Mechanisms and Evolution of Phenotypic Plasticity in Social Insects

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Author’s declaration

I, Lewis Revely, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, or published elsewhere, I confirm that this has been indicated in the thesis.

Abstract

Phenotypic plasticity - the ability of individuals to develop into different phenotypes in response to the environment - is a primary component of biological diversity, permitting organisms to exploit different niches in response to changing environmental cues. The reproductive and non-reproductive roles of social insects are an especially intriguing example of phenotypic plasticity. To better understand the proximate and ultimate basis of variation in phenotypic plasticity (the degree of plasticity, reversibility, and speciality) we must explore across the life cycle of a phenotype within a species, the contrasting plasticity between two closely related species or across many species. By exploring the macroevolutionary patterns of phenotypic plasticity, we can further the understanding of phenotypic plasticity's relationship with fundamental evolutionary frameworks such as social complexity and major evolutionary transitions. This thesis explores the variation in phenotypic plasticity among the shared phenotypes of reproductives (queens) in the Polistes wasps and non-reproductives (workers) in the termites. First, comparative transcriptomics reveals high variation in neuro-transcriptional activity across the adult life cycle of the reproductive phenotype in *P. dominula*. This study emphasises the importance of stress and caste uncertainty in the early stages of the phenotype. Second, a combination of comparative genomics and transcriptomics point toward an expansion of molecular apparatus and conditional gene expression in the socially parasitic wasp *P. sulcifer;* this broadens our understanding of the evolution of phenotypic specialisation. Finally, phylogenetic comparative analyses provide evidence to the diminished importance of obligate sterility in termites for explaining phenotypic and evolutionary social complexity in this group. Collectively, these findings bring to light the complex and diverse nature of both intra- and inter-species phenotypic plasticity within the queen and worker phenotypes of social insects and find greater parallels across the major evolutionary transitions.

Impact statement

Phenotypic plasticity is a key attribute for organisms in a changing environment and is an invaluable process to shed light on the dynamic interplay between genetics and environmental influences. Studying the relationship of plasticity and social complexity also enhances our understanding of the mechanisms driving social organisation and altruistic behaviours between organisms. This thesis provides a greater understanding of plasticity and its relationship with social complexity at different scales. Broadening our understanding of these processes is pivotal for developing more nuanced frameworks and models of evolution, ecology and behaviour. This contributes to mitigating the impacts of environmental changes on biodiversity and human societies.

The first half of the thesis includes an examination of **intraspecific life-time changes in plasticity** within a single phenotype - the reproductive females (queens) of a social paper wasp, *Polistes dominula* - to understand the molecular basis of phenotypic specialisation during the life cycle of a reproductive adult female. It finds potential evidence for canalisation of gene expression through the life cycle, reflecting phenotypic specialisation as the uncertainty of reproductive strategy diminishes. It also uncovers the importance of environmental stress on transcriptional activity early in the adults life-cycle. This shows that single phenotypes are not necessarily static and within phenotype variation should be considered going forward.

Next, it examines an **interspecific evolutionary change in plasticity** exemplified in an inquiline social parasite *Polistes sulcifer*- a species that evolved from a social ancestor but has lost any form of working ability, compared to its social host *P. dominula*. By comparing new, chromosome-level genomes of these two species, together with phenotype-specific brain transcriptomes, this study uncovers an expanded genome in the parasite, together with an overall trend of up-regulated transcription. This likely reflects the phenotypic innovations of their unique lifestyle. This study is the first to explore the molecular mechanistic basis of social parasitism of a species with a simple social ancestor, paving the way for future analyses to explore further the interplay between the proximate and ultimate basis of social parasitism.

The second half of the thesis explores **macroevolutionary patterns of phenotypic plasticity and its relationship with social complexity**, particularly in the reproductive plasticity of the non-reproductive phenotype (workers) in termites. It first provides a **synthesis of phenotypic plasticity** across termites and highlights how degrees of plasticity are often over-simplified. This published work makes use of concepts in cellular developmental plasticity to define a new framework of understanding variation in this plasticity, applicable across any study system.

Finally, a **phylogenetic comparative study** is conducted to determine the interplay of losses and gains in reproductive plasticity with other traits indicative of social complexity. A weak correlation between social complexity and losses in reproductive plasticity is found, suggesting that the social complexity hypothesis may not apply universally to social insects. This work is now in review and will likely impact our understanding of social evolutionary theory through the re-examining of the central role of sterility in phenotypic and evolutionary social complexity; it also provides greater parallels between atypical systems across transitions.

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Table of Contents

Table of Figures

Figure number:

Table of Tables

Table number:

Chapter One:

Introduction

Phenotypic plasticity is fundamental to the diversity of biological forms, allowing organisms to adapt to changing environmental pressures (Pigliucci et al., 2006). A particularly fascinating example of phenotypic plasticity is exhibited within social systems, where discrete reproductive and non-reproductive phenotypes arise from a single genotype (Miura, 2004; Rehan et al., 2018; Revely et al., 2021; Srinivasan & Brisson, 2012; Sumner et al., 2018; Taylor et al., 2021). The plasticity of each phenotype can vary within the life cycle of a species, between closely related species and across many species. It is important that we unravel the evolutionary and mechanistic basis of this variability as it has fundamental ramifications for our understanding of phenotypic plasticity and for broader evolutionary frameworks such as social complexity and major evolutionary transitions (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2023; West et al., 2015).

Our understanding of plasticity and its relationship with sociality has been hindered by the narrow scope of the research in this area – e.g. in the choice of study organism and life-stage (Bourke, 2023; Howe et al., 2022; Revely et al., 2021). Outstanding questions include: to what extent do phenotypes vary within individuals through their various life stages? What are the drivers and mechanisms by which phenotypic plasticity is lost within and among species? In this thesis, I use genomic and phenotypic trait data from across a range of insect species, which exhibit diversity in phenotypic plasticity at different scales. These include plasticity across a reproductive life cycle in a simple social species *Polistes dominula* (chapter 2), between two closely related species with differing phenotypic capacity (*P. dominula* and *Polistes sulcifer*) (chapter 3), and then across the understudied termites to understand the interplay between varying worker reproductive plasticity (chapter 4) and social complexity (chapter 5). First, I will overview our understanding of phenotypic plasticity (section 1.1) and sociality (section 1.2) and the interplay of both (section 1.3).

1.1 Phenotypic plasticity

The following section overviews phenotypic plasticity, the costs of retaining this biological phenomenon (therefore why it might be lost) and the different types of plasticity that can be studied.

Phenotypic plasticity can be defined as ‘the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions’ (Pigliucci et al., 2006). Just as gene expression can change through development, the environment can affect gene expression and interact to alter developmental trajectories (Gilbert, 2005). If the development-changing in response to the environment causes an increase in fitness and is also heritable, morphology, physiology and life history strategies potentially evolve adaptive phenotypic plasticity (DeWitt & Scheiner, 2004; West-Eberhard, 2003). Plasticity may result in an increase in fitness when an environment is heterogenous, unpredictably unstable, or when there is high gene flow among populations with diverging environments. For example, many species have evolved to plastically change their appearance to match the environment, camouflaging themselves from predation (Stevens, 2016).

1.1.1 Costs of Plasticity

There are costs to plasticity that can outweigh the benefits and may cause its loss through evolutionary history or promote specialisation of individuals within their lifetime. The cost to maintain sensory and regulatory machinery can be high (DeWitt et al., 1998). For example, the ability of plants to grow in response to the presence of ethylene requires sensory machinery in the form of ethylene receptor proteins on the plants’ cell membranes (Fusco & Minelli, 2010; Jones, 1994). The energetic and material cost of producing this sensory apparatus would not be needed if the plastic growth response did not occur. There will also be genetic costs related to the negative effects of pleiotropy and epistasis (Fusco & Minelli, 2010). Another cost of plasticity can arise when the cost of production is greater for plastic genotypes compared with those that have fixed genotypes (DeWitt et al., 1998). Therefore, there is an evolutionary trade-off between the adaptive retention of plasticity considering environmental uncertainty and its loss to increase efficiency of the phenotype

(Waddington, 1960; Revely et al., 2021; Waddington, 1942a, 1942b). To understand the evolutionary and mechanistic basis for the losses and gains in plasticity, we must explore across the various types of plasticity seen within, between and across many different species which experience different forms of environmental and developmental conditions.

1.1.2 Types of phenotypic plasticity

Changes in environmental pressures can lead to continuous phenotypic variation or the production of discrete phenotypic variation, called polyphenisms (Nijhout, 2003). A well-known example of a polyphenism is sex determination in reptiles and fish (Leonard, 2013) (Figure 1.1A). Within a number of these ectothermic species, the temperature that is experienced during their development will determine whether the embryo turns into a male or female as an adult (Leonard, 2013). Temperature-dependent sex determination is advantageous when the growth rate is a function of the temperature in the environment and there are differential advantages for males and females with respect to size (Leonard, 2013). Therefore, temperature-dependent sex determination will allow individuals to change to the sex that gives them the highest fitness via reproductive success with respect to the environmental conditions (D. A. Warner & Shine, 2008). Another fascinating text-book example of discrete polyphenisms includes sneaky and fighter males within beetles, which are different morphological strategies to successfully reproduce (Moczek, 2010; Moczek & Emlen, 2000; Valena & Moczek, 2012) (Figure 1.1B). These make exemplar systems to study the mechanistic basis of phenotypic plasticity (West-Eberhard, 2003). By comparing these different phenotypes, the genetic processes leading to these examples of phenotypic plasticity can be elucidated (Beldade et al., 2011; Corona et al., 2016; Evans & Wheeler, 2001; Lo et al., 2018). For instance, comparative transcriptomics has allowed for a greater understanding of the molecular mechanistic basis of discrete phenotypic plasticity (Casas et al., 2016; Cullen et al., 2017; Mulugeta et al., 2017; Taylor et al., 2021).

Even once an individual has reached adulthood, the phenotype it exhibits is not necessarily a static state; changing developmental, ecological, and social conditions may make plastic responses in the single phenotype advantageous (Crispo, 2007; Degnan & Degnan, 2010;

Garland & Kelly, 2006; Moczek, 2010; West-Eberhard, 2003). For instance, when seasonal conditions lead to freezing temperatures in winter, the wood frog (*Rana sylvatica*) goes into dormancy, reducing its metabolism to survive the harsh environment (Costanzo, 2019) (Figure 1.1C). An organism's social environment can also drastically change an individual's phenotype; for example, some fish have been shown to have social sex determination (Godwin, 2009) (Figure 1.1D). When the large dominant male reef fish within a territory is removed, the largest female will be stimulated into becoming a male as they can maximise their reproductive success by doing so. In meerkats, a species of cooperatively breeding mammal, a helping individual has the potential to take over the group as a dominant reproductive if the leader dies, even though it has spent its adult life thus far as a non-reproductive (Duncan et al., 2023) (Figure 1.1E). Some studies have outlined the molecular basis of these changes, highlighting the plastic mechanistic processes at play to create these subtle differences over a life cycle (Denlinger, 2002; Pimsler et al., 2020; Ragland & Keep, 2017).

One of the best-studied examples of polyphenisms are specialist roles within social systems (Miura, 2005; Rehan et al., 2018; Revely et al., 2021; Roisin, 2000; Shibao et al., 2010; Srinivasan & Brisson, 2012; Taylor et al., 2021). The morphological and behavioural differentiation of reproductive and non-reproductive phenotypes can be seen across the whole diversity of life (Boomsma, 2022; Bourke, 2011a; West et al., 2015). From the differentiation of somatic and germline cells in multicellular organisms to the workers (helpers) and queens (and kings) of highly complex social insects, the study of these discrete polyphenisms has yielded novel insights into the nature of phenotypic plasticity (Miura, 2005; Rehan et al., 2018; Revely et al., 2021; Roisin, 2000; Shibao et al., 2010; Srinivasan & Brisson, 2012; Taylor et al., 2021). Insights have been gained mechanistically, where the gene regulatory basis of these polyphenisms are being continuously uncovered (Rehan et al., 2018; Srinivasan & Brisson, 2012), and evolutionarily, suggesting that when plasticity is lost at the individual level it is gained at the colony level (Taylor et al., 2019). Less well-known is how plasticity within these phenotypes vary, particularly within the reproductive phenotype where different environmental factors such as changing developmental, ecological and social conditions could lead to changes within this phenotype (Crispo, 2007;

Degnan & Degnan, 2010; Garland & Kelly, 2006; Moczek, 2010; West-Eberhard, 2003). Understanding the interplay between these subtle changes within a phenotype will provide a greater understanding the evolution of plasticity. Further, the plastic capacity of the non-reproductive phenotypes to become reproductive is pivotal to our understanding and categorization of the complexity of life (Bernadou et al., 2021; Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2023; Howe et al., 2022). To fully understand the proximate and ultimate processes underlying the relationship between plasticity and sociality, we must first understand the proximate and ultimate basis of sociality itself.

Figure 1.1: Exemplar species showing different types of phenotypic plasticity. For A) Alligators with temperature-based sex determination (image from Samantha Bock) B) sneaky and fighter male horned beetles (image from Alex Wild) C) Wood frog surviving freezing temperatures in winter (image from Janet. M. Storey) D) social sex determination in coral reef fish (image from Kevin Bryant) E) reproductive plasticity in cooperatively breeding meerkats (image from shutterstock).

1.2 Sociality

Section 1.3 outlines the outstanding questions relating to the plastic nature of the reproductive and non-reproductive phenotypes for our understanding of sociality. But first, section 1.2 gives an overview of the most important frameworks in social evolutionary theory and explains the variation in sociality at the highest level, in insects; this will contextualise the relationship of plasticity in the reproductive and non-reproductive phenotypes with sociality.

1.2.1 Major Evolutionary Transitions

Life can be seen as an emergent process of the bringing together of biological units into higher level units and the subsequent synthesis of new higher units into integrated collectives. This process has been repeated several times to produce the biological hierarchy that can be observed today (Bourke, 2011a). This hierarchical view of social complexity arose in the late 19th and early 20th centuries (Wheeler, 1911) but it was not until Maynard-Smith and Szathmary (in 1995) that the term 'major transition in evolution' was proposed (Szathmáry & Maynard-Smith, 1995). They set out a comprehensive framework of what constitutes a major evolutionary transition (MET), broadly defined as a significant change in the way that heritable information is stored and transmitted (Szathmáry & Maynard-Smith, 1995), thus allowing for transitions such as to sex and language. Queller (2000) later outlined there are two distinct categories of METs: fraternal, which is the grouping of related individuals, and egalitarian, which is the grouping of non-related individuals (Queller, 2000). An egalitarian transition is exemplified by separate unicells becoming a symbiotic unicell such as in a eukaryotic cell. Examples of fraternal transitions are unicells becoming multicellular organisms such as in plants or animals. This framework constitutes the evolutionary view of social complexity and allows us to better understand the evolutionary basis of the complexity of life. It also provides a novel basis for comparing across these transitions to identify fundamental processes that potentially lead to repeated innovations in the evolution of complex individuals; this includes the variation in plasticity of the reproductive and non-reproductive phenotypes (Boomsma, 2022; Bourke, 2011a; Revely et al., 2021; Taylor et al., 2019; West et al., 2015).

METs (or more broadly evolutionary social complexity) can be defined by two conditions (Bourke, 2011a). Firstly, biological units which were able to replicate independently before the transition are now only able to as part of a higher-level unit, in other words there is complete interdependence. Secondly, there is a lack of within group conflict (alignment of interests) between biological units such that the higher-level unit itself can be thought of as a fitness maximising individual. These transitions are achieved through a two-step pathway of (1) group formation, and (2) group transformation into a cohesive and integrated entity which could be considered an organism in itself (West et al., 2015) (Figure 1.2). The first step is characterised by individual entities performing different tasks (division of labour) but are highly flexible and able to change roles in their lifetime. The second step is characterised by division of labour becoming so specialised that they become irreversibly interdependent (West et al., 2015). Unifying factors, leading to entities transitioning, have been ecological benefit to cooperation and a mechanism which unites the interests of different individuals (West et al., 2015). These have been well documented within most taxa but none more so than in social insects which span the transition from a multicellular organism to a superorganism (Miura, 2005; Rehan et al., 2018; Revely et al., 2021; Roisin, 2000; Shibao et al., 2010; Srinivasan & Brisson, 2012; Taylor et al., 2021).

Figure 1.2: Steps of a major evolutionary transition leading to increases in evolutionary social complexity, adapted from (West et al., 2015).

1.2.2 Inclusive fitness theory

Before overviewing the highest form of evolutionary social complexity, we must understand the theory of why an organism would cooperate in the first place, and in some circumstances even commit completely to one role (reproductive or non-reproductive). This is well described through Hamilton's rule, where individuals are thought to exhibit cooperative behaviour if the gain in fitness via their relatives is greater than their own personal loss in fitness (Hamilton, 1964). Altruistic cooperative behaviour will therefore be favoured when $rb - c > 0$, where b is the fitness benefit to the beneficiary, c is the fitness cost to the altruist, and r is their genetic relatedness (Hamilton, 1964). Like the transition to multicellularity which required clonality, social insects exhibited lifetime monogamy when transitioning to superorganismality (West et al., 2015). This meant that their relatedness within the group allowed for cooperation to proliferate due to beneficial actions being favoured because of genes for these beneficial actions being present in their relatives. This is referred to as kin selection (Hamilton, 1964). By contributing to a relative's reproduction through actions such as caring or protection, the individual is still passing on copies of its genes, even if indirectly. These are important factors working within both the necessary transitional steps to superorganismality. They are also important in understanding the relationship between sociality and plasticity in the reproductive and non-reproductive roles.

1.2.3 Superorganismality

Insects vary massively along a spectrum of evolutionary social complexity; for simplicity, species tend to be described as members of one of three categories (Bourke, 2011a): (1) individuals that have not yet evolved the first step in transitioning to superorganismality, which are solitary breeding insects that reproduce independently and are likely to be the ancestral condition of all social insect lineages. (2) Individuals that have evolved the first step transitioning towards superorganismality have shifted from solitary breeding to cooperative breeding, in which social groups are formed and division of labour occurs. However, at this stage individuals still have high reproductive flexibility. (3) Finally, those that have evolved the second step in the transition are superorganismal species which have extreme morphological division of labour with reproductive and non-reproductive castes (Boomsma, 2022; Bourke, 2011a; Revely et al., 2021; West et al., 2015).

Superorganismality, which is a fraternal transition to individuality, is likely to have arisen multiple times independently throughout the social insects (Boomsma, 2022; Bourke, 2011a; Revely et al., 2021; West et al., 2015). This is best known in Hymenoptera (bees, wasps and ants), and in the termites. Insect societies are unique within the products of the major evolutionary transitions due to the biological components (individual insects) being easily deconstructed and manipulated, thus allowing life's proximate and ultimate processes to be examined (Kennedy et al., 2017). There has been much confusion on the terminology behind highly social insects. Wheeler (1911) first defined superorganismality as colonies with physically polymorphic queen and worker castes (Wheeler, 1911). However, the definition was later misconstrued and then disregarded in the literature. Instead, the term eusociality became prominent in research for social insects (Wilson, 1971). The definition was first used to describe the sociality of halictid bees, in that an organism can be thought of as eusocial if you cooperatively breed in matrifilial nests which are permanent enough for all for multiple generations to be present within one nest (Batra, 1966). Boomsma (2018) outlines that it is only through the use of Wheeler's (1911) definition, where each colony member is committed to a single morphologically distinct adult caste that is fixed during early individual development, that we can fully begin to disentangle the proximate and ultimate processes at play in the transition to superorganismality (Boomsma & Gawne, 2018). There however, has been further debate on the importance of the irreversibility and necessity of a fully committed sterile worker phenotype (loss of reproductive potential in the worker phenotype) for the transition to superorganismality (Bernadou et al., 2021; Boomsma & Gawne, 2018; Bourke, 2023; Howe et al., 2022; Revely et al., 2021).

1.2.4 Sterility and phenotypic social complexity

There are several long-standing assumptions surrounding the reproductive potential of the worker phenotype that make it central to many concepts within social evolution. It has been proposed that once a species has gained sterility (or lost its reproductive potential), they have transitioned through an irreversible barrier in which they will be selected for greater complexity (i.e. an MET) (Bernadou et al., 2021; Boomsma & Gawne, 2018). It is also assumed that there will be a positive relationship between worker sterility and all the other phenotypic social complexity traits, leading to assumptions that all these measures can be conflated (Boomsma & Gawne, 2018; Howe et al., 2022; Michod, 1997; Michod et al., 1996; Michod & Roze, 1997).

Examples of these other phenotypic social complexity traits include colony size, helper polyphenism, and nest complexity (Table 1.1). Colony size quantifies the number of individuals that a colony is composed of (Bonner, 2004; Boomsma & Gawne, 2018; Bourke, 1999; Ferguson-Gow et al., 2014; Holbrook et al., 2011; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019; Kramer & Schaible, 2013; La Richelière et al., 2022). The larger the colony, the more likely they are to specialise due to reduced chances of becoming a primary reproductive. More social interactions and different more complex jobs are undertaken in larger colonies also, leading to complex behaviours and extended phenotypes (Bonner, 2004; Bourke, 1999; Ferguson-Gow et al., 2014; Holbrook et al., 2011; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019; Kramer & Schaible, 2013)(Table 1.1). Helper polyphenism describes the discrete variation in forms within a colony. The greater number of these discrete forms present within a colony allows for the formation of specialised groups that can efficiently perform different tasks, increasing the productivity of the colony (Ferguson-Gow et al., 2014; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019; La Richelière et al., 2022; Sumner et al., 2018) (Table 1.1). Nest complexity signifies the set of behaviours colonies can utilise to build and maintain their nests (Table 1.1). Other traits include colony longevity and queen-worker dimorphism. Understanding the relative strength in association between the reproductive plasticity of the worker (or helper) phenotype and these other phenotypic social complexity traits will aid in our understanding of the role of worker plasticity in the evolution of social systems.

	LOW	HIGH
Helper Polyphenism		
Nest Complexity		
Colony Size	x10	x1000000

Table 1.1 Example phenotypic social complexity traits in termites and representations of the lowest and highest levels of each. Helper polyphenism with either one morph of helpers being low or multiple in a colony being high; Nest complexity with wood nests being low and large above ground mounds being high; Colony size with tens of individuals in a colony being low and millions being high. Polyphenism images collected from NHM London collections; nest complexity image from Bill Sands.

1.3 The Interplay of Plasticity and Sociality

It is important to explore the evolutionary and mechanistic basis for changes in plasticity in both the non-reproductive and reproductive phenotypes to aid in our understanding of key evolutionary processes. There are seemingly distinct differences in the plasticity of these phenotypes between the three categories of insect sociality therefore making insects ideal systems for understanding phenotypic plasticity evolution (Table 1.2). The reproductive tasks that are performed in solitary breeding insects, for instance reproduction and caring, are sequential, with environmental influences such as mortality risk (Javoiš & Tammaru, 2004) or seasonal fluctuations in the availability in resources (Koštál, 2006) dictating the state that will be present. High individual reproductive plasticity allows a single entity to able to do both reproductive and non-reproductive tasks and modify their expression in response to fluctuating environmental and physiological factors (Javoiš & Tammaru, 2004; Koštál, 2006) (Table 1.2). However, this high individual reproductive plasticity will incur a cost due to the trade-off of optimising the two differing states which are limited by the time and production costs required to switch traits (Murren et al., 2015). These costs will be further exacerbated when the individual resides in an unpredictable environment and when the traits related to the two different traits require a lot of time to modify (Gabriel, 2006). This form of within individual plasticity of the reproductive phenotype is exemplified by solitary wasps like the Eumenid *Synagris cornuta and* Sphecid *Ammophila pubescens* which sequentially provision and care for their brood themselves, effectively cycling through periods of reproductive and non-reproductive labour (Baerends, 1941; Field, 1992; West-Eberhard, 1996).

The transition from solitary to cooperative breeding leads to a shift from performing tasks sequentially to in parallel through reproductive division of labour (Peeters & Molet, 2010) (Table 1.2). In that, tasks are no longer performed by a single individual but instead allocated to multiple. Consequently, these cooperative groups have a small number of dominant reproductive individuals and a larger number of non-reproductive (working) individuals that undergo tasks such as foraging, offspring care and nest defence. By switching from sequential to parallel task performance, the trade-off of reproductive and non-reproductive investment can be avoided (Peeters & Molet, 2010). These individuals within cooperative groups still retain reproductive totipotency for the majority of their lives, with non-reproductives able to acquire reproductive traits in the event the dominant reproductive within the colony dies (Korb & Hartfelder, 2008). However, for the reproductive phenotype within these colonies, there may well be some form of specialisation. This is due to inclusive fitness theory predicting that to maximise their direct fitness they should be the most efficient member possible and therefore lose the capacity to work once in an established nest (Bourke, 2011b; Hamilton, 1964; Linksvayer & Wade, 2005), 1964; Linksvayer & Wade, 2005). More research into this is required to understand the queen’s level of specialisation behaviourally and molecularly. Model cooperatively breeding insect systems include Halictid bees and paper wasps (Batra, 1966; Field & Leadbeater, 2016; Turillazzi & West-Eberhard, 1996)

The shift from cooperative breeding to superorganismality involves a reduction in individual plasticity, allowing for strong specialisation in roles (Table 1.2). Consequently, morphological differentiation between the reproductive and non-reproductive phenotypes is present in these superoganismal species (Boomsma, 2022; Boomsma & Gawne, 2018; West et al., 2015). The transition signifies complete interdependence and alignment of interests, leading to higher individuality (Boomsma, 2022; Boomsma & Gawne, 2018; West et al., 2015). Examples of highly specialised caste differentiated species are present across the termites, most ants, Honeybees and Vespine wasps (Boomsma & Gawne, 2018; Revely et al., 2021; Daisy Taylor et al., 2018). At this level the reproductive phenotype is highly specialised and efficient in its role for reproduction, unable to return to working behaviours after colony establishment. However, there has been much debate on the reproductive plasticity of the working phenotype at this level (Bernadou et al., 2021; Boomsma & Gawne, 2018; Bourke, 2023; Howe et al., 2022; Revely et al., 2021). It may be the case that complete interdependence and alignment of interests require a complete loss of reproductive plasticity in the worker phenotype (obligate sterility) (Bernadou et al., 2021; Boomsma & Gawne, 2018). This, however, may not be the case, as highly complex organisms and colonies have been shown to retain only functionally sterile worker phenotypes, leading us to question the assumption that obligate sterility is necessary for superorganismality (Bourke, 2023; Revely et al., 2021; Zhuang et al., 2023). Therefore, exploring the mechanistic and evolutionary basis for the specialisation of the non-reproductive (worker) phenotype is fundamental to our understanding of superorganismality and all social complexity (phenotypic and evolutionary).

Moreover, the levels of plasticity within the reproductive and non-reproductive phenotypes exhibited at each category of sociality is not a static state. They vary within a phenotype’s life cycle, between species over evolutionary time (specifically with a major loss or gain), and more widely across taxa. Understanding the mechanistic and evolutionary basis for these variations will provide us with a greater understanding of plasticity and sociality.

	Solitary	**Cooperative breeding**	**Superorganismal**
			
Reproductive phenotype	Sequential phenotypic plasticity of both worker and reproductive phenotypes	No morphological specialisation but likely behavioural.	Behavioural and morphological specialisation
Worker Phenotype		Totipotent, can plastically switch to reproductive if the chance arises	Morphological specialisation but complete commitment (obligate sterility) is debated

Table 1.2: Plasticity of the reproductive and non-reproductive (worker) phenotypes across the spectrum of sociality; Solitary exemplified by a burying beetle with its brood (image from Rahia Mashoodh), cooperative breeding exemplified by a colony of *Polistes lanio* (image from Owen Corbett), and superorganismal exemplified by a *Macrotermes* colony (image from Bill Sands).

1.3.1 Intraspecific variation in plasticity of reproductive phenotype

The intraspecific variation in reproductive plasticity, which is exemplified by the sequential reproductive and non-reproductive behaviours in solitary insects, occurs at all levels of sociality. For instance, in many socially complex groups, like that of termite colonies, queens must first work to establish a nest and tend young before handing over the work to their brood (George & Wilson, 1978; Shellman-Reeve, 1990). Further, this pathway to established queenhood is fraught with social and abiotic environmental changes (Cervo, 2006; Cini & Dapporto, 2009; Field & Leadbeater, 2016; Gamboa et al., 2004; Gibo, 1972; Jandt et al., 2014; Karsai et al., 1996; Leadbeater et al., 2011; Pepiciello et al., 2018; Starks, 2001). This provides a perfect model system to explore the genotype-phenotype relationship and therefore the molecular basis of plastic phenotypes.

The queen phenotype in cooperatively breeding societies is particularly interesting as it must contend with uncertainty relating to their reproductive fate across their lifecycle (Kronauer & Libbrecht, 2018; Leadbeater et al., 2011; Patalano et al., 2015; West-Eberhard, 1996). Females of these simple societies initially balance reproductive and non-reproductive roles before the colony is established and stabilises, transitioning from a hopeful queen to foundress, to finally a specialised primary reproductive (Bourke, 2011b; Hamilton, 1964; Kronauer & Libbrecht, 2018; Leadbeater et al., 2011; Patalano et al., 2015; West-Eberhard, 1996). Changing environmental variables and progressive phenotypic specialization provide a fascinating source for understanding the molecular basis of phenotypic plasticity. However, very little has been studied on the molecular narrative behind the journey to queenhood (Berens et al., 2014; Field & Leadbeater, 2016; Leadbeater et al., 2011; Patalano et al., 2015; Standage et al., 2016). Instead, most studies have concentrated on the plastic capacity of the working phenotype to become the primary reproductive, due to its connotations with social evolutionary theory (Hunt, 2006; Jandt et al., 2017; Suryanarayanan et al., 2011; Taylor et al., 2021; Turillazzi & West-Eberhard, 1996). Exploring the relative importance of these different environmental variables on the molecular basis behind the steps to becoming an established queen provides an opportunity to further our fundamental understanding of phenotypic plasticity. It will also help understand the extent to which simple social queens actually undergo molecular specialisation.

1.3.2 Interspecific evolutionary change in plasticity

The stepwise progression from solitary to cooperatively breeding and finally superorganismal is a simplistic view of the evolution of the species we see (Linksvayer &

Johnson, 2019). There are many examples of losses and gains in evolutionary social complexity which are a result of a loss in the plasticity of a species. For instance, sweat bees have evolutionarily gained and lost parallel reproductive division of labour, with reductions in evolutionary social complexity when regressing to solitary species (Jones et al., 2023; Schwarz et al., 2007). Within the termites, there are several species that have also lost the ability to produce soldiers, reducing colony polyphenisms and therefore phenotypic social complexity (Bourguignon et al., 2016; Revely et al., 2021; Romero Arias et al., 2021).

A fascinating example of a reversion in sociality can be seen in social parasites. Over evolutionary time these species have lost the worker phenotype and the reproductives ability to work themselves, instead relying on the altruistic behaviours of closely related social hosts (Brandt et al., 2005; Stoldt et al., 2022). At a genomic level, transposable elements selfishly replicate within a host genome (Ågren, 2014). At a cellular level, transmissible cancers reverted from somatic cells and proliferated from their original host to exploit the altruistic resources of other multicellular organisms (Dujon et al., 2020; Ujvari, Gatenby, et al., 2016; Ujvari, Papenfuss, et al., 2016), as in canine transmissible venereal tumour (Baez-Ortega et al., 2019) and Clam leukaemia (Metzger et al., 2015). This reversion in sociality is exemplified by Hymenopteran social parasites and in particular inquiline social parasites that use the workers of their host to raise strictly reproductive offspring (Alleman et al., 2018; Borowiec et al., 2021; Cervo, 2006; Cini et al., 2019; Hines & Cameron, 2010; Schrader et al., 2021; Stoldt et al., 2022)(Figure 5). They have also evolved novel innovations relating to usurping and integrating into the host’s nest, like heightened aggression and chemical mimicry (Cervo, 2006; Cini, Bruschini, Poggi, et al., 2011; Cini, Bruschini, Signorotti, et al., 2011; Cini, Ortolani, et al., 2015; Ortolani & Cervo, 2009). These parasite-host relationships likely evolved from common ancestors, and the host is usually a close relative of the social parasite (Emery, 1909).

This system of closely related species which have a distinct loss in plasticity in the social parasite has become a fantastic model for understanding the molecular basis of phenotypic plasticity (Cini, Patalano, et al., 2015; West-Eberhard, 1989, 2003). There are several non-mutually exclusive hypotheses outlining the mechanistic basis for the loss of the worker phenotype and working behaviours including molecular loss, molecular gain, and conditional gene expression, with some evidence for each hypothesis (Alleman et al., 2018; Cini et al., 2019; Cini, Patalano, et al., 2015; Smith et al., 2015; Stoldt et al., 2022; Sun et al., 2020). No study has tried to explore the relative importance of these hypotheses in a single study system. Moreover, most studies exploring this question have concentrated on parasites of superoganismal societies that likely evolved from ancestors that are superoganismal (Alleman et al., 2018; Schrader et al., 2021; Smith et al., 2015; Stoldt et al., 2022; Sun et al., 2020). No studies have yet explored the molecular basis of social parasitism in species with cooperatively breeding (simple social) ancestors, where the reproductive and non-reproductive phenotypes have retained high phenotypic plasticity (Cervo, 2006; Cini et al., 2019; Cini, Patalano, et al., 2015). This could potentially provide a novel mechanistic narrative for the convergent evolution of social parasitism. Comparative genomics and transcriptomics between a closely related host and social parasite will help us understand this mechanistic basis for the loss of the worker phenotype and reduction in phenotypic plasticity.

Figure 1.3: Exemplary representatives of social parasitism across the Hymenoptera; A) *Bombus rupestris* (a bumblebee) on the right is parasitising its *host Bombus lapidarius* (image by Luca Franzini); B) *Pogonomyrmex anergismus* (an ant) on the left, parasitising its host *Pogonomyrmex barbatus* (image by Elizabeth Cash); C) *Polistes sulcifer* (a wasp), on the left is parasitising its host *Polistes dominula* which is on the right (image by Rita Cervo).

1.3.3 Macroevolutionary patterns of reproductive plasticity in the working phenotype and its relationship with sociality

The complete loss of worker reproductive plasticity (obligate sterility) is thought to have been of central importance in phenotypic and evolutionary social complexity (Bernadou et al., 2021; Boomsma, 2022; Boomsma & Gawne, 2018). However, more recently, studies across the major transitions have cast doubt on the central nature of this (Bourke, 2023; Howe et al., 2022; Revely et al., 2021). For instance, the ability of some multicellular organisms to regenerate their germline, if necessary, casts doubt on the importance of complete early separation of germline and soma (i.e., worker sterility) for obligate multicellularity and, therefore, for every major evolutionary transition (Howe et al., 2022; Pineda-Krch & Lehtilä, 2004; Pineda-Krch & Fagerström 1999). Those species which can regenerate severed gonads, such as starfish (Echinodermata), have somatic cells which are functionally sterile, not obligately sterile (Wessel et al., 2014). It has also been hypothesised that this form of plastic development represents the ancestral metazoan form (Howe et al., 2022). Consequently, it may be that functional sterility of somatic cells, rather than obligate sterility, is more important for the transition to obligate multicellularity (i.e., transitioning from a group of separate cells to a multicellular individual). Similarly, it may be that the presence of functional sterility, rather than obligate sterility, is more important for the transition to superorganismality.

The importance of sterility for evolutionary social complexity is underpinned by its supposed linear association with other phenotypic social complexity traits (Boomsma & Gawne, 2018; Howe et al., 2022; Michod, 1997; Michod et al., 1996; Michod & Roze, 1997). However, studies have recently shown that highly socially complex species of bumblebees still retain reproductive plasticity in the worker phenotype (functional sterility) (Zhuang et al., 2023). More broadly, studies have also questioned the linear nature of all these complexity traits due to variation seen in Hymenoptera across multiple species (Holland & Bloch, 2020).

There has already been a call to broaden of our understanding of multicellularity to include more diverse developmental strategies as seen in some metazoan taxa and modular organisms such as plants (Howe et al., 2022; Pineda-Krch & Lehtilä, 2004; Pineda-Krch & Fagerström, 1999). To explore the overall importance of functional and obligate sterility in explaining evolutionary social complexity we must examine them at the highest levels of sociality in developmentally atypical systems. Further, to truly understand the phenotypic and evolutionary associations between worker reproductive plasticity with social complexity traits, large scale macroevolutionary studies are required across a diverse number of species. Only then can we make broad scale conclusions.

1.4 Novel systems

In this section I outline model systems equipped to answer questions on intraspecific, interspecific and macroevolutionary patterns in plasticity across the sociality spectrum.

1.4.1 *Polistes dominula*: a model system for exploring intraspecific variation in the queen phenotype

Polistine paper wasps (and in particular *Polistes dominula*) have been extensively studied to understand their simple societies and highly plastic division of labour (Kronauer & Libbrecht, 2018; Leadbeater et al., 2011; Patalano et al., 2015; West-Eberhard, 1996) and the molecular basis of this (Cini et al., 2019; Cini, Patalano, et al., 2015; Hunt et al., 2011; Legan et al., 2021; Taylor et al., 2021). Although various studies have explored the plastic capacity of working individuals to become primary reproductives and the molecular basis of this, the molecular steps to queenhood from overwintering hopeful queens has not. The lifecycle of *P. dominula's* queen phenotype however is fascinating and filled with uncertainty, both environmentally and socially, making it an ideal system to explore the mechanistic basis of intraspecific variation in phenotypic plasticity (Field & Leadbeater, 2016; Hunt, 2006; Jandt et al., 2014; Karsai et al., 1996; Kovac et al., 2022; Pardi, 1996; Pepiciello et al., 2018).

Future *P. dominula* queens go through three distinct life stages, each with distinct environmental and social pressures that could potentially shape them at a molecular level (Figure 1.4). At the overwintering stage, these hopeful queens gather in hibernacula which shelter them from rain and predation and allow them to enter diapause (Kovac et al., 2022). During this stage there is high uncertainty of survival due to such harsh conditions and their reproductive role is also undecided (Gamboa et al., 2004; Gibo, 1972; Pratte, 1982; Starks, 2001). By spring, the females emerge and build nests either alone or with co-foundresses (Dapporto, Pansolli, et al., 2004; Field & Leadbeater, 2016). When there are co-foundresses, this stage is one of instability, with fights to decide the position of primary reproductive for the rest of their lifecycle (Leadbeater et al., 2011). Consequently, at this stage there is still uncertainty on their future role as reproductive or non-reproductive on the nest, and therefore both indirect and direct fitness could be possible avenues for them (Field & Leadbeater, 2016; Leadbeater et al., 2011). It is only at the summer stage when the workers emerge from the nest that the dominant female is an established queen on the nest. There, they can be seen as a specialised reproductive individual, certain of their role on the nest as the primary reproductive (Leadbeater et al., 2011). Between these stages, alongside the different physiological changes and reproductive role certainty, the social environment also changes, with a stepwise increase in social interactions and behaviours required for this (Cervo, 2006; Cini & Dapporto, 2009; Field & Leadbeater, 2016; Jandt et al., 2014; Karsai et al., 1996; Pepiciello et al., 2018).

By exploring the molecular narrative across the life cycle of the queen phenotype we can begin to understand the relative importance of these different variables and further our understanding of the plasticity of the journey to queenhood. This will consequently allow us to expand our fundamental understanding of phenotypic plasticity (Cervo, 2006; Field & Leadbeater, 2016; Jandt et al., 2014; Karsai et al., 1996; Pepiciello et al., 2018). This will also provide greater evidence on the extent to which queens in simple societies undergo molecular specialization.

Figure 1.4: *P. dominula* at three distinct life stages; A) Overwintering B) Founding of a nest C) Established summer nest (images from Alessandro Cini and Rita Cervo).

1.4.2 *Polistes sulcifer*: a model system for interspecific variation in plasticity

Although *P. dominula* provides an ideal model system for exploring intraspecific variation in plasticity, it is also part of a fascinating system for understanding interspecific variation in plasticity. The closely related social parasite *Polistes sulcifer* selfishly manipulates the workers of *P. dominula* to look after its brood, parasitising the social host's altruistic behaviours (Cervo et al., 2004; Cervo, 2006; Cini, Bruschini, Poggi, et al., 2011; Cini, Bruschini, Signorotti, et al., 2011; Cini et al., 2014, 2019; Cini, Ortolani, et al., 2015; Cini, Patalano, et al., 2015; Ortolani & Cervo, 2009; Pepiciello et al., 2018; Rozanski et al., 2021). This system has been well documented but lacks molecular analyses to uncover the mechanistic basis for the loss of the worker phenotype in *P. sulcifer* (Cervo, 2006; Cini, Patalano, et al., 2015). Further, it is a novel system to explore the molecular underpinnings of this loss in plasticity from a simple social ancestor.

Alongside the losses, the social parasite also has novel physiological and behavioural gains at each stage of its lifecycle that could be present molecularly (Figure 1.5)(Cervo, 2006). For example, the social parasite must physiologically deal with much more stressful environmental pressures when overwintering due to hibernating at much higher altitudes (Ragland & Keep, 2017). It has also acquired novel behaviours like hyperactivity that seems very important for usurpation (Ortolani et al., 2008), and has evolved increased stroking behaviour to promote chemical integration on the nest (Cervo, 2006; Cini, Bruschini, Signorotti, et al., 2011).

Preliminary comparative transcriptomic studies have explored the differential expression of genes across these two species at the different life stages (Cini, Patalano, et al., 2015). However, comparative genomics and transcriptomics must be used to truly understand the mechanistic basis for these phenotypic losses and gains.

Figure 1.5: The social parasite *P. sulcifer* at three distinct life stages; A) Overwintering B) usurping the host's nest C) in an established summer nest (images from Alessandro Cini and Rita Cervo).

1.4.3 Termites: an atypical system to explore the relative importance of worker reproductive plasticity in phenotypic and evolutionary social complexity

The variation in reproductive plasticity in workers (sterility) across atypical study systems must be explored to gain a true understanding of its importance for phenotypic and evolutionary social complexity. The hemimetabolous termites are an extremely useful system to explore this and for investigating relationships between the different social complexity traits; this is due to them being highly socially diverse and having species with both functional and obligate sterility (Korb & Hartfelder, 2008; Revely et al., 2021; Roisin, 2000; Roisin & Korb, 2011).

