, F.L.W., Wenseleers, T., 2008. Altruism in insect societies and beyond: voluntary or enforced? Trends in Ecology and Evolution 23, 45-52.

Roth, K.M., Beekman, M., Allsopp, M.H., Goudie, F., Wossler, T.C., Oldroyd, B.P., 2014. Cheating workers with large activated ovaries avoid risky foraging. Behavioral Ecology 25, 668-674.

Sladen, F.W.L., 1912. The Humble-Bee. Its Life-History and How to Domesticate It. Macmillan and Co., Limited, London.

Smeets, P., Duchateau, M.J., 2003. Longevity of *Bombus terrestris* workers (Hymenoptera: *Apidae*) in relation to pollen availability, in the absence of foraging. Apidologie 34, 333-337.

Stroeymeyt, N., Brunner, E., Heinze, J., 2007. "Selfish worker policing" controls reproduction in a *Temnothorax* ant. Behavioral Ecology and Sociobiology 61, 1449-1457.

Trivers, R.L., Hare, H., 1976. Haplodiploidy and the evolution of the social insects. Science 191, 249-263.

Tsuji, K., Kikuta, N., Kikuchi, T., 2012. Determination of the cost of worker reproduction via diminished life span in the ant *Diacamma* sp. Evolution 66, 1322-1331.

Van der Blom, J., 1986. Reproductive dominance within colonies of *Bombus terrestris* (L.). Behaviour 97, 37-49.

Van Doorn, A., Heringa, J., 1986. The ontogeny of a dominance hierarchy in colonies of the bumblebee *Bombus terrestris* (Hymenoptera, *Apidae*). Insectes Sociaux 33, 3-25.

Wade, M.J., Breden, F., 1980. The evolution of cheating and selfish behavior. Behavioral Ecology and Sociobiology 7, 167-172.

Wenseleers, T., Badcock, N.S., Erven, K., Tofilski, A., Nascimento, F.S., Hart, A.G., Burke, T.A., Archer, M.E., Ratnieks, F.L.W., 2005a. A test of worker policing theory in an advanced eusocial wasp, Vespula rufa. Evolution 59, 1306-1314.

Wenseleers, T., Helanterä, H., Hart, A., Ratnieks, F.L.W., 2004. Worker reproduction and policing in insect societies: an ESS analysis. Journal of Evolutionary Biology 17, 1035-1047.

Wenseleers, T., Princen, S., Oliveria, R., Oi, C.A., 2021. Conflicts of Interest Within Colonies. Encyclopedia of Social Insects 28, 279-293.

Wenseleers, T., Ratnieks, F.L.W., 2006a. Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. American Naturalist 168, E163-E179.

Wenseleers, T., Ratnieks, F.L.W., 2006b. Enforced altruism in insect societies. Nature 444, 50.

Wenseleers, T., Tofilski, A., Ratnieks, F.L.W., 2005b. Queen and worker policing in the tree wasp *Dolichovespula sylvestris*. Behavioral Ecology and Sociobiology 58, 80-86.

Wilson, E.O., 1971. The Insect Societies. Belknap Press, Cambridge, MA.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Almond, E.J., Huggins, T.J., Jordan, W.C., Bourke, A.F.G., 2012. Reproductive conflict in bumblebees and the evolution of worker policing. Evolution 66, 3765-3777.

Zhao, H., Liu, Y., Zhang, H., Breeze, T.D., An, J., 2021. Worker-Born Males Are Smaller but Have Similar Reproduction Ability to Queen-Born Males in Bumblebees Insects 12.

3 Chapter

Testing the relationship between dominance behaviour and reproductive success with *Bombus terrestris*

Photo taken by Jenny Livesey

3.1 Abstract

In many animal societies, aggressive interactions, and the subsequent dominance hierarchies formed, help regulate the reproductive division of labour. These hierarchies are often thought to be linear, or approximately linear, with the most dominant individual monopolising reproduction, while those below them in rank are increasingly less likely to achieve reproductive success. Yet, while aggression has been shown to play a role in determining an individual's social status in a group, whether or not the level of aggression and rank that an individual displays, in comparison to others in the group, positively correlate with their overall reproductive success (direct fitness) has yet to be confirmed as the general rule across species. For example, in colonies of some eusocial Hymenoptera, including those of bumblebees (*Bombus* spp.), it has been suggested that dominance and reproductive success in male-producing reproductive nestmate workers are not completely aligned. By quantifying rates of aggression, egg-laying, egg-eating (policing) and subsequent egg survivorship in queenless groups of 10 full-sister *B. terrestris* workers, this study first aimed to determine the association between dominance rank and reproductive success at the individual worker level. It then aimed to evaluate the strength of the linear dominance hierarchy in the same queenless groups by the experimental removal of either the alpha worker or a control (subordinate) worker. The results showed that alpha workers within groups had significantly higher rates of aggression, egg-eating and egg survivorship than beta and subordinate workers. Although alpha workers' rates of egg-laying did not differ significantly from those of betas, alphas had the greatest rates of production of unpoliced (surviving) eggs. In alpha-worker removal groups, the pre-treatment beta was disproportionately likely to achieve alpha rank, in contrast to the results in control-worker removal groups in which the pre-treatment alpha was disproportionately likely to retain alpha rank. These results demonstrate that alpha workers achieve the greater share of reproduction but that this occurs not through increased egg-laying rates but through increased egg-policing of other workers' eggs. Therefore, policing of eggs by dominant workers plays a pivotal role in increasing their reproductive success. In addition, the removal results demonstrate that dominance is effective in suppressing the reproductive success of workers subordinate to the alpha. Consequently, the findings strongly support the hypothesis that dominance is positively correlated with reproductive success in *B. terrestris* workers and add to the evidence that, in animal societies in general, increasing direct fitness is the ultimate function of dominance behaviour.

3.2 Introduction

Many animal societies are reported as being structured by a dominance hierarchy whereby individuals within a group are linearly ranked based on differences in their competitive ability (Chase, 1974; Wilson, 1975b; Strauss et al., 2022). In these societies, the victor of the greatest number of dyadic aggressive interactions is considered to be the dominant group member and is placed at the top of the hierarchy (i.e., as the group alpha), while the second highest victor takes the second position (group beta) and so on, until all group members are ranked. High ranking individuals are thought to use physical aggression to gain prime access to group resources (food, nest sites, mates) and to inhibit reproduction in subordinate group members. As a result, the group alpha is expected to monopolise reproduction within a group (Wilson, 1975b; Ellis, 1995). Yet, whether or not an individual's dominance rank positively correlates with its overall reproductive success (direct fitness, i.e., personal offspring production) has yet to be confirmed as the general rule across species. This suggests that the ultimate function of dominance behaviour and dominance hierarchies has yet to be fully understood.

Research has shown that dominance hierarchies help to promote cooperation and group productivity (Wilson, 1975a; Bourke, 2011). However, in multiple animal societies, it is unclear if an individual's dominance rank (i.e., the level and directionality of aggression it displays relative to other group members) has a direct impact on its reproductive success. For example, in social groups of yellow baboons (*Papio cynocephalus*), high-ranking females have more offspring and shorter inter-birth intervals as a result of priority of access to resources (Wasser et al., 2004). Yet in social groups of another primate, the blue monkey (*Cercopithecus mitis),* rank does not affect the probability of female conception (Roberts and Cords, 2013). Empirical data that both support and fail to support a strong positive relationship between dominance and reproductive success can be found not only in social groups of mammals, but also in social groups of birds, fish and insects (reviewed in Ellis, 1995; Stockley and Bro-jørgensen, 2011; Amsalem et al., 2015; Lewis, 2022). Hence, the existence of variation, across multiple taxa, in how an individual's dominance rank correlates with reproductive success raises questions over whether dominance ranks (based on aggression) can accurately explain the structure and dynamics of social groups, or be reliably used to predict an individual's reproductive success (Amsalem et al., 2015; Lewis, 2022).

In eusocial Hymenoptera (ants, wasps and bees) the kin and social structure of colonies, and stage in the colony cycle, are factors that affect (1) the degree of reproductive conflict and (2) the role dominance hierarchies play in determining a group member's reproductive success (Bourke and Franks, 1995; Beekman and Ratnieks, 2003; Shimoji and Dobata, 2022). For example, in 'advanced' monogynous polyandrous eusocial societies (i.e., with a single, multiply-mated queen per colony), such as those of the honeybee (*Apis melifera*), workers are more closely related to queen-derived males than nestmate worker derived-males. This relatedness asymmetry, combined with the threat of worker policing, promotes reproductive self-restraint among workers, ensuring reproductive conflict is minimal (Ratnieks et al., 2006; Wenseleers and Ratnieks, 2006; Bourke, 2011). However, in eusocial societies in which indirect fitness benefits are relatively low, reproductive conflict is common. In such colonies, cooperation and colony stability may rely partly on a subordinate's future opportunities for direct fitness (Leadbeater et al., 2011) and be characterised by dominance hierarchies among workers (Monnin et al., 2003; Molet et al., 2005).

In general, reproductive conflict is highest in annual species, where reproductive opportunities are limited to a single season (Bourke and Ratnieks, 2001; Ratnieks et al., 2006). However, even in these species, dominance hierarchies may affect an individual's reproductive success only at certain stages of the colony cycle. For example, many eusocial bumblebee species (*Bombus* spp.) exhibit an annual cycle in which colonies, each headed by one, monandrous queen, rear one generation of reproductives per year (Alford, 1975; Schmid-Hempel and Schmid-Hempel, 2000; Owen and Whidden, 2013). The colony queen is the only group member capable of mating and laying fertilised eggs, and these develop into workers or gynes (new queens). This is because, although workers possess ovaries, they cannot mate, and so, through haplodiploidy, can lay only unfertilised eggs developing into males (Bourke, 1988b). In such colonies, haplodiploidy also means that workers are more closely related to their sisters (workers and gynes) ($r = 0.75$) than their own sons ($r = 0.5$) (Hamilton, 1964). This relatedness asymmetry means that workers receive higher fitness benefits from rearing the queen's reproductive daughters (gynes) than they would from rearing their own sons. Therefore, in colonies with a queen (queenright), and while the queen is laying fertilised eggs, although workers may have active ovaries, they refrain from egg-laying despite a lack of aggressive interactions with the queen (Duchateau and Velthuis, 1989; Alaux et al., 2007). This is considered to occur through self-restraint, i.e., because workers maximise their inclusive fitness through focusing on aiding the queen during the colony's growth phase (Bourke and Ratnieks, 2001; Almond et al., 2019).

Aggression and the resulting dominance hierarchies are expected to play more of a key role at the stage in the colony cycle when gyne production has been initiated and queens are mainly laying unfertilised eggs that will develop into males, an event termed, in bumblebees, the *switch point* (Duchateau and Velthuis, 1988). At this stage in the colony cycle it is in the fitness interests of workers to compete aggressively with the queen and other workers over male parentage because of their higher relatedness to their sons ($r = 0.5$) over nephews ($r = 0.375$) or the queen's sons ($r = 0.25$). Following the *competition point* (first appearance of worker-laid eggs), colonies in *B. terrestris* are indeed characterised by aggressive interactions, worker egg-laying, and egg policing (egg-eating) by both the queen and workers (Duchateau, 1989; Bourke and Ratnieks, 2001; Zanette et al., 2012). Workers are observed forming dominance hierarchies among themselves (Van Doorn and Heringa, 1986; Duchateau, 1989) and, though the queen may still be the highest-ranking individual directly following the competition point (exhibiting the most aggression and the highest reproductive output), it is not uncommon for workers to aggress the queen (Almond et al., 2019) and, in certain instances, kill her (Bourke, 1994). This is interpreted as being to successfully rear worker-derived males, most of which are produced by queenless colonies (Lopez-Vaamonde et al., 2009).

Yet, while it is clear dominance hierarchies, based on aggression, exist among workers in *Bombus* colonies following the competition point, as in multiple other taxa it is currently still unclear if there is a strong positive relationship between workers' dominance ranks and their reproductive output. So far, research has shown that, in two bumblebee species (*B. impatiens* and *B. terrestris*), workers with active ovaries are significantly more likely to aggress the queen (Almond et al., 2019) or other workers (Van Doorn and Heringa, 1986; Bloch and Hefetz, 1999; Amsalem and Hefetz, 2010; 2011; Amsalem et al., 2014; Princen et al., 2020), than workers with inactive ovaries. Workers with inactive ovaries may even emit a ‘sterility signal’ to signal that they are not reproductively active, in order to render them safe from attack by dominant workers (Amsalem et al., 2013). This suggests there is a positive relationship between ovary activation and aggression.

However, as these studies measured level of reproductivity only as the degree of ovary activation, it is unclear if these dominant workers would have been able to use aggression to monopolise egg-laying in colonies or if another worker would have laid more eggs and, overall, produced more male offspring. For example, previous research has shown that up to 64% of workers may have active ovaries capable of egg-laying within a colony, but only 38% may lay eggs (Alaux et al., 2004). In addition, a study found that ovariectomised workers perform the same aggressive behaviours as workers with intact, activated ovaries (Van Doorn, 1989). This suggests that ovarian activation may not necessarily lead to oviposition and that aggressive behaviours are not limited to ovary-active workers. Indeed, research that has focused on measuring an individual's dominance rank against their egg-laying rate has found no relationship between the levels of aggression a worker exhibited and the number of egg-laying events they performed, both in the presence of the queen (Duchateau, 1989) and in queenless conditions (Sibbald and Plowright, 2013; 2015).

Further to this, it is unclear what role worker policing (by egg eating) plays in determining reproductive success among groups of workers. In queenright colonies, policing involves the colony queen (queen policing) and/or workers (worker policing) reducing the level of successful worker reproduction by eating workers' eggs (oophagy) (Ratnieks, 1988; Zanette et al., 2012). As a result of egg-eating, it is known that the majority of eggs laid by workers are destroyed soon after they have been laid, to the extent that, around 95% of successfully reared colony males are queen-derived (Alaux et al., 2004; Zanette et al., 2012). Hence, measuring ovary activation or the number of egg-laying events alone may not give an accurate indication of a worker's reproductive success, as it fails to consider egg survivorship. In addition, it is not clear if the worker with the highest dominance rank performs the majority of egg-eating events or if egg-eating behaviours are performed regardless of rank. Sibbald and Plowright (2015) noted that in queenless groups of two *B. impatiens* workers, subordinate workers ate 38% of eggs laid by dominant workers, whereas dominant workers did not eat any eggs laid by the subordinates they were paired with. However, as workers in this experiment were kept only in groups of two, it is not clear if this observation was a product of the unnatural social environment whereby the two workers present could interact only with one another rather than with a larger number of workers. For example, in contrast to this result, this thesis (Chapter 2) showed a positive relationship between egg-laying and egg-eating among individual workers in *B. terrestris* colonies of natural size (mean ± SD of 125 ± 27 workers). Overall, in order for the relationship between dominance rank and reproduction to be fully investigated, the interplay of aggression, egg-laying, egg-eating and egg survivorship needs to be considered at the individual and group level.

Therefore, the main aim of this study was to use queenless groups of *B. terrestris* workers to test for a within-group positive association of dominance and reproductive success. In nature, *B. terrestris* colonies are headed by one, singly-mated queen, such that workers eclosing in a colony are all full sisters of one another; in addition, colonies may become queenless through death of the queen (Lopez-Vaamonde et al., 2004; Lopez-Vaamonde et al., 2009; Zanette et al., 2014). To reflect natural queenless conditions, this experiment involved observing 22 queenless groups of 10 individually marked full-sister workers for acts of aggression, egg-laying and egg-eating. First, behavioural observations were carried out over a period of 4 weeks to determine within group dominance hierarchies as well as to observe for egg-laying and egg-eating events. Second, after 4 weeks of observations the highest-ranking worker (alpha) was removed in 11 colonies, before observations continued for another 4 weeks, to investigate the strength of the linear dominance hierarchy and determine if the second highest ranking worker (beta) would transition to the alpha position, as seen in queenless groups of other eusocial Hymenoptera, i.e., the slave-making ant *Harpagoxenus sublaevis* (Bourke, 1988a) and wasp *Mischocyttarus cerberus* (Carvalho da Silva et al., 2020). This study used queenless groups of 10 workers to ensure worker numbers were high enough for group members to interact with multiple individuals, much as they would in natural colonies.

The central hypothesis was that in queenless groups of *B. terrestris* workers there is a positive association at the individual level between rank in the dominance hierarchy, as assessed from the amount and directionality of aggression towards nestmate workers, and direct fitness, as assessed from the number of successful egg-laying events (i.e., the number of eggs laid that are not policed). As a corollary, dominance behaviour was hypothesised to have an inhibitory effect on the aggression level and reproductive success of subordinate workers. This hypothesis made two predictions: (1) within unmanipulated queenless groups of workers, there is a positive association across individual workers between the amount of aggression, the number of egg-eating events and the number of successful egg-laying events; and (2), if the alpha worker is removed, another worker, previously the second-ranked (beta), upregulates its aggression level and egg-laying rate to adopt the rank of alpha.

3.3 Materials and Methods

3.3.1 Formation, rearing and development of *Bombus terrestris* queenless groups

Twelve pre-competition point *Bombus terrestris audax* colonies were obtained from a commercial supplier (Biobest, Westerlos, Belgium). On delivery (20 May 2021), each colony contained 1 queen, a mean (± SD) of 16 ± 3 workers and a small quantity of brood at varying stages of development (e.g., 12-15 clumps of eggs, larvae or pupae). Colonies were transferred into numbered wooden nest-boxes (nos.1-12; internal dimensions 17 cm × 27.5 cm × 16 cm high) with clear, colourless Perspex lids, kept under standard conditions (28°C, 60% relative humidity, constant darkness), and fed *ad libitum* on sugar syrup and fresh pollen (Attracker; Koppert Biological Systems, Haverhill, UK).

Between 22 May 2021 and 1 June 2021, newly-eclosed workers within the 12 pre-competition point colonies were censused and individually marked on the day of eclosion with a unique numbered disc glued to the thorax (Queen Marking Kit, Thorne Ltd., UK). These newly-eclosed workers were used to create 22 queenless groups, each containing 10 workers. To reflect the formation of queenless groups of workers in nature, each experimental queenless group was formed using workers drawn from the same source colony, with each source colony contributing up to three such groups (**Table A3.1**). To ensure all workers within a given group were of a similar age and size, workers were only placed in a group together if (1) they eclosed within 1-4 days of one another and (2) were of a similar size. Noticeably larger and smaller-bodied workers were excluded from all groups.

Workers are thought to use changes in the pheromone output of the queen, associated with her starting to lay queen-destined eggs, to determine when to begin laying haploid eggs (Bourke and Ratnieks, 2001; Alaux et al., 2006). Therefore, in the absence of these queen pheromones (i.e. queenless conditions), *B. terrestris* workers may activate their ovaries and begin egg-laying within 7-8 days (Duchateau and Velthuis, 1989; Alaux et al., 2007). To ensure the oldest workers of a group did not have a chance to activate their ovaries before the youngest workers in a group, all 10 workers forming a given queenless group were removed from the queenright source colony and placed together to create the queenless group on the same day. If 10 suitable workers did not eclose within a source colony on the same day, newly-eclosed marked workers were retained in the source colony (which remained in the pre-competition point queenright phase) until the required 10 workers had eclosed. All workers used to form queenless groups were left for at least 24 hours in their source colony following eclosion before being removed to form queenless groups. This was to ensure that all workers in a group had had at least 24 hours post eclosion to mature prior to queenless groups being formed.

Once 10 suitable workers had been marked and spent at least 24 hours in their colony of origin, they were transferred into a new numbered wooden nest-box (nos. 1-22; internal dimensions 15 cm ×17 cm × 16 cm high) with a clear, colourless Perspex lid. To stimulate oviposition and facilitate nest building (Klinger et al., 2019) on the day of transfer each queenless group was provided with a circular 8-gram patty of pollen dough, consisting of a mixture of pollen and syrup (diameter 22 mm, height 11 mm). Queenless groups were kept under standard conditions (28°C, 60% relative humidity, constant darkness), and thereafter fed *ad libitum* on sugar syrup and given fresh pollen daily (Biobest, Westerlos, Belgium). Because the rate of worker eclosion in source colonies varied, the formation of queenless groups was staggered over seven days between 26 May 2021 and 2 June 2021. The variation in the rate at which workers eclosed in source colonies also meant that source colonies with higher rates of worker eclosion were used to form more queenless groups than other source colonies (**Table A3.1**). Overall, the number of queenless groups that originated from each source colony ranged from 0-3, with a mean of 1.8 queenless groups produced per source colony.

Between 26 May 2021 and 30 July 2021 queenless groups were monitored to (1) check for and count any dead workers and (2) check for, count and remove any newly-eclosed adult males, i.e. males produced by the workers in the queenless group. If either dead workers or newly-eclosed males were found within a queenless group, they were removed on the day of discovery and immediately frozen at -20°C. As adding new workers to the group could have affected any existing dominance hierarchy, workers that died prior to the end of the experiment were not replaced. Therefore, some queenless groups contained fewer live workers at the end of the experiment than others (**Table A3.1**).

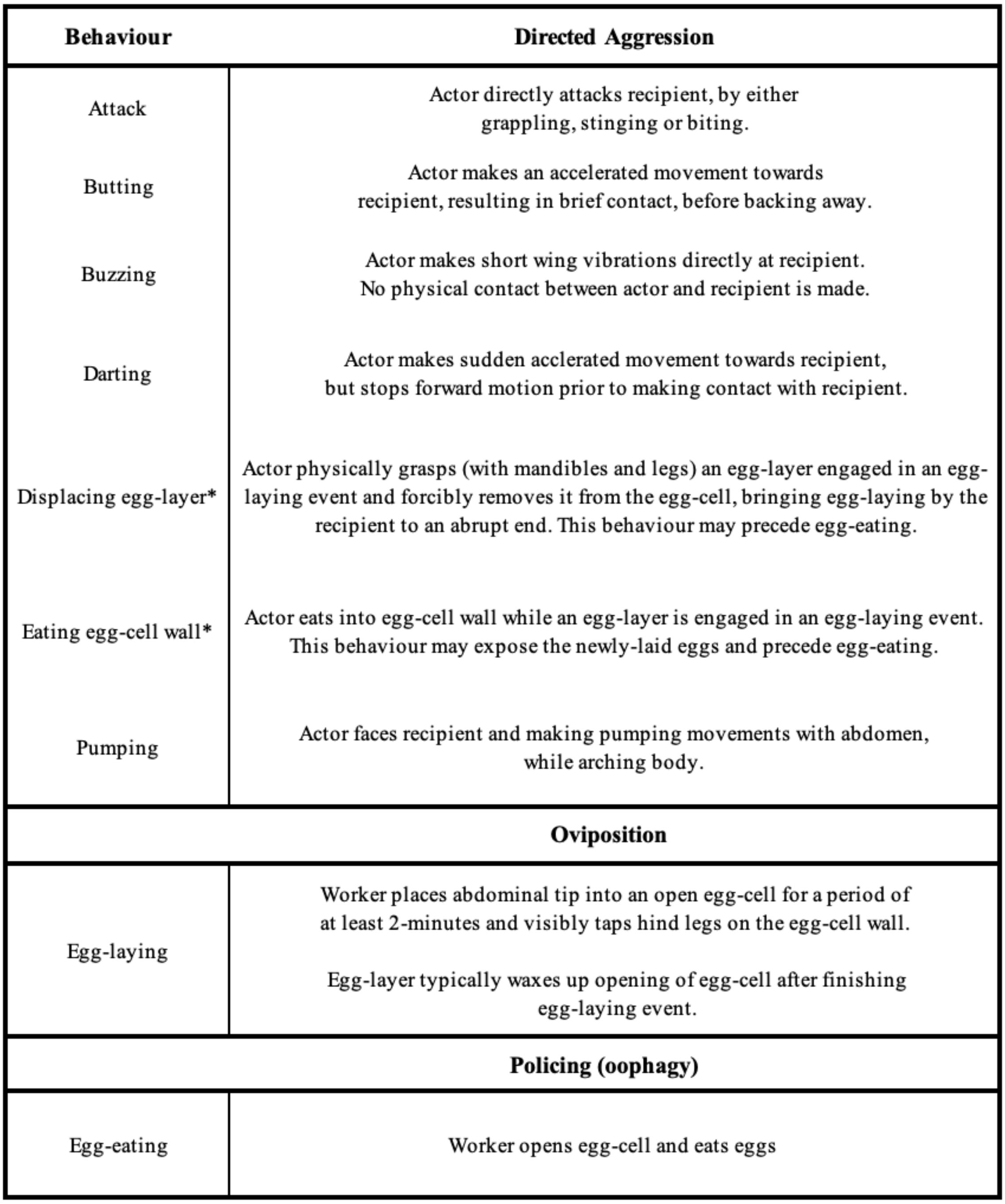
3.3.2 Pre-treatment phase: behavioural observations

The pre-treatment phase of the experiment lasted from 27 May 2021 to 2 July 2022. The queenless groups were observed directly in 1-hour bouts three times a week (12 h per group over a period of 25-29 days) and filmed digitally in 2-hour bouts two times a week (16 h per group over a period of 25-29 days).

In the direct observation bouts, all egg-laying and egg-eating behaviours were recorded, along with the identity of the egg-layer or egg-eater (number on coloured disc) (**Table 3.1**; **Figure 2.2**). If an egg-laying event by a worker in a particular egg-cell was followed by an egg-eating event (without any other event intervening), then the identity of the worker that had laid the eggs eaten was also recorded. In some cases, workers were observed laying into an egg-cell that (1) already visibly contained eggs or (2) could not be determined to be empty at the start of the observed egg-laying event. In these cases, if egg-eating from the cell then occurred, eaten eggs could have included eggs of both known and unknown parentage. Therefore, where required, later analyses distinguished between eggs of known and unknown origin (parentage). For all workers within a queenless group, rates of both egg-laying and egg-eating were quantified as the rate of events observed per individual per hour, where 'event' referred to either an individual being observed to lay eggs into an egg-cell (egg-laying) or to an individual being observed to eat eggs from an egg-cell (egg-eating). During egg-laying events workers typically lay multiple eggs into an egg-cell (**Figure 3.1**; Almond *et al.* 2019). Due to the small size of queenless groups, it was often feasible to count the number of eggs laid during an egg-laying event and the number of eggs eaten during an egg-eating event. However, in some instances the actual numbers of eggs laid or eaten could not be accurately quantified because the depth of the egg-cell and the position of the worker's body prevented eggs from being counting accurately. Therefore, where required, later analyses distinguished between egg-laying and egg-eating events in which the number of eggs were counted accurately and events in which the number of eggs were not counted.

In addition to egg-laying and egg-eating events, occurrences of any directed aggression were recorded during direct observations, along with the identities of the actors and the recipients of each behaviour and the duration of the aggressive act (**Table 3.1**).

For digital film bouts, each colony was filmed using Sony HDR CX240E digital camcorders mounted on tripods so as to film the nest comb from above through the clear Perspex lid of the nest-box. To ensure that the unique numbered disc identifying each individual worker was clearly visible throughout digital recordings, lamps were used to illuminate queenless groups during each recording. As previous preliminary observation had shown that the behaviour of colonies did not differ under red or dim white light, all filming was conducted under 3W LED white lights. In each digital film bout, it was possible to ensure that the entire queenless group was within the camera's field of view and hence that the activity of all workers could be recorded at any one time.



Behaviour	**Directed Aggression**
Attack	Actor directly attacks recipient, by either grappling, stinging or biting.
Butting	Actor makes an accelerated movement towards 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 acclerated movement towards recipient, but stops forward motion prior to making contact with recipient.
Displacing egg-layer*	Actor physically grasps (with mandibles and legs) an egg-layer engaged in an egg-laying event and forcibly removes it from the egg-cell, bringing egg-laying by the recipient to an abrupt end. This behaviour may precede egg-eating.
Eating egg-cell wall*	Actor eats into egg-cell wall while an egg-layer is engaged in an egg-laying event. This behaviour may expose the newly-laid eggs and precede egg-eating.
Pumping	Actor faces recipient and making pumping movements with abdomen, while arching body.
	Oviposition
Egg-laying	Worker places abdominal tip into an open egg-cell for a period of at least 2-minutes and visibly taps hind legs on the egg-cell wall. Egg-layer typically waxes up opening of egg-cell after finishing egg-laying event.
	Policing (oophagy)
Egg-eating	Worker opens egg-cell and eats eggs

Table 3.1. Behaviours of interest performed by workers and directed towards other workers. These behaviours were recorded for all workers within a queenless group during the pre- and post-treatment phases of the experiment. Behaviours marked with * were first classified by myself in Chapter 2 of this thesis. All other behaviours were as described in Duchateau (1989) or Bloch and Hefetz (1999).

3.3.3 Treatments: removal of alpha-worker or control-worker

Once a queenless group had been directly observed and digitally filmed for four weeks, the pre-treatment phase ended. First, workers within a group were assigned a rank in a dominance hierarchy based on the level of aggression exhibited by each worker in the pre-treatment phase, as quantified from acts of directed aggression performed by each worker in each queenless group during direct observations. In all queenless groups, one individual worker performed more directed aggression than any other group member, and so was ranked as the group alpha. The worker that performed the second highest number of acts of directed aggression within each queenless group was assigned the rank of beta. All remaining workers within a queenless group exhibited little or no aggression and so were grouped as subordinates (**Table 3.2**). In queenless group 10, the group alpha was responsible for all acts of directed aggression observed, so the rank of beta could not be assigned to another worker within the queenless group. This meant that in this queenless group there were nine workers ranked as subordinates and no worker ranked as a beta. In all other queenless groups (21/22) each group contained one worker ranked as an alpha, one worker ranked as a beta and eight workers ranked as subordinates (**Table 3.2**).

Queenless groups were allocated equally to one of the two following treatments: (1) alpha-worker removal treatment, in which the worker assigned the rank of alpha was removed from the group; and (2) control-worker removal treatment, in which a worker assigned the rank of subordinate was removed from the group. In all queenless groups the subordinate worker chosen to be removed was a subordinate worker that had not been observed performing egg-laying, egg-eating or directed aggressive behaviours in any of the direct observation bouts of the pre-treatment phase. The subordinate worker was removed as a control for any potential effect on the social structure of the queenless group (in the alpha-worker removal treatment) of reducing group size by one worker. In queenless groups 5 and 22, the alpha died 5-7 days before the planned date of removal. No removals were made from these two queenless groups, and instead they were both allocated to the 'alpha-worker removal treatment', with the date of the alpha's death being used as the start date of the post-treatment phase for the two groups.

Prior to removal, to the extent possible, queenless groups were paired with another queenless group from the same source colony. One of each pair was then randomly assigned to the alpha-worker removal treatment and the other the control-worker removal treatment. Queenless groups were paired based on their source colony to ensure that each treatment had similar numbers of queenless groups from each source colony. This was to account for behavioural and genetic variability across source colonies that may have affected queenless group behaviour. It was possible to pair 18/22 queenless groups with another queenless from the same source colony. For the remaining four queenless groups (groups 8, 11, 14 and 15), each queenless group was paired based on overall levels of aggression observed during direct observations. The two queenless groups with the highest levels of aggression (groups 8 and 15) formed one pair and the two queenless groups with the lowest levels of aggression (groups 11, 14) formed the second pair. This was to ensure each treatment had queenless groups of varying levels of aggressive behaviour.

At the end of the post-treatment phase, the number of aggressive acts performed by each worker during this phase were again counted to re-evaluate each individual's dominance rank. In this phase, it was not possible to assign the rank of beta in nine queenless groups as only one individual worker performed directed acts of aggression (**Table A3.7**). In one queenless group (group 11) no acts of aggression were observed being performed during direct or digital observations and so it was not possible to assign a dominance rank to any worker during the post-treatment phase (**Table A3.7**).

3.3.4 Post-treatment phase: behavioural observations

The post-treatment phase of the experiment lasted from 22 June 2021 to 30 July 2021, with the start of the post-treatment phase (removal of alpha-worker or control-worker) being staggered across the queenless groups to account for differences in the dates at which the groups had been established (**Table A3.1**). Both direct observations and digital filming of the queenless groups were then conducted following methods identical to those used in the pre-treatment phase described in the previous section. During the post-treatment phase, queenless groups were each directly observed for a period of 12 h over 28-34 days. Colonies were also filmed digitally for 16 h over 28-34 days. Once a queenless group had been observed for four weeks, i.e. for approximately as long as the pre-treatment phase, observation ended and the queenless group was retained and frozen at -20°C. Dates at which observations for a queenless group ended and queenless groups were terminated were staggered between 26 July 2021 and 30 July 2021.

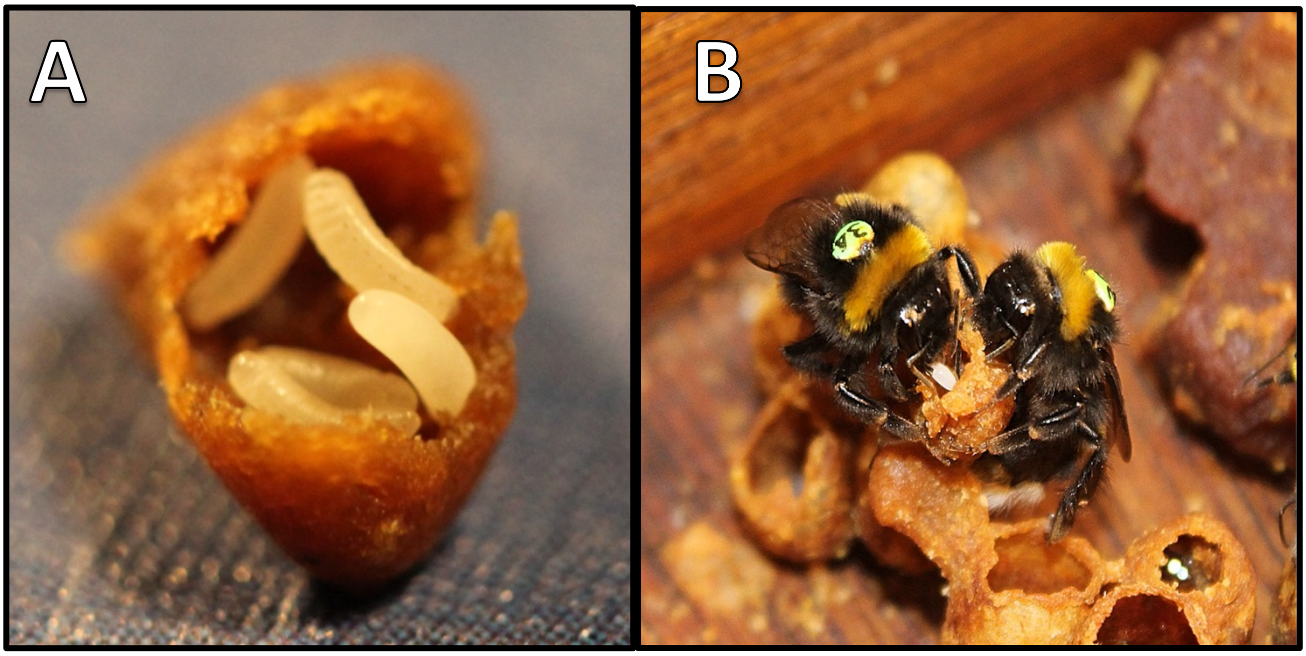


Figure 3.1. Occurrence of worker reproduction and associated egg-eating behaviour in *Bombus terrestris audax* queenless groups. (**A**) Worker-laid eggs (bottom right) and 1st instar larvae (top right) within an egg-cell. Eggs and 1st instar larvae are approximately 4 mm in length. (**B**) Worker (#34 green) eating worker-laid eggs from egg-cell as another worker watches. Photos taken by Jenny Livesey with a Canon DSLR camera and EF-S 18-55 mm lens.

