The behavioural, chemical, and morphological basis of caste regulation in the worker caste of ants

Angelica Lillico-Ouachour

Department of Biology

McGill University

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ABSTRACT

The generation and regulation of castes is critical for the success of a eusocial insect colony. The hyperdiverse ant genus *Pheidole* has a worker caste divided into two subcastes: small-bodied minor workers and big-headed soldiers. The physical distinction between subcastes enables minor workers and soldiers to specialize on different tasks. Minor workers perform the majority of day-to-day tasks, while soldiers preferentially perform particular foraging and defensive tasks. To partition tasks efficiently, colonies regulate the proportion of soldiers and minor workers in the nest in response to a number of factors that influence individual development. My thesis is divided into 3 chapters centered on the worker subcaste and its regulation in *Pheidole*. In Chapter 1, I summarize the ecological, social, and developmental interactions that both regulate subcaste ratios in a colony and facilitate their evolution. This review highlights the significant advances made over the last 100 years and the areas that need more consideration. One such area is to understand how social interactions regulate individual development through pheromones. When soldier numbers are too high in a *Pheidole* colony, a 'soldier inhibitory pheromone' released by adult soldiers is thought to inhibit larvae from developing into soldiers. In Chapter 2, I identified a behavioural mechanism by which soldiers transmit the soldier inhibitory pheromone to larvae and uncovered potential chemical candidates for this pheromone that are compatible with the behavioural model. I found that soldiers transmit the soldier inhibitory pheromone by selectively and passively contacting larvae. As many contact pheromones in insects are cuticular hydrocarbons, I analyzed cuticular hydrocarbon differences between subcastes. I found that soldiers have more saturated hydrocarbons (alkanes) on their cuticle than minor workers. Based on this finding, I propose that alkanes are good chemical candidates for the soldier inhibitory pheromone. Lastly, in Chapter 3, I tested whether cephalic glands which produce many

semiochemicals also differed between subcastes. I compared head structures, including glands, for minor workers and soldiers in 4 *Pheidole* species: *Pheidole dentata, Pheidole hyatti, Pheidole moerens,* and *Pheidol*e *spadonia*. I found that although soldiers have more muscle than minor workers, they have smaller nervous systems and similarly sized cephalic glands. The finding that soldiers have similarly sized cephalic glands to minor workers is consistent with a hydrocarbon-derived soldier inhibitory pheromone because hydrocarbons are produced in individual secretory cells throughout the body. Taken together, the research summarized in my thesis integrates behavioural, chemical, and morphological perspectives to improve our understanding of how complex worker caste systems are regulated.

ABRÉGÉ

La génération et la régulation des castes sont essentielles au succès d'une colonie d'insectes eusociaux. Les fourmis du genre hyperdiverse *Pheidole* sont divisées en deux sous-castes: les ouvrières, distingables par leur petite taille, et les soldats, présentant une large tête. Cette distinction physique entre les sous-castes permet aux ouvrières et aux soldats de se spécialiser dans différentes tâches. Les ouvrières accomplissent la majorité des tâches de base journalières, alors que les soldats s'adonnent plutôt aux tâches liées à la recherche de nourriture et à la défense. Afin de répartir ces tâches efficacement, les colonies régulent la proportion de soldats et d'ouvrières présents dans le nid en réponse à certains facteurs influençant le développement des individus. Ma thèse est divisée en trois chapitres centrés sur la sous-caste ouvrière et sa régulation dans le genre *Pheidole*. Dans le premier chapitre, je résume les intéractions écologiques, sociales et développementales qui permettent de réguler le ratio des sous-castes à l'intérieur d'une colonie et qui facilitent leur évolution. Cette revue de littérature met en évidence les avancées significatives réalisées au cours des 100 dernières années ainsi que les aspects nécessitant davantage d'attention. Un de ces aspects est la compréhension des intéractions sociales qui permettent de réguler le développement individuel par l'entremise des phéromones. Au sein d'une colonie de *Pheidole*, quand le nombre de soldats devient trop élevé, une "phéromone d'inhibition de soldats", relâchée par les soldats adultes, empêcherait les larves de se développer en soldats. Dans le deuxième chapitre, j'ai identifié un mécanisme comportemental par lequel les soldats transmettent la phéromone d'inhibition de soldats aux larves et découvert des composés chimiques candidats pour cette phéromone, compatibles avec le modèle comportemental. J'ai découvert que les soldats transmettaient la phéromone d'inhibition de soldats par des contacts sélectifs et passifs avec les larves. Comme plusieurs phéromones de

contacts retrouvées chez les insectes sont composées d'hydrocarbures cuticulaires, j'ai analysé les différences entre les hydrocarbures cuticulaires des deux sous-castes. J'ai ainsi découvert que les soldats possédaient davantage d'hydrocarbures saturés (alcanes) sur leur cuticule que les ouvrières. En me basant sur ces résultats, je propose que les alcanes sont de bons composés chimiques candidats pour la phéromone inhibitrice de soldats. Enfin, dans le troisième chapitre, j'ai regardé si les glandes céphaliques, qui produisent plusieurs composés semi-chimiques, différaient entre les deux sous-castes. Pour ce faire, j'ai comparé les structures de la tête, y compris les glandes, d'ouvrières et de soldats et ce, pour 4 espèces de *Pheidole*: *Pheidole dentata*, *Pheidole hyatti*, *Pheidole moerens* et *Pheidole spadonia*. J'ai découvert que, bien que les soldats possèdent davantage de muscles que les ouvrières, leur glandes mandibulaires et leur système nerveux sont plus petits. Le fait que les soldats possèdent les glandes de tête de même taille est compatible avec la phéromone d'inhibition des soldats dérivés d'hydrocarbures, les hydrocarbures étants produits dans des cellules sécrétrices individuelles à travers le corps. Ensemble, les recherches présentées dans ma thèse intègrent des perspectives comportementales, chimiques et morphologiques afin d'améliorer notre compréhension du degré de complexité de la régulation des systèmes de sous-castes chez les ouvrières.

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I dedicate this thesis to my biggest role models, my parents.

PREFACE: CONTRIBUTIONS OF AUTHORS

This thesis represents my own original research, organized into three manuscript-based chapters for submission to peer-reviewed journals, of which I am the primary author. I conceived, executed, analyzed, and wrote all chapters with the guidance and support of my supervisor, Dr. Ehab Abouheif. The details regarding the contribution of co-authors for each chapter are listed below:

Chapter 1

Authors: Angelica Lillico-Ouachour and Ehab Abouheif

A version of this chapter is published as "Lillico-Ouachour, A., and Abouheif, E. 2017. Regulation, development, and evolution of caste ratios in the hyperdiverse ant genus *Pheidole*. Current Opinion in Insect Science. 19: 43-51. doi: 10.1016/j.cois.2016.11.003" and is reproduced here. I conceived of and wrote this manuscript.

Chapter 2

Authors: Angelica Lillico-Ouachour, Juergen Liebig, Ehab Abouheif

This chapter is in preparation for submission to a peer-reviewed journal. I conceived the hypotheses tested, designed and performed experiments, analyzed results, and wrote the manuscript. Dr. Juergen Liebig helped design the chemical experiments, taught me how to conduct these experiments in his laboratory, and helped me to analyze their results.

Chapter 3

Authors: Angelica Lillico-Ouachour, Brian Metscher, Tomonari Kaji, Ehab Abouheif

This chapter is in preparation for submission to a peer-reviewed journal. I conceived of the idea, analyzed the data, and wrote the manuscript. Dr. Brian Metscher performed the microCT scans. Dr. Tomonari Kaji taught me how to produce and analyze reconstructions using Imaris software.

In order to unify my thesis, references for each chapter use the same citation format.

Chapter 1: Regulation, development, and evolution of caste ratios in the hyperdiverse ant genus *Pheidole*

Angelica Lillico-Ouachour and Ehab Abouheif

ABSTRACT

Ant colonies are considered complex biological systems because many individuals are divided into different castes that interact to efficiently perform their tasks. Colonies in the hyperdiverse ant genus *Pheidole* have evolved a worker caste with at least 2 subcastes: soldiers and minor workers. The proportion of soldiers and minor workers in a colony has a major impact on the colony's fitness and is tightly regulated. Here, we summarize over 100 years of research on the internal, external, and developmental factors that regulate subcaste production as well as influence subcaste evolution in *Pheidole*. We hope that summarizing these factors into a network of interactions will provide insight into how complex biological systems regulate, develop, and evolve.

INTRODUCTION

Complex systems are ubiquitous, spanning from the biological (individuals, populations, and ecosystems) to the physical (turbulence and weather) to the abstract (free markets and the Internet) (Ladyman et al. 2013; Pagel 2002). Despite this diversity, all complex systems are thought to exhibit similar underlying principles. For a unit to be called a complex system, it must have many interacting components that are responsive to feedback and produce an emergent and robust organization (Ladyman et al. 2013). A fundamental and enduring challenge in biology is to uncover how complex systems develop, evolve, and regulate themselves. We often refer to the organization of complex systems in biology as a 'division of labour,' meaning that different parts within a system efficiently perform tasks (Anderson and McShea 2001; Bonner 1988; 1993). This division of labour plays an important role in the evolution of complex systems because increasing specialization between interacting parts is thought to be a driving force behind several

major evolutionary transitions, where selection begins to act on a higher functional level (Michod 2005; 2007; Szathmary and Smith 1995). Examples range from genes to gene networks, from single cells to multicellular organisms, or from individuals to societies (Michod 2005; 2007; Szathmary and Smith 1995).

Accordingly, a single ant colony is a complex biological system with respect to its organization, development, and regulation and can be used as a model to gain insight into other systems. However, ant colonies provide an advantage over other systems because their individual parts are obvious and their colonies can be taken apart and reassembled, what E.O. Wilson has termed as the 'pseudomutant' technique (Hölldobler and Wilson 2009; Wilson 1981; 1985b; 1996). For example, colonies have long been called 'superorganisms' and are often compared to how multicellular organisms develop and evolve (Kilfoil et al. 2009; Linksvayer et al. 2012; Yang 2007). Ant colonies have a morphological and reproductive division of labour within the colony, where a queen caste primarily functions as the germline, while a worker caste functions as the soma and performs all other tasks (Anderson and McShea 2001; Bourke 1999; Hölldobler and Wilson 2009; Oster and Wilson 1978; Wheeler 1910). Like ant colonies, organismal development also has a division of labour, which unfolds after cells differentiate and acquire their distinct fates, generating an incredible degree of cellular diversity (Alberts et al. 1989; Valentine et al. 1994). Cellular differentiation is thought to be regulated by the action of gene regulatory networks that respond to inputs from both internal and external cues (Davidson 2010; Davidson and Erwin 2006; Davidson and Levine 2008; Davidson et al. 2002). In ants, the eventual caste fate of eggs and larvae is also determined by the action of gene regulatory networks that respond to hormonal, chemical, social, and environmental cues (Abouheif and Wray 2002; Wheeler and Nijhout 1983; 1984). The internal and external cues in ants are

equivalent to the signals that a totipotent (undifferentiated) field of cells receive from short- and long-range signaling molecules, such as the Decapentaplegic morphogen gradient (Affolter and Basler 2007), which induces differentiation and determines the fate of particular cells within the embryo (Linksvayer et al. 2012).

We focus on the hyperdiverse ant genus *Pheidole* because their behaviour, physiology, and development has been studied extensively (Abouheif and Wray 2002; Brown and Traniello 1998; Calabi and Traniello 1989a; 1989b; Mertl and Traniello 2009; Muscedere et al. 2011b; Patel 1990; Rajakumar et al. 2012; Seid and Traniello 2006; Sempo and Detrain 2010; Shbailat et al. 2010; Wheeler and Nijhout 1981a; Wilson 1984). Unlike most ants, the worker caste is completely sterile and is divided into at least 2 morphologically distinct subcastes: 'minor workers' and 'soldiers' (Figure 1.1). Minor workers perform most tasks including maintaining the nest, foraging, and caring for brood, while soldiers defend the nest and help process food, like cracking large seeds (Feener 1987; Hölldobler and Wilson 1990; Huang 2010; Mertl and Traniello 2009; Wilson 2003; Yang et al. 2004). Soldiers are adapted to this role with disproportionately larger heads and mandibles than minor workers (Figure 1.1) (Pie and Traniello 2007; Wilson 2003). The ratio of soldiers : minor workers in a colony ranges from 5% soldiers : 95% minor workers to 25% soldiers : 75% minor workers (Brown and Traniello 1998; Calabi and Traniello 1989b; Huang and Wheeler 2011; Ito and Higashi 1990; Kaspari and Byrne 1995; McGlynn et al. 2012; Ono 1982; Passera 1974; Sempo and Detrain 2004; Wheeler and Nijhout 1984; Wilson 1984). The ability of *Pheidole* colonies to respond plastically to environmental changes on shorter time scales and to evolve different subcaste ratios over longer time scales depends on both genetic and environmental factors. This ability likely played a key

role in the evolutionary and ecological success of *Pheidole*. Here, we review the literature on the factors that affect the regulation, development, and evolution of subcaste ratios in *Pheidole*.

INTERNAL INFLUENCES

Activation of the soldier program: nutrition and juvenile hormone

In *Pheidole*, individuals undergo a series of developmental switches that determine queen and worker castes. The first developmental switch is controlled by haplo-diploid sex determination, where unfertilized (haploid) embryos become males and fertilized (diploid) embryos become females (Crozier 1971). The second developmental switch is mediated by juvenile hormone (JH) and occurs shortly after the first switch, where individuals differentiate into either reproductive queens or sterile workers (Figure 1.2; blue lines) (Passera and Suzzoni 1979). This switch is season-dependent; seasonal cues, such as temperature, are thought to activate JH production to induce the queen program (Fave et al. 2015; Hölldobler and Wilson 1990; Passera and Suzzoni 1979; Schwander et al. 2008). The third developmental switch differentiates worker larvae into either soldiers or minor workers (Figure 1.2; blue lines) (Wheeler and Nijhout 1983). This switch is also mediated by JH and is thought to be largely influenced by nutrition; a protein-rich diet activates JH production to prolong development and induce the soldier program (Figure 1.2; blue lines) (Goetsch 1937; Metzl et al. 2017; Passera 1974; Wheeler and Nijhout 1981b; 1983).

The effect of nutrition on the production of soldiers in a colony was established experimentally by Goetsch (Goetsch 1937; Metzl et al. 2017) in *Pheidole pallidula* after initial observations made by Wheeler (Wheeler 1902) and Emery (Emery 1921), and further investigated by Passera (Passera 1974). Goetsch (Goetsch 1937; Metzl et al. 2017) fed larvae a honey- and sugar water-rich diet (low protein) or a mealworm-rich diet (high protein) at

alternative times during development. Only when larvae were fed a protein-rich diet were soldiers able to be produced (Goetsch 1937; Metzl et al. 2017). This study was the first to suggest that there is a critical period during larval development that mediates the soldier to minor worker switch (Goetsch 1937; Metzl et al. 2017). The confirmation of this critical period and its connection to JH were investigated later by Wheeler and Nijhout (Wheeler and Nijhout 1981b), who applied a JH analogue called methoprene to *Pheidole bicarinata* at different stages of larval development. During a critical period in the 4th larval instar, methoprene was able to induce the soldier developmental pathway (Wheeler and Nijhout 1981b). Methoprene effectively delays metamorphosis by several days such that larvae continue to develop past the point where minor workers normally pupate and, instead, pupate at a larger terminal size (Ono 1982; Wheeler and Nijhout 1981b). To date, nutrition and JH titres have been studied independently in *Pheidole* and associated by the period in which larvae are sensitive to their effects (Metzl et al. 2017). Research that establishes a direct link between a larva's diet and the JH pathway is needed for a thorough physiological understanding of this developmental decision (Metzl et al. 2017).

Inhibition of the soldier program: pheromones

Complex systems rely on positive and negative feedback loops for their generation and maintenance (Ladyman et al. 2013). We have already summarized a key activating influence on soldier production– nutrition–and now we will consider its complementary inhibitory influence, namely the soldiers themselves. Evidence that adult soldiers negatively regulate soldier production came first from Gregg (Gregg 1942), where he perturbed worker subcaste ratios and found that they influence the production of soldiers in the next generation; increasing the proportion of soldiers decreases soldier production, whereas decreasing the proportion of soldiers

increases soldier production (Gregg 1942; Passera 1974). Furthermore, Wheeler and Nijhout (Wheeler and Nijhout 1984) showed that colonies with the most soldier contact suppressed the soldier program more effectively than those with the least contact (Wheeler and Nijhout 1984). They treated *P. bicarinata* larvae with methoprene to induce the soldier program and raised them either in a high soldier contact environment (40 free soldiers and 40 minor workers) or low soldier contact environment (20 free soldiers and 40 minor workers) (Wheeler and Nijhout 1984). The high soldier contact environment inhibited soldier production more than the low soldier contact environment (1984). Together, these experiments show that the higher proportion of soldiers in a colony, the greater the degree of inhibition (Wheeler and Nijhout 1984).

The inhibition of soldier production in response to a larger than normal proportion of soldiers in a colony can be explained by three alternative hypotheses: (1) soldiers may modulate their behaviour or be inherently less effective at caring for and feeding brood, (2) soldiers may emit a signal (either pheromonal, behavioural or morphological) to the minor workers, inducing them to modulate the way they raise the brood, and (3) soldiers may emit an inhibitory pheromone that directly affects larval development. Wheeler and Nijhout (1984) tested the first possibility, whether soldiers adequately feed larvae and rear them to adulthood, and found that soldiers rear 100% of brood through to adulthood and disseminate food rapidly and effectively. This shows that soldiers do not affect the survival of brood or underfeed them (Wheeler and Nijhout 1984). Furthermore, Wheeler and Nijhout (Wheeler and Nijhout 1984) raised larvae treated with methoprene to induce the soldier program in colonies with minor workers and caged soldiers, where soldiers could contact minor workers but could not directly contact brood. Caged soldiers suppress the soldier program in developing larvae which eliminates the first hypothesis (soldier behaviour or ability to care for brood) (Wheeler and Nijhout 1984). Sempo and Detrain

(Sempo and Detrain 2010) provide key evidence to distinguish between the second (modulation of minor worker behaviour) and third (inhibition of larval development by action of a pheromone) hypotheses; they varied subcaste ratios and observed that minor workers do not change their behavioural repertoire, social interactions, or activity levels at different subcaste ratios (Sempo and Detrain 2010). This shows that modulation of minor worker behaviour does not account for soldier inhibition. Therefore, soldiers regulate the numbers of future soldiers produced in a colony through the action of a pheromone that directly inhibits the soldier developmental program in larvae (Figure 1.2; blue lines) (Gregg 1942; Wheeler and Nijhout 1984). Wheeler and Nijhout (Wheeler and Nijhout 1984) propose that the inhibitory pheromone decreases the sensitivity of the larvae to JH, effectively raising the JH threshold for the production of soldiers.