Termites are a complicated but fascinating group of social insects. Originating at least 150 million years ago (Bucek et al., 2019; Evangelista et al., 2019; Thorne et al., 2000), termites are social cockroaches (order Blattodea), sister to the sub-social cockroach genus, *Cryptocercus* (Bourguignon et al., 2014; Bucek et al., 2019; Evangelista et al., 2019; D. J. G. Inward et al., 2007; Lo et al., 2000). They contribute massively to ecosystem services (Jouquet et al., 2011) and disservices (Govorushko, 2019; Rouland-Lefèvre, 2011; Su & Scheffrahn, 2000). The traditional classification of termites is based on symbionts. Lower termites have bacteria and flagellates in their guts whereas higher termites have no flagellates but do have other symbionts (including bacteria). Lower termites include *Cryptotermes* and *Reticulitermes*, which have been extensively studied and are highly successful pest species across the world (Asai, 2018; DeHeer & Vargo, 2008; Korb et al., 2009; Kuhn et al., 2019; Mitaka et al., 2018; Neoh & Lee, 2011; Su et al., 2015; Wu et al., 2016; Zhou et al., 2008). Higher termites, on the other hand, include the Macrotermitinae, a sub-family of fungus-farming termites found in Africa and Southeast Asia (Elsner et al., 2021; Hinze et al., 2002; Lee, 2009; K. B. Neoh & Lee, 2009; Okot-Kotber, 1981; P. Sun et al., 2019). This binary categorization of higher and lower termites, however, is unhelpful for social complexity as there is huge variation within these groupings (Revely et al., 2021).

An alternative way of classifying termite social complexity is through their life history (wood-dwelling or foraging). Wood-dwelling termites, found only in the lower termites

(Bourguignon et al., 2014; Korb & Hartfelder, 2008), have a linear development with working individuals which are not committed to any caste and can develop into reproductives to disperse and found new colonies, and therefore have fully fertile workers (Korb, 2019). Foraging termites, which are found across the lower and higher termites (Figure 5.1) (Bourguignon et al., 2014; Korb & Hartfelder, 2008), have a bifuricated development where there is separation between working individuals and winged dispersing reproductives (Korb, 2019). These working individuals are constrained to being workers or soldiers, and importantly non-dispersing replacement reproductives if one of their parents die, i.e. they are functionally sterile (Korb, 2019). It is only a subsection of species which are foraging termites that are also seen as having obligate sterility, where the workers are unable to become replacement reproductives ever (Korb & Hartfelder, 2008; Revely et al., 2021; Roisin & Korb, 2011)

It may well be that the more widely used trait of obligate sterility (subset of foraging termites) better explains the diversity of phenotypic social complexity across the termites than (at least) functional sterility (all foraging termites). By investigating the relative importance of these traits, utilising phylogenetic comparative analyses, we will allow for greater generalisation and cross clade comparisons to improve our understanding of phenotypic social complexity. Further, by understanding the relationships of obligate sterility and functional sterility with the other traits will inform us on their relative importance for evolutionary social complexity and therefore for the transition to superorganismality (Bernadou et al., 2021; Boomsma & Gawne, 2018; Bourke, 2023).

1.5 Research Aims

Only by exploring phenotypic plasticity at different levels of sociality and in different clades, using different analytical techniques, can we begin to generalise our understanding of the evolutionary and mechanistic basis of phenotypic plasticity and its relationship with both phenotypic and evolutionary social complexity.

Therefore, in the **second chapter** I first look at the **intraspecific change in phenotypic plasticity** within a simple social insect *P. dominula*. I do this by undergoing comparative transcriptomics across the different life stages of this reproductive phenotype to understand the molecular mechanistic narrative to becoming an established summer queen in this species.

In the **third chapter**, I explore the **interspecific mechanistic basis of loss in phenotypic plasticity** between closely related species of Polistes wasps. I do this using comparative genomics and transcriptomics of the host (*P. dominula*) and social parasite (*P. sulcifer*) to understand the molecular mechanistic consequences of losing working behaviours within the social parasite.

In the **fourth chapter**, I **synthesise the diversity of developmental plasticity of termites** and leverage known cellular biology to create a framework for this variation in developmental plasticity. This is done through an exhaustive literature review of termite developmental biology.

Finally in the **fifth chapter** I bring together the data on developmental plasticity alongside other phenotypic social complexity traits within termites to understand the **macroevolutionary patterns of reproductive plasticity and its consequences for our understanding of social complexity**. To do this I undergo phylogenetic comparative analyses to test the relative importance of obligate versus facultative sterility in social complexity of termites.

Chapter Two:

The Road to Royalty: Canalisation in Gene Expression Shapes the Ontogeny of Queenhood in a Social Wasp

2.1 Abstract

Phenotypic diversity is crucial for successful resource exploitation and adaptation to changing environments. Insect societies are excellent models for studying phenotypic diversity and plasticity due to the distinct, contrasting phenotypes that are produced from a shared genome, in the form of queens and workers. Previous studies have revealed the molecular basis of these alternative phenotypes by comparing brain transcription among these phenotypes. However, these studies have largely been limited to analyses of castes within established colonies meaning that caste differentiation is represented as a snapshot from one stage in the life-cycle of these societies. Thus, within-phenotype variation that may occur through the life-cycle remains unexplored. This is important, as the expression of a phenotype is likely to change depending on the social and ecological environment, and so a phenotypic ‘snap-shot’ at a single time point in the life-cycle may not represent the true mechanistic basis of a phenotype. Here, we show that the molecular basis of a single phenotype – the queen - changes substantially throughout the life-cycle. Brain transcriptome analyses unveil large-scale transcriptomic shut-down and reduction in variance among queens of the paper wasp *Polistes dominula* as they transition through three key life-stages (overwintering, nest founding, established summer colony). These findings suggest that canalisation of gene expression plays a critical role in shaping the queen phenotype. The genes involved appear to reflect the complex physiological and cognitive demands on reproductive females in early life when caste roles are uncertain and there is severe environmental stress due to overwintering in high climes. Shifts in gene expression through the life-cycle reveal the putative importance of transcriptomic specialisation when a female is entirely committed to her role as queen. These data also illustrate the dynamic nature of transcription in a single phenotype, and the importance of

accounting for transcriptomic change due to ontogeny in our quest to understand the molecular basis of plastic phenotypes. Our findings have broad implications for the understanding of phenotypic diversity and plasticity, highlighting the importance of studying within phenotypes as well as between them in social organisms.

2.2 Introduction

Phenotypic plasticity shapes the biological diversity within and between species, and even within an individual's life-cycle (West-Eberhard, 1989, 2003). This plasticity, whereby different phenotypes are produced from the same genetic background when exposed to different environmental conditions (Pigliucci et al., 2006), is particularly important for generating within-species variation on which selection can act (Moczek, 2010). Determining the mechanistic basis of this plasticity helps us understand what drives within-species diversity (Pfennig & McGee, 2010), contributes to our understanding of how organisms adapt and persist in the face of ever-changing environmental challenges (Fusco & Minelli, 2010). One of the most remarkable examples of phenotypic plasticity comes in the form of discrete alternative phenotypes, or polyphenisms (Evans & Wheeler, 2001); text-book examples include queen and worker castes in social insects (Miura, 2004; Rehan et al., 2018; Revely et al., 2021; Srinivasan & Brisson, 2012; Sumner et al., 2018; Taylor et al., 2021), as well as fighter and sneaky males in beetles (Moczek, 2010; Moczek & Emlen, 2000; Valena & Moczek, 2012). Such distinctive phenotypes make these organisms popular models for uncovering the mechanistic basis of how phenotypic differentiation can arise from a single genome (West-Eberhard, 2003). Comparisons of alternative phenotypes have revealed how transcriptomic, epigenetic and proteomic processes in shared genomes, expressed in response to environmental triggers, manifest as individuals with contrasting morphologies, physiologies and/or behaviours (Beldade et al., 2011; Corona et al., 2016; Evans & Wheeler, 2001; Lo et al., 2018). Typically, such studies have focused on the most extreme expressions of alternative phenotypes; e.g. adult queens and workers in an established, mature social insect colony (Shibao 2010; Miura, 2004, 2005; Roisin, 2000; Sumner et al., 2018). However, a single phenotype is not a static state; even within an adult's lifetime, the phenotype is shaped by ontogeny, reflecting responses to a number of environmental cues such as changing ecological, social and developmental conditions (Crispo, 2007; Degnan & Degnan, 2010; Garland & Kelly, 2006; Moczek, 2010; West-Eberhard, 2003). Determining within-phenotype variation through the life-cycle of an individual has important implications for our understanding of the mechanistic basis of phenotypic plasticity, and offers untapped insights into the relationship between phenotypes and the environment.

There are a diversity of ways by which adult phenotypes can change during their life-cycle. When the local environment changes drastically it is sometimes necessary for some animals to undergo diapause, a period of dormancy, to survive the harsh abiotic challenges during that time (Costanzo, 2019; Denlinger, 2002; Koštál, 2006; Ragland & Keep, 2017). One such example is the wood frog (*Rana sylvatica*) that slows its metabolism substantially to survive freezing temperatures in winter (Costanzo, 2019). The organism's social environment can also cause changes in their phenotype as seen in locusts switching from solitary to swarming gregarious phases (Cullen et al., 2017; Lo et al., 2018) and fish changing their sex when the social structure changes (Casas et al., 2016). Further, in cooperatively breeding organisms like meerkats, an individual can spend much of its life-cycle as a non-reproductive helper but later become a dominant reproductive (Duncan et al., 2023). The burgeoning use of omics research and in particular transcriptomics has inspired a wealth of research aiming to unpick the molecular mechanistic basis behind discrete sequential phenotypic changes (Casas et al., 2016; Cullen et al., 2017; Mulugeta et al., 2017; Taylor et al., 2021), but less attention has been given to exploring the subtle changes that take place *within* a phenotype over, for example, an adult's life-cycle (Denlinger, 2002; Pimsler et al., 2020; Ragland & Keep, 2017).

There is now a substantial body of research on the molecular basis on the discrete developmental plasticity of castes in complex social insects (Cahan & Keller, 2003; Hawkings et al., 2019; Cahan et al., 2002; Hughes et al., 2003; Qiu et al., 2022; Sun et al., 2019; Warner et al., 2019). Some research has also been done on the molecular relationship between specific environmental factors on a single phenotype, such as diapause (Ragland & Keep, 2017), chemicals (Colgan et al., 2019) and heat stress (Pimsler et al., 2020). However, it is paramount that we understand the relative importance of multiple environmental changes on phenotypic variation across a life-cycle to fully understand the molecular narrative of phenotypic plasticity. The queen caste within social insect colonies provides a perfect model system to explore how individual phenotypes are influenced by the changes in the abiotic, social and seasonal environments that occur through the life-cycle. Such variation in these environmental variables within a single lifetime presents unprecedented opportunities to study the molecular basis of dynamic plastic phenotypes.

The queen phenotype within simple societies (small colonies of totipotent individuals able to become queens or workers as adults) is particularly interesting because, alongside these ubiquitous environmental variables across the life-cycle, these females also experience variation in the degree to which they are phenotypically specialised (Kronauer & Libbrecht, 2018; Leadbeater et al., 2011; Patalano et al., 2015; West-Eberhard, 1996). A foundress queen cannot be sure of her future: will she become a successful queen on an established colony and thus achieve direct fitness? Or will she lose the battle to be the primary egglayer and need to invest in indirect fitness, helping raise the offspring of (hopefully) a relative? The uncertainty of this foundress period means she needs to keep her reproductive options open. But as her colony becomes established and stabilizes, her role evolves from a hopeful foundress (who hedges her reproductive bets) to a specialized primary egg layer who can be sure of her direct-fitness future (Bourke, 2011b; Hamilton, 1964; Kronauer & Libbrecht, 2018; Leadbeater et al., 2011; Patalano et al., 2015; West-Eberhard, 1996). Most transcriptomic studies have concentrated on comparing workers and established queens in these simple societies; they have generally found very little differential expression between them, but with a trend of greater upregulation in the workers (Berens et al., 2014; Jones et al., 2017; Patalano et al., 2015; Standage et al., 2016; Sumner et al., 2006, 2018; Taylor et al., 2023; Toth et al., 2010). There has also been some research focusing on the plastic ability of workers from established colonies to become queens if the opportunity arises (Jones et al., 2017; Taylor et al., 2021). However, we know very little on the molecular basis of this phenotypic specialisation that shapes the journey to queenhood in these simple societies (Berens et al., 2014; Field & Leadbeater, 2016; Leadbeater et al., 2011; Patalano et al., 2015; Standage et al., 2016). In particular, targeting queen brains for transcriptomics studies provides a view of the molecular basis of this plastic, fast responding part of the wasp body that is likely to be key to the phenotypic differences across the life cycle.

Here, we address the question of: How does the brain transcriptome of an individual queen change in response to the changing environment of her life-cycle? This is a broad question that is applicable to any social insect that lives in small societies and for which individuals retain the ability to become queens or workers throughout adulthood; e.g. halictid and allodapine bees, independent founding paper wasps (e.g. *Polistes, Belonogaster, Ropalidia)*. The null hypothesis is that, despite all the changes in the environment of a reproductive female, there may be little variation in the molecular basis of her phenotype (Figure 2.1, Table 2.1: Hyp 0). In other words, a queen is always a queen: her caste identity overrides the influence of other environmental variables. Under this scenario, we expect there to be no significant difference between the life stages in magnitude, variance or identity of brain gene expression. This would indicate selection for insensitivity to fluctuating climatic, social and ecological conditions, and that a thorough understanding of the molecular basis of the queen caste can be adequately captured by studying any single phase in her life-cycle.

An alternative hypothesis is that the molecular basis of the reproductive phenotype changes through the life-cycle, reflecting the dynamic environment an individual is exposed to (Figure 2.1, Table 2.1). There are several non-mutually exclusive predictions for the types of changes that may occur, depending on the environmental factor most at play (Figure 2.1, Table 2.1). 1) Ontogeny – Each life stage may have its own equally influential set of demands due to stage-specific environment and/or ontogeny, such that each stage is a distinct phenotype in its own right (Table 2.1: Hypothesis 1); 2) Physiology – The most profound physiological differences during the life-cycle are diapause (in overwintering females) and reproductive maturation (in founding and established queens). If transcription reflects this, we expect the overwintering stage to be distinct from the other two stages (Table 2.1: Hypothesis 2); 3) Social environment – Typically, females in these simple societies are almost always living in a social context, whether as a group of overwintering females, in a co-foundress nest or as queen on an established nest of workers (although single foundress nests are also common in some species, e.g. *P. dominula)*. However, the complexity of their social environment increases as the life-cycle progresses, from a small number of unstructured interactions at overwintering to a large number of both intra- and interspecific interactions (e.g. fending off socially parasitic wasps that may try and usurp their nest

(Cervo, 2006)). This ensures high reproductivity and dominance on the established nest in the summer. If gene expression reflects this, we expect the summer stage to have the greatest relative upregulation of differentially expressed genes and greatest unique genes being expressed that are involved in cognition and communication. (Table 2.1: Hyp 3). 4) Phenotypic specialization - All overwintering females in these simple societies are hopeful queens, but their reproductive strategy is not certain. Although all females hope to be the egg layer (direct reproduction), helping raise relatives (indirect reproduction) remains important as not all females who survive the winter will get to be queen (Bourke, 2011b; Hamilton, 1964). Only when the colony is established, and the first workers have emerged can the queen be certain that she will remain the primary egg layer; after this point the chances that she will invest in indirect fitness are extremely small. Thus, there is a gradual specialization to a reproductive phenotype, as the fitness equation tips from indirect + direct fitness to just direct fitness. If gene expression reflects this, we expect to see the overwintering stage to have the greatest relative upregulation of differentially expressed genes with founding and summer stages upregulating genes for canalization of expression (Table 2.1: Hyp 4).

In this chapter, we test these predictions using the European paper wasp, *Polistes dominula* (Figure 2.1). *P. dominula* reproductive females (future queens) overwinter in a communal hibernaculum which shelters them from rain and predation. During this time, they enter at least partially into diapause, but during warmer periods they retain some social interactions with those around them (Cini & Dapporto, 2009; Kovac et al., 2022). In the Spring, females emerge and build a nest either alone (around 55% of foundresses) or with other individuals (around 45%, average- 4.5 foundresses) (Miller et al., 2018), who are often (but not always) their sisters, and sometimes with whom they hibernated (Dapporto, Pansolli, et al., 2004; Field & Leadbeater, 2016). On nests with co-foundresses, the founding stage is a period of social instability where each individual fights for the position as primary egglayer; whoever wins this is likely to retain reproductive monopoly through the rest of the colony cycle, as queen (Leadbeater et al., 2011). These individuals, therefore, undergo a change in physiological phenotype, from reproductively dormant to reproductively active. However, those who lose the contest end up acting as helpers for the reproductive foundress (Field &

Leadbeater, 2016; Leadbeater et al., 2011). On average, within polygynous nests, 78% of cofoundresses fail to initially become a dominant queen and must become subordinates in the nest (Miller et al., 2018). Thus, at this stage in the life-cycle, there is considerable uncertainty over reproductive strategies, with both direct and indirect fitness options being realistic alternatives (Field & Leadbeater, 2016; Leadbeater et al., 2011). After the first workers emerge ('summer stage'), the nest is much less likely to fail (established); thus, by this stage in the queen's life-cycle she is effectively committed to being a specialised reproductive and will not act as a helper. During this phase, the social environment becomes even more important, as the nest grows exponentially. She must maintain her position as sole reproductive through aggressive interactions, as well as on constant alert for subordinate and/or worker egg laying; thus, the demands on communication, cognition, and social interactions are substantial (Cervo, 2006; Field & Leadbeater, 2016; Jandt et al., 2014; Karsai et al., 1996; Pepiciello et al., 2018). Accordingly, the life-cycle of a single *P. dominula* queen goes through identifiable changes in her physiological, social and fitness environment within the space of a few months (Field & Leadbeater, 2016; Hunt, 2006; Jandt et al., 2014; Karsai et al., 1996; Kovac et al., 2022; Pardi, 1996; Pepiciello et al., 2018). Sampling queens at different stages in this ontogenetic journey provide an excellent opportunity to explore how changing environments define the molecular narrative of the life-cycle of the queen phenotype. Accordingly, we compare brain transcriptomes of 31 *P. dominula* adult reproductive females representing the three key stages in their life-cycle: overwintering, founding and established summer colonies (see Figure 2.1). We use these data to explore within-phenotype variation in gene expression and test a set of specific predictions about how ontogeny and environment may shape the route to royalty (Table 2.1).

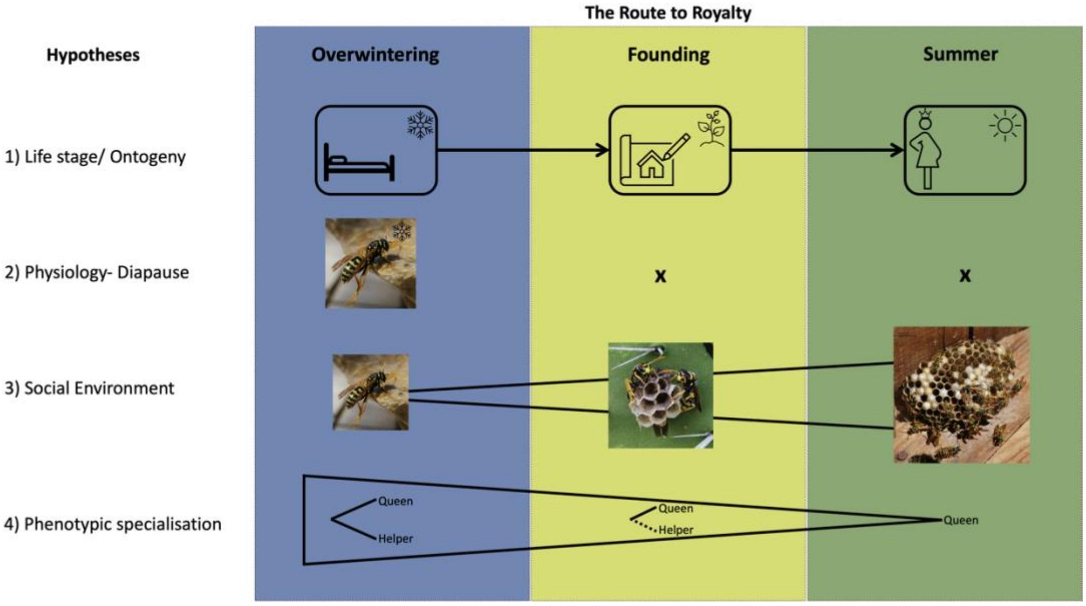
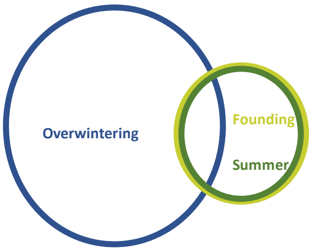
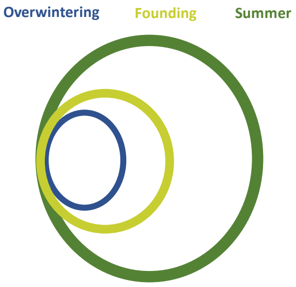
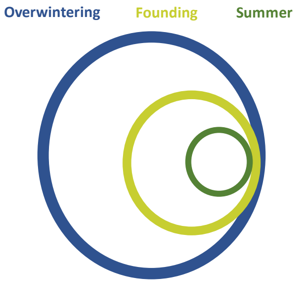


Figure 2.1: The life-cycle of the queen phenotype within a simple social colony like *P. dominula* with different non-mutually exclusive factors that could be the main force explaining variability across the life-cycle. 1) Ontogeny- the distinct life-cycle steps of the reproductive phenotype with arrows signifying the transitions between the distinct steps 2) Physiology- physiological differences during the life-cycle such as diapause (in overwintering females) and reproductive maturation (in founding and established queens) 3) Social environment- the number and complexity of social interactions occurring at each life-cycle stage, progressively increasing in complexity 4) phenotypic specialization- the progressive certainty in commitment to the reproductive role across the life-cycle stages.

Table 2.1: Non-mutually exclusive hypotheses regarding the changes in phenotype that occur through the life of a successful queen, based on the biology of the *P. dominula* life-cycle. Predictions of expected patterns of brain transcription among the three life stages, under each hypothetical scenario. Visualization of the predictions come in the form of coloured circles: blue, overwintering; orange, founding; red, summer. Size of circle relates to the relative magnitude gene expression and thickness of circle variance of expression. Proximity of circles to each other signifies the relationship of gene identities between life stages.

Hypothesis Description and Transcriptomic Predictions	Visual representation
Hyp 0: Null hypothesis – a queen is always a queen **Hyp 0:** The phenotype is overwhelmingly explained by caste identity (queen) such that other potential effects of life stage have little/no influence. **Prediction H0[1]:** Brain transcriptomes of queens are always similar to each other but distinct from workers, irrespective of the stage in the life-cycle. **Prediction H0[2]** Queens sampled through the life-cycle show no discernable differences among them in: (i) magnitude of gene expression; (ii) variance in gene expression; (iii) identity of highly expressed genes; (iv) functional enrichment.	
Hyp 1: Distinct Adult Life-stages **Hyp 1:** Each life stage during adulthood is a distinct phenotype, with its own equally influential set of demands due to stage-specific environment and/or ontogeny, and its own transcriptomic profile. **Prediction H1**: Brain transcriptomes from each stage show distinct, non-overlapping expression patterns, involving different genes and with evidence of relevant functional enrichment.	
Hyp 2: Changing Physiology **Hyp 2:** The phenotype is primarily a reflection of physiological state, due to diapause (in overwintering females) and reproductive maturation (in founding and established queens). **Prediction H2[1]:** Queens in the overwintering stage show neuro-transcription that is distinct from the other two stages. Specifically, we expect to see up regulation of genes involved with diapause (e.g. metabolic, clock, heat shock proteins) during overwintering, relative to the rest of the life-cycle. **Prediction H2[2]:** Little difference in brain transcriptomes of founding and established queens; similar genes are expressed to similar levels; no functional enrichment between them.	
Hyp 3: Increase in Complexity of Social Environment **Hyp 3:** The social environment has a strong influence on the phenotype expressed by a queen; the cognitive demands of sociality increase through the life-cycle as the structure of the society develops, group size increases and the type of social interactions change. **Prediction H3[1]:** If brain transcription reflects this increase in social complexity with development of the society, we expect to see an upregulation in genes involved in cognition and communication through the colony cycle. **Prediction H3[2]:** We also expect greater variance in gene expression, reflecting the progressive need for plastic expression in the face of greater diversity in social challenges.	
Hyp 4: Increased Caste Specialization **Hyp 4:** Queens become more specialized and committed as a reproductive during the life-cycle, as the fitness payoffs tips from indirect + direct fitness to just direct fitness. **Prediction H4[1]**: If brain transcription reflects specialization as a committed reproductive ('queenness'), we expect to see downregulation in genes not associated with queen behaviour across the life-cycle and upregulation of genes associated with reproduction. **Prediction H4[2]:** Variance in expression of the upregulated genes ('queenness') may also decrease if the transcriptional state reflects the phenotypic canalization as a committed reproductive.	

2.3 Methods

2.3.1 Sample collection

A total of 38 *P. dominula* females were collected for analyses from nests originating from the vicinity of Florence (Tuscany, Italy). Overwintering females (n=11) were collected from their hibernacula mid-March 2016, right before the end of their overwintering season (usually late March/April, depending on the weather, (Cervo, 2006)). We checked that no nests were yet built in the sampled population, confirming that the overwintering season was not yet ended. Overwintering females were brought to the lab and kept in glass cages (15 cm × 15 cm × 15 cm) with ad libitum water and sugar, under controlled and low temperature mimicking the environmental overwintering conditions for at least one week until sampling in RNA later (see below).

Foundress colonies were collected in early May with cofoundresses, nest carton and brood (foundress and later summer queens, n=20). No nests had hatched pupal cells, indicating they were still in pre-emergence stage. Collections took place early in the morning before any wasps had left the nest to forage. They were brought back to the laboratory, where they were reared in glass cages (15 cm × 15 cm × 15 cm) under a natural light cycle and temperature conditions with additional illumination from neon lighting with a daily rhythm (L:D 10:14). They were provided with sugar, fly larvae and water *ad libitum;* paper was supplied for nest construction. Four of the ten colonies had two foundresses (polygynous); the remaining six had a single foundress (monogynous). Wasps were individually marked using enamel paint and observed for three days; dominant foundresses were identified based on uni-directional ritualized dominance behaviours, chases and attacks (Jandt et al., 2014; Pardi, 1946; Kenneth G. Ross & Matthews, 1991). After one week in the lab, under controlled conditions, we sampled the dominant foundress from 10 colonies (hereafter F, n=10); none of the colonies had produced workers and nests had on average 37.7 ± 11.2 cells containing immature brood (pupae and/or larvae and eggs) on the nest.

Established summer queens (n=10) were collected from the remaining 10 nests, ten days after the emergence of the first workers. Established nests had on average 49.8 ± 22.7 cells containing immature brood (pupae and/or larvae and eggs) and between 5 and 10 workers. Lab-based collections ensured that there had been no queen turn over, and that environmental conditions were controlled for at least a week before collection. Finally, workers (n=7) known to be at least seven days old, were collected from seven of the established colonies; workers were collected while foraging, to be sure they were active and displaying the typical worker phenotype.

In all cases, individual wasps were collected directly from the nest using forceps and their heads were immediately cut off and immersed in RNAlater in 1.5 ml Eppendorf tubes. Samples were kept at 4°C overnight and then stored at -20°C until RNA extraction. Bodies were stored at -20°C for ovarian dissection to confirm that the overwintering females had no developed ovaries, and that founding and summer females had mature ovaries, indicating that they were active egglayers at the time of collection.

2.3.2 Gene expression quantification

2.3.2.1 RNA extraction sequencing and processing

Brain tissue was dissected from the heads of 38 individual female wasps (OW=11, F=10, S=10, W=7) using a Leica M165 C stereomicroscope (Leica Microsystems, Wetzlar, Germany); RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. Library preparation was performed by Novogene Co. followed by sequencing on an Illumina HiSeq 2000 platform with 150-base pair paired-end reads.

2.3.2.2 RNASeq Reads Processing

We followed a standardized processing pipeline for the RNAseq raw reads using the publicly available nextflow wrapper nf-core/rnaseq v.1.4.2 (DI Tommaso et al., 2017). This involved trimming the raw RNAseq reads with TrimGalore (Krueger et al., 2021) mapping the reads to the *P. dominula* genome annotation 1.0 (Standage et al., 2016) using STAR (Dobin et al., 2013), and finally obtaining GFF feature read counts with FeatureCounts (Liao et al., 2014).

2.3.3 Analyses of Gene Expression Patterns

We used several analytical methods to identify patterns of differential expression among the stages of a queen's life-cycle, as previous work has highlighted the benefits of using a combination of methods when looking for patterns of gene expression, which capture coarse and fine-scale differences (Favreau et al., 2023; Taylor et al., 2021; Wyatt et al., 2023). The first two analyses (clustering (section 2.3.4) and support vector machine learning (section 2.3.5)) examine how similar the different life-stages are in their global brain gene expression. These methods do not impose fold-change parameters, and instead capture the combined role of many genes of small effect. The next set of analyses examines differential expression of genes using fold-changes to identify specific genes of large effect (section 2.3.6), and compares within-phenotype gene-wise expression variance to infer the difference in gene expression between life stages (section 2.3.7). All analyses were conducted in R version 4.2.1 (R Core, 2022).

2.3.4 Clustering analyses

We used a multi-variate data clustering tool to identify how the different phenotypes clustered with respect to variance in their brain gene expression. DAPC simplifies high-dimensional data by identifying patterns of variance, helping to reveal relationships and trends specifically between groups. It condenses variance into a small number of axes, making it ideal for visualizing and interpreting global trends and discriminating between predefined clusters or groups (i.e. *between* life-stages). We used the package Adegenet (Jombart, 2008) for the DAPC analysis, which allows us to identify the optimum number of PCs to be used in a discriminant analysis.

2.3.5 Support Vector Machine Classification Analyses

We used support vector machine (SVM) classification analyses to test the extent to which the different life-stages were similar in their global patterns of brain gene expression. SVMs offer a valuable approach to unraveling the intricate relationships of groups using gene expression data (Favreau et al., 2023; Taylor et al., 2021; Wyatt et al., 2023). Their capacity to handle high-dimensional and noisy gene expression datasets, with the ability to capture non-linear dependencies among genes, helps reveal intricate biological patterns that linear methods like DEseq2 might overlook (Favreau et al., 2023; Taylor et al., 2021; Wyatt et al., 2023). It is a powerful tool for identifying similarities and differences in gene expression of the life stages based on pairwise comparisons against the worker samples, ensuring we are specifically training genes that are differentially expressed from the worker phenotype compared to one other life stage (and therefore can be a measure of queenness per life stage). We then test the classification of queenness (specifically of the trained life stage) on the two other life stages as a test dataset.

We ran three SVMs where each life stage takes its turn being in the training dataset while the other two life stages are in the test dataset. This allowed us to see how any two life stages classify compared to the third life stage and thus test the predictions made by the different hypotheses (Table 2.1). For example, if the null hypothesis is true, in each SVM, queens from any of the life stages will always classify strongly as the training queen phenotype, rather than as workers. Conversely, if each queen phenotype is defined by her life stage (Hyp 2) we expect test queens in all three SVMs to not classify as the training queen nor the workers.

SVMs were conducted in R using the package e1071 (Dimitriadou et al., 2006) using the gene expression data for all queen worker phenotypes. We assessed the ability of the classifier via their threefold cross-validation error rates where superiority was indicated by lower classification error. Scripts followed previous publications using radial kernel function (Favreau et al., 2023; Taylor et al., 2021) but the cost and Y parameters vary depending on the training dataset and are determined using the ‘tune’ function (Overwintering: $\gamma = 10^{-5}$ and $C=2^3$; Founding: $\gamma = 10^{-6}$ and $C=2^5$; Summer: $\gamma = 10^{-6}$ and $C=2^4$). We used feature selection methods to reduce the number of predictor genes to a set that were most informative for the classification with the lowest error rates.

2.3.6 Differential gene expression analysis

Differential gene expression analyses were performed in R using the DESeq2 package (Love et al., 2014). Raw counts were transformed by variance stabilization using the VST function (Love et al., 2014) and pairwise analyses of each phenotype combination were conducted to identify genes that were up- or -down regulated in each phenotype relative to another. We assumed that genes were differentially expressed between phenotypes if $p<0.05$ after false discovery rate correction according to the Benjamini-Hochberg procedure and if the lfc >1. We then used a chi-squared test to understand if there was a significant difference between the life stage's up and down regulation of shared genes.

2.3.7 Variance in gene expression

Differentially expressed genes that show little variance in their expression within a phenotype may be under strong selection to maintain a specific level of expression in order that they help regulate a specific function. For example, it may be critical to the survival of an overwintering female that a heat shock protein is produced in the correct amount and so we would predict very low variance in the expression of the gene encoding these proteins in the transcriptomes of overwintering females compared to later life stages (Hyp 2)(although any distinct pattern of variance for the overwintering stage compared to the others would be in support of Hyp 2). Conversely, high variance within a group in the expression of a gene may indicate that plasticity is beneficial; e.g. foundresses are uncertain whether their future will involve investing in direct fitness (as a queen) or indirect fitness (as a subordinate), and so variance in expression of genes involved in reproduction may be high relative to established queens later in the colony cycle, who are securely committed to being a reproductive (Hyp 4).

An existing analysis to understand relative variation in gene expression of groups was utilized for our study (Kapheim et al., 2020; Schrader et al., 2017). Initially, we preprocessed the data by standardizing the gene count matrix. We then calculated gene variance values for each gene among pairwise comparisons of samples within each group, calculating the squared differences in log2-transformed and normalized gene expression values. This involved using the function 'normalizeBetweenArrays' from the limma package (Ritchie et al., 2015) to adjust for between-sample variability and to ensure comparability of expression measures across the dataset. Gene variance values were obtained as the square root of the sum of squared differences for each gene. We then used t-tests and Wilcoxon tests to assess significance between pairs of treatment groups, adjusting p-values for multiple comparisons using the Benjamini-Hochberg method.

2.3.8 Functional Interpretation of Candidate Genes

To identify the putative functions of genes that are significantly up or downregulated in each life stage, we collated the log fold-change values for all the significant genes (padj<0.05, lfc>1) for each pairwise comparison between the life stages from DEG analyses. For instance, the pairwise comparison of overwintering vs founding will provide positive lfc values which signify upregulated genes for overwintering compared to summer (or downregulated genes for summer compared to overwintering) and negative lfc values which signify downregulated genes for overwintering compared to summer (or upregulated genes for summer compared to overwintering). The negative and positive lfc values for each pairwise comparison are separated and made to be absolute values with their own column so that we have 6 columns with unique lfc values relating to gene IDs. We then filtered the genes of interest based on the average lfc value for the upregulation and downregulation of a focal life stage compared to the other two life stages, with a cutoff of lfc >2. For instance, we took the genes that are both up regulated in overwintering compared to founding and overwintering compared to summer and had an average of more than lfc of 2 for those two comparisons. We acquired the gene ID names from the *P. dominula* gff file (Standage et al., 2016).

We looked for any evidence of functional specialisation among the differentially expressed genes for each phenotype using GO Term enrichment analysis. Differentially expressed genes (padj<0.05, lfc>1) were aligned to their orthologs in the *D. melanogaster* protein data sets using orthofinder v.2.4.0 (Emms & Kelly, 2019), and GO terms of *D. melanogaster* were used to infer gene IDs in *P. dominula* using TopGO v.2.52.0 (Alexa & Rahnenfuhrer, 2023) with the following parameters: terms with at least five annotated genes, classic algorithm, Fisher statistics. We highlight only the most significant GO terms of interest based on the average fdr values for the up and downregulation of a focal phenotype compared to the other two life stages with a cutoff of fdr<0.02.

2.4 Results

We found evidence from across the analytical approaches that the increasing levels of caste specialization (Hyp 4) and changes in physiology (Hyp 2) that occur through the ontogeny of the queen's life-cycle best explain the patterns of brain gene expression. We present evidence from each analysis in turn, and explain how these data either support or refute the different hypotheses.

2.4.1 Clustering Analysis

Data presented in the DAPC plot (Figure 2.2) reveals differences between the phenotypes that may reflect physiology (Hyp 2) and/or caste specialization (Hyp 4). The DAPC revealed 10 PCs as the optimum number for accounting for the variance in our data (97% of conserved variance). There are three clear clusters of phenotypes: i) workers, ii) overwintering females and iii) a combined cluster of founding and summer queens. The differentiation between workers and queens is as expected for this species (as a species with division of reproductive labour and cooperative brood care) and is best explained by DAPC 2, which captures 29% of the variance. Conversely, DAPC1, which explains 68% of the variance, separates overwintering females from all other phenotypes. These clustering patterns provide support for some of the predictions made by Hyp 2 (changing physiology) and 4 (Increased caste specialization) (see Table 2.1). We unpack the reasoning for this here.

DAPC 1 separates overwintering females and workers. These females are all reproductively inactive, and so this separation cannot be attributed to their shared non-reproductive state. This separation is consistent with their contrasting environmental conditions and may therefore reflect the demands of diapause on the overwintering phenotype, as predicted by Hyp 2 (Prediction H2^1).

The clustering of all three queen phenotypes by DAPC2 may reflect a shared core set of genes associated with their role. Although this supports both Predictions H3^1 and H4^1, the tight clustering of founding and summer queens on both DAPC 1 and 2 makes these two phenotypes transcriptomically indistinguishable, despite their contrasting social environments, in which summer queens experience a much more complex social environment compared to founding queens (Hyp 3, Table 2.1). Accordingly, these results do not provide support for Hyp 3. Instead, this tight clustering may reflect their shared active reproductive state, as predicted by Hyp 4 (Prediction H4^1).

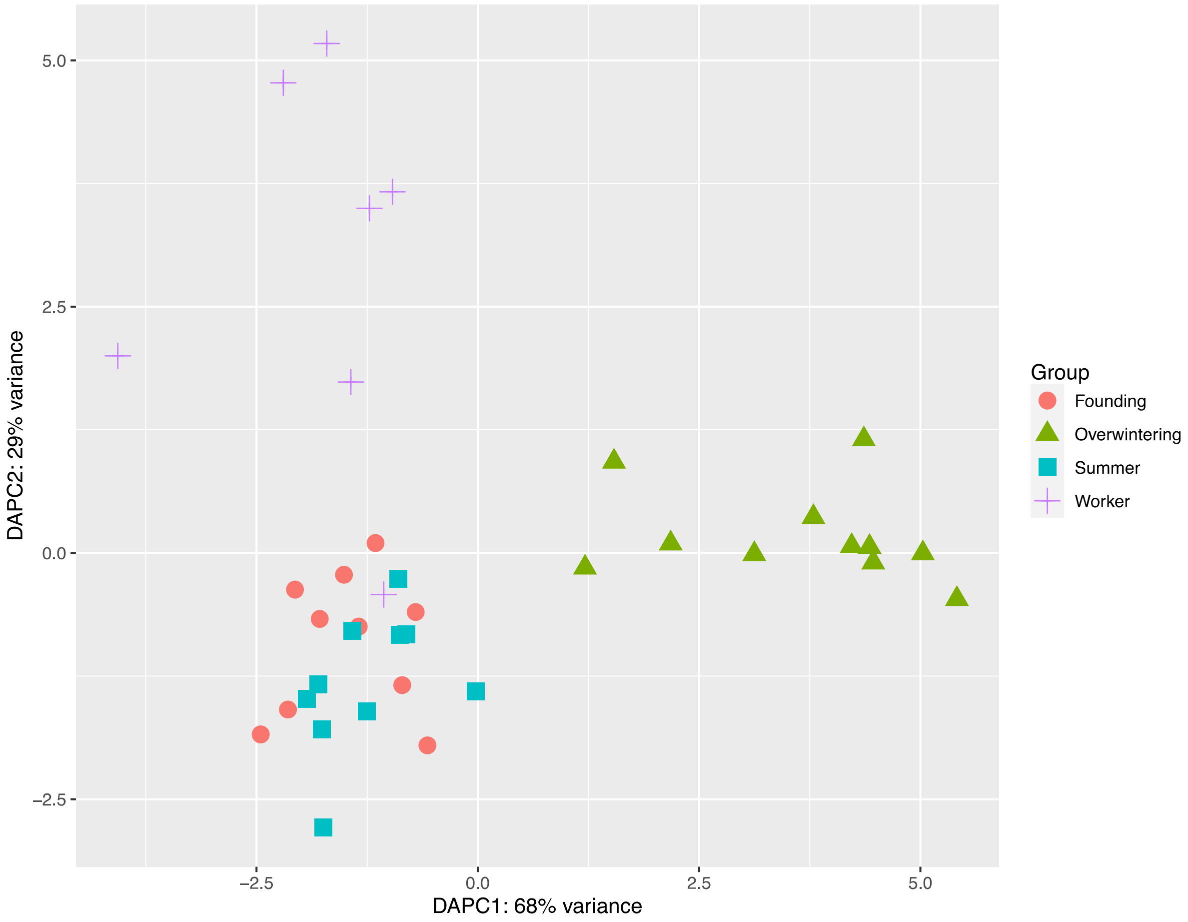


Figure 2.2: Clustering analysis (DAPC) explains most of the variance in the data, providing support for a role of both changing physiology (Hyp 2 – DAPC 1) and increased caste specialization (Hyp 4 – DAPC 2) in brain transcription.

2.4.2 Support Vector Machine Classification Analyses

Further support for the effects of caste specialization (Hyp 4) are apparent from the three SVMs; moreover, these results are inconsistent with the other hypotheses (Hyp 0-3). We explain the rationale for this reasoning.

