PROGRAMMED CELL DEATH AND THE DEVELOPMENT AND EVOLUTION OF MORPHOLOGICAL CASTES IN ANTS

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DEDICATION

I dedicate this work to my partner, James Aretakis, and to my family members.

ABSTRACT

One of the most intriguing questions in biology is how morphological complexity originates and evolves. One-way morphological complexity is thought to have increased is through 'major evolutionary transitions in individuality', where single organisms come together to form a higher-level organism. The evolution of eusocial insect colonies, where solitary individuals integrated into a single colony, are a prime example of a major evolutionary transition. The evolution of eusociality in ants has contributed to their remarkable degree of morphological and social complexity. This was possible due to the highly plastic development of ants where a single genotype, in response to environmental conditions, can give rise to disparate phenotypes (a process termed 'polyphenism' or 'polymorphism'). At the origin of their evolution, ants evolved a wing polyphenism where the queens (reproductive caste) are winged as adults, and the workers (sterile female caste) are wingless as adults. Subsequently during their evolutionary history, some ant lineages further split their worker caste into morphologically distinct subcastes, known as worker polymorphism. Here I focus on the developmental and genetic mechanisms that are contributing to the evolution of this phenotypic plasticity: wing polyphenism and worker polymorphism. Specifically, I examine the role that cell death is playing in the evolution of a universal wingless worker caste and how this same role is necessary to produce worker subcaste complexity in the ant genus *Pheidole*. In Chapter 1 of my thesis, I review the ecological, evolutionary, and developmental factors that led to wing polyphenism in ants, spanning its origin from their wasp ancestors, to fossil evidence, to underlying gene regulatory networks, to genetic accommodation. I then use this information to propose hypotheses and generate testable models. In Chapter 2, I focus on one of the potential developmental mechanisms for regulating wing polyphenism, programmed cell death, and review its ancient origins, widespread and numerous functions, and most importantly, its repeated role in regulating various polyphenisms and sexual dimorphisms found across the tree of life. In Chapter 3, I test the role of programmed cell death in wing polyphenism across the major subfamilies of ants and infer its possible presence at the very origin of the trait during the evolution of ants. In Chapter 4, I test how apoptosis signaling, a form of programmed cell death, in the wing disc rudiment of the big-headed ant, *Pheidole*

dentata, is contributing to the development of a soldier worker subcaste. In Chapter 5, I present preliminary data testing the possible function of the wing disc rudiment in a species that lacks a complex worker caste, *Harpegnathos saltator*. Collectively, the findings presented in my thesis shed light on how two key morphological traits in ants: wing polyphenism and worker polymorphism, are regulated. Specifically, my thesis shows the unexpected role that cell death has in contributing to both of these morphological traits and highlights its undervalued contribution to biological complexity as a whole and the rise of major evolutionary transitions.

ABRÉGÉ

L’une des questions les plus intrigantes en biologie est de savoir comment la complexité morphologique apparaît et évolue. Une manière dont on pense que la complexité morphologique a augmenté est par le biais de ‘transitions évolutives majeures de l’individualité’, où des organismes uniques se réunissent pour former un organisme de niveau supérieur. L'évolution des colonies d'insectes eusociaux, où des individus solitaires sont intégrés dans une même colonie, sont un excellent exemple d'une transition évolutive majeure. L'évolution de l'eusocialité chez les fourmis a contribué à leur remarquable degré de complexité morphologique et sociale. Cela a été possible grâce au développement hautement plastique des fourmis, où un seul génotype, en réponse aux conditions environnementales, peut produire des phénotypes disparates (un processus appelé polyphénisme ou polymorphisme). À l’origine de leur évolution, les fourmis ont développé un polyphénisme alaire où les reines (caste reproductrice) sont ailées à l’âge adulte et les ouvrières (caste des femelles stériles) sont aptères à l’âge adulte. Par la suite, au cours de leur histoire évolutive, certaines lignées de fourmis ont divisé leur caste d'ouvrières en sous-castes morphologiquement distinctes, connues sous le nom de polymorphisme des ouvrières. Je me concentre ici sur les mécanismes développementaux et génétiques contribuant à l’évolution de cette plasticité phénotypique : le polyphénisme alaire et le polymorphisme des ouvrières. Plus précisément, j'examine le rôle de la mort cellulaire dans l'évolution d'une caste universelle d'ouvrières aptères et comment ce même rôle est nécessaire afin de produire une complexité de sous-caste d'ouvrières chez le genre de fourmis *Pheidole*. Dans le chapitre 1, je passe en revue les facteurs écologiques, évolutifs et développementaux qui ont conduit au polyphénisme alaire chez les fourmis, allant de leurs ancêtres guêpes aux preuves fossiles, en passant par les réseaux de régulation génétique sous-jacents et l'accommodation génétique. J'utilise ensuite ces informations pour proposer des hypothèses et générer des modèles testables. Dans le chapitre 2, je me concentre sur l'un des mécanismes développementaux potentiels pour la régulation du polyphénisme alaire, la mort cellulaire programmée et passe en revue ses origines anciennes, ses fonctions répandues et nombreuses et surtout, son rôle répété dans la régulation de divers polyphénismes et dimorphismes sexuels répandus à travers l’arbre de la vie. Dans le chapitre 3,

je teste le rôle de la mort cellulaire programmée par rapport au polyphénisme alaire chez les principales sous-familles de fourmis et déduis sa présence possible à l'origine même du trait au cours de l'évolution des fourmis. Dans le chapitre 4, je teste comment la signalisation de l'apoptose, une forme de mort cellulaire programmée, dans le rudiment du disque alaire de *Pheidole dentata*, contribue au développement d'une sous-caste de soldats. Dans le chapitre 5, je présente des données préliminaires testant la fonction possible du rudiment du disque alaire chez une espèce dépourvue de caste d'ouvrières complexe, *Harpegnathos saltator*. Collectivement, les résultats présentés dans ma thèse mettent en lumière la manière dont deux traits morphologiques clés sont régulés chez les fourmis; le polyphénisme alaire et le polymorphisme des ouvrières. Plus précisément, ma thèse démontre la contribution inattendue de la mort cellulaire à ces deux traits morphologiques et met en évidence sa contribution sous-estimée à la complexité biologique dans son ensemble et à l'apparition de transitions évolutives majeures.

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PREFACE: CONTRIBUTION OF AUTHORS

This manuscript-based thesis comprises my original work, organized into six chapters. I am the primary author of all chapters excepting Chapter 3 of which I am the co-primary author. Chapters 1, 2, and 3 have been published, and Chapters 4 and 5 are in preparation for publication. For Chapters 1-2 and 4-6, I conceived the ideas, conducted all the experiments, analyzed the data, and wrote the manuscript with the guidance of my mentor Ehab Abouheif in all above aspects. For Chapter 3, I wrote the manuscript and oversaw the peer-review procedure. Details of contributions from co-authors are described below:

Chapter 1. The origin of wing polyphenism in ants: an eco-evo-devo perspective

Authors: Lisa Hanna and Ehab Abouheif

A version of this chapter is published as "Hanna, L., and E. Abouheif. (2021). The origin of wing polyphenism in ants: an eco-evo-devo perspective. *Current Topics in Developmental Biology* 141, 279-336". I conducted all the background research for this work, and together with Dr. Ehab Abouheif, conceived the ideas and wrote the manuscript.

Chapter 2. Deep conservation and co-option of programmed cell death facilitates the evolution of alternative phenotypes at multiple biological levels

Authors: Lisa Hanna and Ehab Abouheif

A version of this chapter is published as "Hanna, L. and E. Abouheif. (2022). Deep conservation and co-option of programmed cell death facilitates the evolution of alternative phenotypes at multiple biological levels. *Seminars in Cell and Developmental Biology* 145, 28-41". I conducted all the background research for this work, and together with Dr. Ehab Abouheif, conceived the ideas and wrote the manuscript.

Chapter 3. Inferring a role for programmed cell death during the origin and evolution of wing polyphenism in ants

Authors: Lisa Hanna, Brendon Boudinot, Jürgen Liebig, and Ehab Abouheif

This chapter has been prepaired to be submitted to a peer-review journal. I conducted the

experiments for this work and wrote the manuscript with E.A. B.B. conducted the ancestral state analysis and J.L. provided the ant species *Harpegnathos saltator*.