There are several exciting directions for future research on the soldier inhibitory pheromone. One is to elucidate the nature and chemical composition of the pheromone itself, as well as its transmission. Although we know it is not necessary for soldiers to be in direct contact with the brood for it to be effective, it is unclear whether the chemical is easily diffusible and transmitted passively or whether the chemical is non-volatile and transmitted through contact. If the pheromone is contact-mediated, it will be important to work out what type of contact is required. Once we are able to distinguish between these alternative possibilities, the pheromone's physiological and molecular mode of action on the receiver may be resolved, including its relation to the JH pathway.

Finally, both activation and inhibition of the soldier program contribute to the regulation of subcaste ratios, but it remains unclear how these two factors interact within the social context of the colony. Activation of the soldier developmental program by minor workers can occur in at

least two different ways: first, minor workers may constantly activate particular larvae by feeding them with more protein, or second, minor workers may constantly activate all larvae by feeding them equally. The first possibility implies that activation by minor workers is the primary mechanism for maintaining subcaste ratios and the soldier inhibitory pheromone largely fine tunes them. This raises questions about how minor workers choose these particular larvae and what role larvae play in this choice, i.e., do certain larvae beg for food more than others? Alternatively, the second possibility implies that minor workers are always activating all larvae and that the soldier inhibitory pheromone is the primary mechanism for maintaining subcaste ratios. This raises questions about when soldiers produce the soldier inhibitory pheromone and how larvae sense it. Do soldiers constantly produce the soldier inhibitory pheromone but larvae only respond at a certain threshold by inhibiting soldier development? Or, do larvae always respond to the soldier inhibitory pheromone but soldiers only produce it once they reach a threshold? Formally testing all of these alternative possibilities is an important future direction. Clearly, we have only scratched the surface in terms of our understanding of how these internal influences interact to regulate subcaste ratios in *Pheidole*.

EXTERNAL INFLUENCES

For a complex biological system to be robust it must be able to respond to short- and long-term environmental challenges or the system will be eliminated by natural selection (Buchman 2002; Kitano 2002; Ladyman et al. 2013). Resource availability and competition are two such environmental challenges that stress biological systems in nature (Krebs 2009). Ant colonies are no different; if they cannot buffer against competition, the colony would inevitably be eliminated. For example, one way *Pheidole* colonies can temper this burden is through

behavioural flexibility; if minor workers are lost defending the colony, soldiers will expand their behaviour to include more brood care tasks (Brown and Traniello 1998; Mertl and Traniello 2009; Wilson 1985a). In this section, we will discuss how *Pheidole* subcaste ratio is generally regulated by external influences as a means of modulating environmental challenges on the colony.

Resource availability

Diet is a key activator of the soldier program at the scale of individual development. Therefore, resource availability at an ecological scale could potentially influence activation of the soldier program in a dramatic way and alter the subcaste ratio in colonies (Gregg 1942; Passera 1974). A colony's ability to respond to changes in food supply at an ecological scale would be a testament to the robustness of the superorganism. McGlynn and Owen (McGlynn and Owen 2002) manipulated the food available to *Pheidole flavens* colonies in Costa Rica by providing colonies in the field with clumped or split protein-rich food sources. Food supplementation increased the number of soldier pupae observed in the colony, especially when food was supplemented in a clumped manner (McGlynn and Owen 2002). This treatment may affect subcaste ratios through manipulations of nutrition or through manipulations of soldier presence (Figure 1.2; green lines). If resource availability affects subcaste ratios by way of nutrition, then access to additional protein in the environment is used by the colony to activate the soldier program in a greater number of larvae (McGlynn and Owen 2002). Alternatively, if larger and more abundant resources are available, then soldiers may be recruited for foraging; soldier recruitment lowers the inhibitory influence of soldier presence on larvae in the nest and leads to increased soldier production overall (McGlynn and Owen 2002). Yet, studies which attempt to connect caste ratios

with ecological correlates, like food abundance or limitation, have not found an association between natural resource availability and the proportion of soldiers in a colony (Calabi and Traniello 1989b; Kaspari and Byrne 1995). In fact, Yang (Yang 2006) found that colonies of *Pheidole morrisi* respond to seasonal changes in food distribution with increased fat stores and behaviourally 'replete' soldiers, not with changes in subcaste ratio or body size. It is possible that resource availability was not the limiting factor for soldier production and other ecological correlates are more important in these populations.

Competition

Competition imposes an ecological challenge on any given colony. Because soldiers work to defend the nest, regulating soldier production in response to competitive interactions would confer an adaptive advantage for the colony. To test this possibility, Passera *et al.* (Passera et al. 1996) studied the influence of intraspecific competition on subcaste ratios. In their experiment, *Pheidole pallidula* colonies were made to perceive, but not directly come into contact with, a foreign conspecific colony (Passera et al. 1996). Exposed colonies upregulated the number of soldier pupae and adults over several weeks (Passera et al. 1996). Furthermore, studies have also focused on the effect of interspecific competition on subcaste ratios. Yang *et al.* (Yang et al. 2004) found that *Pheidole morrisi* colonies in geographic areas exposed to more intense interspecific competition from the red fire ant, *Solenopsis invicta,* are composed of more soldiers than colonies in areas with less competition. Yang *et al.* (Yang et al. 2004) was able to then link the proportion of soldiers in a nest with their success at defending against fire ants; having more soldiers decreases the time it takes to kill fire ants. Further support comes from Ito and Higashi (Ito and Higashi 1990), who found that a larger defense zone (an area in which individuals are

likely to encounter competitors) is associated with having more soldiers in a colony. Competition may regulate subcaste ratios, then, by altering subcaste proportions because soldiers are eliminated in combat thereby alleviating the effect of the soldier inhibitory pheromone on larvae or through altering minor worker behaviour to promote soldier production via nutrition (Figure 1.2; green lines). Yet, a study which stressed *Pheidole dentata* with fire ants for 19 weeks did not result in changes in subcaste proportion when compared to controls stressed with a 'non-competitive' species, *Tetramorium caespitum* (Johnston and Wilson 1985). This lack of subcaste ratio regulation in response to fire ants could be real and *P. dentata* may be different from other *Pheidole* species. In support of this explanation, some observational field studies also argue that competition does not influence subcaste composition on *P. dentata* (Calabi and Traniello 1989b). Alternatively, this result may be the consequence of the experimental control used–*P. dentata* colonies may perceive any foreign workers as competition and regulate subcaste ratios accordingly. If so, *T. caespitum* should not be classified as 'non-competitive,' making it invalid as a control. It therefore remains unclear whether the results of these conflicting studies in *P. dentata* can be generalized to other *Pheidole* species. Future research on different species of *Pheidole* and of competitors in a controlled environment is necessary to substantiate this relationship mechanistically and at an ecological level. The association between competition and subcaste proportion is critical to the notion that the demography of the worker caste is adaptive, so this is an important area where much work is still needed.

COLONY DEVELOPMENT AND LIFE CYCLE

Ontogeny and colony size

Subcaste composition is influenced by the development of the colony as it grows from when the queen founds the colony to when it reaches its full size. Winged virgin queens take part in mating flights in spring or summer (Judd 2005; Murdock and Tschinkel 2015), and after they mate, they tear off their wings and attempt to establish a colony (Hölldobler and Wilson 1990). At first, queens produce small minor workers called 'nanitic' workers and gradually produce more and larger minor workers typical of that found in mature colonies (Huang and Wheeler 2011). Similarly, after a minimum number of minor workers have been produced, the queen begins to produce nanitic soldiers and then gradually produces more and larger soldiers typical of that found in mature colonies (Huang and Wheeler 2011). This has been shown in *Pheidole obtusospinosa, Pheidole rhea,* and *Pheidole spadonia*, where the distribution of soldier head width increases as colonies reach their full size (Huang and Wheeler 2011). The effect of colony ontogeny on the proportion and physical size of the soldier subcaste may be partially due to nutrition (Figure 1.2; red lines) (Hölldobler and Wilson 1990). During early founding stages, the amount of resources available to the colony is limited by a small workforce (Hölldobler and Wilson 1990). Once established, the large workforce is able to provide the queen and her brood with a sufficient diet for soldier production (Hölldobler and Wilson 1990).

If this positive relationship between increasing caste size, proportion of soldiers, colony ontogeny and nutrition (Tschinkel 1988) is a general feature of *Pheidole* colonies, then colony ontogeny and colony size should be linked. However, support for this link has been debated. Kaspari and Byrne (Kaspari and Byrne 1995) provide evidence that faster growing colonies of Neotropical *Pheidole* invest more in defense by producing a larger proportion of soldiers, but show that the increased proportion of soldiers is related to colony ontogeny and not colony size. However, they could not track the ontogeny of individual colonies to confirm that there is indeed

a relationship between size and ontogeny because of the general challenges of performing this kind of study in the field (Kaspari and Byrne). Therefore, future studies should track subcaste composition during colony maturation to dissociate the influence of colony size and nutrition on colony ontogeny.

Life cycle and reproductive investment

During spring or early summer, seasonal cues like increased photoperiod and elevated temperature prompt mature colonies to invest in reproductive queens and males (Johnston and Wilson 1985). During this period a 'developmental trade-off' has been proposed where queens and males are produced at the expense of soldiers. The basis of this developmental trade-off is: (1) queens and soldiers are similar in size and are energetically costly; and (2) the developmental switch for queens and workers is prior to that of soldiers and minor workers (Figure 1.2; red lines) (Johnston and Wilson 1985; Passera and Suzzoni 1979; Wheeler and Nijhout 1983). Observations of caste investment in *Pheidole dentata* and *Pheidole morrisi* support the developmental trade-off hypothesis, particularly the observation that queens and soldiers are produced largely at different times of year (Johnston and Wilson 1985; Murdock and Tschinkel 2015). However, this inverse relationship between queen and soldier investment has not been observed across all *Pheidole* species tested and, in some cases, some studies have reported a positive relationship (Ito and Higashi 1990; Kaspari and Byrne 1995). Ito and Higashi (Ito and Higashi 1990) reported no correlation between production of queens and subcaste ratio in the Old World species, *Pheidole fervida* (Ito and Higashi 1990), and in populations of Neotropical *Pheidole*, when there is more queen biomass there is an increase in soldier biomass (Kaspari and Byrne 1995).

How can we account for disagreement between the results of these studies? First, some key methodological differences exist between the studies supporting the developmental trade-off hypothesis and those that do not. Of the two studies that support the hypothesis, one was experimental in nature and conducted under uniform laboratory conditions, while the other was an exhaustive field study where numerous whole colonies were collected via wax-casting (Johnston and Wilson 1985; Murdock and Tschinkel 2015), and the studies that did not support this hypothesis were both correlational field studies (Ito and Higashi 1990; Kaspari and Byrne 1995). Second, species supporting this hypothesis have large colony sizes and live in North America, while those not supporting this hypothesis have small colony sizes or located closer to the equator (Ito and Higashi 1990; Johnston and Wilson 1985; Kaspari and Byrne 1995; Murdock and Tschinkel 2015). Third, because elevated temperature is known to increase soldier production in a laboratory setting (Johnston and Wilson 1985), it is unclear how this would translate to the field where seasonal changes in sexual production correspond to increased temperature. To identify whether temperature and the other factors above contribute to subcaste regulation, it will be important to perform long-term experimental manipulations that identify their effects on life cycle, subcaste size and subcaste ratio.

EVOLUTION

Pheidole colonies have evolved a remarkable degree of developmental plasticity allowing them to dynamically activate (through hormones) and inhibit (through pheromones) the soldier developmental program. On shorter time scales, this allows colonies to regulate subcaste ratios to respond to challenges from their external environment, as well as changes in colony ontogeny and life cycle. Over longer time scales, however, this developmental plasticity allows colonies to

rapidly evolve to adapt to these continual changes and challenges from the environment. Developmental plasticity can mediate evolution of both quantitative and qualitative (novel) changes in subcaste composition through evolutionary mechanisms known as 'genetic assimilation' and 'genetic accommodation (Abouheif et al. 2014; Moczek et al. 2011; Rajakumar et al. 2012; Suzuki and Nijhout 2006; West-Eberhard 2003). These mechanisms describe the evolution of the sensitivity of phenotypes to environmental inputs. Genetic assimilation occurs when initially plastic phenotypes evolve to be less responsive to the environment (Suzuki and Nijhout 2006; West-Eberhard 2003). In contrast, genetic accommodation occurs when phenotypes evolve to be more responsive to the environment (Suzuki and Nijhout 2006; West-Eberhard 2003). These mechanisms can lead to both quantitative changes in subcaste ratio, such as from 10% soldier : 90% minor workers to 15% soldiers : 85% minor workers (McGlynn et al. 2012; Yang et al. 2004), as well as qualitative changes in subcaste composition leading to the evolution of novel subcastes (Molet et al. 2012; Rajakumar et al. 2012).

Quantitative evolution of subcaste ratios

A pioneering study by Yang *et al.* (Yang et al. 2004) provided evidence that populations experiencing intense competition may lead to the evolution of a quantitative increase in subcaste ratio through genetic accommodation. They determined the composition of the worker subcaste in geographically-separated *Pheidole morrisi* populations along the east coast of the USA (New York, North Carolina, and Florida). Colonies co-existing with an ecologically dominant competitor, the fire ant, in Florida have a higher proportion of soldiers compared to colonies in New York and North Carolina without fire ants (Yang et al. 2004). To determine if the relationship between subcaste ratios and geography is the consequence of microevolutionary

divergence or phenotypic plasticity, Yang *et al.* (Yang et al. 2004) performed common-garden experiments in which whole colonies were supplanted into artificial nests in the lab. They found that the differences in soldier ratio between geographically-separated colonies were maintained when *P. morrisi* colonies were removed from their natural environment. This shows that observed differences in subcaste proportion was the consequence of microevolutionary divergence. A similar study was conducted on *P. megacephala*; populations of *P. megacephala* from sites with different competitive environments were sampled for subcaste ratio (Wills et al. 2014). They found that invasion of a Soldiers and minor workers were biggest in the highly competitive habitats and minor workers were smallest in the low competitive habitats, yet these results were not correlated with soldier proportion (Wills et al. 2014). Differences in these two studies could stem from methodology where Yang *et al.* (Yang et al. 2004) sampled whole colonies of *P. morrisi* but Wills *et al.* (Wills et al. 2014) could not feasibly do so because *P. megacephala* are polydomous and expansive. Other explanations for the difference between the two studies could stem from resource limitation (Wills et al. 2014). If *P. megacephala* are not resource limited like *P. morrisi* they may not exhibit developmental trade-offs due to competition (Wills et al. 2014).

Evidence suggests that the quantitative divergence in subcaste ratios between *Pheidole* populations, as shown by Yang *et al.* (Yang et al. 2004), are translated into quantitative divergence in subcaste ratios between species. A study by McGlynn *et al.* (McGlynn et al. 2012) surveyed many species of *Pheidole* and found that species with smaller body sizes produce more soldiers. Species differences in colony composition and individual size are likely the consequence of environmental pressures that drive the evolution of developmental plasticity through genetic assimilation or accommodation (McGlynn et al. 2012).

Evolution of novel subcastes

Complex biological systems can evolve through quantitatively shifting pre-existing components, but how do systems evolve novel components? The evolution of an additional worker subcaste in some *Pheidole* species is an excellent example of how novelty in complex biological systems arises (Rajakumar et al. 2012). At least eight *Pheidole* species have a third worker subcaste called supersoldiers, which are disproportionately larger in head and body size than the soldiers (Wilson 2003) (Figure 1.3). Rajakumar *et al.* (Rajakumar et al. 2012) demonstrated that application of JH on larvae in species without a supersoldier caste was able to environmentally induce the development of supersoldiers. This result shows that the supersoldier caste did not evolve de novo in these eight species, but instead evolved once in the ancestor of all *Pheidole* and the phenotypic expression of supersoldiers was subsequently lost in almost all species in the genus (Rajakumar et al. 2012). However, the ancestral genetic potential was not lost and was retained for 25 to 47 millions of years in the genus, most likely because the same physiological pathways regulate the development of soldier and supersoldier subcastes (Rajakumar et al. 2012; Ward et al. 2015). The supersoldier subcaste re-evolved at least 4 times in the genus after induction of the ancestral potential for supersoldiers was fixed through genetic accommodation (Suzuki and Nijhout 2006; West-Eberhard 2003).This work on the supersoldier ants provides evidence that phenotypic variation induced by the environment can lead to the evolution of new components in biological systems and increase their complexity.

CONCLUSION

From tiny signalling molecules like hormones to major ecological stressors like competition, the factors that influence *Pheidole* subcaste regulation are multifaceted and intricately linked. Not only do these signals induce reactive short-term changes in subcaste number, but they also produce the phenotypic variation required for natural selection to act upon. We hope to have demonstrated that the dynamic relationships between the individuals in a superorganism and the superorganism with its environment are rich and complex. Furthermore, studying these relationships may further our understanding of complex systems as a whole, including the generation of a multicellular individual through organismal development with networks upon networks of regulatory mechanisms governing proliferation and differentiation. More broadly, by studying the tangible complex systems of eusocial insects we can gain insight into other complex biological systems and thereby acquire a deeper understanding of the world around us.