The first SVM (Figure 2.3A) explores to what extent the genes differentiating overwintering females from workers are informative in classifying founding and summer phenotypes. We found no support for this; neither founding (mean= 0.532; sd= 0.116) nor summer (mean= 0.502, sd= 0.0853) phenotypes were classified as overwintering or workers. Their distinctness from the overwintering phenotype is at odds with predictions of the null hypothesis (all queen-like phenotypes are the same – Hyp 0) and Hyp 3 (overwintering is made up of only core genes relating social complexity that are shared with the other stages). However, it is in line with Hyp 1 (each life stage is neuro-transcriptionally distinct), Hyp 2 (distinct neuro-transcription of overwintering phenotype) and Hyp 4 (has additional distinct neuro-transcription related to enhance caste plasticity).

The second (Figure 2.3B) and third (Figure 2.3C) SVMs use respectively the gene set that best distinguishes founding phenotypes (Figure 2.3B) and summer queens (Figure 2.3C) from workers as a training set. In both models, the phenotypes classify well with the queen-like training phenotype, highlighting how these three phenotypes share a core set of 'queenness' genes. This is an assumption that underpins Hyp 4 ($H4^1$) but rejects a scenario where each phenotype represents a distinct adult life-stage (Hyp 1) and where overwintering is transcriptionally distinct from the other two stages (Hyp 2).

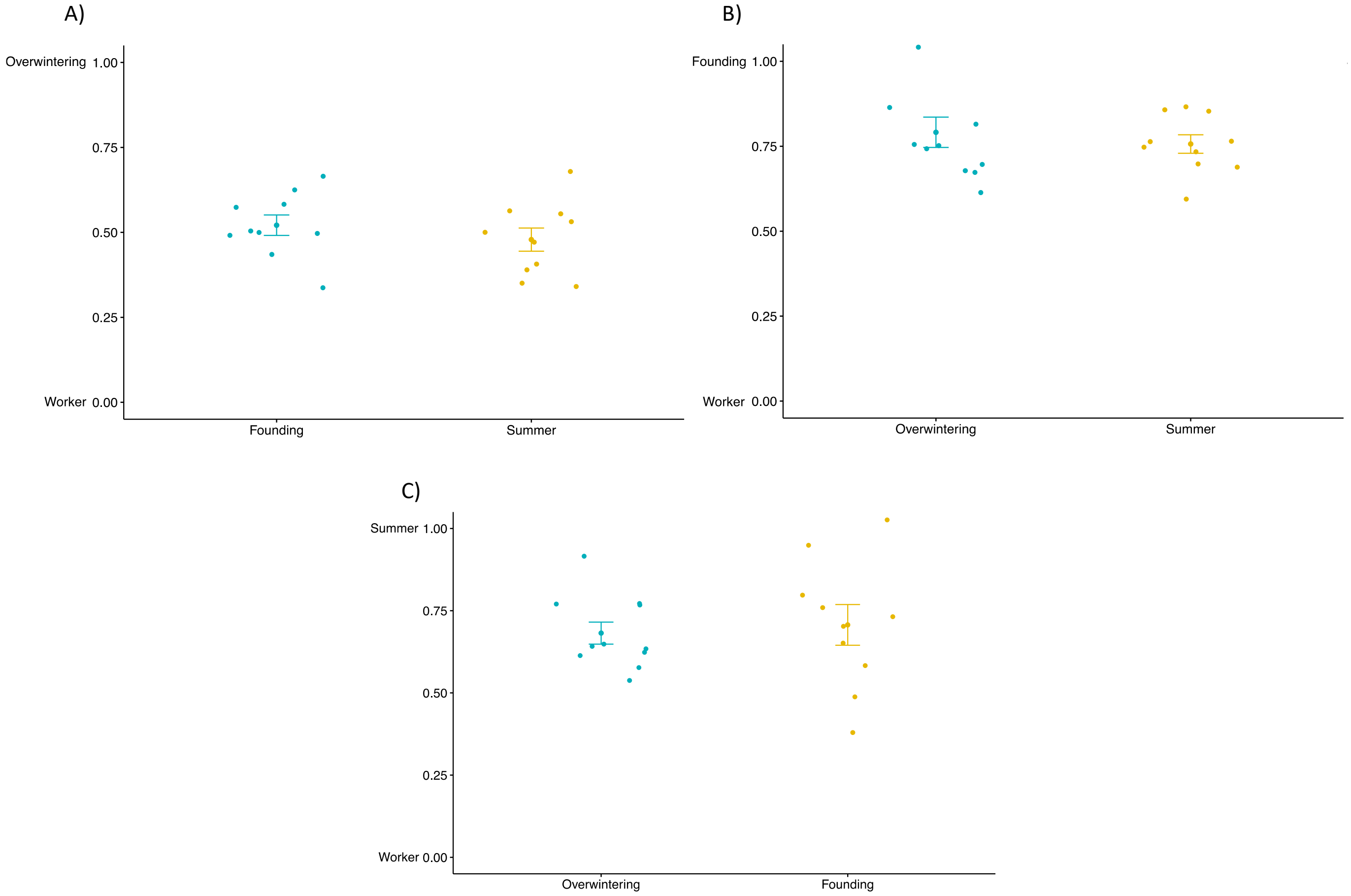


Figure 2.3: A) Classification of datasets from test phenotypes (on x axes) after feature selection when the SVM is trained on overwintering (A; error rate=0.0522, optimal number of genes=1250), founding (B; error rate=0.06379, optimal number of genes=2244) and summer (C; error rate=0.06399, optimal number of genes=4497). All SVMs used workers as a baseline for the training data against which each queen phenotype was compared. In none of the cases did the queen phenotype classify close to being a worker, as expected due to caste differentiation ((Favreau et al., 2023; Taylor et al., 2021; Wyatt et al., 2023); see also Figure 2.2). Y axes indicate the extent to which test phenotypes were classified, with worker classification always indicated by 0.00. E.g. a test phenotype in Panel A that classifies towards 1.00 would indicate strong similarity in gene expression with the reproductive phenotype of interest in the training set (i.e. overwintering females); conversely, classification towards 0.00 would indicate strong similarity to workers. The same logic applies to Panels B & C, with their respective training sets.

2.4.3 Gene expression

2.4.3.1 Reduction in brain transcription with development through the life-cycle

Pairwise comparisons of gene expression between phenotypes indicate that the brain transcriptomes of overwintering females represent an overall up-regulated state relative to any of the other phenotypes (Table 2.2). Founding and summer phenotypes represent down-regulated versions of the overwintering state (Table 2.2), with more genes down- as opposed to up-regulated in summer and founding queens, compared to overwintering than expected by chance (Table 2.2). Moreover, significantly more genes were downregulated through the progression of the life-cycle development (Founding vs overwintering: up=116, down=160; Summer vs overwintering: up=124, down=243; X^2 value=4.23, P-value=0.0398). These patterns are consistent with Hyp 4 which predicts a pattern of reproductive specialization in expression of genes involved in reproduction ($H4^1$). It also aligns with Hyp 2, which predicts that the overwintering stage will have much greater upregulated gene numbers compared to the other two stages ($H2^1$).

Pairwise comparisons	**Up_reg**	**Down_reg**	**p-value**
Founding v Overwintering	116	160	0.008
Summer v Overwintering	124	243	<0.001
Summer vs Founding	6	25	<0.001

Table 2.2: Numbers of genes in that are significantly up and down regulated in pairwise life stage specific comparisons (direction of regulation from the view of the first stage mentioned in the first column). P-values signify the significant difference in up and down regulation at each life stage.

2.4.3.2 Reduction in variance in gene expression through the life-cycle

We compared variance in gene expression among each of the phenotype pairs and found a reduction in variance with progression of the life-cycle. The highest levels of variance were detected in the overwintering phenotypes, followed by founding and then summer phenotypes (Figure 2.4). The pairwise differences were significant between overwintering and founding females (-0.01, padj<0.001), and between overwintering and summer females (-0.021, padj <0.001). The same pattern was observed in founding females versus summer females, but was not significant (-0.012, padj=0.99). These overall patterns of reduction in variance of gene expression through the life-cycle are in line with an effect of caste specialization over time (H4²) and/or an effect of overwintering physiology (Hyp 2). It is not in line with the hypotheses of a strong effect of life-stage (H1²), increasing demands of social complexity (H3³) or all phenotypes were equivalent (Hyp 0).

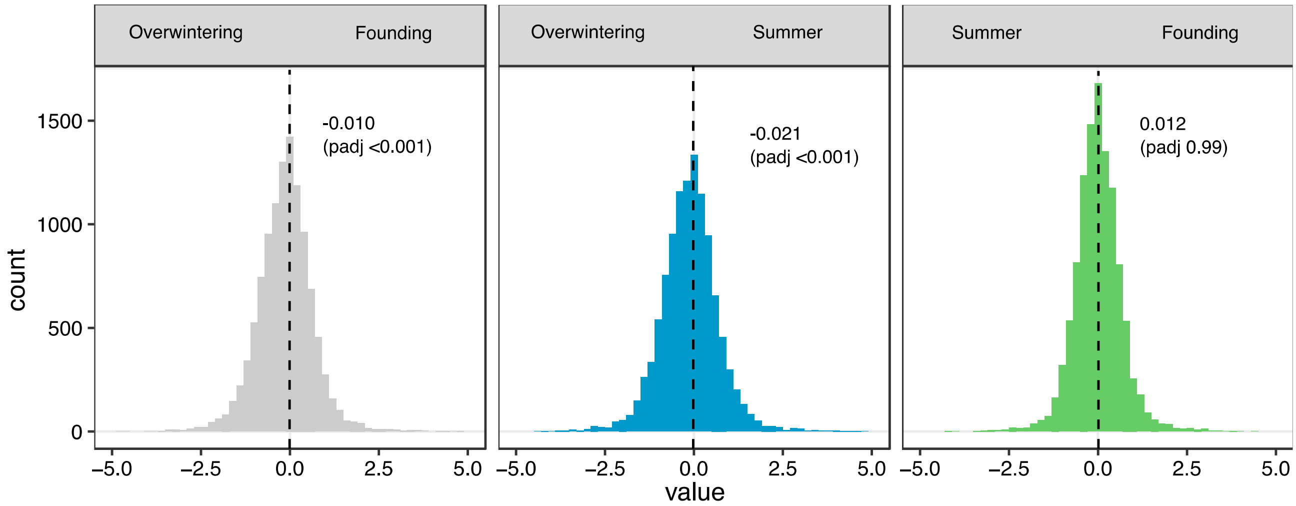


Figure 2.4: Each box represents a pair-wise comparison between life stages, with the x-axis showing the difference in log2-transformed coefficient of variance (CV) values. Dashed vertical lines indicate the mean difference in log2-transformed CVs. The histogram within each box provides a visual representation of the distribution of CV differences. Adjusted p-values, denoted as ‘padj', indicate the significance of differences between CVs. A negative value indicates greater variability for the stage on the left whereas a positive value indicates greater variability on the right.

2.4.3.3 Identities of differentially expressed genes and functional enrichment

The putative functions of differentially expressed genes across the three life stages can aid in uncovering the molecular basis of the queen phenotypes across its life-cycle. Genes that are upregulated in the overwintering life-stage are likely to be important for surviving harsh winters in the high altitudes where hibernacula are found (Hyp 2). We found some evidence for this. GO terms relating to overwintering biased genes (compared to founding and summer) such as hormone metabolic process (GO:0042445) and regulation of ecdysone receptor-mediated signalling pathway (GO:0120141) reflect the slowing of metabolism and growth during overwintering (Figure 2.6). The genes contributing to these functional terms support this further; for instance, a hexamerin gene, arylphorin subunit beta-like (LOC107067185), is upregulated in overwintering females - this is an important storage protein potentially important for assuring energy reserves for the wasps. A gene for (11Z)-Hexadec-11-enoyl-CoA conjugase-like (LOC107073447), is also overwintering biased and is important for fatty acid metabolism likely aiding in tolerance to cold conditions (Figure 2.5). Cytochrome P450 4C1-like (LOC107073689), a gene related to digestion, is however downregulated in overwintering compared to the other stages, reflecting that overwintering wasps are not feeding at this time.

There are also genes that point towards a pattern of specialization through the life stages (Hyp 4), specifically in the progressive importance of genes involved in regulation of gene expression through the life-cycle. GO terms such as chromatin assembly (GO:0031497), organization (GO:0006325), remodelling (GO:0006338), nucleosome assembly (GO:0006334) and organization (GO:0034728) and finally chromosome organization (GO:0051276) are enriched among genes upregulated in founding compared to overwintering females and even more so in genes upregulated in summer compared to overwintering (Figure 2.6). Genes for Histone H2A, H2B-like, H3 and H4-like are founding and summer biased and Histone H4 transcription factor is overwintering and founding biased. Upregulation of molecular machinery involved in gene regulation suggests summer queens are canalized as specialised reproductives.

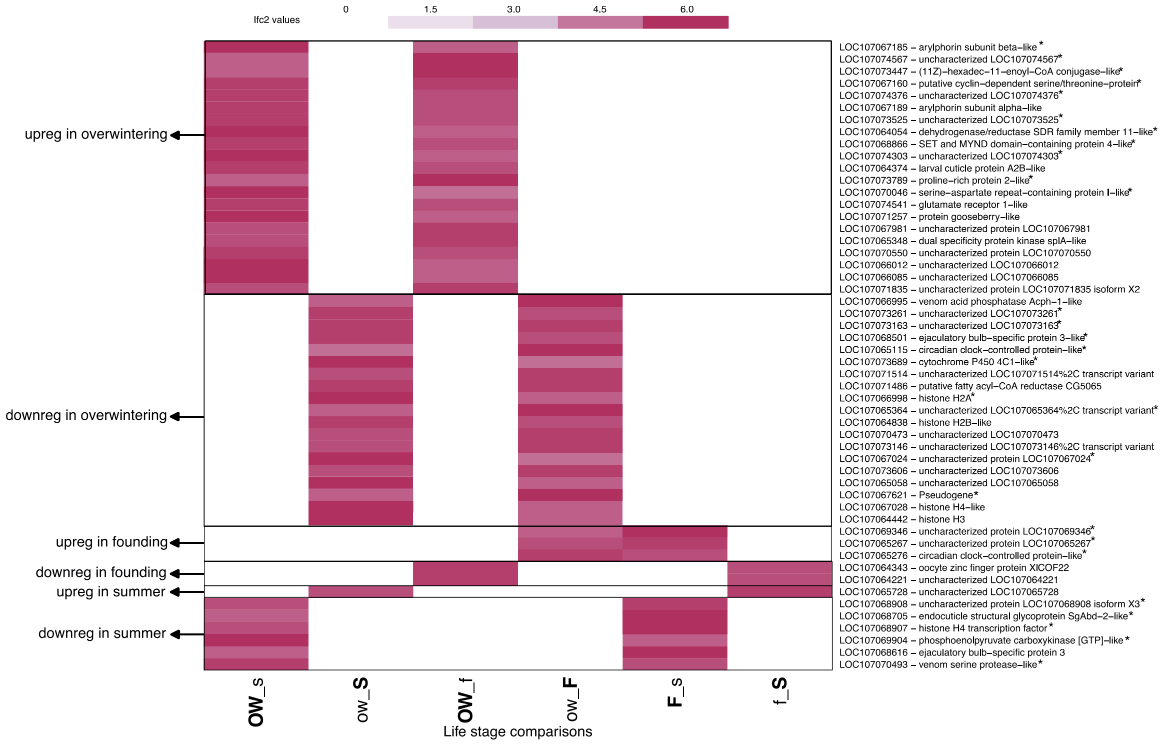


Figure 2.5: Heatmap showing genes (LOC IDs and the individual product names on the right) which have an average lfc2 value of above 2 from DESeq2 comparisons where one life stage is both up regulated or downregulated compared to the other life stages. For instance, the first box surrounding the heatmap outlines the genes that are upregulated in overwintering compared to summer (column 1) and upregulated in overwintering compared to founding (column 2). Genes that are upregulated in one comparison but not the other are not included even if the average is above 2. The column names signify the pairwise comparison that gave rise to the lfc2 values, specifically the capitalized life stage is the up-regulated life stage in this comparison. OW, Overwintering; F, Founding; S, Summer. Genes in comparisons that were above the cutoff threshold of p-value < 0.05 and below lfc2 > 1 are shown as lfc2 of 0.

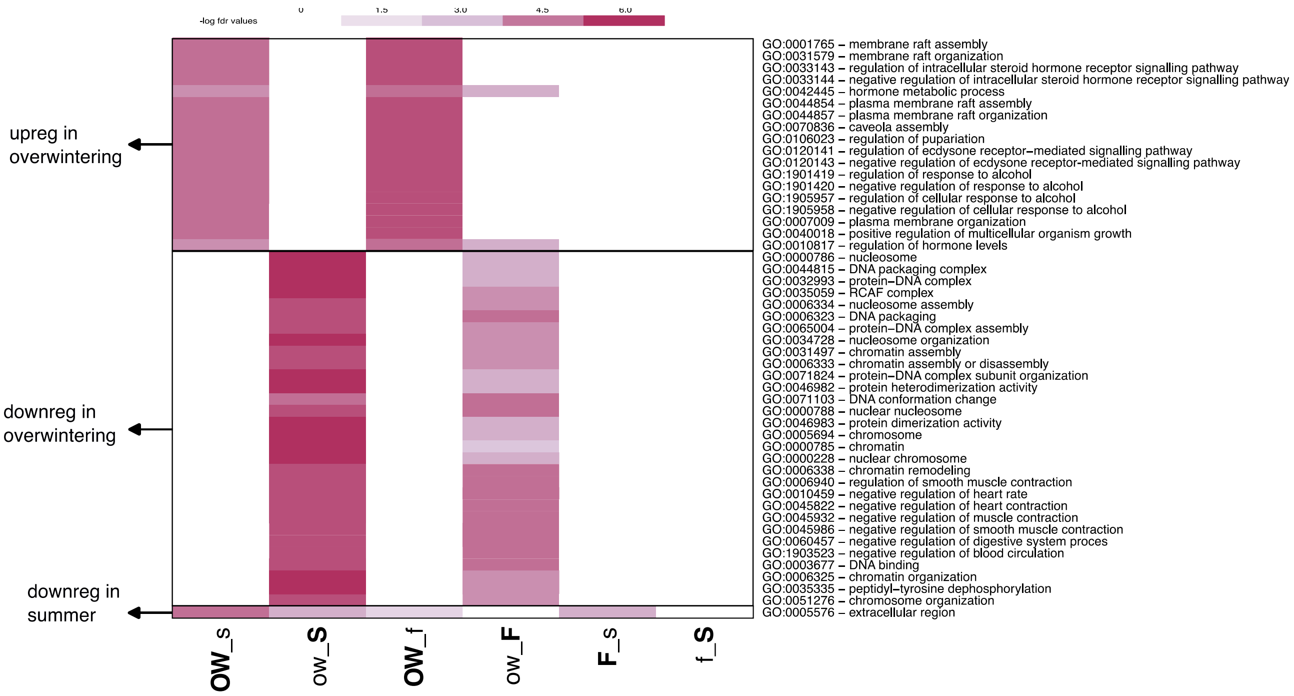


Figure 2.6: Heatmap showing Go terms (Go IDs and their respective terms on the right of the plot) which have an average fdr value of above below 0.02 from GO analysis where one life stage is up regulated or downregulated compared to the other life stages. For instance, the first box surrounding the heatmap outlines the genes that are upregulated in overwintering compared to summer (column 1) and upregulated in overwintering compared to founding (column 2). Genes that are upregulated in one comparison but not the other are not included even if the average is below 0.02. The column names signify the pairwise comparison that gave rise to the fdr values, specifically the capitalized life stage is the up-regulated life stage in this comparison. It is -log fdr values which are visualized in this plot. GO terms that were not significantly enriched in a given comparison are shown as 0.

2.5 Discussion

Determining the mechanistic basis of the phenotypic diversity within and between species is essential for understanding how organisms exploit resources and adapt to changing environments. Insects often produce alternative phenotypes from a shared gene set to exploit different reproductive strategies, and these are well studied at the phenotypic and molecular level; e.g. as castes in social insects or male morphs in beetles (Shibao 2010; Miura, 2005; Moczek, 2010; Moczek & Emlen, 2000; Rehan et al., 2018; Revely et al., 2021; Roisin, 2000; Srinivasan & Brisson, 2012; Taylor et al., 2021; Valena & Moczek, 2012). By contrast, the extent of variation across the ontogeny of an individual is less well studied. Understanding how changes in the extrinsic and intrinsic environment influence the molecular basis of a single phenotype is an important part of the puzzle in understanding the mechanistic basis of phenotypic plasticity, and the relationship between phenotypes and the environment. We examined how neuro-transcription changes through the life-cycle of the reproductive female phenotype in a social wasp, which experience changes in their physiological, social and fitness environment. By analysing brain transcriptomes of the different life-stages in a queen's adult development, we found evidence for two of our hypotheses on the molecular basis to queen ontogeny in *Polistes* and were able to exclude a further three hypotheses. Firstly, we found no evidence for the null hypothesis that caste identity overrides any other effects on brain gene expression; similarly, there is no evidence for each stage in the life-cycle representing a distinct molecular phenotype (Hyp 1); nor did we find any clear evidence that the increasingly complex social environment influences brain gene expression (Hyp 3). The two remaining hypotheses that we explored in this study are physiology (specifically diapause vs reproduction – Hyp 2) and caste specialization (specifically, become a committed egglayer – Hyp 4). They potentially explain brain gene transcriptomes of queen phenotypes through their life-cycle. These two effects are not mutually exclusive. We discuss the implications of these two processes on our understanding of *Polistes* life-history and the role of phenotypic plasticity in the ontogeny of a queen. We also discuss other potential explanations of the patterns we have uncovered.

2.5.1 The Influence of Overwintering on the Reproductive Phenotype

Hyp 2 outlined the possibility that the queen phenotype in *P. dominula* is predominantly a reflection of physiological state typified by diapause in overwintering females. Diapause is associated with different strategies to survive the harsh environments that are making this dormant phase necessary, including a reduction in energy consumption, increased nutrient storage and a shift to anaerobic catabolism (Hahn & Denlinger, 2011). If this hypothesis is true, the overwintering queens would show distinct neuro-transcription compared to the other stages while the founding and summer stages have little to no difference between each other. It is also predicted that most upregulated genes in overwintering queens compared to the other stages would be related to diapause. We found some evidence for this from the clustering analysis, where overwintering queens are distinct from reproductive females at other life-cycle stages, and also workers (Figure 2.2).

Further evidence for Hyp 2 came from the differential gene expression analysis, which identified the up-regulation of genes known to be associated with diapause in insects, highlighting functions related to circadian rhythms (up-regulated), insulin/tor pathways (up and down regulated) and Ecdysone signalling (down-regulated) (Ragland & Keep, 2017). Likewise, hexameric storage proteins are also important for diapause and have also been shown to be upregulated in our study (arylphorin subunit beta-like-LOC107067185)(De Kort & Koopmanschap, 1994; Hunt et al., 2007; Lewis et al., 2002).

Temperate *Polistes* paper wasps have evolved to successfully overwinter in conditions that often include low temperatures. *P. dominula* have adapted to this environmental stress by gathering as clusters of individuals at an overwintering site (hibernaculum) and reducing overall activity (Gobbi et al., 2006; Hunt et al., 1999; Reeve et al., 1991). There is still a lot of uncertainty for survival, however, as many wasps do not make it to spring (Gamboa et al., 2004; Gibo, 1972; Pratte, 1982; Starks, 2001). Although overwintering is one of outward inactivity, our results emphasise the importance of this life stage and therefore indicates the potential need for a significantly greater amount of molecular transcriptional activity to help in ensuring survival over these winter months.

2.5.2 Caste specialization

Hyp 4 describes the journey to becoming a queen as one of progressive specialization and commitment to being a reproductive. This is due to the increased certainty that they can gain direct fitness by becoming a primary reproductive instead of indirect fitness as a worker (Bourke, 2011b; Hamilton, 1964). The predictions are that neuronal transcription would reflect this specialization with progressive downregulation of gene expression alongside a core of shared genes related to reproduction across all the stages. Finally, it is predicted that there would be a stepwise decrease in gene wise variance in these life stages, reflecting the progressive canalization of expression due to becoming a committed reproductive. There is evidence supporting these predictions across all the analyses we ran in our study. Within the clustering analysis, the second axis shows a distinction between reproductive stages (overwintering, founding and summer) compared to non-reproductive (workers), highlighting the potential core set of shared gene expression across the queen phenotype (Figure 2.2). Collectively, the classifications of the life stages when each are the training set within the SVM analyses also support Hyp 4. This aligns with Hyp 4's predictions of a shared set of genes across the life stages but also that overwintering will be the most expanded and unique of the life stages. There is also greater downregulation of gene expression across the life stages also, indicative of specialization in expression (Table 2.2). Finally, the stepwise reduction in gene wise variance seen in the founding and summer compared to overwintering aligns with this hypothesis also (Table 2.2.2).

Commitment or canalization has been long studied and was first hypothesized by Waddington (Lo et al., 2018; Waddington, 1942a). The well-known depiction of a pebble rolling downhill in an epigenetic landscape signified cells losing developmental potential as they progressively commit to different developmental trajectories (Waddington, 1960; Jr & Waddington, 1943; Qiu et al., 2022). This has subsequently been used to explain caste differentiation in social insects (Patalano et al., 2012; Qiu et al., 2022). Inclusive fitness theory predicts behavioural canalization of a reproductive in a social insect colony (Bourke, 2011b; Hamilton, 1964; Linksvayer & Wade, 2005). Within the colony, there is a shared fitness benefit for all those in the colony from a primary reproductive committing to their role. The workers are able to maximise indirect fitness benefits by having an efficient committed queen present on the nest (Shreeves et al., 2003). The reproductive maximises direct fitness by more effectively allocating resources to reproduction only. Therefore, when there is greater certainty of being a reproductive, we expect this specialization behaviorally and molecularly.

Canalization of gene expression via epigenetic processes that regulate genes in response to environmental cues, is a mechanism allowing for this reproductive commitment to occur (Ehrenreich & Pfennig, 2016; Hemberger et al., 2009; Reik et al., 2001; Waddington, 1942a). One such epigenetic mechanism involves the modification of chromatin, the complex of DNA and proteins that forms chromosomes within the nucleus, via histone modifications (Galbraith et al., 2016; Simola et al., 2016; Sumner et al., 2018). This regulatory mechanism has been found to be of importance in castes of ants and honeybees, with greater levels exhibited in the queens of these species (Dickman et al., 2013; Simola et al., 2013; Spannhoff et al., 2011; Sumner et al., 2018). The significant upregulation of genes with GO terms for DNA packaging (GO:0006323) and chromatin remodeling (GO:0006338) in the founding and summer stages of the queen life-cycle emphasizes the importance of canalization of gene expression as they become more certain in their roles. Interestingly, genes that are important for reproduction - such as *vitellogenin* and *transferrin* - are not upregulated in the later life-cycle stages compared to the overwintering stage, suggesting that neuro-transcriptomic processes for reproduction are active prior to physiological activation of the reproductive system (Aumer et al., 2018; Korb et al., 2021).

This narrative of epigenetic canalization reflects the uncertainty of caste fate early in the queen's life within simple societies of *Polistes* wasps (Leadbeater et al., 2011). Within these species, the reproductive caste fate is not determined early in development but progressively through distinct stages where the potential for indirect fitness (as a co-foundress) is removed with greater certainty of becoming a primary reproductive (therefore gaining direct fitness) (Leadbeater et al., 2011). This is an example of a subtle reduction in phenotypic plasticity and commitment to a single caste. This parallels the dramatic specializations found in more complex societies of Hymenoptera (Hughes et al., 2003; La Richelière et al., 2022; Wilson, 1953) and termites (Revely et al., 2021). As Waddington identified, it also closely resembles the specialization occurring in somatic cells within metazoan multicellular organisms (Waddington, 1960; Revely et al., 2021; Waddington, 1942a, 1942b). This reproductive specialization can be seen in its most extreme in a closely related species *Polistes sulcifer* (Cervo, 2006). They have lost the ability to produce a working phenotype over evolutionary time and instead socially parasitises *P. dominula* by killing the queen and manipulating host workers to care for parasite brood (Cervo, 2006). This provides a fascinating model system to understand between species variation in plasticity and the genetic basis of this (Cini, Bruschini, Signorotti, et al., 2011; Cini et al., 2019; Cini, Patalano, et al., 2015).

2.5.3 Uncertainty and Risk

The two hypotheses we have explored are not mutually exclusive. They capture the inherent risk and uncertainty within the overwintering life stage (Gamboa et al., 2004; Gibo, 1972; Pratte, 1982; Starks, 2001) with growing certainty in survival and direct fitness benefits of being a primary reproductive from founding to summer stages (Leadbeater et al., 2011). Our data are consistent with the idea that transcriptional activity across these stages is being shaped by these shifts in risk and uncertainty, with a general pattern of upregulation (Table 2.2) and increased variance in gene expression (Figure 2.4) in the overwintering life stage to account for the risk of survival and uncertainty in fitness. The progressive increase in relative downregulation and variance in gene expression is indicative of this progressive increase in certainty of direct fitness benefits and survival. The canalization of a core set of reproductive genes via chromatin remodelling (histone modifications) and other epigenetic factors in the founding and summer stages ensures the downregulation of genes important for overwintering and the molecular specialization of this queen phenotype to being a refined primary reproductive.

2.5.4 Alternative Hypotheses

In this study, we found no evidence for hypotheses predicting that the stages of a queen's life-cycle would be exactly the same molecularly (Hyp 0), or completely distinct (Hyp 1), nor did we find a molecular pattern reflecting the increasing social complexity in the queen's life-cycle (Hyp 3). We do find some evidence for risk and uncertainty as potential hypotheses behind the molecular patterns but there could be other explanations. Some of the patterns may not be adaptive. For instance, it may be the case that the increased variance in expression in overwintering compared to the other life stages reflects the heightened stress response from the abiotic pressures leading to difficulty maintaining an optimal profile. Further, it may be the process of ageing that is contributing to the canalization of expression. Future analyses should include the subordinate queens in the founding and summer stages to see if their transcriptional profiles differ from their dominant counterparts, indicating that certainty of caste fate is of central importance.

2.6 Conclusions

By looking under the hood of the phenotype, we’ve shown that there are significant changes in the neurotranscriptional machinery that tells us more about the nature of the phenotype, and the biotic and abiotic factors shaping it. We show this through the lens of the reproductive female in a social wasp - typically treated as a single static phenotype known as the ‘queen’. Overwhelmingly, we show that the molecular basis of the ‘queen’ varies depending on when in her lifecycle she is sampled. Our analyses suggest that physiology (specifically the stress of overwintering) and shifts in certainty of fitness strategies influence the nature of the single phenotype. This has important implications on our understanding of phenotypic plasticity, highlighting how the categories we assign to specific phenotypes may often be constructs that mask the true diversity of the phenotype. This has important implications for future analyses surrounding phenotypic plasticity, which should take better account of the effects of environmental variation, life history and individual-level ontogeny.

Chapter Three:

From Socialite to Parasite: Transcriptomic and Genomic Expansion in a Socially Parasitic Wasp

3.1 Abstract

Inquiline (permanent) social parasites rely on the socially-acquired resources of their social hosts, with whom they typically share a recent common social ancestor. Their evolution is characterised by the loss of the worker phenotype, and the gain of specialist parasitic behaviours and modifications. Such losses and gains make them popular models for understanding phenotypic plasticity. Among the Hymenoptera (bees, wasps and ants) inquiline social parasites have at least 57 independent origins from social ancestors. There are three possible (non-mutually exclusive) mechanisms by which the social parasite phenotype could be produced from a shared social ancestral state. The loss (or reduction) of social traits may be reflected in reductions and losses at the genome level; gains in specialised parasitic traits may be reflected through gene family expansions and/or de novo gene evolution; social parasite traits may arise via differential expression of shared genes. There is mixed evidence for each of these mechanisms but we lack a combined comparative analysis of genomes and transcriptomes within any one host-parasite pair to understand their respective contributions. Here, we generate chromosome-level genomes and life-stage-specific brain transcriptomes for the paper wasp social parasite *Polistes sulcifer* and its cooperative breeding social host *Polistes dominula* to test these hypotheses simultaneously in a single host-parasite pair. We found evidence that the social parasite exhibits signatures of genome expansion relative to its host, in terms of size, chromosome number, transposable element (TE) number, and gene family evolution. Further, the social parasite also exhibits a more active transcriptional state relative to its host across all the life-cycle stages, with higher overall patterns of gene up-regulation at each life stage. We explore the putative relationships in the interplay of genomic and transcriptomic processes and identify

a suite of genes of putative importance in the evolution of social parasitism. Our findings contrast with previous studies, which focused on inquilines arising from complex, superorganismal ancestry; by contrast, *P. sulcifer* evolved from a simple, cooperatively breeding social ancestor, raising the possibility that the molecular processes underpinning the evolution of social parasites may differ depending on the social complexity of their social ancestor. This has fundamental implications on how we study social parasitism as a model for understanding losses and gains in phenotypic plasticity and provides unique insights into the interplay between genes and phenotypes.

3.2 Introduction

Sociality provides a framework for understanding how complex systems arise (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2023; West et al., 2015). The focus is typically on examining how higher-levels of social complexity (i.e. superorganismality) evolve (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2011a; Michod & Roze, 1997; West et al., 2015). Reductions in social complexity are less well studied but provide key insights into the mechanisms and evolution of complex traits. Examples include some termites within the Apicotermitinae which have lost the ability to produce a soldier caste (Bourguignon et al., 2016; Revely et al., 2021; Romero Arias et al., 2021) and single celled eukaryotes like chromists and dinoflagellates which have lost their symbiotic chloroplasts (Cavalier-Smith, 1992). Such reversions are, however, rare (Boomsma, 2022; West et al., 2015). One exception is the evolution of social parasites in the Hymenoptera (bees, wasps, and ants) (Alleman et al., 2018; Borowiec et al., 2021; Cervo, 2006; Cini et al., 2019; Hines & Cameron, 2010; Schrader et al., 2021; Stoldt et al., 2022) for which there are least 57 independent origins from social ancestors. The parasites typically evolved from a common social ancestor of their host, meaning host and parasite tend to be close relatives (Emery, 1909). Social parasites are a modified version of the social ancestor; typically, they have partially or fully lost the worker phenotype and instead they exploit the worker force provided by their closely related social host (Brandt et al., 2005; Stoldt et al., 2022). This is an example of a reversion in sociality but with the gain of selfish parasitic traits which make the parasite specialised to exploit their host's system (Cervo et al., 2008; Lenoir et al., 2003; Lorenzi, 2006; S. J. Martin et al., 2008, 2010; Nash & Boomsma, 2008). Comparisons of hosts and social parasites, therefore, offer unprecedented opportunities to discover how losses and gains in phenotypic plasticity evolve.

The extent to which social parasites have lost their worker traits and evolved new parasitic ones varies widely. Facultative social parasites may opportunistically adopt a parasitic lifestyle but retain full capacity for free-living as a social insect; thus, these social parasites show no loss of worker or other social traits. By contrast, obligate social parasites are fully dependent on their hosts for survival; they are unable to complete their life cycle without the host society. This form of social parasitism is characterised by at least a partial loss in function of the worker caste (Stoldt et al., 2022). For example, dulotic (or slave-making) ants exhibit partial loss of worker function as they depend on host workers to feed, forage and nurse (Cervo, 2006; Foitzik et al., 2001; Jongepier et al., 2014). However, their workers have evolved novel raiding behaviours to acquire larvae from host colonies and 'enslave' them as workers in the parasite's nest. Inquiline social parasites typically show complete loss of the worker phenotype (although exceptions include the leafcutting ant social parasite, *Acromyrmex insinuator*, which produces a small number of workers with reduced functionality (Sumner et al., 2003)), with socially parasitic queens exploiting host workers to raise the sexual parasite offspring (Buschinger, 2009; Cervo, 2006; Dronnet et al., 2005). These specialised, lifetime committed social parasites have also evolved novel behaviours enabling them to successfully usurp and integrate into a host's nest, such as increased aggression, specific usurpation behaviours and chemical mimicry (Cervo, 2006; Cini, Bruschini, Poggi, et al., 2011; Cini, Bruschini, Signorotti, et al., 2011; Cini, Ortolani, et al., 2015; Ortolani & Cervo, 2009).

We expect the loss of the worker phenotype, and gain in novel traits in inquiline social parasites to be reflected mechanistically within their genomes. There are several non-mutually exclusive hypotheses for how genomes evolve to produce these changes, relative to the genomes of their social hosts with whom they typically shared a recent common (social) ancestor. The first hypothesis posits that the parasitic genome will be a reduced form of the free-living/social genome, reflecting the reduced functionality required (Cini et al., 2019; Cini, Patalano, et al., 2015; Schrader et al., 2021). This reasoning is based on evidence from non-social parasites, like tapeworms and other human parasites, which exhibit degenerative genomes compared to their hosts; this is thought to reflect their dependence on the host for sustenance, protection and reproduction (Attardo et al., 2014; Benoit et al., 2016; Kirkness et al., 2010; Tsai et al., 2013). There is some evidence for genomic reductions in hymenopteran social parasites. A recent study on *Acromyrmex* inquiline ants (parasites of free-living congeneric leafcutting ant societies) showed signatures of genome-wide erosion and relaxed selection compared to their closely related social host (Schrader et al., 2021). Such gene losses have been attributed to the worker phenotype, and are thought to have occurred via genomic rearrangements, including chromosome fusion (Schrader et al., 2021). However, other social parasites show the opposite: e.g. the socially parasitic bumblebee subgenus *Psithyrus* exhibits an increase in chromosome number and no reduction in genome size (Sun et al., 2020). A study on *Temnothorax* and *Harpagoxenus* dulotic ants found convergent patterns of relaxed selection and loss of chemoreceptor genes across these socially parasitic ants (Jongepier et al., 2022). Similarly, *Psithyrus* and *Acromyrmex* social parasites also exhibited convergent loss of odorant receptors and gustatory genes (Jongepier et al., 2022; Schrader et al., 2021; Sun et al., 2020). This suggests a reduced function of these sensory processes in the transition from social to parasitic lifestyles (Legan et al., 2021; Robertson & Wanner, 2006; Sadd et al., 2015; Yan et al., 2020; Zhou et al., 2012, 2015). Intriguingly, no gene loss was detected in a study on *Pogonomyrmex* and *Vollenhovia* inquiline ants (Smith et al., 2015).

The second way in which social parasite genomes may evolve is through the acquisition of novel genomic features, reflecting the novel selfish behaviours and traits that social parasites use in order to effectively infiltrate, usurp and manipulate their host societies. There is some evidence of such trait evolution at the genomic level (Feldmeyer et al., 2017; Trible et al., 2023). For instance, in dulotic ants, genes relating to circadian rhythms exhibit positive selection compared to their social hosts; precise regulation of activity patterns is required for these parasites to successfully raid host colonies and enslave workers (Feldmeyer et al., 2017). A supergene enriched with Cytochrome p450 was also found to be mutated in a socially parasitic strain of *Ooceraea biroi* (Trible et al., 2023). This indicates this gene may be of importance in the evolution of social parasitism, with mutations leading to its up regulation or expansion driving the parasitic phenotype.

The third mechanistic hypothesis is that social parasite traits arise through conditional expression of genes shared with their hosts. Conditional expression is an important mechanism known to facilitate phenotypic plasticity; any differences in the phenotypes of host and parasite may arise due to contrasting patterns in the expression and/or regulatory networks (West-Eberhard, 1989). This would evolve more easily, and be less costly, than irreversibly losing large parts of the genome or evolving genomic novelty. Indeed, the mechanism of conditional expression for achieving phenotypic plasticity explains how queen and worker phenotypes are produced, in almost all social insects (C. R. Smith et al., 2008; Sumner, 2006). Therefore, it may be unsurprising if conditional expression were a key mechanism in the early stages of social parasite evolution, which may then be supplemented or replaced by genomic modifications via genetic assimilation in older lineages (Price et al., 2003). There is already evidence of differential transcription in social parasite evolution (Aumer et al., 2018; Cini, Patalano, et al., 2015; Smith et al., 2015); for instance, upregulation of queen-specific genes like transferrin and vitellogenin in the cape honeybee may have led to their high fecundity (Aumer et al., 2018; Korb et al., 2021). Further, transcriptome analyses of *Pogonomyrmex* and *Vollenhovia* inquiline ants revealed that genes which are worker biased in their social hosts were also present in these social parasite genomes (Smith et al., 2015).

Given the diversity of mechanisms and current evidence, it is no surprise that social parasites have been highlighted as key model systems for understanding the molecular basis of phenotypic plasticity (Cini, Patalano, et al., 2015; West-Eberhard, 1989, 2003). Comparisons of genomic and/or transcriptomic basis in host-parasites pairs is a powerful approach, especially as they are often very close relatives and thus share a very recent common (social) ancestor (Cini, Patalano, et al., 2015). However, we lack studies that simultaneously compare genomic and transcriptomic processes in hosts and their social parasites; we are currently unable to determine how these three mechanisms collectively contribute to the evolution of social parasites (Stoldt et al., 2022). Moreover, to date, studies have been limited to parasites of superoganismal societies which means that the common ancestor of the parasite and host is likely to have also been superoganismal (Alleman et al., 2018; Schrader et al., 2021; Smith et al., 2015; Stoldt et al., 2022; Sun et al., 2020). Superorganisms are highly complex social organisms with very little individual-level capacity for expressing phenotypic plasticity because social roles are typically determined early in development (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2011a). A social parasite evolving from this ancestral state, therefore, may be at least partially canalised in the mechanisms that can be adapted, evolved, and built on to produce a novel socially parasitic phenotype. We lack any genome-wide studies that compare hosts with social parasites that have evolved from a simple social ancestor, which has retained high phenotypic plasticity due to behavioural caste determination (Cervo, 2006; Cini et al., 2019; Cini, Patalano, et al., 2015).