Chapter 4. Apoptosis signaling in a rudimentary organ is required for the development of a novel soldier subcaste in ants

Authors: Lisa Hanna and Ehab Abouheif

This chapter is in preparation for submission to a peer-review journal. I conceived the ideas, performed the experiments, analyzed the data, and together with E.A. wrote the manuscript.

Chapter 5. Exploring the functional role of rudimentary wing discs in a monomorphic ant species with ancestral characteristics

Authors: Lisa Hanna and Ehab Abouheif

This chapter encompasses results that will be further investigated and submitted to a peer-review journal. E.A. and I conceived the ideas, I performed the experiments, analyzed the data, and wrote the manuscript.

For clarity, section number of published chapters are re-labeled here and references for all chapters are cited using the same format.

Chapter 1: The origin of wing polyphenism in ants: An eco-evo-devo perspective

Lisa Hanna[1] and Ehab Abouheif[1]*

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1.1. Abstract

The evolution of eusociality, where solitary individuals integrate into a single colony, is a major transition in individuality. In ants, the origin of eusociality coincided with the origin of a wing polyphenism approximately 160 million years ago, giving rise to colonies with winged queens and wingless workers. As a consequence, both eusociality and wing polyphenism are nearly universal features of all ants. Here, we synthesize fossil, ecological, developmental, and evolutionary data in an attempt to understand the factors that contributed to the origin of wing polyphenism in ants. We propose multiple models and hypotheses to explain how wing polyphenism is orchestrated at multiple levels, from environmental cues to gene networks. Furthermore, we argue that the origin of wing polyphenism enabled the subsequent evolution of morphological diversity across ants. We finally conclude by outlining several outstanding questions for future work.

1.2. Introduction

Dramatic increases in biological complexity have occurred repeatedly during the history of life. Maynard-Smith and Szathmary (1997; Szathmary & Smith, 1995) proposed that increases in biological complexity originate and evolve through 'major evolutionary transitions in individuality,' which occur when individuals integrate to form a single, higher-level, individual that replicates independently and has a division of labor between its constituent parts (Buss, 2014; Taylor et al., 2019; West et al., 2015). Prime examples of major transitions in individuality include unicellular organisms integrating to form a multicellular organism, distantly related organisms integrating to form an obligate endosymbiosis, and solitary individuals integrating to form a eusocial colony. Although much recent progress has been made, both theoretically and empirically, in understanding the evolutionary pathways towards major evolutionary transitions in individuality (Boomsma & Gawne, 2018; Maliet et al., 2015; Miller & Reeve, 2019; Moran, 2007; Rafiqi et al., 2020; Ruiz-Trillo & Nedelcu, 2015), much work remains in understanding the evolutionary and developmental steps leading to transitions in different taxonomic groups, which have occurred independently across the tree of life.

Major transitions in individuality are often associated with the evolution of polyphenism, which is defined as the ability of a single genome to produce discrete phenotypes in response to environmental cues (Nijhout, 1999; Stearns, 1989). For example, the transition from unicellular to obligate (complex) multicellularity in animals was accompanied by a cellular polyphenism, in which differentiated cell types (e.g., over 200 in humans) are produced from the same genome in response to cues from the internal environment, like morphogen gradients (Marquez-Zacarias et al., 2020). Recent studies suggest that the origins of this cellular polyphenism may have even preceded the major transition to multicellularity in animals because the choanoflagellates, the closest living unicellular relatives of the animals differentiate into five distinct cell types in response to environmental cues (Dayel et al., 2011; Mikhailov et al., 2009). Furthermore, the transition to eusociality, which is defined by individuals that obligately live in colonies with overlapping generations, cooperative brood care, and a reproductive division of labor between the reproductive queen and male caste and non-reproductive worker caste, is also associated with the evolution of polyphenism (Wilson, 1971;

Wilson & Holldobler, 2005a). Both ants (order Hymenoptera) and termites (order Isoptera), for example, have convergently evolved eusociality and a polyphenism in wing development producing winged queens and wingless workers in response to environmental cues (Wilson, 1971). As a consequence, all species of ants and termites are wing polyphenic and eusocial. Therefore, wing polyphenism has been proposed to be associated with, if not the basis for, the convergent origin of eusocial life in these two groups (Abouheif & Wray, 2002; Holldobler & Wilson, 1990; Korb, 2008; Nowak et al., 2010). Finally, in other insect groups where eusociality has evolved, including wasps and bees (order Hymenoptera), thrips (order Thysanoptera), aphids (order Hemiptera), and ambrosia beetles (order Coleoptera), the females are also polyphenic and develop into discrete reproductive or non-reproductive castes based on environment cues (Crespi, 1992; Foster, 1990; Kent & Simpson, 1992). Therefore, studying the evolution of polyphenisms in taxonomic groups before and after they have undergone major transitions in individuality is key to understanding the factors and steps leading to the origin and elaboration of these transitions.

Here we focus on the origin of wing polyphenism in ants. Ants belong to the order Hymenoptera, along with sawflies, bees, and wasps, and are one of the most ecologically dominant and evolutionarily successful groups of insects on the planet. The transition to eusociality occurred several independent times within the Hymenoptera. Bees and wasps display a wide range of social organization, from communal groups to a complex grade of eusocial organization (Michener, 1969; West-Eberhard, 1978; Wheeler, 1928b). In contrast, all species of ants are eusocial and wing polyphenic, where the females develop either into winged queens or wingless workers in response to environmental cues such as temperature and nutrition (Fig. 1) (Holldobler & Wilson, 1990; Wheeler, 1928b).

In the majority of ant species, the establishment of a new colony typically begins with mating flights where unmated winged males and queens emerge from their parental nest, fly, and then mate in the air. After mating, the queens search for a proper nest site to lay their eggs. Once a suitable site is found, the queen sheds her wings and begins to lay her first eggs, which typically develop into female workers to kick start her new colony. Sex-determination in the Hymenoptera occurs through ‘haplodiploidy’ – if the queen fertilizes her eggs, they are

diploid and develop into females; if they are not fertilized, they are haploid and develop into males (Grimaldi & Engel, 2005; Holldobler & Wilson, 1990). The primary role of males is procreation, which is why male offspring are typically produced and present in colonies for only a short period of time once a year just before the mating flight of a colony takes place. In contrast, females are long-lived and their development depends on environmental cues like temperature and nutrition. In response to these environmental cues, female eggs will develop either into winged queens that disperse and establish their own nests, or into wingless workers that remain in the nest and carry out tasks such as foraging for food and caring for the brood.

In early ant evolution, queens and workers were similar in morphology and reproductive potential. The origin of wing polyphenism in ants, therefore, is thought to have prevented the wingless workers from participating in mating flights, while simultaneously allowing winged queens to do so. This may have been one of several factors that either facilitated or maintained the appearance of reproductive division of labor, which is a defining feature of eusociality. After the origin of wing polyphenism and eusociality in ants, a diverse array of mating strategies evolved, including the evolution of wingless queens and when the queen caste is permanently lost, workers mate with males on the ground (Keller et al., 2014; Peeters, 1991).

Studies in evolutionary developmental biology (evo-devo) have shown that morphological diversity in animals arose through changes in a relatively small number of highly conserved developmental regulatory genes, collectively known as the 'genetic toolkit' (Carroll, 2008; Gilbert et al., 1996; Shubin et al., 2009). However, the development of polyphenic traits is largely influenced by environmental and social factors. Therefore, to understand the origins of wing polyphenism in ants it's important to assess the interactions between the environment, genes, development, and evolution. The emerging field of ecological evolutionary development (eco-evo-devo) integrates ecology with evolutionary development in order to explore how the environment interacts with genetic toolkits during development to generate phenotypic diversity over evolutionary time (Abouheif et al., 2014; Gilbert & Epel, 2015; Sultan, 2015; Toth & Rehan, 2017; West-Eberhard, 2003). Here, we take an eco-evo-devo approach to provide a holistic synthesis of the factors that may have led to the origin and subsequent evolution of wing polyphenism in ants. We hope that the knowledge gained by reconstructing the events

leading to wing polyphenism in ants will potentially teach us about other major evolutionary transitions in individuality.