3.3.5 Data extraction from digital films

Following the end of the experiment, data were extracted from the digital films. The data types extracted from each digital film bout viewed included all behaviours recorded in direct observations. This included all egg-laying and egg-eating behaviours, along with the identity of the egg-layer or egg-eater (number on coloured disc). Again, the occurrences of any directed aggressive behaviours were recorded, along with the identities of the actors and the recipients of each behaviour (**Table 3.1**).

For queenless groups 2 and 3, all digital film bouts available for both the pre-treatment and post-treatment phase were viewed. For each of these two queenless groups, this amounted to eight digital film bouts (16 h of digital film) in the pre-treatment phase and eight digital film bouts (16 h of digital film) in the post-treatment phase. From these observations it became clear that the majority of aggressive interactions occurred during the first two weeks of each treatment phase, beyond which aggressive interactions occurred at a lower intensity. (This is in line with findings from previous research that showed workers reduce aggression when a stable dominance hierarchy is in place (Hogeweg and Hesper, 1983; Bloch et al., 1996).) Therefore, for each of the remaining 20 queenless groups (1, 4-22), only digital film bouts recorded in the first two weeks of the pre-treatment phase (4 digital film bouts and 8 h of digital film) and first two weeks of the post-treatment phase (4 digital film bouts and 8 h of digital film) were viewed for data extraction.

All digital film bouts were viewed for data extraction blind to the results produced by direct observations. Therefore, no individual workers were targeted for data collection and instead the behaviour of all workers within a queenless group was recorded. Data for egg-laying and egg-eating events and aggressive interactions recorded from digital film bouts were combined with the data for these events collected from direct observations.

To take into account variation in hours of observation for each queenless group, count data for each type of behaviour (egg-laying, egg-eating, aggression) were used to calculate rates of behaviours per individual per hour. To do this, the number of hours of direct and digital observations for each queenless group was summed for each of the pre-treatment and post-treatment phases.

3.3.6 Data analyses

For each worker in each queenless group, all directed aggressive behaviours performed during direct and digital film observations were pooled within each of the pre- and post- treatment phase (**Table 3.1**). This was done so that an overall level of aggression could be determined in each phase for each worker within each queenless group. Pooled directed aggressive behaviours were classed at 'overall acts of aggression'. Therefore, each worker in each queenless group had overall counts for number of egg-laying events, egg-eating events and overall acts of aggression performed in (1) the pre-treatment phase and (2) the post-treatment phase.

In queenless groups 5 and 22, the premature death of the alpha meant that these two groups had a lower number of direct observations in the pre-treatment phase than all other queenless groups. In the pre-treatment phase, queenless group 5 was directly observed for 9.0 hours and queenless group 22 was directly observed for 8.0 hours. Likewise, as the post-treatment phase started early in these two queenless groups, in this phase queenless group 5 was directly observed for 14.0 hours and queenless group 22 was directly observed for 15.0 hours. In order to determine how often workers of different dominance ranks performed agonistic behaviours directed at workers engaged in an egg-laying event, the number of directed agonistic acts of (1) 'displacing egg-layer' and (2) 'eating egg-cell wall' (**Table 3.1**) performed by each worker in each queenless group were also counted separately in the pre- and post-treatment phase. Again, counts of these two behaviours were pooled in each of the pre- and post-treatment phases, so that an overall level of aggression directed at workers engaged in egg-laying events could be determined in each phase for each worker within each queenless group. These two pooled behaviours were classed as 'acts of aggression directed at workers engaged in an egg-laying event'. Therefore, 'displacing egg-layer' and 'eating egg-cell wall' behaviours for each worker in each queenless group were included in two separate counts: (1) a count of 'overall acts of aggression' performed and (2) a count of 'acts of aggression directed at workers engaged in an egg-laying events'.

Some queenless groups had to be excluded from analyses that concerned the response of pre-treatment beta to post-treatment conditions and their rates of overall acts of aggression (Section: 3.4.5). In two queenless groups (8 and 13), the worker ranked as the pre-treatment group-beta died early in the post-treatment phase. In queenless group 10, all acts of aggression observed in the pre-treatment phase were performed by the worker ranked as the group alpha and so no worker was ranked as a beta in this queenless group. While either the alpha or control-worker were removed from these queenless groups (**Table A3.1**), these three groups were excluded from the analysis concerning changes in the mean rate at which workers ranked as the pre-treatment group beta performed overall acts of aggression, as, in these groups, a pre- and post-treatment comparison could not be assessed.

3.3.7 Statistical Analysis

All statistical analyses were performed using R v 4.2.1 (R Core Team 2022).

As the behavioural rate data consisted of zero-inflated continuous data, the Kruskal-Wallis rank sum test in the *native stats* package (R Core Team, 2022) was used to determine if the rates of behaviours (overall acts of aggression, acts of aggression directed at a worker engaged in an egg-laying event, egg-eating events and egg-laying events) differed between workers of different rank (Alpha, Beta or Subordinate). To identify which workers of different dominance rank (Alpha, Beta or Subordinate) varied in their rates of behaviours, multiple pairwise comparisons were obtained using Dunn’s test in the *FSA* package (Ogle et al. 2022) and a Benjamin-Hochberg (BH) correction was applied to adjust the threshold of significance to correct for multiple comparisons. These statistical methods were used to compare workers of different dominance rank in both the pre- and post-treatment phase.

A generalised linear mixed effects model (GLMM) with a Poisson distribution was constructed using the package *lme4* (Bates et al. 2002) to determine if there was a significant relationship between a worker's dominance rank (i.e., alpha, beta or subordinate) within a queenless group and the number of egg-laying events a worker performed. To do this, the total number of egg-laying events performed by individual workers in each queenless group during the pre-treatment phase was calculated as count data. Count of egg-laying events was used as a fixed effect, with a worker's dominance rank and queenless group used as covariates. An ANOVA was performed before pairwise comparisons were obtained using the *contrast* function in the *emmeans* package (Russel et al 2023). A Tukey correction was applied to pairwise comparisons to adjust the threshold of significance to correct for multiple comparisons. Scaled residuals and overall model fit were assessed using the *DHARMa* package (Hartig 2022).

To determine if survivorship of eggs differed depending on whether they originated from a worker ranked as an alpha, beta or subordinate, a survivorship analysis was performed and visualised with Kaplan-Meir plots using the *survival* package (Therneau, 2022). For this analysis, only eggs of known origin recorded in both direct and digital observations of the pre-treatment phase were used. In addition, any egg-laying/egg-eating events for which it was not possible to count the number of eggs laid or eaten were excluded from analysis. Data on the survivorship of eggs laid by workers ranked at betas were pooled with data on the survivorship of eggs laid by workers ranked as subordinates. This was for two reasons: (1) the survivorship of eggs laid by betas and eggs laid by subordinates did not differ significantly (log rank test: $\chi^2 = 0$, df = 1, $p = 0.9$); and (2) the longest period over which beta-laid eggs were observed during direct and digital observations was 49.5 minutes, whereas in comparison it was possible to observe alpha-laid eggs for 120.0 minutes and subordinate-laid eggs for up to 107.8 minutes. Therefore, beta-laid eggs were pooled with subordinate-laid eggs, so that the survivorship of eggs laid by alpha workers and beta/subordinate workers could be visualised with a Kaplan Meir plot. For all eggs laid, either a time to event recording (the length of time between an egg being laid and eaten) or a censored recording (the length of time between an egg being laid and end of an observation) was taken. In all censored recordings, the survivorship of eggs beyond the point of the observation was unknown. As no eggs laid by workers ranked as alphas were observed being eaten, it was not possible to compare the survivorship of eggs laid by workers of different dominance rank by means of a Cox proportional hazards model. Instead, the survivorship of eggs laid by workers of different dominance rank was analysed using log rank tests in the *survival* package (Therneau, 2022).

The rates at which egg-laying workers of different dominance rank laid eggs that either were not policed or were only partially policed (i.e., only some of eggs laid were eaten) were determined. These egg-laying events were defined as 'unpoliced egg-laying events'. During the pre-treatment phase and post-treatment phase, for each individual worker that performed egg-laying events, the count of egg-laying events performed was adjusted to reflect the number of eggs that were destroyed and the number of eggs that survived. This was done by allocating values of 0, 0.5 and 1.0, respectively, to each egg-laying event in which no eggs laid in the egg-laying event survived to the end of an observation, some of the eggs laid were still present at an end of an observation, or all eggs laid survived to the end of an observation. For each worker in each queenless group the count of unpoliced egg-laying events was converted into a rate of unpoliced egg-laying events per individual per hour. The rates of unpoliced egg-laying events for workers of different dominance rank were then calculated. Rates of unpoliced egg-laying events were further analysed using the methods identical to those used for all other rates of behaviour (see above).

A Spearman rank correlation was conducted using the *stats* package (R Core Team 2020) to assess the relationship between the rates at which workers performed unpoliced egg-laying events and acts of aggression. A Spearman rank correlation was used as the data were not normally distributed (Shapiro-Wilk: rate of aggression: W = 0.37, $p < 0.001$; rate of unpoliced egg-laying events: W = 0.46, $p < 0.001$).

Lastly, paired-sample Wilcoxon tests were used to test the differences in rates of overall acts of aggression and unpoliced egg-laying events performed in the pre- and post-treatment phases by workers ranked as pre-treatment betas.

3.4 Results

3.4.1 Pre-treatment phase: rates of acts of aggression (directed and received) by workers of different dominance rank

In the pre-treatment phase of the experiment, data were collected from a total of 448 h of observation ($n_{\textit{direct observations}} = 258$ h, $n_{\textit{digital observations}} = 190$ h) and a total of 1310 acts of aggression were observed across the 22 queenless groups ($n_{\textit{aggressive acts performed by alphas}} = 1111$, $n_{\textit{aggressive acts performed by betas}} = 115$, $n_{\textit{aggressive acts performed by subordinates}} = 84$; **Figure 3.3, Table 3.2**). The mean rate at which alphas, betas and subordinates performed overall acts of aggression towards group members differed significantly (Kruskal-Wallis test: $\chi^2 = 125.38$, df = 2, $p < 0.001$; **Table 3.2)**. Pairwise comparisons showed alphas performed overall acts of aggression towards group members at a significantly higher mean rate than betas or subordinates (Dunn's test: alpha vs beta: Z = 3.62, $p < 0.001$; alpha vs subordinate: Z = 7.23, $p < 0.001$; **Figure 3.2A**). Betas also performed overall acts of aggression towards group members at a significantly higher mean rate than workers ranked as subordinates (Dunn's test: beta vs subordinate: Z = 3.52, $p < 0.001$; **Figure 3.2A**). Overall, alphas aggressed group members at a mean rate (± SE) (2.44 ± 0.36 acts per hour) that was 8.7 times higher than that of betas (0.28 ± 0.08 acts per hour) and 122 times higher than that of subordinates (0.02 ± 0.01 acts per hour) (**Figure 3.2, Table 3.2**). Therefore, the worker designated as the group 'alpha' within each queenless group was the most aggressive group member by a large factor.

Of the 1310 acts of aggression observed across the 22 queenless groups, a subset of 78 acts of aggression were directed at workers engaged in egg-laying events ($n_{\textit{aggressive acts performed by alphas and directed at workers engaged in an egg-laying event}} = 72$, $n_{\textit{aggressive acts performed by betas and directed at workers engaged in an egg-laying event}} = 6$, $n_{\textit{aggressive acts performed by subordinates and directed at workers engaged in an egg-laying event}} = 0$; **Figure 3.2, Table A3.2**). These behaviours consisted of (1) 'displacing egg-layer' and (2) 'eating egg-cell wall' (**Table 3.1**). The mean rate at which alphas, betas and subordinates performed aggressive acts directed at group members engaged in egg-laying events differed significantly (Kruskal-Wallis test: $\chi^2 = 164.51$, df = 2, $p < 0.001$; **Figure 3.2B, Table A3.2**). Pairwise comparisons showed alphas performed acts of aggression directed at group members engaged in egg-laying events at a significantly higher rate than either betas or subordinates (Dunn's test: alpha vs beta: Z = 3.78, $p < 0.001$; alpha vs subordinate: Z = 5.00, $p < 0.001$; **Figure 3.2B**, **Table A3.2**). In contrast, the rate at which betas and subordinates performed acts of aggression directed at group members engaged in egg-laying events did not differ significantly (Dunn's test: beta vs subordinate: Z = 1.17, $p < 0.24$; **Figure 3.2B**, **Table A3.2**). Overall, alphas aggressed group members engaged in egg-laying events at a mean rate (± SE) (0.16 ± 0.04 acts per hour) that was 16 times higher than that of betas (0.01 ± 0.01 acts per hour). Subordinates performed such acts at a mean rate of 0.00 ± 0.00 acts per hour (**Table A3.2**). Overall, during the pre-treatment phase alphas, betas and subordinates were responsible for 92.3%, 7.7% and 0.0% of aggressive acts directed towards group members engaged in egg-laying events, respectively. This result suggests acts of aggression towards workers engaged in egg-laying events are predominately performed by alphas in queenless groups.

Within queenless groups, betas were the main recipient of (1) overall acts of aggression and (2) the subset of aggressive acts directed at workers engaged in egg-laying events ($n_{\textit{overall acts of aggression received by alphas}} = 49$, $n_{\textit{acts of aggression received by alphas while engaged in egg-laying event}} = 1$, $n_{\textit{overall acts of aggression received by betas}} = 210$, $n_{\textit{acts of aggression received by betas while engaged in egg-laying event}} = 15$, $n_{\textit{overall acts of aggression received by subordinates}} = 1051$, $n_{\textit{acts of aggression received by subordinates while engaged in egg-laying event}} = 62$; **Figure 3.2, Tables A3.2, A3.3**). Workers ranked as group betas received overall acts of aggression at a mean (± SE) rate (0.50 ± 0.10 acts received per hour) that was 4.5 times higher than that of alphas (0.11 ± 0.03 acts received per hour) and 1.7 times higher than that of subordinates (0.29 ± 0.04 acts received per hour) (**Figure 3.2B, Table A3.2**). Likewise, betas were the recipient of aggression while engaged in an egg-laying event at a mean (± SE) rate (0.04 ± 0.01 acts received per hour) that was 2.0 times higher than that of subordinates (0.02 ± 0.00 acts received per hour), while alphas were never recipients of this form of aggression (0.00 ± 0.00 acts received per hour) (**Figure 3.2B**, **Table A3.2**).

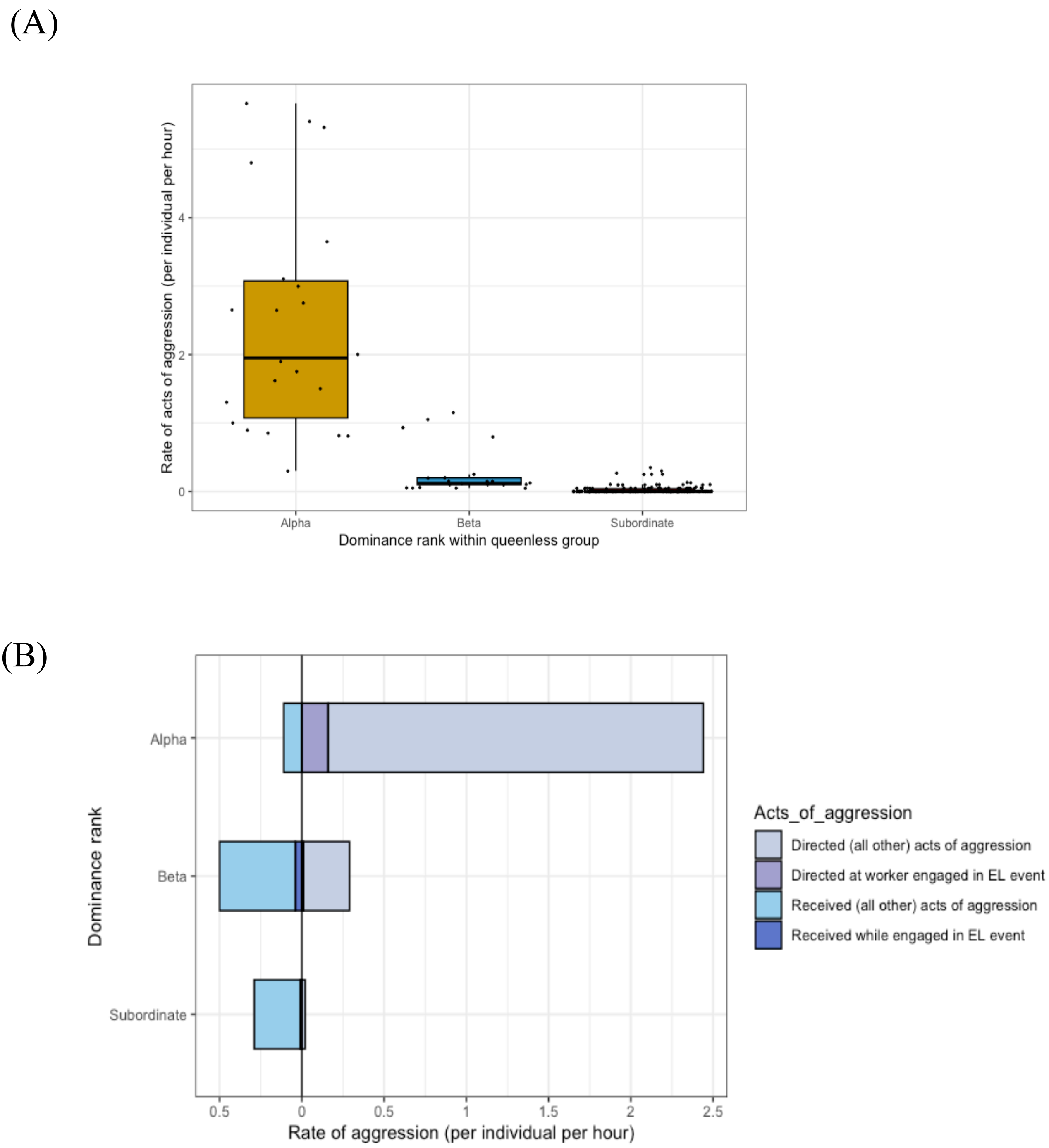


Figure 3.2. Rates of acts of aggression performed by, directed to, or received from, workers of different dominance ranks during the pre-treatment phase (n $_{alpha}$ = 22, n $_{beta}$ = 21, n $_{subordinate}$ = 177). (**A**) Mean rates of overall acts of aggression performed by workers of different dominance ranks. Thick horizontal bars, medians; boxes, interquartile range; whiskers, range; filled black circles, 'jittered' raw data, with each point representing a single individual within each rank of worker. (**B**) Mean rates of all other acts of aggression (attack, buzzing, darting, pumping) and acts of aggression directed at workers engaged in egg-laying (EL) events (displacing egg-layer, eating egg-cell wall), directed to, and received from, workers of different dominance ranks. Data collected from workers in 22 *Bombus terrestris* queenless groups during the pre-treatment phase Rates of behaviours (per individual per hour) are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations (mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital observations per queenless group)

	Rate of overall aggressive acts performed per individual per hour			Rate of egg-laying events performed per individual per hour			Rate of egg-eating events performed per individual per hour		
Colony	**By Alpha**	**By Beta**	**By Subordinate**	**By Alpha**	**By Beta**	**By Subordinate**	**By Alpha**	**By Beta**	**By Subordinate**
1	1.90	0.10	0.01	0.10	0.05	0.01	0.15	0.00	0.00
2	3.65	0.12	0.03	0.12	0.08	0.05	0.31	0.00	0.00
3	5.32	0.11	0.01	0.07	0.11	0.05	0.36	0.00	0.01
4	2.65	0.20	0.01	0.00	0.05	0.03	0.25	0.00	0.00
5	0.82	0.06	0.01	0.00	0.00	0.01	0.06	0.00	0.00
6	2.65	0.80	0.04	0.05	0.15	0.08	0.80	0.05	0.00
7	1.62	0.10	0.02	0.10	0.05	0.03	0.14	0.05	0.00
8	0.85	0.15	0.01	0.00	0.00	0.02	0.00	0.00	0.00
9	0.30	0.15	0.03	0.00	0.00	0.00	0.00	0.00	0.00
10	0.81	**NA**	0.00	0.10	**NA**	0.01	0.10	**NA**	0.00
11	0.90	0.10	0.01	0.05	0.00	0.01	0.05	0.00	0.00
12	3.10	1.05	0.12	0.05	0.00	0.03	0.10	0.00	0.00
13	2.00	0.25	0.04	0.00	0.00	0.00	0.00	0.00	0.00
14	3.00	0.15	0.01	0.05	0.05	0.00	0.00	0.05	0.00
15	1.75	0.05	0.00	0.05	0.00	0.01	0.10	0.10	0.01
16	5.40	1.15	0.10	0.15	0.10	0.07	0.50	0.00	0.00
17	4.80	0.20	0.03	0.00	0.05	0.03	0.25	0.00	0.00
18	1.30	0.05	0.00	0.10	0.00	0.00	0.00	0.00	0.00
19	1.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20	2.75	0.10	0.01	0.10	0.05	0.01	0.05	0.00	0.00
21	1.50	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22	5.67	0.93	0.07	0.07	0.27	0.03	0.33	0.00	0.01
Mean	2.44	0.28	0.02	0.05	0.05	0.02	0.16	0.01	0.00
SE	0.35	0.08	0.01	0.01	0.01	0.00	0.04	0.01	0.00
Mean no. of workers per dominance rank	1.00	0.95	7.90	1.00	0.95	7.90	1.00	0.95	7.90

Table 3.2. Rates of overall aggressive acts, egg-laying events and egg-eating events in 22 *Bombus terrestris* queenless groups of workers, during the pre-treatment phase. In each queenless group, individual workers were assigned to either alpha, beta, or subordinate dominance rank categories. Rates of behaviour were calculated from a combination of 1-hour long direct observations and 2-hour long digital film observations (mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital film observations per queenless group).

3.4.2 Pre-treatment phase: rates of egg-laying and egg-eating events by workers of different dominance rank

During the pre-treatment phase, egg-laying events were observed in 18 of 22 queenless groups. A total of 119 egg-laying events were observed ($n_{\text{egg-laying events performed by alphas}} = 24$, $n_{\text{egg-laying events performed by betas}} = 20$, $n_{\text{egg-laying events performed by subordinates}} = 75$; **Figure 3.3A, Table 3.2**). Pairwise comparisons suggested that the mean rates at which alphas, betas and subordinates, within the 22 queenless groups, performed egg-laying events did not vary significantly (Dunn's test: alpha vs beta: Z = 0.92, *p* = 0.53; alpha vs subordinate: Z = 1.78, *p* = 0.22; beta vs subordinate: Z = 0.84, *p* = 0.39 ; **Table 3.2**). Overall, there was no difference in the mean (± SE) rate at which alphas (0.05 ± 0.01 events per hour) and betas (0.05 ± 0.01 events per hour) performed egg-laying events (**Figure 3.3A; Table 3.2**). In addition, although alphas and betas within queenless groups performed egg-laying events at a mean rate that was 2.5 times higher than group subordinates (0.02 ± 0.00 events per hour), this was not a significant difference (**Table 3.2**).

As the rates at which individual workers of different dominance ranks performed egg-laying events were relatively low in comparison to the rates of overall acts of aggression and egg-eating events (**Table 3.2**), the relationship between egg-laying and dominance rank was further assessed using count data (**Section 3.3.7**). At the queenless group level, a significant relationship was found between worker dominance rank and the number of egg-laying events performed (ANOVA: $\chi^2 = 7.50$, df = 2, *p* = 0.02; **Table A3.4**), with pairwise comparisons showing that, within queenless groups, the alpha performed significantly more egg-laying events than subordinates (pairwise comparisons: alpha vs subordinate: Z = 2.691, *p* = 0.02; **Table A3.4**). However, alphas did not perform significantly more egg-laying events than betas (alpha vs beta: Z = 0.429, *p* = 0.91; **Table A3.4**) and betas did not perform significantly more egg-laying events than subordinates (beta vs subordinate: Z = 2.309, *p* = 0.06; **Table A3.4).**

In summary, these results suggested that the high-levels of aggression exhibited by an alpha did not prevent the beta or subordinate workers performing egg-laying events within queenless groups. In addition, alphas did not perform egg-laying events at a higher rate than betas but did perform egg-laying events at a higher rate than subordinates.

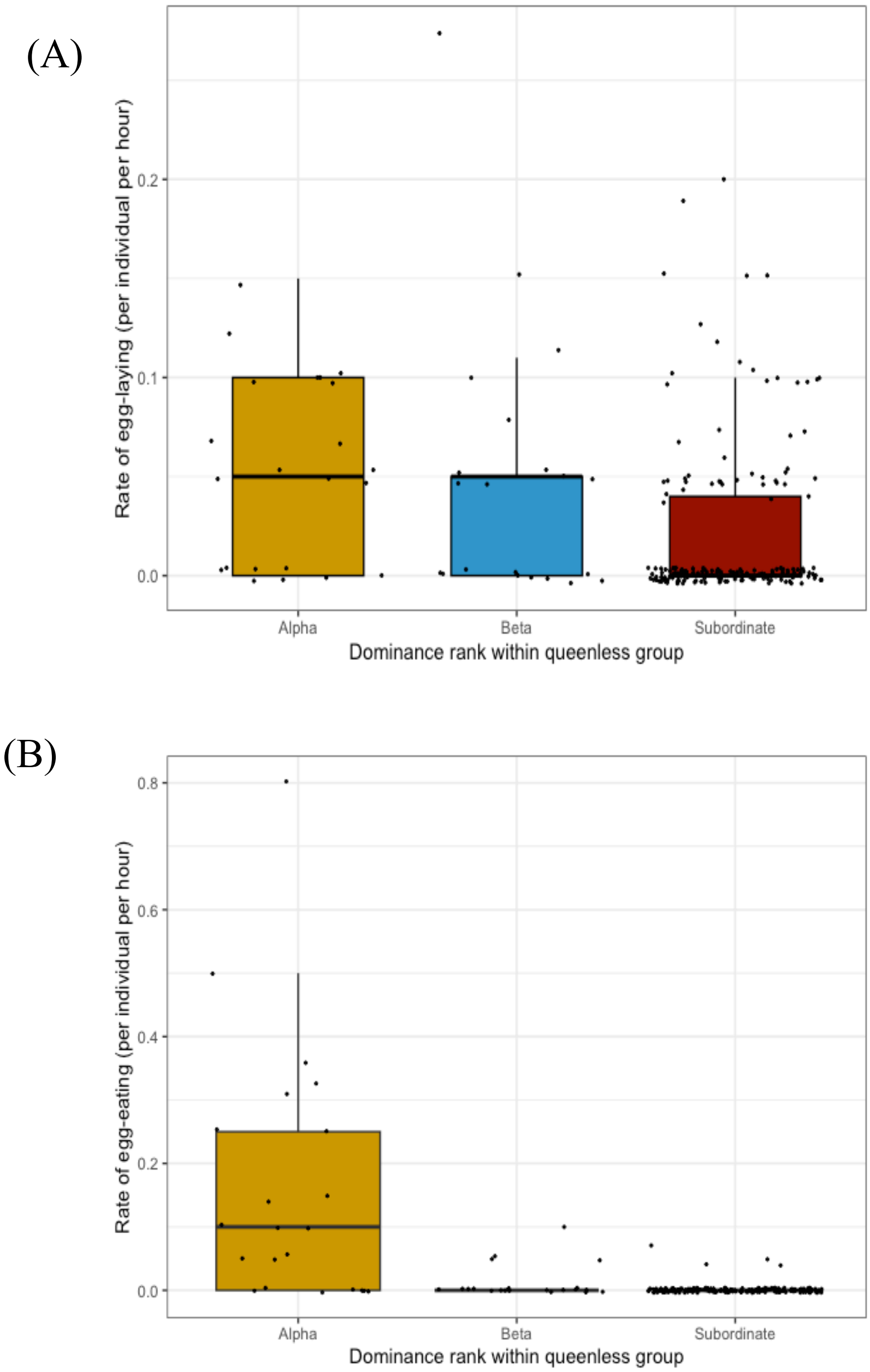


Figure 3.3. Mean rates of (**A**) acts of egg-laying ($n_{\textit{egg-laying events performed by alphas}} = 24$, $n_{\textit{egg-laying events performed by betas}} = 20$, $n_{\textit{egg-laying events performed by subordinates}} = 75$) and (**B**) egg-eating events ($n_{\textit{egg-eating events performed by alphas}} = 74$, $n_{\text{egg-eating events performed by betas}} = 5$, $n_{\text{egg-eating events performed by subordinates}} = 4$) performed by workers of different dominance rank in 22 *Bombus terrestris* queenless groups during the pre-treatment phase ($n_{\textit{alpha}} = 22$, $n_{\text{beta}} = 21$, $n_{\textit{subordinate}} = 177$). Rates of behaviours (per individual per hour) are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations (mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital observations per queenless group). Thick horizontal bars, medians; boxes, interquartile range; whiskers, range; filled black circles, 'jittered' raw data, with each point representing a single individual within each rank of worker.

In the pre-treatment phase, in 16 of the 22 queenless groups, egg-eating events were observed in which eggs laid by one group member were eaten (policed) by another group member. In total, 83 egg-eating events were observed in this phase ($n_{\textit{egg-eating events performed by alphas}} = 74$, $n_{\text{egg-eating events performed by betas}} = 5$, $n_{\text{egg-eating events performed by subordinates}} = 4$; **Figure 3.3B, Table 3.2**). Of the 23 workers observed performing egg-eating events during the pre-treatment phase, 18 (78.3%) were themselves observed egg-laying during this phase. Of the remaining five workers (21.7%), three (13.0%) were observed egg-laying in the post-treatment phase (and were thus reproductive at some point during their lifetime) and two (8.7%) were not observed egg-laying in either phase. The mean rates at which workers ranked as alphas, betas and subordinates performed egg-eating events differed significantly (Kruskal-Wallis test: $\chi^2 = 164.11$, df = 2, $p < 0.001$; **Figure 3.3B, Table 3.2**). Pairwise comparisons showed that alphas performed egg-eating events at a significantly higher rate than betas and subordinates (Dunn's test: alpha vs beta: Z = 3.87, $p < 0.001$; alpha vs subordinate: Z = 4.39, $p < 0.001$; **Figure 3.3B**). In contrast, betas did not perform egg-eating events at a significantly higher rate than subordinates (beta vs subordinate: Z = 0.47, $p = 0.64$; **Figure 3.3B**). Overall, alphas ate other workers' eggs (performed egg-eating events) at a mean (± SE) rate (0.16 ± 0.04 events per hour) that was 16 times higher than that of betas (0.01 ± 0.01). Subordinates were not observed eating other workers eggs during the pre-treatment phase (mean rate of 0.00 ± 0.00 acts per hour) (**Table 3.2**). In summary, alphas performed egg-eating events at the highest individual rates in queenless groups, performing 93.5% of all egg-eating events observed in the pre-treatment phase, while betas and subordinates performed 6.5% and 0.0%, respectively. This supported prediction (1) of the hypothesis by showing an association between directed aggression and egg-eating (policing).

3.4.3 Pre-treatment phase: effect of dominance rank on survivorship of eggs laid

In the pre-treatment phase, it was possible to continuously monitor the survival of 299 eggs of known parentage for up to 120 minutes ($n_{\textit{eggs laid by alphas}} = 81$, $n_{\textit{eggs laid by betas}} = 47$, $n_{\textit{eggs laid by subordinates}} = 171$; **Figure 3.4**). These eggs fell within 17/24 (70.8%), 12/24 (50.0%)| and 57/74 (77.0%) of the egg-laying events performed by alphas, betas and subordinates, respectively. None of the alpha-laid eggs continuously monitored were observed being eaten during observations. In contrast, 33 eggs laid by betas, and 132 eggs laid by subordinates, were observed being eaten (**Figure 3.4**). Beta-laid eggs had a median survival time of 7.7 minutes (95% CI [6.68, 11.6]), and subordinate-laid eggs had a median survival time of 13.8 minutes (95% CI [8.52, 17.0]). The median survival time of eggs laid by betas and subordinates did not differ significantly (log rank test: $\chi^2 = 0$, df = 1, $p = 0.9$) and, when combined, beta- and subordinate-laid eggs had a median survival time of 10.4 minutes (95% CI [8.45,14.3]) (**Figure 3.4**). Overall, of eggs continuously monitored, 100% of the eggs laid by workers ranked as alphas survived up to the end of the monitoring period of 120-minutes. In contrast, only

29.8% of eggs laid by betas and 22.8% of eggs laid by subordinates survived up to the end of this period. Eggs laid by alphas were therefore significantly more likely to survive than eggs laid by betas or subordinates (log rank test: $\chi^2 = 122$, df = 1, $p < 0.001$; **Figure 3.4**). These results show a positive association between dominance rank and egg survivorship, supporting prediction (1) of the hypothesis.