We address this knowledge gap using the simple social host *Polistes dominula* and its closely related social parasite *Polistes sulcifer* (Cervo et al., 2004; Cervo, 2006; Cini, Bruschini, Poggi, et al., 2011; Cini, Bruschini, Signorotti, et al., 2011; Cini et al., 2014, 2019; Cini, Ortolani, et al., 2015; Cini, Patalano, et al., 2015; Ortolani & Cervo, 2009; Pepiciello et al., 2018; Rozanski et al., 2021). The life history and behaviours of these species are well documented but little genomic work has been conducted (Figure 3.1) (Cervo, 2006; Cini, Patalano, et al., 2015). *P. sulcifer* is a typical inquiline social parasite in that it has completely lost the worker phenotype; it has also lost the ability to found a nest (Cervo, 2006). The social parasite has distinct, specialised behaviours at each of its major life stages that may manifest at the molecular level (Cervo, 2006). First, it overwinters at high altitudes and for around two months longer than its host stage; this more stressful environment may have selected for molecular differences to allow it to survive (Figure 3.1)(Ragland & Keep, 2017). Second, at the usurpation stage (or founding stage for the host), the social parasite has acquired a novel behaviour of ‘restlessness’ or hyperactivity that is vital for successful usurpation (Ortolani et al., 2008). It has also modified the host’s alarm pheromones into propaganda pheromones to distract the defending workers (Bruschini & Cervo, 2011) and synced with the host’s circadian rhythms (Ortolani & Cervo, 2009). Once in the nest the parasite has evolved an increased rate of stroking behaviour to allow for chemical integration (Cervo, 2006; Cini, Bruschini, Signorotti, et al., 2011), as well as retention of dominance behaviours such as aggressive interactions which ar shared with the host (Cervo, 2006). Finally, the brood of *P. sulcifer* has novel appeasement pheromones to prevent eviction from the nest by reducing host aggression and promoting tolerance to the parasite brood (Elia et al., 2018). There is already evidence that two species differ in the expression of shared genes at the overwintering, founding/usurping and summer stages of their life cycle (Figure 3.1) (Cini, Patalano, et al., 2015). This suggests some support for phenotypic plasticity being an important mechanism in the evolution of the social parasite. However, the extent to which the parasite genome exhibits losses and gains associated with these parasitic traits remains unexplored.

In this study, we provide new, high-quality chromosome-level genome sequences for the host (*P. dominula*) and social parasite (*P. sulcifer*), as well as brain transcriptomes for the three major life stages in reproductive females of both species (Overwintering, Usurping/Founding, Summer, Figure 3.1). We conduct comparative genomic analyses to identify differences between the genomes of the host and social parasite and interpret these within the context of genomic losses (Hypothesis 1) and gains (Hypothesis 2); we use measures of stage-specific brain transcriptional activity to examine the extent to which conditional expression explains phenotypic evolution (Hypothesis 3). Finally, we examine how differential transcription corresponds to regions of genomic evolution in order to identify the key processes in social parasite evolution. Accordingly, these combined analyses afford the first test of the three main mechanistic pathways by which social parasitism evolves, within a single host-parasite pair.

Figure 3.1: Adapted from (Cini, Patalano, et al., 2015). The annual life cycles of the host, *Polistes dominula* (red line) and social parasite *Polistes sulcifer* (blue line) (Cervo, 1996ab; Pardi, 1996). Host colonies are founded in spring (March-April) by one or more foundresses. Dominance interactions allow for a reproductive hierarchy to occur in those that have multiple foundresses (Pardi, 1946). At the end of May/early June the first brood emerges; these females become workers helping raise the brood of the single reproductive foundress (queen). At the end of summer males and queen-destined females emerge, leave the colony and mate. Mated females cluster in sheltered areas to overwinter, emerging the following spring to found new colonies (Pardi, 1996). The social parasites overwinter alone in the mountains and do not emerge until late April/early May (Cervo, 1996a). Using visual and chemical stimuli (Cervo et al., 1996; Cini, Bruschini, Signorotti, et al., 2011), they migrate from their montane overwintering sites to lower altitudes where hosts have built foundress nests (Cervo, 1996ab, 2006). Parasites must usurp the host reproductive from the nest within a short window of time in late May-early June); this involves violent fights between the host queen and incoming parasite (Cini, Bruschini, Poggi, et al., 2011; Ortolani et al., 2008; Turillazzi et al., 1990). If the social parasite wins the fight, she takes on the behaviours and chemical signatures of the host queen and becomes the sole egg-layer on a nest supported by host workers (Dapporto, Cervo, et al., 2004; Sledge et al., 2001; Turillazzi et al., 2000). In the summer months the social parasite experiences the same social and environmental conditions with that of an un-parasitised host queen.

3.3 Methods

3.3.1 Sample Collection for Genome and Transcriptome Sequencing

A total of 70 wasps were collected for individual-level brain transcriptome sequencing, in order to capture the neuronal gene expression of females across the three life-cycle stages (overwintering, founding (usurping) and summer (established)) for both the host *(P. dominula)* and the parasite *(P. sulcifer)*. Sampling conditions for the two species are described below (sections 3.3.1.1; 3.3.1.2). In all cases, individual wasps were collected directly from the hibernaculum or nest using forceps and their heads were immediately cut off and immersed in RNAlater in 1.5 ml Eppendorf tubes. Samples were kept at 4°C overnight and then stored at -20°C until RNA extraction. Bodies were stored at -20°C for ovarian dissection to confirm that overwintering females had no developed ovaries, and that founding/usurping and summer females had mature ovaries, indicating that they were active egglayers at the time of collection.

Samples of adult male host and parasites were collected directly onto dry ice and flash frozen before shipping to Dovetail for genome sequencing (see Section 3.3.3).

3.3.1.1 Host Stage-specific Phenotype Collections

A total of 38 P. *dominula* females were used for transcriptome sequencing; they were all collected in the vicinity of Florence (Tuscany, Italy).

Overwintering *P. dominula* females (n=11) were collected from their hibernacula mid-March 2016, right before the end of their overwintering season (usually late March/April, depending on the weather, (Cervo, 2006)); we confirmed that the overwintering season by checking that no nests had yet been built in the sampled population. Overwintering females were brought to the lab and kept in glass cages (15 cm × 15 cm × 15 cm) with ad libitum water and sugar, under controlled and low temperature, mimicking the environmental overwintering conditions for at least one week until sampling in RNA later (see below).

Foundress *P. dominula* colonies (n=10; with co-foundresses, nest carton and brood) were collected in early May; we confirmed that the nests were in the pre-emergence stage by the absence of hatched pupal cells. Collections took place early in the morning before any wasps had left the nest to forage. They were brought back to the laboratory, where they were reared in glass cages (15 cm × 15 cm × 15 cm) under a natural light cycle and temperature conditions with additional illumination from full spectrum day-light-mimicking lighting with a daily light cycle (Light:Dark 10:14). They were provided with sugar, fly larvae and water *ad libitum;* paper was supplied for nest construction. Four of the ten colonies had two foundresses (polygynous); the remaining six had a single foundress (monogynous). Once in the laboratory we individually marked all wasps on each nest with enamel paint and observed them for a few days; we identified the dominant foundress based on uni-directional ritualized dominance behaviours, chases, and attacks (Jandt et al., 2014; Pardi, 1946; K. G. Ross & Matthews, 1991). After one week in the lab, under controlled conditions, we sampled the dominant foundress from ten colonies (n=10); no colony had yet produced workers and nests had on average 37.7 ± 11.2 cells containing immature brood (pupae and/or larvae and eggs).

Established (summer) queens (n=10) were collected from the remaining 10 nests, ten days after the emergence of the first workers. Established nests had on average 49.8 ± 22.7 cells containing immature brood (pupae and/or larvae and eggs) and between 5 and 10 workers. Lab-based collections ensured that there had been no queen turn over, and that environmental conditions were controlled for at least a week before collection. Finally, workers (n=7) known to be at least seven days old, were collected from seven of the established colonies; workers were collected while foraging, to be sure they were active and displaying the typical worker phenotype.

3.3.1.2 Social Parasite Stage-specific Phenotype Collections

A total of 32 P. *sulcifer* females were used for transcriptome sequencing; the overwintering samples were collected from Sibillini Mountains (Central Italy) and the usurping and summer stages were collected from the laboratory in the vicinity of Florence (Tuscany, Italy). *P. sulcifer* is a rare species, making it challenging to sample the different phases of the life-cycle in the field. We thus used a protocol developed by Cini et al. 2011 (Cini, Bruschini, Poggi, et al., 2011) to obtain the adequate sample size of each of the three life-cycle stages (overwintering, usurping and established summer social parasites). We collected *P. sulcifer* females from their overwintering sites in central Italy during spring (late April), before their emergence from the winter diapause. We brought them to the laboratory and kept them under overwintering-like conditions (4 °C, light:dark cycle 8:16 h). After one week 10 females (n=10) were sampled as indicated above. These wasps did not show any sign of activation (increase levels of locomotion, see (Ortolani et al., 2008); usurpation restlessness), confirming they were still in the overwintering phase.

The remaining social parasites were activated in May (when usurpation usually occurs, (Cervo, 2006)) by a 'warming treatment' (as defined in (Ortolani et al., 2008), i.e. natural light:dark conditions with additional light bulb from 0800 to 2000 hours). This treatment simulates the field conditions in which parasites usurp the nests (Ortolani et al., 2008; Ortolani & Cervo, 2009). After activation, the parasites were introduced into host colony cages and only those parasites directly approaching the nest were considered for the analyses. This laboratory procedure, compared to a field collection, guarantees that the analysed parasites had not yet usurped any nest and permits sampling of social parasites in the exact moment of usurpation. Accordingly, 11 social parasites were sampled during usurpation.

Some usurping parasites were left to conquer the host colony and integrate into it. Successful integration was confirmed by the observation that the social parasites has dominated all the other cofoundresses, (Cervo, 2006; Dapporto, Cervo, et al., 2004; Pardi, 1946). One week after successful usurpation, 11 established parasite females were collected as indicated below (n=11).

3.3.2 Transcriptome Sequencing: RNA extraction, sequencing and processing.

Brain tissue was dissected from the heads of 38 individual female *P. dominula* (host) samples (Overwintering=11, Founding=10, Summer=10, Worker=7), and 32 individual female P. sulcifer (social parasite) samples (Overwintering=10, Usurping=11, Summer=11) using a Leica M165 C stereomicroscope (Leica Microsystems, Wetzlar, Germany). RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Library preparation was performed by Novogene Co. followed by sequencing on an Illumina HiSeq 2000 platform with 150-base pair paired-end reads.

3.3.3 Genome Sequencing: DNA extraction, sequencing, assembly and annotation

3.3.3.1 Dovetail Omni-C Library Preparation and Sequencing

The sequencing and scaffolding were undertaken by Dovetail Genomics (Cantata Bio, CA, USA). For each Dovetail Omni-C library, chromatin was fixed in place with formaldehyde in the nucleus and then extracted. Fixed chromatin was digested with DNAse I, chromatin ends were repaired and ligated to a biotinylated bridge adapter followed by proximity ligation of adapter containing ends. After proximity ligation, crosslinks were reversed and the DNA purified. Purified DNA was treated to remove biotin that was not internal to ligated fragments. Sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The library was sequenced on an Illumina HiSeqX platform to produce approximately 30x sequence coverage. HiRise used MQ>50 reads for scaffolding.

3.3.3.2 Scaffolding the Assembly with HiRise

The input de novo assembly and Dovetail OmniC library reads were used as input data for HiRise, a software pipeline designed specifically for using proximity ligation data to scaffold genome assemblies (Putnam et al., 2016). Dovetail OmniC library sequences were aligned to the draft input assembly using bwa (https://github.com/lh3/bwa). The separations of

Dovetail OmniC read pairs mapped within draft scaffolds were analysed by HiRise to produce a likelihood model for genomic distance between read pairs, and the model was used to identify and break putative mis-joins, to score prospective joins, and make joins above a threshold. Hi-C contact maps were produced to allow for quantification of chromosome number also.

To gain the chromosome sizes for comparative analyses we used the GFF files for the host and social parasite, then parsed the start and end positions of each chromosome (scaffold), then calculated the maximum end position for each chromosome as its length.

3.3.3.3 Gene Ontology and enrichment

To assign gene ontology (GO) terms to each gene we ran a custom Nextflow pipeline (https://github.com/chriswyatt1/Goatee/), which takes genome '.fasta' files along with annotation '.gff' files, as well as a list of Ensembl RefSeq IDs of species with annotated GO terms to use as a guide (see Insect_data/go_files in Goatee). The pipeline takes the genomes/annotations and runs gffread to produce protein fasta files for each species, which are then input into Orthofinder (Emms & Kelly, 2019), to obtain orthogroups (genes that shared orthology). Using these groups, GO terms from the RefSeq species are assigned to our new species gene lists. To test for enrichment, we used topGo (Alexa & Rahnenfuhrer, 2010), to obtain fdr p-values for enrichment of gene lists against a background where appropriate.

3.3.4 Analyses for detection of Losses and Gains in Genomes of Host and Social Parasite (Hypotheses 1 &2)

3.3.4.1 Synteny

To measure syntenic links between species we ran a custom nextflow pipeline (https://github.com/chriswyatt1/jcvi-nextflow), that uses the jcvi package (Tang et al., 2006) to take input genomes/annotations for each species and run a pairwise analysis of the syntenic regions of two genomes. This pipeline uses MCScan (Tang et al., 2006) which detects syntenic blocks. We kept the default settings to produce the output files that were used by a custom perl script (synteny_GO.pl) to identify the specific genes and GO terms for a species that were in (syntenic with at least 5 species) and out (syntenic with less than 2 species) of synteny (Figure 3.4). We also used MCScan to produce the pairwise karyotype plot (Figure 3.5).

This was used to identify both macrosyntenic (viewed at the chromosome level) and microsynetic (viewed at the gene level) change across genomes. It detects structural changes including: fission events which are the splitting of chromosomes into multiple other chromosomes; fusion events which are the joining of chromosomes into a single chromosome; inversions which are the rearrangements of segments of a chromosome or singular gene by reversing end to end, this can be partial (only a section of the chromosome) or complete (the whole chromosome has been flipped); translocations which are the movement of chromosome segments or genes from one position to another, either within a chromosome or between.

3.3.4.2 Transposable element identification

Repetitive or transposable elements were identified using Earl Grey, a TE annotation pipeline (https://github.com/TobyBaril/EarlGrey) (Baril et al., 2021). Earl Grey ran RepeatMasker (Smit, AFA, Hubley, R & Green, 2013-2015) using Dfam (Hubley et al., 2015) to mask known repeats prior to the de novo TE annotation. RepeatModeler2 (Flynn et al., 2020) was then used to identify and optimise the de novo TEs using the automated “BLAST, Extract, Extend” process (Platt et al., 2016). The de novo and known repeat libraries were combined after redundant consensus sequences were removed. Earl Grey then processed the annotations to resolve overlaps and defragment repeat loci. R version 4.2.1 (R Core, 2022) was then used for the quantification and visualisation of TEs within both species. This includes kimura plots that visualise the distribution of TEs present in the genome over evolutionary time. Peaks signify TE bursts which indicate high levels of TE mobilisation throughout the genome. Troughs signify repression periods where the activity and mobility of TEs are significantly reduced, likely due to cellular mechanisms suppressing TE movement.

3.3.4.3 Gene expansion and contraction

Using a custom Nextflow pipeline (https://github.com/chriswyatt1/Goatee/; café flag), we took our 6 species genomes and annotations (See Figure 3.2, starred species which had the best quality genomes and were closely related to host and social parasite) and ran Orthofinder (Emms & Kelly, 2019) to obtain fine grained groups of genes with shared evolutionary relationships (Hierarchical orthogroups, HOGs), referred to as gene families for the rest of this chapter, and the complementary species tree. Orthofinder, which used the STAG programme for tree inference (Emms et al., 2018), was previously run using an expanded species list to create the phylogeny of Figure 3.2 (13 species including the starred species used for later analyses, other closely related species and outgroups). These files could then be used as the input of CAFÉ (Han et al., 2013), which calculates the expected numbers of gene expansions and contractions per gene family across the phylogenetic tree. We used gamma k3 and otherwise default settings. We then used a custom Perl script (sum_cafe.pl) to summarise the number of significant gene families and genes that were expanded and contracted in the species of interest (Table 3.1). This script also provides files of the gene names of the expanded and contracted gene families for each species that could be used for further comparative analyses including GO analysis (Figure 3.6).

3.3.4.4 Gene Selection analyses

Analyses exploring signatures of selection were run only on single-copy orthologues (SCOs) across the 6 highlighted species of interest (Figure 3.2). SCOs were identified using Orthofinder (Emms & Kelly, 2019), a custom Perl script (protein2DNA.perl) generated equivalent CDS sequences for the protein sequences produced in Orthofinder. Subsequently, we used prank that is a multiple sequence alignment (MSA) tool to align the SCOs (Löytynoja, 2014). This is run through Guidance2 with default settings which accurately detects unreliable alignment regions, masking them where appropriate (Sela et al., 2015). The codon alignments are then ready to run through models to test for signatures of selection. We tested for relaxed and intensified selection in 5253 SCOs by running RELAX (Wertheim et al., 2015) with an inferred species tree from Orthofinder (Emms & Kelly, 2019). Relaxed selection indicates a reduction in selection pressure on genes, increasing variation and potential for loss or novel innovations. Intensified selection indicates a heightening of selective pressure, reducing variation, and therefore honing the gene for a specific existing function. We ran the social parasite as the test group and the other social species as the reference group to understand whether there has been a change in selection intensity in the social parasite. RELAX does this by fitting three DN/DS rate categories under the alternative model to the test and reference branch sets, inferring a "selection intensity parameter" K, to understand if there is relaxed or intensified selection in the test branch compared to the reference branch. For comparing average evolutionary rates across the test and reference branches we removed genes that had DN/DS >10 on average in at least one branch. The remaining number of SCOs was therefore 3801 (72.4%). Scripts were modified from (Schrader et al., 2021) to visualise the genome-wide pattern of selection intensity (Figure 3.7). We also identified genes showing positive selection using adaptive branch-site tests (M. D. Smith et al., 2015) using the processed SCOs and the inferred species tree from Orthofinder (Emms et al., 2018; Emms & Kelly, 2019). After processing, 5460 SCOs were analysed. Lists of genes under significant positive selection in both the social parasite and host (FDR<0.1) as well as relaxed (with k<1, FDR<0.1) and intensified selection (with k>1, FDR<0.1) for the social parasite were identified. GO analysis was performed using topGO on these gene lists using FDR correction (Alexa & Rahnenfuhrer, 2010).

3.3.5 Quantifying Life-stage Specific Differential Gene Expression Within & Between Species (Hypothesis 3)

3.3.5.1 Gene Expression Quantification

We followed a standardized processing pipeline for the RNAseq raw reads using the publicly available next w wrapper nf-core/rnaseq v.1.4.2 (DI Tommaso et al., 2017). This involved trimming the raw RNAseq reads with TrimGalore (Krueger et al., 2021) mapping the reads to the new reference genomes using STAR (Dobin et al., 2013), and finally obtaining GFF feature read counts with FeatureCounts (Liao et al., 2014). For cross-species analyses we used the species orthologues identified using Orthofinder (Emms & Kelly, 2019) and filtered genes out that were not SCO's.

3.3.5.2 Differential Gene Expression Analyses

Differential gene expression analyses were performed in R version 4.2.1 (R Core, 2022) using the DESeq2 package (Love et al., 2014). We ran this analysis both with the social parasite (13,877) and host (12,536) only gene counts and the orthologous (8105) gene counts. Gene numbers after filtering (fpkm<1) were 10,440 (from a total of 13,877) genes for the social parasite, and 9,393 (from a total of 12,526) genes for the host. The raw counts were transformed by variance stabilization using the VST function in DESeq2 (Love et al., 2014). DESeq2 was run on all the groups and then contrasts were calculated for each pair of groups. We assumed that genes were differentially expressed between conditions if $p<0.05$ after false discovery rate correction according to the Benjamini-Hochberg procedure and if the lfc >1. We obtained the results of the contrasts such as overwintering queen versus summer queen and were able to identify the number of genes up regulated in say overwintering compared to summer queen and up regulated in summer queen compared to overwintering queen. These significant DGE lists were then used to create the Venn diagrams in Figure 3.9 using the R package 'nVennR' (Pérez-Silva et al., 2018).

3.4 Results

We first describe and compare the genomic features (Section 3.4.1) of the host and social parasite to test to what extent genomic contraction/losses (Hypothesis 1) and genomic expansions/gains (Hypothesis 2) explain the evolution of the social parasite phenotype. We then outline the transcriptomic features (Section 3.4.2) – specifically, the patterns of transcriptional activity across the two species at each of the major reproductive female life stages - and use these to test to what extent conditional gene expression explains the phenotypic differences. Finally, we combine the genome and transcriptomic datasets to determine to what extent the contrasting genomic features are associated with differential transcriptional activity (Section 3.4.3).

3.4.1 Genomic Features

3.4.1.1 Expanded genome size and composition in the social parasite

The social parasite shows traits suggesting it has an expanded genome, compared to other social insects, and especially compared to its host. First, the genome is large (277.1Mb), compared to the social host (213.6Mb). In fact, it has the largest genome of the all the 13 species represented in the phylogeny, apart from fire ant *Solenopsis invicta* and *Nasonia vitripennis* (a parasitoid wasp) (Figure 3.2). A recent comparative genomic analysis in bumblebees showed that closely related species generally have a maximum of 20Mb difference (Sun et al., 2020); the >60MB difference between the two genomes we have studied is, therefore, large for closely related congenera.

There are some subtle differences in chromosomal properties between the social parasite and its host. Hi-C contact heatmaps identify 24 chromosomes in the social parasite and 22 chromosomes in the host (Figure 3.4A). Chromosome sizes are also generally larger in the social parasite (mean=11,528,406 bp, SE=-/+ 924,559bp) compared to the host (mean=9,626,345bp, SE=-/+ 995,917bp).

Transposable elements (TEs) in the social parasite and host genomes differ in several ways. As a proportion of the genome, the social parasite exhibits almost twice as many TEs (33.3% of total genome) as the host (18.8% of total genome; Figure 3.3A). We found a positive correlation between chromosome size and TE content (Figure 3.3B); this relationship has been previously reported, with larger chromosomes harbouring a higher proportion of TEs than smaller chromosomes (A. H. Nguyen et al., 2022). The large representation of TEs in the social parasite genome may explain why its chromosomes tend to be so much larger on average than in the host (Figure 3.3B). Both genomes have a high proportion of potentially recently acquired TEs (<25 for kimura distance; Figure 3.3C-D). However, the social parasite exhibits multiple distinct transposition bursts (mainly via LTR's and a most recent LINE burst) as well as repression periods (See methods for definitions of these)(Figure 3.3C). This suggests there have been multiple events in evolutionary time where TEs within the social parasite have undergone extreme mobilisation and inactivity (Figure 3.3C). These events were not detected in the host, where TEs appear to have been accumulated in a much more gradual manner over evolutionary time.

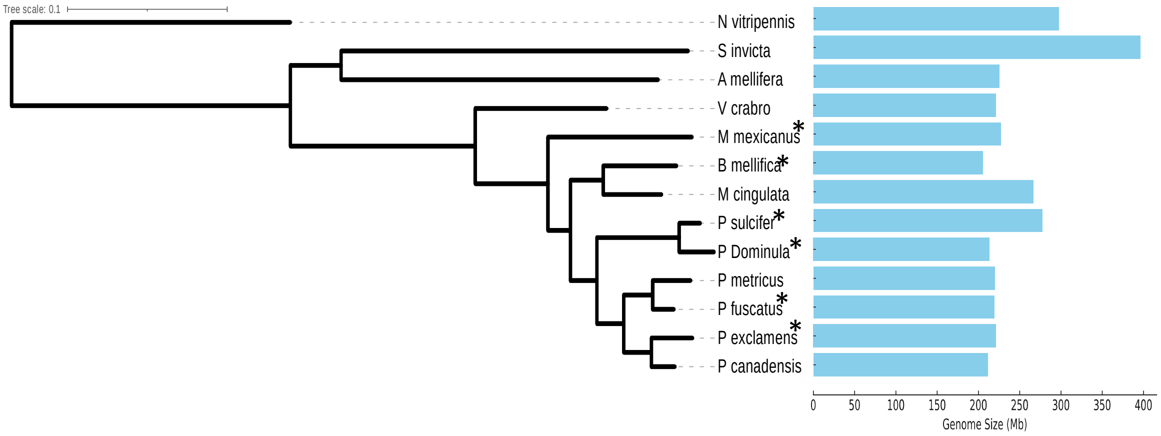


Figure 3.2: phylogeny emphasising the closely related nature of host and the social parasite. * denotes species that are included in further comparative genomic analyses.

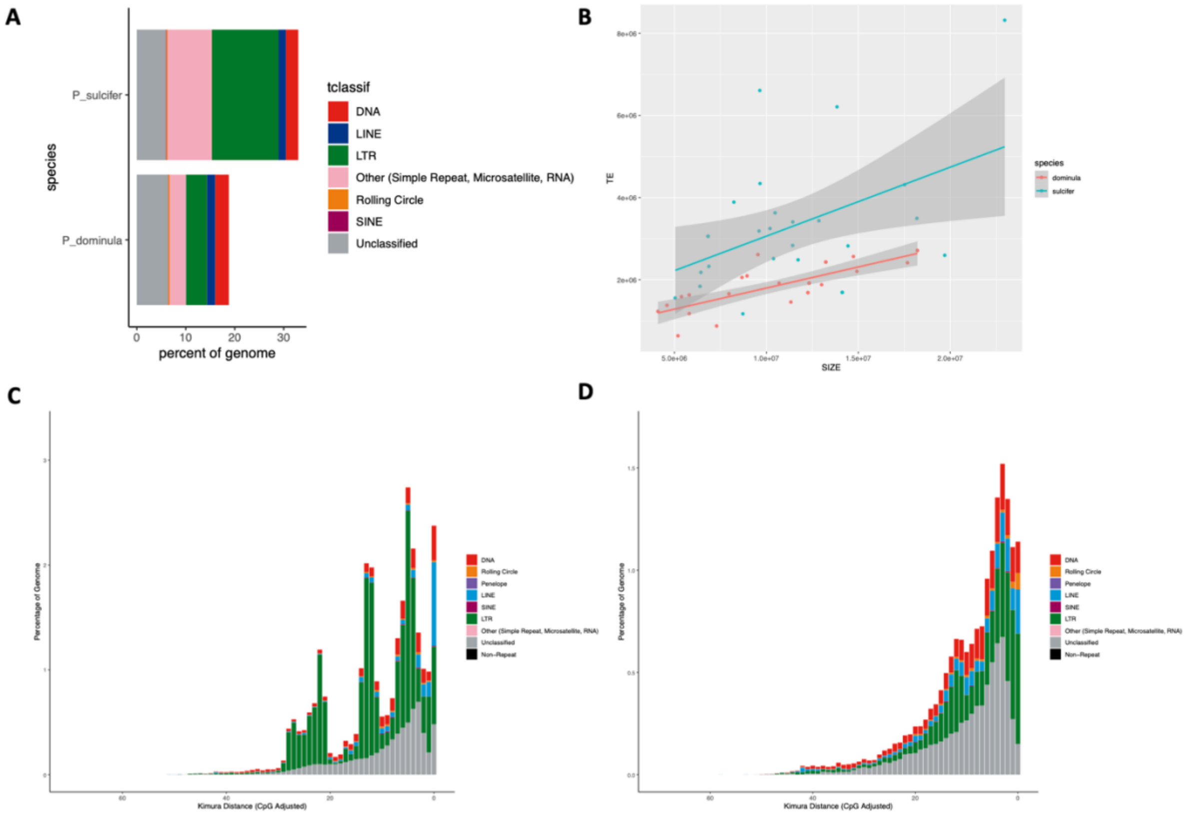


Figure 3.3: TE composition and patterns within the social parasite and host genomes. A) Composition of TE types for each species of interest and their percentage of their genome. B) Number of TEs per chromosome size in both the social parasite (blue) and host (red). C-D) Divergence of TEs for the social parasite (C) and the host (D), using Kimura distance; clustered based on kimura distances to their corresponding consensus sequence (x-axis: <25 are potentially more evolutionary recent TE events, >25 are potentially older TE events).

3.4.1.2 Contrasting Chromosomal Architecture between Social Parasite and Host

We identified orthologous scaffolds between the host (21) and social parasite (24) which revealed contrasting features in chromosomal architecture between the two species at the macrosyntenic (chromosome view) and microsyntenic (gene view) level (Figure 3.4B).

At the macrosyntenic level, we detected four fission events and two fusion events in the social parasite's chromosomes relative to those of the host (Figure 3.4B). An example of fission is host chromosome 1 which is orthologous to the social parasite's chromosomes 7 and 8 (Figure 3.4B). An example of a fusion event is social parasite chromosome 6 which is orthologous to a block in host chromosomes 5 and 19. Host chromosome 5 also has orthology to social parasite chromosome 22, indicating another fission event. Thus, fusion and fission events are inextricably linked across the genomes.

Secondly, we found evidence of multiple inversions between the host and social parasite (Figure 3.4B). Some chromosomes have segments reordered (partial inversions) as seen between host chromosome 1 and social parasite chromosome 4. However, 9 of the orthologous chromosomes exhibit the flipping of the entire chromosome order (complete inversions), for instance between chromosome 2 for both host and social parasite (Figure 3.4B). This could be an artefact of how the chromosomes were sequenced or a true complete inversion of the chromosome.

Thirdly, we detected a potentially novel chromosome in the host (chromosome 22) (Fig 3A). Pairwise synteny analyses across other social species with our host and social parasite showed the orthologous chromosomes relative to the paired species. The host's novel chromosome was not orthologous to any of the other species' scaffolds, suggesting it is a novel gain in the host as opposed to a loss in the social parasite. The Hi-C plot indicates a similarly sized chromosome 22 to chromosome 21 (4100kb) in the host. However, chromosome 22 is in fact very small (105kb), with only 3 genes, of which only one has a blast match (g12491- smad nuclear interacting protein 1). Earlier work on chromosome number in *P. dominula* predicted 21 chromosomes (Pardi, 1940). Therefore, more high-resolution chromosome-level genomes of other *Polistes* species are required to explore this putative novel chromosome further and understand the directionality of these fission and fusion events.

At the microsyntenic level, we identified 981 non-syntenic genes in the social parasite (relative to the host) and 847 in the host (relative to the social parasite). Non-syntenic genes in the social parasite are significantly enriched for three main functional categories (Fig 4): (i) regulation: these include GATA zinc finger domain-containing protein 14-like, transcription factor Sox-2-like (Figure 3.4B), zinc finger X-chromosomal protein-like, Protein

MAATS1-like; (ii) sensory perception: e.g. putative fatty acyl-CoA reductase CG8306, odorant receptor 13a-like, cytochrome P450 4C1-like; (iii) stress tolerance: e.g. heatshock protein 83 (Figure 3.4B). GO analyses of these genes identified terms such as chemical sensing, as well as ribosomal genes and gene regulation (Figure 3.5).

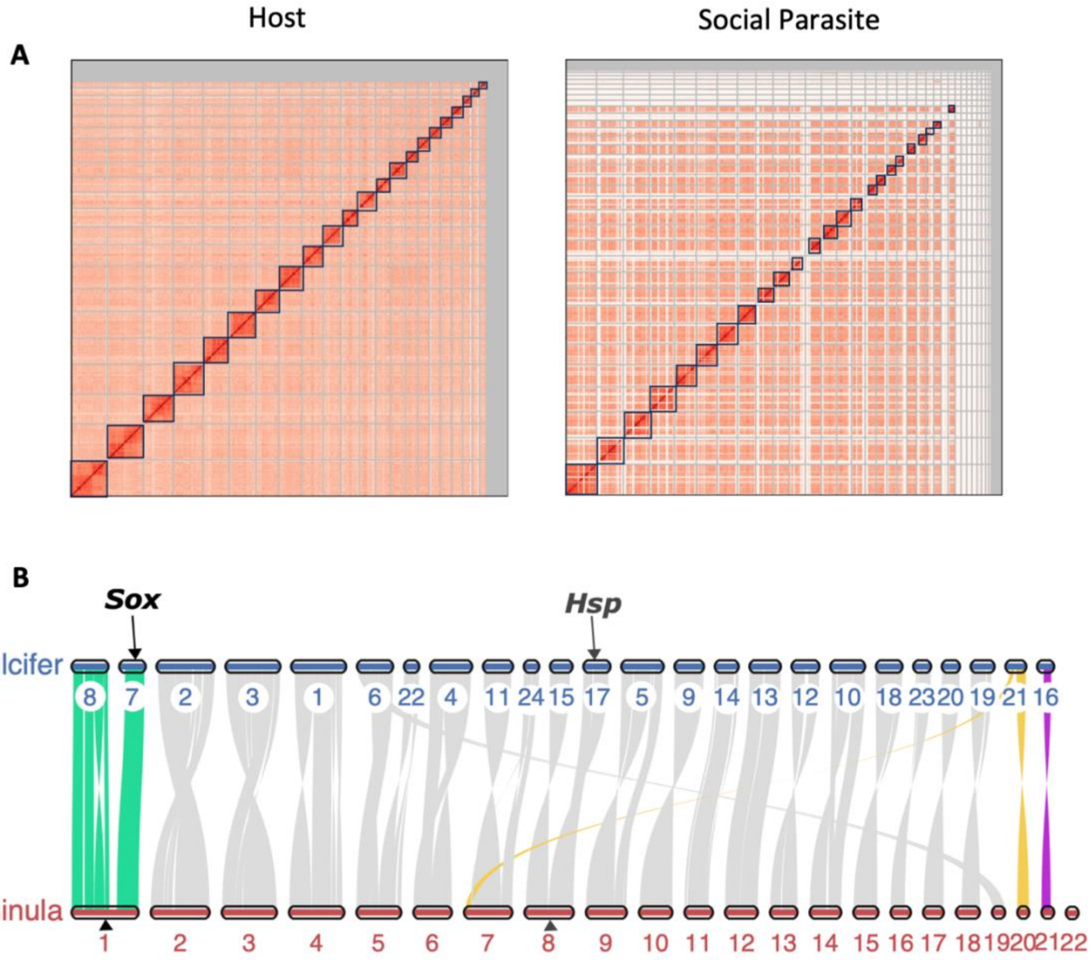


Figure 3.4: Chromosome number and structural changes between host and social parasite. A) Hi-C contact maps for host (left) and social parasite (right), with scaffold numbers increasing from left to right. B) chromosome structural evolution showing host (red) and social parasite (blue) undergoing fission events (e.g. green), fusion events (e.g. yellow) and conserved chromosome structures (e.g. purple). Examples of microsyntenic differences shown by black arrows and two genes, Sox (transcription factor Sox-2-like) and Hsp (Heat shock protein 83).

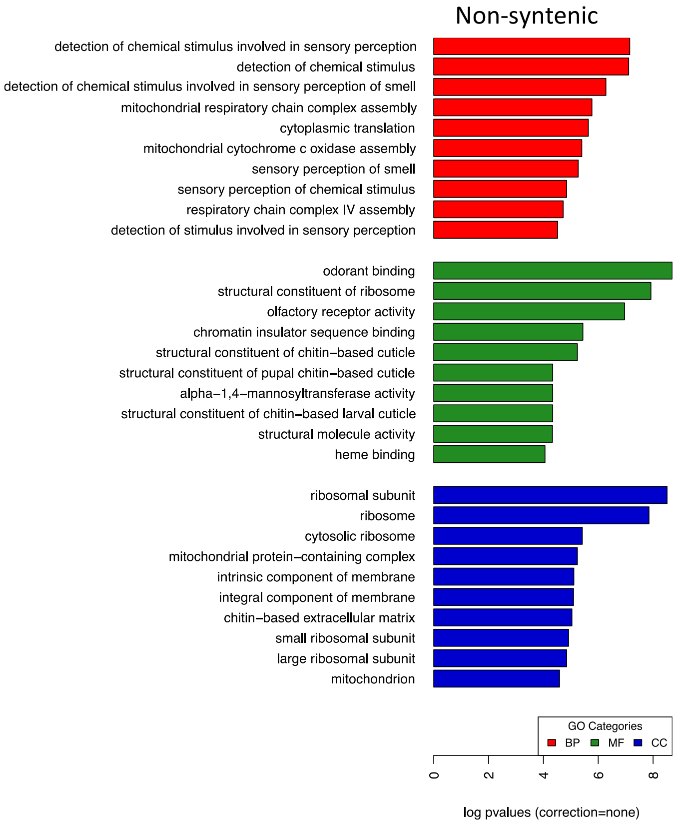


Figure 3.5: Significant enrichment of Gene Ontology terms among genes that are out of synteny in the social parasite compared to the host.

3.4.1.3 Gene Expansions and Contractions in the Social Parasite

We found evidence of gene family expansions and contractions in the social parasite, with 252 expanded gene families compared to the host's 73, and 155 contracted gene families compared to the host's 229 (Table 3.1). These patterns are significantly different, with the social parasite having more gene family expansions and fewer gene family contractions than the host (X-squared = 97.965, df = 1, p-value < 0.001). The prevalence of expanded gene families in the social parasite holds in the wider comparison with other social wasp species (see Table 3.1); moreover, the host-parasite pair each exhibit at least an order of magnitude more contracted gene families than the other social wasp species examined. This is unlikely to be due to the resolution and quality of the genomes: although *P. fuscatus, P. exclamans* and *M. mexicanus* are not chromosome-level genomes, *B. mellifica* is and yet displays some of the lowest levels of gene family evolution (see Table 3.1).

Some of the gene families that are expanded in the social parasite are similar in identity and function to observations in other social parasites; e.g. Cytochrome p450 9e2like (4 copies compared to the host's 3) was observed in a socially parasitic ant mutant (Trible et al., 2023) and also found to be important in honeybee gonad evolution (Lago et al., 2023). Other gene families indicate the importance of regulation of gene expression and developmental processes, e.g. histone acetyltransferase (2 copies compared to the host's 1), GATA zinc finger domain-containing protein 10-like (2 copies compared to the host's 1) and homeobox protein onecut (2 copies compared to the host's 1). Finally, the expansion of circadian clock-controlled protein (2 copies compared to the host's 0 and other social species' 1) (Feldmeyer et al., 2017) may reflect the importance of circadian rhythm behaviours in the life-history of this particular social parasite (Ortolani & Cervo, 2009). In line with regions which have undergone syntenic breaks, gene families related to sensory perception have undergone expansions in the social parasite; e.g. toll like receptor 8 (2 copies compared to the host's 1) and 13 (8 copies compared to the host's 4), gustatory receptor (3 copies compared to the host's 2), chymotrypsin (2 copies compared to the host's 1), odorant receptor 85c-like (2 copies compared to the host's 1), odorant receptor 13a-like (1, host has 0 other have 1).

Interestingly, expansions of olfactory machinery have been found only rarely in other social parasites (Sun et al., 2020), as seen in socially parasitic bumblebees which exhibits both expansions and contractions of olfactory machinery. It is much more common to observe a *loss* of olfactory receptor genes (Alleman et al., 2018; Schrader et al., 2021; Smith et al., 2015; Stoldt et al., 2022; Sun et al., 2020). We found some evidence of this in *P. sulcifer*, with multiple reductions in gene families with putative sensory functions, including odorant receptor 94a-like (0 copies compared to the host's 1), odorant receptor 83a-like (0 copies compared to the host's 1) and odorant receptor 4 like (0 copies compared to the host's 1). Other sensory related reductions include putative fatty acyl-CoA reductase CG5065 (1 copy compared to the host's 6), fatty acid synthase-like (1 copy compared to the host's 2). This is alongside contracted gene families which are associated with regulation; e.g. histone H4 like (1 copy compared to the host's 3), histone-lysine N-methyltransferase SETMAR-like (1 copy compared to the host's 3) (Santos et al., 2018), and structural maintenance of chromosome protein2 (0 copies compared to the host's 1).

Gene ontology terms were produced from genes that exhibited significant expansion (Figure 3.6A) and the gene orthologues of those contracted (Figure 3.6B) in the social parasite. Expanded genes are significant enrichment for GO terms relating to olfactory and chemical reception. Contracted genes are enriched for GO terms related to neurological functioning.

Species	Expanded	Contracted
B.mellifica	29	13
M.mexicanus	94	7
P.fuscatus	68	17
P.exclamens	120	6
P.sulcifer	252	155
P.dominula	73	229

Table 3.1: CAFÉ results showing the number of significantly expanded or contracted gene families for each species.

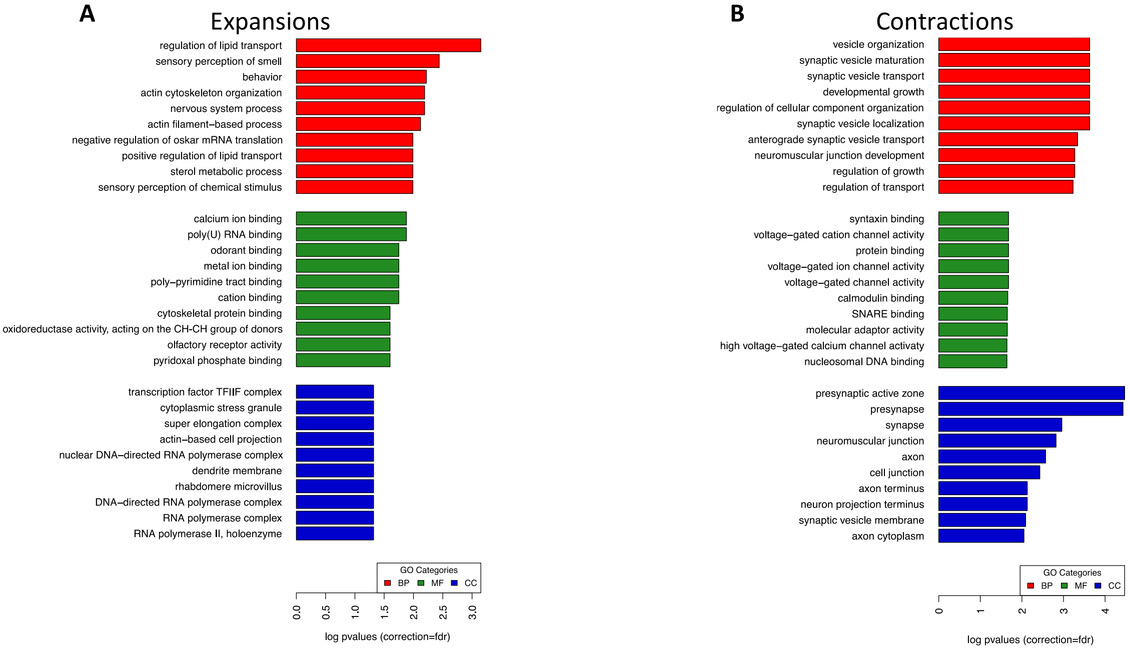


Figure 3.6: A) GO terms relating to significant gene family expansion for the social parasite B) GO terms relating to significant gene family contractions for the social parasite.