1.3. Hymenopteran clues for the origin of wing polyphenism in the Formicidae

Within the Hymenoptera, the ants, vespid wasps, crabronid wasps, and halictid bees all evolved eusociality independently (see stars in Fig. 2). Among these eusocial groups, ants are the only family (Formicidae) in which all species are both eusocial and wing polyphenic with winged queen and wingless worker castes within a colony. In this section, we explore whether wing polyphenism is a unique feature that originated in ants, or if it also evolved in other Hymenopteran groups. Furthermore, we informally explore the possible phylogenetic correlations between the origin of wing polyphenism and sexual wing dimorphism, a feature that is widespread among the Hymenoptera. We raise the possibility that the developmental capacity to generate sexual wing dimorphism was co-opted to generate a wing polyphenism among the ants. Finally, we explore environmental and ecological factors, as well as possible selection pressures, that may have facilitated the origin of wing polyphenism.

By mapping the occurrence of wing polyphenism on a phylogeny of the Hymenoptera, we discovered that wing polyphenism is not unique to ants, but instead, evolved several times independently in a small number of species belonging to four different wasp families (see squares in Fig. 2). In the family Bethylidae (bethylid wasps), wing polyphenism in the females (winged and wingless morphs) evolved in two species, *Sclerodermus puparia* and *Sclerodermus guani* (Wei et al., 2016; Yang et al., 2012). In the superfamily Ichneumonoidea (ichneumon wasps), wing polyphenism evolved in males of the species *Gelis corruptor* where they can develop either into a fully winged morph or a morph with minute rudimentary wings (Salt, 1952). In the superfamily Chalcidoidea (chalcid wasps), wing polyphenism is found in all species of the genus *Melittobia* (family Eulophidae) and in various species of fig wasps (family Agaonidae). In *Melittobia*, females have a wing polyphenism such that they develop into a short-winged morph or a fully winged morph (Matthews & Gonzalez, 2008; Matthews et al., 2009; Schmieder, 1933). The Agaonidae (fig wasps) have commonly evolved a wing polyphenism in males, with an estimate of five independent origins across the family (Cook et al., 1997). Furthermore, some species of fig wasps produce only wingless males (Hamilton,

1979). Overall, our informal character mapping of wing polyphenism shows that has it has evolved several times independently across the Hymenoptera in taxa that are distantly related to ants (Fig. 2). Despite this repeated evolution of wing polyphenism across the Hymenoptera, ants remain the only group in which it is a universal trait.

Sexual wing dimorphism refers to genetically induced variation in wing development between the sexes (Mayr, 1963). Because sexual wing dimorphism has frequently evolved within the Hymenoptera, we also informally mapped its occurrence to explore the phylogenetic correlation between the independent origins of sexual wing dimorphism and wing polyphenism (see black and grey circles and squares in Fig. 2). Sexual wing dimorphism has evolved in at least 19 wasp families, and in at least 8 of these families, it is present in all species (Fig. 2; Goulet & Huber, 1993; Reid, 1941). Among the sawflies, sexual wing dimorphism is extremely rare and found in about four species belonging to the Tenthredinoidea superfamily (Smith, 1993). In almost all cases where a sexual wing dimorphism has evolved, the females are the sex that is wingless or short-winged, whereas the males are winged. Majority of the taxa that develop a sexual wing dimorphism are part of the Apocrita suborder, which includes wasps, bees, and ants (Fig. 2). Within the Apocrita, the subclade Aculeata, with the exception of the superfamily Apoidea, contains the largest concentration of families where a sexual wing dimorphism has evolved (Fig. 2). Our informal mapping suggests that the evolution of a sexual wing dimorphism coincides with the evolution of a wing polyphenism in 2 wasp taxa: the genus *Melittobia* (superfamily Chalcidoidea) and the species G. corruptor (superfamily Ichneumonoidea). In *Melittobia*, the males are always short-winged, whereas the females can be short-winged or fully-winged (Matthews & Gonzalez, 2008; Matthews et al., 2009; Schmieder, 1933). In *G. corruptor,* the females are always wingless, whereas the males can be short-winged or fully-winged (Salt, 1952). In ants, wingless males have evolved infrequently, notably in the species *Hypoponera bondroiti* and in the genus *Cardiocondyla* (Heinze et al., 2005; Oettler et al., 2010; Yamauchi et al., 1996). These species represent instances where the evolution of sexual wing dimorphism (winged female queens and wingless males) and wing polyphenism (winged queen and wingless workers) also overlap in ants. Moreover, these observations reveal that, for the majority of cases, the females are the wingless morph in both the evolution of sexual wing

dimorphism across the Hymenoptera and wing polyphenism in ants. Furthermore, two out of four wasp taxa that have wing polyphenism within one sex also develop sexual wing dimorphism between the sexes.

Ants are a sister-taxon to the Apoidea superfamily, which constitutes sphecid wasps and bees (Fig. 2; Branstetter, Danforth, et al., 2017; Johnson et al., 2013; Peters et al., 2017). Since sexual wing dimorphism is rare in Formicidae and Apoidea (Fig. 2), we can infer that sexual wing dimorphism was absent in the ancestral ant lineages and their immediate wasp relatives. However, there may have existed an ancestral developmental potential, which is a latent genetic program for a specific trait that evolved in the ancestors of a group of organisms and is retained in the descendants. Such potentials can be subsequently realized resulting in the independent or parallel evolution of a trait in response to certain mutations or environmental perturbations (Fave et al., 2015; Rajakumar et al., 2012; West-Eberhard, 2003). The frequent and repeated evolution of sexual wing dimorphism in the Apocrita (see circles in Fig. 2) raises the possibility that the ancestral lineage leading the origin of ants may have possessed a latent developmental potential to generate a sexual wing dimorphism that was retained from the ancestor of the Apocrita. If the developmental potential to produce sexual wing dimorphism was present in early ants, or even in the common ancestor of Formicidae and Apoidea, then we propose the bold hypothesis that this potential may have been co-opted to give rise to wing polyphenism within the female caste of ants. A transition from genetically determined sexual dimorphism to an environmentally-induced wing polyphenism is possible if the developmental mechanism underlying the regulation of sexual dimorphism becomes sensitive to environmental stimuli (Schwander & Leimar, 2011). In the section titled “Evolution of wing polyphenism via genetic accommodation”, we propose a mechanism through which such a transition could have evolved as well as a mechanism for the de novo origin of wing polyphenism in the absence of developmental potentials in the ancestral lineages that led to the evolution of ants.

Additionally, we explored if there are similar environmental factors and selective pressures that led to the parallel or convergent evolution of wing polyphenism across the five Hymenopteran groups (including ants). Temperature, photoperiod, nutrition, and brood size

appear to be involved in regulating wing polyphenism across these five groups (Consoli & Vinson, 2004; Hamilton, 1979; Heinze, 2017; Innocent et al., 2010; Wang et al., 2016; Wheeler, 1986). This would not be surprising given that these environmental factors are also found to trigger the development of a wing polyphenism in species outside of the Hymenoptera, such as aphids, rice planthoppers, and crickets (reviewed in Zhang et al., 2019). Furthermore, in many cases, the selection pressure behind the origin of a wing polyphenism is a trade-off between remaining in the nest or dispersing. In *Mellitobia* wasps, fig wasps, and *Cardiocondyla* ants, wing polyphenism is associated with a trade-off between different mating strategies – mating with conspecifics in the confines of the parental nest or dispersing to mate outside of the parental nest. Specifically, in the wasp genus *Mellitobia*, winged or wingless morph development depends on nutritional levels where decline in nutrition (due to an increase in brood size) leads to the production of winged females that disperse and mate outside of the parental nest (Consoli & Vinson, 2004; Innocent et al., 2010; Schmieder, 1933). Fig-pollinating wasps nest in the ovaries of fig flowers where the males have evolved a wing polyphenism linked to different mating strategies. The male fig wasps develop either into winged morphs that disperse or wingless morphs that mate inside the confines of the nest. The development of wingless males is found to be correlated with a large brood size (Hamilton, 1979). Finally, in the ant genus *Cardiocondyla*, the production of winged or wingless males is also due to selection between mating in the natal nest and dispersal (reviewed in Heinze, 2017). This is similar to the female wing polyphenism in ants where winged queens disperse and wingless workers remain in the parental nest.