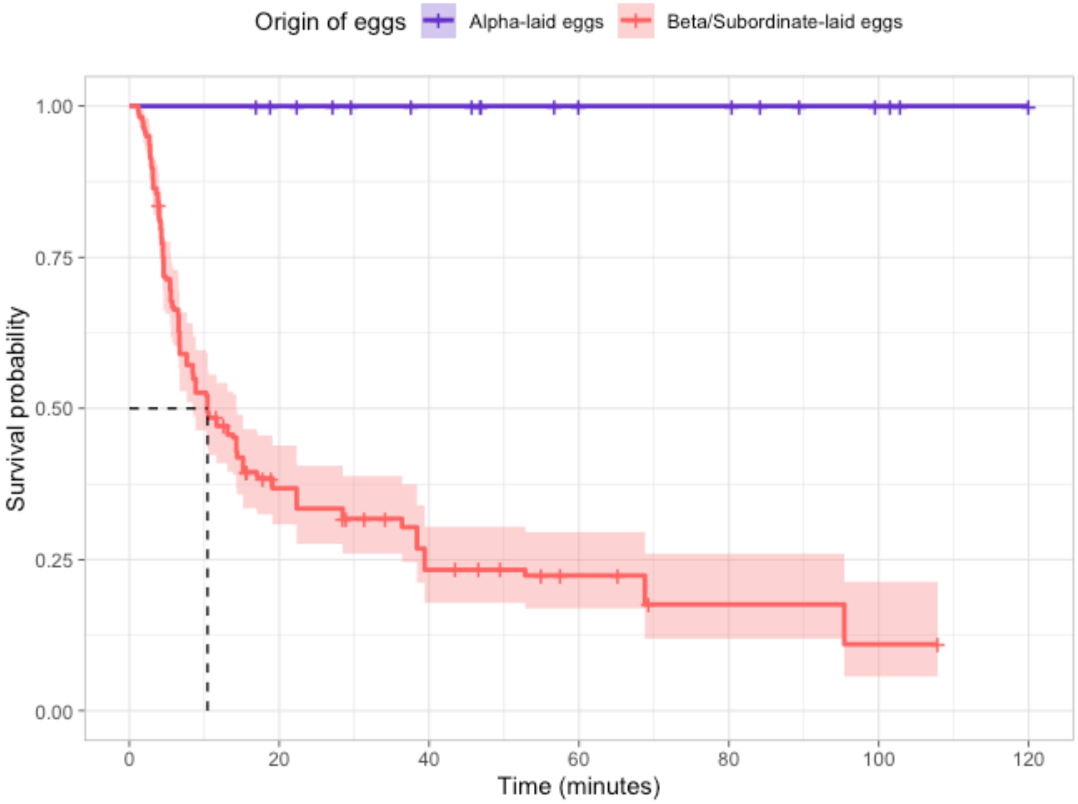


Figure 3.4. Survivorship curves (Kaplan-Meyer curves, ± 95 CI) for eggs laid by alpha workers and beta/subordinate workers for up to 120 minutes of observation following egg-laying event, in the pre-treatment phase (censored recordings: $n_{\textit{alpha-laid eggs}} = 81$, $n_{\textit{beta/subordinate-laid eggs}} = 60$; time to event recordings: $n_{\textit{alpha-laid eggs}} = 0$, $n_{\textit{beta/subordinate-laid eggs}} = 158$). Egg mortality was caused by egg eating (policing). Data were collected across 22 *Bombus terrestris* queenless groups from multiple 1-hour-long direct observations and 2-hour long digital film observations (pre-treatment phase: mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital observations per queenless group).

3.4.4 Pre-treatment phase: rates of unpoliced egg-laying events performed by workers of different dominance rank

In total, 50 unpoliced egg-laying events were observed ($n_{\textit{unpoliced egg-laying events performed by alphas}} = 24$, $n_{\textit{unpoliced egg-laying events performed by betas}} = 6.5$, $n_{\textit{unpoliced egg-laying events performed by subordinates}} = 19.5$; **Figure 3.5**). Alphas performed unpoliced egg-laying events at a mean rate that was significantly higher than that of either betas or subordinates (Dunn's test: alpha vs beta: Z = 3.10, $p = 0.002$; alpha vs subordinate: Z = 6.76, $p < 0.001$; beta vs subordinate: Z = 2.53, $p = 0.01$; **Figure 3.5, Table A3.5**). Betas also performed unpoliced egg-laying events at a significantly higher rate than subordinates (beta vs subordinate: Z = 2.53, $p < 0.001$; **Figure 3.5, Table A3.5**). In addition, a strong positive relationship was found between the mean rate at which an individual worker performed overall acts of aggression and the mean rate at which an individual worker performed unpoliced egg-laying events (Spearman rank correlation: $\rho = 0.48$, $n = 220$, $p = 0.04$; **Figure 3.6, Tables 3.2, A3.5**). In summary, these results suggested that alphas performed unpoliced (i.e., successful) egg-laying events at a higher rate than all other workers within a queenless group. In addition, these results show a strong relationship between directed aggression, egg-eating and successful egg-laying, supporting prediction (1) of the hypothesis.

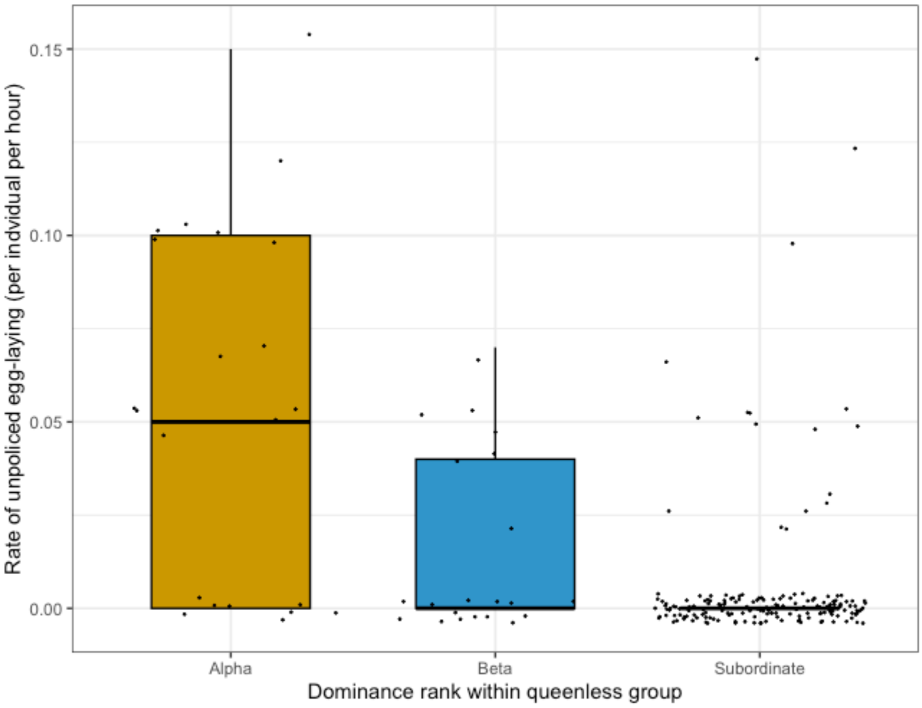


Figure 3.5. Rates of unpoliced egg-laying events performed by workers of different dominance ranks in 22 *Bombus terrestris* queenless groups during the pre-treatment phase ($n_{\text{unpoliced egg-laying events performed by alphas}} = 24$, $n_{\text{unpoliced egg-laying events performed by betas}} = 6.5$, $n_{\text{unpoliced egg-laying events performed by subordinates}} = 19.5$). Rates of unpoliced egg-laying events (per individual per hour) were calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations (mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital observations per queenless group). Thick horizontal bars, medians; boxes, interquartile range; whiskers, range; filled black circles, 'jittered' raw data, with each point representing a single individual within each rank of worker.

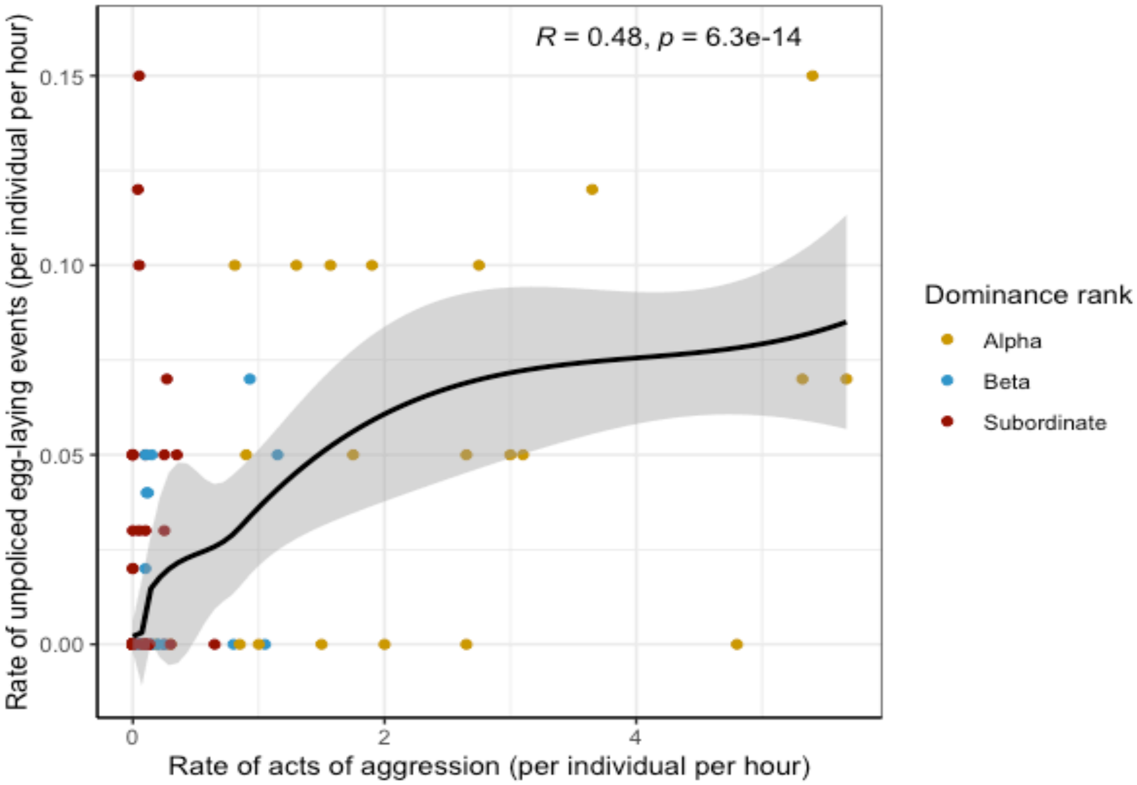


Figure 3.6. Relationship between the mean rate (per individual per hour) at which alpha, beta and subordinate workers performed unpoliced egg-laying events and overall acts of aggression in the pre-treatment phase ($n_{alpha} = 22$, $n_{beta} = 21$, $n_{subordinate} = 177$). All data were obtained from 22 *Bombus terrestris* queenless groups. Rates of behaviours are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations (mean ± SD of 11.73 ± 1.28 h of direct observations and 8.64 ± 2.08 h of digital observations per colony). Spearman rank correlation: $\rho = 0.48$, n = 220, $p < 0.001$. Analysis is based on 220 workers, but each data point may represent multiple workers because of overlapping points; colours represent workers of different dominance rank. Fitted curve is plotted from the correlation analysis, with grey areas representing 95% confidence intervals.

3.4.5 Post-treatment phase: response of pre-treatment betas to post-treatment conditions and rates of overall acts of aggression

Data used in analyses for the post-treatment phase were collected from a total of 455 h of observation (n $_{direct\ observations}$ = 273 h, n $_{digital\ observations}$ = 182 h). In total, 11 queenless groups were assigned to the alpha-worker removal treatment (i.e., the worker ranked as alpha was removed from the group) and 11 queenless groups were assigned to the control-worker removal treatment (i.e., a worker ranked as subordinate was removed from the group) (**Table A3.1**).

In the alpha-worker removal treatment, one queenless group (group 10) had to be excluded from the analysis concerning the response of pre-treatment betas to the post-treatment phase as, due to low levels of aggression, no worker was assigned the rank of beta in the pre-treatment phase (**Sections: 3.3.3, 3.3.6**). In the remaining 10 queenless groups assigned to the alpha-worker removal treatment, no acts of aggression were observed in 1/10 queenless groups following the start of the post-treatment phase. In the remaining nine queenless groups, the former beta in the pre-treatment phase became the group alpha in the post-treatment phase in 5/9 groups (55.6%), while a former subordinate worker in the pre-treatment phase became the group alpha in the post-treatment phase in the remaining 4/9 groups (44.4%). Given the null hypothesis that the post-treatment alpha arose at random from all workers remaining (range, 7-9 workers; **Table A3.1**) within each of the 9 groups in which a post-treatment alpha occurred, one would expect the pre-treatment beta to have been the observed post-treatment alpha in 5 groups with a probability of 0.0013-0.0041 (from the binomial formula with $n = 9$, $k = 5$ and $p = 0.111$ to 0.143). Since this probability range is very low, the null hypothesis of random selection is rejected. Therefore, within a post-treatment queenless alpha-worker removal group, the former pre-treatment beta was, on average, the worker most likely to become the alpha, consistent with prediction (2) of the hypothesis.

In the control-worker removal treatment, the worker ranked as the alpha in the pre-treatment phase maintained its alpha rank in the post-treatment phase in 7/11 queenless groups (63.6%). In contrast, despite the pre-treatment alpha still being present, the former beta in the pre-treatment phase became the alpha in the post-treatment phase in 1/11 groups (9.1%), while a former subordinate in the pre-treatment phase became the alpha in the post-treatment phase in the remaining 3/11 groups (27.3%). Given the null hypothesis that the post-treatment alpha arose at random from all workers remaining (range, 7-9 workers; **Table A3.1**) within each of the 11 control-worker removal groups, one would expect the pre-treatment alpha to have been the observed post-treatment alpha in 7 groups with a probability of 0.00004-0.00022 (from the binomial formula with $n = 11$, $k = 7$ and $p = 0.111\text{-}0.143$). Therefore, within a post-treatment queenless control-worker removal group, the pre-treatment alpha was, on average, the worker most likely to be the alpha. This result suggests the changes in dominance ranks observed between phases cannot be solely attributed to the decrease in group size resulting from the removal of a worker. Hence, these results reinforce the interpretation of the results in the alpha-worker removal groups as supporting prediction (2) of the hypothesis. Nonetheless, either the former beta or a former subordinate became the alpha in four control-worker removal groups, suggesting the occurrence of some dynamic, temporal turnover in dominance rank.

Overall, in the alpha-worker removal treatment, workers that held the rank of beta in the pre-treatment phase (i.e., former pre-treatment betas) performed overall acts of aggression in the post-treatment phase at a mean (± SE) rate (0.28 ± 0.13 acts per hour) similar to, and not significantly different from, the rate at which they performed overall acts of aggression in the pre-treatment phase (0.27 ± 0.12 acts per hour) (alpha-worker removal treatment: $n_{\textit{aggressive acts performed in pre-treatment phase by former pre-treatment betas}} = 50$, $n_{\textit{aggressive acts performed in post-treatment phase by former pre-treatment betas}} = 58$) (paired Wilcoxon test: alpha-worker removal treatment, V = 28, *p* = 1.00; **Figure 3.7, Table A3.6**). Additionally, in the alpha-worker removal treatment, former pre-treatment betas performed egg-eating events in the post-treatment phase at a mean (± SE) rate (0.07 ± 0.04 acts per hour). This was higher than the rate at which they performed egg-eating events in the pre-treatment phase (0.00 ± 0.00 acts per hour) (alpha-worker removal treatment: $n_{\textit{egg-eating events performed in pre-treatment phase by former pre-treatment betas}} = 1$, $n_{\textit{egg-eating events performed in post-treatment phase by former pre-treatment betas}} = 14$; **Table A3.6**), though this difference was also not significant (paired Wilcoxon test: alpha-worker removal treatment V = 17.5, *p* = 0.17; **Table A3.6**).

In the control-worker removal treatment, two queenless group (groups 8, 13) had to be excluded from the analysis concerning the response (rate of overall acts of aggression and egg-eating) of former pre-treatment betas to the post-treatment phase as in both queenless groups the former pre-treatment beta died early in the post-treatment phase, preventing a comparison from being made (**Sections: 3.3.3, 3.3.6**). In contrast to the alpha-worker removal treatment, in queenless groups assigned to the control-worker removal treatment, former pre-treatment betas performed overall acts of aggression in the post-treatment phase at a mean rate (0.13 ± 0.13 acts per hour), that was less than half the rate at which they performed acts of aggression in the pre-treatment phase (0.31 ± 0.13 acts per hour) (control-worker removal treatment: $n_{\textit{aggressive acts performed in pre-treatment phase by former pre-treatment betas}} = 57$, $n_{\textit{aggressive acts performed in post-treatment phase by former pre-treatment betas}} = 32$; **Figure 3.7**, **Table A3.6**). Additionally, in the control-worker removal treatment, former pre-treatment betas performed egg-eating events in the post-treatment phase at a mean (± SE) rate (0.03 ± 0.03 acts per hour) that was 1.5 times higher than the rate at which they performed egg-eating events in the pre-treatment phase (0.02 ± 0.01 acts per hour) (control-worker removal treatment: $n_{\textit{egg-eating events performed in pre-treatment phase by former pre-treatment betas}} = 4$, $n_{\textit{egg-eating events performed in post-treatment phase by former pre-treatment betas}} = 6$**; Table A3.6**). However, for both behaviours, the difference in rates across phases was not significant (paired Wilcoxon test: control-worker removal treatment overall acts of aggression V = 29, *p* = 0.14; control-worker removal treatment egg-eating events: V = 6, *p* = 0.9).

Overall, in terms of rates of overall acts of aggression, the response of former pre-treatment betas to the post-treatment phase differed significantly between treatments

(Fisher's exact test: $p = 0.02$). In the alpha-worker removal treatment, the former pre-treatment betas maintained their rates of overall acts of aggression following the removal of the pre-treatment alpha whereas, in contrast, in the control-worker removal treatment former pre-treatment betas displayed lower rates of overall acts of aggression compared to the pre-treatment phase. Similarly, in the alpha-worker removal treatment, the former pre-treatment betas upregulated their rates of egg-eating to a greater extent than the former pre-treatment betas in the control-worker removal treatment. However, the changes in rates of egg-eating events performed by former pre-treatment betas (between the pre- and post-treatment phase) varied between queenless groups, leading to no significant differences between treatments (Fisher's exact test: $p = 0.12$).

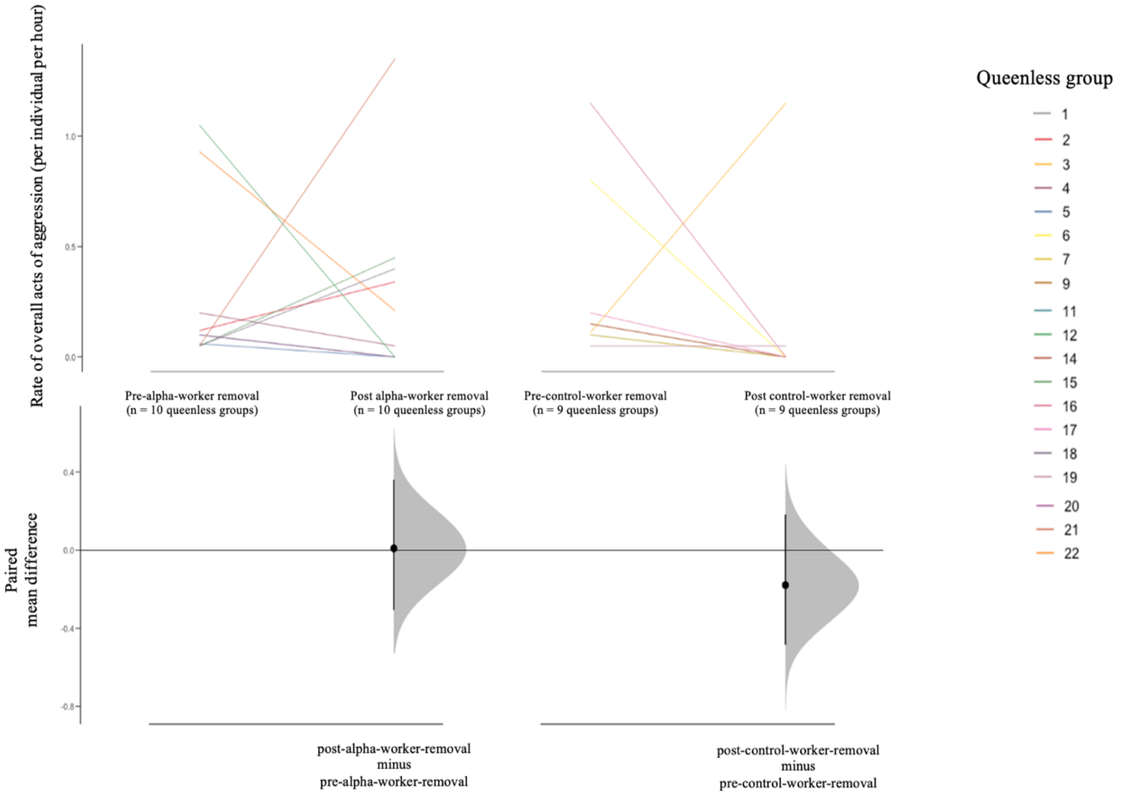


Figure 3.7. Comparison of mean rates of overall acts of aggression performed in the pre-treatment phase and post-treatment phase by workers ranked as pre-treatment betas in 19 *Bombus terrestris* queenless groups. Queenless groups were assigned to the alpha-worker removal treatment (left) ($n_{\text{aggressive acts performed in pre-treatment phase by former betas}} = 50$, $n_{\text{aggressive acts performed in post-treatment phase by former betas}} = 58$) or control-worker removal treatment (right) ($n_{\text{aggressive acts performed in pre-treatment phase by former betas}} = 57$, $n_{\text{aggressive acts performed in post-treatment phase by former betas}} = 32$). Rates of overall acts of aggression (per individual per hour) were calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations taken during the pre-treatment phase (mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital observations per queenless group) and post-treatment phase (mean ± SD of 12.41 ± 1.41 h of direct observation and 8.27 ± 2.78 h of digital observations per queenless group). Raw data are plotted at top, with a coloured line (one per colony) connecting each paired data set; paired mean difference is plotted directly below raw data as a bootstrap sampling distribution. For both the alpha- and control-worker removal treatment, the difference in pre- and post-treatment phase means is depicted as a filled black circle with a vertical black line representing the 95% confidence interval. Each sideways grey Gaussian curve represents the probability distribution of the difference between the pre- and post-treatment phase means.

3.4.6 Post-treatment phase: rates of unpoliced egg-laying events in contrasting treatment groups

In the alpha-worker removal treatment, a total of 21.5 unpoliced egg-laying events were observed ($n_{\text{unpoliced egg-laying events performed by alphas}} = 13.5$, $n_{\text{unpoliced egg-laying events performed by betas}} = 3.5$, $n_{\text{unpoliced egg-laying events performed by subordinates}} = 4.5$). Overall, in this treatment, post-treatment alphas performed unpoliced egg-laying events at a significantly higher mean (± SE) rate (0.05 ± 0.02 acts per hour) than post-treatment betas (0.02 ± 0.02 acts per hour) or subordinates (0.00 ± 0.00 acts per hour) (Dunn's test: post-treatment alpha vs post-treatment beta : Z = 2.74, *p* = 0.02; post-treatment alpha vs post-treatment subordinate : Z = 2.77, *p* = 0.02; **Table A3.7**). In contrast, post-treatment betas did not perform unpoliced egg-laying events at a rate significantly different from that of post-treatment subordinates (post-treatment beta vs post-treatment subordinate: Z = -0.05, p = 1.00; **Table A3.7**).

In the control-worker removal treatment, a total of 11 unpoliced egg-laying events were observed ($n_{\text{unpoliced egg-laying events performed by alphas}} = 6.5$, $n_{\text{unpoliced egg-laying events performed by betas}} = 2.5$, $n_{\text{unpoliced egg-laying events performed by subordinates}} = 2$). Again, in this treatment, the post-treatment alpha performed unpoliced egg-laying events at a significantly higher mean (±SE) rate (0.03 ± 0.01 acts per hour) than post-treatment betas (0.02 ± 0.02 acts per hour) or subordinates (0.01 ± 0.00 acts per hour) (Dunn's test: post-treatment alpha vs post-treatment beta : Z = 2.45, *p* = 0.02; post-treatment alpha vs post-treatment subordinate : Z = 2.93, *p* = 0.01; **Table A3.7**). In addition, post-treatment betas did not perform unpoliced egg-laying events at a rate significantly different from that of post-treatment subordinates (post-treatment beta vs post-treatment subordinate: Z = -0.24, p = 0.81; **Table A3.7**). In summary, post-treatment alphas performed unpoliced egg-laying events at significantly higher rates than any other worker rank in both treatments, suggesting a consistent association between alpha rank (based on directed aggression) and unpoliced egg-laying, i.e. an association that occurs even in groups in which the rank of alpha transitioned from one worker to another.

In the alpha-worker removal treatment, former pre-treatment betas performed unpoliced egg-laying events in the post-treatment phase at a mean (± SE) rate (0.03 ± 0.01 acts per hour) that was not significantly different from the rate at which they performed unpoliced egg-laying events in the pre-treatment phase (0.02 ± 0.01 acts per hour) (**Figure 3.7, Table A3.6**) (paired Wilcoxon test: V = 9.5, *p* = 0.68). In the control-worker removal treatment, former pre-treatment betas performed unpoliced egg-laying events in the post-treatment phase at a mean rate (0.01 ± 0.01acts per hour) not significantly different from the rate at which they performed unpoliced egg-laying events in the pre-treatment phase (0.02 ± 0.01acts per hour) (**Figure 3.7**, **Table A3.6**) (paired Wilcoxon test: V = 1, *p* = 0.20). Therefore, although in the alpha-worker removal groups the post-treatment alpha performed unpoliced egg-laying events at a significantly higher rate than post-treatment betas or subordinates (see above), this did not involve pre-treatment betas upregulating their absolute rates of performing unpoliced egg-laying events, most likely because not all became alphas and/or overall rates of performing these events fell. In the control-worker removal groups, the lack of difference in the rate at which former pre-treatment betas performed unpoliced egg-laying events between pre- and post-treatment was as expected from prediction (2) of the hypothesis, as in these groups the former pre-treatment betas was in the continued presence of the pre-treatment alpha.

3.5 Discussion

3.5.1 Overview

This study aimed to investigate the ultimate function of dominance behaviour in animal societies by determining if a within-group positive association exists between dominance and reproductive success (direct fitness) in coexisting egg-laying workers of a *Bombus* species. In total, 22 queenless groups of full-sister *B. terrestris* workers were utilised to measure individual rates of aggression, egg-laying, egg-eating (policing) and subsequent egg survivorship. The primary objective was to determine rank in the within-group dominance hierarchy and reproductive success at the individual worker level. Additionally, the study manipulated queenless groups by removing either the alpha worker or a control (subordinate) worker to test for the effect of dominance on subordinates and evaluate the strength of the linear dominance hierarchy. As discussed below, the findings of this study supported both predictions of the focal hypothesis. Specifically, they demonstrate a clear positive association between aggression, policing and egg survivorship and provide evidence that higher rank inhibits the reproductive success of workers of subordinate rank. Consequently, the findings strongly support the hypothesis that, among *B. terrestris* workers, dominance rank is positively correlated with reproductive success.

3.5.2 Association between dominance and reproductive success

Consistent with previous research (Duchateau, 1989; Cnaani et al., 2007; Amsalem and Hefetz, 2011), I found that dominant (alpha) workers did not completely suppress egg-laying in workers of lower rank (**Figure 3.3A),** despite performing significantly higher rates of overall acts of aggression, and acts of aggression directed at group members engaged in egg-laying events, than either betas or subordinates (**Figure 3.2A; Table A3.2**). Consequently, alphas were responsible for only 19.6% of egg-laying events observed in the pre-treatment phase, while betas and subordinates accounted for 18.6% and 61.8% (7.7% per individual subordinate) of events, respectively (**Table 3.2**). However, examining individual egg-eating rates (**Figure 3.3B**) and subsequent egg survivorship (**Figure 3.4**) revealed a clear positive association between dominance rank and reproductive success in terms of production of surviving eggs (**Figures 3.5, 3.6**). Specifically, in the pre-treatment phase, alphas performed egg-eating events at significantly higher rates than betas or subordinates (**Figure 3.3B**), performing 93.5% of events observed (**Table 3.2**). This resulted in a relatively short median survival time of 10.4 minutes for eggs laid by betas and subordinates, whereas all eggs laid by alphas remained unpoliced, leading to significantly higher survival rates for eggs laid by alphas (**Figure 3.4**). The outcome was a strong, significant positive correlation emerging between alpha rank and successful (unpoliced) egg-laying (**Figures 3.5, 3.6**). Overall, therefore, these findings supported prediction (1) of the hypothesis, providing strong evidence for an association between dominance and realised reproductive success in *B. terrestris* workers.

These results provide several novel insights into the regulation of reproduction within worker hierarchies in *Bombus* species. First, in contrast to those of previous studies, the findings demonstrate that dominance and reproductive success are positively associated in reproductive workers of *Bombus* species (**Figure 3.6**). However, the results show, for the first time, that this association comes about through a combination of levels of aggression, egg-laying and egg-eating by the alpha worker. As stated above, within queenless groups the alpha was unable to completely suppress egg-laying in betas or subordinates and so did not monopolise egg-laying within queenless groups (**Figure 3.3A**). Previous research has found similar results, with the alpha unable to supress ovary activation and/or egg-laying in groups of two or three workers (Sibbald and Plowright, 2013; 2014) or in groups of five or more workers (Bloch and Hefetz, 1999; Amsalem and Hefetz, 2011), leading to the suggestion that multiple workers share reproduction (Duchateau, 1989; Cnaani et al., 2007; Amsalem and Hefetz, 2011). The conclusions of these studies have been used to suggest that dominance hierarchies and reproductive hierarchies are partially decoupled in *Bombus* species (Sibbald and Plowright, 2014; Amsalem et al., 2015). However, in these studies reproductive output was measured as either the degree of ovary activation (Cnaani et al., 2007; Amsalem and Hefetz, 2011; Sibbald and Plowright, 2014) or number of egg-laying events (Duchateau, 1989; Sibbald and Plowright, 2013), and so acts of policing and egg survivorship were not taken into account. As I found that egg-eating events performed by the group alpha, who selectively ate eggs of betas and subordinates at a high rate, played a pivotal role in reducing the reproductive success of beta and subordinate workers within a queenless group (**Figure 3.4**), it is likely that these studies overestimated the reproductive success of betas and subordinate workers and so underestimated the reproductive monopoly of the dominant (alpha) worker.

Second, the results are relevant to the concept of worker policing in general. The presence of selfish worker policing in queenless groups of *B. terrestris* workers is predicted by inclusive fitness theory, as in colonies of eusocial Hymenoptera headed by one, singly-mated queen, workers are more related to their sons (r = 0.5) than they are to their nephews (r = 0.375) or to the queen's sons, their brothers (r = 0.25) (**Figure 2.1**) (Trivers and Hare, 1976). As a result, reproductive workers are selected to eat other workers' eggs in queenless groups, to reduce reproductive competition and secure a greater share of resources for their own sons (**Chapter 2**). In general, selfish worker policing has been well documented in eusocial Hymenoptera with similar colony kin structures (Wenseleers et al., 2005; Bonckaert et al., 2011; Zanette et al., 2012). However, the association between dominance rank and reproductive success as mediated through worker policing of other workers' eggs has not been previously reported.

In addition, in the current study a strong association was found between the rates of aggression, egg-eating and egg-survivorship, with alphas exhibiting the highest rates for all three metrics (**Figures 3.2, 3.3B, 3.4**). These findings support results reported and discussed in Chapter 1 of this thesis (**Section 2.5.4**) and indicate that aggressive workers are significantly more likely to perform policing events than workers of lower rank. Therefore, the results from both chapters provide empirical data suggesting that a workers' rate of policing is associated with their rate of aggression, with dominant workers utilising both mechanisms to inhibit the reproduction of other workers for direct fitness gains. Similar positive associations between dominance and policing (or infanticide) have been reported in other kin-based social groups, such as queenless ants (Dinoponera quadriceps; Monnin and Peeters, 1997) and several species of mammal (Lukas and Huchard, 2019), highlighting the crucial role policing can play in maintaining the reproductive monopoly of dominant individuals in animal societies.

3.5.3 The importance of directed aggression

While it is evident that directed aggression alone is insufficient for dominant workers to suppress the reproduction of other workers in *B. terrestris* (**Figures 3.2, 3.3A**), the findings of this study suggest that directed aggression may contribute to the regulation of reproduction among workers in ways that previous research has not fully considered. For example, during the pre-treatment phase, alphas exhibited significantly higher rates of aggression directed towards workers engaged in egg-laying events compared to other group members (**Figure 3.2**), accounting for 92.3% of observed events. This suggest that alphas physically interfere with the egg-laying attempts of lower-ranking workers. Therefore, it is highly likely that these aggressive interruptions reduced the reproductive success of recipient subordinate workers by impeding the number of eggs they could lay.

Furthermore, it is possible that, in the pre-treatment phase, the lower rates of aggression and egg-eating displayed by betas compared to alphas (**Figures 3.2, 3.3B**), despite their equal potential for egg-laying (**Figure 3.2A**), could be attributed to their consistent experience of defeat in aggressive interactions. Betas were found to be the primary recipients of aggression, implying they consistently experienced defeat in such interactions (**Section 3.4.1**). This observation aligns with extensive empirical evidence, in eusocial Hymenoptera and other taxa, that suggests an individual's previous experience of winning or losing in aggressive encounters proximately impacts their subsequent behaviour and performance in future contests (Chase et al., 1994; Chase et al., 2002; Dugatkin and Druen, 2004; Bang and Gadagkar, 2016; Ge et al., 2021; Shimoji and Dobata, 2022). Overall, it is clear that the outcomes of aggressive interactions play an important role in shaping the formation and structure of dominance hierarchies in multiple animal societies.