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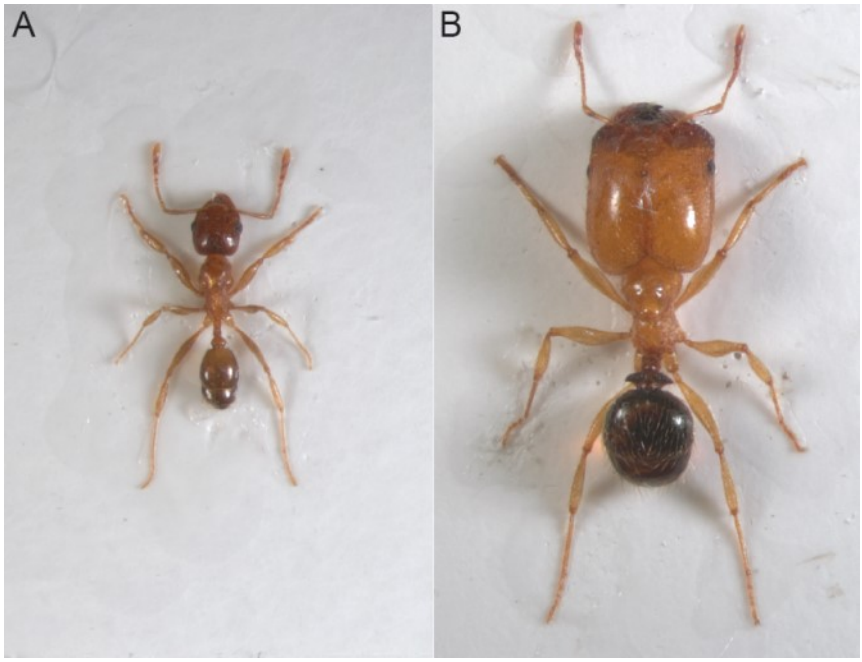

Figure 1.1 Subcastes of *Pheidole spadonia*. (A) Minor worker and (B) soldier. Images to scale.

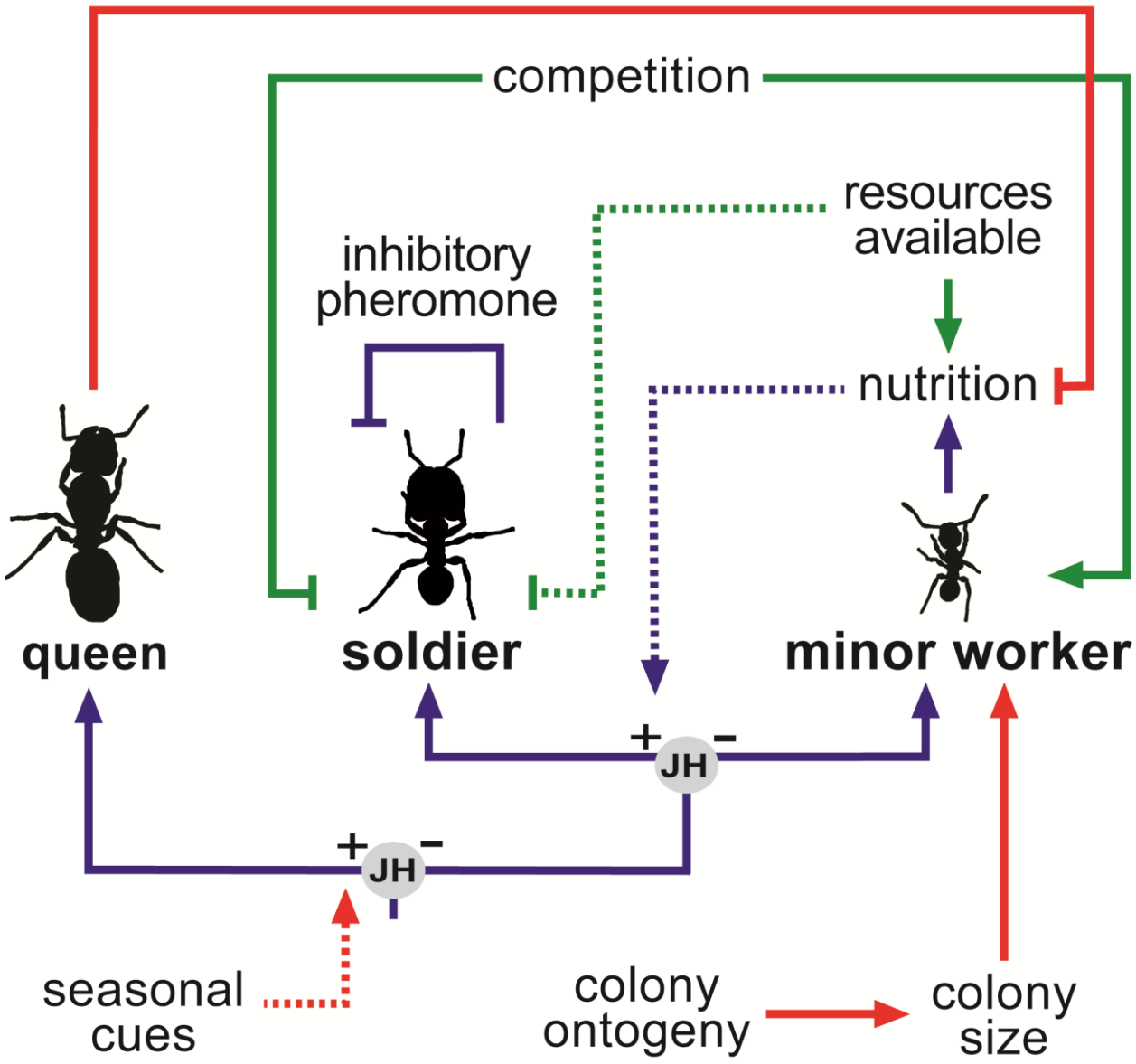
competition
resources available
inhibitory pheromone
nutrition
queen
soldier
minor worker
+
JH
–
+
JH
–
seasonal cues
colony ontogeny
colony size

Figure 1.2. Network model of the major factors regulating subcaste ratios in *Pheidole*. Solid lines indicate regulatory mechanisms supported by sufficient evidence, while dashed lines indicate regulatory mechanisms supported by evidence but requiring further study. Lines with arrowheads indicate activation, while lines with perpendicular bars indicate repression. *Internal influences (blue lines).* Two developmental switches are mediated by JH: an early switch in embryogenesis determines queen or worker fate, while a late switch in larvae determines soldier or minor worker fate. Nutrition promotes soldier production by activating JH. Soldiers suppress soldier production though the soldier inhibitory pheromone. *External influences (green lines)*. Resources available in the habitat promote soldier production by providing more nutrition to the colony or effectively decreasing the numbers of soldiers in the colony by recruiting them to large finds. Competition increases soldier production by promoting the feeding of larvae by minor workers or increasing the death of adult soldiers and thereby alleviating the effect of the soldier inhibitory pheromone on larvae. *Colony development and life cycle influences (red lines)*. Seasonal cues increase the production of virgin queens by promoting JH. Virgin queens reduce the amount of nutrition available to activate the soldier program, thereby reducing soldier production. As a colony ages (colony ontogeny), the colony's size and workforce increases. This effectively promotes soldier production because more minor workers can finally provide sufficient nutrition to larvae.

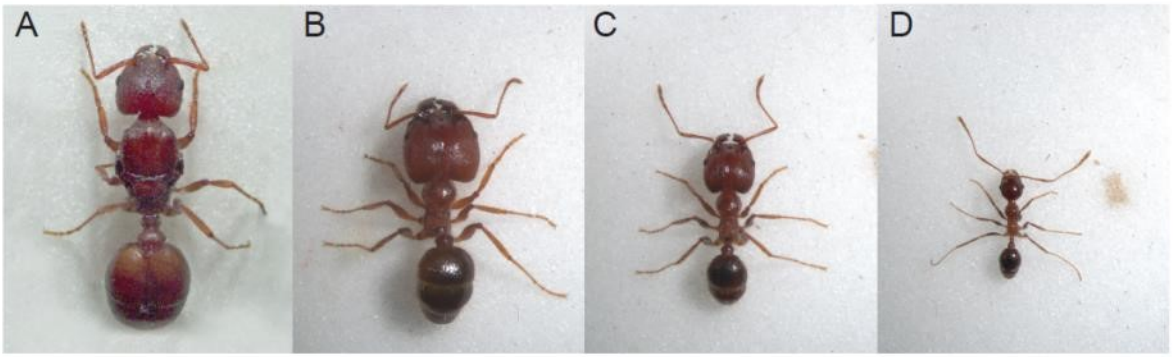

Figure 1.3. Castes and subcastes of *Pheidole obtusospinosa*. (A) Queen, (B) supersoldier, (C) soldier, and (D) minor worker. Images to scale.

Connecting Statement

In Chapter 1, I summarize the many interconnected factors that regulate subcaste ratios in *Pheidole*. This summary provides insight into each component of the system and addresses gaps in our understanding. A major area which needs further study is the negative regulatory influence of soldiers on soldier production. The last study on this influence was conducted in 1984 by Wheeler and Njihout, which established the existence of a soldier inhibitory pheromone. This study raised many questions about the pheromone, including how it is transmitted to brood and what its chemical composition is. In the next chapter, I address these specific questions using a combination of behavioural and chemical techniques.

Chapter 2: Worker caste ratio is regulated through passive and selective contact with soldiers in the ant *Pheidole dentata*

Angelica Lillico-Ouachour, Juergen Liebig, and Ehab Abouheif

ABSTRACT

The generation and regulation of distinct castes is critical for the success of a eusocial insect colony. The ant, *Pheidole dentata,* has two worker subcastes: soldiers and minor workers. Colonies are able to regulate the proportion of soldiers and minor workers depending on both nutritional and pheromonal influences. If larvae consume a high protein diet, the soldier developmental program will initiate. However, if there are already a large number of soldiers in the colony, the soldier program will not initiate. Previous evidence shows that this social regulation occurs through a pheromone produced by adult soldiers in the colony called the 'soldier inhibitory pheromone.' Here, we identify a behavioural mechanism by which soldiers transmit the soldier inhibitory pheromone and uncover potential chemical candidates for the pheromone. We found that transmission occurs through idle contact with specific larval types. This suggests that the inhibitory pheromone is most likely composed of cuticular hydrocarbons, which are the basis of many contact-mediated pheromones known in insects. We analyzed subcaste specific cuticular hydrocarbon profiles and found that minor workers and soldiers are quantitatively but not qualitatively different, with alkanes being over-represented on the soldier cuticle. This raises the possibility that one or some of these alkanes constitute the soldier inhibitory pheromone. Our study marks the first step in uncovering the behavioural and chemical basis of social subcaste regulation in *Pheidole*.

INTRODUCTION

Complex worker caste systems in ants, where the worker caste is composed of physical subcastes that differ in size and morphology, has evolved several times independently during ant evolution (Hölldobler and Wilson 1990). Evolution of complex worker caste systems is generally

associated with ecologically dominant or highly speciose genera, including the big-headed ant (*Pheidole*), the leaf cutter ant (*Atta*), and the carpenter ant (*Camponotus*), and therefore their evolution is thought to be a key factor driving success (Hölldobler and Wilson 1990; Wilson 1979; 1980; 1983; 1984; 2003). Distinct subcastes are thought to allow for greater specialization of each subcaste on particular tasks (Szathmary and Smith 1995). Accordingly, the ratio of different physical subcastes within the colony is dynamically and tightly regulated in response to the ecological and social environment. This response occurs by changing the developmental trajectory of individual workers in the colony because the subcaste fate of an individual worker is determined during development in response to environmental factors like nutrition and pheromones (Nijhout 2003). Therefore, at any given time subcaste ratios are shaped by the dynamic interplay between the ecological environment, social interactions, and individual development (Chapter 1). Yet, we still know little about the behavioural and chemical mechanisms through which social interactions and individual development interact to regulate complex worker caste systems (Chapter 1).

To address this question, we focus on the social regulation of the worker caste in the hyperdiverse ant genus *Pheidole*, which have two female castes: reproductive 'queens' and sterile 'workers.' The worker caste is further subdivided into at least 2 subcastes: 'minor workers' and 'soldiers.' Both minor workers and soldiers carry out day-to-day tasks in the colony (e.g., brood care, foraging, grooming), but minor workers take on the majority of this workload (Mertl and Traniello 2009; Muscedere and Traniello 2012a; Patel 1990; Sempo and Detrain 2010; Wilson 1984). Soldiers preferentially perform specialized defense behaviours and tasks related to food processing, like seed cracking, which minor workers cannot physically

execute (Wilson 1985a). Because of their varied roles, regulating the proportion of each subcaste in a colony becomes important for its success as a whole.

In *Pheidole,* two critical periods determine whether an individual will become a queen, soldier, or minor worker (Figure 2.1). The first critical period occurs during embryonic development where springtime seasonal cues elicit an endocrine response via the juvenile hormone pathway to induce queen development (Passera and Suzzoni 1979). The second critical period occurs during larval development where high quality nutrition elicits a similar endocrine response to induce soldier development (Wheeler and Nijhout 1981b; 1983). However, if the number of soldiers exceeds its colony baseline, which is typically around 5-10% of the colony, the soldier developmental program may be suppressed, resulting in minor workers (Gregg 1942; Passera 1974; Wheeler and Nijhout 1984). This social feedback is thought to be due to a pheromone released by soldiers called the 'soldier inhibitory pheromone' (Wheeler and Nijhout 1984).

The first studies to show that soldiers have an inhibitory effect on soldier production came from studies that perturbed worker subcaste ratios (Gregg 1942; Passera 1974) . When soldiers are in low numbers, the colony increases the number of new soldiers produced (Gregg 1942; Passera 1974). Likewise, when soldiers are in high numbers, the colony decreases the number of new soldiers produced (Gregg 1942; Passera 1974). Wheeler and Nijhout (1984) then confirmed that soldiers are responsible for repressing soldier production by treating larvae with methoprene, a juvenile hormone analogue, and raised these larvae with minor workers in one of three conditions: (1) free soldiers with access to minor workers and brood, (2) caged soldiers with access to minor workers but no access to brood, and (3) no soldiers (Wheeler and Nijhout 1984). Both 'soldier' conditions, including the 'caged' condition, were able to repress soldier

production when compared to the 'no soldier' condition (Wheeler and Nijhout 1984). Because soldiers do not necessarily need direct contact with brood to elicit an inhibitory response, this suggests that soldier inhibition is mediated by an inhibitory pheromone. This pheromone can directly trigger a physiological response in individuals during development to alter their developmental trajectory, or alternatively, it can trigger a behavioural response in minor workers such that minor workers provide larvae with lower quality nutrition to facilitate minor worker development (Butler 1967; Wheeler and Nijhout 1984; Wilson 1963). However, soldiers take care of brood of late stage larvae with similar efficiency to minor workers and perturbations of subcaste ratios do not affect the behavioural repertoire, activity level, or social behaviour of minor workers (Brown and Traniello 1998; Sempo and Detrain 2010; Wheeler and Nijhout 1984). Therefore, current evidence supports that the soldier inhibitory pheromone is a 'primer pheromone' that directly affects larval physiology (Butler 1967; Wheeler and Nijhout 1984).

Primer pheromones are important for the regulation of social insect colonies (Butler 1967; Wilson 1963). For example, several are known to regulate the reproductive division in eusocial insects, including the queen inhibitory pheromone in honeybees, ants, and termites (Butler et al. 1962; Endler et al. 2006; Endler et al. 2004; Holman et al. 2010; Matsuura et al. 2010). However, primer pheromones that regulate non-reproductive castes remain understudied. The best documented case is from termites, where the soldier caste emits a primer pheromone to inhibit other individuals from developing into soldiers (Haverty and Howard 1981; Lefeuve and Bordereau 1984; Lüscher 1972; Park and Raina 2003; Park and Raina 2004).

We therefore investigated the behavioural and chemical mechanisms underlying the soldier inhibitory pheromone in *Pheidole dentata*. First, we attempt to uncover how the soldier inhibitory pheromone is behaviourally transmitted from soldiers to the brood by identifying (a)

whether soldiers actively interact with brood or passively contact brood, and (b) whether soldiers are selective with respect to the developmental stage of larvae they interact with. To do this, we tracked and monitored the behavioural interactions of workers in small colonies that consist of 100% minor workers or 100% soldiers with larvae prior to the critical period (bipotential larvae) and larvae slightly after initiation of the soldier program (soldier-destined larvae) (Figure 2.1). Second, we analyzed differences between chemicals produced by minor workers and soldiers that fit with our behavioural transmission model to reveal potential candidates for the soldier inhibitory pheromone.

MATERIALS AND METHODS

Ant collection and colony care

For both our behavioural and chemical work, we used whole colonies of *P. dentata* collected from Gainesville, Florida, USA. The colonies were kept in plastic boxes lined with Fluon®. Artificial nests were made from glass test tubes filled half-way with water and plugged with cotton. Ants were fed meal worms, fruit flies, sugar water, and Bhatkar-Whitcomb diet (Bhatkar and Whitcomb 1970). Colonies were kept in a Conviron environmental chamber (Controlled Environments Ltd., Winnipeg) maintained at 27°C, 70% humidity, and 12 hour day:night cycle.

Larval classifications in **P. dentata**

To test when the critical period that mediates minor worker and soldier development occurs in *P. dentata*, we topically treated 3 different larval size classes with a juvenile hormone analogue, methoprene (5 mg/mL; Sigma-Aldrich). We chose these size classes based on critical periods found in other *Pheidole* species: 0.7-0.9 mm, 1.0-1.29 mm, and 1.3-1.59 mm (Rajakumar et al.

2012; Wheeler 1982). As a control, we applied acetone. We reared the methoprene- and acetone-treated larvae in replicate boxes; each of which contained, at minimum, a 1 minor worker for every larva in the replicate. We took measurements of the head (mm) and body size (mm) for the resulting pupae using a Zeiss Discovery V12 stereomicroscope with Zeiss Axiovision software. Because allometry of the head:body is the defining feature of the soldier subcaste, we characterized induction of the soldier program as successful if methoprene was able to significantly increase the head:body ratio of the resulting pupae compared to acetone treatment. We analyzed head:body measurements using a Student's t-test. Soldier-destined larvae were classified as such if they were in the 3rd larval instar and bigger than minor workers in the pre-pupal stage (Wheeler 1982).

Behavioural set-up and video monitoring

We constructed small colonies in a pair-wise design of (1) 100% minor workers and (2) 100% soldiers, each with equal numbers of bipotential and soldier-destined larvae to compare whether minor workers and soldiers treat these types of brood differently. Specifically, minor workers or soldiers, bipotential, and soldier-destined larvae were isolated from source colonies and put into circular arenas (6 cm diameter) in 10:5:5 or 8:4:4 ratios, respectively. The side of each arena was lined with Fluon® and the bottom of each arena had a thin layer of dental plaster (approximately 1 cm). After a 1 hr acclimation period, 30 min videos were taken on a Canon HD Camcorder VIXIA HF M52 with ring light every 3 hr, starting at 9:00 a.m., for 48 hr total. We chose a 48 hr monitoring period because this is approximately the amount of time it takes for soldier-destined larvae to move into the 'pre-pupae' developmental stage and be fully determined (Wheeler 1982). Dental plaster was rehydrated with water every 3 hr and ants were fed with half of a

mealworm every 12 hr. For the duration of monitoring, we kept replicates in a growth chamber maintained at 27°C, 70% humidity, and 12 hour day:night cycle.