The ecology and life-history traits of the Apocrita clade may have facilitated the evolution of sexual wing dimorphism. The Apocrita are characterized by having a restriction in their abdomen (known as 'wasp-waist') and a parasitoid lifestyle (see red triangles in Fig 2). The females use their wasp-waist to bend their abdomen and lay their eggs on or in the larvae of other insects (Whitfield, 2003). The parasitoid female wasps commonly locate host larvae that are well concealed in ground nests, tree bark, or plant galls – a behavior suggested having evolved as selection against parasitizing exposed larvae that would be vulnerable to predators (Whitfield, 1998). Although this idea has not been formally tested, the evolution of a sexual

wing dimorphism is correlated with a parasitoid lifestyle where the females burrow through soil or small spaces in search of host larvae (Deyrup, 1988; Kimsey, 1991; Quicke, 2015). Furthermore, it has been previously suggested that wingless females evolved as an adaptation to burrowing through cryptic spaces as (1) wings hamper movement and become abraded; and (2) energy used for development of flight muscles can be instead allocated towards muscles used for digging (Deyrup, 1988; Evans, 1969; Quicke, 2015). Therefore, a parasitoid lifestyle which involves navigating through concealed environments in search of hosts may have been a strong selective pressure for the repeated evolution of sexual wing dimorphism in wasps. This selection pressure is different from that which induces wing polyphenism in wasps (trade-off between mating strategies, see above). In ants, however, wing polyphenism and wing shedding behavior in queens after mating, is in part associated with their ability to inhabit ground and subterranean strata. Therefore, the selective pressure for generating sexual wing dimorphism in wasps (wingless female wasps) and wing polyphenism in ants (wingless worker caste) in ants may have evolved in response to similar environmental factors and selective pressures.

In the Apoidea superfamily, which includes sphecid wasps and bees, the evolution of sexual wing dimorphism is rare (Fig. 2). Ecological or behavioral changes that occurred in Apoidea may account for the decreased selection for evolving wingless females in this group. The majority of sphecid wasps construct nests where they provision their young with hosts to consume as they develop (see yellow triangles in Fig. 2). Since the host or prey is translocated to the nest, the sphecid wasps no longer need to burrow through cryptic habitats to locate hosts for their young. In fact, many sphecid wasps parasitize adult insects as opposed to their well-concealed larvae (Goulet & Huber, 1993). Perhaps it was this ecological innovation (i.e., nest building and provisioning) that contributed to lowering the selection pressure to evolve a sexual wing polyphenism. In bees, in addition to nest building, the parasitoid lifestyle was completely substituted with provisioning the young with pollen and nectar (see green triangles in Fig. 2; Michener, 2000). Consequently, this suggests that there was no selection for developing a sexual wing dimorphism since flight is essential in this group for provisioning the larvae with food. Nest-building and pollen-collecting have also evolved in some species of vespid wasps (family Vespidae), which also coincides with the absence of sexual wing

dimorphism in this group (Fig. 2; Goulet & Huber, 1993). Finally, the transition from a parasitoid lifestyle to that of gall-forming (where females lay their eggs on a plant and the developing larvae induce the formation of a protective gall where they feed on the plant tissue) and fig-pollinating (where females lay their eggs in the ovaries of fig flowers while simultaneously pollinating them) occurs in lineages where the evolution of a sexual wing dimorphism is sporadic (see orange and blue triangles in Fig. 2; Whitfield, 2003). Taken together, compared with a parasitoid lifestyle, the evolution of nest-building and provisioning, pollen-collecting, gall-forming, and fig-pollinating may have decreased the selective pressure to evolve a sexual wing dimorphism in Apoidea and other hymenopteran families.

1.4. Ant fossils provide evidence that wing polyphenism and eusociality in ants arose together during the Early Cretaceous

The oldest ant fossils found to date are from the Early Cretaceous dating approximately 100 million years ago (mya) (Barden, 2016; Perrichot, Lacau, et al., 2008). The majority of ant fossils found in the Early Cretaceous are members of extinct and early-branching subfamilies. In addition, several of the Early Cretaceous fossils discovered belong to the crown group of ants, which are extant and derived ant lineages (Grimaldi & Agosti, 2000; Perrichot, 2019). These fossil findings, in addition to molecular analyses, place the origin of the crown group of ants between 111 and 169 mya (Borowiec, 2019; Brady et al., 2006; Moreau & Bell, 2013; Moreau et al., 2006). Therefore, the extinct and early-branching subfamilies of ants (stem group) must have originated earlier than 111-169 mya. These Early Cretaceous fossils, although rare, have been instrumental in revealing the origin and ancestral characteristics of ants, particularly the origins of wing polyphenism and eusociality (Perrichot, Nel, et al., 2008; Wilson, 1987; Wilson et al., 1967a).

The main morphological synapomorphies used to define ants (with the exception of secondary losses in certain taxa) include a metapleural gland, a petiole (constriction of the second abdominal segment), elongated scape (the first antennal segment), and elbowed antennae (Fig. 3a; Barden, 2016; Engel & Grimaldi, 2005; Wilson et al., 1967a). Additionally, some authors use non-morphological synapomorphies such as eusocial behavior and the presence of a wingless worker caste (Bolton, 2003; LaPolla et al., 2013). The metapleural gland,

which is a uniquely evolved feature of ants, serves numerous functions from communication to antisepsis and hygiene (Yek & Mueller, 2011). It is regarded by many paleomyrmecologists as the primary synapomorphy that defines true ants (Barden, 2016; Bolton, 2003; Engel & Grimaldi, 2005; Ward, 2007; Wilson et al., 1967a)

The first appearance of what might be considered the earliest ants are the Armaniids (Fig. 3b). This group is known exclusively from poorly preserved rock impressions of winged individuals from the Albian (~100-110 mya), part of the Early Cretaceous (Dlussky, 1983, 1999). These specimens have ant-like characteristics such as a poorly developed petiole and a short scape, but the poor preservation of impression fossils has made the presence of a metapleural gland in Armaniids extremely difficult to ascertain. This uncertainty regarding the presence of the metapleural gland has inspired an on-going debate on whether Armaniids should be considered 'true' ants, representing the most basal subfamily or whether they should be given a family rank and placed as an extinct sister family to the Formicidae (Bolton, 2003; Engel & Grimaldi, 2005; Wilson, 1987). Furthermore, no wingless specimens of Armaniids have been found to date that would indicate the presence of a worker caste (Dlussky, 1983, 1999). If Armaniids are considered to be ants, then the absence of a wingless worker caste from the fossil record can be explained by one of the following: (1) these early ants were solitary; (2) they lived in groups but did not have a social structure; (3) there was a social structure but the females were not morphologically differentiated; and (4) they were morphologically differentiated (i.e. presence of wingless worker caste) but conditions did not allow workers to be fossilized. These types of fossils that are formed in lake sediments are biased to include aquatic insects or insects that fell into the lake while flying above (LaPolla et al., 2013). Therefore, if a wingless worker caste did exist in Armaniids then it is improbable that it would have been fossilized in lake sediment.

One of the oldest unequivocal ant fossils is *Sphecomyrma freyi* (Fig. 3c) in New Jersey amber (dating ~92 mya) (Wilson et al., 1967a). This was the first discovery of a Cretaceous fossil that undoubtedly had a metapleural gland (Agosti et al., 1998). The primitive characteristics of *S. freyi*, in addition to its remarkable combination of ant-like and wasp-like features, led to the establishment of the Sphecomyrminae subfamily (Wilson et al., 1967a, 1967b). Given the

uncertainty surrounding the status of the Armaniidae, the Sphecomyrminae is considered by some myrmecologists to be the most basal subfamily in Formicidae and the closest relative to all extant ants (Wilson et al., 1967a). The fact that *S. freyi* is known from two wingless workers preserved in the same resin strongly indicates that individuals in the colonies of this species were wing polyphenic and eusocial because it is improbable for two ants to be trapped in the same resin unless they were foraging together (LaPolla et al., 2013; Perrichot, Lacau, et al., 2008). This fossil discovery, in addition to the fact that wing polyphenism is present in all extant ant lineages, suggests that wing polyphenism evolved either just prior to or shortly after the origin of ants.