3.5.4 The strength of the dominance hierarchy

In the alpha-worker removal treatment groups, it was observed that in in 5/9 groups (55.6%), the beta worker from the pre-treatment phase assumed the role of the new alpha (**Section 3.4.5**). This outcome matched prediction (2) of the hypothesis, which stated that the beta of a group was, on average more likely to ascend to the alpha position compared to the subordinates. Similarly, in the control-worker removal treatment groups, the alpha of the pre-treatment phase retained its rank in 7/11 groups (63.6%) (**Section 3.4.5**). This showed that rank changes in the alpha-worker removal treatment groups were not attributable just to the reduction in group size caused by removing a worker, and so again helped support prediction (2) of the hypothesis. These findings are also consistent with previous research, indicating that once reproductive competition begins, dominance hierarchies among workers of *Bombus* species tend to remain relatively stable (Hogeweg and Hesper, 1983; Van Doorn and Heringa, 1986; Duchateau, 1989; Bloch et al., 1996). Nonetheless, in some control-worker removal treatment groups, a former beta or subordinate worker became the alpha (**Section 3.4.5**), suggesting a low level of dynamic, temporal turnover in dominance rank.

Although the removal of the former alpha did not lead to significant changes in the rates of aggression displayed by former pre-treatment betas within the alpha-worker removal treatment groups (**Figure 3.7**), the response of former pre-treatment betas to the post-treatment phase did vary significantly between treatments. In the alpha-worker removal treatment groups, former pre-treatment betas maintained their overall aggression level, while in the continued presence of the pre-treatment alpha (in control-worker removal groups) former pre-treatment betas downregulating the rates of aggression they exhibited (**Section 3.4.6**). Similarly, former pre-treatment betas in the alpha-worker removal groups upregulated their rates of egg-eating and unpoliced egg-laying relative to former pre-treatment betas in the control-worker removal treatment groups (**Sections 3.4.6, 3.4.7**). These result again supports predictions (2) of the hypothesis and the studies mentioned above by suggesting worker dominance hierarchies are relatively stable in *Bombus* species and only prone to heightened aggression following changes in group membership (i.e., the death/removal of the alpha).

At the proximate level, the comparative lack of increased aggression by former pre-treatment betas in the alpha-removal treatment could also be attributed to the presence of increasing amounts of brood, since brood has been demonstrated to reduce aggression and egg-laying behaviours in workers and trigger rearing behaviours in various eusocial Hymenoptera, including ants (Heinze et al., 1996; Ulrich et al., 2016; Schultner et al., 2017), wasps (Solís and Strassmann, 1990) and bees (Sibbald and Plowright, 2014; Amsalem et al., 2015; Starkey et al., 2019). In the current study, queenless groups produced a mean (± SD) of 72.3 ± 22.5 males each. As many of these males were present as eggs and larvae at the start of the treatment phase, this may have reduced the likelihood of intense reproductive conflict following the removal of the alpha.

Despite no significant differences in the rate of aggression and policing between phases in both treatments, a clear association between dominance and reproductive success was still observed in both treatment groups (**Section 3.4.7**). Post-treatment alphas exhibited significantly higher rates of successful (unpoliced) egg-laying events compared to betas and subordinates in both the alpha-worker removal treatment and control-worker removal treatment groups. This suggests that, in unmanipulated queenless groups, an alpha is capable of maintaining its reproductive monopoly for extended periods. Furthermore, the findings indicate that an association between aggression and successful (unpoliced) egg-laying can exist even in queenless groups where the alpha rank transitions from one worker to another (**Figure 3.6; Section 3.4.7**).

3.5.5 Other aspects of study

As continuous observations of queenless groups throughout the entire experiment were not possible, it is plausible that some behavioural events (i.e., aggressive interactions, egg-laying, egg-eating) may have been missed. This could have led to workers being assigned the wrong rank during the pre-treatment phase. However, observations were extensive, totalling approximately 903 hours over a period of 2 months. The substantial volume of observations increase the likelihood that the behaviours observed in individual workers accurately represent the overall behaviours and interactions within each queenless group. Consequently, the assignment of worker ranks in each phase of the experiment is considered reliable and reflective of the actual dynamics within the queenless groups. As the assigned alpha retained its rank in the 7/11 of the control-worker removal groups, this further suggests that, within queenless groups, the assignment of the alpha rank was accurate.

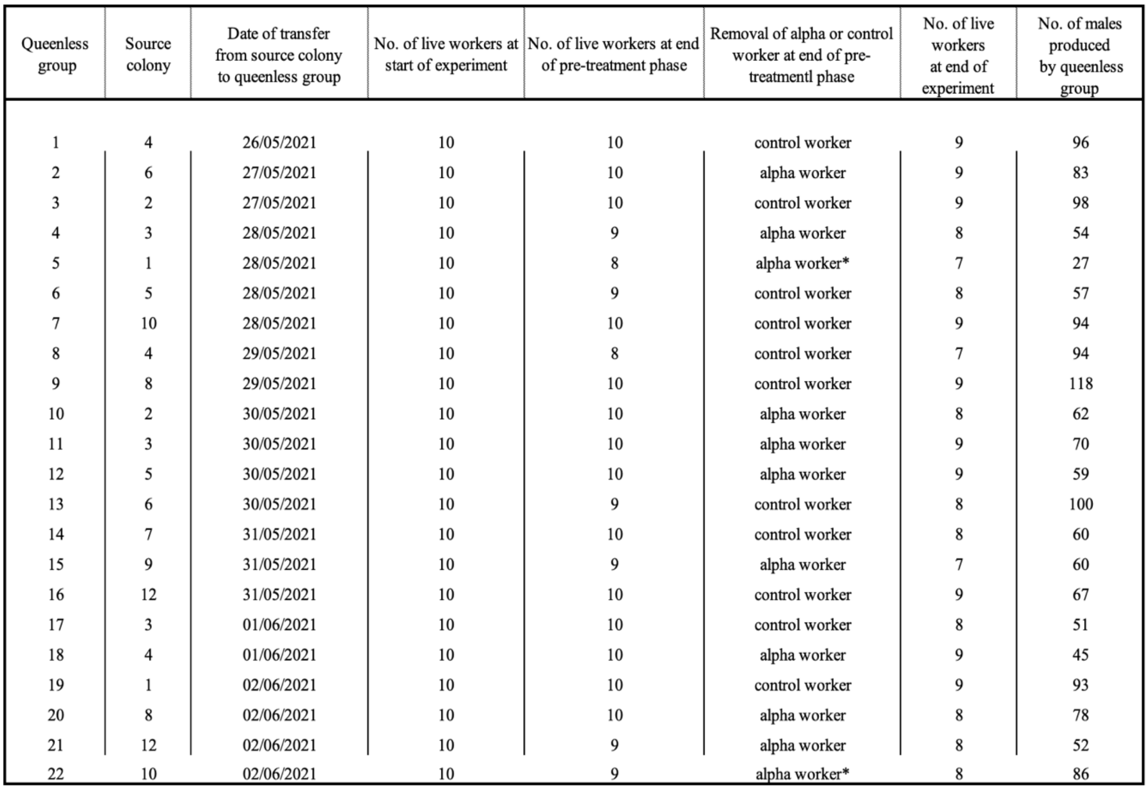
In addition, as a parentage analysis was not conducted on adult males that eclosed within queenless groups, the output of adult sons by individual workers was not determined in the current study. Instead, the reproductive success of workers was inferred based on the survival rate of eggs after the initial few hours following their laying. Previous research by Zanette et al. (2012) has shown that worker-laid eggs that survive policing during the first 2 hours of life are typically raised to adulthood. Hence, it is highly likely the calculated rate of egg-survivorship and successful (unpoliced) egg-laying employed in the current study served as a reliable indicator of realised reproductive success among workers.

3.5.6 Conclusions

In conclusion, this study provides strong evidence for a positive association, at the individual level, between dominance and reproductive success among reproductive workers in a *Bombus* species. The findings demonstrate that dominant workers (alphas) employ directed aggression and policing of other workers' eggs to reduce the reproductive success of subordinate workers, in order to maintain their rank and promote their own reproductive success.

The findings of this study also enhance understanding of the function and dynamics of dominance hierarchies, shedding light on the pivotal role policing plays in maintaining the reproductive monopoly of dominant workers in *Bombus* species and, in all likelihood, in eusocial Hymenoptera in general. The study also confirms the stability of dominance hierarchies in the face of disruption, with betas typically ascending to the alpha position in the absence of the former alpha. Overall, this research contributes valuable insights into the regulation of reproduction within worker hierarchies and the mechanisms governing worker reproductive success in bumblebee colonies. By demonstrating an association of dominance behaviour and realised reproductive success in this system, it also adds to evidence from across animal societies as a whole (see Introduction) that the ultimate function of dominance is to promote an individual's direct fitness.

3.6 Appendix



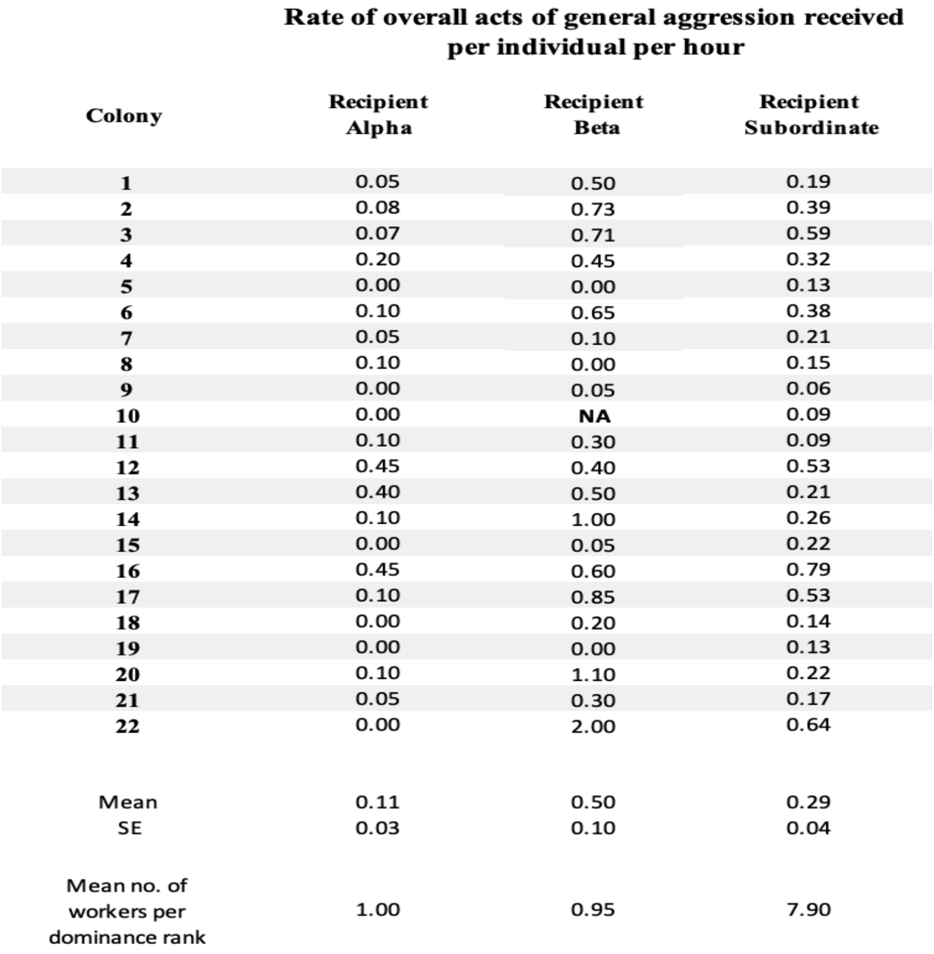
Queenless group	Source colony	Date of transfer from source colony to queenless group	No. of live workers at start of experiment	No. of live workers at end of pre-treatment phase	Removal of alpha or control worker at end of pre-treatmentl phase	No. of live workers at end of experiment	No. of males produced by queenless group
1	4	26/05/2021	10	10	control worker	9	96
2	6	27/05/2021	10	10	alpha worker	9	83
3	2	27/05/2021	10	10	control worker	9	98
4	3	28/05/2021	10	9	alpha worker	8	54
5	1	28/05/2021	10	8	alpha worker*	7	27
6	5	28/05/2021	10	9	control worker	8	57
7	10	28/05/2021	10	10	control worker	9	94
8	4	29/05/2021	10	8	control worker	7	94
9	8	29/05/2021	10	10	control worker	9	118
10	2	30/05/2021	10	10	alpha worker	8	62
11	3	30/05/2021	10	10	alpha worker	9	70
12	5	30/05/2021	10	10	alpha worker	9	59
13	6	30/05/2021	10	9	control worker	8	100
14	7	31/05/2021	10	10	control worker	8	60
15	9	31/05/2021	10	9	alpha worker	7	60
16	12	31/05/2021	10	10	control worker	9	67
17	3	01/06/2021	10	10	control worker	8	51
18	4	01/06/2021	10	10	alpha worker	9	45
19	1	02/06/2021	10	10	control worker	9	93
20	8	02/06/2021	10	10	alpha worker	8	78
21	12	02/06/2021	10	9	alpha worker	8	52
22	10	02/06/2021	10	9	alpha worker*	8	86

Table A3.1. Demographic, productivity and experimental data for 22 queenless groups of *Bombus terrestris* workers used for data analyses (colony nos. 1-22). Queenless groups were produced from 12 queenright source colonies received on 20/05/2021 and formation of queenless groups was started on 26/05/2021. Removal of alpha or control (subordinate) workers (see text) was staggered in line with the start of observations for each queenless group. In two queenless groups (marked *) the alphas died prior to the planned date of removal; in these cases, date of death was used as a proxy for date of alpha removal. The number of adult males produced includes all males that eclosed in queenless groups from the start of the experiment to the date of termination. Dates are given in the format dd/mm/yyyy.

Rate of aggression directed at workers engaged in an egg-laying event performed per individual per hour

	Actor of aggression			Recipient of aggression		
Colony	**Alpha**	**Beta**	**Subordinate**	**Alpha**	**Beta**	**Subordinate**
1	0.25	0.00	0.00	0.00	0.10	0.02
2	0.38	0.00	0.00	0.00	0.04	0.04
3	0.25	0.00	0.00	0.00	0.04	0.03
4	0.40	0.00	0.00	0.00	0.10	0.04
5	0.00	0.00	0.00	0.00	0.00	0.00
6	0.20	0.10	0.00	0.00	0.10	0.03
7	0.19	0.10	0.00	0.00	0.00	0.04
8	0.00	0.05	0.00	0.00	0.00	0.01
9	0.00	0.00	0.00	0.00	0.00	0.00
10	0.05	**NA**	0.00	0.00	**NA**	0.01
11	0.10	0.00	0.00	0.00	0.00	0.01
12	0.05	0.00	0.00	0.00	0.00	0.01
13	0.05	0.00	0.00	0.00	0.05	0.00
14	0.00	0.00	0.00	0.00	0.00	0.00
15	0.05	0.00	0.00	0.00	0.00	0.01
16	0.55	0.05	0.00	0.05	0.00	0.07
17	0.30	0.00	0.00	0.00	0.05	0.03
18	0.00	0.00	0.00	0.00	0.00	0.00
19	0.00	0.00	0.00	0.00	0.00	0.00
20	0.10	0.00	0.00	0.00	0.05	0.01
21	0.00	0.00	0.00	0.00	0.00	0.00
22	0.60	0.00	0.00	0.00	0.27	0.04
Mean	0.16	0.01	0.00	0.00	0.04	0.02
SE	0.04	0.01	0.00	0.00	0.01	0.00
Mean no. of workers per dominance rank	1.00	0.95	7.90	1.00	0.95	7.90

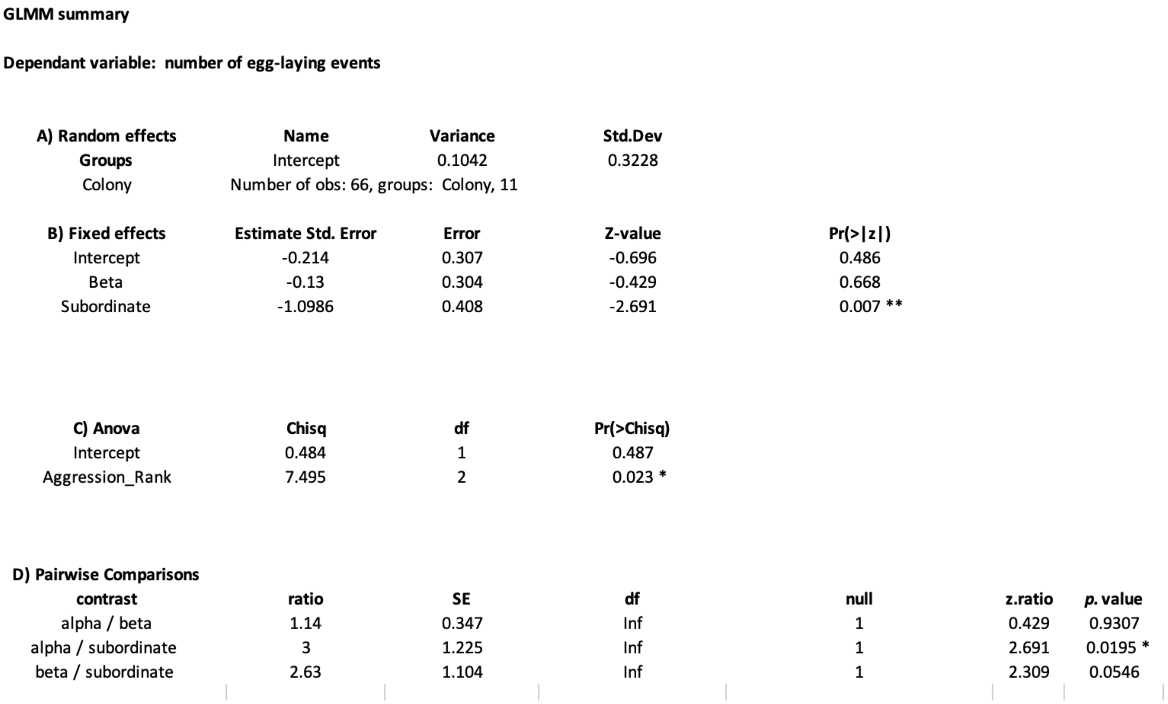
Table A3.2. Data from the pre-treatment phase on rates of acts of aggression towards workers engaged in egg-laying events performed and received as a function of dominance rank in 22 *Bombus terrestris* queenless groups. Rates of behaviours are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations(pre-treatment phase: mean ± SD of 11.73 ± 1.28 h of direct observations and 8.64 ± 2.08 h of digital observations per colony)



Rate of overall acts of general aggression received per individual per hour

Colony	Recipient Alpha	Recipient Beta	Recipient Subordinate
1	0.05	0.50	0.19
2	0.08	0.73	0.39
3	0.07	0.71	0.59
4	0.20	0.45	0.32
5	0.00	0.00	0.13
6	0.10	0.65	0.38
7	0.05	0.10	0.21
8	0.10	0.00	0.15
9	0.00	0.05	0.06
10	0.00	**NA**	0.09
11	0.10	0.30	0.09
12	0.45	0.40	0.53
13	0.40	0.50	0.21
14	0.10	1.00	0.26
15	0.00	0.05	0.22
16	0.45	0.60	0.79
17	0.10	0.85	0.53
18	0.00	0.20	0.14
19	0.00	0.00	0.13
20	0.10	1.10	0.22
21	0.05	0.30	0.17
22	0.00	2.00	0.64
Mean	0.11	0.50	0.29
SE	0.03	0.10	0.04
Mean no. of workers per dominance rank	1.00	0.95	7.90

Table A3.3. Data from the pre-treatment phase on rates of overall acts of aggression received by individual workers of different dominance ranks in 22 *Bombus terrestris* queenless groups. Rates of behaviours are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations (pre-treatment phase: mean ± SD of 11.73 ± 1.28 h of direct observations and 8.64 ± 2.08 h of digital observations per colony).



GLMM summary

Dependant variable: number of egg-laying events

A) Random effects	**Name**	**Variance**	**Std.Dev**
Groups	Intercept	0.1042	0.3228
Colony	Number of obs: 66, groups: Colony, 11		

B) Fixed effects	**Estimate Std. Error**	**Error**	**Z-value**	**Pr(>|z|)**
Intercept	-0.214	0.307	-0.696	0.486
Beta	-0.13	0.304	-0.429	0.668
Subordinate	-1.0986	0.408	-2.691	0.007 **

C) Anova	**Chisq**	**df**	**Pr(>Chisq)**
Intercept	0.484	1	0.487
Aggression_Rank	7.495	2	0.023 *

D) Pairwise Comparisons

contrast	**ratio**	**SE**	**df**	**null**	**z.ratio**	***p.* value**
alpha / beta	1.14	0.347	Inf	1	0.429	0.9307
alpha / subordinate	3	1.225	Inf	1	2.691	0.0195 *
beta / subordinate	2.63	1.104	Inf	1	2.309	0.0546

Table A3.4. GLMM model summary for analyses of number of egg-laying events performed by individual workers of different dominance ranks in the pre-treatment phase. Group of workers ranked as alphas used as reference level. Analysis included all egg-laying events performed by workers in direct and digital observations of the pre-treatment phase in 22 colonies of *Bombus terrestris*. ***$p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Pairwise tests were performed on the log odds ratio scale.

Colony	Rate of unpoliced egg-laying events performed per individual per hour		
	Alpha	**Beta**	**Subordinate**
1	0.10	0.00	0.00
2	0.12	0.04	0.02
3	0.07	0.04	0.01
4	0.00	0.00	0.00
5	0.00	0.00	0.00
6	0.05	0.00	0.01
7	0.10	0.02	0.02
8	0.00	0.00	0.02
9	0.00	0.00	0.00
10	0.10	**NA**	0.00
11	0.05	0.00	0.01
12	0.05	0.00	0.01
13	0.00	0.00	0.00
14	0.05	0.05	0.00
15	0.05	0.00	0.01
16	0.15	0.05	0.01
17	0.00	0.00	0.00
18	0.10	0.00	0.00
19	0.00	0.00	0.00
20	0.10	0.05	0.00
21	0.00	0.00	0.00
22	0.07	0.07	0.01
Mean	0.05	0.02	0.01
SE	0.01	0.01	0.00
Mean no. of workers per dominance rank	1.00	0.95	8.05

Table A3.5. Data from the pre-treatment phase on rates of unpoliced egg-laying events performed by individual workers of different dominance ranks in 22 *Bombus terrestris* queenless groups. Rates of behaviour are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations (pre-treatment phase: mean ± SD of 11.73 ± 1.28 h of direct observations and 8.64 ± 2.08 h of digital observations per colony).

Colony	Phase and treatment	Rate of overall acts of aggression performed by pre-treatment betas (per individual per hour)	Rate of unpoliced egg-laying events performed by pre-treatment betas (per individual per hour)	Rate of egg-eating events performed by pre-treatment betas (per individual per hour)
1	Pre control-worker removal treatment	0.10	0.00	0.00
1	Post control-worker removal treatment	0.00	0.00	0.00
2	Pre alpha-worker removal treatment	0.12	0.04	0.00
2	Post alpha-worker removal treatment	0.34	0.10	0.00
3	Pre control-worker removal treatment	0.11	0.04	0.00
3	Post control-worker removal treatment	1.15	0.06	0.05
4	Pre alpha-worker removal treatment	0.20	0.00	0.00
4	Post alpha-worker removal treatment	0.05	0.00	0.00
5	Pre alpha-worker removal treatment	0.06	0.00	0.00
5	Post alpha-worker removal treatment	0.00	0.00	0.00
6	Pre control-worker removal treatment	0.80	0.00	0.00
6	Post control-worker removal treatment	0.00	0.00	0.00
7	Pre control-worker removal treatment	0.10	0.02	0.05
7	Post control-worker removal treatment	0.00	0.00	0.10
9	Pre control-worker removal treatment	0.15	0.00	0.00
9	Post control-worker removal treatment	0.00	0.00	0.00
11	Pre alpha-worker removal treatment	0.10	0.00	0.00
11	Post alpha-worker removal treatment	0.00	0.00	0.00
12	Pre alpha-worker removal treatment	1.05	0.00	0.05
12	Post alpha-worker removal treatment	0.00	0.00	0.00
14	Pre control-worker removal treatment	0.15	0.05	0.22
14	Post control-worker removal treatment	0.00	0.00	0.00
15	Pre alpha-worker removal treatment	0.05	0.00	0.03
15	Post alpha-worker removal treatment	0.45	0.00	0.10
16	Pre control-worker removal treatment	1.15	0.05	0.00
16	Post control-worker removal treatment	0.00	0.00	0.00
17	Pre control-worker removal treatment	0.20	0.00	0.00
17	Post control-worker removal treatment	0.00	0.00	0.00
18	Pre alpha-worker removal treatment	0.05	0.00	0.00
18	Post alpha-worker removal treatment	0.40	0.05	0.00
19	Pre control-worker removal treatment	0.05	0.00	0.00
19	Post control-worker removal treatment	0.05	0.00	0.00
20	Pre alpha-worker removal treatment	0.10	0.05	0.00
20	Post alpha-worker removal treatment	0.00	0.00	0.00
21	Pre alpha-worker removal treatment	0.05	0.00	0.05
21	Post alpha-worker removal treatment	1.35	0.10	0.00
22	Pre alpha-worker removal treatment	0.93	0.07	0.45
22	Post alpha-worker removal treatment	0.21	0.00	0.04

Table A3.6. Data on rates of overall acts of aggression unpoliced egg-laying events and egg-eating events performed in the pre-and post- treatment phases by workers ranked as pre-treatment betas in 19 *Bombus terrestris* queenless groups. Rates (per individual per hour) are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations taken during the pre-treatment phase (mean ± SD of 11.73 ± 1.28 h of direct observation and 8.64 ± 2.08 h of digital observations per queenless group) and post-treatment phase (mean ± SD of 12.41 ± 1.41 h of direct observation and 8.27 ± 2.78 h of digital observations per queenless group).

		Post-treatment phase					
		Rate of overall acts of aggression performed per individual per hour			Rate of unpoliced egg-laying events performed per individual per hour		
Colony	**Treatment**	**By Alpha**	**By Beta**	**By Subordinate**	**By Alpha**	**By Beta**	**By Subordinate**
1	Control-worker removal	0.48	0.19	0.01	0.00	0.00	0.01
2	Alpha-worker removal	0.34	0.07	0.00	0.10	0.00	0.00
3	Control-worker removal	1.15	0.52	0.03	0.06	0.09	0.02
4	Alpha-worker removal	0.76	0.05	0.00	0.02	0.00	0.00
5	Alpha-worker removal	0.40	0.04	0.01	0.07	0.00	0.00
6	Control-worker removal	0.11	**NA**	0.00	0.05	**NA**	0.00
7	Control-worker removal	1.05	0.07	0.01	0.00	0.00	0.01
8	Control-worker removal	0.74	**NA**	0.00	0.11	**NA**	0.00
9	Control-worker removal	0.10	**NA**	0.00	0.00	**NA**	0.01
10	Alpha-worker removal	3.65	0.60	0.12	0.13	0.13	0.01
11	Alpha-worker removal	0.00	**NA**	0.00	0.00	**NA**	0.00
12	Alpha-worker removal	0.05	**NA**	0.00	0.00	**NA**	0.00
13	Control-worker removal	0.05	**NA**	0.00	0.00	**NA**	0.00
14	Control-worker removal	0.20	0.10	0.00	0.00	0.00	0.00
15	Alpha-worker removal	0.45	**NA**	0.00	0.00	**NA**	0.00
16	Control-worker removal	0.10	**NA**	0.00	0.05	**NA**	0.01
17	Control-worker removal	0.50	**NA**	0.00	0.05	**NA**	0.01
18	Alpha-worker removal	1.75	0.33	0.14	0.10	0.03	0.01
19	Control-worker removal	0.20	0.05	0.00	0.00	0.00	0.00
20	Alpha-worker removal	0.85	**NA**	0.00	0.01	**NA**	0.00
21	Alpha-worker removal	1.35	0.05	0.00	0.10	0.00	0.00
22	Alpha-worker removal	0.21	0.10	0.04	0.02	0.00	0.01
	Mean	0.66	0.18	0.02	0.04	0.02	0.00
	SE	0.17	0.06	0.01	0.01	0.01	0.00
	Mean no. of workers per dominance rank	0.95	0.55	6.82	0.95	0.55	6.82

Table A3.7. Data from the post-treatment phase on rates of overall acts of aggression and unpoliced egg-laying events performed by individual workers of different dominance rank in 22 *Bombus terrestris* queenless groups. Rates of behaviours are calculated from a combination of multiple 1-hour-long direct observations and multiple 2-hour long digital film observations(mean ± SD of 12.41 ± 1.41 h of direct observation and 8.27 ± 2.78 h of digital observations per queenless group)

3.7 References

Alaux, C., Boutot, M., Jaisson, P., Hefetz, A., 2007. Reproductive plasticity in bumblebee workers (*Bombus terrestris*) - reversion from fertility to sterility under queen influence. Behavioral Ecology and Sociobiology 62, 213-222.

Alaux, C., Jaisson, P., Hefetz, A., 2004. Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies. Insectes Sociaux 51, 287-293.

Alaux, C., Jaisson, P., Hefetz, A., 2006. Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal. Behavioral Ecology and Sociobiology 60, 439-446.

Alford, D.V., 1975. Bumblebees. Davis-Poynter, London.

Almond, E.J., Huggins, T.J., Crowther, L.P., Parker, J.D., Bourke, A.F.G., 2019. Queen longevity and fecundity affect conflict with workers over resource inheritance in a social insect. American Naturalist 193, 256-266.

Amsalem, E., Grozinger, C.M., Padilla, M., Hefetz, A., 2015. The physiological and genomic bases of bumble bee social behaviour. Advances in Insect Pysiology 48, 37-93.

Amsalem, E., Hefetz, A., 2010. The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers. Behavioral Ecology and Sociobiology 64, 1685-1694.

Amsalem, E., Hefetz, A., 2011. The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*. PLoS One 6, e18238.

Amsalem, E., Kiefer, J., Schulz, S., Hefetz, A., 2014. The effect of caste and reproductive state on the chemistry of the cephalic labial glands secretion of *Bombus terrestris*. Journal of Chemical Ecology 40, 900-912.

Amsalem, E., Shpigler, H., Bloch, G., Hefetz, A., 2013. Dufour's gland secretion, sterility and foraging behavior: correlated behavior traits in bumblebee workers. Journal of Insect Physiology 59, 1250-1255.

Bang, A., Gadagkar, R., 2016. Winner-loser effects in a eusocial wasp. Insectes Sociaux 63, 349-352.

Beekman, M., Ratnieks, F.L.W., 2003. Power over reproduction in social Hymenoptera. Philosophical Transactions of the Royal Society of London Series B 358, 1741-1753.

Bloch, G., Borst, D.W., Huang, Z.-Y., Robinson, G.E., Hefetz, A., 1996. Effects of social conditions on Juvenile Hormone mediated reproductive development in *Bombus terrestris* workers. Physiological Entomology 21, 257-267.

Bloch, G., Hefetz, A., 1999. Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. Behavioral Ecology and Sociobiology 45, 125-135.

Bonckaert, W., Tofilski, A., Nascimento, F.S., Billen, J., Ratnieks, F.L.W., Wenseleers, T., 2011. Co-occurrence of three types of egg policing in the Norwegian wasp *Dolichovespula norwegica*. Behavioral Ecology and Sociobiology 65, 633-640.

Bourke, A.F.G., 1988a. Dominance orders, worker reproduction, and queen-worker conflict in the slave-making ant *Harpagoxenus sublaevis*. Behavioral Ecology and Sociobiology 23, 323-333.

Bourke, A.F.G., 1988b. Worker reproduction in the higher eusocial Hymenoptera. Quarterly Review of Biology 63, 291-311.

Bourke, A.F.G., 1994. Worker matricide in social bees and wasps. Journal of Theoretical Biology 167, 283-292.

Bourke, A.F.G., 2011. Principles of Social Evolution. Oxford University Press, Oxford.

Bourke, A.F.G., Franks, N.R., 1995. Social Evolution in Ants. Princeton University Press, Princeton.

Bourke, A.F.G., Ratnieks, F.L.W., 2001. Kin-selected conflict in the bumble-bee *Bombus terrestris* (Hymenoptera: Apidae). Proceedings of the Royal Society of London Series B 268, 347-355.

Carvalho da Silva, R., Prato, A., Oi, C.A., Turatti, I.C.C., Nascimento, F.S., 2020. Dominance Hierarchy, Ovarian Activity and Cuticular Hydrocarbons in the Primitively Eusocial Wasp *Mischocyttarus cerberus* (Vespidae, Polistinae, Mischocyttarini). Journal of Chemical Ecology 46, 835–844.

Chase, I.D., 1974. Models of hierarchy formation in animal societies. Behavioral Science 19, 374-382.

Chase, I.D., Bartolomeo, C., Dugatkin, L.A., 1994. Aggressive interactions and inter-contest interval: how long do winners keep winning? . Animal Behaviour 48, 393-400.

Chase, I.D., Tovey, C., Spangler-Martin, D., Manfredonia, M., 2002. Individual differences versus social dynamics in the formation of animal dominance hierarchies. Proceedings of the National Academy of Sciences, U.S.A. 99, 5744-5749.

Cnaani, J., Wong, A., Thomson, J.D., 2007. Effect of group size on ovarian development in bumblebee workers (Hymenoptera: Apidae: *Bombus*). Entomologia Generalis 29, 305-314.

Duchateau, M.J., 1989. Agonistic behaviors in colonies of the bumblebee *Bombus terrestris*. J. Ethol. 7, 141-151.

Duchateau, M.J., Velthuis, H.H.W., 1988. Development and reproductive strategies in *Bombus terrestris* colonies. Behaviour 107, 186-207.

Duchateau, M.J., Velthuis, H.H.W., 1989. Ovarian development and egg laying in workers of *Bombus terrestris*. Entomologia Experimentalis et Applicata 51, 199-213.