Scoring of behaviour and tracking of movement

We made instantaneous behavioural and interactional observations (1 sec) for every adult every 5 min (at 1, 6, 11, 16, 21, 26 min) in each 30 min video based on Table 2.1. 'Behaviour' refers to the behavioural task performed and 'interaction' refers to the social or physical interaction of workers. These observations were recorded in J Watcher Version 1.0. We analyzed the tracking of individual movement using EthoVision XT 11.5 for 3 pairs of soldier and minor worker replicates. Zones were drawn around the brood (brood zones) and the cumulative time that nurses spent within each zone for a 5 min period was recorded and depicted in a heatmap. We analyzed these results with a two-way ANOVA, followed by a Student's t-test for planned comparisons between bipotential and soldier-destined larvae or a Tukey's honestly significantly different (HSD) post hoc test for all other unplanned comparisons.

Chemical extraction of worker profiles and analysis

To identify potential chemical candidates, we analyzed the cuticular hydrocarbon profiles of soldiers and minor workers for 11 different colonies by hexane extraction. We used 30 minor workers and 10 soldiers to obtain a comparable biomass for analysis. Samples were frozen at -80°C and placed in a Teflon-capped borosilicate glass vial containing 50 µl hexane (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. High-purity nitrogen was used to dry the extracts. Once completely dry, the extracts were re-suspended in 4 µl hexane. 1 µl aliquots of the hexane extracts were injected into an Agilent 6890N GC (Agilent, Santa Clara, CA, USA) coupled with

an Agilent 5975 mass selective detector, operated in the electron impact ionization mode. The GC was operated in splitless injection mode with helium as carrier gas at 1ml min^{-1} flow rate. It was fitted with a 30m x 0.25mm (ID) x 0.1µm DB-1MS non-polar column (Agilent). The oven temperature was programmed to rise from 60 to 200° C at 40° C min^{-1} after an initial delay of 2 min including a splitless time of 0.5 min. Subsequently, the temperature rose from 200 to 320° C at 5° C min^{-1}. Injector temperature was 260° C, MS quad 150° C, MS source 230° C, and transfer line 300° C. Using typical fragmentation patterns, we identified alkanes, alkenes and alkadienes in the mass spectra and their peak areas determined. For each sample, we summed up the peak areas for alkanes and for alkenes separately, calculated the index total alkane/(total alkane plus total alkenes), and analyzed these proportions using a Student's t-test.

RESULTS

Determination of critical periods for bipotential and soldier-destined larvae in **P. dentata**

Our behavioural experiments require being able to reliably identify 'bipotential' and 'soldier-destined' larvae. Although the critical period for soldier determination has been identified in other *Pheidole* species, this period remains unknown in *P. dentata*. We therefore applied methoprene, a juvenile hormone analogue, to varying larval size classes to determine this critical period. We found that soldiers developed after application of methoprene in all three larval size classes (Table 2.2). In a closely-related *Pheidole* species the critical period has been narrowed down a span of 0.6 mm in range during the 4th larval instar (Rajakumar et al. 2012), whereas the period we only narrowed down the critical period to a span of 0.9 mm (0.7 to 1.59 mm). Therefore, we used a narrower, more conservative size range (0.9 to 1.39 mm), to classify larvae as 'bipotential' for our behavioural experiments because this corresponds to the mid-range of *P.*

denata and is consistent with range found in other *Pheidole* species. In contrast, we classified larvae as 'soldier-destined' if they were in the last larval instar and larger than minor workers in the pre-pupal stage, which was approximately from 2.2-2.75 mm.

Subcastes have similar behavioural repertoires but differ in time allocated to each task

To determine if there are differences between how soldiers treat bipotential and soldier-destined larvae from how minor workers treat them, we recorded a total of 8003 behavioural interactions for minor workers and 9072 behavioural interactions for soldiers. Minor workers performed 26 types of compound behaviours, while soldiers performed 24 types (an overlap of 92% of the minor worker repertoire), excluding ambiguous interactions with unclear larvae. Although the behavioural repertoires of minor workers and soldiers are similar, the proportion of time that minor workers and soldiers spend on each behavioural interaction varies (Table 2.3).

Soldiers passively contact brood through idling and aggregating on the brood pile

We asked whether transmission of the inhibitory pheromone to the brood relies on contact with soldiers. Therefore, we tracked how much time soldiers spend in the vicinity of the brood. We found that soldiers tend to spend more cumulative time in the brood zone than minor workers ($p = 0.0068$; Figure 2.2A-C). This result is confirmed by analyzing the amount of direct contact made by each subcaste; soldiers contact brood more than minor workers overall ($p < 0.001$; Figure 2.2D). Therefore, we conclude that soldiers spend more time contacting brood than minor workers.

To determine whether soldiers actively interact with brood or passively contact them, we compared the brood-associated behaviours of minor workers and soldiers. If soldiers transmit the

inhibitory pheromone through active interactions with brood, then in colonies consisting of 100% soldiers, we expect soldiers to perform at least one directed interaction—grooming, feeding, or carrying—with larvae more than when colonies consist of 100% minor workers. In contrast, if soldiers transmit the pheromone through passive contact with brood, then when colonies consist of 100% soldiers we expect soldiers to contact brood haphazardly by more walking and idling on brood than when colonies consist of 100% minor workers. We found that minor workers and soldiers are not significantly different in their active behavioural interactions with brood, like feeding, carrying, and grooming ($p < 0.05$). In contrast, we discovered significant differences in passive brood contact between minor workers and soldiers. Minor workers walk on larvae more than soldiers ($p = 0.009$) and soldiers idle on larvae more than minor workers ($p < 0.001$; Figure 2.3A). However, there is a dramatic difference between soldiers and minor workers with respect to idling but not walking ($p < 0.05$; Figure 2.3B). These results show that soldiers transmit the soldier inhibitory pheromone by engaging in persistent and passive contact with brood.

Our analyses reveal that soldiers interact passively with brood more than minor workers. This suggests that soldiers are aggregating passively on the brood pile. To test this hypothesis, we analyzed other behaviours and interactions which would support an aggregation hypothesis. Soldiers spend more time in contact with nestmates and idling, whereas minor workers spend more time alone and walking ($p < 0.05$; Figure 2.4). The close contact between soldiers with their nestmates and brood shows that soldiers transmit inhibitory pheromone to brood through passive idling and aggregation behaviour on the brood pile.

Soldiers are passively and selectively contacting soldier-destined larvae

To test whether soldiers show bias in the larvae that they contact, we asked whether workers and soldiers treat bipotential or soldier-destined larvae differently. If soldiers are biased in the type of brood they contact, then in the 100% soldier experiment: (1) soldiers should interact with soldier-destined larvae more than bipotential larvae, which evaluates whether there is differential brood treatment at all; and (2) the 100% soldier experiment should show a greater difference in brood treatment than in the 100% minor workers experiment, which evaluates whether soldier-destined larvae inherently require more attention than bipotential larvae. Both criteria must be fulfilled to support a hypothesis of selective transmission of the inhibitory pheromone. Alternatively, non-biased transmission is supported if one or both criteria are not met.

We found that soldiers interact with soldier-destined larvae more than bipotential larvae (Figure 2.5A). Although this relationship is also significant for minor workers ($p < 0.001$; Figure 2.5A). Because we previously determined that soldiers interact with brood passively through idling, we analyzed whether this bias was dependent on idling behavior. Soldiers only interact more with soldier-destined larvae than bipotential larvae through idling ($p < 0.001$; Figure 2.5B). This is also the case for absolute amount of time spent with soldier-destined and bipotential larvae ($p < 0.05$; Figure 2.6). Again, we found that minor workers interact relatively more with soldier-destined larvae through passive interactions, like walking and idling, but they spend less absolute time idling with these larvae compared to soldiers ($p < 0.05$; Figure 2.5B, 2.6). Together, we can infer that there is differential treatment through passive contact by both subcastes, where soldier-destined larvae are favoured over bipotential larvae.

To test whether differential larval treatment is due to the inherent nature of caring for the larger-sized soldier-destined larvae, we compared minor worker and soldier interactions with the larval types. Because idling was the only behavioural interaction for soldiers that drove

differential larval treatment, we analyzed whether the difference in treatment was greater than when colonies consist only of minor workers. In fact, the difference between bipotential larvae and soldier-destined larvae for minor workers and soldiers is significantly different ($p = 0.021$; Figure 2.5C). Our results therefore show a true preference for soldier-destined larvae by soldiers that is not due to the inherent nature of soldier-destined larvae themselves.

Minor workers and soldiers differ quantitatively in their cuticular hydrocarbon profiles

The passive soldier contact with brood suggests that soldiers are transmitting a contact pheromone. Because cuticular hydrocarbons are often contact pheromones in insects, we analyzed the cuticular hydrocarbons of soldiers and minor workers to reveal potential chemical candidates that fit with our behavioural model. Minor workers and soldiers have qualitatively similar cuticular hydrocarbons profiles, as described in Figure 2.7 and legend. However, minor workers and soldiers differ quantitatively between the relative amount of alkanes (saturated hydrocarbons) and alkenes (unsaturated hydrocarbons) on their cuticle in their profiles (Figure 2.8). Specifically, soldiers have a significantly higher percentage of alkanes to total hydrocarbons compared to minor workers ($p < 0.001$; Figure 2.8). This quantitative difference suggests that alkanes may be potential candidates for the soldier inhibitory pheromone.

DISCUSSION

The primary goal of our study was to identify the behavioural and chemical mechanisms underlying transmission of the soldier inhibitory pheromone to brood. Behaviourally, we approached this question by studying the types of interactions soldiers have with larvae and identifying potential larval biases that they have. From our results, both soldiers and minor

workers devote more interactions to soldier-destined larvae compared to bipotential larvae. When we break down the behaviours included within these interactions, we see that soldiers spend more time idling on soldier-destined larvae than bipotential larvae. Our results support a 'passive selective' transmission of the inhibitory pheromone because: (1) soldiers interact with soldier-destined larvae more with respect to idling behaviours than bipotential larvae, and (2) these interactions are significantly different from minor workers. Idling is not an involved larval centric interaction (thus, 'passive'), yet differential treatment is evident and appears to be due to the aggregation of soldiers on the brood pile and soldier-destined larvae in particular. This passive selective mechanism is supported by analyses of movement into the brood zone and other social interactions of workers. First, soldiers tend to spend more cumulative time on or near the brood than minor workers. Second, soldiers dedicate a greater proportion of their total interactions to brood and other nestmates (usually idle), whereas minor workers spend a greater proportion of time alone (usually exploring the arena). Combined, this implies that soldiers tend to cluster on the brood more than minor workers and reveals a general pattern of inactivity of soldiers compared to minor workers.

A passive selective mode of pheromonal transmission that relies heavily on idling suggests that the transmission of the soldier inhibitory pheromone is rooted in general contact, as Wheeler and Nijhout (1984) originally proposed. Brood has the potential to act as an attractant for workers in social insects (Sempo et al. 2006; Walsh and Tschinkel 1974) and workers exhibit some ability to recognize them (Brian 1975; Free and Winder 1983; Hare 1996). In *Pheidole pallidula,* Sempo et al. (2006) have shown that brood indeed enhances and nucleates worker aggregation and suggest the aggregation of soldiers is driven by attraction to soldier-destined larvae. Furthermore, Sempo and Detrain (2010) found differences in the way that *Pheidole*

pallidula workers treat brood depending on developmental stage. In our study, all workers were attracted to brood and both minor workers and soldiers displayed a particular affinity for soldier-destined larvae over bipotential larvae. This suggests that not only do minor workers and soldiers have the ability to recognize brood and modulate their aggregation response but they also have the ability to discriminate between them as well. However, in *Pheidole* it remains unclear whether the distinction is based on specific or relative concentrations of pheromones, neediness, size, and/or morphology of larvae (Brian 1975; Robinson and Cherrett 1974). Furthermore, because soldiers interact with brood more than minor workers in general, soldiers may have a lower threshold for any of these hypothetical signals, resulting in a pattern of aggregation and heightened transmission of the soldier inhibitory pheromone through contact.

'Contact chemoreception' is contingent upon relatively non-volatile surface pheromones that must be transmitted through direct contact in order to be perceived by the intended receiver (Butler 1967; Städler 1984; Wilson 1963). These pheromones are implicated in a number of types of behaviours including mating (Ginzel et al. 2003; Ginzel and Hanks 2003; Gleeson 1980; Hegdekar and Dondale 1969; Snell et al. 1995), foraging (Leoncini et al. 2004), food exchange (Ali and Morgan 1990; Wilson 1965), and recognition (Singer 1998; Slessor et al. 2005; Walsh and Tschinkel 1974). More importantly, contact pheromones can elicit the physiological responses of primer pheromones. The most notable is perhaps the honeybee queen mandibular pheromone, which is responsible for suppressing worker reproductive potential (Slessor et al. 2005). Although most honeybee pheromones (e.g., alarm and swarming signals) diffuse and transmit readily in air, the queen requires direct contact in the form of grooming and antennation with her workers for effective transmission of the queen mandibular pheromone (Naumann et al. 1991; Slessor et al. 2005). In the carpenter ant, *Camponotus floridanus*, the cuticular

hydrocarbons of queen-laid brood signal queen presence and regulate the reproductive status of workers (Endler et al. 2004). Because these semiochemicals are waxy surface substances, they are likely transmitted through passive contact between brood and nurses. It is conceivable that communication of the soldier inhibitory pheromone in *P. dentata* requires a passive means of contact chemoreception, similar to the carpenter ant.

As many contact-mediated pheromones rely on chemoreceptors recognizing waxy substances on the cuticle of individuals (Wilson 1965), we analyzed the cuticular hydrocarbon profiles of minor workers and soldiers to reveal potential candidates for the soldier inhibitory pheromone. These chemicals are produced directly in the epidermis by secretory cells called 'oenocytes' (Diehl 1973; 1975; Fan et al. 2003; Makki et al. 2014; Romer 1980), and are subsequently the largest chemical class found on the insect cuticle (Nelson and Blomquist 1995). Although we did not identify any unique cuticular hydrocarbons in soldiers, we did find relative quantitative differences between the proportion of alkanes and alkenes in their profiles: minor workers have a higher proportion of unsaturated alkenes, whereas soldiers have a higher proportion of saturated alkanes. Relative differences in hydrocarbons are used by many social insects to gather information and trigger particular responses (Conte and Hefetz 2008; Greene and Gordon 2007; Howard and Blomquist 2005). For example, behavioural castes in the red harvester ant, *Pogonomyrmex barbatus*, differ in the mixture of hydrocarbons on their cuticle: patrolling foragers have more alkanes compared to alkenes and branched hydrocarbons than nest maintenance workers (Wagner et al. 1998). Individuals have the ability to discern morphological castes based on this profile and appropriately adjust their behaviour (e.g., in response to a patroller's profile entering the nest, the colony will increase its foraging activity) (Greene and Gordon 2003; Wagner et al. 1998; Wagner et al. 2000; Wagner et al. 2001). Studies in ants and

other eusocial insects support the signalling of cuticular hydrocarbons for similar behavioural and physiological purposes (Dani et al. 2001; Dietemann et al. 2003; Endler et al. 2004; Lahav et al. 1999; Thomas et al. 1999).

So far, the only work on primer responses in ants to cuticular hydrocarbons comes from studies on reproductive regulation (Liebig 2010). This work focuses on how the queen caste suppresses worker fertility to maintain a reproductive division of labour (de Biseau et al. 2004; Dietemann et al. 2003; Endler et al. 2006; Endler et al. 2004; Liebig 2010; Smith et al. 2008). In contrast with other ants (Dietemann et al. 2003; Smith et al. 2009; Smith et al. 2008), highly dimorphic societies like those of *C. floridanus* and *Linepithema humile* appear to regulate worker fertility by way of queen-derived alkanes (de Biseau et al. 2004; Endler et al. 2006; Endler et al. 2004; Liebig 2010). Our behavioural and chemical work suggests that soldier-produced alkanes have the potential to repress the development of soldiers in a similar way. Because *Pheidole* workers do not have ovaries and are completely sterile, if the alkanes of soldiers are found to be truly inhibitory, our data may suggest that alkanes have been co-opted for a secondary communicative function in polymorphic societies. This novel role may mirror the co-option of an additional juvenile hormone sensitive period that gives rise to soldiers during development in *Pheidole*. Future studies will need to assess the role of alkanes on individual development to test this hypothesis. The most pertinent experiment would be to test whether application of soldier cuticular hydrocarbons can indeed repress soldier development in larvae. If this is true, the next logical step would be to identify which hydrocarbons, alkanes or otherwise, are responsible for the inhibitory effect.

In conclusion, we have uncovered a potential behavioral mechanism for the transmission of the solider inhibitory pheromone in *Pheidole*: express contact with soldier-destined larvae.

This behavioural mode of transmission by soldiers raises questions about the relative attractiveness of brood to workers and suggests that the type of chemical being communicated is likely, to some extent, non-volatile. We have also identified relative differences in the non-volatile cuticular hydrocarbon signatures of soldiers from minor workers that could potentially trigger a primer response in larvae. In the future, it will be important to conduct bioassays that link the cuticular hydrocarbons of soldiers to a developmental inhibitory function for validation of this proposed mechanism. Our research brings us one step closer to elucidating the socio-environmental factors—behavioural and chemical—that generate and regulates the subcaste composition in *Pheidole,* a key factor in the ecological and evolutionary success of this hyperdiverse genus.