Following the discovery of the Cretaceous ant fossil *S. freyi*, further support for the early origin of wing polymorphism in ants came from the discovery of wingless females in numerous other Cretaceous fossils (Agosti et al., 1998; Barden & Grimaldi, 2014; Barden & Grimaldi, 2016; Engel & Grimaldi, 2005; Wilson et al., 1967a). Wilson's (1987) analysis of various wingless ant fossils showed that they morphologically group with modern ants and hence are part of a worker caste as opposed to their grouping with solitary wingless female wasps (see section 2). Perhaps the strongest support for the existence of winged and wingless castes is the discovery of winged queens or males, and most importantly, the discovery of both winged queens or males and wingless workers from the same species (Barden & Grimaldi, 2012; Perrichot, Nel, et al., 2008). Numerous fully-winged males and females, as well as dealate females (queens that have shed their wings), have been discovered and classified into at least 6 distinct genera (Barden & Grimaldi, 2012; Barden & Grimaldi, 2016; Grimaldi et al., 1997; Perrichot, Nel, et al., 2008). Among these, a winged queen was discovered preserved in French amber (dating ~ 100 mya) alongside two wingless workers belonging to the Sphecomyrminae species *Haidomyrmodes mammuthus* (Perrichot, Nel, et al., 2008). This finding removes any uncertainty about the presence of wing polyphenic female caste in early ants with a eusocial lifestyle.

The fossils of *H. mammuthus* also suggest that the ancestral condition of ants are colonies with queens and workers with little size dimorphism and the principal morphological difference between them is the wing polyphenism (Barden & Grimaldi, 2016; Perrichot, Nel, et al., 2008). This is also supported by less derived ant subfamilies, such as the Ponerinae and

Leptanillinae, which are generally characterized by colonies in which the queen and worker caste also show little size dimorphism or other morphological differences between them (Brady et al., 2006; Masuko, 1990; Peeters, 1997; Wilson & Holldobler, 2005b). This contrasts with the derived subfamilies, where the queen can be up to ten times larger than the workers (Peeters, 1997). The few species for which data is available show there is a correlation between the timing of caste determination and morphological divergence between castes; the earlier caste determination occurs, the more divergent queen and worker morphologies become (Fjerdingstad & Crozier, 2006; Wilson, 1954). Consequently, if the ancestral condition of ant colonies is the absence of pronounced queen-worker size dimorphism, then we can infer that caste determination in early ants may have occurred during a late stage of larval development. This makes less derived ant subfamilies with low queen-worker dimorphism a good model to infer the developmental steps to the origin of wing polyphenism ants. This topic will be further discussed in Section 5.

Finally, early Cretaceous fossils also provide evidence on the nature of eusociality in these early ant lineages. First, studies in crown-group ants (derived lineages) show that the primary role for the metapleural gland is in colony hygiene and chemical defense (Yek & Mueller, 2011), and therefore, its presence in early ants suggests that its evolution allowed them to live and defend their colonies in the ground or in rotting wood above the ground. Second, Barden and Grimaldi (2016) reported two remarkable samples of amber each containing an aggregation of 6 and 11 *Gerontoformica spiralis* workers. This finding, along with the presence of a prey item in one of the amber samples, led the authors to suggest that these ants were social and performed group search and recruitment of prey. Finally, a notable piece of amber also reported by Barden and Grimaldi (2016) contains two workers from different species engaged in a fight. This suggests the presence of warfare between different species and aggression towards invaders, which is a common feature of a social or cooperative lifestyle. Collectively, in all fossils where a social organization is apparent, the presence of wingless workers is also found, indicating that wing polyphenism and eusociality originated within a very short period of evolutionary time of one another.

1.5. The implications of originating first: wing polyphenism versus eusociality

Although there is little doubt that the early ant lineages were both eusocial and wing polyphenic (Barden & Grimaldi, 2016; Perrichot, Nel, et al., 2008), it remains unclear which of these key traits originated before the other. If wing polyphenism followed the evolution of eusociality then it may have been instrumental to maintaining or reinforcing eusociality. On the other hand, if wing polyphenism originated first, then it may have facilitated the origin of eusociality. And of course, it remains possible that, although these two events evolved very close to one another in evolutionary time, they may have evolved independently without mutually influencing each other. Here we explore the implications of three possible scenarios: (1) eusociality evolves before wing polyphenism; (2) eusociality evolves after wing polyphenism; or (3) eusociality evolves at the same time as wing polyphenism.

In all three scenarios (Fig. 4a-c-1), we can assume that the ancestral wasp lineage leading to the ants was solitary, preyed on arthropods, and constructed/inhabited nests similar to the sphecid wasps, which are the closest relatives to ants (see section 2; Branstetter, Danforth, et al., 2017; Johnson et al., 2013; Peters et al., 2017). In the first scenario or model, which we term the 'eusociality first model', the formation of groups composed of parents and their daughter offspring may have evolved immediately for the purpose of sharing and defending a nest (Fig. 4a-2; Nowak et al., 2010). Subsequent to the formation of such groups, cooperation and reproductive division of labor appeared leading to the origin of eusociality (Fig. 4a-3). Although at this stage workers may have had the capacity to reproduce, social regulation by the queen suppressed worker reproduction. Next, the evolution of eusociality, where daughters remained in the nest and foraged on the ground, may have contributed to the origin of wing polyphenism in ants. The reproductive females (gynes) developed wings whereas the non-reproductive females (workers) did not (Fig. 4a-4). The rise of a wingless worker caste may have enabled ants to inhabit new niches particularly underground strata. Finally, we propose that the origin of wing polyphenism further reinforced the reproductive division of labor within the colony by preventing workers from participating in mating flights and dispersing far from the nest (Fig. 4a-5). Therefore, in this scenario, the origin of wing polyphenism is thought to have reinforced eusociality.

In the second scenario, which we term the 'wing polyphenism first model', wing development evolved to response to different environmental conditions, such as nutritional levels, rearing density, and temperature. This would have led offspring from the same nest to develop either into winged or wingless morphs depending on environmental cues (Fig. 4b-2). The development of daughter offspring without wings would have prevented them from dispersing, and as a consequence, they would have remained in the parental nest and mated with nestmates (Fig. 4b-3). The sharing of a nest by parent-offspring groups eventually led to cooperation and reproductive division of labor, giving rise to the origin of eusociality (Fig. 4b-4). Therefore, in this scenario, the origin of wing polyphenism first is thought to have directly influenced the formation of parent-offspring groups, ultimately leading to the origin of eusociality.

Finally, in the third scenario, which we term 'simultaneous origins model', wing polyphenism and eusociality evolved simultaneously. First, the formation of parent-offspring groups (Fig. 4c-2) may have evolved for the purpose of sharing and defending a nest (Fig. 4a-2; Nowak et al., 2010). Ecological and/or social factors may have then led to the simultaneous evolution of wing polyphenism and eusociality (Fig. 4c-3). Wing polyphenism may have either evolved because of ecological factors or through the manipulation of larval nutrition by the mother. At the same time, a reproductive division of labor may have evolved either through the aggression of dominant individuals towards 'subordinate' individuals or through aggression of the mother towards her daughters. In this scenario, it remains unclear whether wing polyphenism and eusociality evolved independently and without influencing each other or whether they had mutual influence on each other and were causally linked.