Dugatkin, L.A., Druen, M., 2004. The social implications of winner and loser effects. Proc R Soc Lond B (Suppl) 271, S488-S489.

Ellis, L., 1995. Dominance and reproductive success among nonhuman animals: a cross-species comparison. Ethology and Sociobiology 16, 257-333.

Ge, J., Zhou, X., Ge, Z., Zhu, D., Nie, X., Wang, X., 2021. Phased contests allow rapid hierarchy formation in paired bumblebee workers. Animal Behaviour 179, 125-138.

Hamilton, W.D., 1964. The genetical evolution of social behaviour I, II. Journal of Theoretical Biology 7, 1-52.

Heinze, J., Trunzer, B., Oliveira, P.S., Hölldobler, B., 1996. Regulation of reproduction in the Neotropical ponerine ant, *Pachycondyla villosa*. J. Insect Behav. 9, 441-450.

Hogeweg, P., Hesper, B., 1983. The ontogeny of the interaction structure in bumble bee colonies: a MIRROR model. Behavioral Ecology and Sociobiology 12, 271-283.

Klinger, E.G., Camp, A.A., Strange, J.P., Cox-Foster, D.L., Lehmann, D.M., 2019. Bombus Microcolonies as a Tool for Biological Understanding and Pesticide Risk Assessment. Environ. Entomol. 48, 1249-1259.

Leadbeater, E., Carruthers, J.M., Green, J.P., Rosser, N.S., Field, J., 2011. Nest inheritance is the missing source of direct fitness in a primitively eusocial insect. Science 333, 874-876.

Lewis, R.J., 2022. Aggression, rank and power: why hens (and other animals) do not always peck according to their strength. Philosophical Transactions of the Royal Society B.

Lopez-Vaamonde, C., Koning, J.W., Brown, R.M., Jordan, W.C., Bourke, A.F.G., 2004. Social parasitism by male-producing reproductive workers in a eusocial insect. Nature 430, 557-560.

Lopez-Vaamonde, C., Raine, N.E., Koning, J.W., Brown, R.M., Pereboom, J.J.M., Ings, T.C., Ramos-Rodriguez, O., Jordan, W.C., Bourke, A.F.G., 2009. Lifetime reproductive success and longevity of queens in an annual social insect. Journal of Evolutionary Biology 22, 983-996.

Lukas, D., Huchard, E., 2019. The evolution of infanticide by females in mammals. Philosophical Transactions of the Royal Society B 374.

Molet, M., Van Baalen, M., Monnin, T., 2005. Dominance hierarchies reduce the number of hopeful reproductives in polygynous queenless ants. Insectes Sociaux 52, 247-256.

Monnin, T., Peeters, C., 1997. Cannibalism of subordinates' eggs in the monogynous queenless ant *Dinoponera quadriceps*. Naturwissenschaften 84, 499-502.

Monnin, T., Ratnieks, F.L.W., Brandao, C.R.F., 2003. Reproductive conflict in animal societies: hierarchy length increases with colony size in queenless ponerine ants. Behavioral Ecology and Sociobiology 54, 71-79.

Owen, R.E., Whidden, T.L., 2013. Monandry and polyandry in three species of North American bumble bees (*Bombus*) determined using microsatellite DNA markers. Canadian Journal of Zoology 91.

Princen, S., V., O.A., van Zweden. J. S, Wenseleers, T., 2020. Worker dominance and reproduction in the bumblebee *Bombus terrestris*: when does it pay to bare one's mandibles? Animal Behaviour 166, 41-50.

Ratnieks, F.L.W., 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. American Naturalist 132, 217-236.

Ratnieks, F.L.W., Foster, K.R., Wenseleers, T., 2006. Conflict resolution in insect societies. Annual Review of Entomology 51, 581-608.

Roberts, S., Cords, M., 2013. Group size but not dominance rank predicts the probability of conception in a frugivorous primate. Behavioral Ecology And Sociobiology 67, 1995–2009.

Schmid-Hempel, R., Schmid-Hempel, P., 2000. Female mating frequencies in *Bombus* spp. from Central Europe. Insectes Sociaux 47, 36-41.

Schultner, J., Oettler, J., Helantera, H., 2017. The role of brood in eusocial Hymenoptera. Quarterly Review of Biology 92, 39-78.

Shimoji, H., Dobata, S., 2022. The build-up of dominance hierarchies in eusocial insects. Philosophical Transactions of the Royal Society B 377, 20200437.

Sibbald, E.D., Plowright, C.M.S., 2013. On the relationship between aggression and reproduction in pairs of orphaned worker bumblebees (*Bombus impatiens*). Insectes Sociaux 60, 23-30.

Sibbald, E.D., Plowright, C.M.S., 2014. Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (*Bombus impatiens)*. Behavioral processes 103, 150-155.

Sibbald, E.D., Plowright, C.M.S., 2015. Reproductive potential and its behavioural consequences in orphaned bumblebee workers (*Bombus impatiens*). Apidologie 46, 618-627.

Solís, C.R., Strassmann, J.E., 1990. Presence of brood affects caste differentiation in the social wasp, *Polistes exclamans Viereck* (Hymenoptera: *Vespidae*). Functional Ecology 4, 531-541.

Starkey, J., Brown, A., Amsalem, E., 2019. The road to sociality: brood regulation of worker reproduction in the simple eusocial bee *Bombus impatiens*. Animal Behaviour 154, 57-65.

Stockley, P., Bro-jørgensen, J., 2011. Female competition and its evolutionary consequences in mammals. Biological Reviews 86, 341-366.

Strauss, E.D., Curley, J.P., Shizuka, D., Hobson, E.A., 2022. The centennial of the pecking order: current state and future prospects for the study of dominance hierarchies. . Philosophical Transactions of the Royal Society B 377, 20200432.

Trivers, R.L., Hare, H., 1976. Haplodiploidy and the evolution of the social insects. Science 191, 249-263.

Ulrich , Y., Burns, D., Libbrecht, R., Kroemer, G., 2016. Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner. Behavioral Ecology And Sociobiology 7, 1011 - 1018.

Van Doorn, A., 1989. Factors influencing dominance behaviour in queenless bumblebee workers (*Bombus terrestris*). Physiological Entomology 14, 211-221.

Van Doorn, A., Heringa, J., 1986. The ontogeny of a dominance hierarchy in colonies of the bumblebee *Bombus terrestris* (Hymenoptera, *Apidae*). Insectes Sociaux 33, 3-25.

Wasser, S.K., Norton, G.W., Kleindorfer, S., Rhine, R.J., 2004. Population Trend Alters the Effects of Maternal Dominance Rank on Lifetime Reproductive Success in Yellow Baboons (*Papio cynocephalus*) Behavioral Ecology And Sociobiology 56, 338-345.

Wenseleers, T., Ratnieks, F.L.W., 2006. Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. American Naturalist 168, E163-E179.

Wenseleers, T., Tofilski, A., Ratnieks, F.L.W., 2005. Queen and worker policing in the tree wasp *Dolichovespula sylvestris*. Behavioral Ecology and Sociobiology 58, 80-86.

Wilson, D.S., 1975a. A theory of group selection. Proceedings National Academy of Sciences, U.S.A. 72, 143-146.

Wilson, E.O., 1975b. Sociobiology: The New Synthesis. Belknap Press, Cambridge, MA.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Almond, E.J., Huggins, T.J., Jordan, W.C., Bourke, A.F.G., 2012. Reproductive conflict in bumblebees and the evolution of worker policing. Evolution 66, 3765-3777.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Lopez-Vaamonde, C., Bourke, A., 2014. Bumble bee workers drift to conspecific nests at field scales. Ecological Entomology 39, 347-354.

4
Chapter
Quality, reproductivity and longevity in *Bombus terrestris* workers

Picture taken by Jenny Livesey

4.1 Abstract

Sociality has been hypothesised to influence the relationship between reproduction and longevity, modifying, and even reversing, the typical negative fecundity-longevity association (trade-off) observed in many organisms. However, while advanced eusocial societies appear to exhibit a true abolition of this trade-off, with queens exhibiting a positive fecundity-longevity association, in intermediate eusocial systems like that of the bumblebee *Bombus terrestris*, queens and reproductive workers show evidence of costs associated with reproduction. This suggests that the apparent absence of the fecundity-longevity trade-off in intermediate eusocial systems may be contingent upon individual quality, with only high-quality, well-resourced individuals being able to overcome the costs of reproduction. By manipulating the quantity of nutrition provided to worker-destined *B. terrestris* larvae, this study set out to test the hypothesis that positive associations between fecundity and longevity among adult workers in *B. terrestris* arise from within-colony variation in intrinsic worker quality, which in turn result from rearing conditions during their development, and specifically from the level of larval nutrition provided. The results showed that the expected differences in longevity, egg-laying rate, egg-eating rate, and aggression between workers fed a high quantity of food as larvae and workers fed a low quantity of food as larvae were not statistically significant. Furthermore, workers from both the high-feeding and low-feeding treatments did not differ in adult body size and the proportion of workers from both treatments that were observed performing egg-laying behaviours did not differ significantly. These findings indicate that the manipulation of larval nutrition did not effectively generate sets of workers with distinct body sizes and so did not create the intended variations in intrinsic quality between treatments. Nevertheless, associations between reproductive behaviours and longevity were still evident. Combining data from all experimental (focal) workers showed that there was a positive relationship between egg-laying and longevity, with workers that laid eggs having significantly greater longevity and larger body size than non-laying workers. Additionally, the data revealed significant associations among workers between egg-laying, egg-eating, aggression, and body size. These findings support the hypothesis that variations in intrinsic worker quality, as reflected in body size, underlie positive associations between fecundity and longevity in *B. terrestris* workers. The results also provide further evidence of the links between reproductive dominance, egg-laying behaviour, and aggression in *B. terrestris* and comparable social systems. However, future investigations should explore the effects of larval nutrition on body size and intrinsic quality experimentally to gain deeper insights into these relationships.

4.2 Introduction

Survival and fecundity are considered the two main components of evolutionary fitness. However, in most organisms, both cannot be maximised simultaneously. As a result, in many species, a negative fecundity-longevity association is thought to occur (Stearns, 1989; Kirkwood and Rose, 1991). This trade-off is proposed to arise as organisms only have limited resources for growth, maintenance, and reproduction and, as natural selection prioritises reproductive success over survival, the allocation of resources towards reproduction comes at the expense of maintenance and repair mechanisms that promote longevity. The fecundity-longevity trade-off may, at the proximate level, result in molecular damage accumulating in an organism over time, resulting in a decreased probability of survival per unit time beyond a certain age, i.e., ageing (Williams, 1957; Hamilton, 1966; Kirkwood, 1977; Stearns, 1992; Edward and Chapman, 2011).

However, the fecundity-longevity trade-off is not universally applicable to all species. For example, current research indicates that, in multiple taxa, sociality can alter the relationship between reproduction and longevity, with reproductive individuals that live in cooperatively breeding groups, or eusocial societies, having significantly longer lifespans compared to their non-social relatives (Kim et al., 2011; Hammers et al., 2019; Korb and Heinze, 2021). Evidence of the absence of the fecundity-longevity trade-off is perhaps most apparent in eusocial Hymenoptera (ants, bees, wasps) and termites, in which reproductive castes (queens and kings) have been shown to have significantly longer lifespans in comparison to non-reproductive, or less-reproductive, castes (workers) (Keller and Genoud, 1997; Bourke, 2007; Lopez-Vaamonde et al., 2009; Kramer et al., 2015; Southon et al., 2015; Rodrigues and Flatt, 2016). In addition, research has suggested a positive fecundity-longevity relationship may even exist among reproductive individuals within the worker caste of eusocial insects. For example, research has shown that in species of ants (Negroni et al., 2021), honeybees (Dixon et al., 2014) and bumblebees (Blacher et al., 2017), workers that activate their ovaries and/or reproduce (via asexual reproduction of haploid sons) live longer than workers that do not reproduce during their lifetime.

Yet, while there is clear evidence for a positive longevity-fecundity relationship in reproductive and worker castes of some eusocial species that exhibit an 'advanced' level of eusociality and a perennial life cycle (Dixon et al., 2014; Schrempf et al., 2017), recent research has suggested that in eusocial insects that exhibit an intermediate level of eusociality and an annual life-cycle, the reversal of the fecundity-longevity trade-off in queens and workers may be condition dependent, with only high quality, well-resourced individuals able to overcome the costs of reproduction (Blacher et al., 2017; Collins et al., 2023).

Given the high relatedness observed among nest-mate workers of *B. terrestris* and other eusocial Hymenoptera with monandrous queens, whereby workers are more closely related to their sisters (workers and gynes [new queens]) (r = 0.75) than to their own sons (r = 0.5) (Hamilton, 1964; Trivers and Hare, 1976), the question arises as to how they might differ so substantially in intrinsic quality, to the extent that some are able to overcome the costs of reproduction while others cannot. It therefore seems possible that genetic differences alone cannot explain the significant variation in intrinsic quality observed. Instead, it is possible that environmental factors play a crucial role in determining a worker's ability to overcome the costs of reproduction. Accordingly, Blacher et al. (2017) suggested that the variation in workers' intrinsic quality could be attributed to differences in their rearing conditions during development. Specifically, they hypothesised that the workers receiving a higher quantity of larval nutrition during development would eclose as adults with larger body sizes. In turn, this would contribute to enhanced reproductive success and longevity, as larger body sizes provide these workers with superior physical conditions and resource-holding abilities, compared to smaller workers. As research has already shown that, in *Bombus* species, worker-destined larvae receive differential resource allocation, depending on their location within the colony (Couvillon and Dornhaus, 2009), this suggestion is plausible. Indeed, previous research has shown that workers' adult body size can vary greatly (Goulson, 2003) and that this variation in size is at least partially influenced by such factors as the quantity of food provided during development and the rate of larval feeding (Pereboom, 2001; Couvillon and Dornhaus, 2009; Shpigler et al., 2013). Furthermore, a link between larval nutrition and adult fecundity has previously been suggested in honeybee workers (Hoover et al., 2006) and a recent study has also show that in ants, workers' longevity was affected by the ratio of protein to carbohydrate in the larval diet (Choppin et al., 2023). This suggests that larval nutrition can have a significant impact on workers' size, reproductivity and longevity in various eusocial Hymenoptera societies.

However, while it is clear that the quantity of food provided during larval development can influence a worker's body size in *B. terrestris,* it is currently unclear if body size influences a worker's reproductive status. In the study by Blacher et al. (2017), it was observed that workers with activated ovaries were significantly larger and exhibited greater longevity than workers with inactive ovaries. However, previous research on *Bombus* species has indicated that ovarian activation does not necessarily result in a worker performing egg-laying events during its lifetime (Alaux et al., 2004; Sibbald and Plowright, 2014). Furthermore, studies that have investigated the relationship between egg-laying rate and body size of workers have found no correlation, suggesting that body size may not play a crucial role in determining a worker's likelihood of becoming an egg-layer (Duchateau and Velthuis, 1989). Consequently, it remains unclear if a relationship between body size, reproduction and longevity would still be found in *B. terrestris* workers if reproduction were measured based on the rate of egg-laying, rather than the degree of ovarian activation alone.

Further to this, it is unclear if intrinsic quality affects the rates at which *B. terrestris* workers perform egg-eating events and acts of aggression. In general, workers are only selected to perform egg-laying events late in the colony cycle when gyne production has been initiated and queens are mainly laying unfertilised eggs that will develop into males, an event termed, in bumblebees, the *switch point* (Duchateau and Velthuis, 1988). At this stage in the colony cycle, it is in the fitness interests of workers to compete aggressively with the queen and other workers over male parentage because of their higher relatedness to their sons (r = 0.5) over nephews (r = 0.375) or the queen's sons (r = 0.25). Following the *competition point* (first appearance of worker-laid eggs), colonies of *B. terrestris* are indeed characterised by aggressive interactions, worker egg-laying, and egg policing (egg-eating) by both the queen and workers (Duchateau, 1989; Bourke and Ratnieks, 2001; Zanette et al., 2012). Yet, *B. terrestris* workers have been shown to vary widely in the rates at which they perform egg-laying, egg-eating and aggressive behaviours (Zanette et al., 2012, Chapter 2, Chapter 3), and results from this thesis (Chapters 2 and 3) suggest that there is a positive association between egg-laying, egg-eating, aggression and reproductive success. If reproductive success is determined not only by the rate of egg-laying but also by the rate of egg-eating and aggression, as evidenced in this thesis, it would be expected that the highest quality workers would perform all three behaviours at the highest rates. However, it is currently unclear if egg-eating and aggression are indicators of high worker quality or if workers, that perform all three behaviours, exhibit higher longevity than workers that performed egg-laying events but do not perform egg-eating events.

Therefore, the primary objective of the current study was to test the hypothesis that positive associations between fecundity and longevity among adult workers in *B. terrestris* arise from within-colony variation in intrinsic worker quality resulting from rearing conditions during larval development and specifically from the level of larval nutrition. To address this, we conducted an experiment in which the quantity of pollen provided to larvae during development was manipulated, to assess its impact on the behaviour, egg-laying rate and longevity of the adult workers developing from these larvae. The experimental design involved dividing colonies into two compartments, each representing a different treatment group. In one treatment compartment, food supply was manipulated with the aim of worker-destined larvae receiving 70% of the daily per capita requirement of pollen throughout their development. In the other treatment group, food supply was manipulated with the aim of worker-destined larvae receiving 130% of the daily per capita pollen requirement. After reaching adulthood, the workers eclosing from the larvae receiving these different feeding treatments were individually marked and observed for acts of aggression, egg-laying and egg-eating. Additionally, we tested for the existence of a positive relationship between egg-laying and longevity. The hypothesis predicted that adult workers that, as larvae, received higher levels of nutrition, would have larger body sizes, show increased rates of egg-laying and display behaviours associated with higher reproductive success (i.e., egg-eating and aggression). Furthermore, it predicted that these workers would live longer than workers that received lower levels of nutrition during larval development.

4.3 Materials & Methods

4.3.1 Rearing and development of *Bombus terrestris* colonies and treatment assignment

Twelve pre-competition point *Bombus terrestris audax* colonies were obtained from a commercial supplier (Biobest, Westerlos, Belgium). On delivery (10 February 2022), each colony contained 1 queen, a mean (± SD) of 25.3 ± 5.1 workers and a small quantity of brood. Colonies were randomly assigned a unique number (nos. 1-12) and transferred on 11 February 2022 into wooden nest-boxes (internal dimensions 17 cm × 27.5 cm × 16 cm high) with clear Perspex lids. Prior to transfers, each nest-box was marked with a horizontal line to serve as a reference for where nest-boxes could later be partitioned to produce two compartments of equal size (internal dimension 8.5 cm long × 17 cm wide × 16 cm high).

In each colony, brood from a given colony was transferred to each compartment of that colony's wooden nest-box. During transfers, for a given colony, each compartments' group size and composition were equalised. For this, in each colony, only wax-cells assessed from their external appearance to contain eggs were selected for transfer, being placed randomly and equally into the centre of the floor of each of the two marked-out compartments. Wax-cells containing larvae in the 1st- 4th instars were excluded from transfers as the development of any larvae present on delivery had already been influenced by external factors, such as temperature and food availability, prior to the start of the treatment phase of the experiment. Throughout the experiment, focal workers were defined as the adult workers developing from the larvae exposed to the nutritional treatments, and non-focal workers were defined as all other workers. To ensure sufficient non-focal workers were present within a colony to perform brood care behaviours (thermoregulation and larval feeding) for future larvae that would develop into focal workers, during the set-up of the compartments all pupae present on delivery were randomly and equally distributed between the two marked-out compartments. Following brood transfer, the colony queen and adult workers (non-focal workers) were transferred and, between the 11 February 2022 and 13 February 2022, allowed to move freely between both nest-box compartments. During this 2-day period colonies were provided with *ad libitum* sugar syrup and fresh pollen (Biobest, Westerlos, Belgium) to allow colonies to adjust to laboratory conditions. All colonies were kept under standard conditions (28°C, 60% relative humidity, constant darkness) for the duration of the experiment.

On 13 February 2022, non-focal workers were removed from each colony and a sheet of fine wire mesh (17 cm × 16 cm) was fixed vertically along the marked central partition of the nest-box, effectively separating the two compartments by preventing the passage of the queen and workers between compartments, while maintaining the two compartments of a given colony in a common airspace. Each compartment within a nest-box was then randomly allocated to one of the following treatments: (1) a high-feeding treatment with the aim of supplying larvae with 130% of the mean per capita per day larval pollen and syrup intake and (2) a low-feeding treatment with the aim of supplying larvae with 70% of the mean per capita per day larval pollen and syrup intake (**Figure 4.1**; **Section 4.3.2**).

The non-focal workers present within a colony were then randomly split into two equal-sized groups and allocated to one of the two treatments. If there was an unequal number of non-focal workers present, a single worker was removed and frozen to ensure two equal-sized treatment groups were produced. To facilitate the identification of non-focal workers, in each colony, non-focal workers assigned to the high-feeding treatment group were marked on the thorax with a non-toxic purple marking paint (Queen Marking Paint, Thorne Ltd., UK), while those assigned to the low-feeding treatment group were marked with a non-toxic blue marking paint. As worker-destined pupae require 9.9 ± 0.9 days to fully develop and eclose as an adult worker (Cnaani et al., 2000; Tian and Hines, 2018), any workers that eclosed within the first 12 days following the colony transfer (i.e., from the pupae placed in compartments at the start of the experiment) was considered to be a non-focal worker. Depending on the treatment (high- or low-feeding) group from which they eclosed, newly-eclosed non-focal workers were marked on the thorax with either blue or purple marking paint before being returned to the treatment compartment of origin (**Figure 4.1**).

Previous research has suggested that *B. terrestris* workers and diploid (female) larvae require direct contact with the queen in order to detect her presence and pheromone output (Alaux et al., 2004). If adult workers cannot detect the queen, they may activate their ovaries and begin egg-laying within 7-8 days (Duchateau and Velthuis, 1989; Alaux et al., 2007). Moreover, the regulation of caste-determination in diploid larvae is likely to be pheromonally mediated by the queen, as such uncommitted diploid larvae (under 5 days old) may develop into gynes if they are unable to detect the queen (Cnaani et al., 1997; Bourke and Ratnieks, 2001; Alaux et al., 2005). Therefore, to minimise the effects of queen absence, and ensure non-focal workers and uncommitted diploid larvae in each compartment were equally exposed to the queen, colony queens were transferred between treatment groups (i.e., from one compartment to the other) three times a day. For each of the 12 colonies, the colony queen was initially randomly assigned to one of the treatment groups, with six queens starting in the high-feeding treatment compartment and six queens starting in the low-feeding treatment compartment. Colony queens were then transferred across the mesh partition into the compartment with the opposing treatment group every 6-12 hours (transferred at 08:00, 14:00 and 20:00) for the duration of the treatment phase, resulting in a 12-hour presence of the queen in one treatment compartment overnight one night of two successive days, and in the opposing treatment compartment overnight the following night.

4.3.2 Treatment phase: feeding regime and mixing regime

The treatment phase of the experiment was split into two phases (1) a feeding regime phase: in which the nutritional treatment was applied to all larvae and non-focal workers present; and (2) a mixing regime phase: in which all eclosed focal workers and non-focal workers were mixed between treatment compartments every 48 hours. The feeding regime of the treatment phase took place in the period 13 February 2022 – 4 March 2022. Each treatment group in each colony was monitored daily in order to (1) census and individually mark newly-eclosed non-focal and focal workers, (2) census wax cells containing eggs and larvae, and (3) determine daily syrup and pollen consumption rates. During the daily inspections, any dead non-focal workers were removed, with the date of death and compartment of origin recorded.

During the feeding regime, to determine the daily quantity of pollen and syrup required by each treatment group within a colony, all brood and adult workers within each treatment were individually counted daily between 08:00 and 10:00. As eggs and 1st - 3rd instar larvae reside in communal wax-cells (**Figure 4.2**), it was not possible to count individual eggs or 1st- 3rd instar larvae directly. However, *Bombus* queens have been shown to lay approximately 8-10 eggs into an egg-cell during an egg-laying event (Alford, 1975; Duchateau and Velthuis, 1988; Zanette et al., 2012). Therefore, it was estimated that there were nine eggs in any wax-cell containing eggs and, likewise, any wax-cells containing 1st – 3rd instar larvae were estimated to contained nine individual larvae (**Figure 4.2**). As 4th instar larvae reside in individual wax-cells, it was possible to count larvae in this stage of development at the individual level (**Figure 4.2**). From these census data, it was calculated how much pollen and syrup each compartment would receive that day according to the assigned treatment (see below). To minimise disturbance to colonies, in each treatment in each colony, pollen dishes and syrup dispensers were changed daily at 14:00, i.e., at the same time at which queens were transferred between treatment compartments. The in-going and out-going pollen dishes and syrup dispensers were weighed on an analytical balance (to the nearest 0.001 g) daily. The out-going pollen and syrup measurements were then subtracted from the in-going measurements from the day prior (i.e., 24 hours earlier) to calculate the quantity of pollen and syrup consumed during a 24-hour period, within a treatment compartment. Using the census data from the day before (number of larvae) it was therefore possible to calculate daily per capita estimated consumption rates for larvae in each treatment compartment in a colony.

Over the period 14 February 2022 – 18 February 2022, we used the median values of daily syrup and pollen consumption for individual workers and individual 1st-4th instar worker-destined larvae taken from Gradish et al. (2019) to calculate the standard quantity of pollen and syrup required daily by each treatment group within each colony, based on the number of non-focal workers and larvae present. These values were then manipulated to ensure that (1) groups assigned to the high-feeding treatment received 130% of median per capita per day larval pollen and syrup intake and (2) groups assigned to the low-feeding treatment received 70% of median per capita per day larval pollen and syrup intake. These nutritional levels (130% and 70%) were selected to create levels of variation in adult worker body size between the treatment groups no larger than that observed naturally within *B. terrestris* colonies (Owen, 1988; Goulson et al., 2002; Goulson, 2003). In both high and low feeding treatment groups, non-focal workers present were supplied with an estimated 100% of median per capita per worker pollen and syrup intake (Gradish et al 2019). This was to ensure that the non-focal workers rearing the larvae in both treatment groups had sufficient resources available to achieve the daily energy requirements needed to effectively perform brood care tasks such as larval feeding and thermoregulation (Heinrich, 2004).

From 19 February 2022, the standard quantity of pollen and syrup required per treatment per colony was re-calculated to account for a surplus of pollen and shortage of syrup that were observed in the daily out-going feeder measurements from both treatment groups. Using the data available, the mean estimated pollen and syrup consumption per larva across all colonies from the preceding 48-hour period was calculated daily and used as the new mean standard value of pollen and syrup required per larva. This per-capita consumption factor was therefore adjusted daily. For each treatment group in each colony, the mean standard value of pollen and syrup amount required was multiplied by the number of larvae present to calculate the per capita per day larval pollen and syrup requirement. These values were then again manipulated to ensure (1) groups assigned to the high-feeding treatment received 130% of mean per capita per day larval pollen and syrup intake and (2) groups assigned to the low-feeding treatment received 70% of mean per capita per day larval pollen and syrup intake. As adult *Bombus* workers prioritise carbohydrate intake and have a relatively low requirement for protein (Stabler et al., 2015), we continued to use the median value of daily pollen consumption taken from Gradish et al. (2019) to calculate the standard quantity of pollen required daily per non-focal worker. However, we increased the standard quantity of syrup required daily per worker by 34.6% to 318.3 mg (i.e., 2/3 of the range of values from Gradish et al. 2019).

To calculate the mean daily estimated consumption of pollen by individual focal worker larvae over the course of the feeding regime, for each treatment group within a colony the daily estimated consumption of pollen by non-focal workers was subtracted from the actual observed daily pollen consumption. The remaining value of actual observed daily pollen consumption was then divided by the total number of 1^{st}-4^{th} instar larvae present on the day of consumption, to provide a mean daily estimated consumption of pollen per individual larvae. The daily mean estimated consumption of pollen per individual larva over the course of the feeding regime period was then calculated for each treatment compartment within a colony. Using this data, the mean estimated consumption of pollen over the course of the feeding regime was calculated for each treatment.

Once a treatment compartment within a colony had reached a threshold of 15 or more focal worker pupae, the feeding treatment assigned to that specific compartment ended and all adult workers (non-focal and newly-eclosed focal workers) and brood within the treatment compartment were instead fed *ad libitum* (**Figure 4.1**). This marked the end of the feeding regime for that specific treatment compartment. Given that focal worker larvae underwent pupation at different time points both within, and between, treatment compartments of a colony, the eclosion of focal workers occurred over several days within a colony. Consequently, to accurately determine whether newly-eclosed focal workers belonged to the high- or low-feeding treatment, the wire mesh partition remained in place until all focal workers within both treatment compartments of a colony had eclosed and been appropriately marked (see below). During this time, colony queens continued to be transferred between treatment compartments every 6-12 hours until the mesh partition was removed.

Once focal workers began to eclose within a treatment compartment of a colony, the mixing regime of the treatment phase was implemented (**Figure 4.1**). The mixing regime phase took place from 02 March 2022 to 15 March 2022. For each colony, the specific start date of the mixing regime phase was determined by the date at which the first focal worker eclosed within a treatment compartment of the colony. Conversely, the end date of the mixing regime phase was determined by the date at which all focal workers had eclosed within a colony. All newly-eclosed focal workers were first censused and individually marked on the day of eclosion with a unique numbered disc glued to the thorax (Queen Marking Kit, Thorne Ltd., UK). To allow focal workers from the low and high feeding treatment group to be easily differentiated, within colonies it was ensured that focal workers that eclosed in opposing treatments were marked with different coloured numbered discs. Then every 48 hours all non-focal and marked focal workers were mixed equally between the two treatment compartments. The measure was taken to ensure that newly eclosed focal workers were exposed to comparable social environments as adults in both treatment compartments prior to the removal of the mesh partition (**Figure 4.1**).

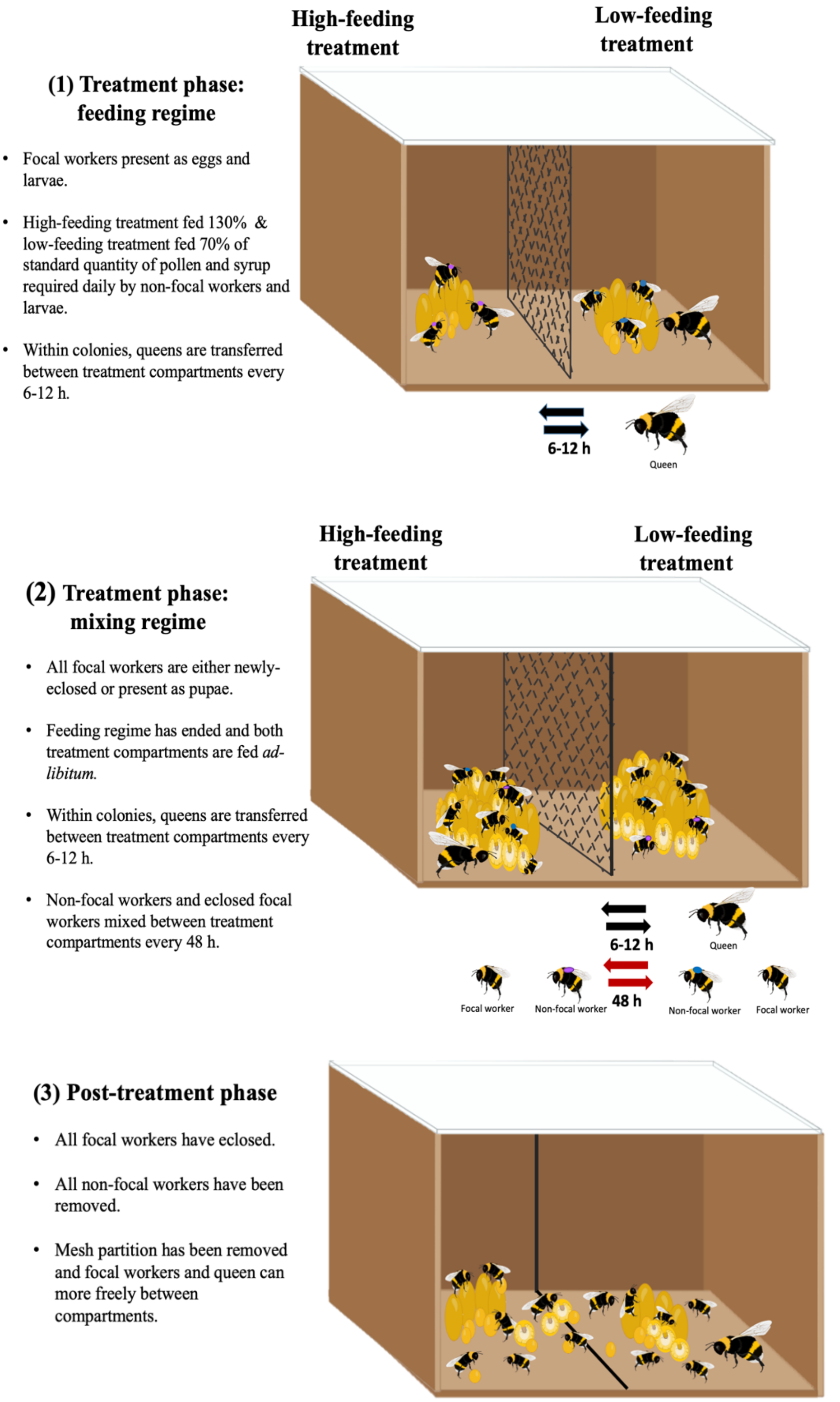
4.3.3 Treatment phase: behavioural observations

From the start of the feeding regime of the treatment phase, colonies were observed directly in 1-hour bouts once a day, five days a week (total of 13-14 h per colony over a period of 16-18 days). Using direct observations, it was possible to record the behaviour of non-focal workers in both treatment compartments of a colony during a single observation bout. For each colony, all larval feeding events (i.e., instances of adult workers providing food to larvae) in each treatment compartment were recorded (**Table 4.1**). Once focal workers began to eclose, they were monitored for behaviours of interest (see below); however, no focal workers were observed performing any egg-laying, egg-eating or aggressive behaviours prior to the mesh partition being removed.