3.4.1.4 Evidence of Gene Evolution in the Social Parasite

Diverse patterns of gene evolution have been detected in the genomes of ant social parasites, including relaxed genome-wide selection (intensity of selection), reduced genome-wide positive selection (direction of selection) (Schrader et al., 2021) and no significant change (Smith et al., 2015).

Here, we find overall no significant difference in selection pressure between the social parasite compared to a background of social species (Figure 3.7). Using the RELAX model from HYPHY, we are able to understand the intensity of selection on orthologous genes of the social parasite as the focal species compared to a background of social species (including the host, *P. dominula*). Among the refined list of 3801 orthologous genes (see Methods), we found no significant deviation from 0 in genome-wide patterns of selection pressure in the social parasite, relative to social species (including the host) (P-value= 0.5895, Figure 3.7). This suggests that *P. sulcifer* has not experienced genome-wide differences in selection pressure compared to its social relatives.

However, at the individual gene level, a small number of genes exhibited significant levels of selection (Table 3.2). Among all 5253 orthologous genes, 6 genes exhibited significant relaxed selection (fdr <0.1) and 9 genes exhibited significant intensified selection (fdr <0.1) in the social parasite compared to the other social species (Table 3.2). Most of these genes (4 out of 6 relaxed genes; 7 out of 9 intensified genes) are located in regions of the social parasite chromosomes that have undergone fission events (Table 3.2, Figure 3.7). Genes under relaxed selection were significantly enriched for terms associated with histone methylation (Figure 3.8B) and include genes putatively related to regulation of gene expression (e.g. histone lysine methyltransferase) and digestion (e.g. trypsin-1 like). Genes under intensified selection are significantly enriched for terms such as metabolism and eclosion (Figure 3.8A), and include genes such as pantothenate kinase 4, which is important for CoA synthesis and therefore metabolism. Finally, 18 genes exhibited significant levels of positive selection in the social parasite (fdr<0.1). These genes are enriched for functions related to photoreception and signalling (Figure 3.8C). A further 25 genes exhibited significant positive selection in the host (fdr<0.1); these were enriched for processes relating to stress response, cellular localisation mechanisms and reproduction (Figure 3.8D).

Orthogroup	sulcifer ID	Selection pressure	Gene name	Sul_scaffold	Dom_scaffold
OG0005562	g6147	Relaxed	cleavage and polyadenylation specificity factor subunit 1 isoform X1 [Polist	HiC_scaffold_8	HiC_scaffold_1
OG0005070	g6048	Relaxed	protein kinase C-binding protein NELL1-like isoform X1 [Polistes dominula]	HiC_scaffold_8	HiC_scaffold_1
OG0005553	g6236	Relaxed	RNA polymerase-associated protein CTR9 homolog [Polistes dominula]	HiC_scaffold_8	HiC_scaffold_1
OG0006155	g5730	Relaxed	histone-lysine N-methyltransferase ash1 isoform X1 [Polistes dominula]	HiC_scaffold_7	HiC_scaffold_1
OG0004789	g1523	Relaxed	trypsin-1-like [Polistes canadensis]	HiC_scaffold_2	HiC_scaffold_2
OG0007679	g12452	Relaxed	protein disulfide-isomerase [Polistes dominula]	HiC_scaffold_20	HiC_scaffold_17
OG0007373	g6458	Intensified	jmjC domain-containing protein 4 isoform X2 [Polistes dominula]	HiC_scaffold_8	HiC_scaffold_1
OG0006019	g5862	Intensified	pantothenate kinase 4 [Polistes dominula]	HiC_scaffold_7	HiC_scaffold_1
OG0006433	g2059	Intensified	ankycorbin isoform X1 [Polistes dominula]	HiC_scaffold_2	HiC_scaffold_2
OG0007289	g6586	Intensified	transcription factor IIIB 90 kDa subunit [Polistes dominula]	HiC_scaffold_8	HiC_scaffold_1
OG0004267	g7889	Intensified	inactive dipeptidyl peptidase 10 isoform X1 [Polistes dominula]	HiC_scaffold_11	HiC_scaffold_7
OG0005344	g11127	Intensified	regulatory-associated protein of mTOR isoform X3 [Polistes dominula]	HiC_scaffold_17	HiC_scaffold_8
OG0005377	g11166	Intensified	BAI1-associated protein 3 isoform X1 [Polistes dominula]	HiC_scaffold_17	HiC_scaffold_8
OG0006174	g5587	Intensified	leucine-rich repeat protein soc-2 homolog [Polistes dominula]	HiC_scaffold_7	HiC_scaffold_1
OG0006606	g2023	Intensified	cGMP-specific 3',5'-cyclic phosphodiesterase isoform X1 [Polistes dominul	HiC_scaffold_2	HiC_scaffold_2

Table 3.2: Genes under selection in the social parasite compared to the five reference social wasps.

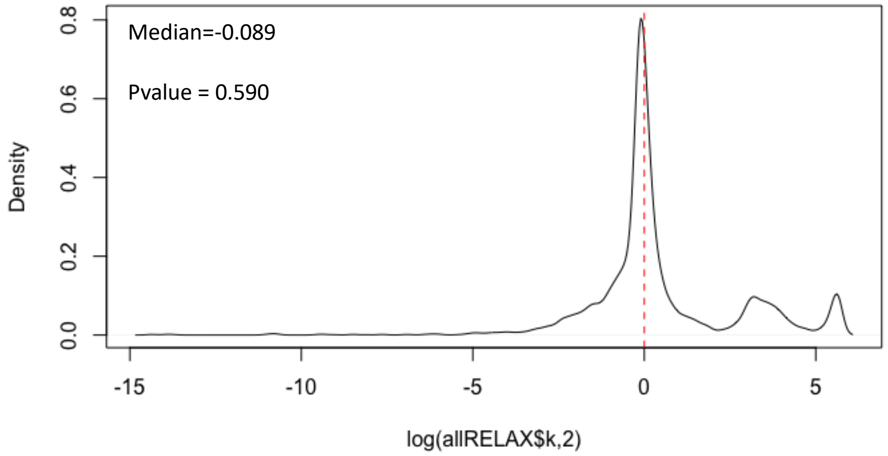


Figure 3.7: Significant levels of relaxed (negative logK2 values) and intensifying (positive logK2 values) among orthologous genes in the social parasite. Two-sided one sample Wilcoxon-Mann-Whitney test indicates that logK2 values are significantly different from 0.

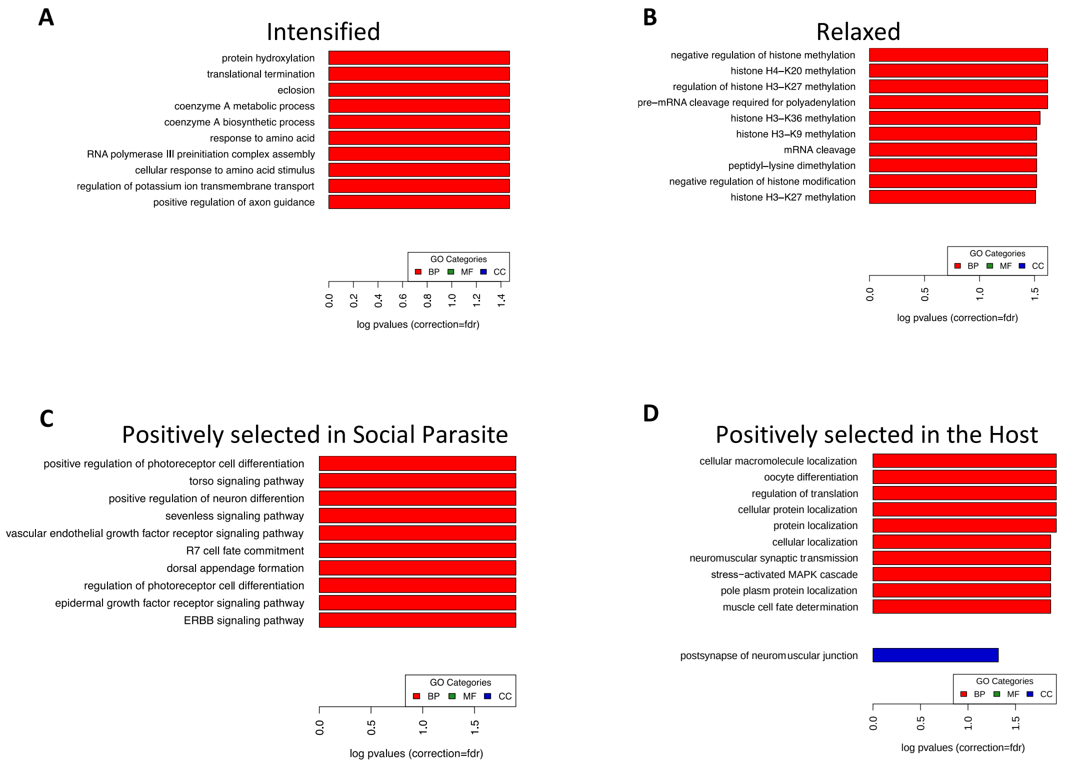


Figure 3.8: Functional enrichment (GO terms) of genes under selection in the social parasite and host, using GO enrichment analysis. A) Genes under intensified selection in the social parasite compared to a background of social species. B) Genes under relaxed selection in the social parasite compared to a background of social species. C) Genes under positive selection in the social parasite. D) Genes under positive selection in the host.

3.4.2 Differential Gene Expression as a Mechanism for Social Parasite Evolution

In order to determine the role of conditional expression as a mechanism for social parasite evolution (Hypothesis 3), we defined three sets of differentially expressed genes (DEGs) that are associated with the life histories of the two species. Two sets of genes were those that are differentially expressed among brain transcriptomes for adult females across the main life stages (overwintering, founding and established summer nesting phase): DEG Set 1: life-stage DEGs within species – these included single copy orthologous genes (SCOs) found in both species (candidates for conditional expression) but also multi-copy orthologous genes and taxon-restricted genes that were uniquely expressed in either the host or social parasite (indicators of mechanisms other than conditional expression); DEG set 2: life-stage specific DEGs between species (limited, to shared, single copy orthologues (SCO) – candidates for conditional expression), for example the DEGs from comparing the overwintering stages of each species. DEG Set 3 were those that are upregulated in host workers, relative to the host queen life-stages: this set of DEGs can be used to directly test the hypothesis on the extent to which the social parasite has lost the worker-phenotype regulatory machinery (indicators for conditional expression and other mechanisms).

DEG Set 1 reveals several consistent patterns in transcription among different life-stages, with some marked differences between species. Firstly, there is a general pattern of elevated transcriptional activity across life-stages in the social parasite relative to the host, with over 5-fold more DEGs among the social parasite life-stages than among host life-stages (Figure 3.9A). Secondly, a large proportion of DEGs in both species were non-SCOs: they represented 50-53% of DEGs in the host, and 35-38% in the social parasite. These genes could be multi-copy orthologues or taxon-restricted genes. Those taxon-restricted genes may potentially contribute to the evolution of social parasitism through mechanisms other than conditional gene expression. Thirdly, a small but significant set of single copy orthologs (SCOs) were up-regulated in a stage-specific manner across the two species (Hypergeometric test of gene overlap: Overwintering: 8% of SCO DEGs, Pvalue<0.001. Founding/Usurping: 3.6% of SCO DEGs, Pvalue<0.001. Summer: 3.8% of SCO DEGs, Pvalue<0.001) (Figure 3.9A). These genes are the candidates for a mechanism of conditional expression in the evolution of the social parasite.

The second set of DEGs are those that shared SCOs between the two species but were differentially expressed between corresponding life-stages of the social parasite and host. Of the 8105 single-copy orthologous genes, 23.6% (n=1914 genes) were differentially expressed between corresponding life-stages of the social parasite and host (DEG Set 2). Several clear patterns are apparent. Firstly, significantly more genes (52%; n=1013 genes) were upregulated in the overwintering stage, compared to founding/usurping (25%; n=485, Pvalue<0.001) and summer (22%; n=416, Pvalue<0.001). Secondly, significantly more genes were upregulated in the social parasite than host, in each of the three life stages (Overwintering: 167 more upregulated genes in the social parasite, Pvalue< 0.001. Founding/Usurping: 158 more upregulated genes in the social parasite, Pvalue< 0.001.

Summer: 46 more upregulated genes in the social parasite, Pvalue< 0.024) (Figure 3.9B). This highlights the potential importance of conditional expression of shared SCOs in the evolution of the social parasite.

The final set of DEGs (DEG Set 3) are those that are associated with worker-like behaviours in the host: we expect these to be down-regulated or absent in the social parasite. To obtain a holistic list of worker-biased genes, we selected all the genes that were up-regulated in workers related to the queens at each host life stage (overwintering, founding and summer/established) as DEG Set 3. Of these 431 genes, 90 were non-SCOs (21%) and 341 (79%) were SCOs. Of the SCOs, 320 (74%) were detected as transcribed genes in the social parasite (average fpkm>1) and 100 (23%) were DEGs in the social parasite (Figure 3.9C). This suggests that the social parasite has retained the majority of the worker genomic machinery, but that only a small proportion of it is used functionally for regulating life-cycle behaviours.

In conclusion, in each of these DEG sets, we found evidence to support the hypothesis that conditional expression of shared genes is an important mechanism in the evolution of social parasitism (Hypothesis 3). However, the non-SCOs in DEG sets 1 and 3 reiterate the importance of additional processes generated by genomic evolution, via some of the mechanisms identified in Section 3.4.1.

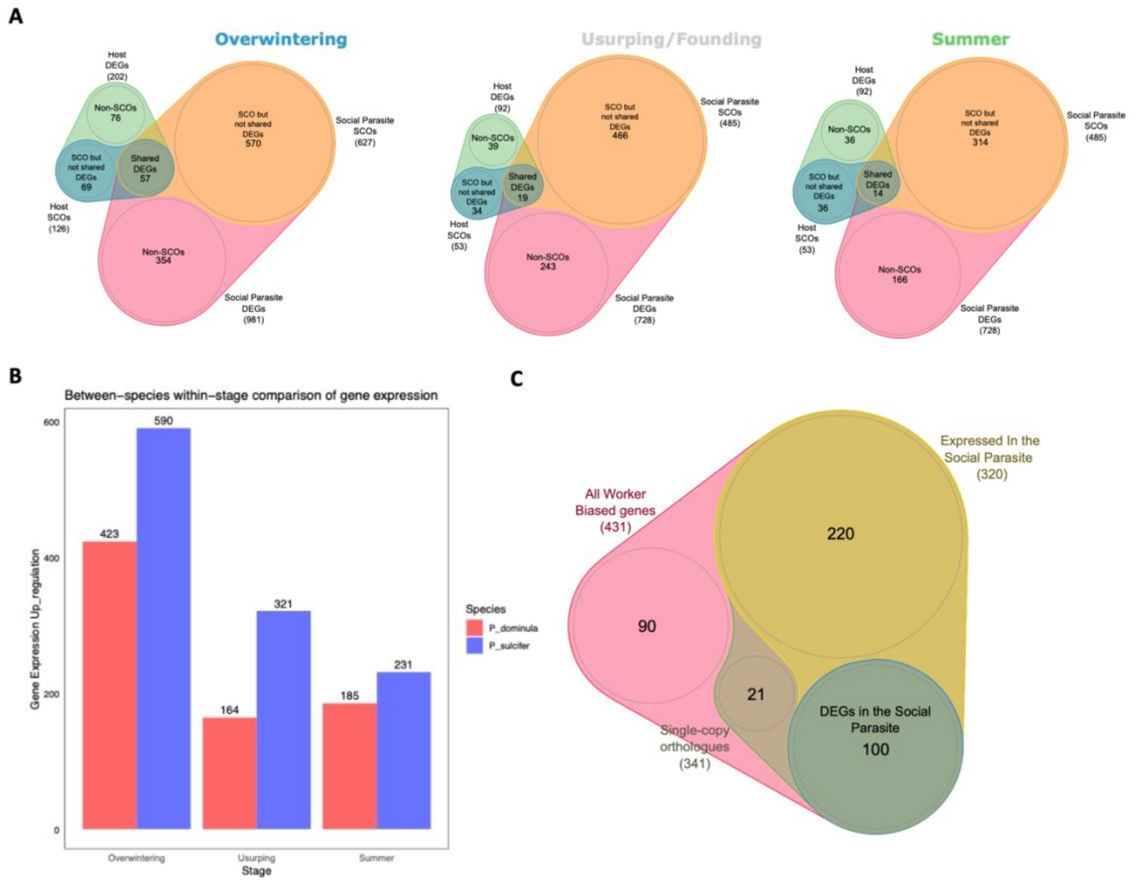


Figure 3.9: Exploration of life stage specific DEGs both within and between species. A) DEG Set 1: Venn diagrams comparing within-species life-stage specific DEGs (up regulated in one stage compared to at least one other) in host (left circles) and social parasite (right circles). Non-SCOs (single-copy orthologues) could be multi-copy orthologues or taxon-restricted genes. SCOs are the DEGs that have shared orthology with the other species and shared DEGs are SCO genes upregulated in both species. B) DEG Set 2: The number of significantly species biased gene expression in the pairwise life stage specific comparisons of SCO DEGs where the host (*P. dominula*) is red and the social parasite (*P. sulcifer*) is blue. C) DEG set 3: nested Venn diagram for number of worker biased genes that are single copy orthologues, expressed in the social parasite (fpkm>1) and also DEGs in the social parasite.

3.4.3 Differential Transcription is Associated with Genome Evolution

Here we combine the findings on differential transcription (Section 3.4.2) with the signatures of genomic evolution (Section 3.4.1) in order to identify the key processes in social parasite evolution. Identifying where life-history related differential transcription overlaps with genomic differences provides the strongest evidence for functional genomic evolution in the adaptation of the social parasite phenotype.

3.4.3.1 Syntenic Breaks are Associated with Between-Species Differential Gene Expression

We found evidence linking microsyntenic (gene view) changes with the genes that were differentially expressed between corresponding life-stages of the two species (DEG Set 2). Specifically, genes that are social parasite biased are significantly more likely to be out of synteny than genes that are host biased. This result was true for up-and down-regulated genes. Using DEG set 2 (DEGs between species for each life stage), upregulated DEGs in the social parasite were more likely to be non-syntenic (49/981 non-syntenous genes, 764 social parasite upreg DEGs) compared to the host (13/847 non-syntenous genes, 507 host upreg DEGs) (X-squared = 8.920, Pvalue = 0.003). Similarly, downregulated DEGs in the social parasite were more non-syntenic (34/981 non-syntenous genes, 507 social parasite upreg DEGs) compared to the host (15/847 non-syntenous genes, 764 host upreg DEGs) (X-squared = 17.237, Pvalue < 0.001). This suggests that breaks in synteny may be important for changes in gene regulation in the social parasite and therefore for differences in phenotypes.

There was, however, no evidence that transcriptomic differences between the species were associated with the macrosyntenic (chromosome view) differences. The genes that were differentially expressed between corresponding life-stages of the two species (DEG Set 2) had no evidence of being significantly associated with non-syntenic or fissioned (splitting) chromosomes (Figure 3.10, t = -1.022, Pvalue = 0.326). Due to the fused chromosomes not being independent of the fission events we did not include them in the analysis.

3.4.3.2 Genes Under Selection are Differentially Expressed

Genes identified to be under positive selection in the host were significantly over-represented in DEG Set 2 (orthologous genes that were differentially expressed between the host and social parasite with respect to life history) but not over-represented in the social parasite. Four of these DEGs from DEG set 2 were in the social parasite (4/18 positively selected genes, X-squared = 0.768, df = 1, Pvalue = 0.381) which is the expected number of DEGs, and 11 in the host (11/25 positively selected genes, X-squared = 16.716, df = 1, Pvalue < 0.001) which is significantly more than expected. Of the four genes that were positively selected in the social parasite, only two had social parasite biased gene expression (both uncharacterised proteins), the other two were host biased.

Of the 15 genes identified to be under intensified or relaxed selection in the social parasite relative to the host, four were differentially expressed (4/15, DEG set 2, X-squared = 1.648, df = 1, Pvalue = 0.199) which is the expected number. Three have undergone intensified selection, and one relaxed selection (being either host or social parasite biased, see Table 3.4). One of the DEGs under intensified selection is upregulated in the social parasite and is putatively involved in metabolic stress adaptation (Regulatory-Associated Protein of mTOR). The gene that is relaxed in the social parasite (Protein Kinase C-Binding Protein NELL1-Like) is also social parasite biased. The protein coded by this gene has been found in Honeybee queens as a venom trace molecule, hinting at this gene's role in interspecific differences in venoms (Danneels et al., 2015).

3.4.3.3 Differentially Expressed Genes and Expanded Gene Families

Some of the expanded gene families in host and parasites were also species-specific DEGs: these included 96 of the 1796 genes that were DEGs across the life stages of the social parasite and 19 of the 1007 genes that were DEGs across host life stages. These rates were not significantly different between host (DEG set 1 and 3; out of 247 gene families in the host; (X-squared = 0.040, df = 1, Pvalue = 0.841) or social parasite (DEG set 1; out of 753 gene families in the social parasite; X-squared = 0.040, df = 1, Pvalue = 0.842): there is no evidence that DGEs are more likely to be in expanded gene families than expected by chance. The social parasite specific DEGs include zinc finger protein jing, putatively involved in repression of transcription and multiple epidermal growth factor-like domains protein 10 (megf10) which is involved in muscle development (Draper et al., 2018). Eight social parasite specific DEGs were both part of expanded gene families *and* non-syntenic (Table 3.3). They included genes like homeobox onecut-like which has an important role in neuronal differentiation in *Drosophila* (D. N. T. Nguyen et al., 2000).

3.4.3.4 Host Worker-Biased Genes as Targets of Genome Evolution in the Social Parasite

Almost 80% of worker-biased genes were detected in the social parasite genome (see above Section 3.4.2), which suggests there has not been strong selection to lose worker-specific machinery (lack of support for Hypothesis 1). We examined whether these DEGs had been subject to genome evolutionary change, which might indicate an evolved function of these genes - co-option of worker machinery for new functions in the social parasite. We did find some evidence that these host-worker-biased genes have been subjected to genome evolution in the social parasite. One gene was under relaxed selection in the social parasite: g949, histone-lysine N-methyltransferase ash1 (ASH1). This gene is involved in the methylation of histone H3 lysine 36 (H3K36), which is necessary for maintaining the balanced transcriptional output (Gregory et al., 2007). Twelve host specific DEGs corresponded to contracted gene families in the social parasite genome, (DEG set 1, 12/1007 DEGs, 205 contracted genes). Of these 12 DEGs, 3 are upregulated in workers compared to "queen" life stages (DEG set 3) (Table 3.5). Finally, 2 genes were non-syntenic; one gene is of unknown function; the other - arginine kinase – is putatively involved in cellular energy metabolism and was found to be important for energy consumption and production in workers of red imported fire ant (*Solenopsis invicta*) (Wang et al., 2009).

One hundred of the worker-biased genes were also DEGs in the social parasite. Indeed, none of the genes related to contractions, relaxation of selection or being out of synteny are present in these one hundred genes. GO term analysis identified genes related to energy synthesis and metabolism, potentially pointing towards the heightened energy requirements of both workers and social parasites (Figure 3.11). They are candidates for functional evolution in the social parasite phenotype, however more research is needed to explore their stage specific expression in the social parasite.

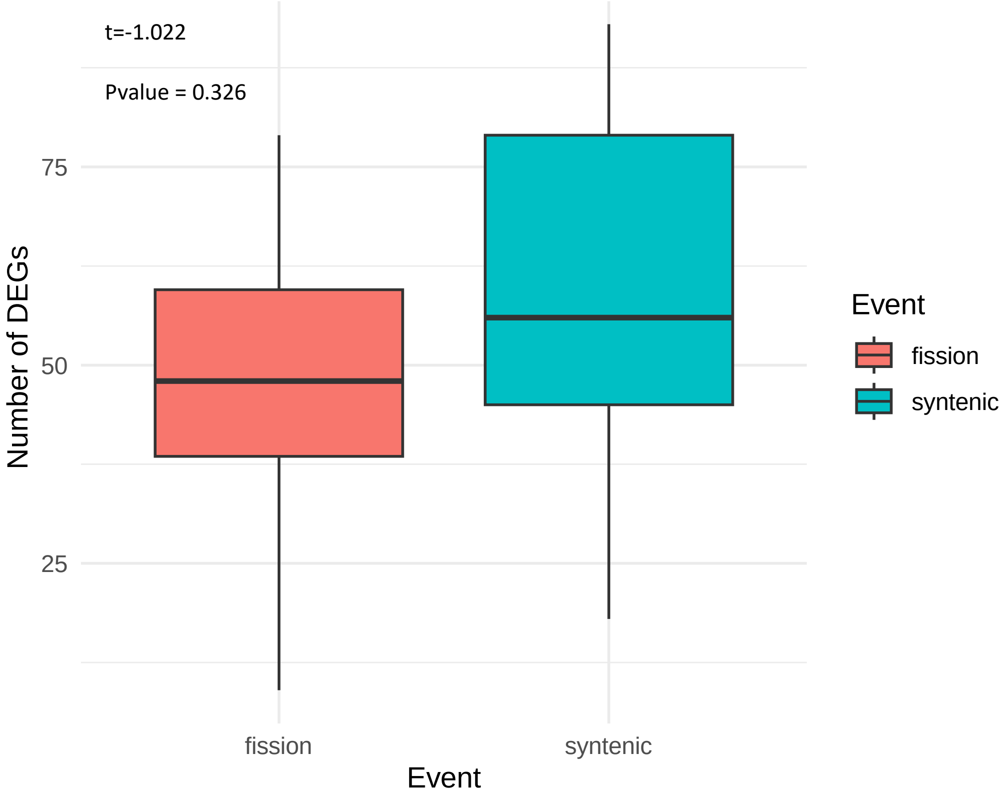


Figure 3.10: No significant difference in number of DEGs on chromosomes that have fissioned in the social parasite but not the host (left) compared to chromosomes that are in synteny between both species (right).

id	predictions		OW_s	ow_S	OW_f	ow_F	F_s	f_S	scaffold.y
g12109	protein phosphatase 1 regulatory subunit pprA-like [Polistes dominula]		0	0	0	3.492	0	0	HiC_scaffold_19
g2242	A disintegrin and metalloproteinase with thrombospondin motifs 9 isoform X1 [Polistes dominula]		1.340972	0	0	0	0	0	HiC_scaffold_3
g4432	uncharacterized protein LOC107067685 [Polistes dominula]		0	1.06509	0	1.1024	0	0	HiC_scaffold_5
g4456	uncharacterized protein LOC107067685 [Polistes dominula]		0	1.06509	0	1.1024	0	0	HiC_scaffold_5
g4976	homeobox protein onecut-like [Polistes dominula]		1.458803	0	1.7666	0	0	0	HiC_scaffold_6
g4981	homeobox protein onecut-like [Polistes dominula]		1.458803	0	1.7666	0	0	0	HiC_scaffold_6
g5946	KAI4475654.1 hypothetical protein M0802_015080 [Mischocyttarus mexicanus]		2.323844	0	0	0	2.9115	0	HiC_scaffold_8
g6624	KAI4475654.1 hypothetical protein M0802_015080 [Mischocyttarus mexicanus]		2.323844	0	0	0	2.9115	0	HiC_scaffold_8

Table 3.3: DEGs of the social parasite that are non-syntenic and part of expanded gene families. Abbreviated life stage pairs for the columns signify the pairwise comparison that gave rise to the fdr values, specifically the capitalized life stage is the up-regulated life stage in this comparison, e.g. OW_s is the fdr values for overwintering biased genes compared to summer.

selection pressure	id	OW_dom	OW_sul	U_dom	U_sul	S_dom	S_sul	predictions
Intensified	g1224	0	1.084	0	0	0	0	ankycorbin isoform X1 [Polistes dominula]
Intensified	g5996	0	1.54685	0	1.8873	0	1.8457	inactive dipeptidyl peptidase 10 isoform X1 [Polistes dominula]
Intensified	g6695	0	1.10516	0	0	0	0	regulatory-associated protein of mTOR isoform X3 [Polistes dominula]
Relaxed	g80	0	1.1472	0	0	0	0	protein kinase C-binding protein NELL1-like isoform X1 [Polistes dominula]

Table 3.4: Life-stage specific DEGs for host and social parasite with evidence of significant selection in the social parasite. Abbreviated life stage pairs for the columns signify the pairwise comparison that gave rise to the fdr values, specifically the capitalized life stage is the up-regulated life stage in this comparison, e.g. F_s is the fdr values for founding biased genes compared to summer.

id	OW_S_up	OW_S_down	OW_F_up	OW_F_down	F_S_up	F_S_down	W_OW_up	W_OW_down	W_F_up	W_F_down	W_S_up	W_S_down	predictions	scaffold
g10891	0	0	0	0	0	0	0	0	0	1.750951	0	1.45268	solute carrier family 41 member 3-like isoform X2 [Polistes dominula]	HiC_scaffold_16
g12176	0	0	0	0	0	0	0	1.4933717	0	0	0	0	uncharacterized protein LOC107071777 [Polistes dominula]	HiC_scaffold_20
g1949	0	0	0	0	0	0	1.29695	0	0	0	0	0	copper homeostasis protein cutC homolog [Polistes dominula]	HiC_scaffold_2
g4373	0	0	0	0	0	0	1.34948	0	0	0	0	0	lysoplasmalogenase-like protein TMEM86A [Polistes dominula]	HiC_scaffold_5
g4380	3.48469	0	0	0	4.5	0	0	0	0	0	4.2406	0	histone H4 transcription factor [Polistes dominula]	HiC_scaffold_5
g5524	0	0	0	0	0	0	0	0	0	3.571593	0	0	pol polyprotein [Lasius niger]	HiC_scaffold_7
g775	0	0	1.15212	0	0	0	0	0	0	0	0	0	RNA-binding protein 28-like [Polistes dominula]	HiC_scaffold_1
g8407	0	1.5587534	0	0	0	0	0	0	0	0	0	1.74951	fas apoptotic inhibitory molecule 1 [Polistes dominula]	HiC_scaffold_11
g986	0	0	0	0	1.139	0	0	0	0	0	0	0	sodium-coupled monocarboxylate transporter 2-like [Polistes dominula]	HiC_scaffold_1

Table 3.5: Host DEGs that belong to gene families that were contracted in the social parasite. Abbreviated life stage pairs for the columns signify the pairwise comparison that gave rise to the fdr values, specifically the capitalized life stage is the up-regulated life stage in this comparison, e.g. ow_S is the fdr values for summer biased genes compared to overwintering.

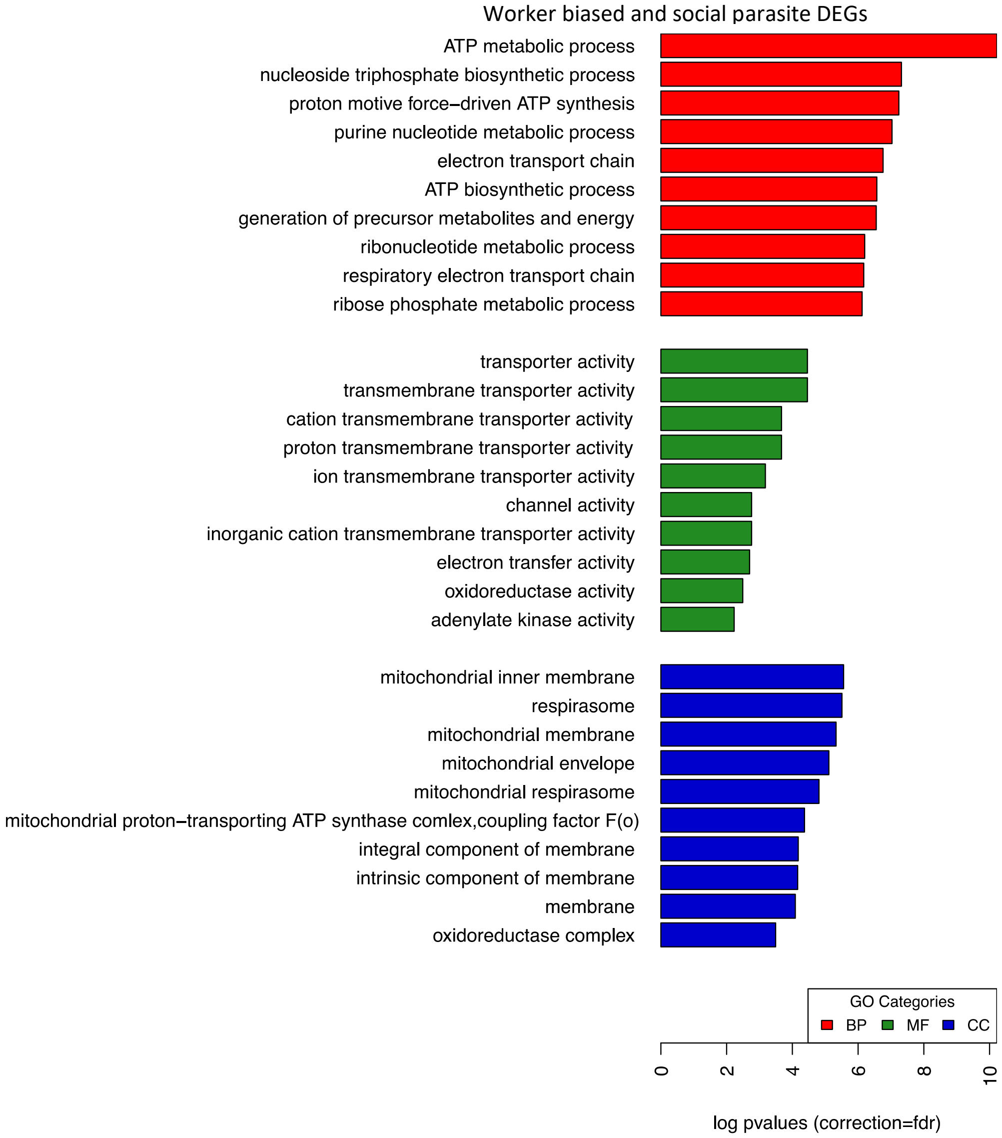


Figure 3.11: Functional enrichment (GO terms) of genes that are worker biased and differentially expressed between life stages of the social parasite, using GO enrichment analysis.

3.5 Discussion

A fascinating example of phenotypic evolution is found within socially parasitic insects that have lost their capacity to work and instead evolved as specialists that parasitise the altruistic brood care behaviour of their close relatives. Several non-mutually exclusive hypotheses predict different mechanistic patterns defining the evolution of social parasitism: genomic loss (Hypothesis 1), genomic gain (Hypothesis 2) and conditional gene expression (Hypothesis 3). There is mixed support in the literature for these hypotheses; however, no study has simultaneously analysed genome and transcriptome data in order to understand the relative importance of these three processes. Moreover, genome-level research to date has been limited to social parasites that evolved from a highly complex, superoganismal ancestor which have already experienced extreme losses and gains of phenotypic evolution (Kronauer & Libbrecht, 2018; Taylor et al., 2019). Thus, we lack any information on the mechanistic basis of a social parasite evolving from a simple social ancestor, with high levels of phenotypic plasticity. Here we explored the molecular evolution of the social parasite *P. sulcifer*, which shares a recent common ancestor with its cooperatively breeding host, *P. dominula*. By combining high-resolution genome sequencing with life-stage-specific brain transcriptomes, we were able to examine the roles of genomic losses, gains and conditional expression in the evolution of the social parasite. We find evidence for all three, and discuss these in the wider context of phenotypic evolution.

3.5.1 Which hypotheses explain the genome-wide patterns

There are a number of examples of a reduction in a species' phenotypic capacity from a single genome (Bourguignon, Šobotník, Dahlsjö, Roisin, et al., 2016; Cavalier-Smith, 1992; Cervo, 2006; Jones et al., 2023; Roff, 1994). For instance, a number of species of insects have independently lost the ability to fly, due to the energetic costs of producing the wing apparatus outweighing its benefits in some circumstances (e.g. if they can acquire food without flight) (Roff, 1994). Further, some species of Apicotermitinae, a group of termites, have lost the ability to produce soldiers in their colony (Bourguignon, Šobotník, Dahlsjö, Roisin, et al., 2016). Similarly, in parasites and social parasites, they have significantly lost phenotypic capacity by being dependent on their host for resources aiding their survival (Attardo et al., 2014; Benoit et al., 2016; Cervo, 2006; Stoldt et al., 2022; Tsai et al., 2013). These losses may well be met with genome-wide relaxation of selection and consequently large-scale gene loss of once important genes relating to the lost phenotypes. Hypothesis 1 predicts a streamlining of the genome in response to this perceived loss of importance of large sections of molecular machinery. For instance, in sweat bees, the loss of eusociality is met with large scale relaxation of selection in their genomes (Jones et al., 202323). In social parasites in particular, there have been some theoretical and empirical evidence towards little change or large-scale erosion of the genome (Schrader et al., 2021; Smith et al., 2015; Sun et al., 2020). In this study however, we find very little gene loss, and instead find evidence towards genome-wide expansions (Hypothesis 2) and conditional gene expression (Hypothesis 3).

3.5.1.1 Expansion

The pattern of expansion is multifaceted; we can see increases in genome size, chromosome number and expanded gene numbers. They all potentially explain the same thing, an increase in functions and potential for innovation at the genomic level. For each of these genomic measures we will highlight the existing patterns in social parasite literature and beyond to compare with our research:

Genome size is highly variable across organisms with ranges of 2.3Mbp to as large as 150 Gbp in eukaryote species; this is predominantly driven by the accumulation of TEs (Elliott & Gregory, 2015). Recent studies have found that an increased genome sizes with hyperaccumulation of TEs are associated with sociality in snapping shrimps (Chak et al., 2021). In fact, they find that the increases in TEs fuel the larger genomes and transitions to eusociality (Chak et al., 2021). Therefore, a loss in sociality may lead to the opposite effect. However, in this study we find that the social parasite has one of the largest genomes of the wasps studied and is much larger than the closely related host (Section 3.4.1.1, Figure 3.2). This increase in genome aligns with a greater number of TEs, but there are still greater numbers of genes in the social parasite. This hints towards the mechanistic basis of this social parasitism not simply being a reversal in molecular patterns found in the transition to eusociality (Chak et al., 2021). More fundamental analyses are required to explore the potential mechanistic basis for the hyperaccumulation of the TEs and their causal role in the evolution of social parasitism (Chak et al., 2021; van der Bijl, 2018).

Chromosome number is correlated with recombination rates, as a greater number of chromosomes lead to increases in possible genotypes due to independent assortment during meiosis (Ross et al., 2015). Therefore, understanding the changes in chromosome number will help understand the evolution of genetic diversity in a species. Chromosome number has also been associated with the transition to more complex sociality (Ross et al., 2015). Across the Hymenoptera, eusocial species have greater variability in chromosome number than solitary species (Ross et al., 2015). Interestingly, the solitary Euglossini have evolutionarily lost sociality but have a higher average chromosome number than their social sister group (Ross et al., 2015). This is also the case with socially parasitic ants and in the bumblebee subgenus *Psithyrus*, which all increased in chromosome number (Ross et al., 2015; Sun et al., 2020). We also find this pattern of chromosome number change in our social parasite, likely due to fission events (Section 3.4.1.2, Figure 3.4). It has not been made clear why these social parasites exhibit a convergent expansion in chromosome number but it has been hypothesised that antagonistic coevolution between host and social parasite could select for an increase in recombination, which in turn provides genetic diversity for novel parasite innovations (Ross et al., 2015). Further studies should explore the causal relationships of this genomic trait with TEs.

It has long been suggested that gene duplications and gene family expansions generate phenotypic variation and novel traits through subsequent functional divergence (Ohno, 1970; Rubenstein et al., 2019). For instance, duplicated genes could undergo neofunctionalization, where they assume a new adaptive function compared to the original gene. This is exemplified in the sub-social beetle *Nicrophorus vespilloides* that exhibits parental care (Mehlferber et al., 2017). During the feeding of offspring a certain gene increases in expression, whereas a copy of this gene that has been duplicated is decreased during this state. Therefore, it is thought that this family expansion has led to neofunctionalization, allowing for regulation of parental care (Mehlferber et al., 2017; Rubenstein et al., 2019). Therefore, it could be hypothesised that when a species undergoes phenotypic loss, they would exhibit greater gene family contractions than expansions. In other social parasites this is certainly the case, when compared to their social relatives (Schrader et al., 2021; Sun et al., 2020). However, in this study we find that there are much greater numbers of expanded gene families in the social parasite compared to closely related social species (Section 3.4.1.3, Table 3.1). Much of the parallels between the genomic changes in parasitism and social parasitism have concentrated on gene loss and relaxation (Hypothesis 1)(Schrader et al., 2021; Stoldt et al., 2022). However, there are several parasitic insects, trematodes and nematodes that in fact exhibit some significant gene family expansions to do with digestive proteases or xenobiotic detoxification (Chougule et al., 2005; Dvořák et al., 2005; Rispe et al., 2008; Zhang et al., 2020). These are innovations to a novel environment, and it could be the case that these gene family expansions are allowing for phenotypic innovations in the social parasite also (Hypothesis 2). Examples of particular expanded gene families and their potential role in novel parasitic traits are explored in sections 3.5.2.2 and 3.5.2.3. This result hints towards greater impact on the genome by these phenotypic innovations compared to the perceived loss in the worker phenotype.