The ancestral habitat of the early ants, which may have contributed to both the origin of wing polyphenism and eusociality, regardless of which of the three scenarios may be true, remains largely unknown. One hypothesis put forth by Wilson and Holldobler (2005b), called the "Dynastic Succession" hypothesis, proposes that ants evolved in leaf-litter and soil surface habitats and then subsequently spread to other strata (underground and canopy) during the rise of the angiosperms. This is supported by the presence of large eyes in the extinct ant genus *Sphecomyrma* indicating that early ants foraged above ground (Wilson et al., 1967a). In

contrast, Lucky et al. (2013) put forth an alternative hypothesis called the “Out of the Ground” hypothesis. Lucky et al. (2013) performed an ancestral state reconstruction of habitat transitions in ants and concluded that the ancestral ants were subterranean (nested and foraged below the surface). Therefore, colonization of leaf-litter and other strata would have evolved only subsequently.

The metapleural gland, a defining feature of ants that is present in all early ant fossils (Agosti et al., 1998; Barden, 2016), may provide insight into the ancestral habitat as well as the relationship between wing polyphenism evolution and eusociality. In extant ants, the gland has been shown to secrete antibiotic substances essential to overcoming the pressures from microbes and parasite transmission associated with living in colonies as well as nesting under the soil or in decomposing leaf-litter or rotting wood (Boomsma et al., 2005; Holldobler & Wilson, 1990; Yek & Mueller, 2011). Although there have been no studies on the function of the metapleural gland in early-branching ant lineages, it is reasonable to infer that its original function was for colony hygiene and immunity against soil microbes. The metapleural gland is located on the metapleuron plate of the thorax (Holldobler & Wilson, 1990), where its secretions can be easily accessed and spread to the body through coordinated leg movements (Fernandez-Marin et al., 2006). We propose that the presence of wings on the thorax of workers would have blocked access to the metapleural gland, and as a consequence, would have hindered its evolution. If our hypothesis is correct, then the suppression of wings from the thorax of workers (origin of wing polyphenism) may have preceded, and was a necessary condition for, the evolution of the metapleural gland. This would also apply to queens because they shed their wings after mating. It therefore follows that if the metapleural gland is a necessary condition for the origin of eusociality because its antimicrobial and immune functions were necessary for group / colony living, then this would support the ‘wing polyphenism first model,’ where wing polyphenism preceded the origin of eusociality. Furthermore, if nesting in subterranean environments (under the soil surface) also depends on the presence of a metapleural gland, and if we assume that the development of the metapleural gland was made possible by evolving a wing polyphenism, then wing polyphenism must have preceded underground nesting. This raises the possibility that the ancestral ants arose aboveground and

moved to soil secondarily (as proposed by the Dynastic Succession hypothesis). These hypotheses regarding the metapleural gland can be tested by investigating whether or not there are biomechanical constraints placed on the metapleural gland by the presence of wings.

1.6. A model for the molecular and developmental mechanisms regulating wing polyphenism in basal ant lineages

Polyphenism typically begins with sensing an environmental cue, which is then converted into internal signals that alter the developmental trajectory of an organism (Nijhout, 1999). Various types of environmental cues have been shown to regulate wing polyphenism in insects, such as social interactions, pheromones, photoperiod, temperature, nutrition quality, and rearing density (Rajakumar et al., 2018; Zhang et al., 2019). In ants, nutrition, temperature, and social interactions are key environmental cues known to induce worker-queen determination and wing polyphenism (Penick & Liebig, 2012; Wheeler, 1986; Wheeler & Nijhout, 1984). After sensing the ecological or social cue, internal signals such as insulin signaling and juvenile hormone are released during a sensitive period in development. This sensitive period can be as early as embryogenesis, such as in the genus *Pheidole* (Passera & Suzzoni, 1979), or as late as the last larval instar, such as in the ponerine species *Harpegnathos saltator* (Penick et al., 2012).

In ants, wings as well as other adult structures such as legs and antennae, develop from clusters of cells in the larvae called 'imaginal discs.' These imaginal discs proliferate during larval development, particularly the last larval instar, and terminally differentiate and evaginate during pupal development to become the adult structures (Held, 2002). Queen and male ants develop fully functional adult wings from wing imaginal discs (hereafter wing discs). However, adult workers, although completely wingless, develop vestigial or rudimentary wing discs during larval development (Fig. 5a; Dewitz, 1878; Wheeler & Nijhout, 1981a). The size and shape of the worker rudimentary wing discs varies between species, varies within subcaste of the same species, and correlates with the timing of queen-worker determination (Abouheif & Wray, 2002; Shbailat et al., 2010; Wheeler & Nijhout, 1981b).

Molecular studies investigating the genetic and developmental mechanisms underlying ant wing polyphenism have been predominantly conducted in the two most species-rich and

highly-derived subfamilies: the Formicinae and the Myrmicinae (Fig. 6). These subfamilies are part of the Formicoid clade, which is composed of taxa that have derived developmental and social characteristics (Fig. 6; Borowiec, 2019; Brady et al., 2006). The Poneroid clade, on the other hand, is composed of subfamilies (particularly the Ponerinae) that exhibit less divergent characteristics such as predatory behavior, low queen-worker dimorphism, and small colony size (Peeters, 1997; Wilson & Holldobler, 2005b). These two clades, the Poneroid and Formicoid, have had the same amount of time to diverge from the ancestral phenotype, yet they drastically differ in their evolutionary rates and developmental and social characteristics. The less divergent characteristics of the Poneroid clade, especially the subfamily Ponerinae, reflect the presumed ancestral condition because they are similar to those found in the most basally branching clade of ants: the subfamilies Leptanillinae and Martialinae, and the extinct subfamily Sphecomyrminae (Masuko, 1990; Perrichot, Nel, et al., 2008; Perrichot et al., 2016; Rabeling et al., 2008). Unfortunately, these early-branching subfamilies are rarely found in nature and have limited life history information and no developmental studies. Here, we use all available information from ants to propose a model of developmental mechanisms that may have facilitated the origin of wing polyphenism. Due to the limited number of studies on wing polyphenism in ants, we will also base our model on the mechanisms of wing development known from *Drosophila* and Lepidoptera, as well as from other insects that evolved a wing dimorphism.

1.6.1 Interruption in the wing gene regulatory network (GRN)

In insects, wing development is regulated by a series of transcription factors and signaling molecules (morphogens) that together comprise the wing gene regulatory network (GRN; Fig. 7a; Held, 2002). This GRN has been conserved in insects for at least 400 million years (Abouheif & Wray, 2002; Brisson et al., 2010; McCulloch et al., 2019; Tomoyasu et al., 2009). Regulation of the wing GRN is well understood in the fruit fly *Drosophila melanogaster*. In *D. melanogaster*, the wing GRN in the wing discs begins with the activation of the transcription factors *apterous* (*ap*) and *engrailed* (*en*), which specify the formation of the dorsal-ventral (D-V) and the anterior-posterior (A-P) compartments, respectively (Cohen, 1993; Diaz-Benjumea & Cohen, 1993). In the D-V compartment, ap initiates the activation of *serrate* (*ser*) which then

activates *wingless* (*wg*), while in the A-P compartment, *en* activates *hedgehog* (*hh*) which in turn activates *decapentaplegic* (*dpp*) (Diaz-Benjumea & Cohen, 1995; Kim et al., 1995; Zecca et al., 1995). *Dpp* activates *spalt* (*sal*), which controls notum and hinge development, as well as vein positioning in the *D. melanogaster* wing discs (de Celis & Barrio, 2000; Grieder et al., 2009). Additionally, *dpp* suppresses the expression of *brinker* (*brk*), which when upregulated, suppresses the activity of *Dpp* targets, such as *sal*, to prevent wing disc development (Campbell & Tomlinson, 1999; Martin et al., 2004; Minami et al., 1999; Winter & Campbell, 2004). Ultimately, the D-V and A-P compartments together control the growth of the wing discs through the activation of the wing-specific selector gene complex *vestigial* (*vg*) and *scalloped* (*sd*) (Cohen, 1996; Couso et al., 1995; Halder et al., 1998; Kim et al., 1997; Simmonds et al., 1998; Williams et al., 1991).