4.3.4 Treatment phase: removal of mesh wire partition and non-focal workers

Once all focal workers had eclosed within both treatment compartments of a colony, the mixing regime of the treatment phase ended, and the mesh wire partition separating the two treatment compartments was removed, allowing focal workers and the colony queen unrestricted movement between the two halves of the colony, which previously represented separate treatment compartments (**Figure 4.1**)

In *Bombus* species, workers are observed forming dominance hierarchies among themselves by means of aggressive interactions and these hierarchies are thought to influence the reproductive success of individual workers (Chapter 2, Chapter 3). It has been suggested that workers from the first and second brood produced by the queen in the colony cycle monopolise the highest ranks (Van Doorn and Heringa, 1986; Duchateau, 1989) and may inhibit the ovary activation and reproductive success of younger workers (Van Doorn and Heringa, 1986; Duchateau and Velthuis, 1989; Bloch and Hefetz, 1999). Therefore, to ensure the behaviour and egg-laying rate of focal workers was not influenced by the presence of (older) non-focal workers, in each colony all non-focal workers were removed from the colony and frozen at -20°C once all focal workers had eclosed and been marked in a colony.



(1) Treatment phase: feeding regime

- Focal workers present as eggs and larvae.
- High-feeding treatment fed 130% & low-feeding treatment fed 70% of standard quantity of pollen and syrup required daily by non-focal workers and larvae.
- Within colonies, queens are transferred between treatment compartments every 6-12 h.

(2) Treatment phase: mixing regime

- All focal workers are either newly-eclosed or present as pupae.
- Feeding regime has ended and both treatment compartments are fed *ad-libitum.*
- Within colonies, queens are transferred between treatment compartments every 6-12 h.
- Non-focal workers and eclosed focal workers mixed between treatment compartments every 48 h.

(3) Post-treatment phase

- All focal workers have eclosed.
- All non-focal workers have been removed.
- Mesh partition has been removed and focal workers and queen can more freely between compartments.

Figure 4.1. Schematic visualisation of experimental set-up and stages. (1) Treatment phase (feeding regime): focal workers present as eggs and larvae and treatment groups separated by mesh partition. High-feeding treatments supplied daily with 130% of the mean per capita per day larval pollen and syrup intake and low-feedings treatment with 70% of mean per capita per day larval pollen and syrup intake. Colony queen transferred between treatments every 6-12 hours. (2) Treatment phase (mixing regime): all focal workers present as pupae or newly-eclosed adults. Feeding treatments have ended and both compartments are fed *ad libitum*. Queen transferred every 6-12 hours and adult workers mixed every 48 hours. (3) Post-treatment phase, following point at which all focal workers have eclosed. Non-focal workers have been removed, the mesh partition has been removed, and colonies are each directly observed 1 hour a day, 5 days a week.

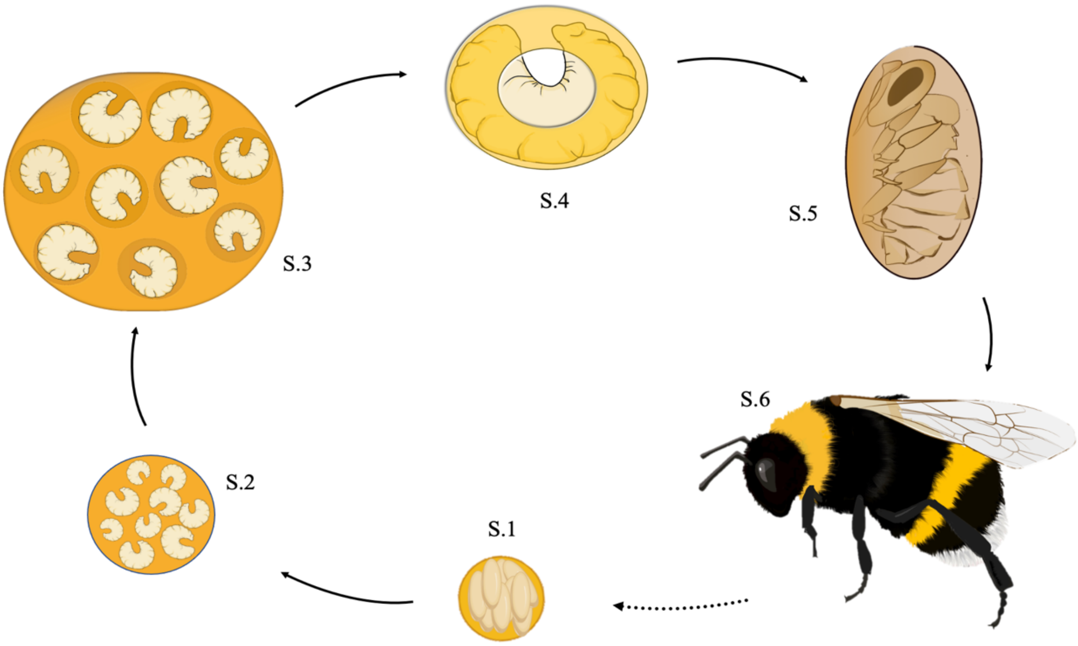
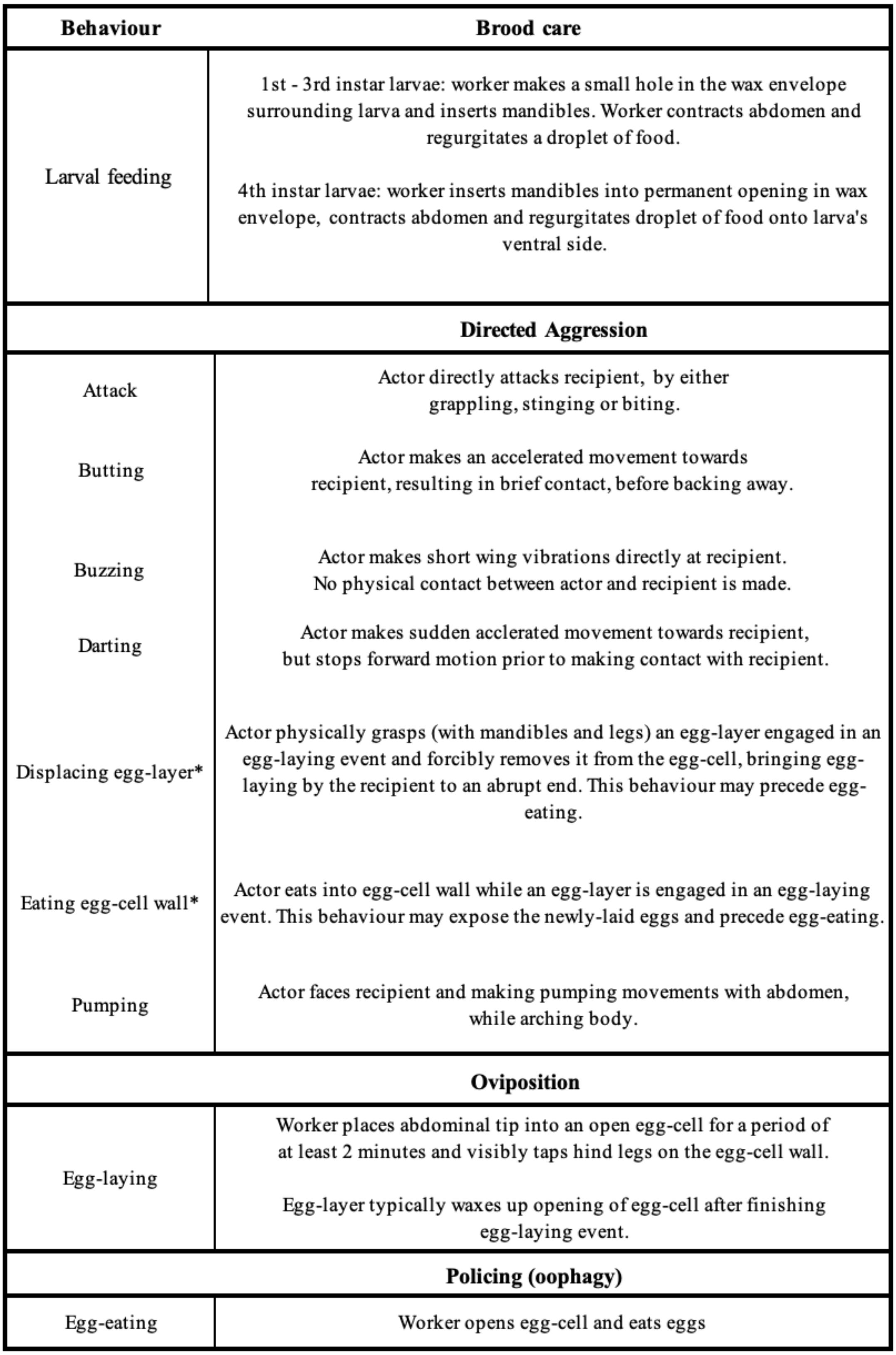


Figure 4.2. The developmental stages (S) of a *Bombus* adult (both castes and sexes). (S.1) egg clump in communal wax-cell; (S.2) 1st -2nd instar larvae in communal wax-cell; (S.3) 3rd instar larvae in communal wax-cell; (S.4) 4th instar larva in individual wax-cell, with permanent opening; (S.5) pupa in silken cocoon; (S.6) eclosed adult. All eggs and larvae in 1st-3rd instars are covered in a wax envelope; however, for the purposes of illustrating these developmental stages, this is absent in the figure. Arrow with dotted line indicates that only queens are able to lay diploid eggs (developing into queens and workers) and only queens and workers are able to lay haploid eggs (developing into males).



Behaviour	Brood care
Larval feeding	1st - 3rd instar larvae: worker makes a small hole in the wax envelope surrounding larva and inserts mandibles. Worker contracts abdomen and regurgitates a droplet of food. 4th instar larvae: worker inserts mandibles into permanent opening in wax envelope, contracts abdomen and regurgitates droplet of food onto larva's ventral side.
	Directed Aggression
Attack	Actor directly attacks recipient, by either grappling, stinging or biting.
Butting	Actor makes an accelerated movement towards 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 acclerated movement towards recipient, but stops forward motion prior to making contact with recipient.
Displacing egg-layer*	Actor physically grasps (with mandibles and legs) an egg-layer engaged in an egg-laying event and forcibly removes it from the egg-cell, bringing egg-laying by the recipient to an abrupt end. This behaviour may precede egg-eating.
Eating egg-cell wall*	Actor eats into egg-cell wall while an egg-layer is engaged in an egg-laying event. This behaviour may expose the newly-laid eggs and precede egg-eating.
Pumping	Actor faces recipient and making pumping movements with abdomen, while arching body.
	Oviposition
Egg-laying	Worker places abdominal tip into an open egg-cell for a period of at least 2 minutes and visibly taps hind legs on the egg-cell wall. Egg-layer typically waxes up opening of egg-cell after finishing egg-laying event.
	Policing (oophagy)
Egg-eating	Worker opens egg-cell and eats eggs

Table 4.1. Behaviours in *Bombus terrestris* workers recorded during the treatment and post-treatment phase of the experiment. Behaviours marked with * were first classified by myself in Chapter 2 of this thesis. All other behaviours are as described in Duchateau (1989), Bloch and Hefetz (1999) or den Boer and Duchateau (2006).

4.3.5 Post-treatment phase: behavioural observations

The post-treatment phase of the experiment took place between 4 March 2022 and 22 August 2022. From 4 March 2022 to 16 June 2022 colonies were again directly observed once a day, five times a week (total of 52-54 hours per colony over a period of 93-94 days). In two colonies (nos. 7 and 8), all brood that eclosed within treatments consisted of either gynes or males. As no focal workers were produced in either of these two colonies, both colonies were excluded from the experiment. Therefore, all direct observations in the post-treatment phase occurred across 10 colonies (nos. 1-6, 9-12) (**Table A4.1**).

In the direct observation bouts, all egg-laying and egg-eating behaviours were recorded, along with the identity of the egg-layer or egg-eater (number and colour of disc) (**Table 4.1**; **Figure 2.2**). For all focal workers within a colony, rates of both egg-laying and egg-eating were quantified as the rate of events observed per individual per hour, where 'event' referred to either an individual being observed to lay eggs into an egg-cell (egg-laying) or to an individual being observed to eat eggs from an egg-cell (egg-eating). The actual numbers of eggs laid, and eggs eaten per hour could not be accurately quantified, because egg-cells typically contain multiple eggs (Zanette *et al.* 2012; Almond *et al.* 2019; **Figure 4.2**), with more than one egg being laid or eaten per event, and during most egg-laying and egg-eating events the depth of the egg-cell and the position of the workers' body prevented the number of eggs laid or eaten being accurately counted.

In addition to egg-laying and egg-eating events, occurrences of any directed aggressive behaviours were recorded during direct observations, along with the identities of the actors and the recipients of each behaviour and the duration of the aggressive act (**Table 4.1**). Direct observations in the post-treatment phase ended on 16 June 2022, as focal workers within all colonies had by then largely stopped performing the behaviours of interest.

4.3.6 Post-treatment phase: measurement of focal worker longevity

To measure longevity in focal workers each colony was observed for 30 s once a day from the beginning of the post-treatment phase until the last focal worker’s death. Any dead focal workers observed were removed. The identity of the worker, the treatment group of origin and the date of death were recorded. Worker longevity was defined as the interval in days between a worker's eclosion and death. All dead focal workers were individually stored at -20 °C in order to take marginal wing cell length measurements at a later date.

4.3.7 Body size measurements

After the end of the experiment, the marginal wing cell length in a forewing was measured in all available focal workers in each colony under a *Zeiss SteREO Discovery.V12* dissection microscope. For each focal worker, a forewing was photographed at 15 × magnification using an AxioVison camera. The marginal wing cell of the forewing was then measured in the image using AxioVison software. In *Bombus*, the length of the marginal cell in the forewing is a reliable index of total body size (Duchateau, 1989; Owen, 1989; Goulson et al., 2002). All wing cell measurements were performed blindly with respect to worker longevity and behaviour.

4.3.8 Statistical analyses

All statistical analyses were performed using R v 4.2.1 (R Core Team 2022).

A generalised linear mixed effects model (GLMM) with a binomial distribution was constructed to analyse if the proportion of focal workers that were observed egg-laying differed depending on whether the focal worker developed (from egg to eclosed adult) in the high-feeding or low-feeding treatment. The GLMM was implemented using the package *lme4* (Bates *et al.* 2015). Numbers of egg-laying and non-laying focal workers were used as fixed effects, with treatment of origin (high-feeding or low-feeding) as a covariate and colony as a random effect. A type 'II' (sum of squares) ANOVA was performed before pairwise comparisons were obtained using the *contrast* and *lsmeans* functions in the *lsmeans* package (Lenth, 2016). A Benjamini-Hochberg correction was applied to adjust the threshold of significance to correct for multiple comparisons. Scaled residuals and overall model fit were assessed using the *DHARMa* package (Hartig 2022).

In order to determine if there was an association between body size, reproductive status and treatment of origin, an analysis was implemented to compare the length of marginal wing cell (index of total body size) between egg-laying and non-laying focal workers. A linear mixed model (LMM) was constructed using the packages described above. However, the model was fitted with a gaussian distribution. To account for variation in the distribution of the residuals, marginal wing cell was used as a log-transformed fixed effect. A focal workers' egg-laying status (egg-laying/non-layer) and treatment were used as covariates and colony of origin was used as a random effect. A type 'III' (sum of squares) ANOVA was performed followed by pairwise comparisons using the *contrast* and *lsmeans* functions from the *lsmeans* package (Lenth, 2016). A Benjamini-Hochberg correction was applied for pairwise comparisons to adjust the threshold of significance to correct for multiple comparisons. In order to determine if there was a significant association between focal worker body size and display of reproductive behaviours (egg-laying and/or egg-eating), a second analysis comparing the length of the marginal wing cell between different classes of focal worker (**Table 2.2**) was also implemented as described above. However, in this analysis the model fit the data well, without needing to transform the length of the marginal wing cell. In both analyses, scaled residuals and overall model fit were assessed using the *DHARMa* package (Hartig 2022).

A final LMM was constructed with a gaussian distribution to analyse if the longevity of focal workers differed depending on the treatment of origin and class of worker (**Table 2.2**). The LMM was implemented using the packages described above. Focal worker longevity and class of worker were used as fixed effects, with treatment as a covariate and colony as a random effect. A type 'III' (sum of squares) ANOVA was performed before pairwise comparisons were obtained. A ' Benjamini-Hochberg' correction was applied to adjust the threshold of significance to correct for multiple comparisons before scaled residuals and overall model fit were assessed using the *DHARMa* package (Hartig 2022).

To analyse rates of egg-laying performed by focal workers, for each focal worker in each colony all egg-laying events performed during direct observations were pooled together and converted into rates in units of events per hour for each individual. The same procedure was followed for egg-eating events to determine rates of egg-eating. All acts of aggressive behaviour were pooled together for each focal worker in each colony. The pooled aggressive behaviours were classed as 'acts of aggression'. Again, acts of aggression were converted into rates of events/h per individual. As the behavioural rate data consisted of zero-inflated continuous data, the comparison of rates of behaviour (egg-laying events, egg-eating events, acts of aggression) between egg-laying workers in each treatment was analysed using unpaired two-sample Wilcoxon tests in the *stats* package (R Core Team, 2022). Similarly, to analyse rates of egg-laying, egg-eating and aggression by focal workers of different class, count data taken from direct observations were pooled together for each individual focal worker and converted into rates of events/h per individual. Comparisons between, respectively, the rates of egg-laying and egg-eating by LEEs and LNEEs and LEEs and NLEEs, as defined in Chapter 2 (**Table 2.2**), were tested using unpaired two-sample Wilcoxon tests. A comparison of the rate of aggression performed by focal workers of different class was tested using the Kruskal-Wallis rank sum test in the *native stats* package (R Core Team, 2022). Multiple pairwise comparisons were obtained using Dunn’s test in the *FSA* package (Ogle et al. 2022) and a ' Benjamini-Hochberg ' correction was applied to adjust the threshold of significance to correct for multiple comparisons.

To test if there was an effect of treatment of origin on the longevity of focal workers, two survival analyses were performed and visualised with Kaplan-Meir plots using the *survival* package (Therneau, 2022). All focal workers that did not have a date of death recorded were excluded from analyses ($n_{\text{number of focal workers excluded for survival analyses}} = 34$, $n_{\text{number of focal workers included in survival analyses}} = 371$). For all other focal workers, either a time to event recording (the length of time between an eclosion and date of natural death) or a censored recording (the length of time between an eclosion and date of unnatural death) was used. Focal workers were considered to have had an unnatural death if they died of accidental causes (e.g., drowned in syrup feeders). The first survival analysis compared the longevity of all focal workers from the high-feeding treatment against all focal workers from the low-feeding treatment. The second analysis compared only the longevity of egg-laying focal workers in both treatments (i.e., all focal workers that were not observed performing egg-laying events were excluded from analysis). Cox proportional-hazards models were then constructed with the *survival* package to test the effects of treatment and colony on the longevity of (1) all focal workers in both treatments and (2) only egg-laying focal workers in both treatments. Initially an inspection of Schoenfeld residuals revealed the categorical variable 'Colony' did not meet the proportional-hazards assumption; however, the stratification of this variable allowed for the assumptions of the proportional-hazards model to be met. Following the procedures above, two further survival analyses were performed to test (1) if there was an effect of egg-laying behaviour on the longevity of focal workers, regardless of treatment, and (2) if there was a further effect of class of worker (**Table 2.2**) on the longevity of focal workers.

4.4 Results

4.4.1 High-feeding treatment vs low-feeding treatment: focal worker longevity

Following the end of the treatment phase, each of the 10 colonies used in analyses produced a mean (± SD) of 20.0 ± 11.6 focal workers in the high-feeding treatment (H-workers) and 19.8 ± 13.2 focal workers in the low-feeding treatment (L-workers) with a total of 399 focal workers marked overall ($n_{\text{total number of H-workers across colonies}} = 200$, $n_{\text{total number of L-workers eclosed across colonies}} = 199$; **Table A4.1**). Overall, the estimated mean daily consumption of pollen by individual focal workers during larval development was significantly higher for focal workers that eclosed in the high-feeding compartment, compared to focal workers that eclosed in low-feeding compartment (Wilcoxon test: W = 88, *p* = 0.004; **Table A4.1**).

It was possible to continuously monitor the survival of 371 (93.2%) individual focal workers from the date of eclosion to the date of death ($n_{\text{H-workers}} = 199$, $n_{\text{L-workers}} = 172$). When all 371 focal workers were considered, irrespective of individual rates of behaviour, there was no relationship between a focal worker's longevity and the treatment of origin (Cox's proportional hazards analysis: hazard ratio = 0.85, 95% Cl [0.68, 1.07], *p* = 0.2; **Figure 4.3A**), with H-workers having a median survival time (longevity) of 95 days (95% CI [89, 105]) and L-workers a median survival time (longevity) of 100 days (95% CI [92, 113]). An analysis that compared only the longevity of egg-laying focal workers in both treatments ($n_{\text{H-workers observed egg-laying}} = 79$, $n_{\text{L-workers observed egg-laying}} = 64$) also found no significant difference in the longevity of focal workers observed egg-laying in either treatment (Cox's proportional hazards analysis: hazard ratio = 0.86, 95% Cl [0.59, 1.27], $p = 0.5$; **Figure 4.3B**). Egg-laying H-workers were found to have a median survival time (longevity) of 113 days (95% CI [107, 119]) and egg-laying L-workers had a median survival time (longevity) of 116 days (95% CI [104, 129]). Therefore, the prediction that focal workers in the high-feeding larval treatment would be longer lived as adults was not supported, either for all focal workers or for egg-laying focal workers alone.

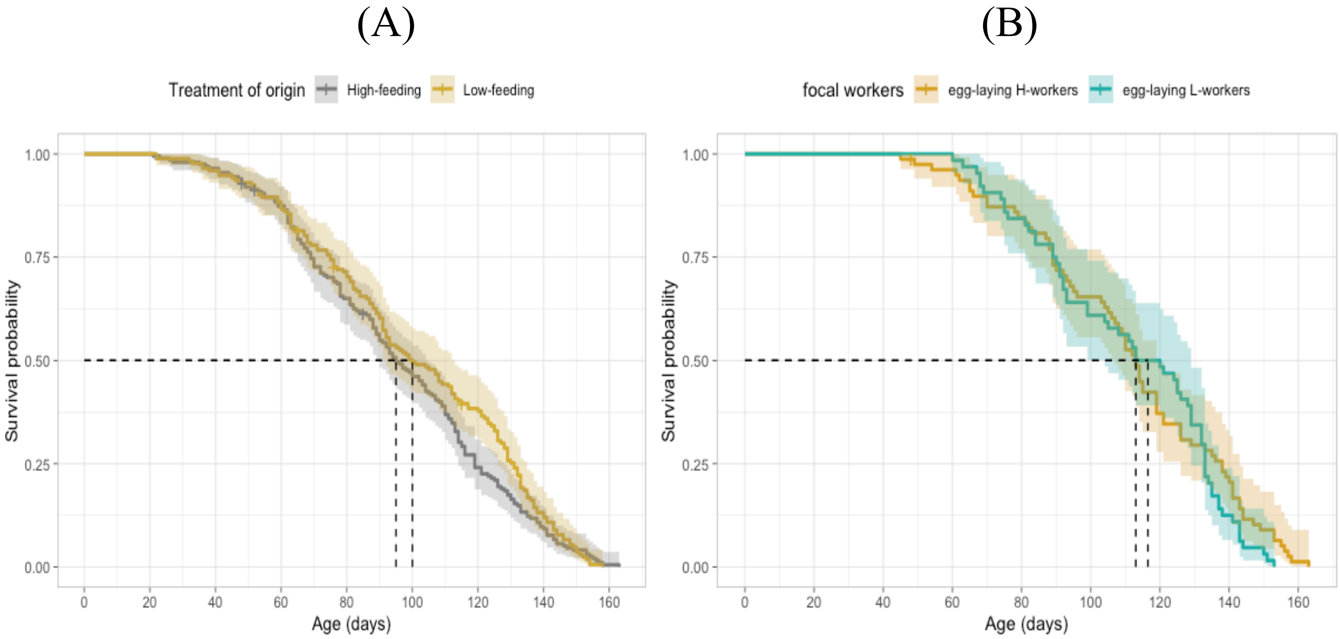


Figure 4.3. Relationship between longevity and treatment of origin in *Bombus terrestris* workers. (A) longevity (days between focal worker eclosion and death) of all H-workers (from the high-feeding treatment) and all L-workers (from the low-feeding treatment) (censored recordings: $n_{H\text{-}workers} = 3$, $n_{L\text{-}workers} = 3$; time to event recordings: $n_{H\text{-}workers} = 181$, $n_{L\text{-}workers} = 157$). (B) longevity of egg-laying H-workers and egg-laying L-workers (censored recordings: $n_{egg\text{-}laying\ H\text{-}workers} = 1$, $n_{egg\text{-}laying\ L\text{-}workers} = 0$; time to event recordings $n_{egg\text{-}laying\ H\text{-}workers} = 71$, $n_{egg\text{-}laying\ L\text{-}workers} = 60$). Dashed lines represent the median survival time (longevity) for each treatment group, which corresponds to a survival probability of 50%. Data were obtained from 10 *B. terrestris* colonies. Data include censored data and were collected during the post-treatment phase of the experiment, over a period of 171 days.

4.4.2 High-feeding treatment vs low-feeding treatment: rates of egg-laying, egg-eating, and aggression performed by focal workers

Behavioural data was collected from a total of 540 h of observations, with each colony being observed for a mean (± SD) of 54.0 ± 0.9 hours over a period of 93-94 days, following the start of the post-treatment phase. Overall, 279 egg-laying events were observed within colonies ($n_{\textit{egg-laying events performed by H-workers}} = 151$, $_{\textit{egg-laying events performed by L-workers}} = 128$), with a mean of 40.0% and 32.0% of H-workers and L-workers observed performing egg-laying events, respectively. Though the proportion of L-workers that were observed egg-laying was lower than the corresponding proportion in H-workers, the difference was not significant ($n_{\textit{H-workers observed egg-laying}} = 81$, $_{\textit{L-workers observed egg-laying}} = 66$) (GLMM: egg-laying H-workers vs egg-laying L-workers; Z = -1.54, *p* = 0.12; **Table A4.2**).

The mean rate at which egg-laying H- and L-workers performed egg-laying events did not differ significantly (Wilcoxon test: H-workers vs L-workers, W = 49, *p* = 0.76; **Figure 4.4**, **Table A4.3**), being 0.04 ± 0.00 and 0.04 ± 0.00 events per individual per hour for H-workers and L-workers, respectively. Of the H-workers and L-workers observed performing egg-laying events, 23 (28.4%) of the egg-laying H-workers and 22 (33.3%) of the egg-laying L-workers were also observed performing egg-eating events during direct observations. In total, H- and L-workers observed egg-laying were also observed performing 101 egg-eating events during direct observations ($n_{\text{egg-eating events performed by egg-laying H-workers}} = 54$, $_{\text{egg-eating events performed by egg-laying L-workers}} = 47$). However, as with egg-laying events, the mean rate at which these H- and L- workers performed egg-eating events did not differ significantly (Wilcoxon test: H-workers vs L-workers, W = 36.5, *p* = 0.50; **Figure 4.4**, **Table A4.3**), being 0.01 ± 0.00 and 0.02 ± 0.00 events per individual per hour for H-worker and L-workers, respectively. Of the H-workers and L-workers observed performing egg-laying events, 47 (58.0%) of the egg-laying H-workers and 41 (62.1%) of egg-laying L-workers were also observed performing acts of aggression. In total, egg-laying H- and L-workers were observed performing 229 acts of aggression during direct observations ($n_{\text{egg-eating events performed by egg-laying H-workers}} = 109$, $_{\text{egg-eating events performed by egg-laying L-workers}} = 120$). As with the rate of egg-laying and egg-eating events, the mean rate at which egg-laying H- and L-workers performed acts of aggression did not differ significantly (Wilcoxon test: H-workers vs L-workers, W = 38, *p* = 0.59; **Figure 4.4**, **Table A4.3**), being 0.03 ± 0.01 and 0.03 ± 0.01 events per individual per hour for H-worker and L-workers, respectively. Therefore, the predictions that focal workers in the high-feeding larval treatment would be more likely to become egg-layers and, if egg-layers, would show greater rates of egg-laying, egg-eating, and aggression than focal workers in the low-feeding larval treatment, were not supported.

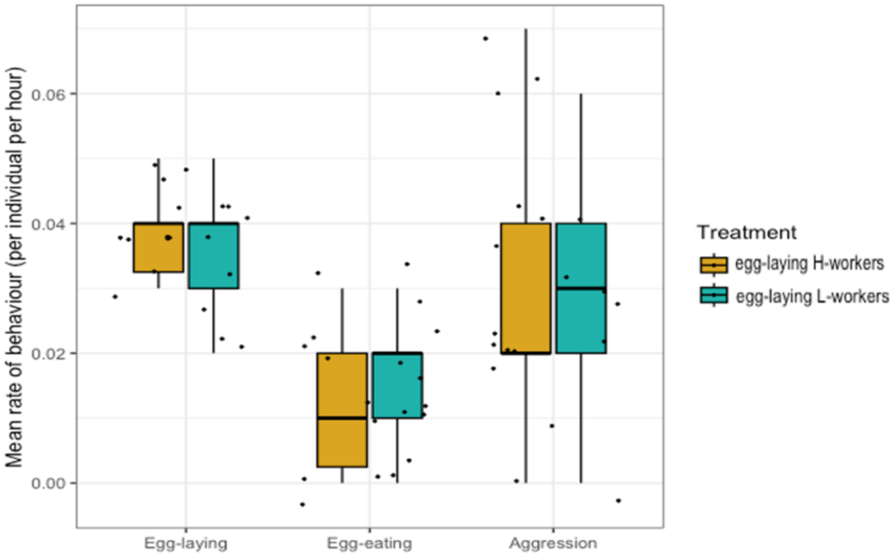


Figure 4.4. Mean rates of egg-laying, egg-eating and acts of aggression performed by egg-laying focal workers of *Bombus terrestris* from the high (H-workers) and low (L-workers) feeding treatments of 10 colonies. Rates of behaviours (per individual per hour) are calculated from multiple 1-hour-long direct observations (mean ± SD of 54.0 ± 0.9 h of direct observation per colony). Thick horizontal bars, medians; boxes, interquartile range; filled black circles, 'jittered' raw data, with each point representing the mean rate of behaviour for egg-laying workers within a single colony.

4.4.3 Egg-laying focal workers vs non-laying focal workers: variations in longevity and body size

As stated, the analyses conducted above revealed no significant differences in the longevity of focal workers from either the high or low feeding treatment. Similarly, there were no significant differences in the proportion of egg-laying focal workers in either treatment, or in the rates at which these egg-laying focal workers performed behaviours of interest. These results suggest that treatment had either a very small effect on focal worker quality or no effect at all.

As there were no significant differences between H-workers and L-workers found with respect to the metrics investigated, both groups of focal workers were combined in order to determine (with increased power) if a relationship between fecundity and longevity existed in focal workers, irrespective of their treatment of origin. Of the combined 399 focal workers, 147 (36.8%) were recorded performing at least one egg-laying event during observations, while the remaining 252 (63.2%) focal workers were not recorded performing any egg-laying event during observations throughout the experiment (**Table A4.4**). The overall mean (± SE) longevity of focal workers, regardless of treatment or reproductive status, was 97.4 ± 1.7 days ($n_{\textit{egg-laying focal workers}}$ = 143, $_{\textit{non-laying focal workers}}$ = 228). There was a significant positive relationship between egg-laying and longevity, resulting in non-laying focal workers having a 52%

increased risk of mortality in comparison to egg-laying focal workers (Cox's proportional hazards analysis: hazard ratio = 1.52, 95% Cl [1.21, 1.89], $p < 0.001$; **Figure 4.5A).** Overall, egg-laying focal workers had a median survival time (longevity) of 113 days (95% CI [108, 121]) and non-laying focal workers had a significantly lower median survival time (longevity) of 88 days (95% CI [81, 95]) (**Figure 4.5A**). Therefore, these results suggest that, when pooling focal workers from all colonies and both treatments, egg-laying was associated with increased worker longevity (**Figure 4.5A**).

Measurements of marginal wing cell length (as an index of total body size) were obtained from 344 (86.2%) focal workers across 10 colonies ($n_{\textit{egg-laying focal workers}}$ = 132, $n_{\textit{non-laying focal workers}}$ = 212). Among these focal workers, it was found that egg-laying focal workers were significantly larger than non-laying focal workers (LMM: $\chi^2 = 33.21$, df = 1, $p < 0.001$; **Table A4.6**), with the mean (± SE) marginal wing cell length for egg-laying and non-laying workers being 2.78 ± 0.06 mm and 2.59 ± 0.04 mm, respectively (**Table A4.5**). However, there was no significant association between marginal wing cell length and treatment of origin, with no significant difference occurring in marginal wing cell length for focal workers from the high-feeding treatment compared to those from the low-feeding treatment (LMM: $\chi^2 = 1.92$, df = 1, $p = 0.17$; **Table A4.6).**

These results suggest that the larval feeding treatment did not affect the body size of focal workers as adults. However, from the analyses pooling focal workers across treatments, there were significant positive associations between focal worker egg-laying, longevity and body size, such that egg-laying focal workers were longer-lived and were larger than non-laying focal workers.