Although some of the patterns of genomic changes in the social parasite can be explained by hypothesis 2 (genomic gain), there are other patterns which are likely to be explained more so by conditional gene expression (Hypothesis 2).

3.5.1.2 Conditional expression

Phenotypic differences across species are not always one of genomic gain or loss, but could instead be due to plastic conditional expression of shared genes (West-Eberhard, 1989). This is exemplified in social insects where the reproductive and non-reproductive phenotypes arise through differential expression (Smith et al., 2008; Sumner, 2006). The plastic nature of simple societies means that the molecular machinery has to be readily equipped to allow for phenotypic changes from a non-reproductive (worker) role to a reproductive role as an adult if the opportunity arises (Taylor et al., 2021). It may be that the evolution of social parasitism has been facilitated, in some way, by this form of conditional expression of existing genes for a parasitic context, which is a less costly strategy than acquiring completely new genes (Stoldt et al., 2022). In fact, in *Pogonomyrmex* and *Vollenhovia* inquilines which found little to no changes between the genomes of social and socially parasitic ants (Smith et al., 2015). In our study we also find some evidence towards conditional expression.

Our results of gene evolution support hypothesis 3; the social parasite does not have a significant difference in genome-wide selective pressure compared to the other social species we tested (Section 3.4.1.4, Figure 3.7). Further, only a small number of individual genes have been found to be positively selected, and under intensified or relaxed selection (Table 3.2). This indicates that the perceived loss of the worker phenotype and non-reproductive behaviours in this social parasite has not caused a significant change in selective pressure in the social parasite's genome. Therefore, conditional gene expression (Hypothesis 3) may be important in causing the phenotypic differences between the host and social parasite. This is reiterated within DEG set 2, where there is also a pattern of greater upregulation at every queen life stage in the social parasite when compared to its host for the shared SCOs (Section 3.4.2, Figure 3.9). This highlights its expanded transcriptional repertoire compared to the host, supporting the hypothesis that conditional gene expression is the mechanistic basis for the social parasite phenotype (Hypothesis 3). It also indicates that the social parasite phenotype is not one of simple loss, whether it be by conditional expression or not. Instead, it requires greater numbers of highly expressed genes to enable survival in winter, usurpation of the host's nest, and finally the maintenance of social dominance in the established summer phase.

This highlights the highly plastic nature of the social parasite genome to retain most of its genome without any relaxation or loss in light of a seemingly significant loss in phenotypic plasticity. The plastic capacity of individuals within simple societies to switch between worker and reproductive phenotypes in adulthood may equip them with the gene regulatory machinery to, over evolutionary time, specialise as social parasites without the loss of significant parts of its genome. This hints towards the lack of costs in retaining seemingly unimportant molecular machinery, or their co-option into other novel means (Waddington, 1960; Waddington, 1942a, 1942b, 1942c). Further analyses are required to test the potential for this social parasite to work, either via nest building or by becoming a worker on the nest experimentally.

3.5.2 Specific Genes for Phenotypic Loss and Gain

In section 3.5.1 we have explored the genome-wide patterns that are exhibited in the social parasite compared to its host, and uncovered support for the genomic gain (Hypothesis 2) and also conditional gene expression (Hypothesis 3) hypotheses. However, a small number of specific genes may be having a significant effect on phenotypic evolution by either being lost, gained or under conditional expression in the genome. For instance, in the human parasite *Cimex lectularius* (bed bug), chemosensory genes have been lost in its genome due to a reduced need for this form of host detection; novel genes have been gained related to blood digestion (Benoit et al., 2016). In cave fish (*Astyanax mexicanus*), the molecular basis for loss of sight involves repression of gene expression via methylation of a small number of eye-related genes (Gore et al., 2018). We now explore the identities of particular genes to further understand the abiotic and biotic pressures that are shaping this species and its genome.

3.5.2.1 Worker biased genes

The major phenotypic loss exhibited by the social parasite is in its inability to have a worker phenotype or any working ability (Cini, Patalano, et al., 2015). The loss in phenotypic potential is a consequence of a novel dependence on another species for its survival. This is similar to non-social parasites like tapeworms which have lost specific genes relating to metabolic activity in light of their dependence on their hosts for similar processes (Tsai et al., 2013). It may be the case that the worker phenotype is lost through structural (syntenic) changes, genomic loss and relaxation relating to a set of worker specific genes (Hypothesis 1). However, we do not find a significant relationship between syntenic changes in the SCOs (single copy orthologues) of the worker biased genes in the social parasite (Section 3.4.3.4).

One gene was shown to be relaxed, and a small number of contracted genes in the social parasite were also shown to be worker biased genes (Section 3.4.3.4, DEG set 3, Table 3.5). This emphasises that worker biased genes are mostly still present and functional within the social parasite. Another study that compared genomic patterns in the social parasite to worker biased gene expression also similarly found little to no worker gene loss (Smith et al., 2015). This goes against hypothesis 1 which predicts both genome-wide losses but also losses relating to worker specific genes. This more fundamentally provides evidence that losses phenotypically aren't necessarily reflected at the genomic or even transcriptomic level.

3.5.2.2 Novel phenotypic traits of the social parasite

Novel gains and biased gene expression of specific genes potentially emphasise the relative importance of novel behaviours and environments in shaping the molecular machinery for a phenotype. For instance, in the bed bug, *C. lectularius,* genes related to blood digestion have significantly expanded, reflecting their novel dependence on host blood for survival (Benoit et al., 2016). Further, they help identify potential candidates of interest for the evolution of social parasitism (Cini, Patalano, et al., 2015; Stoldt et al., 2022).

We predicted candidate genes for social parasitism to be highlighted in our study, such as reproductive genes (vitellogenin and transferrin, (Aumer et al., 2018; Korb et al., 2021)) and cytochrome p450 genes (putatively importance for mutant socially parasitic ants (Trible et al., 2023)). We also predicted species-specific candidate genes for the novel behaviours found in this social parasite such as the restlessness behaviour, their heightened aggressiveness at the usurping stage, or their matching of circadian rhythm during usurpation (Cervo, 2006; Ortolani et al., 2008; Ortolani & Cervo, 2009). Our study only partially aligned with these predictions; we do see expanded cytochrome p450 genes (Section 3.4.1.3) (Trible et al., 2023). Also, we found expanded genes for circadian clock proteins, which may correspond to the social parasite's ability to match the hosts circadian rhythm to increase usurpation success (Section 3.4.1.3) (Ortolani & Cervo, 2009). These results highlight, however, that not all phenotypic differences and novel behaviours can be clearly reflected in the molecular machinery of a species of interest.

3.5.2.3 Losses and gains in olfactory receptor genes

Within insects, olfactory receptors are important for detecting volatiles such as sex pheromones and food cues and are especially important in social species for nestmate recognition and communication (Yan et al., 2020). Therefore, losses and gains in genes coding for these receptors can inform us on the changing selective pressure for these apparatus in the species of interest. For instance, these genes have been shown to be plastically lost in species of parasitic fig wasps due to their sensory specificity to particular hosts (Yu et al., 2023). Similarly, the reduction in social behaviours in the social parasites also means they are theorised to not require as complex a repertoire of sensory apparatus (Schrader et al., 2021). In fact, in studies exploring gene family expansions and contractions in social parasites, they find a common pattern of losses of olfactory receptor genes, and this study is no different (Jongepier et al., 2022; Schrader et al., 2021; Sun et al., 2020). The olfactory receptor genes are among the contracted gene families of the social parasites. However, expansions of olfactory receptor genes have also been exhibited in the social parasite in this study. Although the parasite no longer helps via foraging and nursing it still requires sensory apparatus to find their host nests (Cervo et al., 1996; Cini, Bruschini, Signorotti, et al., 2011). Further they still take part in social interactions during the summer life stages (Cervo, 2006). Therefore, it is unsurprising they have acquired novel olfactory receptor genes. This emphasises the plastic nature of olfactory receptor genes and that both losses and gains can occur depending on the changing environmental pressure on the organism.

3.5.3 Broadening Our Scope of Social Parasitism

This study is a first step towards uncovering the mechanistic basis for the evolution of social parasitism in *P. sulcifer*. However, to understand the fundamental patterns behind reversions in sociality, more sequencing and analyses are required. First, other wasp models, specifically social parasites with simple social ancestral states, will broaden our understanding of reversions in sociality from a plastic state. For instance, there are several facultative and obligate socially parasitic Polistines which are likely to have had simple ancestral states and therefore could be analysed to help understand if there are any genomic similarities with the current study (Cervo, 2006). Further, allodapin*e* and halictid bees have numerous independent origins of social parasitism, likely with ancestral states of various social complexities (Gibbs, 2009; Gibbs et al., 2012; Tierney et al., 2008). We should sequence these social parasites and their closely related hosts, alongside examples across the spectrum of social complexity in *Dolichovespula*, *Vespula* and *Vespa* social parasites, and other ant and bee species (Carpenter & Perera, 2006; Cini et al., 2019; Gibbs et al., 2012; Hines & Cameron, 2010; Huang & Dornhaus, 2008; Smith et al., 2013; Stoldt et al., 2022; Tierney et al., 2008). This will provide greater understanding of the fundamental patterns relating to reversions in sociality, and if the ancestral state of the social parasite contributes to variation in these patterns.

To generalise our understanding, we should broaden our scope of interest as wide as possible. Interestingly, social parasitism is not restricted to just insects, and in fact spans the major evolutionary transitions (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2011a; West et al., 2015). At the cellular level, transmissible cancers have selfishly reverted from somatic cells of their now host species to exploit altruism of these multicellular organisms (Dujon et al., 2020; Ujvari, Gatenby, et al., 2016; Ujvari, Papenfuss, et al., 2016). This can be seen in domestic dogs with canine transmissible venereal tumours (Baez-Ortega et al., 2019) and in clams with clam leukaemia (Metzger et al., 2015). Even at the genomic level, selfish units have reverted to a socially parasitic state to exploit and replicate in the host genome in the form of transposable elements (Ågren, 2014). By exploring the proximate and ultimate mechanisms behind reversions in sociality across the major evolutionary transitions, we may be able to identify general commonalities that occur in all these instances (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2011a; West et al., 2015). For example, it may be that there are structural, selective and functional patterns that are ubiquitous when a loss of working capacity occurs, as in transmissible cancers (Dujon et al., 2020; Strakova & Murchison, 2015; Ujvari, Gatenby, et al., 2016; Ujvari, Papenfuss, et al., 2016). However, from the small number of species that have been explored to date, it seems there is variation across all aspects of genomic traits. To better understand the potential explanations behind this, we must pair comparative genomics with phenotypic traits of interest using phylogenetic comparative analyses. From this, causal relationships can be inferred to explain the variation in genomic patterns within these social parasites (Thorson & van der Bijl, 2023; van der Bijl, 2018).

3.6 Conclusion

There are several hypotheses to explain the molecular basis for phenotypic loss in an organism (genomic loss, genomic gain and conditional expression). Socially parasitic insects provide an ideal model system to test these hypotheses and there has been some evidence to support each of them. However, to date, no study has explored the relative importance of all three hypotheses. Further, research has concentrated on species which have evolved from superorganismal societies and therefore already have mechanisms limiting phenotypic plasticity early in development. In contrast no comprehensive molecular analyses have been done on a social parasite that has evolved from a simple society which has plastic behavioural castes. In this study we have explored the genomic and transcriptomic mechanisms that signify the loss of the worker phenotype and non-reproductive behaviours in the social parasite *P. sulcifer*, compared to its closely related host *P. dominula*. We find patterns of genomic expansion with increases in genome size, chromosome number and TE number as well as greater gene family expansions. Further, we find that the social parasite exhibits a much higher transcriptional state at all life stages compared to the host. The potential interplay of genomic and transcriptomic processes is also explored. From this we can find parallels from the literature to point towards putative genes of importance for the phenotypic loss and gain in this social parasite. This highlights the diversity of mechanistic changes that could enable the convergent evolution of social parasitism across species with different social origins and more generally the molecular basis for major losses in plasticity in a species. To generalise our understanding further, we must expand our analyses to other socially parasitic Hymenoptera and even across major evolutionary transitions.

Chapter Four:

The Plasticity and Developmental Potential of Termites

4.1 Abstract

Phenotypic plasticity provides organisms with the potential to adapt to their environment and can drive evolutionary innovations. Developmental plasticity is environmentally induced variation in phenotypes that arise from a shared genomic background during development. Social insects are useful models for studying the mechanisms of developmental plasticity, due to the phenotypic diversity they display in the form of castes. However, the literature has been biased towards the study of developmental plasticity in the holometabolous social insects (i.e. bees, wasps and ants); the hemimetabolous social insects (e.g. the termites) have received less attention. Here, we review the phenotypic complexity and diversity of termites as models for studying developmental plasticity. We argue that the current terminology used to define plastic phenotypes in social insects does not capture the diversity and complexity of these hemimetabolous social insects. We suggest that terminology used to describe levels of cellular potency could be helpful in describing the many levels of phenotypic plasticity in termites. Accordingly, we propose a conceptual framework for categorising the changes in potential of individuals to express alternative phenotypes through the developmental life stages of termites. We compile from the literature an exemplar dataset on the phenotypic potencies expressed within and between species across the phylogeny of the termites and use this to illustrate how the potencies of different life stages of different species can be described using this framework. We highlight how this conceptual framework can help exploit the rich phenotypic diversity of termites to address fundamental questions about the evolution and mechanisms of developmental plasticity. This conceptual contribution is likely to have wider relevance to the study of other hemimetabolous insects, such as aphids and gall-forming thrips, and may even prove useful for some holometabolous social insects which have high caste polyphenism.

4.2 Introduction

Phenotypic plasticity describes how different phenotypes or forms can arise from a single genome, and can manifest as diversity in behaviour, physiology or morphology. The ability to express such phenotypic diversity can be considered an individual's 'potency'. Phenotypic plasticity can produce novelty, facilitating adaptation and evolution, with effects on biodiversity, from populations to ecological communities (Whitman & Agrawal, 2009). Understanding the proximate (mechanisms by which plasticity arises) and ultimate (effects on fitness and function) causes underpinning phenotypic plasticity is therefore of paramount importance in ecology and evolution (Whitman & Agrawal, 2009). Insects are popular study subjects for phenotypic plasticity, where it has contributed to their ecological success due to their ability to change morphology during development, in response to environmental cues. Such developmental plasticity produces morphologically distinct phenotypes within a species, often called polyphenisms (Lo et al., 2018; Moczek, 2010). Social insects have proven a useful study group where their societies exhibit different phenotypes in the form of reproductive 'queen' and non-reproductive 'worker' castes; caste polyphenisms are well studied in some groups (e.g. Hymenoptera), they are less well understood in others, particularly the termites. This is surprising as termites exhibit enormous diversity in phenotypic plasticity and levels of potency. In this paper we discuss how the many 'phenomes' exhibited by termite castes provide us with both complexities and opportunities to further our understanding of the nature of plasticity and developmental potential and propose a conceptual framework that could facilitate future research in this area.

Developmental plasticity can be described in quantitative terms by the number of phenotypes that can be produced from a single genome – its 'potency'. Examples of this include the distinct queen and worker phenotypes in ant and social bee and wasp colonies; these hymenopteran species undergo complete metamorphosis, where there is a distinct transformation from a single pre-adult form (the larva) to an adult form (See Box 4.1): only the pre-adult form can express developmental plasticity and alter its ultimate caste fate, meaning that no external morphological change (i.e. change occurring through a moult) is possible once they are adults. As a result, potency of developmental plasticity in social

Hymenoptera is largely binary: individuals are either ‘totipotent’, i.e. they can become *any* of the caste options available to that species, or they are ‘committed’, i.e. their caste fate is sealed, usually irreversibly, and they have lost plasticity (Box 4.1) (Boomsma & Gawne, 2018; Crespi & Yanega, 1995). There is of course variation within this dichotomy; e.g. simple social species, like *Polistes* paper wasps, lack any form of commitment and plasticity is retained into adulthood (Jandt et al., 2014); caste is determined genetically in all species for males such that even an egg is committed to a specific caste, and this is also the case in females for a rare number of species (Cahan et al., 2002; Cahan & Keller, 2003; Julian et al., 2002; Schwander et al., 2010; Volny & Gordon, 2002). But largely, in the social Hymenoptera, an individual has the potential to become *any* caste until some point in development, after which it is developmentally committed to a specific caste. It is the relative simplicity in caste potency that has made Hymenoptera castes popular for studies of phenotypic plasticity, and as a consequence the concept of insect caste polyphenisms is largely considered a dichotomy in terms of potency.

A number of social insect taxa which do not conform to this dichotomy are gall-forming thrips (Crespi, 1992), aphids (Stern & Foster, 1996), and termites which are the focus of our study. The hemimetabolous life history of species like the termites means that they undergo incomplete metamorphosis, changing their form gradually over multiple pre-adult moults before finally producing a developmentally inflexible adult (Box 4.1). Each moult provides an opportunity to change their level of developmental plasticity. In other words, unlike most of the social Hymenoptera, there is not a single switch point in termite development during which an individual’s potency tips from completely plastic (totipotent) to not plastic (committed). As a result, they exhibit more complex levels of potency both within and between species, across their phylogeny (Lo et al., 2018; Toubiana & Khila, 2016). This difficulty in applying the mainstream binary terminology makes it challenging to study the mechanisms and evolutionary processes underpinning phenotypic plasticity in this group (Corona et al., 2016; Lo et al., 2018). However, if we are able to create a framework which allows us to utilise this complexity, we will be able to gain a deeper understanding of these processes. This is important as our current understanding is largely based on mechanisms and evolution derived from holometabolous insects; it is little known whether the same processes underpin the more complex plasticity found in other taxa. The same can be said of the theorised conditions surrounding major evolutionary transitions to superorganismality, namely the need for every colony member to be committed to a single morphologically distinct adult caste during early development, which is largely Hymenoptera-focused and therefore does not fully take into account hemimetabolous modes of development (Boomsma & Gawne, 2018; Szathmáry & Maynard-Smith, 1995). It is only when the complexity arising from hemimetabolous systems is incorporated into such theories that we can truly understand the processes underpinning the evolution of higher levels of individuality as superorganisms. A key stumbling block in achieving this, however, is the ability to translate descriptors of phenotypic complexity across social taxa.

Here, we provide a new conceptual framework for classifying phenotypic diversity in termites. We explain the challenges that termites bring to the current terminology used to describe developmental plasticity. We apply terminology from cell biology literature to better categorise the complexities of termite phenotypes and discuss how these many stages of potency makes them important for understanding developmental plasticity. We apply these categories to examples from the termite literature to increase the value of these hemimetabolous (eusocial) insects for testing fundamental questions on the evolution and mechanisms of developmental plasticity. In doing so, we also generate a comprehensive, albeit not exhaustive, dataset on the levels of potency found in 73 species of termites from the literature, which allows us to highlight promising groups for future study, using this new framework. Finally, we explore the future research that can be generated from our study which promise to advance our understanding on the proximate and ultimate mechanisms of caste determination in termites, and on the fundamental nature of phenotypic plasticity found across all taxa. We suggest that our framework may also provide a useful conceptual framework for understanding diverse polyphenisms in other taxa such as aphids and gall-forming thrips (Crespi, 1992; Stern & Foster, 1996). Indeed, any rare examples within the holometabolous social insects which don’t conform to the dichotomous categorisation will also benefit from this new more fine-grained framework.

Box 4.1 Glossary

Adult- The final stage after the last ecdysis. Therefore, any committed termite workers are adults, and anything not committed is as a juvenile.

Alate- Winged reproductives that undergo nuptial flights before shedding their wings and forming new colonies.

Apterous- wingless

Apterous neotenics- (Ergatoids) Neotenic reproductive that develops from a worker in higher termites or from false workers in lower termites (See Neotenic reproductive).

Brachypterous neotenics- (Nymphoids) Neotenic reproductive that develop from nymphal instars in both lower and higher termites (see Neotenic reproductive). They have small, non-functional, wings.

Brachypterous- reduced wings

False workers- individuals that have not diverged from the winged line and can therefore still become Nymphs, but that help the colony to a greater or lesser extent

Foraging termites- (Formally referred to as separate-piece or multiple piece nesters) Species which live in well-defined nest where workers, at some point in the colony-cycle, will leave the nest to forage. This means the colony longevity is not limited to the availability of food. All foraging termites have true workers which can be thought to have reduced potential. These are found in the Mastotermitidae, Hodotermitidae, most Rhinotermititdae and all Termitidae.

Hemimetabolous- (*Sensu lato*) species which undergo incomplete metamorphosis due to no pupal stage. These species progress through a series of morphologically distinct stages separated by a moult, gradually acquiring adult characteristics. Under this definition, any species with hemimetabolous (S*ensu stricto*) and paurometabolous development are included.

Holometabolous- species which undergo complete metamorphosis. This involves four stages: egg, larva, pupa and adult.

Larvae- instars which do not have externally visible wing buds and are dependent on others for survival and do not take part in work within the colony.

Moult, Progressive- The gradual development from egg via several instars into an adult. This type of moult involves an increase in body size and morphological development. This is the default developmental program in all hemimetabolous and holometabolous insects.

Moult, Regressive- This type of moult involves a decrease in body size and/or regression of morphological development, generally with a reduction in wing bud size in nymphal instars.

Moult, Stationary- an intermittent moult which involves no increase in body size and morphological development. This has been exhibited in several insect species and is associated with periods of food shortage, when a larva or nymph is not capable of passing a critical mass threshold in an instar. In some termites it might be associated with replacing worn mandibles.

Neotenic reproductive- reproductives with either no wings or small non-functional wings, that develop within a colony from any instar after L3. Gonads grow and they develop some imaginal characters while maintaining an otherwise larval appearance; some characters, like wing pads, may regress but usually lack compound eyes and have a less sclerotized cuticle.

Nymph- Instars with externally visible wing buds.

Presoldier- A single transitional instar during development from previous phenotype to soldier.

Pseudergates- Nymphs which have regressively moulted back into a worker-like form without wing buds.

Soldier- Sterile altruistic caste which are generally morphologically and behaviourally specialised for defence.

Termite, Higher- Made up of only termite species within the family Termitidae, which only have non-flagellate gut symbionts.

Termite, Lower- All termites other than the Termitidae. They have both flagellates and non-flagellate gut symbionts.

True workers- Individuals that have diverged and are part of a separate wingless line.

Winged line- (nymphal/pterous) developmental pathway where winged phenotypes develop.

Wingless line- (apterous) developmental pathway where wingless phenotypes develop.

Wood-dwelling termites- (Formally one-piece nesters) species where a colony will live in a single piece of wood which serves as both food and nest source. Only the winged sexuals leave the nest and when their only food source is exploited the colony will die. Species within this life type are thought to have highly flexible development and false workers. These are found within the Termposidae, Kalotermitidae and some species within the Rhinotermitidae.

Worker- individuals which are now independent and able to help the colony but still do not have externally visible wing buds.

4.3 The Challenges of Classifying Developmental Plasticity in Termites

Originating at least 150 million years ago (Bucek et al., 2019; Evangelista et al., 2019; Thorne et al., 2000), termites are social cockroaches (order Blattodea), sister to the sub-social cockroach genus, *Cryptocercus* (Bourguignon et al., 2014; Bucek et al., 2019; Evangelista et al., 2019; Inward et al., 2007; Lo et al., 2000). A traditional classification of termites is based on symbionts: "lower termites" have bacteria and flagellates in their guts (Figure 4.1C). This is true of all termite families except the Termitidae ("higher termites"), which have no flagellates but other gut symbionts, mostly bacteria (Figure 4.1C). Termites are key cellulose decomposers in hotter ecosystems (Takamura, 2001), can feed on many substrates (grass, wood, leaf litter or soil) (Eggleton, 2011) and have important roles in enhancing ecosystem resistance to drought (Ashton et al., 2019). The value of their ecosystem services (Jouquet et al., 2011) and the disservices they can cause to buildings and crops (Rouland-Lefèvre, 2011; Su & Scheffrahn, 2000), makes them important species to study (Govorushko, 2019). As a result, there is a considerable body of literature on the natural history of termites, providing a foundation of information on phenotypic potency and plasticity. In this section we review the different facets of termite life-history that provide the means to classify phenotypes based on their level of plasticity, illustrating the difficulties of classifying potency from current literature and highlighting the need for new terminology to explain variation in potency.

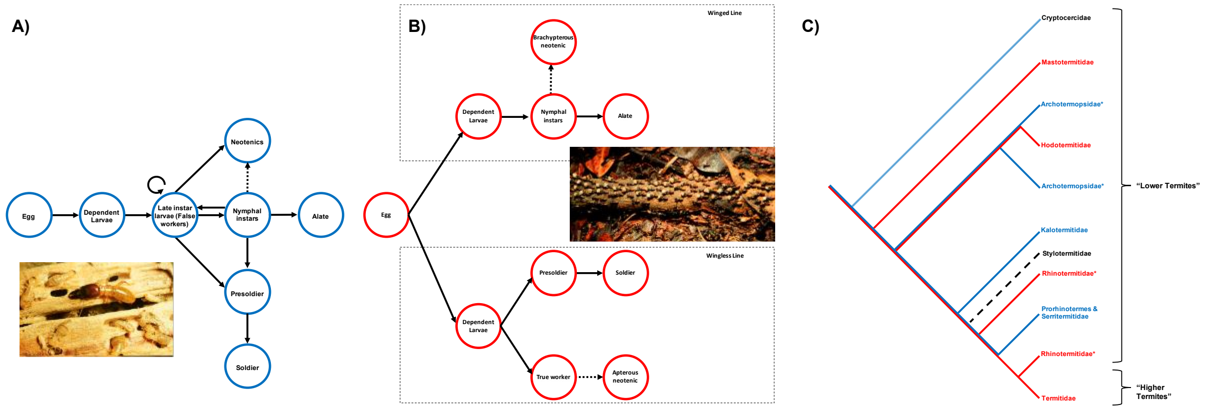


Figure 4.1: Simplified developmental pathway of A) wood-dwelling termites and B) foraging termites. Solid arrows, progressive moults; dashed arrows, potential regressive moults in some species; circular arrow, stationary moult. Modified after (Korb & Hartfelder, 2008). C) Phylogenetic relationships of termite families (cladogram) using the sister taxon Cryptoceridae as outgroup, highlighting the phylogenetic uncertainty of life types and ambiguity surrounding which came first in evolutionary terms. Blue, Wood-dwelling termite species; Red, Foraging termite species; Black dashed, not enough is known about this family to definitively categorise but they are likely to be wood-dwelling species, * signifies paraphyletic families. Modified after (Korb, 2019), (See Box 4.1 for definitions). (photos by Bill Sands, NHM London collection: A) Zootermopsis B) Hospitalitermes.

4.3.1 Developmental stage and phenotypic potency

All species of termites exhibit a range of phenotypic potency which is shared across species and largely based on their stage in development (Bourguignon, Šobotník, et al., 2012; Haifig & Costa-Leonardo, 2016; Miura et al., 2000). Phenotypic states exhibit plasticity along a gradient. First, high plasticity, where an individual has the potential to become (almost) any other phenotype, under environmental cues; e.g. first and second instar larvae (Box 4.1). Second, lower levels of plasticity, where an individual may have limited ability to change into a small number of phenotypes; e.g. nymphs and later instar larvae. Finally, no plasticity, where an individual is committed to a phenotypic state and cannot change, irrespective of the environmental cue; e.g. primary reproductives, neotenic reproductives and soldiers (Box 4.1). However, there are important exceptions. Both workers and nymphs (Box 4.1) show variation in their plasticity both within and between species (Bourguignon, Šobotník, et al., 2012; Lainé & Wright, 2003; Noirot, 1955; Roisin, 2000). Moreover, both egg and larval potency can vary across species and often between sexes (Roisin & Lenz, 1999).

In some species, phenotype is determined by sex and this can influence phenotypic potency. This consequently makes categorising potency based on developmental stage purely more difficult. For instance, high sexual size dimorphism has led to the loss of soldier production in one sex in a number of species as well as ancestral loss in one sex in the higher termites which have led to the majority presence of single sex soldiers (Bourguignon, Hayashi, et al., 2012). In *Coptotermes lacteus,* only females can become soldiers, and so male egg and larval instars cannot become soldiers (Roisin & Lenz, 1999). Therefore, a sex within a species may have a lower level of phenotypic potency at all developmental stages than the complementary sex. However, more experimental evidence is required to determine whether the constraints on potency of a single sex is genetically or epigenetically determined, and if epigenetically determined, at which developmental stage soldier production is inhibited. Consequently, defining potency of termite phenotypes based on their developmental stage should not be used as the only criterion.

4.3.2 Influence of life-history on phenotypic potency

Two main life-history types are found within termites, classified by the relationship between foraging and nest site (Abe, 1987; Korb, 2019)*;* these life-history types are associated with fundamental differences in developmental plasticity. **Wood-dwelling** (*Box 4.1*) species nest and feed in the same piece of dead wood (Korb, 2019) *(Figure 4.1A, Box 4.1).* Species within this life type are found throughout lower termite families (Bourguignon et al., 2014; Korb & Hartfelder, 2008) (Figure 4.1C). Wood-dwelling termites have high levels of developmental plasticity, with progressive, regressive and stationary moults (Box 4.1) allowing reversible morphological change into any phenotype, in response to ecological and social cues (Figure 4.1A). The immatures (larvae) exhibit highly plastic phenotypes and are a stage from which all other more committed phenotypes, for example soldiers, nymphs, primary reproductives and neotenic reproductive, develop. Because of this high plasticity, and lack of commitment, working individuals of the wood-dwelling termites are often referred to as ‘false workers’ (See (d), Box 4.1).

The second type of life-history is the **foraging** termites (Abe, 1987), which have well defined nests, but foraging takes place away from these nests (Korb, 2019) (Figure 4.1B). Families with this life-history include Mastotermitidae, Hodotermitidae, most Rhinotermitidae and all Termitidae (Korb & Hartfelder, 2008) *(Figure 4.1C).* Foraging termites have lower levels of developmental plasticity than wood-dwelling species. Seasonal cues dictate whether early instar larvae moult into winged or wingless lines (Figure 4.1B, Box 4.1). Therefore, there is an irreversible early commitment into either the winged line, where winged sexuals develop from nymphal instars, or the wingless line, which leads to 'true' workers and soldiers. Consequently, individuals within the wingless line are unable to become primary winged reproductives (Figure 4.1B).

However, the seeming dichotomy in developmental potential between wood-dwelling to foraging termites and 'false' to 'true' workers is an underestimation of the variation in plasticity between species. For instance, there are some species with 'true' workers that are not in fact committed to sterility and are able to moult into wingless reproductives under particular conditions, some species with 'true' workers which can only become soldiers, and some species with 'true' workers which are at a final state. Therefore, they exhibit differing levels of potential and should not be defined purely as 'true' workers when discussing phenotypic potency. Instead of a purely "totipotent versus committed" view of plasticity, we envisage a multi-level change in plasticity of certain phenotypes between and within species. We need to find an alternative descriptive framework that accounts for this variation in order to facilitate a better understanding of the complexity in plasticity across the termites.

4.4 Employing Cell Biology Terms as Descriptors of Hemimetabolous Potency

There is currently no terminology sufficient to capture the multiple levels of plasticity, and the nuances among different categories of 'committed' and 'plastic' states within hemimetabolous insects. We suggest, therefore, borrowing terms from cell biology. Comparisons have already been made with the mechanistic basis for ageing in termites and germline cells (Elsner et al., 2018), therefore extending this comparison to potency may help us to better comprehend termite plasticity and consequently hemimetabolous insects. Both hemimetabolous insects and stem cells have potential to change into a range of phenotypes across many periods of development, as they are not restricted by a developmental barrier, as with metamorphosis in holometabolous insects. This is exemplified in the polymorphic social aphids, gall-forming thrips, and particularly termites (Crespi, 1992; Stern & Foster, 1996). By having a unifying framework applicable to all hemimetabolous insects, comparisons can be made across all the levels of social complexity; from the highly complex termites, to the subsocial Cryptocercidae and even to solitary hemimetabolous insects like locusts which exhibit high individual plasticity (Lo et al., 2018; Nalepa, 2010). Here, we outline the different stem cell potency terms, specific to mammals, and explore how they can be used to describe termites' and more generally hemimetabolous insect phenotypic diversity.

Stem cells are clone-producing cells, capable of both self-renewal and multilineage differentiation (Metcalf & Moore, 1971; Till & McCulloch, 1961). There is variation in the capacity of different types of stem cells to differentiate into certain mature cell lines (Weissman, 2000), with a terminology used to describe this variation fully (Figure 4.2). First, **Totipotent** describes a cell that can turn into any type of cell: within a developing multicellular organism, this is the zygote, and it is the first cell to form after fertilisation (Figure 4.2). **Pluripotent** is used to describe cells that retain the ability to renew indefinitely and to differentiate into cells of all three germ layers (mesoderm, endoderm, ectoderm): these are embryonic stem cells produced from the inner cell mass of mammalian blastocysts (Figure 4.2). Pluripotent stem cells differ from totipotent cells in that they cannot become extraembryonic cells (Evans & Kaufman, 1981; Martin, 1981): they have lost one facet of plasticity.

The next level of potency is **multipotent** cells, which are able to differentiate into multiple organ and tissue-specific lineages (Figure 4.2). These are organ- and tissue-specific stem cells which are present eight days post-fertilisation and throughout adulthood within multicellular organisms; they include haematopoietic stem cells (Weissman, 2000), and bone marrow cells which can self-renew and differentiate into all blood cell lineages (Becker et al., 1963; Till & McCulloch, 1961; Wu et al., 1968). Multipotent cells transition through multiple stages of irreversible maturation, before becoming progenitor cells which cannot renew themselves. This further loss of potency is described as **oligopotent** (Figure 4.2) (Weissman et al., 2001). Oligopotent cells are common myeloid progenitors or common lymphocyte progenitors (Akashi et al., 2000). They are committed to a single lineage of a small number of mature cell fates (Figure 4.2). Of these, only **multipotent** is needed to describe potency levels within hemimetabolous insects.

A final level of potency in cells is termed **unipotent**: these are cells that are only able to differentiate into one fully differentiated cell type; this term is limited in use to spermatogonial stem cells, which differentiate into sperm cells (Figure 4.2) (de Rooij, 2001). The **committed** state is a fully differentiated cell which is are no longer able to change into any other form; it can be considered a mature cell and it is committed to this final state (Figure 4.2). For hemimetabolous insects, it may be more appropriate and useful to use “committed” as it better describes their development than the more cell-centric “fully differentiated” used within stem cell research.

High potency

Potency Classification	Cell Description	Examples	Termite Description
Totipotent	The ability to differentiate into all possible cell types	Zygote formed at egg fertilization and the first few cells that result from the division of the zygote	The ability to moult into all alternative phenotypes
Pluripotent	The ability to differentiate into all cell types apart from extraembryonic cells.	Embryonic stem cells and cells that are derived from the mesoderm, endoderm, ectoderm germ layers that are formed in the beginning stages of embryonic stem cell differentiation	The ability to moult into both reproductive and non-reproductive phenotypes but cannot moult into at least one phenotype
Multipotent	The ability to differentiate into a closely related family of cells	Hematopoietic (adult) stem cells that can become red and white blood cells or platelets	The ability to moult into either reproductive or non-reproductive phenotypes
Oligopotent (progenitor)	The ability to differentiate into a small number of closely related subfamily of cells	(adult) Lymphoid or myeloid progenitor cells	
Unipotent	The ability to only produce cells of their own type	(adult) Spermatagonial stem cells	The ability to moult or morphologically and physiologically change into one form which is of very close relation to their current form
Fully Differentiated/ Committed	Incapable of changing into any other cell type or renewing	Skin cells	Incapable of moulting into any other phenotype

Low potency

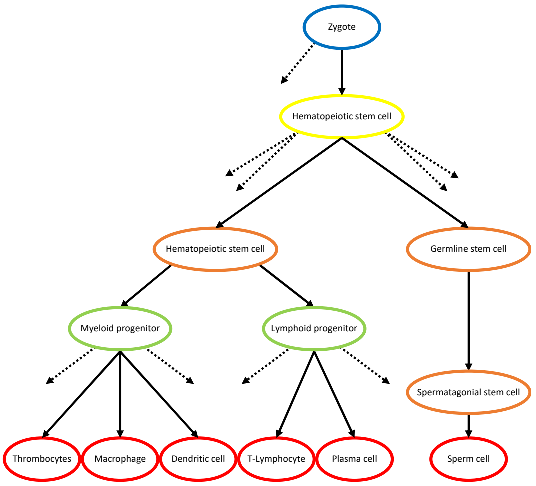


Figure 4.2: Table explaining the terminology used to describe levels of potency within cells with examples, adapted from (Kalra & Tomar, 2014), and simplified cellular developmental pathway showing the different levels of developmental plasticity. Everything but the zygote and embryonic stem cells are adult cells. Full arrows, pathway from one cell type to another; dashed arrows, presence of other pathways not shown. Blue, Totipotent; Yellow, Pluripotent; Green, multipotent; Light Green, Oligopotent (progenitor); Orange, Unipotent; Red, fully differentiated.

4.5 Cell Potency Terminology Applied to Termites

Here, we illustrate how cell potency terminology can be aligned with levels of developmental plasticity in hemimetabolous insects, particularly termites. To do this, literature data was collated on the plastic potential of different phenotypes within and between termite species, across developmental stages. The developmental pathways of 73 species were identified using a Web of Science search for each family name and development stage, refining the search by using lower taxonomic units where necessary. Confidence in assigning a particular level of plasticity for a given phenotype depends on the evidence type: observational studies, using morphometric and moulting data, are less conclusive than experimental studies, where potency has been experimentally instigated, e.g. by colony orphaning. Social and environmental pressures can limit plasticity among termite phenotypes leaving them functionally less plastic; furthermore, the stage in the colony cycle may influence the expression of plasticity (Chouvenc & Su, 2014). Many of our classifications, therefore, will require confirmatory manipulation experiments and more data collection. Despite this, the study shows how aligning potency terminology from the stem cell literature with potency levels in termites provides a useful framework for studying the evolution and mechanisms of plasticity in termites and hemimetabolous insects generally. We now describe each level of potency, explaining their trends across the phylogeny and developmental stages, provide examples of differing levels of plasticity within and between species and then highlight exceptions to these trends that exemplify the need for a framework that transcends above the traditional metrics of plasticity categorisation (which are discussed in section 4.3).

	Developmental Stage								
Potency classification	**Egg**	**Larvae**	**Worker**	**Soldier**	**Nymph**	**Alate**	**Primary Reproductive**	**Apterous Reproductive**	**Brachypterous Reproductive**
Totipotent	Can change into anything	Can change into anything	Can change into anything	N/A	Can regress into workers, change into soldiers, brachypterous neotenics and alates	N/A	N/A	N/A	N/A
Pluripotent	Can change into anything except a soldier/worker/Neotenic. Or can only be Worker or PR	Can change into anything except a soldier/worker/Neotenic. Or can only be Worker or PR	Can change into soldiers and apterous neotenics/ W and AN but not soldiers	N/A	Can change into soldiers/ workers, alates and brachytperous neotenic. Or worker/soldier and alate	N/A	N/A	N/A	N/A
Multipotent	Can only be primary or brachypterous reproductives	Can change into workers which can become soldiers or other worker moults/ into nymphs which can become BN or PR	Can change into other worker moults and soldiers	N/A	Can change into alates or brachypterous neotenics	N/A	N/A	N/A	N/A
Unipotent	Can only become primary reproductives	Can change into workers or just presoldiers which become the terminal state/into nymphs which can become PR	Can change into other worker moults	Presoldiers	Can only change into alates	Can change into primary reproductives /adultoids	N/A	Preapterous reproductives	N/A
Committed	N/A	N/A	Terminal state	Terminal state	N/A	N/A	Terminal state	Terminal state	Terminal state

Table 4.1: Applying cell potency terminology to the different developmental stages of termites; note that a single developmental stage may exhibit different levels of potency, depending on the species; furthermore, a single level of potency can be exhibited by a range of developmental stages, across species. N/A is denoted when there are no instances of the particular phenotype exhibiting the potency level.

4.5.1 Totipotent

In termites, individuals can be thought of as totipotent when they are able to moult into all alternative phenotypes: winged or wingless reproductives, workers or soldiers (Table 4.1, Box 4.1). Totipotent individuals include all workers (See Figure 4.3A – blue) and dependent larval stages in the Termopsidae, Kalotermitidae, Serritermitidae and some Rhinotermitidae (e.g. *Prorhinotermes inopinatus, Termitogeton planus, Psammotermes hybostoma*). It also includes nymphal stages when they are able to regressively moult back into workers; totipotent nymphs are restricted to the lower termites (Box 4.1) – the Termopsidae, Kalotermitidae and a number of Rhinotermitidae.

Eggs, and first and second instar larvae in most species of lower termite are totipotent; exceptions are females in *Glossotermes occulatus* (Bourguignon et al., 2009), *Serritermes serrifer* (Barbosa & Constantino, 2017) and *Hodotermes mossambicus* (Roisin, 2000) and males in *Coptotermes lacteus* (Roisin & Lenz, 1999), which cannot become soldiers and females in *Anacanthotermes ahngerianus* (Roisin, 2000) which cannot become soldiers or workers. These sex specific developmental trajectories are more prominent in the higher termites. Consequently, most higher termites have either only one sex which have totipotent eggs and early instar larvae or none at all, due to one sex not being able to produce soldiers or neither being able to produce neotenics, respectively. For instance, in *Acanthotermes acanthothorax* (Noirot, 1955), males are unable to become soldiers and therefore eggs and the first larval instar of males already have reduced developmental potential.