Abouheif and Wray (2002) conducted the first study investigating the differences in the wing GRN between winged queens and wingless workers in ants. In this study, the authors initially predicted that a common mechanism for interrupting wing development was present in all workers since wing polyphenism originated once in ants. An interruption is defined as any change in activation, suppression, or expression pattern (spatial/temporal) of any gene in the network when compared to its expression in winged queens or males of that species. Expression analysis of six genes in the wing GRN across four species belonging to the subfamilies Formicinae and Myrmicinae (Fig. 6) showed that different genes were interrupted in the wingless worker caste of different species, between the wingless subcastes (minors and majors) within a single species, and between the forewings and hindwings within a single caste (Fig. 6 blue circles; Fig. 7b). For example, *en* expression was absent in the species *Neoformica nitidiventris*, *Crematogaster lineolata*, and minor workers of *Pheidole morrisi*, but was expressed in the soldier caste of *P. morrisi* (Abouheif & Wray, 2002). This finding established that, despite the fact that wing polyphenism evolved once at the origin of ants, the genetic mechanism underlying its regulation is labile, even across relatively short time scales.

Since only a proportion of all the genes comprising the wing GRN were sampled, the presence of conserved non-labile genes that mediate wing suppression remains a possibility. *brk* was predicted to be such a gene since it prevents wing disc growth when upregulated

(Martin et al., 2004) and is a repressor of *sal* (Winter & Campbell, 2004), which is the only interrupted gene in the soldier subcaste of *P. morrisi* (Abouheif & Wray, 2002). Nahmad et al. (2008) constructed a mathematical model to simulate the effect of upregulation or downregulation of *brk*, in addition to other genes in the network, on the growth of the rudimentary wing discs in wingless worker ants. The simulations showed that a 5- or 10-fold increase in *brk* expression led to a significant reduction in *sal* expression as well as a reduction in the size of the rudimentary wing discs. Based on this model, it was predicted that expression of *brk* would be upregulated in the rudimentary wing discs in worker larvae, and thus repressing genes such as *sal* to prevent wing disc growth. However, when formally tested, instead of upregulation, *brk* expression was found to be absent in the rudimentary wing discs of *P. morrisi* soldiers (Fig. 7b; Shbailat et al., 2010). Analysis of *brk* expression in the worker caste of three additional species, *Crematogaster lineolata*, *Tetramorium caespitum*, and *Lasius niger*, consistently showed its absence in the rudimentary wing discs of the worker caste (Fig. 7b; Shbailat & Abouheif, 2013). However, a study in the genus *Mystrium* (Subfamily Amblyoponinae, Poneriod clade; Fig. 6) showed the presence of *brk* expression (Fig. 7b; Behague et al., 2018), suggesting that interruption in *brk* expression (absence) is a common feature that evolved in derived lineages.

Analysis of *brk* and eight additional genes in the Poneroid ant genus *Mystrium* showed a similar expression pattern between the wing discs in queens and workers, suggesting a lack of interruption in the core part of the wing GRN of the worker caste of less-derived lineages (Fig. 6 yellow circle; Fig. 7b; Behague et al., 2018). However, the possibility remains that an interruption in the Poneroid clade may occur downstream of the network. If future studies in the Poneroid clade show that interruption indeed occurs downstream of the network, then it would reveal a compelling evolutionary pattern: late interruption in less-derived lineages and early interruption in more-derived lineages. Furthermore, if interruption does occur downstream of the network in the Poneroid clade, then it might account for the large size of the larval rudimentary wing discs in this group since the wing discs would substantially grow and differentiate before an interruption occurs that halts further development. In contrast, in the Formicoid subfamilies, which generally develop small rudimentary wing discs compared to

Poneriod subfamilies, early interruption in the GRN does not allow the rudimentary wing discs enough time to proliferate considerably. However, an evolutionary reversion to this pattern is observed in the rudimentary wing discs in the genus *Pheidole* (subfamily Myrmicinae) (Rajakumar et al., 2018; Rajakumar et al., 2012). Another intriguing possibility is that in the Poneroid subfamilies there is no interruption in the wing GRN and an alternate mechanism, such as apoptosis, may be responsible for the elimination of the rudimentary wing discs. The two possibilities are not mutually exclusive and further studies in this clade are needed to reveal the precise developmental regulation of wing polyphenism in less-derived ant lineages that have ancestral-like characteristics.

1.6.2 Apoptosis

Apoptosis, also known as programmed cell death, is an important developmental mechanism underlying morphological evolution, and particularly is involved in the production of various polyphenic and dimorphic traits. For instance, apoptosis is involved in the degeneration of pupal wings in female tussock moths (Lobbia et al., 2003), resorption of pupal horns in female dung beetles (Kijimoto et al., 2010), and elimination of male-specific gonads during embryonic development of female fruit flies (DeFalco et al., 2003); in all of these cases, apoptosis facilitates the production of sexual dimorphism. Is apoptosis also involved in generating a wing polyphenism in ants? And if so, is apoptosis induced by the interruption in the wing GRN? A study by Sameshima et al. (2004) showed that apoptosis is present in the rudimentary wing discs of the soldier subcaste of *Pheidole megacephala*. Moreover, in the species *P. morrisi*, Shbailat et al. (2010) showed that the induction of apoptosis is spatially and temporally correlated with a change in expression patterns of two genes in the wing GRN: sal and dpp. Over five decades, genetic manipulations in *D. melanogaster* have shown that appendage patterning genes can induce apoptosis and that mutations in genes belonging to the wing GRN (such as *ap*, *wg*, *vg*) can induce cell death in the tissue of the wing discs (Fristrom, 1969; Giraldez & Cohen, 2003; James & Bryant, 1981; Sedlak et al., 1984). Therefore, it is possible that interruption in the wing GRN may induce apoptosis to eliminate rudimentary wing discs. Furthermore, if multiple genes in the wing GRN are able to induce apoptosis then this may explain why interruption points are evolutionarily labile. Thus far, only two species across

the ants (belonging to Myrmicinae subfamily) have been tested for the presence of apoptosis in the rudimentary wing discs of the worker caste (Fig. 6 purple square; Sameshima et al., 2004; Shbailat et al., 2010). Investigating the prevalence of apoptosis across the Formicidae is critical to determine if it is a conserved mechanism across the phylogeny, and if so, it may have important implications in elucidating the mechanism responsible for generating a wing polyphenism during the early origin of ants.

1.6.3 Differential gene expression outside the wing GRN

Many other genes outside of the wing GRN may be differentially expressed between queens and worker ants in order to initiate and regulate caste-specific development. Here we will examine some of these genes and infer what role they may have on the specific regulation and evolution of wing polyphenism.

A study by Klein et al. (2016) analyzed the expression of the sex-determining gene, *doublesex* (*dsx*), and showed that it was alternatively spliced between males and females and differentially expressed between the female caste (i.e., queens and workers) and between the male morphs (winged and wingless) during all life stages of the ant *Cardiocondyla obscurior* (subfamily Myrmicinae). This led to the hypothesis that *dsx* is co-opted from its function in the development of sex-specific traits to a novel function in modulating polyphenic traits within one sex. A similar function of dsx was also reported in the dung beetle *Onthophagus taurus*, where dsx promotes differential horn development between sexes (horned males and hornless females) and within the males (horned large males and hornless small males) (Kijimoto et al., 2012). *Dsx* has also been shown to control sex-specific traits by altering sensitivity to juvenile hormone (JH) in the stag beetle *Cyclommatus metallifer* (Gotoh et al., 2014), suggesting that its role in ant wing polyphenism may also be through modulating JH sensitivity (Klein et al., 2016). Loehlin et al. (2010) showed that wing size reduction in the wasp *Nasonia* is correlated with an increase in *dsx* expression. Future studies should investigate the regulatory role of *dsx* in wasp wing dimorphism and polyphenism, as well as ant wing polyphenism. These studies raise the possibility that there is a shared proximate mechanism underlying sexual wing dimorphism in wasps and wing polyphenism in ants.