4.4.4 Class of worker: rates of behaviour, longevity and worker size

To determine if longevity and worker size were associated with egg-eating and acts of aggression, all 399 focal workers (pooled across treatments) were classified into one of four classes based on whether or not they were observed egg-laying or egg-eating in direct observations. The four classes were as defined in Chapter 2 (**Table 2.2**) and were as follows: LEE (laying egg-eating worker), LNEE (laying non-egg-eating worker), NLEE (non-laying egg-eating worker) and NLNEE (non-laying non-egg-eating worker) ($n_{\textit{LEEs}}$ = 48, $n_{\textit{LNEEs}}$ = 99, $n_{\textit{NLEEs}}$ = 26, $n_{\textit{NLNEEs}}$ = 226). Overall, in each colony, LEEs, LNEEs, NLEEs and NLNEEs made up 13.1%, 24.3%, 7.1% and 55.5% of all focal workers, respectively (**Table A4.4**). Within colonies, both LEEs and LNEEs performed egg-laying events ($n_{\textit{egg-laying events performed by LEEs}}$ = 111, $n_{\textit{egg-eating events performed by LNEEs}}$ = 167). The mean rate (± SE) at which individual LEEs and LNEEs performed egg-laying events differed significantly (Wilcoxon's test: W = 3126.2, p = 0.001; **Table A4.4**), being 0.05 ± 0.00 and 0.03 ± 0.00 events per individual per hour, respectively (**Table A4.4**). Overall, LEEs were responsible for 44.69% of egg-laying events observed within colonies during direct observations and LNEEs were responsible for 55.31% of events (**Table A4.4**). In addition, both laying and non-laying workers were observed eating the eggs of other workers, with a total of 143 egg-eating events observed being performed by focal workers classed as LEEs and NLEEs during direct observations ($n_{\textit{egg-eating events performed by LEEs}} = 101$, $n_{\textit{egg-eating events performed by NLEEs}} = 42$). The rate at which individual LEEs performed egg-eating events did not differ significantly from that of individual NLEEs (Wilcoxon's test: $W = 728.5$, $p = 0.2$; **Table A4.4**), being 0.04 ± 0.00 and 0.03 ± 0.00 events per individual per hour, respectively (**Table A4.4**). However, overall, because they made up a greater proportion of all workers, LEEs were responsible for 70.36% of egg-eating events observed within colonies, whereas NLEEs were responsible for only 29.64% of events (**Table A4.4**). Hence, these results suggest that, among workers, LEEs laid eggs at the highest individual rates and performed the majority of egg-eating events .

All four classes of worker were observed performing acts of aggression towards other group members, with a total of 347 acts of aggression being observed across the 10 colonies ($n_{\textit{aggressive acts performed by LEEs}} = 143$, $n_{\textit{aggressive acts performed by LNEEs}} = 116$, $n_{\textit{aggressive acts performed by NLEEs}} = 26$, $n_{\textit{aggressive acts performed by NLNEEs}} = 58$). The mean (± SE) rate at which LEEs, LNEEs, NLEEs and NLNEEs performed acts of aggression towards group members differed significantly (Kruskal-Wallis test: $\chi^2 = 4133$, df = 11, $p < 0.001$; **Table A4.4).** being 0.05 ± 0.01, 0.02 ± 0.00, 0.02 ± 0.01 and 0.01 ± 0.00 acts of aggression per individual per hour, respectively (**Table A4.4**). Pairwise comparisons showed that individual LEEs performed acts of aggression towards group members at a significantly higher mean rate than individuals in all other classes of worker (Dunn's test: LEE vs LNEE: $Z = 4.04$, $p < 0.001$; LEE vs NLEE: $Z = 3.37$, $p = 0.002$; LEE vs NLNEE: $Z = 9.08$, $p < 0.001$; **Table A4.4**). Though the rate at which individual LNEEs and NLEEs performed acts of aggression did not differ significantly, both performed acts of aggression towards group members at a significantly higher mean rate than individual NLNEEs (Dunn's test: LNEEs vs NLEEs: $Z = 0.50$, $p = 0.62$; LNEEs vs NLNEEs: $Z = 6.07$, $p < 0.001$; NLEEs vs NLNEEs: $Z = 3.00$, $p = 0.005$; **Table A4.4**). Overall, LEEs, LNEEs, NLEEs and NLNEEs were responsible for 39.35%, 30.84%, 7.58% and 22.23% of acts of aggression observed during direct observations. Overall, these results suggest there is an association between egg-laying, egg-eating and aggression.

Worker longevity was found to differ significantly between the classes of worker (LMM: $\chi^2= 60.7$, df = 3, $p < 0.001$; **Table A4.7**) but, as found in the previous analysis (**Section 4.4.1**), not as a function of treatment (GLMM: $\chi^2= 0.01$, df = 1, $p = 0.94$; **Table A4.7**). While pairwise comparison showed that the longevity of LEEs did not differ significantly from that of either LNEEs or NLEEs (LEEs vs LNEEs, $t =2.07$, $p = 0.06$; LEEs vs NLEEs, $t = 0.65$, $p = 0.52$; LNEEs vs NLNEEs, $t = -0.92$, p = 0.43; **Figure 4.5B**, **Table A4.7**), all these three classes of worker had significantly increased longevity relative to NLNEEs (LEEs vs NLNEEs, $t = 6.31$, $p < 0.001$; LNEEs vs NLNEEs, $t = 5.22$, $p < 0.001$; NLEEs vs NLNEEs, $t = 4.08$, p < 0.001; **Figure 4.5B, Table A4.7**). Overall, LEEs had a median survival time (longevity) of 129 days (95%

CI [113, 135]), LNEEs had a median survival time (longevity) of 110 days (95% CI [95, 119]), NLEEs had a median survival time (longevity) of 114 days (95% CI [103, 132]) and NLNEEs had a median survival time (longevity) of 82 days (95% CI [78, 91]) (**Figure 4.5B**). This result suggests that longevity is associated with both egg-laying and egg-eating.

Additionally, an analysis of body size across the classes of focal worker showed that marginal wing cell length varied significantly among them (LMM: χ^2= 47.8, df = 3, $p < 0.001$; **Table A4.9**). However, the results again found that marginal wing cell length of focal workers did not differ significantly between treatments (LMM: χ^2= 2.10, df = 1, $p = 0.15$; **Table A4.9**). Of the 344 focal workers from which wing cell measurements were taken, 44 (91.7%) were classed as LEEs, 88 as LNEEs, 23 as NLEEs and 189 as NLNEEs. This accounted, respectively, for 91.7%, 88.9%, 88.5% and 83.6% of LEEs, LNEEs, NLEEs and NLNEEs observed throughout the experiment. Pairwise contrasts showed that LEEs had significantly greater marginal wing cell length than all other classes of worker (LMM: LEEs vs LNEEs, *t.ratio* = 2.75, $p = 0.01$; LEEs vs NLEEs, *t.ratio* = 2.17, $p = 0.04$; LEEs vs NLNEEs, *t.ratio* = 6.29, p < 0.001; **Table A4.9**). LNEEs and NLEEs did not differ significantly in marginal wing cell length, but both had significantly greater marginal wing cell length than NLNEEs (LNEEs vs NLEEs, *t.ratio* = 0.171, $p = 0.86$; LNEEs vs NLNEEs, *t.ratio* = 4.23, $p < 0.001$; NLEEs vs NLNEEs, *t.ratio* = 2.34, $p = 0.03$; **Table A4.9**). Overall, the mean marginal wing cell length for individual LEEs, LNEEs, NLEEs and NLNEEs was 2.79 ± 0.07 mm, 2.74 ± 0.06 mm, 2.64 ± 0.06 mm and 2.59 ± 0.04 mm, respectively (**Table A4.8**)

These results suggest that worker size is associated both with egg-laying and egg-eating, with workers that performed both behaviours (i.e., LEEs) having the largest body size.

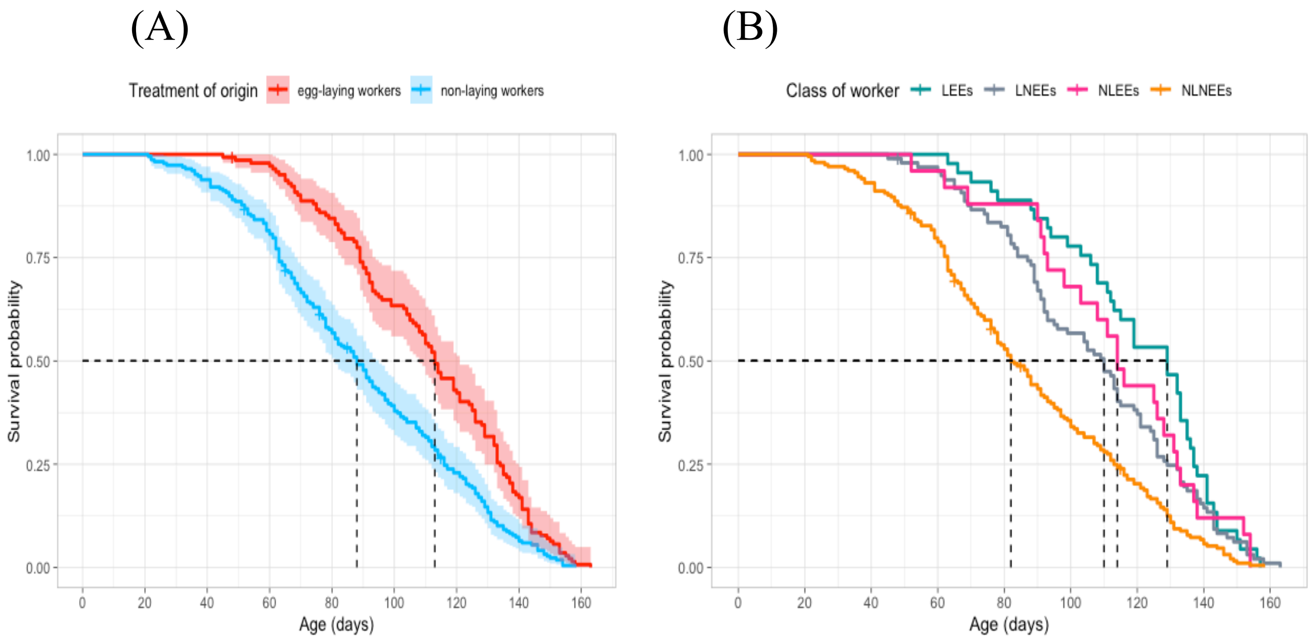


Figure 4.5. Relationship between egg-laying, egg-eating and longevity in *Bombus terrestris* workers. (**A**) longevity (days between worker eclosion and death) of all egg-laying and non-laying focal workers (censored recordings: $_{\textit{egg-laying focal workers}}$ = 1, n $_{\textit{non-laying focal workers}}$ = *5*; time to event recordings: n $_{\textit{egg-laying focal workers}}$ = 132*,* n $_{\textit{non-laying focal workers}}$ = 207). (**B**) longevity of workers of different classes (LEEs, LNEEs, NLEEs, NLNEEs) (censored recordings: n $_{\textit{LNEEs}}$ = 1, $_{\text{NLEEs}}$ = 0, n $_{\textit{NLNEEs}}$ = 0; time to event recordings n $_{\textit{LEEs}}$ =*44,* n $_{\textit{LNEEs}}$ = 87, $_{\text{NLEEs}}$ = 23, $_{\text{NLNEEs}}$ = 184*).* Dashed lines represent the median survival time (longevity) for each group, which corresponds to a survival probability of 50%. Data were obtained from 10 *B. terrestris* colonies. Data include censored data and were collected during the post-treatment phase of the experiment, over a period of 171 days.

4.5 Discussion

4.5.1 Overview

This study reports results of an experiment with two treatments, a high-feeding treatment and low-feeding treatment, the aim of which was to manipulate the quantity of nutrition provided to worker-destined *B. terrestris* larvae during their development. The primary objective was, using the *Bombus terrestris* system, to test the hypothesis that positive fecundity-longevity associations among workers arise from within-colony variation in workers' intrinsic quality, which in turn stems from differences in the rearing conditions experienced by individual workers and their resulting adult body sizes. Accordingly, the study sought to determine if a positive relationship between egg-laying and longevity existed in *B. terrestris* workers of higher quality and, in addition, it examined if behaviours such as egg-eating and aggression were also associated with enhanced intrinsic quality. The results showed that the prediction that workers in the high-feeding treatment would be longer-lived as adults than workers in the low-feeding larval treatment was not supported, and neither were the predictions that workers in the high-feeding treatment would be more likely to become egg-layers than workers in the low-feeding larval treatment and, if egg-layers, would show greater rates of egg-laying, egg-eating, and aggression. However, it was also found that workers did not differ between treatments in adult body size. This suggested that, as discussed below, the treatments manipulating larval nutrition did not succeed in their intended aim of creating two sets of adult workers within colonies of differing average body size. Nonetheless, as also discussed below, it was possible then to use the data from pooling all focal workers regardless of treatment to demonstrate novel associations in workers between reproductivity and longevity, and specifically between egg-laying, egg-eating, aggression and longevity.

4.5.2 High-feeding treatment vs low-feeding treatment

The results showed that there were no significant difference in the longevity of workers that eclosed from the high-feeding treatment (H-workers) compared to those from the low-feeding treatment (L-workers). The median survival of all H-workers and L-workers differed by only 5 days (**Figure 4.3A**), and when only the subsets of H-workers and L-workers observed egg-laying were considered, the median survival of H-workers and L-workers differed by only 3 days (**Figure 4.3B**). Furthermore, the proportion of H-workers and L-workers observed egg-laying did not differ significantly (**Table A4.2**), and nor did the rates at which egg-laying H-workers and L-workers performed egg-laying events, egg-eating events or acts of aggression (**Figure 4.4**).

Several possible explanations could account for the absence of significant differences in longevity and behaviour between H-workers and L-workers. One possibility is that, in general, the quantity of food received during larval development does not have a significant impact on the intrinsic quality of workers. However, this explanation appears unlikely given previous studies have demonstrated that, in *Bombus* species, nutritional inequalities during larvae development cause variation in worker body size, with workers that receive the lowest quantity of food having the smallest body size (Pereboom, 2000; Couvillon and Dornhaus, 2009; Rotheray et al., 2017). Therefore, it seems likely that larval nutrition does in reality affect adult worker body size.

However, the current study also found no significant differences in the length of the marginal wing cell of H-workers and L-workers, showing that adult workers from both treatments were similar in body size (**Table A4.5, A4.7**). This outcome was surprising considering the significantly higher mean daily pollen consumption estimated for H-workers during larval development compared to L-workers. It suggests that the variations in the quantity of food provided in each treatment were not substantial enough to induce noticeable differences in the body size of emerging H-workers and L-workers. As a result, the lack of significant difference in worker quality, and its hypothesised correlates, between H-workers and L-workers might be attributed to the treatment having failed to manipulate larval nutrition in the manner intended.

One potential explanation for the lack of variation in body size between H-workers and L-workers, despite the intended differences in food quantity, is related to the feeding regime applied to each compartment, which, as well as larvae destined to develop as focal workers, contained adult non-focal workers. The non-focal workers were provided with daily pollen and syrup quantities calculated to provide 100% of the median per capita nutritional requirement (Gradish et al 2019). This approach ensured that all workers in both treatment groups had access to sufficient resources to achieve the daily energy requirements needed to effectively perform brood care tasks, such as larval feeding and thermoregulation (Heinrich, 2004). However, research has shown that larvae emit signals that enable workers to perceive their nutritional status. In response to larvae showing signs of hunger or starvation, workers will increase the quantity of pollen they feed to them (Pereboom et al., 2003; den Boer and Duchateau, 2006). It is therefore plausible that non-focal workers in the low-feeding treatment may have reallocated the nutritional resources provided to their compartment by feeding any available pollen to the larvae, instead of consuming it themselves. This could have mitigated the expected effects of reduced food quantity on focal worker body size and quality in the low-feeding treatment. Future versions of a direct experiment test of the hypothesis would therefore somehow need to prevent workers caring for larvae from reallocating resources provided in this way.

4.5.3 Positive relationship between egg-laying and longevity

Although workers did not exhibit significant differences between treatments in size or the hypothesised indicators of quality, the experiment nonetheless generated a large dataset (created by pooling all focal workers regardless of treatment) in which the egg-laying, egg-eating and aggression rates of workers over their entire adult lives were all known at the individual level, along with their individual longevities. This then allowed tests of some of the associations predicted by the hypothesis to be made at a high level of resolution. Previous comparable datasets are few and no previous study has acquired data combining individual-level measures of behaviour over workers' entire lifetimes, fecundity measures based on egg-laying rate, and longevity data. For example, in the study of Blacher et al. (2017), which demonstrated a positive fecundity-longevity association in unmanipulated workers of *B. terrestris*, fecundity was measured not as egg-laying rate but as degree of ovarian activation, which is a less direct measure of fecundity because some workers may activate their ovaries but not lay eggs (Duchateau et al. 1989).

The analysis of the pooled focal worker data demonstrated a clear positive relationship between egg-laying and longevity. Workers that were observed egg-laying had significantly greater longevity compared to workers that were not observed egg-laying within their lifetime (median longevities of 113 vs. 88 days, respectively) (**Figure 4.5A**). Furthermore, egg-laying workers were found to be significantly larger than non-laying workers (**Table A4.5**). These findings support the hypothesis by showing that there is a positive association between fecundity (as measured from egg-laying behaviour) and longevity in *B. terrestris* workers, so providing a demonstration of this association more direct than, and independent of, the one in Blacher et al. (2017). They also support the hypothesis by demonstrating a significant association of egg-laying behaviour and longevity with body size, which was hypothesised by Blacher et al. (2017) to underpin intrinsic quality.

4.5.4 Association between egg-laying egg-eating aggression and longevity

To investigate whether egg-eating behaviours and acts of aggression were indicators of intrinsic quality in *B. terrestris* workers, the pooled focal worker dataset was also used to classify each worker into one of four distinct classes based on its observed behaviours: LEE (laying egg-eating worker), LNEE (laying non-egg-eating worker), NLEE (non-laying egg-eating worker) and NLNEE (non-laying non-egg-eating worker). As workers were not observed continuously, it is possible that some of these classifications were made with error. However, classifications were based on 540 total hours of observations and so are likely to have captured workers' true status with reasonable accuracy. In addition, as only a single observed egg-laying event and a single observed egg-eating event were sufficient to define a worker as a LEE, all workers found to be in this class will have been correctly classified, though some true LEEs may have remained in the other classes through either egg-laying or egg-eating by them having occurred outside the observations. Therefore, specific comparisons between LEEs and other classes would be between workers that were all correctly classified as LEEs and other classes that were likely to have incorrectly included only a small number of LEEs.

Consistent with the findings presented in Chapter 2 and Chapter 3 of this thesis, comparisons between LEEs, LNEEs, NLEEs and NLNEEs provided further evidence of strong associations between the rates of egg-laying, egg-eating and aggression performed by workers (**Table A4.4**). Specifically, workers classed as LEEs demonstrated significantly higher rates of egg-laying events and acts of aggression compared to all other worker classes, although they exhibited rates of egg-eating similar to those of NLEEs (**Table A4.4**). In addition, laying workers as a class had significantly greater longevity than non-laying workers as a class (**Figure 4.5A**). Dividing workers further into the four classes showed that, while LEEs did not differ significantly in longevity from either LNEEs or NLEEs, all these three classes of worker had longevities significantly greater than those of NLNEEs (**Figure 4.5B**). LEEs also had significantly larger body sizes than workers in all other classes and, although LNEEs and NLEEs were not significantly different in size from one another, they were both significantly larger than NLNEEs

These results provide support for the hypothesis developed by Blacher et al. (2017) that, within *B. terrestris* colonies, and by extension in eusocial Hymenoptera with comparable social structures, worker quality varies such that higher-quality workers are larger, more reproductive and longer-lived. This support comes from the findings that LEEs, among all four worker classes, had the greatest body size and rate of egg-laying, and that egg-laying workers as a class had greater longevity than non-laying workers as a class, with NLNEEs being the least long-lived of the four separate worker classes. Therefore, these results support the component of the hypothesis of Blacher et al. (2017) that interprets positive-fecundity associations as occurring in workers because of differences in intrinsic quality, with high-quality workers being able to overcome costs of reproduction and exhibit high-levels of both fecundity and longevity.

As LEEs had the greatest rates of aggression to other workers, the results also confirm the association between egg-laying and aggression demonstrated in Chapters 2 and 3 of the current thesis and in previous studies (e.g., Bloch and Hefetz 1999; Zanette et al. 2012). As regards egg-eating, the similar rates of egg-eating of LEEs and NLEEs could also be interpreted as supporting the conclusion from Chapter 2 that NLEEs are largely would-be selfishly policing workers.

Lastly, the high rate of egg-laying by LEEs also aligns with the hypothesis that only individuals which strong resource-holding abilities should engage in policing behaviours (Frank, 1996; 2003). This may help explain the relatively small proportion of egg-laying workers (37%) observed engaging in egg-eating events in the current study. Overall, the results support the idea that LEEs, as high-quality and high-ranking individuals, possess the necessary resources and capabilities to maintain their reproductive dominance through strategies involving egg-eating and aggression.

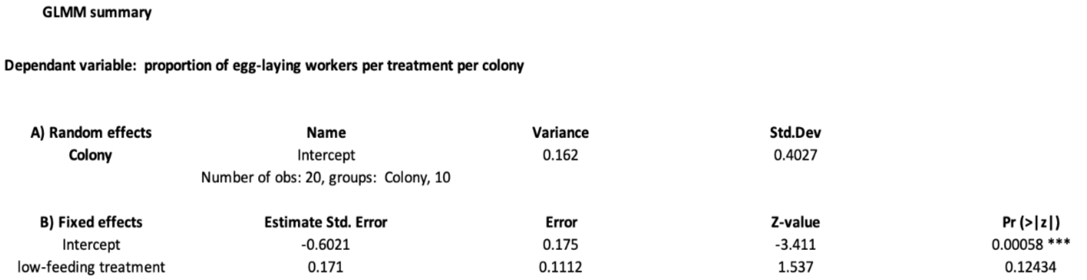
4.5.5 Conclusions

In summary, this study's findings confirm the presence of positive associations between fecundity and longevity among workers of eusocial Hymenoptera and extend previous demonstrations of this relationship by showing that, in *B. terrestris* workers, the relationship holds when fecundity is measured as egg-laying rate over workers' entire lifetimes. However, because the experimental manipulation of larval diet did not achieve its intended aim of producing sets of adult workers of different average body sizes, the exact influence of rearing conditions on variations in worker intrinsic quality remains to be experimentally demonstrated, warranting further investigations into the impact of larval nutrition on workers' body size and intrinsic quality. Although the study did not, therefore, directly address this aspect of the hypothesis, the results still provide valuable insights into the likely underpinnings of within-colony variation in quality among workers by suggesting that, as the hypothesis proposed, body size is associated with workers' degree of reproductivity (egg-laying, egg-eating and aggression) and longevity. The results also add to the evidence from Chapters 2 and 3 of this thesis of the strong links between egg-laying, egg-eating and aggression in social systems like those of *B. terrestris*. Therefore, the results help elucidate not only the basis of reproductive dominance in workers in eusocial Hymenoptera but also the basis of positive-fecundity longevity relationships and hence how sociality affects life history and ageing across organisms in general.

4.6 Appendix

Colony	Feeding treatment	Number of days in feeding regime of treatment phase (prior to pupation of all focal workers)	Assumed mean daily consumption of pollen by indvidual larvae (1st - 4th instar) (mg)	Date of mesh removal	Number of focal workers eclosed
1	H	17	7.52	14/03/2021	3
1	L	19	5.30		3
2	H	17	7.07	14/03/2021	28
2	L	17	5.20		11
3	H	17	6.71	14/03/2021	17
3	L	17	5.41		42
4	H	17	7.07	14/03/2021	21
4	L	17	5.48		6
5	H	19	7.33	14/03/2021	6
5	L	19	3.82		4
6	H	17	6.58	15/03/2021	40
6	L	19	4.20		22
9	H	17	7.72	14/03/2021	34
9	L	17	4.76		31
10	H	17	4.46	15/03/2021	17
10	L	17	3.76		23
11	H	17	6.94	15/03/2021	20
11	L	17	4.03		29
12	H	19	4.44	15/03/2021	14
12	L	17	5.04		27

Table A4.1. Demographic, productivity, and experimental data for 10 colonies of *Bombus terrestris* workers used for data analyses (colony nos. 1-6, 9-12). During the treatment phase (see text), each colony was split by a mesh partition into a high-feeding treatment (H) and a low-feeding treatment (L). The feeding regime ended in treatment compartments following the pupation of all focal workers. The start of the post-treatment phase (see text) began on the date of removal of the mesh partition. The date of mesh removal occurred only following the eclosion of all focal workers within both treatment groups of a colony. Dates are given in the format dd/mm/yyyy.



GLMM summary

Dependant variable: proportion of egg-laying workers per treatment per colony

A) Random effects	**Name**	**Variance**	**Std.Dev**	
Colony	Intercept	0.162	0.4027	
	Number of obs: 20, groups: Colony, 10			
B) Fixed effects	**Estimate Std. Error**	**Error**	**Z-value**	**Pr (>|z|)**
Intercept	-0.6021	0.175	-3.411	0.00058 ***
low-feeding treatment	0.171	0.1112	1.537	0.12434

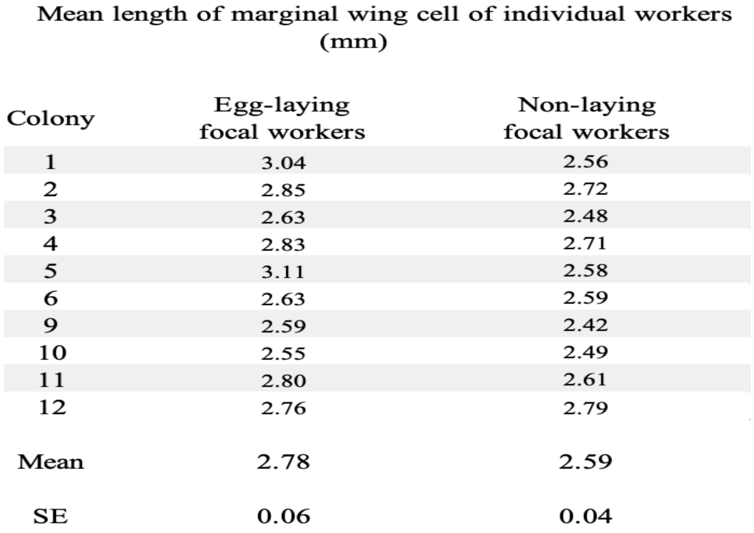
Table A4.2. GLMM model summary for analyses comparing the proportion of focal workers observed egg-laying that eclosed in either the high-feeding or low-feeding treatment, in 10 queenright colonies (nos. 1-6, 9-12) of *Bombus terrestris*. Analysis included the number of egg-laying and non-laying focal workers observed in both treatments in each colony. All observations of egg-laying were collected from direct observations taken in the post-treatment phase (mean ± SD of 54.0 ± 0.9 h of direct observation per colony). *** $p < 0.001$.

Colony	Rate of egg-laying events performed per individual per hour		Rate of egg-eating events performed per individual per hour		Rate of acts of aggression performed per individual per hour	
	High-feeding treatment	Low-feeding treatment	High-feeding treatment	Low-feeding treatment	High-feeding treatment	Low-feeding treatment
1	0.05	0.02	0.02	0.00	0.02	0.00
2	0.04	0.03	0.02	0.00	0.04	0.02
3	0.04	0.04	0.01	0.03	0.04	0.06
4	0.04	0.03	0.00	0.03	0.02	0.03
5	0.02	**NA**	0.00	**NA**	0.00	**NA**
6	0.04	0.05	0.01	0.02	0.02	0.06
9	0.04	0.04	0.02	0.02	0.04	0.03
10	0.03	0.04	0.00	0.02	0.01	0.02
11	0.05	0.04	0.01	0.01	0.02	0.04
12	0.03	0.04	0.03	0.01	0.07	0.03
Mean	0.04	0.04	0.01	0.02	0.03	0.03
SE	0.00	0.00	0.00	0.00	0.01	0.01

Table A4.3. Rates of egg-laying, egg-eating and acts of aggression performed by egg-laying focal workers that eclosed in either the high-feeding or low-feeding treatment of 10 queenright *Bombus terrestris* colonies (nos. 1-6, 9-12). Rates of behaviours (per individual per hour) were collected from the post-treatment phase and calculated from multiple 1-hour-long direct observations (mean ± SD of 54.0 ± 0.9 h of direct observation per colony). Colony queens and non-laying focal workers were excluded from analyses.

Colony	Rate of egg-laying events performed per individual per hour		Rate of egg-eating events performed per individual per hour		Rate of acts of aggression performed per individual per hour			
	By LEEs	**By LNEEs**	**By LEEs**	**By NLEEs**	**By LEEs**	**By LNEEs**	**By NLEEs**	**By NLNEEs**
1	0.05	0.02	0.02	**NA**	0.02	0.00	**NA**	0.00
2	0.03	0.04	0.05	0.02	0.04	0.03	0.05	0.00
3	0.05	0.03	0.04	0.03	0.08	0.03	0.02	0.00
4	0.03	0.04	0.03	0.04	0.03	0.02	0.01	0.02
5	**NA**	0.02	**NA**	0.02	**NA**	0.00	0.00	0.00
6	0.04	0.04	0.03	0.04	0.05	0.02	0.02	0.00
9	0.04	0.04	0.05	0.03	0.05	0.02	0.02	0.01
10	0.06	0.03	0.06	0.02	0.06	0.01	0.00	0.00
11	0.07	0.03	0.03	0.04	0.06	0.02	0.01	0.01
12	0.04	0.03	0.05	0.04	0.06	0.04	0.03	0.02
Mean	0.05	0.03	0.04	0.03	0.05	0.02	0.02	0.01
SE	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00
Mean no. of workers per class per colony	4.8	9.9	4.8	2.6	4.8	9.9	2.6	22.6

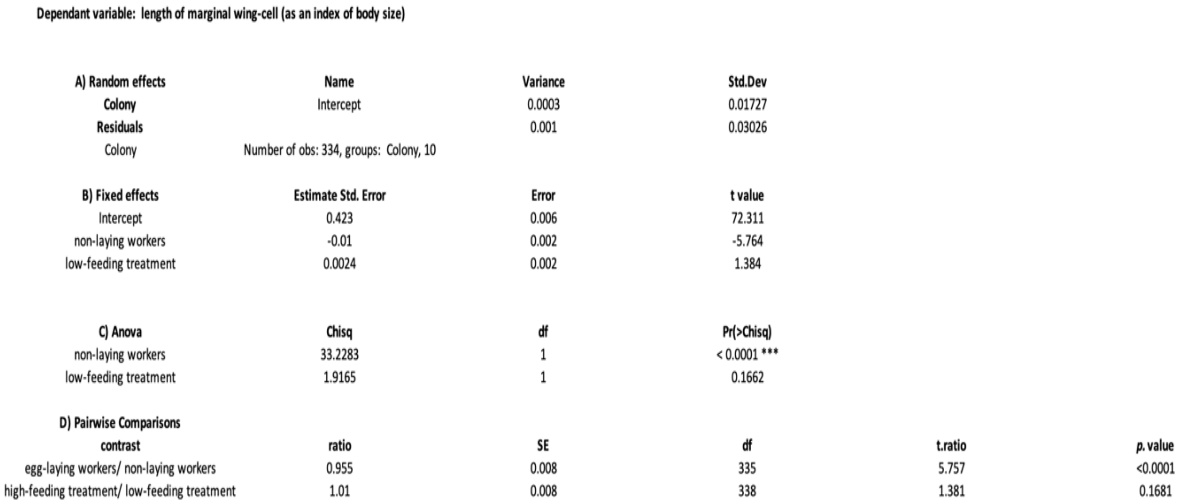
Table A4.4. Rates of egg-laying, egg-eating and acts of aggression performed by focal workers of different class in 10 queenright *Bombus terrestris* colonies (nos. 1-6, 9-12). For each colony, rates of behaviours (per individual per hour) were collected from the post-treatment phase and calculated from multiple 1-hour-long direct observations (mean ± SD of 54.0 ± 0.9 h of direct observation per colony). Colony queens were excluded from analyses. See **Table 2.2** for full definitions of classes of workers.



Mean length of marginal wing cell of individual workers (mm)

Colony	Egg-laying focal workers	Non-laying focal workers
1	3.04	2.56
2	2.85	2.72
3	2.63	2.48
4	2.83	2.71
5	3.11	2.58
6	2.63	2.59
9	2.59	2.42
10	2.55	2.49
11	2.80	2.61
12	2.76	2.79
Mean	2.78	2.59
SE	0.06	0.04

Table A4.5. Mean length of marginal wing cell of egg-laying and non-laying focal workers in 10 queenright *Bombus terrestris* colonies (nos. 1-6, 9-12) (n $_{egg\text{-}laying\ focal\ workers}$ = 132, n $_{non\text{-}laying\ focal\ workers}$ = 212) .



Dependant variable: length of marginal wing-cell (as an index of body size)

A) Random effects	**Name**	**Variance**	**Std.Dev**		
Colony	Intercept	0.0003	0.01727		
Residuals		0.001	0.03026		
Colony	Number of obs: 334, groups: Colony, 10				
B) Fixed effects	**Estimate Std. Error**	**Error**	**t value**		
Intercept	0.423	0.006	72.311		
non-laying workers	-0.01	0.002	-5.764		
low-feeding treatment	0.0024	0.002	1.384		
C) Anova	**Chisq**	**df**	**Pr(>Chisq)**		
non-laying workers	33.2283	1	< 0.0001 ***		
low-feeding treatment	1.9165	1	0.1662		
D) Pairwise Comparisons					
contrast	**ratio**	**SE**	**df**	**t.ratio**	***p.* value**
egg-laying workers/ non-laying workers	0.955	0.008	335	5.757	<0.0001
high-feeding treatment/ low-feeding treatment	1.01	0.008	338	1.381	0.1681

Table A4.6. LMM model summary for analyses comparing the length of the marginal wing cell (as an index of body size) of focal workers as a function of whether they were laying or non-laying workers and whether they were produced in the high-feeding or low-feeding treatment. Egg-laying workers were used as the reference level. Analysis included all focal workers from which marginal wing cell measurements were taken in 10 colonies (nos. 1-6, 9-12) of *Bombus terrestris*. *** $p < 0.001$. Pairwise tests were performed on the log odds ratio scale.