4.5.2 Pluripotent

Pluripotency is a small reduction in developmental potential in comparison with totipotency, therefore they still have very high plasticity. An example is the pluripotent workers (See Figure 4.3B) which have diverged from the winged line and so cannot become winged reproductives. However, they still have the ability to become wingless reproductives and take over a nest when the primary queen or king dies. Therefore, their plasticity is not so different from totipotent workers as they are still able to span both sterile and reproductive phenotypes. Most commonly, therefore, pluripotency is signified by the removal of the ability to become one particular phenotype (Table 4.1). This would apply also to nymphal instars which cannot become soldiers but can regressively moult to become a worker, therefore are able to switch to a sterile fate (Table 4.1). A number of species which do not produce neotenics will therefore have pluripotent eggs and L1, such as the egg and L1 stages in *Termes baculi* (Noirot, 1955). Furthermore, in some species one sex cannot become particular phenotypes (e.g. soldiers); therefore, they have pluripotent eggs and L1; e.g. the males of *Silvestritermes euamignathus* (Haifig & Costa-Leonardo, 2016)(Figure 4.3B). A less common form of pluripotency is when an individual has lost the ability to express two of the possible phenotypes, but the phenotypes that still can be expressed span both sterility and reproduction. An example of this can be seen in male workers of *Silvestritermes euamignathus* (Haifig & Costa-Leonardo, 2016) (Figure 4.3B). Here, they can only become sterile workers or apterous neotenics.

Examples of pluripotent workers are known throughout both lower and higher termites, including Mastotermitidae, Rhinotermitidae and a number of species within the Termitidae. Pluripotent nymphal instars have only been shown within both *Reticulitermes lucifugus* and *Reticulitermes flavipes* (Lainé & Wright, 2003). Their nymphal instars cannot moult into soldiers but can become every other phenotype.

4.5.3 Multipotent

Multipotent describes the loss of plasticity with respect to a specific developmental pathway - i.e. wingless (non-reproductive) or winged (reproductive) of the foraging termites (Figure 4.1B). Individuals still show plasticity and can moult into another form *within* the pathway, but they are committed to either a reproductive or non-reproductive pathway. For example, nymphs of foraging termites are multipotent as they can moult into winged alates, which are committed to dispersing, or brachypterous neotenics, which are committed to non-dispersal; but they are distinct from pluripotent or totipotent nymphs as they cannot regress back into workers or soldiers: they are committed to a reproductive fate (Table 4.1). Workers, and the larvae that produce these workers, are multipotent when they can only moult into soldiers or other worker forms and are therefore committed to a non-reproductive fate (Table 4.1).

Multipotent phenotypes are mainly found within the foraging termites. However, there may be more species-specific complexities than initially apparent. For example, *Psammotermes hybostoma* potentially has both totipotent and multipotent workers, with earlier worker instars potentially becoming apterous neotenics, nymphs or soldiers and the later instars only able to become soldiers (Bourguignon, Šobotník, et al., 2012) *(See Figure 4.3C).* Further experimental work is required to determine the potency of these and many other species (Table 4.1).

Female eggs and early instar larvae in the foraging termite, *Anacanthotermes ahngerianus,* can be considered multipotent because they are only able to become either primary reproductives or brachypterous neotenics (Roisin, 2000). Therefore, females in this species exhibit severely reduced developmental potential compared with males as they are totipotent at the egg and early larval instar stages. When soldier production is sex specific, it is possible that one sex will have multipotent workers and the other sex will have unipotent and committed workers, as seen in multipotent female workers and committed male workers in *Macrotermes natalensis* (Noirot, 1955).

4.5.4 Unipotent

Individuals are unipotent when they are only able to change into one form that is similar to their current form. For example, presoldiers, alates and pre-apterous reproductives in all species can only become soldiers, primary reproductives and apterous reproductives respectively. They are limited to a phenotype within their own specific lineage and so are unipotent. Unipotent workers are the instars preceding the final committed worker form: they cannot moult to become soldiers (Table 4.1). For instance, large worker instars L2-4 in *Microcerotermes beesoni* are unipotent as they are only able to moult into other worker forms (See Figure 4.3D) (Rasib & Akhtar, 2012). Similarly, unipotent nymphs are those which can only moult into alates and therefore are only able to become winged reproductives (Table 4.1, Figure 4.3D).

Unipotent workers are mainly found in higher termites, such as *Amitermes* and *Microcerotermes* species, and a number of the Nasutitermitinae which have a particular sex which cannot become a soldier. Only *Coptotermes lacteus* (Roisin & Lenz, 1999)*,* *Coptotermes formosanus* (Chouvenc & Su, 2014) and *Hodotermes mossambicus* (Roisin, 2000) in the lower termites have been identified as having unipotent workers. Unipotent nymphs can be seen in some lower termites, which seemingly lack the ability to produce brachypterous neotenics, but are most prevalent in the higher termites. One instance of unipotent eggs and L1 has been shown in *Cornitermes walkeri* as females only become primary reproductives (Roisin, 1992) (Table 4.1).

4.5.5 Committed

Individuals are "committed" when they are incapable of moulting into any other form: this includes soldiers, primary reproductives, apterous neotenics and brachypterous neotenics. Species where workers are the final form and cannot moult into soldiers are also committed (Table 4.1); these are observed only in a small number of species within the higher termites such as the Macrotermitinae, and in some cases only one sex has committed workers whilst the other sex produces multipotent workers able to become soldiers. The Apicotermitinae are predominantly soldierless species, and all appear to have committed workers. However, they are poorly studied, due to the difficulty in identification without the soldier caste (Bourguignon et al., 2016) (See Figure 4.3D).

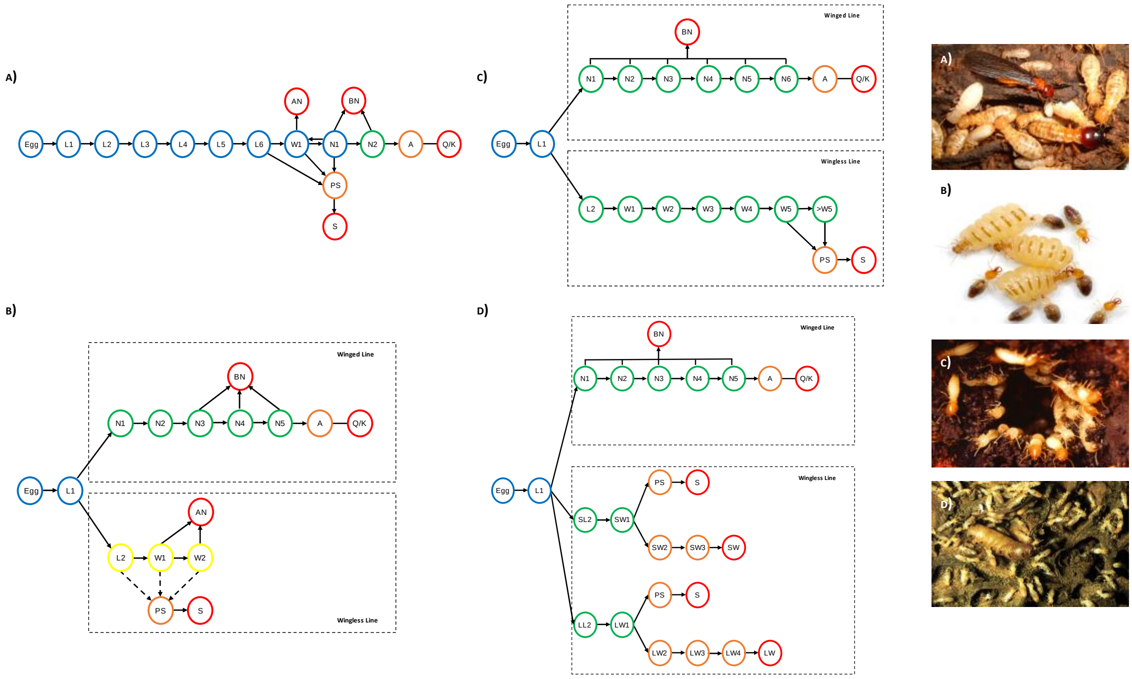


Figure 4.3: Developmental pathways of A) the wood-dwelling termite Hodotermopsis sjostedti, Modified after (32); B) Females in the foraging termite Silvestritermes euamignathus (both solid and dashed arrows) and males in the foraging termite Silvestritermes euamignathus (only solid arrows), Modified after (34); C) females in the foraging termite Coptotermes lacteus, Modified after (38); D) the foraging termite Microcerotermes beesoni, Modified after (57). Arrows symbolise moults. Forward arrows, progressive moults; Backward arrows, Regressive moults; dashed lines; only one sex can moult into this phenotype. Lines symbolise morphological and physiological change without moult. L1, Larval instar 1; W1, worker instar 1; N1, nymphal instar 1; AN, Apterous Neotenic; BN, Brachypterous Neotenic; A, alate; PS, presoldier; S, soldier; Q/K, queen/king. Blue, Totipotent; Yellow, Pluripotent; Green, Multipotent; Orange, Unipotent; Red, Committed. (photos: A) Hodotermopsis sjostedti- https://polyphenism.wordpress.com/2006/04/01/japanese-damp-wood-termite/hodotermopsis-sjostedti/, B) Silvestritermes minutus- Robert Hanus, C) Coptotermes formosanus, D) Microcerotermes- Bill Sands, NHM London collection)

4.6 Future Directions

Termites show enormous phenotypic diversity due to developmental plasticity. They therefore make excellent study systems for fundamental questions surrounding the nature, mechanisms and evolution of phenotypic plasticity, which are applicable to all taxa *(sections 4.6.1, 4.6.3)*. These questions in turn help shine some light on longstanding questions surrounding termite evolution *(sections 4.6.2, 4.6.4)*. The conceptual framework we propose is made possible through an interdisciplinary translation of terminology from the cellular biology world into the world of termite phenotypic plasticity. We anticipate that the formalisation of how to classify individual-level potency in termites will facilitate further research on the proximate and ultimate bases of termite caste evolution, and ultimately the nature of phenotypic plasticity. We unpack a few examples here, to illustrate the utility of our framework and to demonstrate the research potential from identifying the parallels between cellular and termite developmental plasticity.

4.6.1 Are there conserved mechanisms that characterise changes in potency of phenotypes across different organisms?

Our new framework opens opportunities to examine the mechanistic basis for loss and gains in plasticity at a finer scale and allows for comparisons with other taxa, such as Hymenoptera. A phylogenetically directed comparative analysis across termite species would reveal where and when losses and gains in the different forms of plasticity occurred and afford the opportunity to detect patterns of correlated environmental and ecological traits that may explain their evolution. Much work has been done relating the ecological context of wood-dwelling and foraging termites which leads them to have totipotent and more developmentally restricted workers respectively (Korb, 2019; Korb & Thorne, 2017). Potentially multiple losses and gains of totipotent workers have occurred throughout the phylogeny (Legendre et al., 2013), providing us with a good study system to elucidate the mechanistic basis for these losses and gains. The classifications in our framework provide the categorical variables required for such comparative analyses to be performed. Moreover, the lowering in potency for one sex, from multipotent to unipotent or committed, due to their inability to become soldiers has been observed in a number of species. These differences have been attributed to high sexual size dimorphism leading to the favouring of the sex which has the appropriate size for defence of the nest (Bourguignon, Hayashi, et al., 2012). It has however been shown that in some species which have low to no sexual size dimorphism, both sex soldiers have evolved again. Therefore, gains in potency through evolutionary time can occur (Bourguignon, Hayashi, et al., 2012). This provides another avenue within termites to explore the fundamental mechanistic basis for losses and gains in plasticity. Furthermore, by comparing these findings to those from Hymenopteran and cellular studies, we may be able to find common molecular processes which have been conserved relating to losses and gains in plasticity.

4.6.2 What came first, totipotent or the more developmentally committed workers?

By understanding the proximate mechanisms behind the changes in developmental plasticity which arise because of varying ecological pressures, we will be able to elucidate questions relating specifically to termite evolution. For instance, there has been over 30 years of debate over whether the ancestral state of termites was a linear developmental pathway with totipotent workers or a bifurcated pathway with more developmentally restricted workers, with no conclusive evidence to provide an answer (Legendre et al., 2013; Noirot, 1985; Watson & Sewell, 1985) (Figure 4.1C). We see from Legendre's (2013) phylogenetic analyses that there have been potentially multiple emergences of more developmentally restricted worker caste formations. Interestingly, there has also potentially been the loss of more developmentally restricted worker castes and the emergence of more plastic totipotent worker species in the phylogeny. It may be that these are examples of rapid evolution under strong ecological pressure, whereby once foraging is required in a species, a more developmentally restricted worker will follow. However, the binary 'totipotent or not' categorisation has inevitably hindered our ability to elucidate the ancestral developmental state due to its coarse categorisation. Instead, by using our new framework we will be more informed on the transitions we see across the phylogeny, be it totipotent to pluripotent or totipotent to multipotent. The new framework will provide greater context to what these transitions between linear and bifurcated pathways entail and therefore break apart the erroneous notions that all the transitions seen on the phylogeny are the same. By bringing new light to the debate, we will be better equipped to tackle this fundamental evolutionary question in termite biology.

4.6.3 What mechanisms underpin different types of plasticity, and are these mechanisms the same across independent evolutionary lineages?

As we have shown, termites have high variation in potential developmental trajectories, analogous to the developmental potential in stem cells. Our framework benefits from the extensive cellular literature, which defines this variation in developmental potential. Clearly defined categories of 'potency' allow us to select precise termite phenotypes for studying the underlying molecular mechanisms. There is already a body of research on the molecular basis of termite plasticity, predominantly based on the lower termites (Korb, 2015; Korb &

Hartfelder, 2008; Zhou et al., 2006), from which a suite of molecular signatures can be identified, highlighting the importance of juvenile hormone and its associated gene pathways (e.g. the hexamerin genes) in caste regulation (Korb, 2015; Zhou et al., 2006). However, research is beginning to look at the higher termites, highlighting the importance of caste-biased gene expression, such as for vitellogenin genes, which have been repeatedly co-opted for diverse functions across different castes in termites (Sun et al., 2019; Weil et al., 2007). The new framework allows the exploration of commonalities within potency level across phenotypes and species of termites and even across other hemimetabolous insects like aphids, gall-forming thrips and locusts (Crespi, 1992; Lo et al., 2018; Stern & Foster, 1996). For instance, the soldier form is shared across the termites and some aphids and gall-forming thrips (Tian & Zhou, 2014). In the parthenogenetically reproductive social aphid, *Tuberaphis styraci*, soldiers are determined in late first instar female nymphs, meaning before this the female nymphs are pluripotent, able to become either a primary reproductive or soldier (Kutsukake et al., 2004; Shibao et al., 2010). After the late first instar stage, individuals moult into either a soldier which is committed or a second instar nymph which is unipotent, before moulting into a primary reproductive which is committed. Males in this species can only become primary reproductives so are unipotent until they moult into adults and are then committed. It will likely also prove useful for comparing across other taxa such as Hymenoptera, due to there being, albeit more restricted, variation between individuals and between species in potency level pre-metamorphosis. For example, it may be that some exceptional hymenopteran species, like *Pogonomyrmex barbatus* which have genetic caste determination and worker polypmorphism, lose their ability to become reproductives but retain their ability to become one of a number worker phenotypes and therefore could be seen as multipotent at a certain stage of development (Cahan et al., 2002; Cahan & Keller, 2003; Julian et al., 2002; Volny & Gordon, 2002). Also, since every male in Hymenopteran species are genetically determined to be reproductives only, the pre-adult stages can be seen as unipotent transitioning to committed. Moreover, a fundamental further question is whether the molecular signatures that dictate termite potency are the same as those molecular signatures that dictate cell potency. The mechanisms may be conserved across independent evolutionary events or occur via novel mechanisms. To date, there has been very little research drawing comparisons between termite biology and cellular biology (Elsner et al., 2018). However, this new framework opens a great breadth of potential interdisciplinary research due to identifying the amazing parallels between cellular and termite developmental plasticity. Only by creating this framework can we now go on to study further whether this is more than merely analogy.

4.6.4 What constitutes a termite superorganism?

Termite colonies can be considered to have organism-like characteristics (Elsner et al., 2018). Our study has identified major conceptual parallels in development between cellular and termite colony development, therefore, further work potentially identifying more homologous mechanisms shared between these will support this organism-like quality (where units making up a greater whole are interdependent and have aligned interests) of a termite colony. This inevitably has connections with superorganismality, leading us to ask what characteristics a termite superorganism would have (Boomsma & Gawne, 2018). The question of commitment is important to any theory of phenotypic plasticity and also for identifying candidates for superorganismality (Boomsma & Gawne, 2018). The mainly binary development within Hymenoptera makes it easier to establish likely candidates for superorganismality. Research has clarified caste potentials and therefore their relative commitment to colony roles. The true and false worker categorisation in termites is a useful classification that do show contrasts in social complexity probably due to changes in ecology. However, they do not encapsulate the most important trait of workers which are needed to be classed as superorganismal: that they are sterile and are committed to their worker fate (Boomsma & Gawne, 2018). Although species with true workers have irreversibly diverged from the winged line (meaning these workers cannot moult into winged reproductives ever), some have workers which are still able to go on to become apterous neotenic replacement reproductives, and therefore have not completely committed (Haifig & Costa-Leonardo, 2016; Moura et al., 2011; Noirot, 1955, 1969; Roisin & Pasteels, 1987; Thorne & Noirot, 1982; Vieau, 1991, 1994; Watson & Abbey, 1977). Consequently, species with these workers possibly should not be classed as superorganismal as these have not transcended to a higher level of individuality (West et al., 2015). This current categorisation used to define whether a termite has reproductively committed workers, and therefore is superorganismal or not, is insufficient to precisely identify potential candidates for superoganismality. Our new framework allows us to more accurately distinguish between workers which are reproductively committed (multipotent, unipotent, committed) and those which are not (totipotent, pluripotent). Therefore, using the most recent definition of superorganismality (Boomsma & Gawne, 2018), future research surrounding the transition to superorganismality within termites should concentrate on the transition between pluripotent and more developmentally committed workers.

However, it seems that within termites, the prerequisite of reproductively committed worker castes (multipotent, unipotent and committed workers) for superorganismality may leave out highly socially complex species such as in the Nasutitermitinae. Even the less socially complex species with high plasticity have a committed soldier caste and therefore have high inclusive fitness acting upon them. It may be that Hymenoptera-centric definition of superorganismality will need to be revised to allow the full extent of insect sociality to be acknowledged. Furthermore, clearly hemimetabolous insect development show greater parallels with cellular development within an organism so it may be that when revising this definition, termite sociality should be given greater consideration.

4.7 Conclusion

Developmental plasticity drives the vast diversity in form and behaviour seen within and between all taxa. Research on the patterns of phenotypic plasticity have investigated both holometabolous and hemimetabolous insects but there is still a bias towards the holometabolous. This bias is clear in the work on social insects, where holometabolous research dominates our understanding of developmental plasticity and social evolutionary processes (Boomsma & Gawne, 2018; Evans & Wheeler, 2001; Toth & Rehan, 2016; West-Eberhard, 2003). The existing dichotomous view of potency, derived from social Hymenoptera, is insufficient to provide a useful framework for termite developmental plasticity. Moreover, the current categories for explaining potency in termites, be it life history or developmental stage, falls short in encapsulating the nuances of developmental plasticity, both within and between termite species. To address this, we have highlighted parallels in developmental potency of hemimetabolous insects with stem cell plasticity and suggested how we might borrow cell potency terminology to categorise potency among the diverse phenotypes exhibited by termite species. In doing so, a comprehensive dataset has been created on the known potencies of phenotypes across the developmental pathways of 73 species of termites which represent the diversity in life histories and evolutionary relationships. We anticipate that in proposing this new terminology and framework, that termites may become more accessible as a resource for advancing our understanding of mechanisms and evolution of developmental plasticity across all taxa. Future work may discover more direct mechanistic parallels relating to plasticity in cellular and termite development systems.

Chapter Five:

The Diversity of Social Complexity in Termites

5.1 Abstract

Sociality underpins major evolutionary transitions and provides the scaffold from which complex ecosystems are built. Social insects, seen as the pinnacle of this, have traits like obligate sterility that are considered ‘master traits’, used as single phenotypic measures of this complexity. However, evidence is mounting that assuming complete alignment of both phenotypic and evolutionary social complexity, and having obligate sterility central to both, is erroneous. We hypothesise that obligate and even functional sterility will be insufficient in explaining the diversity of phenotypic social complexity and will not have as strong an association with other social complexity traits as expected. To test this, we explore the relative importance of these sterility traits in an understudied but diverse taxon: the termites. We compile the largest termite social complexity dataset to date, using specimen and literature data. We find that although functional and obligate sterility explain a significant proportion of variance, neither trait are adequate singular proxies for the phenotypic social complexity of termites. Further, we show both traits have only a weak association with the other social complexity traits within termites. These findings have fundamental ramifications for our general comprehension of the frameworks of phenotypic and evolutionary social complexity and their relationship with sterility.

5.2 Introduction

Life comes in a bewildering diversity of forms. One of the most striking and pervasive attributes of living organisms is their astonishing array of social interactions. Chromosomes live cooperatively with other chromosomes to form cells, cells with other cells to form multicellular organisms, and individual insects live with other, related, insects to form cohesive colonies (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2011a, 2023; West et al., 2015). The variety and depth of these cooperations can be thought of as social complexity. This complexity forms a framework for both the evolution of forms and the ecology of communities (Boomsma, 2022; Boomsma & Gawne, 2018; Bourke, 2011a, 2023; West et al., 2015).

There are several ways to describe, compare, and understand this variation in social complexity. Here, we focus on two: evolutionary and phenotypic views of social complexity. The evolutionary viewpoint (Box 5.1) asks why social complexity exists at all and investigates the factors determining how individual units cooperate. Under an evolutionary social complexity framework, the diversity of life can be explained by a stepwise progression whereby solitary individuals (e.g. bacteria or mason bees) exist on their own but can form social groups (e.g. slime moulds or *honeybees*). In turn, social groups can come together and form interdependent fitness-maximising individuals (all units working together as a the whole). These shifts from one level of sociality to another are termed major evolutionary transitions (e.g. as seen in red algae or mound building termites) (Bourke, 2011a). Crucially, this stepwise progression can be recursive, creating a nested hierarchy of cooperating units, all working as part of the highest-level individual (Box 5.1)(West et al., 2015). For instance, some social insects are highly integrated societies composed of many individuals, which are themselves made up of cooperating cells, which in turn are collections of cooperating genes. Therefore, evolutionary social complexity at a given level can be seen as the degree to which a group has transitioned to a fitness maximising individual. These transition stages provide us with a valuable tool for understanding the intricacies of social behaviour and how it evolves.

An alternative perspective on social complexity is a phenotypic viewpoint. Phenotypic views of social complexity focus on how social organisms look and function now, rather than on how they have evolved per se. In this sense, phenotypic social complexity can be defined as the extent to which a system is made up of many specialised and interacting parts which come together to contribute to a function (Box 5.1)(Holland & Bloch, 2020). Many of the phenotypic measures of phenotypic social complexity have been derived from the study of social insects. Four readily quantifiable phenotypic measures are colony size, helper polyphenism (i.e. the degree of polymorphism among helper individuals), nest complexity and worker sterility. Colony size quantifies the number of individuals within a colony (Bonner, 2004; Boomsma & Gawne, 2018; Bourke, 1999; Ferguson-Gow et al., 2014; Holbrook et al., 2011; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019; Kramer & Schaible, 2013; La Richelière et al., 2022). The larger the colony of fertile workers, the less likely the individuals are to reproduce and the more likely they are to be selected to specialise within a colony instead. Further, larger colony sizes lead to there being more individuals to interact and perform different jobs and generate complex behaviours and extended phenotypes (Bonner, 2004; Bourke, 1999; Ferguson-Gow et al., 2014; Holbrook et al., 2011; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019; Kramer & Schaible, 2013). Helper polyphenism captures the distinct physical variation observed among the workers and soldiers of a colony. The presence of a diverse array of worker and soldier morphs within a colony enables the formation of specialized groups that can tackle various tasks, thereby enhancing the overall functioning and productivity of the colony (Ferguson-Gow et al., 2014; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019; La Richelière et al., 2022; Sumner et al., 2018). Nest complexity signifies the repertoire of behaviours colonies use to create and manage their nests (colony centres).

Evolutionary and phenotypic views of social complexity are thought to be linked by the concept of sterility due to it being an indicator of high complexity in both views (Boomsma & Gawne, 2018). Within social insects, for instance, it is assumed that there is a positive relationship between worker sterility and all the other phenotypic social complexity traits, such that all social complexity traits can be conflated (Boomsma & Gawne, 2018; Howe et al., 2022; Michod, 1997; Michod et al., 1996; Michod & Roze, 1997). This potential relationship leads to an assumption that worker sterility can act as a proxy measure for social complexity overall. Further, it has been proposed that obligate sterility is the prerequisite of a major evolutionary transition (i.e. evolutionary social complexity). Once a species has irreversibly gained sterility, it will be selected for greater complexity in all traits in a positive feedback process (Bernadou et al., 2021; Boomsma & Gawne, 2018). This is due to obligate sterility removing the potential for reproductive conflict within a colony and therefore allowing for complete interdependence and alignment of interests (Boomsma, 2022; Boomsma & Gawne, 2018; West et al., 2015). Therefore, obligate sterility is seen as the key trait aligning both phenotypic and evolutionary views of social complexity.

Despite the perceived importance of sterility, however, there are two key issues that complicate its use and interpretation as a general proxy for social complexity at large. The first is that phenotypic social complexity is likely to be a more complex and multivariate concept than previously thought. For instance, variation in traditional phenotypic measures of social complexity such as colony size, colony longevity, and worker size variation within different Hymenoptera taxa demonstrate that these measures do not always correlate with each other (Bergman & Beehner, 2015; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019). Furthermore, within bumblebees, some species can display high levels of phenotypic social complexity despite the presence of fertile workers (Zhuang et al., 2023). The second issue is that sterility itself is complicated. For instance, some multicellular organisms can regenerate their germline (i.e. Echinoderms) and therefore have functionally sterile somatic cells, rather than obligately sterile somatic cells. This casts doubt on the importance of complete early separation of germline and soma for obligate multicellularity and, therefore, for major evolutionary transitions (Howe et al., 2022; Pineda-Krch & Lehtilä, 2004; Pineda-Krch & Fagerström, 1999). Multicellular species that have a functionally sterile somatic cell line, rather than an obligately sterile one, show that although there is potential reproductive conflict, it has not prevented complete interdependence and alignment of reproductive interests (Bourke, 2023; West et al., 2015). These datapoints highlight that functional sterility, rather than obligate sterility, may be of more relevance for both evolutionary and phenotypic views of social complexity and major evolutionary transitions (in both multicellularity and social insects). We require a greater understanding of atypical developmental systems if we are to fully appreciate the role of sterility in generating or maintaining social complexity (Bourke, 2023; Howe et al., 2022).

To explore the overall importance of functional and obligate sterility in explaining phenotypic and evolutionary social complexity, we must first examine them at the highest levels of sociality within developmentally atypical systems (Korb & Hartfelder, 2008; Roisin & Korb, 2011). The hemimetabolous termites are an extremely useful system to do this. Termites are of huge ecological and economic importance across the globe (Govorushko, 2019; Jouquet et al., 2011; Rouland-Lefèvre, 2011; Su & Scheffrahn, 2000) and display a wide range of social complexities – from simple colonies to complex agricultural societies. Traditionally they are grouped into the “higher” and “lower” termites; this distinction separates those species which have flagellates in their guts (lower termites) from those that do not (higher termites). This separation has been claimed as a defining biological difference. In terms of social complexity, however, this distinction is not particularly useful as both higher and lower termites exhibit a range of phenotypically complex and simple societies (Carrijo et al., 2023; Revely et al., 2021). Crucially, termites have a complex relationship with sterility – making them an ideal model taxon to investigate how this trait links to other measures of social complexity (Revely et al., 2021). Some termites retain fully fertile workers (often called “wood-dwellers”, one or single-piece nesters (Abe, 1987)), some have functionally sterile workers which can become fertile, if necessary (“foraging” termites or separate piece nesters (Abe, 1987)), and some have fully sterile workers (also within “foraging” termites or separate piece nesters (Abe, 1987)) (Korb, 2019; Korb & Hartfelder, 2008; Revely et al., 2021; Roisin & Korb, 2011).

Here, we use trait data from the large termite collection of the Natural History Museum London, supplementing pre-existing data, to produce the most comprehensive phenotypic social complexity trait dataset for termites to date. By doing so, we will test whether obligate sterility, functional sterility, or neither, are able to explain variation in termite phenotypic social complexity. We make use of the dataset to answer the following questions that arise from the problems discussed above:

1) **Can either trait, functional or obligate sterility, be used a singular proxy for phenotypic social complexity in termites?**

2) **Regardless of their proxy power, are there any significantly positive associations between functional and obligate sterility and the other social complexity traits?**

3) **Is obligate sterility reliable as a central concept allowing phenotypic and evolutionary social complexity to be unified? Is obligate sterility necessary for higher evolutionary social complexity?**

Box 5.1: Glossary

Conflict- This would refer to evolutionary conflict of interest between members of a social group over actions like reproduction. This can be potential conflict when there is differing inclusive fitness optima, such as having higher relatedness of workers to their own offspring compared to their siblings. This may not become actual conflict however (for instance, when there is worker reproduction within a colony while the queen is present).

Fertile workers- workers that can become dispersing primary reproductive that form their own colony and replacement reproductives able to take over a nest if a parent dies.

Foraging termites- (also referred to as separate-piece or multiple piece nesters) Species which live in well-defined nest where workers, at some point in the colony-cycle, will leave the nest to forage. This means the colony longevity is not limited to the availability of food. All foraging termites have true workers which can be thought to have reduced potential and therefore are at least functionally sterile. These are found in the Mastotermitidae, Hodotermitidae, most Rhinotermititdae and all Termitidae.

Functional sterility- When the working unit in a group can become a reproductive only under extreme circumstances. For instance, in termites the apterous (working/somatic) and nymphal (reproductive/germline) lines have incomplete separation where workers can still become reproductives if for example a parent dies but are unable to become dispersing reproductives. Some species with functional sterility, like *Mastotermes* and *Reticulitermes*, have worker derived reproductives in the nest while the primary reproductive is still present but the ability of a worker to become a worker is still much reduced compared to species with fertile workers.

Individual- A collective that adheres to the conditions needed for a major evolutionary transition, i.e. a group whose lower units are interdependent and have aligned interests.

Major evolutionary transition- A change in the way that heritable information is stored and transmitted, concentrating on transitions that lead to a new form of individual. This requires two conditions to be met: 1) entities capable of independent replication before the transition can replicate only as part of a larger unit after it- interdependence of these units. 2) there is a lack of within-group conflict such that the larger unit can be thought of as a fitness maximising individual in its own right- there is long term alignment of interests. Also called evolutionary social complexity.

Phenotypic social complexity- The extent to which a system is made of from many interacting parts all coming together to contribute to a function. This is a measure of phenotypic complexity within each level of transitionary level and can be explained via multiple traits.

Phenotypic social complexity traits:

Colony Size (CS)- The larger the colony the less likely the chance of individuals to become reproductive, therefore they're selected to specialize instead. Also, there are more individuals in the first place to interact to do different jobs. From 1-7, the number of units within a colony for a given species, logged.

measured in this study.

Functional sterility (FS)- This trait is important for separating termite species which have workers able to disperse to become primary reproductives (fertile workers/wood-dwelling) and those that cannot (functionally & obligately sterile workers/foraging).

Helper Polyphenism (HM)- The greater number of morphs of workers and soldiers the greater number of specialised groups in a colony. 1-4 morphs have been measured in this study.

Nest Complexity (NC)- An extended phenotype signifying the complexity of behaviour (polyphenisms) required to create the nests around them three separate levels: 1) no structure, 2) subterranean structure, 3) above ground structure.

Obligate sterility (OS)- Individuals are committed to their role as workers and therefore will be solely selected to be the most specialized and efficient worker they can be. 0 or 1 where 1 is a species which has workers unable to take over the colony or found their own and 0 is anything else.

Soldier- Sterile altruistic caste which are generally morphologically and behaviourally specialised for defence.

Termite, Higher- Made up of only termite species within the family Termitidae, which only have non-flagellate gut symbionts.

Termite, Lower- All termites other than the Termitidae. They have both flagellates and non-flagellate gut symbionts.

True workers- Individuals that have diverged and are part of a separate wingless line.

Evolutionary social complexity- The extent to which a group has become a fitness maximising individual in its own right, with long term alignment of interest and complete interdependence.

Wood-dwelling termites- (Formally one-piece nesters) species where a colony will live in a single piece of wood which serves as both food and nest source. Only the winged sexuals leave the nest and when their only food source is exploited the colony will die. Species within this life type are thought to have highly flexible development and false workers which can also be described as fertile workers. These are found within the Termposidae, Kalotermitidae and some species within the Rhinotermitidae.

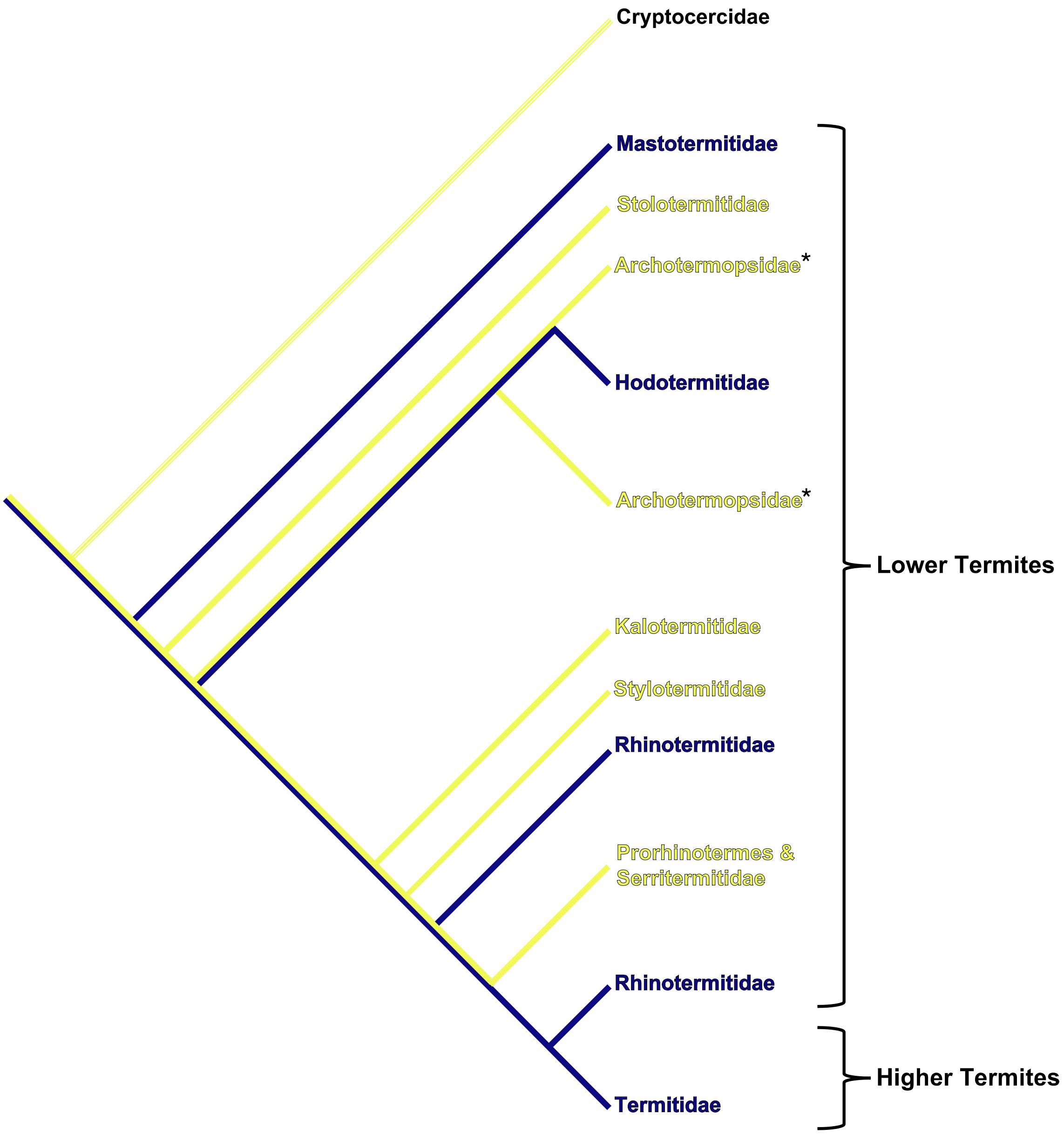


Figure 5.1 Cladogram showing the higher and lower termites as well as the major families within the termites and whether they are wood-dwelling (yellow) or foraging (blue). Cryptocercidae is the outgroup sister taxa to all termites. Asterisks signify non-monophyly

5.3 Methods

5.3.1 Data Collection

We collected morphological trait data from termites preserved in ethanol at the Natural History Museum, London. We used head width, hind femur length and front tibia length to predict the number of worker and soldier morphs in a species (helper polyphenism). Head width is used as a proxy of body size and the two leg measures capture potential limb allometries related to foraging mode and task allocation (Wilson, 1953). We sampled from 300 species in total with every termite genus in the collection represented but with several genera, such as *Macrotermes*, having a greater representation to allow for their geographical spread and species richness. Where possible, 30 workers and at least 10 soldiers were sampled from each species, preferably with 3 individual workers and 1 soldier from 10 different colonies to prevent colony level bias. We photographed each termite specimen twice to allow for digital measurements of their morphology. To do this, we placed individual termites on blobs of K-Y© jelly (Thornton & Ross LTD) within a petri dish to maintain their posture and covered them with ethanol to prevent them from drying out. We then used an Axio Zoom.v16 (Zeiss) to automate the photographing of each individual. We oriented individuals for a profile and a dorsal photograph. In total, we took 18,900 photographs. We used ImageJ Version 1.53a to measure the head width, hind femur length, and hind tibia length directly (Schneider et al., 2012).

Once these measures were collected, we used the clustering analysis tool *DBscan* in R version 4.2.1 (Hahsler et al., 2019; R Core, 2022) to identify the number of distinct groups of workers and soldiers within each species using the morphometric measures. This would act as a measure of helper polyphenism. Within the DBscan function, MinPts (the minimum number of samples seen together that can be defined as a cluster) was set at 4 so that potential human error in measuring a single individual would not cause a mistaken morph number. The functions kNNdist and kNNdisplot, from within the DBscan package, were used to calculate the k-nearest neighbour distances and plot them to identify the most appropriate eps value. The eps value dictates how close points should be to each other to be considered a part of a single cluster. To complement our estimates of helper polyphenism, we cross-referenced them with existing literature and changed the estimates where there was greater evidence for a different estimate within the literature.

The acquisition of data for obligate sterility (OS), functional sterility (FS), colony size (CS) and nest complexity (NC) involved the systematic search of Web of Science for each species that was measured from the museum collections. This search was expanded to genus level when there was no data as species level. In total, 99 of the 300 species photographed could be used for these analyses due to many having incomplete data relating to these traits.

Most colony size data were acquired from a single paper which has compiled the existing literature on this trait (Mizumoto & Bourguignon, 2021). To process the data, we rounded the maximum colony size values to the nearest power of 10 and then applied the logarithm base 10 function to them. Much of the data on sterility was also previously acquired from studies compiling developmental plasticity and worker fertility data across the termites (Myles, 1999; Revely et al., 2021). We define obligate sterility as a species which has workers unable to take over the colony or found their own under any circumstances (Box 5.1). There are varying levels of reliability with the data, as some are long-term observation data stating that no replacement reproductive were present, some were field-based colony orphaning experiments and others lab-based colony orphaning (Myles, 1999; Revely et al., 2021). We can only be totally certain that a species has actual obligate sterility when doing these in-depth colony orphaning experiments. These experiments would help with understanding absolute developmental potential, not for understanding whether these species naturally produce replacement reproductives from workers in the field. Nest complexity data was taken from the literature and defined as three separate levels: no structure, subterranean structure, above ground structure. Species which reside in wood are making use of pre-existing structures so do not require as many building behaviours to create their nest, whereas the subterranean structures of soil dwelling termites certainly require constructions behaviours as well as related behaviours required when creating their own nest and some defence. The creation of above ground structures requires substantially more construction behaviours as well as defence behaviours due to potentially being more vulnerable to predators.

5.3.2 Phylogeny

We estimated a termite phylogeny from 637 termite species and nine outgroup *Cryptocercus* cockroach species as created using PyPHLAWD (Smith & Walker, 2019). This is an open-source python package that creates molecular tree-building datasets from publicly available genetic data from GenBank (Benson et al., 2013), NCBI BLAST (Altschul et al., 1997) and uses a Markov clustering approach (Schaeffer, 2000) to infer a RAxML tree (Stamatakis, 2014). This allowed us to combine DNA from many genes for all termites that had a genus and species name in GenBank. Recent termite phylogenies informed us on the constraints needed on the tree (Bourguignon et al., 2017; Bucek et al., 2019; Buček et al., 2022; Cameron et al., 2012; Legendre & Grandcolas, 2018; Romero Arias et al., 2021): Mastotermitidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Serritermitidae, Stylotermitidae and Termitidae are monophyletic families; Rhinotermitidae and Archotermopsidae are not. Subfamilies Rhinotermitinae, Apicotermitinae, Foraminitermitinae, Macrotermitinae and Nasutitermitinae form monophyletic groups; Heterotermitinae, Termitinae, Cubitermitinae, and Syntermitinae do not. Following tree reconstruction, 14 taxa were trimmed that were known to be incorrectly placed, likely due to previous misidentification. Species present in the tree but not in the data were then removed. Any remaining species missing from the tree but present in our data were manually added either to the already present genus (48 sister species added) and giving the same branch length as their sister species or when no other member of the same genus is present, made use of pre-existing termite phylogenies to add the remainder of the species (11 newly added genera). This was done using treegraph2 (Stöver & Müller, 2010). Consequently, the final phylogeny we used for further analysis included 99 species (Bourguignon et al., 2017; Bucek et al., 2019; Buček et al., 2022; Cameron et al., 2012; Legendre & Grandcolas, 2018; Romero Arias et al., 2021). Their branch lengths were equal to their single sister species, or an average of multiple closely related species already present in the tree (Figure 5.2).