ACKNOWLEDGEMENTS

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Table 2.1. Operational definitions of behaviour types with potential interactions. 'Behaviour type' refers to the behavioural task performed and 'potential interaction type' refers to its logical social or physical interaction.

behaviour type	operational definition	potential interaction type
aggression	displays aggressive behaviour	nestmate
antennate	displays antennal contact	bipotential larva, soldier-destined larva, nestmate, dead nestmate, food
carry	transports 'potential interaction type' in its mouth or rolls it around	bipotential larva, soldier-destined larva, nestmate, dead nestmate, food
feed	trophallaxis or consumes solid food	bipotential larva, soldier-destined larva, nestmate, self, food
groom	runs mouthparts across 'potential interaction type'	bipotential larva, soldier-destined larva, nestmate, self
idle	remains stationary	bipotential larva, soldier-destined larva, nestmate, dead nestmate, self, food
walk	walks	bipotential larva, soldier-destined larva, nestmate, dead nestmate, self, food

Table 2.2. The effect of methoprene on larval development by size class. Comparisons between acetone and methoprene treatments made per size class.

size range (mm)	treatment	mean head:body ratio	standard error	sig ($p < 0.05$)
0.70 - 0.99	acetone	0.49	0.01	yes
0.70 - 0.99	methoprene	0.63	0.05	
1.0 - 1.29	acetone	0.51	0.01	yes
1.0 - 1.29	methoprene	0.53	0.01	
1.3 - 1.59	acetone	0.49	0.00	yes
1.3 - 1.59	methoprene	0.55	0.01	

Table 2.3. Fraction of behaviours performed by minor workers and soldiers in each treatment.

behaviour by interaction type	minor	soldier
bipotential larva	**0.080**	**0.14**
aggression	0.0000	0.0000
antennate	0.00012	0.00034
carry	0.0016	0.00058
feed	0.0000	0.0000
groom	0.0055	0.0028
idle	0.047	0.12
walk	0.027	0.014
soldier-destined larva	**0.18**	**0.26**
aggression	0.0000	0.0000
antennate	0.0030	0.00090
carry	0.0022	0.0010
feed	0.0000	0.0000
groom	0.016	0.010
idle	0.11	0.22
walk	0.048	0.02
nestmate	**0.16**	**0.36**
aggression	0.0000	0.0000
antennate	0.014	0.0036
carry	0.0000	0.00097
feed	0.0019	0.00042
groom	0.0026	0.0064
idle	0.067	0.30
walk	0.078	0.054
self	**0.51**	**0.21**
feed	0.0000	0.0000
groom	0.055	0.034
idle	0.18	0.11
walking	0.28	0.064
food	**0.062**	**0.023**
antennate	0.0059	0.0013
carry	0.00011	0.0000
feed	0.0011	0.00029
idle	0.0099	0.0099
walk	0.0450	0.0117
dead nestmate	**0.0031**	**0.00043**
antennate	0.00093	0.0000
carry	0.0000	0.0000
groom	0.0000	0.0000
idle	0.00020	0.0000
walk	0.0020	0.00043
unclear larva	**0.0000**	**0.0067**
aggression	0.0000	0.0000
antennate	0.0000	0.0000
carry	0.0000	0.0000
feed	0.0000	0.0000
groom	0.0000	0.00011
idle	0.0000	0.0065
walk	0.0000	0.0000

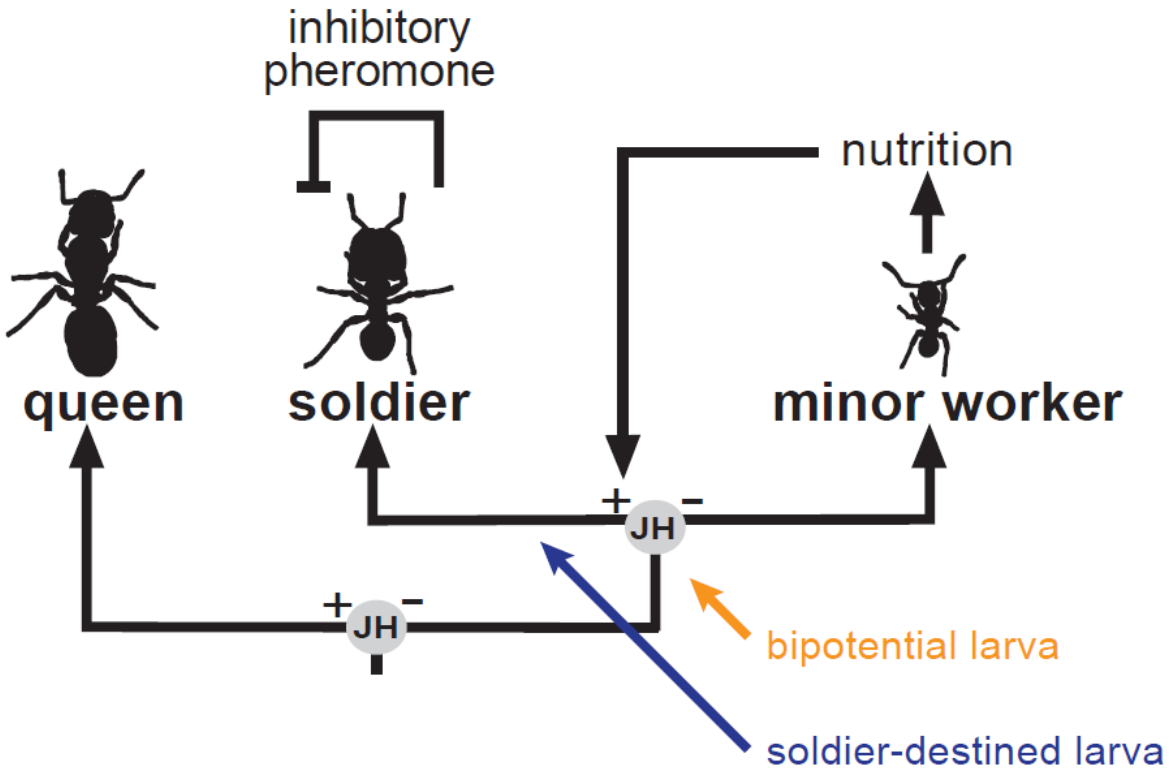

Figure 2.1. Individual development in *Pheidole.* Females undergo a series of developmental switches mediated by levels of juvenile hormone (JH), where '+' indicates JH has surpassed the threshold of activation and '–' indicates JH is below the threshold of activation. Black lines with arrowheads indicate activation, while black lines with perpendicular bars indicate repression. The orange arrow indicates the period where larvae are termed 'bipotential' and the blue arrow indicates the period where larvae are termed 'soldier-destined.'

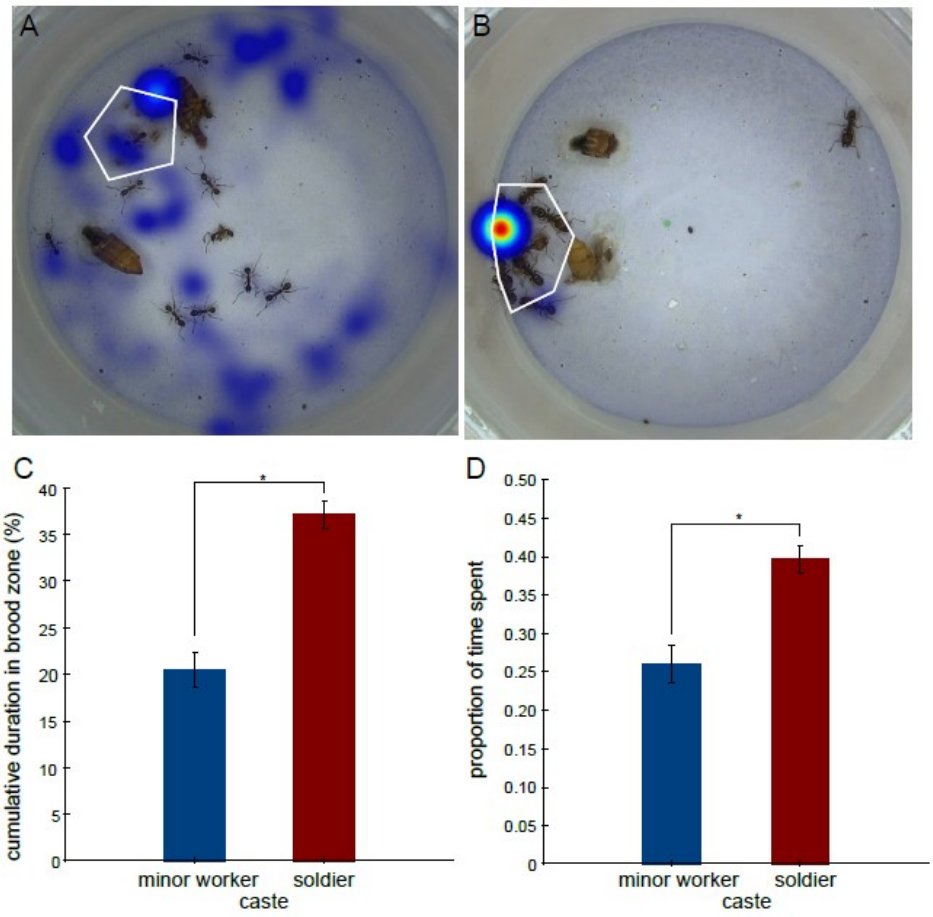

Figure 2.2. Soldiers spend more time with brood than minor workers. (A) Heatmap visualization of a single soldier's activity. White lines mark the 'brood zone.' The colour gradient in the heatmap begins with blue to represent low activity and ends with red to represent high activity. (B) Heatmap visualization of a single minor worker's activity. White lines mark the 'brood zone.' The colour gradient in the heatmap begins with blue to represent low activity and ends with red to represent high activity. (C) Cumulative time minor workers and soldiers spend in the brood zone. Significant differences between means are indicated with an asterisk and based on a Student's t-test ($p < 0.05$). (D) Proportion of time directly interacting with brood for minor workers and soldiers. Significant differences between means are indicated with an asterisk and based on a Student's t-test ($p < 0.001$).

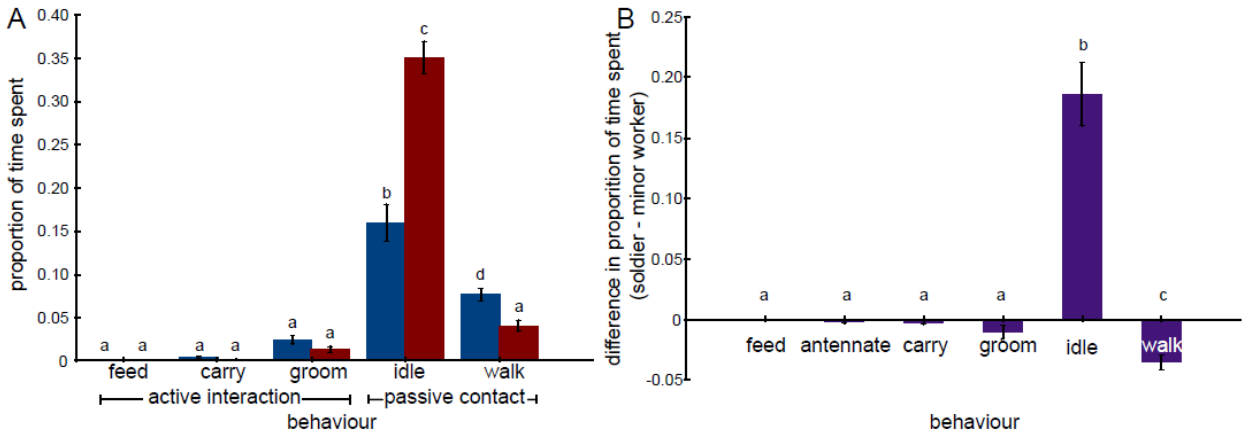

Figure 2.3. Soldiers spend more passive time with brood than minor workers. (A) Proportion of time devoted to active larval interactions, including feeding, grooming, and carrying, and passive larval interactions, including idling and walking. Blue bars indicate minor workers. Red bars indicate soldiers. Means with the same lowercase letter are not significantly different based on a Student's t-test analysis ($p < 0.05$). (B) Difference in proportion of time spent on larvae for soldiers and minor workers. Means with the same lowercase letter are not significantly different based on a Student's t-test analysis ($p < 0.05$).

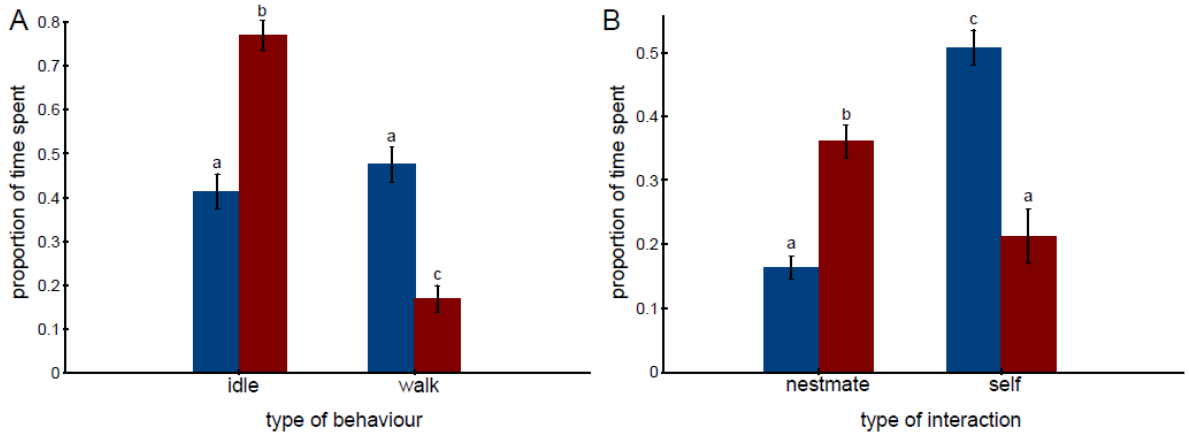

Figure 2.4. Soldiers aggregate on the brood. (A) Proportion of time devoted to idling and walking behaviours of minor workers and soldiers. (B) Proportion of time devoted to interactions with nestmates compared to time spent alone. Blue bars indicate minor workers. Red bars indicate soldiers. Means with the same lowercase letter are not significantly different based on a Tukey HSD test ($p < 0.05$).

Figure 2.5. Soldiers are biased with respect to the brood that they interact with. (A) Proportion of time devoted to interactions with bipotential and soldier-destined larvae. Blue bars indicate minor workers. Red bars indicate soldiers. Means with the same lowercase letter are not significantly different based on a Student's t-test analysis ($p < 0.05$). (B) Proportion of time devoted to idling behaviours with bipotential and soldier-destined larvae. Orange indicates bipotential larvae. Dark blue indicates soldier-destined larvae. Means with the same lowercase letter are not significantly different based on a Student's t-test analysis ($p < 0.05$). (C) Difference in idling behaviours of minor workers and soldiers for bipotential vs. soldier-destined larvae. Significant differences between means are indicated with an asterisk and based on a Student's t-test ($p < 0.001$).

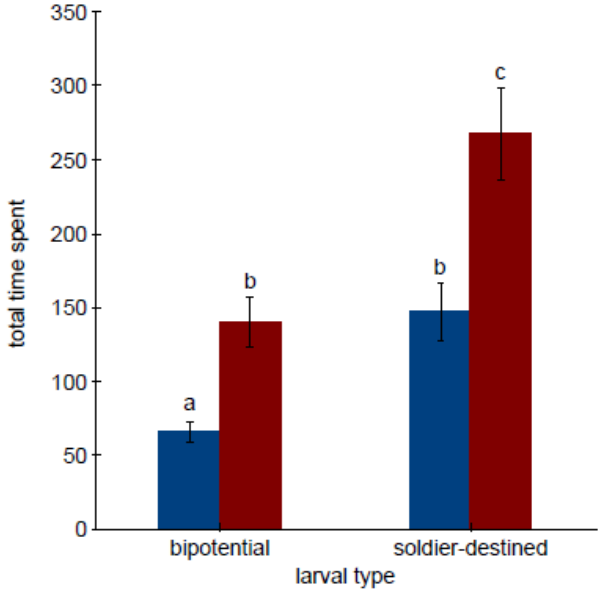

Figure 2.6. Soldiers spend more time with soldier-destined brood. Absolute time devoted to interactions with bipotential and soldier-destined larvae. Blue bars indicate minor workers. Red bars indicate soldiers. Means with the same lowercase letter are not significantly different based on a Student's t-test analysis ($p < 0.05$).

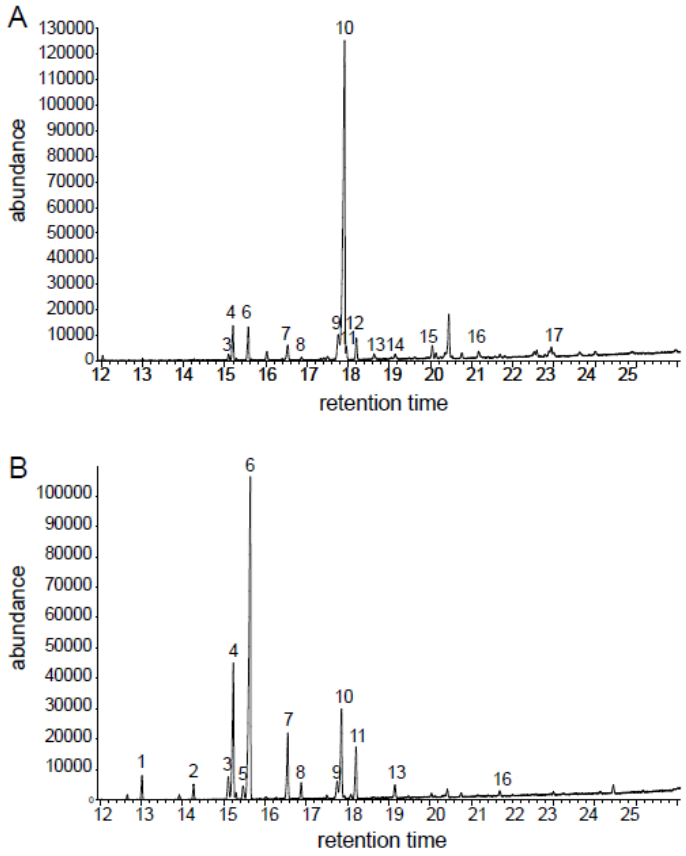

Figure 2.7. Chromatograms representing the cuticular hydrocarbons of (A) minor workers and (B) soldiers. The compounds have been identified on the basis of retention times. *1*, *n*-tricosane; *2*, *n*-tetracosane; *3*, x,y-dipentacosene; *4,* x-pentacosene; *5,n*-pentacosane; *6*, 3-methyl-pentacosane; *7*, *n*-hexacosane; *8*, x,y-diheptacosene; *9,* x-heptacosene; *10, n*-heptacosane; *11,* 13-methyl-heptacosane; *12,* 3-methyl-heptacosane; *13*, x,y-dinonacosene; *14,* x-nonacosene; *15*, 3-methyl-nonacosane; and *16*, x-hetriacontene.