GLMM summary

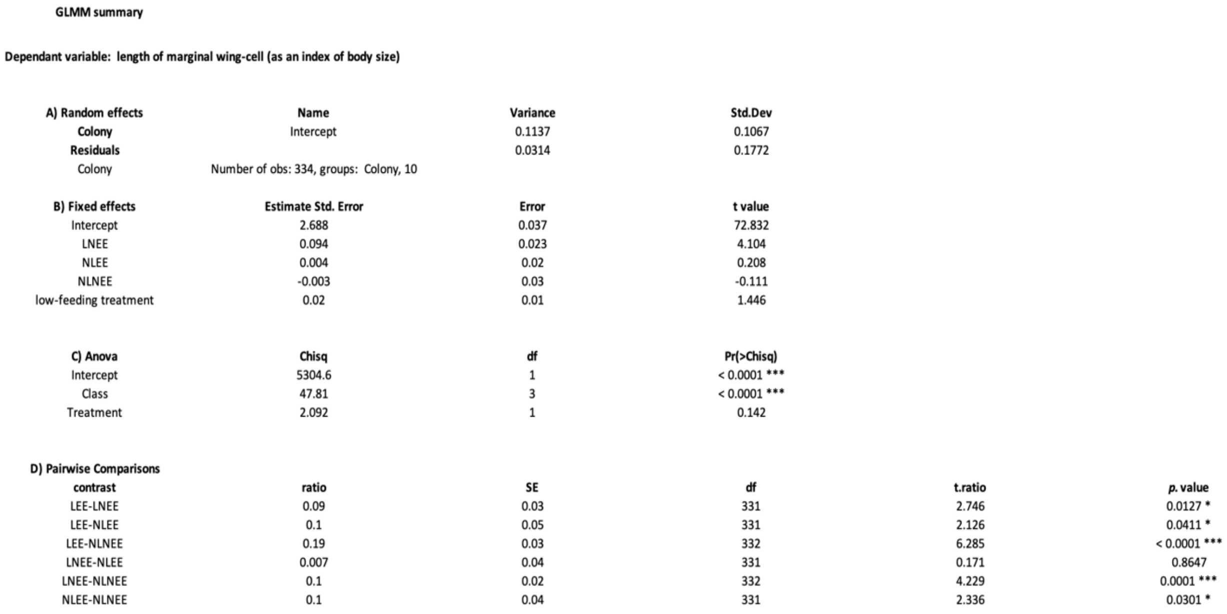
Dependant variable: longevity of workers

A) Random effects	**Name**	**Variance**	**Std.Dev**		
Colony	Intercept	140	11.83		
Residuals		784.6	28.01		
Colony	Number of obs: 334, groups: Colony, 10				
B) Fixed effects	**Estimate Std. Error**	**Error**	**t value**		
Intercept	105.08	4.389	23.94		
LNEE	11.372	3.632	3.131		
NLEE	0.611	2.948	0.207		
NLNEE	6.679	4.615	1.447		
low-feeding treatment	-0.128	1.59	-0.081		
C) Anova	**Chisq**	**df**	**Pr(>Chisq)**		
Intercept	573.141	1	< 0.0001***		
Class	60.7	3	< 0.0001***		
Treatment	0.0066	1	0.9352		
D) Pairwise Comparisons					
contrast	**ratio**	**SE**	**df**	**t.ratio**	***p*. value**
LEE-LNEE	10.76	5.21	332	2.067	0.06
LEE-NLEE	4.69	7.25	332	0.647	0.5182
LEE-NLNEE	30.04	4.76	334	6.31	< 0.0001 ***
LNEE-NLEE	-6.07	6.6	332	-0.919	0.4305
LNEE-NLNEE	19.27	3.69	334	5.217	< 0.0001 ***
NLEE-NLNEE	25.34	6.21	331	4.08	0.0001 ***

Table A4.7. LMM model summary for analyses comparing the longevity (number of days between eclosion and death) of focal workers of different classes and treatment. LEEs were used as the reference level. Analysis included all focal workers from which longevity data were taken in 10 colonies (nos. 1-6, 9-12) of *Bombus terrestris*. *** p < 0.001. Pairwise tests used worker class as an independent variable and were performed on the log odds ratio scale.

	Mean length of marginal wing cell of individual workers (mm)			
Colony	LEEs	LNEEs	NLEEs	NLNEEs
1	3.22	2.86	**NA**	2.56
2	2.83	2.85	2.73	2.71
3	2.69	2.59	2.52	2.48
4	2.68	2.86	2.76	2.70
5	**NA**	3.11	2.30	2.62
6	2.69	2.59	2.71	2.57
9	2.63	2.56	2.58	2.40
10	2.60	2.54	2.53	2.49
11	2.94	2.73	2.74	2.59
12	2.86	2.72	2.89	2.77
Mean	2.79	2.74	2.64	2.59
SE	0.07	0.06	0.06	0.04

Table A4.8. Mean length of marginal wing cell of individual workers of different class in 10 queenright *Bombus terrestris* colonies (nos. 1-6, 8-12) (n $_{LEEs}$ = 44, n $_{LNEEs}$ = 88, n $_{NLEEs}$ = 23, n $_{NLNEEs}$ = 189). See **Table 2.2** for full definitions of classes of workers.



GLMM summary

Dependant variable: length of marginal wing-cell (as an index of body size)

A) Random effects	**Name**	**Variance**	**Std.Dev**
Colony	Intercept	0.1137	0.1067
Residuals		0.0314	0.1772
Colony	Number of obs: 334, groups: Colony, 10		

B) Fixed effects	**Estimate Std. Error**	**Error**	**t value**
Intercept	2.688	0.037	72.832
LNEE	0.094	0.023	4.104
NLEE	0.004	0.02	0.208
NLNEE	-0.003	0.03	-0.111
low-feeding treatment	0.02	0.01	1.446

C) Anova	**Chisq**	**df**	**Pr(>Chisq)**
Intercept	5304.6	1	< 0.0001 ***
Class	47.81	3	< 0.0001 ***
Treatment	2.092	1	0.142

D) Pairwise Comparisons

contrast	**ratio**	**SE**	**df**	**t.ratio**	***p.* value**
LEE-LNEE	0.09	0.03	331	2.746	0.0127 *
LEE-NLEE	0.1	0.05	331	2.126	0.0411 *
LEE-NLNEE	0.19	0.03	332	6.285	< 0.0001 ***
LNEE-NLEE	0.007	0.04	331	0.171	0.8647
LNEE-NLNEE	0.1	0.02	332	4.229	0.0001 ***
NLEE-NLNEE	0.1	0.04	331	2.336	0.0301 *

Table A4.9. LMM model summary for analyses comparing the length of the marginal wing cell (as an index of body size) of focal workers as a function of their class (LEE, LNEE, NLEE or NLNEE as defined in **Table 2.2**) and as a function of whether they were produced in the high-feeding or low-feeding treatment. LEEs were used as the reference level. Analysis included all focal workers from which marginal wing cell measurements were taken in 10 colonies (nos. 1-6, 9-12) of *Bombus terrestris*. *** p < 0.001, * p < 0.05. Pairwise tests used worker class as an independent variable and were performed on the log odds ratio scale.

4.7 References

Alaux, C., Boutot, M., Jaisson, P., Hefetz, A., 2007. Reproductive plasticity in bumblebee workers (*Bombus terrestris*) - reversion from fertility to sterility under queen influence. Behavioral Ecology and Sociobiology 62, 213-222.

Alaux, C., Jaisson, P., Hefetz, A., 2004. Queen influence on worker reproduction in bumblebees (*Bombus terrestris*) colonies. Insectes Sociaux 51, 287-293.

Alaux, C., Jaisson, P., Hefetz, A., 2005. Reproductive decision-making in semelparous colonies of the bumblebee *Bombus terrestris*. Behavioral Ecology and Sociobiology 59, 270-277.

Alford, D.V., 1975. Bumblebees. Davis-Poynter, London.

Blacher, P., Huggins, T.J., Bourke, A.F.G., 2017. Evolution of ageing, costs of reproduction and the fecundity-longevity trade-off in eusocial insects. Proceedings of the Royal Society B - Biological Sciences 284, 20170380.

Bloch, G., Hefetz, A., 1999. Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. Behavioral Ecology and Sociobiology 45, 125-135.

Bourke, A.F.G., 2007. Kin selection and the evolutionary theory of aging. Annual Review of Ecology, Evolution and Systematics 38, 103-128.

Bourke, A.F.G., Ratnieks, F.L.W., 2001. Kin-selected conflict in the bumble-bee *Bombus terrestris* (Hymenoptera: Apidae). Proceedings of the Royal Society of London Series B 268, 347-355.

Choppin, M., Feldmeyer, B., Foitzik, S., 2023. Protein-rich diet decreases survival, but does not alter reproduction, in fertile ant workers. . Frontiers in Ecology and Evolution 10, 1098245.

Cnaani, J., Borst, D.W., Huang, Z.Y., Robinson, G.E., Hefetz, A., 1997. Caste determination in *Bombus terrestris*: differences in development and rates of JH biosynthesis between queen and worker larvae. Journal of Insect Physiology 43, 373-381.

Cnaani, J., Robinson, G.E., Hefetz, A., 2000. The critical period for caste determination in *Bombus terrestris* and its juvenile hormone correlates. Journal of Comparative Physiology A 186, 1089-1094.

Collins, D.H., Prince, D.C., Donelan, J.L., Chapman, T., Bourke, A.F.G., 2023. Costs of reproduction are present but latent in eusocial bumblebee queens. BMC Biol. 21, 153.

Couvillon, M.J., Dornhaus, A., 2009. Location, location, location: larvae position inside the nest is correlated with adult body size in worker bumble-bees (*Bombus impatiens*). Proceedings of The Royal Society B - Biological Sciences 276, 2411-2418.

den Boer, S., Duchateau, M.J.H.M., 2006. A larval hunger signal in the bumblebee *Bombus terrestris*. Insectes Sociaux 53, 369-373.

Dixon, L., Kuster, R., Rueppell, O., 2014. Reproduction, social behavior, and aging trajectories in honeybee workers. Age 36, 89-101.

Duchateau, M.J., 1989. Agonistic behaviors in colonies of the bumblebee *Bombus terrestris*. J. Ethol. 7, 141-151.

Duchateau, M.J., Velthuis, H.H.W., 1988. Development and reproductive strategies in *Bombus terrestris* colonies. Behaviour 107, 186-207.

Duchateau, M.J., Velthuis, H.H.W., 1989. Ovarian development and egg laying in workers of *Bombus terrestris*. Entomologia Experimentalis et Applicata 51, 199-213.

Edward, D.A., Chapman, T., 2011. Mechanisms underlying reproductive trade-offs: costs of reproduction, in: Flatt, T., Heyland, A. (Eds.), Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs, Oxford University Press, Oxford, pp. 137-152.

Frank, S.A., 1996. Policing and group cohesion when resources vary. Animal Behaviour 52, 1163-1169.

Frank, S.A., 2003. Repression of competition and the evolution of cooperation. Evolution 57, 693-705.

Goulson, D., 2003. Bumblebees: their Behaviour and Ecology. Oxford University Press, Oxford.

Goulson, D., Peat, J., Stout, J.C., Tucker, J., Darvill, B., Derwent, L.C., Hughes, W.O.H., 2002. Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? Animal Behaviour 64, 123-130.

Hamilton, W.D., 1964. The genetical evolution of social behaviour I, II. Journal of Theoretical Biology 7, 1-52.

Hamilton, W.D., 1966. The moulding of senescence by natural selection. Journal of Theoretical Biology 12, 12-45.

Hammers, M., Kingma, S.A., Spurgin, L.G., Bebbington, K., Dugdale, H.L., Burke, T., Komdeur, J., Richardson, D.S., 2019. Breeders that receive help age more slowly in a cooperatively breeding bird. Nature Communications 10, 1301.

Hoover, S.E.R., Higo, H.A., Winston, M.L., 2006. Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. Journal of Comparative Physiology B 176, 55-63.

Keller, L., Genoud, M., 1997. Extraordinary lifespans in ants: a test of evolutionary theories of ageing. Nature 389, 958-960.

Kim, E.B., Fang, X.D., Fushan, A.A., Huang, Z.Y., Lobanov, A.V., Han, L.J., Marino, S.M., Sun, X.Q., Turanov, A.A., Yang, P.C., Yim, S.H., Zhao, X., Kasaikina, M.V., Stoletzki, N., Peng, C.F., Polak, P., Xiong, Z.Q., Kiezun, A., Zhu, Y.B., Chen, Y.X., Kryukov, G.V., Zhang, Q., Peshkin, L., Yang, L., Bronson, R.T., Buffenstein, R., Wang, B., Han, C.L., Li, Q.Y., Chen, L., Zhao, W., Sunyaev, S.R., Park, T.J., Zhang, G.J., Wang, J., Gladyshev, V.N., 2011. Genome sequencing reveals insights into physiology and longevity of the naked mole rat. Nature 479, 223-227.

Kirkwood, T.B.L., 1977. Evolution of ageing. Nature 270, 301-304.

Kirkwood, T.B.L., Rose, M.R., 1991. Evolution of senescence: late survival sacrificed for reproduction. Philosophical Transactions Royal Society of London B 332, 15-24.

Korb, J., Heinze, J., 2021. Ageing and sociality: why, when and how does sociality change ageing patterns? Philosophical Transactions of the Royal Society B 376, 20190727.

Kramer, B.H., Schrempf, A., Scheuerlein, A., Heinze, J., 2015. Ant colonies do not trade-off reproduction against maintenance. PLoS One 10, e0137969.

Lopez-Vaamonde, C., Raine, N.E., Koning, J.W., Brown, R.M., Pereboom, J.J.M., Ings, T.C., Ramos-Rodriguez, O., Jordan, W.C., Bourke, A.F.G., 2009. Lifetime reproductive success and longevity of queens in an annual social insect. Journal of Evolutionary Biology 22, 983-996.

Negroni, M.A., Macit, M.N., Stoldt, M., Feldmeyer, B., Foitzik, S., 2021. Molecular regulation of lifespan extension in fertile ant workers. Philosophical Transactions of the Royal Society B 376.

Owen, R.E., 1988. Body size variation and optimal body size of bumble bee queens (Hymenoptera: *Apidae*). Can. Entomol. 120, 19-27.

Owen, R.E., 1989. Differential size variation of male and female bumblebees. Journal of Heredity 80, 39-43.

Pereboom, J.J.M., 2000. The composition of larval food and the significance of exocrine secretions in the bumblebee *Bombus terrestris*. Insectes Sociaux 47, 11-20.

Pereboom, J.J.M., 2001. Size dimorphism in bumblebees; a result of caste specific differences in fat body metabolism? Netherlands Journal of Zoology 51, 323-333.

Pereboom, J.J.M., Velthuis, H.H.W., Duchateau, M.J., 2003. The organisation of larval feeding in bumblebees (Hymenoptera, *Apidae*) and its significance to caste differentiation. Insectes Sociaux 50, 127-133.

Rodrigues, M.A., Flatt, T., 2016. Endocrine uncoupling of the trade-off between reproduction and somatic maintenance in eusocial insects. Current Opinion in Insect Science 16, 1-8.

Rotheray, E.L., Osborne, J.L., Goulson, D., 2017. Quantifying the food requirements and effects of food stress on bumblebee colony development. J. Apic. Res. 56, 288-299.

Schrempf, A., Giehr, J., Röhrl, R., Steigleder, S., Heinze, J., 2017. Royal Darwinian demons: enforced changes in reproductive efforts do not affect the life expectancy of ant queens. American Naturalist 189, 436-442.

Shpigler, H., Tamarkin, M., Gruber, Y., Poleg, M., Siegel, A.J., Bloch, G., 2013. Social influences on body size and developmental time in the bumblebee *Bombus terrestris*. Behavioral Ecology Sociobiology 67, 1601–1612.

Sibbald, E.D., Plowright, C.M.S., 2014. Social interactions and their connection to aggression and ovarian development in orphaned worker bumblebees (*Bombus impatiens*). Behavioral processes 103, 150-155.

Southon, R.J., Bell, E.F., Graystock, P., Sumner, S., 2015. Long live the wasp: adult longevity in captive colonies of the eusocial paper wasp *Polistes canadensis* (L.). PeerJ 3, e848.

Stabler, D., Paoli, P.P., Nicolson, E., Wright, G.A., 2015 Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. Journal of Experimental Biology 218.

Stearns, S.C., 1989. Trade-offs in life-history evolution. Functional Ecology 3, 259-268.

Stearns, S.C., 1992. The Evolution of Life Histories. Oxford University Press, Oxford.

Tian, L., Hines, H.M., 2018. Morphological characterization and staging of bumble bee pupae. PeerJ 6, e6089.

Trivers, R.L., Hare, H., 1976. Haplodiploidy and the evolution of the social insects. Science 191, 249-263.

Van Doorn, A., Heringa, J., 1986. The ontogeny of a dominance hierarchy in colonies of the bumblebee *Bombus terrestris* (Hymenoptera, *Apidae*). Insectes Sociaux 33, 3-25.

Williams, G.C., 1957. Pleiotropy, natural selection, and the evolution of senescence. Evolution 11, 398-411.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Almond, E.J., Huggins, T.J., Jordan, W.C., Bourke, A.F.G., 2012. Reproductive conflict in bumblebees and the evolution of worker policing. Evolution 66, 3765-3777.

5 Chapter

General conclusions

Photo taken by Jenny Livesey

5.1 Thesis summary

As stated in the introduction (Chapter 1), the objective of this thesis is to advance the understanding of how societies remain stable in the face of internal conflict. Specifically, through the use of quantitative experiments involving colonies of the Buff-tailed bumblebee *Bombus terrestris,* this thesis aims to contribute to the understanding of the exact origins of worker policing in eusocial societies and the ultimate function of dominance behaviours in animal societies. Moreover, this thesis also seeks to determine the underlying factors behind reproductive dominance in workers in eusocial Hymenoptera, focusing on elucidating the reasons for within-colony variation in worker quality in eusocial societies that exhibit intermediate complexity.

In the current chapter, I aim to provide a brief integrated summary of the main findings of the experimental chapters of this thesis, discuss their implications, and potential future directions for further research.

5.2 Thesis findings

5.2.1 The origin of non-reproductive worker policing remains unclear

In **Chapter 2** of this thesis, the primary aim was to discriminate between the two main hypotheses - the public goods hypothesis and the selfish policing hypothesis - proposed for the origin of non-reproductive worker policing (egg-eating) in eusocial Hymenoptera. The results confirm the existence of worker policing by non-reproductive workers, termed NLEEs (non-laying egg-eating workers), in *B. terrestris* colonies, as initially observed by Zanette et al. (2012). However, the results also revealed that NLEEs occurred at low frequencies, with only 25 workers (2.7%) across 15 colonies classified as NLEEs in the presence of the queen. In **Chapter 4** of this thesis, where NLEEs were not the main focus but were also observed, NLEEs similarly occurred at low frequencies, with 26 workers (7.2%) across 22 colonies classified as NLEEs. In addition, of the NLEEs observed in queenright conditions in **Chapter 2**, 58% started egg-laying (as predicted by the selfish policing hypothesis) following the removal of the queen. These observations highlight that the majority of egg-eating events are primarily carried out by queens and reproductive workers within colonies of *B. terrestris*. Therefore, this suggests that the majority of worker policing events performed in *B. terrestris* colonies are selfish in origin.

However, 42% of NLEEs observed in queenright conditions in **Chapter 2**, neither ate nor laid eggs following the removal of the queen. Additionally, the NLEEs observed in **Chapter 4** were not observed laying eggs throughout their entire lifetime (i.e., from eclosion to natural death). This suggests that some workers, that are non-reproductive throughout their lifetime, do police worker-laid eggs in this system. It's possible NLEEs that consumed the eggs of other workers in queenright conditions did so in order to enhance the production of queen-derived sexuals (as predicted by the public goods hypothesis). However, in **Chapter 2** it was found that worker policing did not induce egg-laying workers to cease egg-laying activities, therefore it is unclear how, or if, worker policing by non-reproductive workers enhances colony productivity and, thus, if this can be the underlying reason worker policing is selected for in non-reproductive workers in this system. Hence, the origin of non-reproductive worker policing in eusocial systems remains unclear.

However, as bumblebee species (*Bombus* spp.) represent an intermediate level of eusociality (Collins et al., 2017) and share a common ancestor with honeybee species, which represent an advanced level of eusociality (Cardinal and Danforth, 2011), bumblebees offer insights into the ultimate mechanisms that underlie the maintenance of intermediate eusociality and the potential origin of advanced eusociality from intermediate eusociality (Amsalem et al., 2015). In the context of worker policing, it seems clear *B. terrestris* colonies offer a unique opportunity to observe the transition from purely selfish policing to policing as a public good (as seen in colonies of honeybees) (Ratnieks, 1988). Data from **Chapter 2** confirms that, at an intermediate level of eusociality, policing by reproductive workers and non-reproductive workers can coexist. Therefore, bumblebee species provide an excellent model species for future research to explore the dynamic interplay of these two forms of policing and how they contribute to social stability and reproductive success in eusocial Hymenoptera of different levels of complexity.

5.2.2 Reproductive workers destroy both worker- and queen-laid male-destined eggs

In **Chapter 2**, I found that queens were aggressed while engaged in an egg-laying event at a significantly higher rate than any worker, and, in contrast to the findings of Zanette et al. (2012), that workers regularly consumed queen-laid eggs following the competition point. Specifically, data from **Chapter 2** showed 17.4% of egg-eating events performed by workers involved the consumption of queen-laid male-destined eggs. In addition, **Chapter 2** also showed that 96.4% of the workers observed eating queen-laid eggs were themselves reproductive.

Previous research has only mentioned anecdotal observations of worker-to-queen aggression or queen-laid egg destruction in *Bombus* species (Sladen, 1912; Alford, 1975; Van der Blom, 1986) or has found workers ate queen-laid eggs at very low rates (approximating zero) (Zanette et al., 2012). Consequently, the quantification of the rate at which workers eat queen-laid eggs and engage in aggression towards the queen in *B. terrestris* colonies, as presented in **Chapter 2**, provides a novel insight into the degree of actual queen-worker conflict over male parentage in this system. In addition, the findings suggest worker policing of queen-laid eggs is an extension of selfish worker policing, with reproductive workers attempting to enhance their own reproductive success by eating both queen-laid and worker-laid male-destined eggs. This contributes to the understanding of the complex interactions and reproductive conflicts within bumblebee colonies and the role selfish policing plays in regulating reproduction in *Bombus* species.

It seems key that workers would need to be able to differentiate between haploid and diploid queen-laid eggs to avoid destroying diploid eggs, which may be reared into gynes, in error. However, while it is known queen- and worker-laid eggs have chemical differences (Ayasse et al., 1999) it is not known if *Bombus* queens mark their eggs with chemical cues, as seen in species of wasps (Oi et al., 2015). While plausible, it is unlikely *Bombus* queens would be selected to use these chemical cues to differentiate between diploid and haploid eggs, as this would put queen-laid male-destined eggs at a greater risk of being destroyed by workers. It may be that workers use nest wax (Rottler-Hoermann et al., 2016) or queen pheromones (Alaux et al., 2006; Lopez-Vaamonde et al., 2007; Kocher and Grozinger, 2011) to determine, not only when to initiate egg-laying, but also whether to rear or destroy queen-laid eggs. However, further research is needed to draw any definitive conclusions in this area. Overall, investigating the chemical cues and communication mechanisms that underlie worker decisions regarding policing behaviours in *Bombus* species would be a valuable area of future study to gain a deeper understanding of the proximate mechanisms that underly cooperation and conflict within these eusocial insects.

5.2.3 Direct fitness is the ultimate function of dominance behaviour in *Bombus* species

In **Chapter 3** of this thesis, the primary aim was to investigate the ultimate function of dominance behaviour in animal societies, specifically by determining if a within-group positive association exists between dominance and reproductive success (direct fitness) in queenless groups of nest-mate *B. terrestris* workers. I found that within queenless groups the alpha was unable to completely suppress egg-laying in betas or subordinates and so did not monopolise egg-laying events. However, alphas exhibited significantly higher rates of aggression and policing (egg-eating) than all other group members. As a consequence, the survivorship of eggs laid by alpha workers was significantly higher than those laid by beta or subordinate workers. This indicates that the policing of eggs by dominant *B. terrestris* workers plays a pivotal role in enhancing their reproductive success. Overall, the findings of **Chapter 3** strongly support the hypothesis that, among *B. terrestris* workers, dominance rank is positively correlated with reproductive success. Adding to the evidence that, in animal societies in general, increasing direct fitness is the ultimate function of dominance behaviour. Furthermore, the study sheds new light on the significance of policing in resolving reproductive conflicts and ensuring the high reproductive success of alpha workers of *Bombus* species and, in all likelihood, in eusocial Hymenoptera in general.

In **Chapter 3**, reproductive success of workers was determined by the survival rate of eggs after the initial few hours following their laying. As previous research by Zanette et al. (2012) has shown that worker-laid eggs that survive policing during the first 2 hours of life are typically raised to adulthood, the calculated rate of egg-survivorship and successful (unpoliced) egg-laying employed in the current study serves as a reliable indicator of realised reproductive success among *Bombus* workers. However, the original aim of **Chapter 3** was to take the investigation a step further by developing and implementing a panel of Single Nucleotide Polymorphisms (SNPs) (Helyar et al., 2011; Galbraith et al., 2016) to conduct individual-level parentage analyses of adult-worker produced males, reared within each queenless group. Unfortunately, due to disruptions caused by the 2020-2021 global pandemic, the development of this panel of SNPs could not be completed within the timeframe of this thesis. Therefore, future research could include further efforts to develop this panel of SNPs, as it would prove to be a valuable tool for accurately determining the realised reproductive output of workers in *Bombus* species.

5.2.4 There is a positive association between egg-laying, egg-eating, aggression, and worker body size among *B. terrestris* workers, and this may indicate variation in intrinsic quality.

The results from **Chapter 2**, **Chapter 3** and **Chapter 4** consistently demonstrated a significant correlation between aggression, policing, and egg-laying and/or successful (unpoliced) egg-laying among workers. In **Chapter 2** and **Chapter 4**, workers were classified based on egg-laying and egg-eating behaviours. The four classes were defined in **Chapter 2** (**Table 2.2**) and were as follows: LEE (laying egg-eating worker), LNEE (laying non-egg-eating worker), NLEE (non-laying egg-eating worker) and NLNEE (non-laying non-egg-eating worker). In **Chapter 2**, workers classed as LEEs demonstrated significantly higher rates of egg-laying and egg-eating events and acts of aggression, compared to all other worker classes. Similarly, in **Chapter 4**, LEEs again exhibited significantly higher rates of egg-laying and acts of aggression, compared to all other worker classes. Moreover, in **Chapter 4,** it was also found that LEEs had significantly larger body sizes than workers in all other classes and, although LNEEs and NLEEs were not significantly different in size from one another, they were both significantly larger than NLNEEs.

The significant differences found in behaviour and body size may be indicative of variations in intrinsic quality among worker classes, with LEEs potentially being high-quality and high-ranking workers with the necessary resources to maintain reproductive dominance through strategies involving aggression and egg-eating. Further support for this suggestion comes from data in **Chapter 4** that suggests egg-laying workers, as a class, have greater longevity than non-laying workers as a class, with NLNEEs being the least long-lived of the four separate worker classes. Overall, these results support the component of the hypothesis proposed by Blacher et al. (2017) that interprets positive-fecundity associations as occurring in workers because of differences in intrinsic quality, with high-quality workers being larger in body size and also able to overcome costs of reproduction in order to exhibit high-levels of both fecundity and longevity.

Furthermore, the findings from **Chapter 2** and **Chapter 4** that show a relatively small proportion of egg-laying workers engaged in egg-eating events (11.3%-32.7%) could be attributed to differences in intrinsic worker quality. LEEs, with their larger body sizes and higher rates of reproductive behaviours and aggression, may represent a subset of egg-laying workers with strong resource-holding abilities, while LNEEs may lack the necessary resources to exhibit similar rates of aggression and policing behaviours. This aligns with the hypothesis proposed by Frank (1996; 2003), which suggests only individuals with strong resource-holding abilities should engage in policing behaviours, highlighting that even workers that freely choose to lay eggs can vary greatly in intrinsic quality. Overall, the results provide valuable insights in to the significance of intrinsic worker quality in regulating reproductive behaviour and dominance in workers in eusocial Hymenoptera.

To further investigate the proximate causes of variation in worker intrinsic quality, future research could explore differences in hormone levels and gene expression among workers classified as LEEs, LNEEs, NLEEs, and NLNEEs. Hormone levels and gene expression have already been shown to play crucial roles in regulating behaviour and reproductive traits in social insects (Bloch et al., 1996; Shpigler et al., 2010; Amsalem et al., 2015; Collins et al., 2023). Therefore, by researching these physiological factors, deeper insights can be gained into the proximate mechanisms that underlie worker behaviour, dominance, and reproductive success.

5.2.5 The effect of larval nutrition on worker intrinsic quality remains unclear

The main aim of **Chapter 4** was to investigate the relationship between fecundity and longevity among adult workers in *B. terrestris* and determine if this association arises from within-colony variation in intrinsic worker quality, which in turn is influenced by larval nutrition. However, the results of the study did not find substantial differences in body size among emerging workers from the different larval nutrition treatments (high-feeding and low-feeding). This suggests that the variations in the quantity of food provided in each treatment were not sufficient to induce noticeable differences in worker body size.

To further explore the effects of larval nutrition on worker intrinsic quality and body size, future research could consider repeating the experiment described in **Chapter 4,** but with alterations to the quantity of food provided to each treatment. By creating greater extremes in larval nutrition between treatments, it may be possible to observe more pronounced differences in worker body size and potentially establish a clearer relationship between larval nutrition, worker quality, and the associations between fecundity and longevity.

Additionally, future studies could also investigate other factors that may influence worker intrinsic quality, such as genetic factors (as described above) or environmental conditions during development, such as temperature. By examining a broader range of potential influences, a more comprehensive understanding of the factors that contribute to worker intrinsic quality and the ultimate reproductive success of individuals within bumblebee colonies, and other eusocial Hymenoptera, could be gained.

5.3 Concluding remarks

Overall, this thesis makes significant contributions to the understanding of how societies remain stable in the face of internal conflict, specifically by showing a significant relationship between dominance and reproductive success and further elucidating the role selfish policing plays in regulating queen-worker and worker-worker reproductive conflict in *Bombus* species. Furthermore, the observed correlations between body size and rates of egg-laying, egg-eating, and aggression highlight the significance of within-colony variation in worker intrinsic quality, as indicated by body size, in determining the reproductive dominance of individual workers.

5.4 References

Alaux, C., Jaisson, P., Hefetz, A., 2006. Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal. Behavioral Ecology and Sociobiology 60, 439-446.

Alford, D.V., 1975. Bumblebees. Davis-Poynter, London.

Amsalem, E., Grozinger, C.M., Padilla, M., Hefetz, A., 2015. The physiological and genomic bases of bumble bee social behaviour. Advances in Insect Pysiology 48, 37-93.

Ayasse, M., Birnbaum, J., Tengö, J., Van Doorn, A., Taghizadeh, T., Francke, W., 1999. Caste- and colony-specific chemical signals on eggs of the bumble bee, Bombus terrestris L. (Hymenoptera: *Apidae*). Chemoecology 9, 119-126.

Bloch, G., Borst, D.W., Huang, Z.-Y., Robinson, G.E., Hefetz, A., 1996. Effects of social conditions on Juvenile Hormone mediated reproductive development in *Bombus terrestris* workers. Physiological Entomology 21, 257-267.

Collins, D.H., Mohorianu, I., Beckers, M., Moulton, V., Dalmay, T., Bourke, A.F.G., 2017. MicroRNAs associated with caste determination and differentiation in a primitively eusocial insect. Scientific Reports 7, 45674.

Collins, D.H., Prince, D.C., Donelan, J.L., Chapman, T., Bourke, A.F.G., 2023. Costs of reproduction are present but latent in eusocial bumblebee queens. BMC Biol. 21, 153.

Frank, S.A., 1996. Policing and group cohesion when resources vary. Animal Behaviour 52, 1163-1169.

Frank, S.A., 2003. Repression of competition and the evolution of cooperation. Evolution 57, 693-705.

Galbraith, D.A., Kocher, S.D., Glenn, T., Albert, I., Hunt, G.J., Strassmann, J.E., Queller, D.C., Grozinger, C.M., 2016. Testing the kinship theory of intragenomic conflict in honey bees (*Apis mellifera*). Proceedings of the National Academy of Sciences, U.S.A. 113, 1020-1025.

Helyar, S.J., Hemmer-Hansen, J., Bekkevold, D., Taylor, M.I., O'gden, R., Limborg, M.T., Cariani, A., Maes, G.E., Diopere, E., R., C., Nielsen, E.E., 2011. Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. Molecular Ecology 11, 123–136.

Kocher, S.D., Grozinger, C., 2011. Cooperation, conflict, and the evolution of queen pheromones. Journal of Chemical Ecology 37, 1263–1275.

Lopez-Vaamonde, C., Brown, R.M., Lucas, E.R., Pereboom, J.J.M., Jordan, W.C., Bourke, A.F.G., 2007. Effect of the queen on worker reproduction and new queen production in the bumble bee *Bombus terrestris*. Apidologie 38, 171-180.

Oi, C.A., Van Oystaeyen, A., Oliveira, R.C., Millar, J.G., Verstrepen, K.J., van Zweden, J.S., Wenseleers, T., 2015. Dual effect of wasp queen pheromone in regulating insect sociality. Current Biology 25, 1638-1640.

Ratnieks, F.L.W., 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. American Naturalist 132, 217-236.

Rottler-Hoermann, A.-M., Schulz, S., Ayasse, M., 2016. Nest wax triggers worker reproduction in the bumblebee *Bombus terrestris*. Royal Society Open Science 3, 150599.

Shpigler, H., Patch, H.M., Cohen, M., Fan, Y.L., Grozinger, C.M., Bloch, G., 2010. The transcription factor *Krüppel homolog 1* is linked to hormone mediated social organization in bees. BMC Evol. Biol. 10, 120.

Sladen, F.W.L., 1912. The Humble-Bee. Its Life-History and How to Domesticate It. Macmillan and Co., Limited, London.

Van der Blom, J., 1986. Reproductive dominance within colonies of *Bombus terrestris* (L.). Behaviour 97, 37-49.

Zanette, L.R.S., Miller, S.D.L., Faria, C.M.A., Almond, E.J., Huggins, T.J., Jordan, W.C., Bourke, A.F.G., 2012. Reproductive conflict in bumblebees and the evolution of worker policing. Evolution 66, 3765-3777.