5.3.3 Statistical Analysis

All analyses used R version 4.2.1 (R Core, 2022). We investigated the ability of the functional and obligate sterility traits to explain the variance in the other social complexity traits, as a test of their proxy power, by running a phylogenetic MANOVA where functional and obligate sterility (binary traits) were each in turn explanatory variables and the response variables were helper polyphenism, colony size and nest complexity (discrete traits). First, we used principal coordinate analyses' (PCoAs) to summarise the discrete response data as three continuous axes of variation using the *ape* package (Paradis & Schliep, 2019). These axes were used as response variables in a phylogenetic MANOVA which required the *phytools* and *vegan* packages (Oksanen et al., 2022; Revell, 2012).

We also tested the associations between functional and obligate sterility, and the other social complexity traits (Nest complexity, Colony size and Helper polyphenism). To account for non-independence due to common ancestry and the discrete nature of the data, we used a Bayesian phylogenetic mixed model approach from the package MCMCglmm version 2.34 (Hadfield, 2010). This package uses a Markov chain Monte Carlo (MCMC) estimation approach and places the phylogenetic relationships among species as a random variable to account for the non-independence of closely related species (Healy et al., 2014). The number of iterations, thinning and burn-in period for each pairwise comparison was by default 100,000, 50 and 5,000 respectively. We used a mixed model with a threshold distribution where functional and obligate sterility are the response variables and the predictors are helper polyphenism, colony size and nest complexity. A weakly informative Gelman prior was used for fixed effects and an inverse Wishart prior for random effects, fixing the residual variance to 1 as this cannot be estimated from binary data (Gelman et al., 2008; Hadfield et al., 2013; Ross et al., 2013). We ran multiple chains and tested for convergence using the gelmon.plot function from the coda package (Plummer et al., 2006). We report the significance of a relationship using overlap of the upper and lower 95% CLs with 0.

5.4 Results

We found that the functional sterility trait accounts for ~40% of the variation in the other social complexity traits (R^2= 0.404, Table 5.1). However, obligate sterility explains much less with only 13% variation explained in these social complexity traits (R^2= 0.131, Table 5.1). Although both traits, and in particular functional sterility, explain a significant proportion of variance in the other social complexity traits, neither obligate nor functional sterility appear to be sufficient singular proxies for understanding social complexity in termites more broadly.

Genera that have high overall social complexity in all the traits include the Macrotermitinae and Syntermitinae (Figure 5.2). Whereas low overall social complexity families include Kalotermitidae, Stolotermitidae and Archotermopsidae (Figure 5.2). There are examples of species, such as *Trinervitermes bettonianus,* which have a large colony size and high polyphenism but without obligately sterile workers. We also see species such as those in the Apicotermitinae (Figure 5.2) with small colony sizes and low polyphenism (due to their loss of the soldier caste) but workers with obligate sterility. We statistically explored the individual relationships of the different social complexity traits using MCMCglmm analysis. We found that functional sterility only had a significantly positive relationship with nest complexity, having CL 95% not overlapping 0 [l-95%; 1.114, u-95%; 3.428] (Figure 5.3, Table 5.2). Obligate sterility had no significant relationship with any of the other traits (Figure 5.3, Table 5.2).

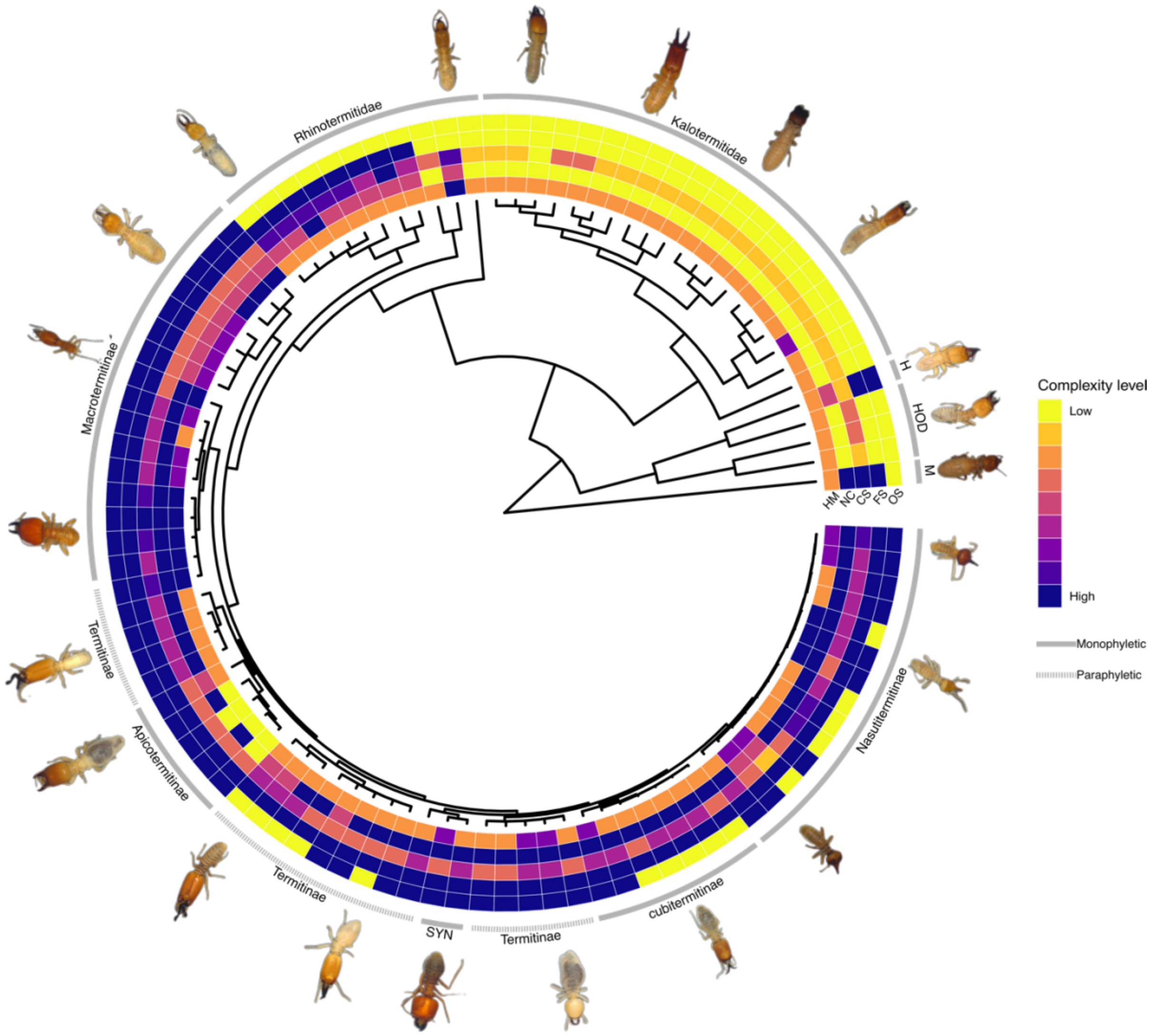


Figure 5.2: Phylogeny with the 99 species of termites used in analyses and their status in each of the five complexity traits standardised from 0 to 1 where yellow is 0 (low complexity) and blue is 1 (high complexity) in each trait: HM, helper polyphenism; NC, nest complexity; CS, colony size; FS, functional sterility; OS, obligate sterility. Abbreiviated sub/family names: M, Mastotermitidae; S, Stolotermitidae; ARC, Archotermopsidae; H, Hodotermitidae; SYN, Syntermitinae. Photographs are from some of the specimens used in this study from NHM London.

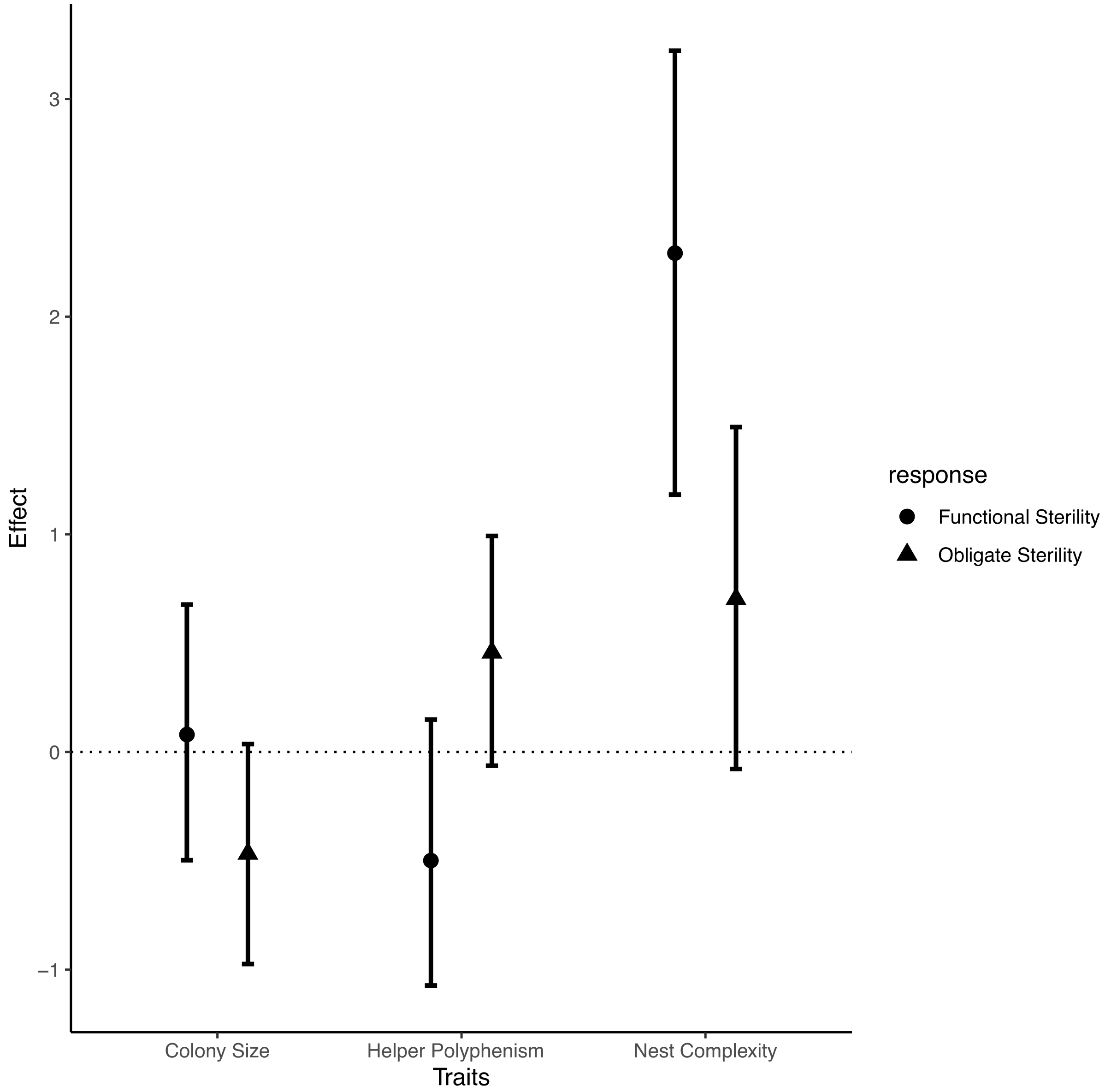


Figure 5.3: Effect plot with explanatory variables are on the x-axis and effect size is on the y-axis. The mean effect sizes with 95% CI's are plotted where an overlap with 0 signifies a lack of significance in the variable's relationship with the response variable (circle, obligate sterility; triangle, functional sterility). This shows results from all three chains from each relationship. Summaries of results for one chain allowing for Pmcmc values are presented in Table 5.2.

Explanatory variable	Df	SumOfSqs	R2	F	Pr(>f)
Functional sterility	1	97.269	0.3998	65.29	0.001
Obligate sterility	1	31.906	0.131	14.668	0.001

Table 5.1: Phylogenetic MANOVA Results A) functional sterility is the explanatory variable B) obligate sterility is the explanatory variable, with helper polymorphism, colony size and nest complexity being the response variables in both.

A	post.mean	L-95% CI	U-95% CI	eff.samp	pMCMC
(Intercept)	-0.809	-4.719	2.325	1900	0.643
Colony size	0.073	-0.623	0.737	1900	0.827
Nest complexity	2.295	0.984	3.418	1101	0.004**
Helper polyphenism	-0.505	-1.2	0.233	1900	0.173

B	post.mean	L-95% CI	U-95% CI	eff.samp	pMCMC
(Intercept)	-1.4167	-6.6117	3.0581	2254	0.551
Colony size	-0.4719	-1.0867	0.1647	1900	0.14
Nest complexity	0.7011	-0.1982	1.7043	1900	0.153
Helper polyphenism	0.4724	-0.1894	1.0653	1725	0.127

Table 5.2: Summary results from a MCMCglmm chain A) results where the response variable is functional sterility, B) results where the response variable is obligate sterility.

5.5 Discussion

5.5.1 Can sterility type explain termite phenotypic social complexity?

Here, we found that although functional and obligate sterility traits explain a significant proportion of the variance in phenotypic social complexity, they cannot be used as a single proxy to explain the diversity of all other social complexity traits (Table 5.1). Instead, we argue that we should use a multivariate view of phenotypic social complexity. This is already gaining traction within the Hymenoptera literature, and clearly would be beneficial in a general case across the social insects and potentially across all forms of sociality (Bergman & Beehner, 2015; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019). The usefulness of complexity traits such as obligate sterility to represent the phenotypic social complexity of species is clearly exaggerated. It would be better to use functional sterility to explain the complexity of termites, but a more nuanced multivariate approach would capture the most variance. An overall phenotypic social complexity score for each species based upon the bringing together of the complexity traits we have explored may allow us to incorporate this greater detail. However, before this can be done in termites, traits such as colony longevity, queen-worker dimorphism and age polyethism will need to be collected. There will also need to be a greater number of species represented. This will allow the truest picture possible of overall phenotypic social complexity (Bergman & Beehner, 2015; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019). Further exploration is needed to determine whether to assign different weights to the traits when developing this score (Bergman & Beehner, 2015; Holland & Bloch, 2020; Kappeler, 2019; Kappeler et al., 2019). It is likely that there will be no single approach for every question using these frameworks and data. Instead, traits may be weighted differently depending on the question of interest. But only through greater discussion are we able to decide the relative importance of each trait in evolutionary social complexity. Finally, to make our results more robust, we should try and gain more termite species trait data to alleviate the issues with having only a relatively small number of independent transitions to obligate sterility.

5.5.2 The relationships of functional and obligate sterility with other social complexity traits

Although our data suggest that functional and obligate sterility traits cannot be used as a single proxy to explain the diversity of social complexity in termites, it could still be the case that they have significant relationships with individual traits. We found that this is only the case with nest complexity and functional sterility. The strong positive relationship we found between functional sterility and nest complexity is likely due, in part, to the first category of nest complexity being ‘nests which have no structure’, which is the case in all wood-dwelling species. This highlights the importance of reduced worker reproductive capacity in the evolution of more complex nesting capabilities. The lack of significance in the relationships between obligate sterility and the other phenotypic traits goes against several studies that assert that obligate sterility should cause an increase in complexity or that complexity is needed to achieve obligate sterility (Boomsma & Gawne, 2018; Howe et al., 2022; Michod, 1997; Michod et al., 1996; Michod & Roze, 1997). We cannot completely rule out the potential that the lack of association between obligate sterility and these other traits could be due to having so few examples of obligate sterility in our present study compared to species with functional sterility or fertile workers. Further, it may well be the case that the presence of obligate sterility is still important for evolutionary social complexity by being the major prerequisite for a major evolutionary transition. However, based on the current data we have at hand, the lack of any relationship with these traits means that it does not have fundamental value for quantifying phenotypic social complexity in termites.

5.5.3 Redefining the importance of sterility in phenotypic social complexity and therefore questioning the inseparability of phenotypic and evolutionary social complexity.

The difference in importance of obligate sterility to phenotypic and evolutionary views of social complexity has already been highlighted (Bernadou et al., 2021). Here, it has been hypothesised that high phenotypic social complexity is not necessary for an increase in evolutionary social complexity (MET) to occur if obligate sterility is present (Bernadou et al., 2021). This is an important and necessary step towards separating these two concepts. We clearly find that there is not a significant association between a species' level of overall phenotypic social complexity and their likelihood of having transitioned to a higher level of evolutionary social complexity, especially when this is defined by obligate sterility. Some species within the Apicotermitinae (Figure 5.2), which have only the worker caste, small colony size and low nest complexity, have obligate sterility. It could be the case that they acquired obligate sterility while having low phenotypic social complexity. Alternatively, it could be that phenotypic complexity was high but was secondarily reduced after acquiring obligate sterility. Either way, this means there is not a clear permanent positive relationship between phenotypic and evolutionary social complexity. We should instead view these as related but separate measures of complexity. By doing so, we are better able to incorporate species that do not conform to the rigid view that phenotypic and evolutionary social complexity fully align at every step. We will see that a colony that has small group size and only one morph with little to no nest structure could still have interdependence and an alignment of interests and therefore be seen as a higher individual (West et al., 2015).

It seems likely that each trait has its own independent selective pressures which cause the changes in their respective complexities. Further analyses exploring the relative importance of biotic and abiotic factors in selecting for these social complexity traits could provide greater understanding on the adaptive reasons for species evolving traits such as worker reproductivity or high levels of polyphenism (La Richelière et al., 2022).

5.5.4 Decoupling obligate sterility from evolutionary social complexity

We must be more inclusive to developmentally atypical organisms when outlining the prerequisites for a major evolutionary transition if we are to create a generalised framework for all life (Bourke, 2023; Howe et al., 2022). This includes systems that seemingly harbour potential conflict, but which have found ways to prevent this from becoming actual conflict over evolutionary time, while still allowing for increases in evolutionary social complexity (Bourke, 2023; Howe et al., 2022). Within cellular groups, the presence of units able to become germline or soma at any point in the group’s life traditionally would prevent them from being defined at a higher level of individuality (Howe et al., 2022). However, it has been shown in some metazoan lineages that species which clearly have interdependence and aligned interests do not always have complete segregation of germline and soma, i.e. have early separation of a germline (Howe et al., 2022). This is also the case in termites, which often have workers within highly complex colonies able to reproduce if the queen or king dies. We have shown in this study that obligate sterility is not an adequate singular proxy for phenotypic social complexity, nor does it correlate with any of the present social complexity traits within termites. It may also be the case that obligate sterility is unnecessary to allow complete interdependence and long-term alignment of interests within the group. The closed nature of these systems, whereby the replacement reproduction by a worker is only occurring with other family members if the king or queen dies, means there is less potential conflict compared to replacement worker reproduction seen in Hymenoptera (Boomsma, 2022). Further, these hemimetabolous insects have much more control over worker self-determination since most are arrested in development for their whole lives, so although potential conflict is present, actual conflict is not possible (Bourke, 2023). Consequently, the evolutionary retention of worker reproductivity could be a group level adaptation to variable environments, present in many atypical systems like the termites and basal metazoans.

Furthermore, the alternative strategies to interdependence and alignment of interests which are not reliant on obligate sterility, shown in developmentally modular organisms like plants, should also be incorporated into our frameworks (Howe et al., 2022; Pineda-Krch & Lehtilä, 2004; Pineda-Krch & Fagerström, 1999). It may be the case that the highly modular but highly complex siphonophores, Hydrozoans within the phylum Cnideria, are another novel example of a higher individual (Boomsma, 2022; Dunn, 2009; Munro et al., 2022). They seemingly have complete interdependence and aligned interests of multicellular replicated units (zooids) coming together for the higher individual (Dunn, 2009; Munro et al., 2022). The more inclusive we are to these developmentally atypical organisms, the more inclusive we become to alternative strategies which can produce a higher individual. Going forward, we must include as many clades as possible to understand the true spectrum of individuality (Bourke, 2023). Wood-dwelling termites have been shown to generally have the lowest levels of social complexity in all the traits we have explored in this study, but this is only compared to other termite species. Their social complexity in traits such as helper polyphenism and colony size align with some Hymenoptera that have obligate sterility and are therefore classed as higher individuals (Boomsma, 2022; Boomsma & Gawne, 2018; Geraghty et al., 2007). The presence of totipotent 'workers' leads many to believe they are simple societies (Bernadou et al., 2021; Boomsma, 2022; Boomsma & Gawne, 2018; Korb, 2019). However, again these 'workers' are arrested in development and may well not have self-determination, with the colony deciding their fate of being a sterile soldier or a reproductive. This has parallels with totipotent cells in plants and some metazoans, which are a colony adaptation for greater developmental flexibility (Howe et al., 2022; Pineda-Krch & Lehtilä, 2004; Pineda-Krch & Fagerström, 1999). Therefore, it may be the presence of one sterile caste (such as the soldier caste in termites) paired with arrested development in other totipotent units allows for a significant step towards alignment of interests and interdependence. Comparisons that include the full spectrum of sociality within the Blattodea may shed more light on this discussion.

5.6 Conclusions

Creating a framework where we can compare the phenotypic and evolutionary processes by which the complexity of life on earth has evolved is invaluable. However, such a unifying concept cannot be explained so simply if we are to include the diversity of all life. Here, we have shown that the diversity of phenotypic social complexity traits such as colony size, nest complexity and worker polyphenism cannot be explained fully by functional and obligate sterility traits. Therefore, instead of a singular proxy for phenotypic social complexity, we must use a multivariate approach to explain its true diversity within termites and more broadly across all sociality if we are to step closer towards this unifying concept. Furthermore, we find that there is a lack of significant association between either functional or obligate sterility and the other social complexity traits, so we should not conflate these traits. Consequently, we outline that phenotypic and evolutionary social complexity (based on individuality) are not necessarily fully in line but instead should be seen as distinct but interacting frameworks if we are to fully understand what is required to transition to higher individuality. By turning our sights to the developmentally atypical termites, we broaden this understanding which allows us to find greater and more accurate parallels across major evolutionary transitions, like that between termites and siphonophores as superorganisms and plants and some metazoan lineages as multicellular organisms.

Chapter Six:

Conclusions and Future Research

This thesis has explored phenotypic plasticity at different scales (intraspecific, interspecific and at a macroevolutionary level), using different systems (*P. dominula*, *P. sulcifer* and termites) and different analytical techniques (comparative transcriptomics, comparative genomics and phylogenetic comparative analyses). This provides a broad basis for understanding common trends surrounding phenotypic plasticity and its interplay with sociality. In this chapter, I outline these commonalities and key conclusions that can be taken from our research into the different scales of plasticity and its interplay with social complexity. I also provide avenues for further research that have arisen from our analyses.

6.1 Synthesis

6.1.1 More complex than first thought

We gain a much greater understanding of phenotypic plasticity and its relationship with phenotypic and evolutionary social complexity when we widen our interest. This is exemplified in this thesis by including the whole adult life-cycle in molecular comparative analyses (**Chapter 2**), by exploring the molecular basis of social parasitism from a novel simple social origin (**Chapter 3**) and finally by exploring reproductive plasticity and sociality in an understudied system (**Chapters 4-5**). I now outline the greater complexities that have been uncovered in this thesis and the new avenues researchers could explore to further our understanding of phenotypic plasticity and sociality.

6.1.1.1 It's not all about summer

To better understand the nuances of phenotypic plasticity, this thesis first aimed to understand the molecular responses of a phenotype in the face of changing environmental conditions across its adult life-cycle. Therefore, in **Chapter 2**, I explored the intraspecific variation in phenotypic plasticity within the queen phenotype of the simple societies of *P.*

dominula. Most molecular analyses to date have only concentrated on the established summer stage of the adult queen's lifecycle in simple societies, assuming there was little to no variation in gene expression compared to the other adult life stages (Ferreira et al., 2013; Patalano et al., 2015; Standage et al., 2016; Sumner et al., 2006; Taylor et al., 2021). However, chapter 2 shows that the narrative is much more complex. The importance of risk from the physiological challenges and uncertainty in reproductive role in the overwintering stage is reflected at the molecular level by the expanded gene expression at this stage with progressive reduction to the founding and established summer stages (Figure 2.2-5, Table 2.2). This provides some evidence towards the reproductive phenotype within a simple society undergoing molecular specialisation. This is an important start in revealing the true complexity of the journey to queenhood in *P. dominula* but more research must be done to push this further.

Due to the difficulties of examining the wasps during the winter months, the behaviour and development during that time is not well understood (Cini & Dapporto, 2009; Kovac et al., 2022). This life stage has clear importance for their plastic response to the social and abiotic environment; therefore, future work should focus on taking a more fine-grained lens observationally and molecularly before, during, and after this life stage. This will help us understand more clearly when individuals undergo canalization of gene expression towards queenhood (Waddington, 1942b). Further, by investigating their physiology through their fat content, ovaries and other structures, we will be able to infer the changes and variation of this life stage (Tibbetts et al., 2011). We would also benefit from exploring the transcriptional activity of the subordinates at the founding and summer stages to try and understand their degree of molecular specialization considering they are still balancing both indirect fitness and potential direct fitness benefits (Field & Leadbeater, 2016; Leadbeater et al., 2011). By having an outgroup like the subordinate foundresses, we are able to truly test our predictions that molecular specialization is due to the certainty of caste fate at the summer life stage. Collectively, these future analyses will bolster our new found understanding on the molecular basis of phenotypic plasticity across the reproductive phenotype's life-cycle in a simple society; it may well provide even more nuance to the journey to queenhood also.

6.1.1.2 A novel path to parasitism

This thesis also endeavoured to uncover the mechanistic basis of major losses in phenotypic plasticity across closely related species. Importantly, I wanted to understand if the level of sociality of the ancestral state of the species which exhibited this loss would affect the mechanistic basis for the loss. Therefore, in **chapter 3**, I explored the loss of the worker phenotype between the social parasite *P. sulcifer* and its social host *P. dominula*. There was mixed evidence of gene gains and genome-wide losses (Cini et al., 2019; Cini, Patalano, et al., 2015; Schrader et al., 2021). This chapter, however, outlined a much more complex story, with genomic and transcriptional expansions, some gene contractions and conditional gene expression all contributing to the proximate and ultimate basis of this social parasite (Figure 3.4-3.9, Table 3.1). This study is a first step towards uncovering the mechanistic basis for the evolution of social parasitism in *P. sulcifer*, hinting to the potential expanded molecular capacity of the social parasite, without any significant losses.

By showing that there has been no major reduction in molecular capacity in the social parasite, questions arise surrounding the species' capacity to exhibit working behaviours. Manipulation experiments of individuals in the field will help us understand whether they have irreversibly lost their capacity to have a working phenotype and founding behaviours. Similar experiments have already been done in *P. dominula* workers, showing their plastic capacity to switch from one phenotype to another and the transcriptomic basis of this (Taylor et al., 2021). By also doing more fine scale transcriptomic analyses with manipulation experiments, we can begin to understand the phenotypic and molecular capacity of the social parasite. This will consequently allow us to better understand the true relationship between molecular changes and the capacity of species to express phenotypes (indicating either a complete loss or only a conditional loss).

To be able to make more robust conclusions about social parasitism in wasps and for those that have evolved from simple social species, we need to analyse more species. Even within the Polistines there are a number of both facultative and obligate social parasites (Cervo, 2006). We must sequence these species to gain a greater understanding of the genomic patterns that underlie social parasitism in this group. To understand if there are any fundamental commonalities in the mechanistic basis of social parasitism, we should also analyse *Dolichovespula*, *Vespula* and *Vespa* social parasites, as well as many more ant and bumblebee species (Carpenter & Perera, 2006; Cini et al., 2019; Gibbs et al., 2012; Hines & Cameron, 2010; Huang & Dornhaus, 2008; J. A. Smith et al., 2013; Stoldt et al., 2022; Tierney et al., 2008). This will also more generally help us understand the relative importance of genomic losses, gains and conditional gene expression relating to major losses in phenotypic plasticity.

6.1.1.3 Sterility is not fundamental for phenotypic or evolutionary social complexity

In **Chapters 4 and 5**, the diversity of developmental plasticity in termites was synthesised and macroevolutionary patterns of reproductive plasticity and its consequences on our understanding of social complexity was explored. Broadly, it was assumed that obligate sterility was the predominant proxy for all other phenotypic social complexity traits and the main prerequisite for evolutionary social complexity (Boomsma & Gawne, 2018; Howe et al., 2022; Michod, 1997; Michod et al., 1996; Michod & Roze, 1997). Further, in termites, social complexity generally has been seen in a binary light, either lower and wood-dwelling termites (low complexity) vs higher and foraging termites (high complexity) (Boomsma & Gawne, 2018; Bourke, 2011a). This thesis has shown that the diversity of phenotypic and evolutionary social complexity across the termites is much more varied and cannot be described so simply. Further, it has been shown that obligate sterility (complete loss of reproductive plasticity), at least in termites, cannot be used as a singular proxy to explain phenotypic social complexity and may not be a prerequisite for evolutionary social complexity (METs). More generally, this has shown that the costs of retaining this reproductive plasticity do not seem to prevent the evolution of these highly complex traits like numerous helper morphs (polyphenisms), incredibly complex nests and large colony sizes. Nor does losing reproductive plasticity (obligate sterility) ensure that the colony will exhibit these complex traits either. It is likely their hemimetabolous nature allows for the retention of high levels of plasticity, like other developmentally atypical and highly plastic species (Crespi, 1992; Lo et al., 2018; Stern & Foster, 1996).

To gain a more generalised understanding on the importance of reproductive plasticity in workers (relative sterility), these analyses must be applied to more taxa across the full spectrum of sociality. Firstly, comparisons that include the full spectrum of sociality within the Blattodea may shed more light on this discussion (Daegan Inward et al., 2007; Legendre & Grandcolas, 2018; Nalepa, 2010). Other hemimetabolous insects should also been included like aphids and thrips (Crespi, 1992; Kutsukake et al., 2004; Shibao et al., 2010; Stern & Foster, 1996; Tian & Zhou, 2014). Furthermore, the alternative strategies to interdependence and alignment of interests which are not reliant on obligate sterility, shown in developmentally modular organisms like plants, should also be incorporated into our frameworks (Howe et al., 2022; Pineda-Krch & Lehtilä, 2004; Pineda-Krch & Fagerström, 1999). It may be the case that the highly modular but highly complex siphonophores, Hydrozoans within the phylum Cnidaria, are another novel example of a higher individual (Boomsma, 2022; Dunn, 2009; Munro et al., 2022). Therefore, we should explore the possibility that these, and other atypical species, have transitioned to become higher individuals. By doing this, we will be better equipped to understand the relative importance of obligate sterility (loss of reproductive plasticity) in major evolutionary transitions and therefore more generally the relationship between plasticity and sociality.

6.1.2 The relationship between plasticity and social complexity is more complicated than first thought

This thesis has endeavoured to move away from a rigid view of the relationship between plasticity and sociality and instead highlight the nuances of the plasticity of reproductive and worker phenotypes across the spectrum of sociality. The molecular specialisation found in the journey to queenhood of *P. dominula* provides evidence towards specialisation in the reproductive phenotype being a common occurrence in simple societies (cooperative breeders). This therefore furthers our understanding of plasticity at this stage of sociality.

Moreover, the convergent evolution of social parasitism across the Hymenoptera clearly does not have a singular molecular narrative. By examining this fascinating example of loss in plasticity, I have highlighted that the sociality of the parasite's ancestor may be of importance, due to the simple society's high reproductive plasticity.

In the worker phenotype, at least in termites, complete loss of reproductive plasticity is not necessary for the evolution and maintenance of high phenotypic social complexity. Our results also hint towards its diminished importance in transitions to superorganismality and therefore evolutionary social complexity. Collectively, this shows that the plasticity of these phenotypes is not constrained by their sociality and the relationship between these two fundamental evolutionary processes (plasticity and social complexity) cannot be simply categorised.

6.1.3 A broader view allows for greater parallels between METs

To truly generalise our understanding of plasticity and its relationship with sociality, we must look across major evolutionary transitions and find novel parallels. Therefore, in the thesis, METs have been used to provide greater parallels across transitions and therefore uncover commonalities that transcend levels of evolutionary social complexity.

The thesis has profited from using the MET framework to understand patterns of specialisation that were originally seen within metazoan cellular specialisation and applying it to the molecular narrative of the queen phenotype in *P. dominula* (**Chapter 2**) (Waddington, 1942b). Only by looking across the breadth of the queen's lifecycle were these parallels possible, however.

Likewise, comparing across METs to find parallels in plasticity between hemimetabolous termites and metazoan cellular development has been invaluable (**Chapter 4**)(Revely et al., 2021). By expanding our study to atypical systems like the termites, our understanding of the commonalities across the METs can be clarified. For instance, we can see greater parallels between the plasticity and sociality of atypical obligate multicellular systems like echinoderms and the hemimetabolous termites (**Chapter 5**) (Howe et al., 2022; Revely et al., 2021; Wessel et al., 2014).

Finally, I have touched on the potential parallels seen across METs with reversions in sociality, like with our insect study system *P. sulcifer* but also in transmissible cancers and transposable elements (**Chapter 3**)(Ågren, 2014; Baez-Ortega et al., 2019; Dujon et al., 2020; Epstein et al., 2016; Metzger et al., 2015; Ujvari, Gatenby, et al., 2016; Ujvari, Papenfuss, et al., 2016). However, much more needs to be done to create an inclusive evolutionary framework that incorporates these reversions so that it can be properly taken advantage of as a comparative tool to identify fundamental patterns in the evolution of life. Moreover, using the MET framework doesn't have to just allow greater understanding of insect biology. There has been extensive research on the biology of socially parasitic insects (Aumer et al., 2019; Brandt et al., 2005; Cervo, 2006; Cini et al., 2014, 2019; Dronnet et al., 2005; Ortolani et al., 2008; Ortolani & Cervo, 2009; Sledge et al., 2001; Smith et al., 2015; Stoldt et al., 2022). Therefore, it would be opportune to use this knowledge to further our understanding of the biology of transmissible cancers, among other reversions in sociality (Ågren, 2014; Baez-Ortega et al., 2019; Dujon et al., 2020; Epstein et al., 2016; Metzger et al., 2015; Ujvari, Gatenby, et al., 2016; Ujvari, Papenfuss, et al., 2016).

6.2 Usefulness of exploring plasticity at different scales

This thesis has explored plasticity and sociality at different scales, be it intraspecific, interspecific or at a macroevolutionary level. This allows distinct but complementary questions to be broached, contributing to our broader understanding of phenotypic plasticity and sociality. It would be useful to continue to apply this multi-scale view on the study systems, using both phylogenetic comparative analysis as well as comparative genomics and transcriptomics that I have covered in this thesis.

The **intraspecific** variation in phenotypic plasticity in the worker phenotype of a termite species could be explored to provide a novel study system for understanding the dynamic molecular changes in a phenotype with changing plasticity. For instance, *Psammotermes hybostoma* is a fascinating termite species with various worker potencies from totipotent, pluripotent and multipotent (Figure 6.1) (Bourguignon, Šobotník, et al., 2012). It is seen as a transitionary developmental state between wood-dwelling and foraging termites (Bourguignon, Šobotník, et al., 2012). They have a high level of polyphenisms but not much is known about the purpose of the later multipotent instars (W8-10) (Figure 6.1). These workers and the soldiers that moult from them are much larger, up to 5 times the size of the previous instars and are seemingly obligately sterile (Bourguignon, Šobotník, et al., 2012). There has been some speculation as to the function of these late instar workers but at the very least they are a source for the production of large soldiers (Bourguignon, Šobotník, et al., 2012). Another potential function of these workers could be as a source of metabolic water for the colony as is it has been shown that the increased size derives from fat stores which can be converted into metabolic water (Arrese & Soulages, 2010). Behavioural and molecular studies could uncover the true function of these instars. It also provides an ideal system to explore the mechanistic basis of discrete losses in reproductive potential, from W1-4 which are totipotent (fully fertile), to W5-7 which are pluripotent (functionally sterile) and finally W8 onwards which are multipotent (obligately sterile). It may well be the case that there are common molecular patterns exhibited here as in *P. dominula* queens regarding caste specialisation.

Understanding the mechanistic basis for the **interspecific** loss and gain of phenotypic plasticity can also be explored in the termites. Specifically, they are a fantastic system to understand the mechanistic basis for the loss of helper phenotypes (worker and soldiers in termites). Termites exhibit the whole spectrum of sexual division of labour (SDL), ranging from: no differences behaviourally and morphologically between sexes, to examples where only one sex can moult into the soldier caste in a colony, to examples of complete sexual division of labour where one sex has completely lost the ability to become a helper (Hojo et al., 2004; Revely et al., 2021; Roisin, 2000; Roisin & Pasteels, 1987). This complete SDL has only been found in a few species and in all of them it is the male, rather than the female in Hymenoptera, that does all the work (Moura et al., 2011; Noirot, 1985; Roisin, 1992). This is a striking example of convergent (or parallel) evolution of complete SDL. However, we do not know exactly how the diploid termites have arrived at this system. The species with complete SDL are closely related to species which have greater plasticity in both sexes and therefore provide a novel interspecific system to explore the genomic and transcriptomic differences leading to the evolutionary loss of the helper phenotypes. More fundamentally, it provides another system to explore how the phenotypic loss is reflected molecularly.

I have already discussed the need to explore the mechanistic basis of social parasitism across more species. This should be extended to applying phylogenetic comparative analyses to explore **macroevolutionary patterns** of change in phenotypic plasticity across socially parasitic wasps and their close relatives. This will further our understanding of the driving traits behind the convergent evolution of this loss in social complexity and plasticity. These can be ecological, life history and molecular traits that are compared to understand their relative importance in the evolution of social parasitism. Including the ancestral social state of the social parasite will also contribute in understanding the interplay of this with the mechanistic basis of social parasitism. Further, novel analyses can be used to try and understand the causality of these different traits (Thorson & van der Bijl, 2023; van der Bijl, 2018). Beyond social parasitism, this provides a novel avenue for understanding the key drivers leading to plasticity losses and whether the mechanistic basis of these losses are contingent on the sociality and therefore plasticity of the ancestral state.

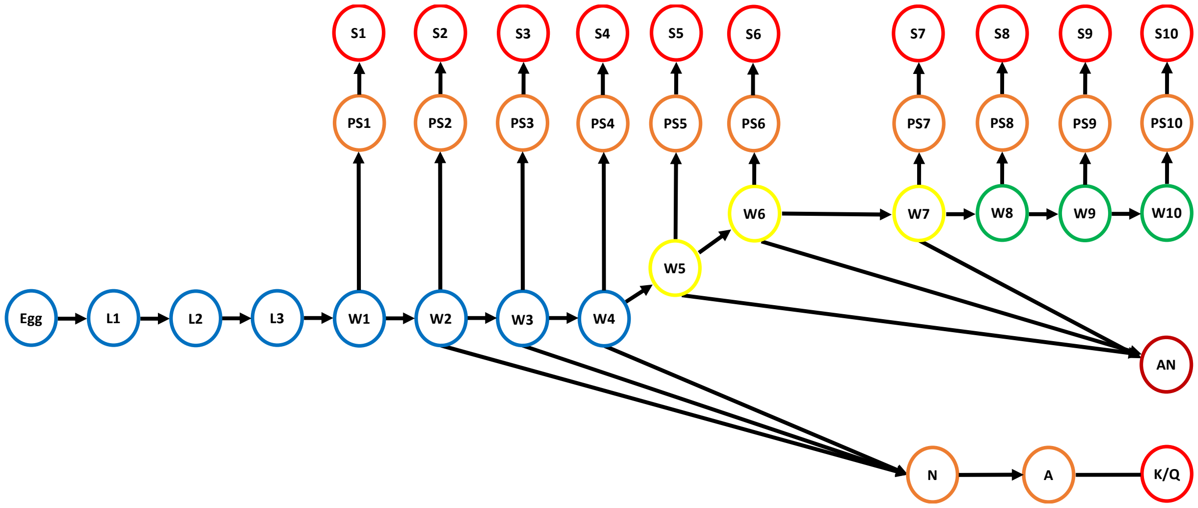


Figure 6.1: Developmental pathways of *Psammotermes hybostoma*. Arrows symbolise moults, Lines symbolise morphological and physiological change without moult. L1, Larval instar 1; W1, worker instar 1; N, nymphal instar; A, alate; PS, presoldier; S, soldier; Q/K, queen/king. Blue, Totipotent; Green, Pluripotent; Yellow, Multipotent; Orange, Unipotent; Red, Committed (Bourguignon, Šobotník, et al., 2012).

6.3 Final conclusions

This thesis has utilised the reproductive and non-reproductive phenotypes of social insects to broaden our understanding of the variation in phenotypic plasticity and its relationship with sociality. Studying plasticity and sociality at different scales (intraspecific, interspecific, and macroevolutionary) has enabled distinct but complementary questions to be broached surrounding these processes and has consequently provided novel results. The thesis has contributed to the fundamental understanding of the mechanistic and evolutionary basis of social parasitism, sociality in termites, and the plasticity of Polistine wasps. But more generally, it has pushed our understanding on the mechanistic and evolutionary basis of phenotypic plasticity and its relationship with sociality.

First, the thesis finds dynamic molecular change in the face of physiological stress and shifts in certainty of fitness strategies in a single phenotype. This has important implications on our understanding of the variability of these single adult phenotypes and the relative importance of different environmental factors. Second, major phenotypic losses were found to be reflected in major genomic expansions and conditional gene expression. This potentially reflects the plastic nature of the ancestral state of the species that has underwent the phenotypic loss. Finally, the reproductive plasticity of the non-reproductive (worker) phenotype is not universally associated with phenotypic or evolutionary social complexity. In that, species which retained some form of reproductive plasticity have not prevented complex social systems that have large colony sizes, complex nests and a large number of morphs (polyphenisms).

Collectively, the chapters of this thesis emphasise the nuanced nature of the relationship between sociality and the plasticity of the reproductive and non-reproductive phenotypes. The framework of major evolutionary transitions has also enabled novel parallels to be drawn to further our fundamental understanding of plasticity and sociality. Future work should continue to utilise the multiple scales of analysis I have used in this thesis to truly broaden our understanding of these important processes.

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