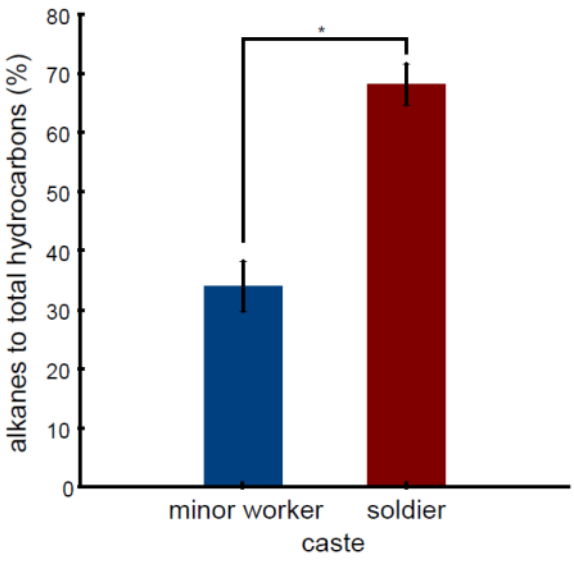

Figure 2.8. Soldiers have more alkanes on their cuticles than minor workers. Blue bars indicate minor workers. Red bars indicate soldiers. Significant differences between means are indicated with an asterisk based on a Student's t-test analysis ($p < 0.001$).

Connecting Statement

In Chapter 2, I established that the transmission of the soldier inhibitory pheromone is through direct contact with larvae. This suggests that the soldier inhibitory pheromone is derived from saturated cuticular hydrocarbons, which are produced in secretory cells called 'oenocytes' (Diehl 1973; 1975; Fan et al. 2003; Romer 1980). However, many pheromones are produced from glands in the insect head, such as the mandibular gland in honeybees (Attygalle and Morgan 1984; Billen 2009; Jackson and Morgan 1993). This gland is responsible for the production of the queen mandibular pheromone, which inhibits worker reproduction (Butler et al. 1962; Huang et al. 1998; Naumann et al. 1991). It may, therefore, be possible that the soldier inhibitory pheromone is produced within the mandibular glands in the soldier head. In the next chapter, I test this possibility by comparing the glands of the head between the minor workers and soldiers to identify whether there are any differences that may account for the production of soldier inhibitory pheromone. If soldiers have significantly larger glands, like the mandibular glands, compared to minor workers, then this finding would be consistent with a soldier inhibitory pheromone that is chemically more similar to the honeybee queen mandibular pheromone. Alternatively, if soldiers have smaller or similar-sized glands compared to minor workers, then this finding would be consistent with my finding in Chapter 2 that the soldier inhibitory pheromone is a cuticular hydrocarbon. Although my goal was to compare the glands, I also compared other structures in the head, which resulted in a manuscript that broadly describes internal head morphology between subcastes across four *Pheidole* species.

Chapter 3: Internal head morphology of minor workers and soldiers in the hyperdiverse ant genus *Pheidole*

Angelica Lillico-Ouachour, Brian Metscher, Tomonari Kaji, and Ehab Abouheif

ABSTRACT

The hyperdiverse ant genus *Pheidole* is remarkably successful with over 1015 species in a single genus. A defining feature shared by all species of *Pheidole* is its worker caste that has evolved a 'minor worker' subcaste and an evolutionarily novel 'soldier' subcaste. Soldiers are bigger in size than minor workers and have disproportionately large heads in comparison. Head and body size are one the most evolutionary variable traits between species in *Pheidole*, and therefore, the evolution of the big-headed soldiers in this genus is thought to be a key factor contributing to its success. Research on the morphological distinction and evolution of subcastes has largely been limited to external anatomy. Here, we compare the internal anatomy of heads within 4 *Pheidole* species: *P. dentata, P. hyatti, P. moerens,* and *P. spadonia*. Using microCT scans, we reconstruct the major glands of the head as well as the musculature, nervous system, and digestive organs. We found that muscle is larger in soldiers and comes at the expense of the nervous system. Not only do we hope that our research increases interest in explaining these patterns in *Pheidole*, but we also hope that our work generates more interest in the study of internal allometric relationships.

INTRODUCTION

Ants have an incredible amount of phenotypic diversity both within and between species because of morphological allometry, or the scaling relationship between body parts (Gould 1966; Lande 1979; Mirth et al. 2016; Shingleton et al. 2007). Every ant colony has a reproductive division of labour and consists of the 'queen' caste, primarily responsible for reproduction, and the 'worker' caste, primarily responsible for all other tasks in the colony (Hölldobler and Wilson 1990; 2009). In addition to a reproductive division of labour, several taxa have evolved

morphologically distinct subcastes within the worker caste (Hölldobler and Wilson 1990; Wilson 1979; 1980; 1983; 1984; 2003). These physical worker subcastes are characterized by size and allometric differences, which allow workers to more effectively perform specific tasks in the colony (Hölldobler and Wilson 1990; Pie and Traniello 2007; Schmid-Hempel 1992; Wilson 1979; 1984; 1985b; Yang et al. 2004).

In the hyperdiverse ant genus *Pheidole*, the worker caste has, at minimum, a 'minor worker' and a 'soldier' subcaste (Hölldobler and Wilson 1990; Wilson 2003). Minor workers perform the majority of tasks within a colony, including foraging, brood care, and nest maintenance, while soldiers usually concentrate on defense and specific foraging-associated duties (Brown and Traniello 1998; Hölldobler and Wilson 1990; Sempo and Detrain 2010; Wilson 1985a). Physically, minor workers and soldiers differ in size, where minor workers are smaller than soldiers; however, the defining feature of these subcastes lies with their heads (Hölldobler and Wilson 1990; Pie and Traniello 2007; Wilson 2003). Although soldiers are relatively large, their heads are disproportionately even more large (Hölldobler and Wilson 1990; Pie and Traniello 2007; Wilson 2003).These massive heads empower soldiers to specialize on tasks that require the strength of their sizable jaws, like the crushing of seeds for food (Hölldobler and Wilson 1990). The difference is even more dramatic when we consider those *Pheidole* species that have evolved a third 'supersoldier' subcaste with even larger heads (Huang 2012; Wilson 2003). For these reasons, the evolution of soldiers is thought to be a key factor contributing *Pheidole*'s success and has even led to the group's nickname as the 'big-headed ants' (Hölldobler and Wilson 1990; Wilson 2003).

Myrmecologists have primarily focused on the external size differences between *Pheidole* subcastes (Hölldobler and Wilson 1990; Pie and Traniello 2007; Wilson 2003). With

advances in technology in recent years, some researchers have begun to enumerate the many differences in internal anatomy as well (Ilieş et al. 2015; Muscedere and Traniello 2006; Muscedere et al. 2011a; Seid et al. 2008; Seid et al. 2005; Seid and Traniello 2005). This work has focused on describing anatomical and physiological differences in muscle and brain structure because of their importance in executing tasks required for division of labour (Ilieş et al. 2015; Muscedere and Traniello 2006; Muscedere et al. 2011a; Seid et al. 2008; Seid et al. 2005; Seid and Traniello 2005). However, cephalic glands have been largely neglected. These glands are important for the production of digestive enzymes and social pheromones, including alarm and recognition pheromones, which also influence worker behaviour and physiology (Ali et al. 1988; Attygalle and Morgan 1984; Billen 2009).

Here, we compare the heads of minor workers and soldiers in 4 *Pheidole* species (*Pheidole dentata, Pheidole hyatti, Pheidole moerens,* and *Pheidole spadonia*) to identify whether disproportionate external size differences in subcastes correspond to internal anatomy. Specifically, we use microCT scans to reconstruct the cephalic glands (mandibular, propharyngeal, and postpharyngeal), pharynx, esophagus, nervous system, and muscle. We predicted that the size of the digestive and nervous systems would not differ between soldiers and minor workers because they are vital organs, while auxiliary structures would differ because they may influence behavioural specialization. For example, we expected that soldiers would have relatively more muscle than minor workers in order to support their large mandibles. Likewise, because the subcastes need to produce and interpret different hormonal and social signals, we expected that at least some of the glands would differ between subcastes.

MATERIALS AND METHODS

Ant collection and colony care

We collected whole colonies of *P. spadonia* from Tuscon, Arizona, USA; *P. hyatti* from Pinto Creek, Arizona, USA; *P. dentata* from Gainesville, Florida, USA; and *P. moerens* from Gainesville, Florida, USA. The colonies were kept in plastic boxes lined with Fluon®. Artificial nests were made from glass test tubes filled half-way with water and plugged with cotton. Ants were fed meal worms, fruit flies, sugar water, and Bhatkar-Whitcomb diet (Bhatkar and Whitcomb 1970). Colonies were kept in a Conviron environmental chamber (Controlled Environments Ltd., Winnipeg) maintained at 27°C, 70% humidity, and 12 hour day:night cycle.

MicroCT preparation and analysis

In order to quantify subcaste differences in size allocation to the different cephalic structures, we examined the heads for our 4 species with microCT analysis. Because microCT analysis is an expensive and time-consuming process, we performed our study on 2 minor workers and 2 soldiers from each species. Ants were chosen haphazardly from each colony and fixed in alcoholic Bouin's solution (1:1 Bouin's:ethanol) for 3-6 days. These samples were then transferred to ethanol (96-100%) for at least 1 day and then stained overnight or longer in 1% iodine in ethanol (I2E) (Metscher 2009). For microCT imaging, we transferred samples to ethanol and mounted in 0.5% agarose in 200µl micropipette tips (Metscher 2011). High-resolution scans were made with the Zeiss/Xradia MicroXCT X-ray microtomography system in the Department of Theoretical Biology, University of Vienna. This system uses a tungsten source and secondary optical magnification of the scintillation detectors. Projection images were collected at 0.2° steps over a half rotation, with a source voltage of 80 kV at 50 µA (4 W), no beam filter, and exposure times of 8-15 sec with 10x magnification or 28-50 sec with 20x

magnification. Tomographic sections were reconstructed with isotropic voxel sizes of 2.0-4.4 μm using the Xradia XMReconstructor software and volume images were exported as stacks of 8-bit or 16-bit TIFFs.

Head reconstructions and analysis

We created three-dimensional reconstructions using Imaris x64 8.2.0 (Bitplane AG, Switzerland). These reconstructed images were arranged with Adobe Illustrator CS5 for figures. We used a paired t-test to assess whether there are statistically significant differences between soldiers and minor workers across the 4 *Pheidole* species in the volume of internal head structures. To determine whether any significant differences were due to caste and/or head capsule volume, we performed an ANCOVA analysis on the regression of the allometric relationship between each organ and head size in JMP 13.

Because we are testing for significant differences across species we need to account for phylogenetic history, which may introduce phylogenetic non-independence between species data points. To test whether each species can be treated as a statistically independent data point for the paired t-test, we used Abouheif's test of phylogenetic independence (Abouheif 1999) for each trait and applied it to the *Pheidole* phylogeny of Moreau (2008), using 1000 permutations to calculate the C-mean. For both of our statistical analyses, we compensated for our low sample size and power by using a conservative *p*-value of 0.10 to assess significance.

RESULTS

Overview of internal head anatomy in **Pheidole**

We found 6 structures in the heads of all species: muscle tissue, the nervous system, the esophagus-pharynx, the propharyngeal glands, the postpharyngeal glands, and the mandibular glands. The pharynx is located in the anterior-most part of the head at the base of its mouthparts. The posterior of the pharynx is connected to the esophagus, which is a simple tubular structure that runs through to the posterior-most part of the head. These digestive structures are connected, therefore we will refer to them together as the 'esophagus-pharynx.' The esophagus is paired with a second tubular structure, the ventral nerve cord (VNC), that runs ventral to the esophagus. The VNC is connected anteriorly to the central brain. Optic nerves extend distally from the brain to the ommatidia. Antennal nerves were also found to extend into the antennae from the brain. Because the brain, VNC, optic nerves, and antennal nerves are connected nervous tissue, we will refer to them together as the 'nervous system.' The propharyngeal glands are small structures located posterior to the pharynx, while the postpharyngeal glands are large sac-like structures located posterior to the pharynx and ventral to the anterior-most esophagus. The mandibular glands are spherical structures on either side of the head, posterior to the clypeus. The muscle extends from just behind the mandibular glands to fill the remainder of the head cavity, dorsally, ventrally, and posteriorly. A second bundle of muscles extends into the head capsule from the ventral mandibular glands and a third bundle of muscles is located dorsal to the pharynx.

Allocation of head space for each subcaste across species

We tested whether the internal anatomy of the head differed allometrically in a similar way to the external anatomy for each species. To do this, we compared volume measurements of each organ in the 4 *Pheidole* species that we reconstructed (Figure 3.1 – 3.4). The muscle and nervous system are the largest organs in the head in both minor workers and soldiers. However, for each

species we consistently found that the nervous system was larger in minor workers than in soldiers, while the muscle was the larger in soldiers than in minor workers. The other structures were slightly more variable in terms of size rank and allocation across species. To verify whether the data points are phylogenetically independent of one another, we performed the Abouheif test and found that all traits are phylogenetically independent ($p > 0.10$; Table 3.1).

We next asked whether there were subcaste differences in the volume of these organs so we pooled all minor worker and soldier measurements in order to assess these differences. We did not find a difference between the volume of the mandibular glands, propharyngeal glands, postpharyngeal glands, and the esophagus-pharynx (Figure 3.5, 3.6E-F). In contrast, muscle was larger in soldiers ($p = 0.0016$; Figure 3.6A-B) and the nervous system was larger in minor workers ($p = 0.0028$; Figure 3.6C-D). Differences in organ size allocation between castes may be attributed to the difference in head size between soldiers and minor workers; therefore, we tested whether caste differences for the nervous system and muscle were dependent on caste and/or head size using an ANCOVA analysis. We found that while muscle was dependent on head size ($p = 0.024$), the nervous system was dependent on caste only ($p = 0.014$).

DISCUSSION

The goal of our study was to identify whether allometric scaling of cephalic organs differed between minor workers and soldiers. Broadly, we found no change in the size of the major head glands and the esophagus-pharynx. In contrast, we did find that the nervous system was larger in minor workers than soldiers, while muscle was larger in soldiers than minor workers. For the remainder of our discussion, we analyze these key structures as separate modules and, when possible, propose potential explanations for our findings.

Digestive organs

Food consumption is a vital process for all organisms, yet the structures that enable this process have been largely overlooked in *Pheidole*. We analyzed 3 digestive structures in the head: the propharyngeal glands, postpharyngeal glands, and the esophagus-pharynx. We did not find a difference in the volumetric size of these organs, which are together an integral part of food processing and produce enzymatic molecules that aid digestion (Billen 2009; Eelen et al. 2006; Fluri et al. 1982; Jackson and Morgan 1993). This finding suggests that minor workers and soldiers have similar digestive requirements and likely process food in a similar manner.

Mandibular glands

Mandibular glands are among the most diverse glands found in all insects. In ants, mandibular glands have taken on both defensive and pheromonal functions, producing a variety of chemicals including sulphides, alcohols, ketones, aldehydes, terpenes, and benezoids (Attygalle and Morgan 1984). In Myrmicines specifically, these glands have largely taken a defensive role as alarm pheromones (Attygalle and Morgan 1984). We found that in *Pheidole*, minor workers and soldiers have similar-sized mandibular glands. At first, this result appears to be counterintuitive because soldiers have traditionally been considered the subcaste specialized in combat. Yet, minor workers are the most active subcaste both inside and outside the nest (Sempo and Detrain 2010). Because of this, minor workers are likely the 'first responders' to communicate the need for back-up when faced with competition or predation. As such, both castes likely require an alarm pheromone response. Research that details the compounds produced by the mandibular

glands in soldiers and minor workers, as well as their pheromonal function, will need to be conducted in order to test this hypothesis.

Nervous system

Of all the internal cephalic anatomy we analyzed, *Pheidole* brains have received the most attention in recent years because of their importance for behavioural flexibility and division of labour. For example, as minor workers mature and expand their behavioural repertoire, they undergo remodelling of synapses (Seid et al. 2005), altering of biogenic amines (Seid and Traniello 2005), and increasing immunoreactivity to serotonin (Seid et al. 2008). These changes primarily affect neuronal networks associated with learning and memory (mushroom bodies), sensing (antennal lobes), and the central nervous system (Seid et al. 2008), which are important for behavioural task performance. Similar associations have been made for *Pheidole* subcastes. Muscedere and Traniello (2012b) compared dissected brains of minor workers and soldiers from *P. dentata, Pheidole morrisi,* and *Pheidole pilifera.* These species broadly differ with respect to life history, colony size, subcaste composition, and behavioural plasticity (Muscedere and Traniello 2012b). When controlling for brain size, Muscedere and Traniello (2012b) found that the brain was configured as a mosaic and different subregions of the brain varied in scale between minor workers and soldiers (subesophageal ganglion, antennal lobe, and mushroom bodies). Furthermore, they found that the relative central brain volume to head width varied was larger for minor workers than soldiers and varied between species (Muscedere and Traniello 2012b). Although the brain is a mosaic, soldiers tend to have more integrated brains than minor workers, with a strong effect of species ecotype and sociobiology (Ilieş et al. 2015; Muscedere and Traniello 2012b). Our study uses a slightly different calculation but finds a similar pattern

using the nervous system volume, where minor workers allocate more to the nervous system than soldiers. Furthermore, this is dependent on caste only and not head size, which suggests that minor workers may require more brain capacity to perform complex tasks. Of course, our data is a broad look at all the nervous tissue in the head and we did not make a distinction between the subregions of the brain or separate nerves from the central brain so we cannot comment on its degree of brain modularity for the species that we studied.

Muscle

According to Gronenberg et al. (1997), the mandibles of insects are akin to the hands of humans as they are both indispensable appendages adapted for a variety of roles. In ants, not only can the mandibles themselves evolve different shapes to specialize on particular tasks but also the head muscle can evolve to control mandibular velocity and force for task specialization (Gronenberg et al. 1997). For example, predators like the Indian jumping ant have fast-acting muscles which allow the mandibles to quickly catch prey (Gronenberg et al. 1997). In *Pheidole*, soldiers are thought to have large heads and complementary large mandibles in order to defend the colony against competitors and mill seeds. For these roles, *Pheidole* do not require rapid mandibular action but instead rely on cephalic mandibular closer muscles to provide the force to carry out powerful crushing actions (Gronenberg et al. 1997). Muscedere et al. (2011b) compared three different cephalic muscle groups in *Pheidole dentata*: the mandibular closer muscles which are important for nursing, foraging, and defense; the pharynx dilator muscles which are important for feeding; and the antennal muscles which are important for collecting sensory stimuli. They found that *P. dentata* soldiers have thicker muscle fibres than minor workers and older workers generally have thicker muscle fibres than younger workers. We found that soldiers in 4 species

studied, including *P. dentata*, have more muscle by volume than minor workers, supporting the findings of Muscedere et al. (2011b). In our study, this too seems to be attributed to the large mandibular closer muscles of the heads. It is apparent that muscle plays an incredibly important role in the diversification of caste specialization in *Pheidole*. Because *Pheidole* soldiers may specialize on a variety tasks related to mandible use like seed crushing and combat, it will be important to elucidate how subtle differences in muscle (e.g., fibre thickness and muscle type) may relate to different tasks by comparing species with diverse ecological lifestyles and varied measures of musculature development.

CONCLUSION

Our study marks the first step in describing both the internal head anatomy of subcaste heads in *Pheidole*. Our allometric data support previous research on the structure and physiology of the brain and muscle that find trade-offs between subcastes. This finding leads to a number of exciting behavioural and chemical hypotheses to explain why muscle is larger in soldiers and the nervous system is larger in minor workers. Furthermore, we add to the body of work on *Pheidole* subcastes by extending our research to the digestive organs and cephalic glands of the head. We hope that our research increases interest in the worker caste system of *Pheidole* and, more broadly, incites interest in the study of internal allometry.

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Table 3.1. No phylogenetic signal in head traits. *C*-mean and *p*-values generated using the Abouheif test of phylogenetic independence.

	structure	***C*-mean**	***p*-value**
minor worker	esophagus-pharynx	-0.21	0.17
	mandibular glands	0.0033	0.42
	muscle	0.2	0.32
	nervous system	0.24	0.24
	postpharyngeal glands	-0.17	0.32
	propharyngeal glands	-0.086	0.48
soldier	esophagus-pharynx	0.11	0.31
	mandibular glands	0.046	0.38
	muscle	-0.25	0.12
	nervous system	0.076	0.56
	postpharyngeal glands	-0.12	0.24
	propharyngeal glands	0.12	0.47

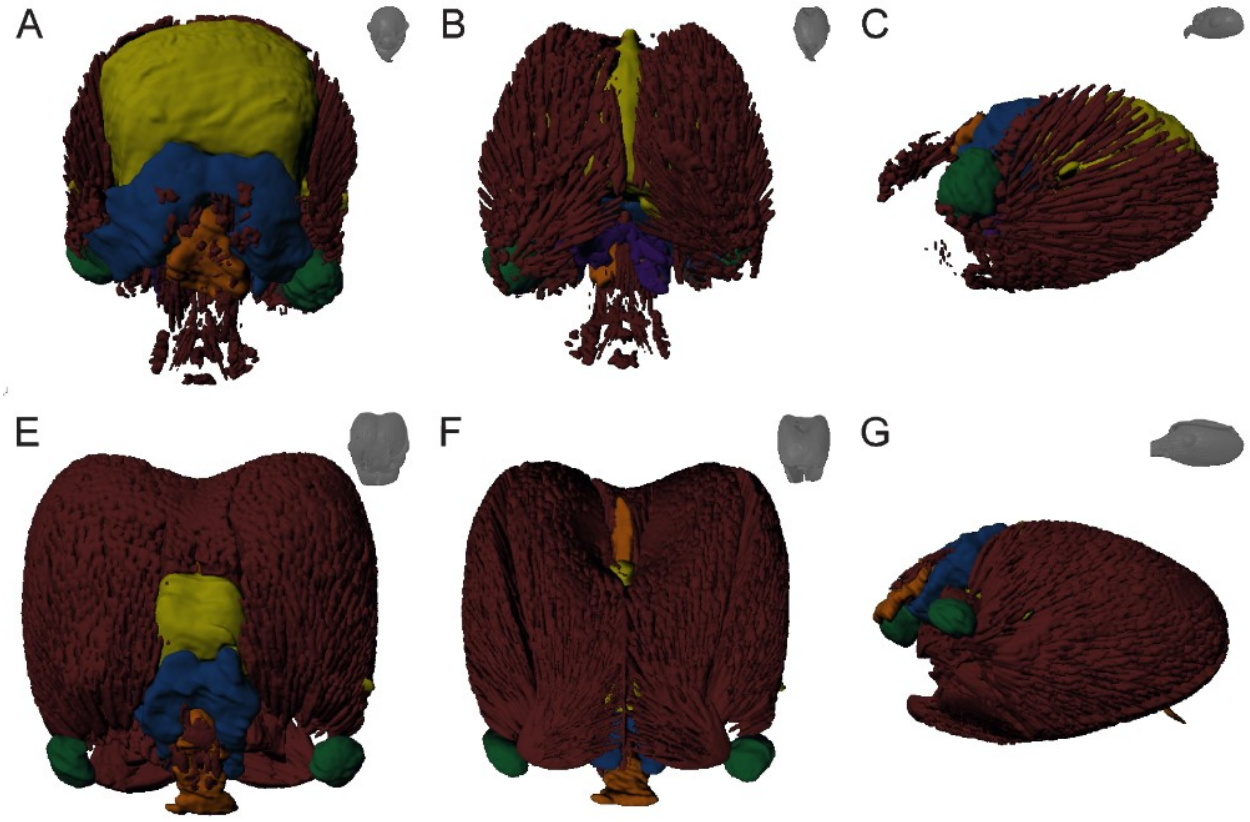

Figure 3.1. Internal anatomy of minor worker and soldier heads in *P. dentata*. The dorsal minor worker (A), ventral minor worker (B), lateral minor worker (C), dorsal soldier (D), ventral soldier (E), and lateral soldier (F) are shown. Orange represents the esophagus-pharynx, green represents the mandibular glands, red represents muscle, yellow represents the nervous system, blue represents the postpharyngeal glands, and purple represents the propharyngeal glands. Orientation and scale for each figure are depicted with the external image of the head in the top right-hand corner.

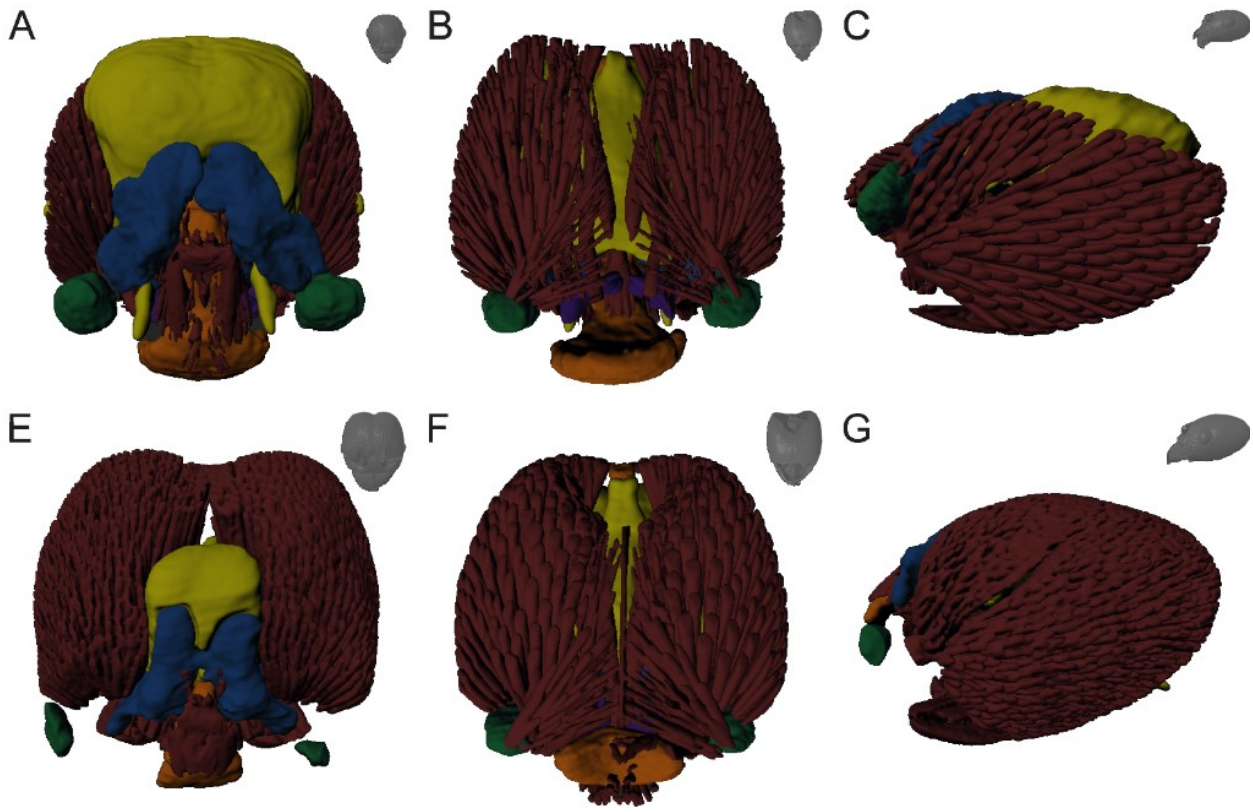

Figure 3.2. Internal anatomy of minor worker and soldier heads in *P. hyatti*. The dorsal minor worker (A), ventral minor worker (B), lateral minor worker (C), dorsal soldier (D), ventral soldier (E), and lateral soldier (F) are shown. Orange represents the esophagus-pharynx, green represents the mandibular glands, red represents muscle, yellow represents the nervous system, blue represents the postpharyngeal glands, and purple represents the propharyngeal glands. Orientation and scale for each figure are depicted with the external image of the head in the top right-hand corner.

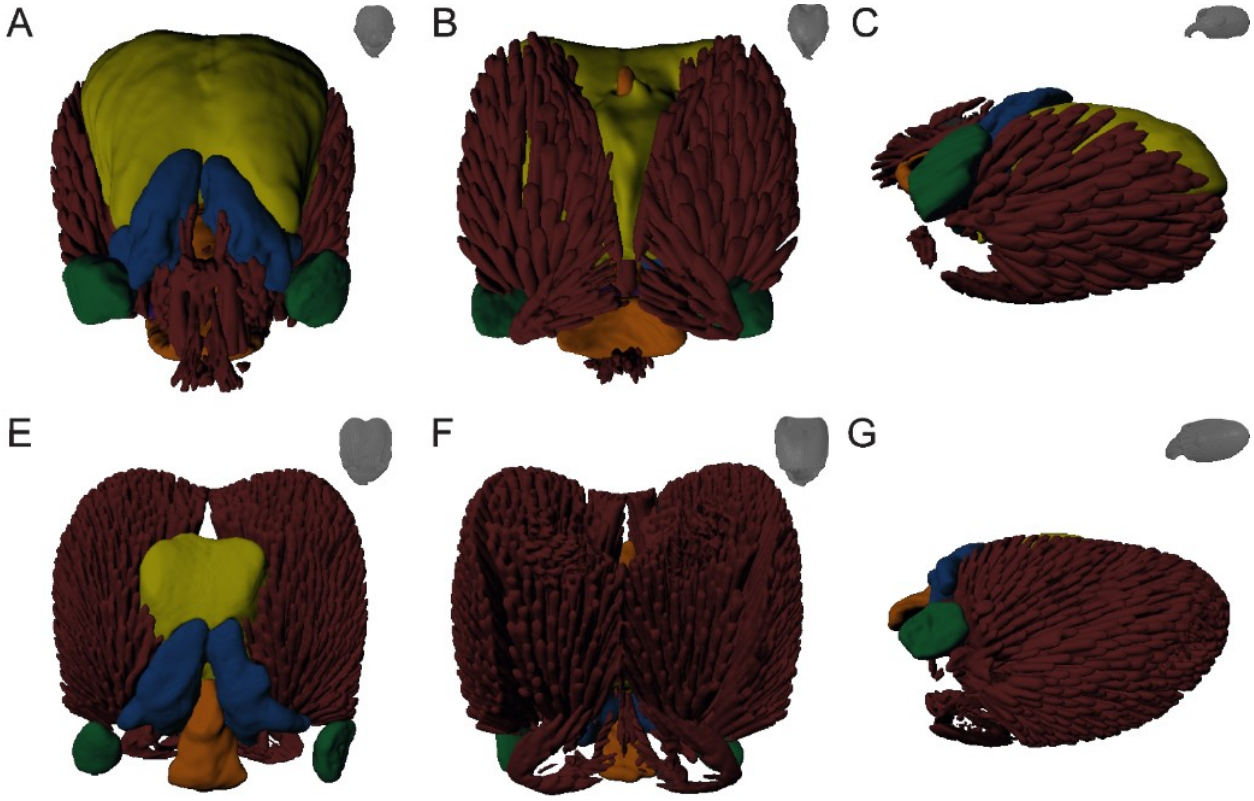

Figure 3.3. Internal anatomy of minor worker and soldier heads in *P. moerens*. The dorsal minor worker (A), ventral minor worker (B), lateral minor worker (C), dorsal soldier (D), ventral soldier (E), and lateral soldier (F) are shown. Orange represents the esophagus-pharynx, green represents the mandibular glands, red represents muscle, yellow represents the nervous system, blue represents the postpharyngeal glands, and purple represents the propharyngeal glands. Orientation and scale for each figure are depicted with the external image of the head in the top right-hand corner.

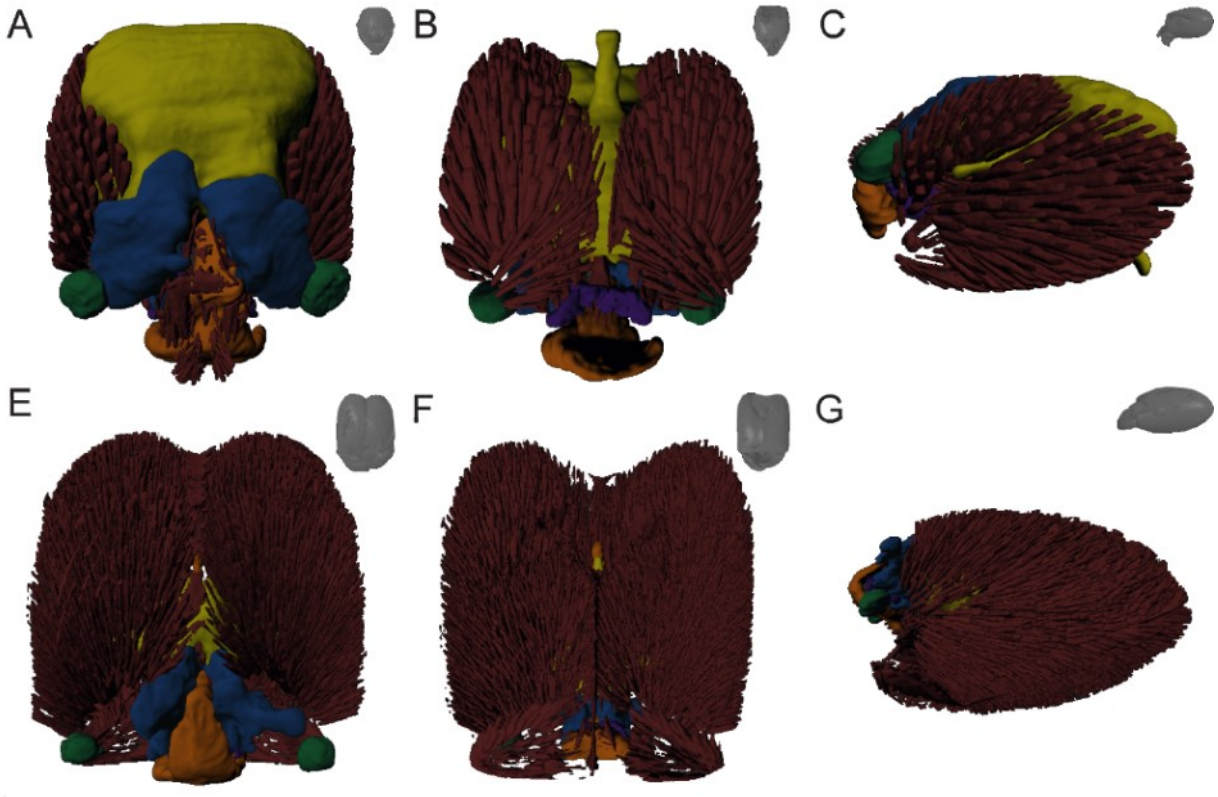

Figure 3.4. Internal anatomy of minor worker and soldier heads in *P. spadonia*. The dorsal minor worker (A), ventral minor worker (B), lateral minor worker (C), dorsal soldier (D), ventral soldier (E), and lateral soldier (F) are shown. Orange represents the esophagus-pharynx, green represents the mandibular glands, red represents muscle, yellow represents the nervous system, blue represents the postpharyngeal glands, and purple represents the propharyngeal glands. Orientation and scale for each figure are depicted with the external image of the head in the top right-hand corner.

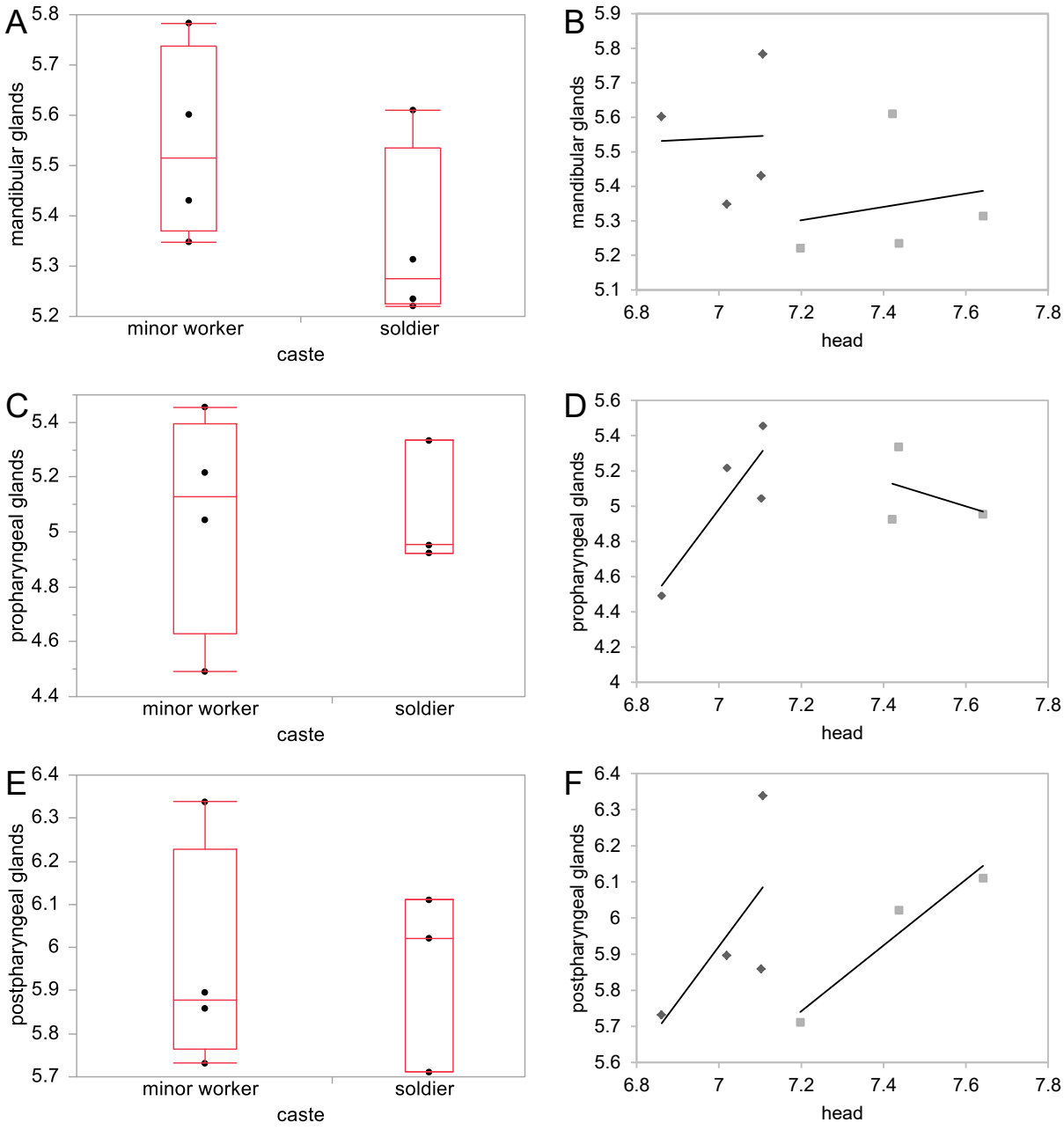
A
mandibular glands
5.8
5.7
5.6
5.5
5.4
5.3
5.2
minor worker
soldier
caste
B
mandibular glands
5.9
5.8
5.7
5.6
5.5
5.4
5.3
5.2
5.1
6.8
7
7.2
7.4
7.6
7.8
head
C
propharyngeal glands
5.4
5.2
5
4.8
4.6
4.4
minor worker
soldier
caste
D
propharyngeal glands
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
6.8
7
7.2
7.4
7.6
7.8
head
E
postpharyngeal glands
6.4
6.3
6.2
6.1
6
5.9
5.8
5.7
minor worker
soldier
caste
F
postpharyngeal glands
6.4
6.3
6.2
6.1
6
5.9
5.8
5.7
5.6
6.8
7
7.2
7.4
7.6
7.8
head

Figure 3.5. Size of cephalic glands in minor workers and soldiers. Volume for the (A) mandibular glands, (C) propharyngeal glands, and (E) postpharyngeal glands is expressed in a logarithmic scale by subcaste. Significant differences between means are indicated with an asterisk and based on a paired t-test ($p < 0.05$). Allometric scaling of the (B) mandibular glands, (D) propharyngeal glands, and (F) postpharyngeal glands to the head is shown for each subcaste. Minor workers are represented by dark grey points and soldiers are represented by light grey points.

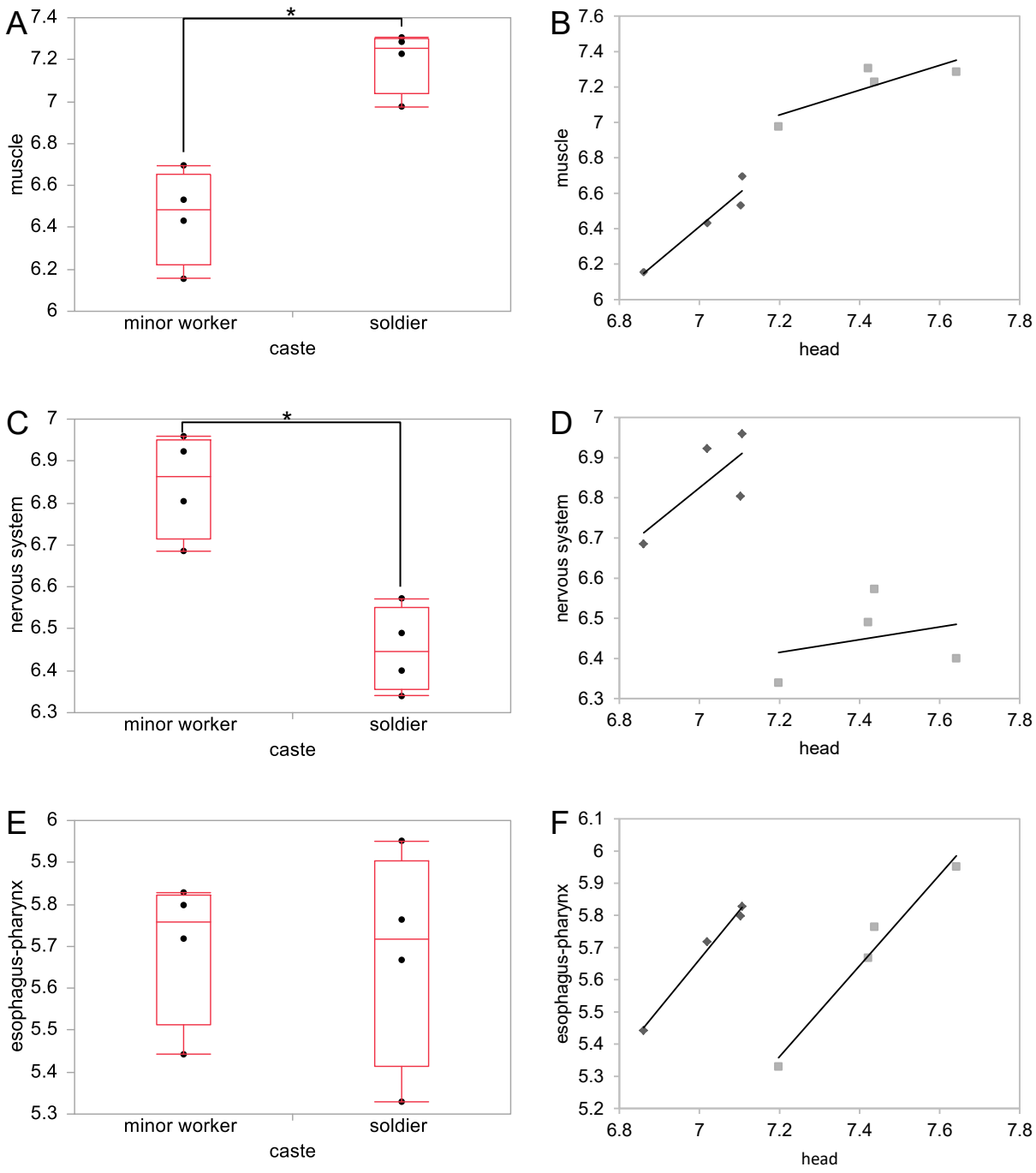
A
B
C
D
E
F
muscle
nervous system
esophagus-pharynx
caste
minor worker
soldier
head
*

Figure 3.6. Size of other cephalic organs in minor workers and soldiers. Volume for the (A) muscle, (C) nervous system, and (E) esophagus-pharynx is expressed in a logarithmic scale by subcaste. Significant differences between means are indicated with an asterisk and based on a paired t-test ($p < 0.05$). Allometric scaling of the (B) muscle, (D) nervous system, and (F) esophagus-pharynx to the head is shown for each subcaste. Minor workers are represented by dark grey points and soldiers are represented by light grey points.

Concluding Remarks

The regulation of the complex worker caste system in ants is essential for colony function and may shed light on the underlying principles of complex systems as a whole. In my thesis, I consider the social component of worker caste regulation from a behavioural, chemical, and morphological perspective in *Pheidole.* My work specifically focuses on how soldiers alter the developmental trajectory of brood by way of the 'soldier inhibitory pheromone.' Behaviourally, I found that soldiers transmit the soldier inhibitory pheromone by passively and selectively interacting with larvae. Chemically, I found key differences in the saturated hydrocarbons produced by minor workers and soldiers, which may contain the solider inhibitory pheromone. Morphologically, I found that soldiers have similar-sized cephalic glands to minor workers. This is consistent with a hydrocarbon-based pheromone as hydrocarbons are produced in secretory cells called 'oenocytes' throughout the body. My research has only scratched the surface of this regulatory mechanism and leads to many more questions. Perhaps the most important of these is to determine whether there is a causal link between the cuticular hydrocarbon profile of soldiers and soldier inhibition. This would definitively establish that at least one cuticular hydrocarbon constitutes the soldier inhibitory pheromone and is currently in the process of being tested in the Abouheif Lab. If so, it will be imperative to look into the chemicals that oenocytes throughout the body produce. If not, comprehensive studies on the chemicals produced by each gland in soldiers may reveal other candidates for the soldier inhibitory pheromone. Once a causal link has been established, the next logical step will be to test what physiological and genetic changes are occurring in larvae in response to this signal. Of course, like any complex system, the worker caste system is intricate and multifaceted, so these are only some of the many questions waiting to be discovered. I believe that my work has taken an initial step in bridging these gaps in our

knowledge and demonstrates the value of integrating many scientific disciplines to tackle a single problem. In my work, by fusing multiple perspectives, I have illuminated a process by which collective behaviour influences individual development and phenotypic diversity. More broadly, by fusing multiple perspectives, we as a scientific community can tease apart the building blocks of the complex phenomena we seek to understand.

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Appendix

Supplemental Table 1. Proportion of time spent on each behavioural interaction for each replicate (1-10).

caste	behaviour	interaction type	1	2	3	4	5	6	7	8	9	10
minor	antennate	bipotential larva	0.0000	0.0000	0.0000	0.0012	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	carry	bipotential larva	0.0087	0.0000	0.0025	0.0000	0.0020	0.0015	0.0000	0.0000	0.0011	0.0000
minor	feed	bipotential larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	groom	bipotential larva	0.0044	0.0109	0.0086	0.0081	0.0051	0.0090	0.0013	0.0000	0.0077	0.0000
minor	aggression	bipotential larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	bipotential larva	0.0189	0.0402	0.0295	0.0476	0.0499	0.0705	0.0394	0.0491	0.0718	0.0495
minor	walking	bipotential larva	0.0160	0.0206	0.0197	0.0325	0.0316	0.0135	0.0394	0.0383	0.0287	0.0247
minor	antennate	dead nest mate	0.0000	0.0033	0.0000	0.0000	0.0000	0.0000	0.0000	0.0061	0.0000	0.0000
minor	carry	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	feed	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	groom	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	aggression	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0014
minor	walking	dead nest mate	0.0000	0.0022	0.0000	0.0000	0.0031	0.0000	0.0000	0.0077	0.0000	0.0069
minor	antennate	soldier-destined larva	0.0087	0.0043	0.0037	0.0081	0.0010	0.0000	0.0000	0.0015	0.0011	0.0014
minor	carry	soldier-destined larva	0.0000	0.0011	0.0098	0.0000	0.0010	0.0000	0.0025	0.0000	0.0066	0.0014
minor	feed	soldier-destined larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	groom	soldier-destined larva	0.0218	0.0043	0.0381	0.0302	0.0153	0.0060	0.0038	0.0077	0.0265	0.0055
minor	aggression	soldier-destined larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	soldier-destined larva	0.0146	0.0858	0.1488	0.1266	0.1385	0.1709	0.0534	0.0936	0.1481	0.1552
minor	walking	soldier-destined larva	0.0378	0.0434	0.0332	0.0592	0.0591	0.0315	0.0584	0.0460	0.0608	0.0536
minor	antennate	food	0.0116	0.0087	0.0049	0.0105	0.0061	0.0060	0.0000	0.0061	0.0022	0.0027
minor	carry	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0011	0.0000
minor	feed	food	0.0000	0.0000	0.0000	0.0093	0.0000	0.0000	0.0000	0.0000	0.0000	0.0014
minor	groom	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	aggression	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	food	0.0029	0.0054	0.0062	0.0163	0.0092	0.0045	0.0089	0.0046	0.0122	0.0288
minor	walking	food	0.0961	0.0304	0.0209	0.0244	0.0305	0.0330	0.0889	0.0460	0.0298	0.0495
minor	antennate	nest mate	0.0131	0.0358	0.0111	0.0290	0.0071	0.0165	0.0127	0.0092	0.0044	0.0027
minor	carry	nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	feed	nest mate	0.0029	0.0000	0.0000	0.0046	0.0041	0.0000	0.0025	0.0000	0.0022	0.0027
minor	groom	nest mate	0.0015	0.0011	0.0049	0.0058	0.0020	0.0030	0.0000	0.0031	0.0033	0.0014
minor	aggression	nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	nest mate	0.0247	0.0706	0.0615	0.0523	0.0784	0.0450	0.0826	0.0936	0.0685	0.0893
minor	walking	nest mate	0.0466	0.0695	0.0381	0.0488	0.1018	0.0495	0.1144	0.1380	0.0785	0.0989
minor	antennate	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

minor	carry	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	feed	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	groom	self	0.0830	0.0608	0.0689	0.0534	0.0448	0.0570	0.0419	0.0368	0.0552	0.0522
minor	aggression	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	self	0.1164	0.1737	0.2509	0.1940	0.2057	0.2414	0.1245	0.1196	0.2000	0.1442
minor	walking	self	0.4702	0.3279	0.2386	0.2381	0.2037	0.2414	0.3253	0.2929	0.1901	0.2266
minor	antennate	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	carry	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	feed	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	groom	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	aggression	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	idle	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
minor	walking	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
soldier	antennate	bipotential larva	0.0010	0.0012	0.0000	0.0000	0.0000	0.0009	0.0000	0.0000	0.0000	
soldier	carry	bipotential larva	0.0000	0.0012	0.0007	0.0000	0.0000	0.0009	0.0013	0.0011	0.0000	
soldier	feed	bipotential larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	groom	bipotential larva	0.0048	0.0048	0.0015	0.0000	0.0000	0.0009	0.0038	0.0080	0.0015	
soldier	aggression	bipotential larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	bipotential larva	0.1017	0.0860	0.1673	0.1287	0.1440	0.1085	0.1186	0.1135	0.0990	
soldier	walking	bipotential larva	0.0029	0.0155	0.0052	0.0189	0.0127	0.0157	0.0242	0.0149	0.0177	
soldier	antennate	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	carry	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	feed	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	groom	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	aggression	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	walking	dead nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0038	0.0000	0.0000	
soldier	antennate	soldier-destined larva	0.0038	0.0024	0.0000	0.0009	0.0000	0.0000	0.0013	0.0000	0.0000	
soldier	carry	soldier-destined larva	0.0029	0.0000	0.0030	0.0000	0.0000	0.0017	0.0000	0.0011	0.0000	
soldier	feed	soldier-destined larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	groom	soldier-destined larva	0.0304	0.0119	0.0134	0.0063	0.0111	0.0017	0.0026	0.0080	0.0074	
soldier	aggression	soldier-destined larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	soldier-destined larva	0.2196	0.1983	0.2942	0.1881	0.2593	0.2415	0.1964	0.2317	0.1743	
soldier	walking	soldier-destined larva	0.0076	0.0239	0.0097	0.0270	0.0247	0.0332	0.0434	0.0310	0.0236	
soldier	antennate	food	0.0010	0.0060	0.0007	0.0018	0.0000	0.0009	0.0000	0.0000	0.0015	
soldier	carry	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	feed	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0011	0.0015	
soldier	groom	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	aggression	food	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	food	0.0019	0.0060	0.0075	0.0054	0.0270	0.0122	0.0013	0.0172	0.0103	
soldier	walking	food	0.0010	0.0084	0.0037	0.0225	0.0064	0.0105	0.0102	0.0206	0.0222	

soldier	antennate	nest mate	0.0057	0.0143	0.0052	0.0009	0.0016	0.0000	0.0013	0.0000	0.0030	
soldier	carry	nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0009	0.0000	0.0000	0.0000	
soldier	feed	nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0023	0.0015	
soldier	groom	nest mate	0.0067	0.0084	0.0007	0.0072	0.0064	0.0052	0.0026	0.0115	0.0089	
soldier	aggression	nest mate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	nest mate	0.4030	0.1792	0.4130	0.2835	0.3930	0.2913	0.2321	0.3028	0.1713	
soldier	walking	nest mate	0.0276	0.0275	0.0164	0.0522	0.0326	0.0647	0.0880	0.0688	0.1064	
soldier	antennate	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	carry	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	feed	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	groom	self	0.0257	0.0597	0.0142	0.0558	0.0072	0.0262	0.0370	0.0206	0.0576	
soldier	aggression	self	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	self	0.0589	0.2473	0.0224	0.1458	0.0621	0.1155	0.1390	0.0894	0.1507	
soldier	walking	self	0.0342	0.0980	0.0209	0.0549	0.0119	0.0674	0.0931	0.0562	0.1418	
soldier	antennate	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	carry	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	feed	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	groom	unclear larva	0.0010	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	aggression	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	idle	unclear larva	0.0589	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
soldier	walking	unclear larva	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	

Supplemental Table 2. Absolute volumes (µm³) for each cephalic structure in *Pheidole spp.*

caste	organ	*P. dentata*	*P. hyatti*	*P. moerens*	*P. spadonia*
minor worker	esophagus-pharynx	1255000	1758351.5	366867.5	1030000
	head	25299200	29050000	9595000	20600000
	mandibular glands	538092.5	1539714.5	528615	439397
	muscle	7051905.613	12978407.13	2021256.328	5326492.408
	nervous system	12761950	20200000	6415000	16500000
	postpharyngeal gland	1486435	5500000	753729.5	1550000
	propharyngeal gland	187334.7	655405	39774.2	324821
soldier	esophagus-pharynx	557900.5	616876	175942.5	957236
	head	26400000	39500000	13100000	46900000
	mandibular glands	175043.45	514543	135752.25	220325
	muscle	17973553.66	29230311.96	7740000	20635625.57
	nervous system	3590000	4165000	1785000	2690000
	postpharyngeal gland	996270	734349.9934	441000	1380000
	propharyngeal gland	177905	115958	NA	95902.5