A transcriptomics analysis in *C. obscurior* also identified a series of genes involved in sphingolipid metabolism to be differentially regulated between sexes and within castes (Schrader et al., 2015). Sphingolipids, found on cell membranes, function in various metabolic processes such as fat storage and nutrient utilization to control growth and cell death (reviewed in Kraut, 2011). Specifically, the genes lace and schlank, responsible for sphingolipid synthesis, were revealed to be weakly expressed in workers and wingless males in *C. obscurior* (Schrader et al., 2015). Interestingly, studies in *Drosophila* have shown that lace and schlank are required for the development of wing discs, where lace mutants exhibit incision in the wing margin and a high number of dead cells (Adachi-Yamada et al., 1999), and downregulation of schlank affects wg activity, increases levels of apoptosis, and reduces cell size of the wing discs (Pepperl et al., 2013). Therefore, downregulation of these genes in worker ants and wingless males may be affecting wing development that leads to wingless morphs. Transcriptomics studies are likely to provide many more candidate genes that should be functionally tested for their role in regulating wing polyphenism in ants.

1.6.4 Juvenile hormone, ecdysone, and insulin signaling

JH and ecdysone regulate molting and metamorphosis in insects (Nijhout, 1994; Truman & Riddiford, 2002). Ecdysone is a steroid hormone that stimulates molting events, which represent the transition from one larval stage to the next, as well as metamorphosis, which represents larval to pupal transition. The level of JH during an ecdysone pulse determines whether this pulse will cause molting or metamorphosis: high JH results in molting, while low or absent JH results in metamorphosis (Emlen & Allen, 2004; Nijhout, 1994; Nijhout & Wheeler, 1982; Truman & Riddiford, 2002). JH and ecdysone, in addition to insulin signaling, also influence the body and imaginal disc growth in insects (reviewed in Edgar, 2006; Martin & Shearn, 1980; Mirth et al., 2009; Shingleton et al., 2005; Tobler & Nijhout, 2010; Truman et al., 2006), and are involved in the regulation of various polyphenisms including caste polyphenism in social Hymenoptera and termites, male horn dimorphism in beetles, winged/wingless and sexual/asexual phases in aphids, solitary and gregarious phases in locusts, and color polymorphism in butterflies (reviewed in Hartfelder & Emlen, 2012; Nijhout & McKenna, 2018; Nijhout & Wheeler, 1982). Direct studies on the influence of JH, ecdysone, and insulin signaling

on wing polyphenism in the worker caste of ants are limited relative to information about their role in other wing dimorphic insects (this topic was recently reviewed by Zhang et al., 2019). We will therefore synthesize what is known about the interactions between the environment, endocrine hormones, GRN interruption, and apoptosis in ants and other insects to propose a model for the developmental and genetic mechanisms that may have been present during the origin of wing polyphenism in ants.

In ants, as well as other social Hymenoptera, there is ample evidence that high levels of JH during a sensitive period can induce queen development (reviewed in Nijhout & Wheeler, 1982; Wheeler, 1986). Induction of queen development by JH would suggest that JH is positively regulating the development of wings among other queen-specific traits. In support of this, Rajakumar et al. (2012) showed that the application of the JH analog, methoprene, stimulates the growth of the rudimentary wing discs in the soldier subcaste of *P. morrisi*. In contrast, studies in Lepidoptera and *Drosophila* have demonstrated that JH inhibits the growth, differentiation, and evagination of the wing discs (Chihara et al., 1972; Miner et al., 2000; Tobler & Nijhout, 2010; Truman et al., 2006). Furthermore, studies in insects with a wing dimorphism, such as the cricket *Gryllus rubens*, the brown treehopper *Nilaparvata lugens*, the stonefly *Zelandoperla fenestrate*, and the aphid *Megoura crassicauda*, collectively have shown that presumptive short-winged (in crickets and brown treehoppers) or wingless (in aphids and stoneflies) morphs have elevated levels of JH titers and that topical application of JH, or its analog, inhibits wing development and results in the production of short-winged or wingless morphs (Ayoade et al., 1999; Bertuso et al., 2002; Ishikawa et al., 2013; Iwanaga & Tojo, 1986; McCulloch et al., 2019; Zera, 2004, 2006). The reversed role of JH that evolved in ants as compared with other insects from numerous orders suggests that when JH was co-opted to control queen-specific development in social Hymenoptera, it was also co-opted to positively regulate wing development in queens during the origins of ants and the evolution of a wing polyphenism.

Unlike JH, the molting hormone ecdysone has received less attention in caste-determination studies. The limited number of studies investigating its role have shown that in ants, worker-biased eggs and worker larvae have higher ecdysone levels than queen-biased

eggs and queen larvae (Schwander et al., 2008; Suzzoni et al., 1980, 1983). Despite this correlation, the role of ecdysone in caste determination and ant wing polyphenism has yet to be investigated. Its role on wing development has been well elucidated from studies in *Drosophila* and the moth *Manduca sexta*, where it is found to induce growth and evagination of the wing discs (Chihara et al., 1972; Nijhout et al., 2007). Furthermore, ecdysone is known to upregulate numerous genes in the wing GRN during *Drosophila* metamorphosis (Li & White, 2003). However, this positive regulation of wing development is contrary to its role in wing morph differentiation in the pea aphid *Acyrthosiphon pisum*. A study by Vellichirammal et al. (2016) conducted functional testing of ecdysone, as well as its antagonists, and consistently showed that higher maternal ecdysone levels resulted in an increased percentage of wingless aphid progeny. This suggests that high ecdysone levels contribute to wing suppression. In several species of sexually wing dimorphic moths (winged males and wingless females) the injection of ecdysone was found to induce apoptosis in the female wing discs leading to their degeneration (Lobbia et al., 2003; Niitsu et al., 2008; Niitsu et al., 2014). Similarly, in the beetle genus *Onthophagus*, which exhibits a horn dimorphism, ecdysone-induced apoptosis is implicated in regulating horn development in species and sex-specific manner (Kijimoto et al., 2010). Therefore, the role of ecdysone in ants may be similar to its role in regulating dimorphic traits in these insects. In taxa where GRN interruption is absent (Fig. 6 yellow circle; Behague et al., 2018), we propose that high levels of ecdysone in worker-destined larvae may be inducing apoptosis to eliminate or halt the growth of the wing discs.

The insulin/insulin-like signaling pathway (IIS) is a highly conserved nutrient-sensing pathway that serves various functions in metabolism, reproduction, longevity, cell size and, growth (reviewed in Edgar, 2006; Wu & Brown, 2006). IIS and its downstream target the TOR (target of rapamycin) pathway are known to be regulators of polyphenisms that are triggered by differential nutrient levels and involve differences in body and organ size (reviewed in Nijhout & McKenna, 2018). Specifically, insulin signaling regulates caste determination in the honey bee *Apis mellifera*, where high expression of IIS and TOR are found in queen-destined larvae, and their knockdown results in worker-like morphologies (de Azevedo & Hartfelder, 2008; Mutti et al., 2011; Patel et al., 2007; Wheeler et al., 2006; Wheeler et al., 2014; Wolschin

et al., 2011). Furthermore, knockdown of insulin receptors and TOR leads to reduced levels of JH in queen-destined larvae and a conversion to worker-like morphologies, revealing that JH is downstream of the nutrient-sensing pathways in establishing caste-determination (Mutti et al., 2011). In ants, studies investigating reproductive division of labor have revealed that insulin signaling is upregulated in the ovaries of adult reproductives in the genus *Diacamma* (Okada et al., 2010), and in the brains of adult reproductives from seven species sampled from the Poneroid and Formicoid clades (Chandra et al., 2018). These studies suggest that reproductive adults with high IIS emerge from larva and pupa that received high-quality nutrition and thus had high IIS levels. However, a study in the fire ant *Solonopsis invicta* has shown that IIS receptor expression levels may fluctuate between the larval and the adult stage (Lu & Pietrantonio, 2011). These disparate findings indicate that the role of IIS in ants is complex and requires further and more rigorous exploration.

1.6.5 A model for the developmental processes underlying the origin of wing polyphenism in early ants

Here, we synthesize known interactions between the environment, endocrine hormones, the wing GRN, dsx, and apoptosis in a model for the regulation of wing polyphenism at the origin of ants (Fig. 8). Although here we focus on the major endocrine hormones that regulate trait development, we acknowledge that other numerous signaling pathways are present and should be integrated into future models. The first component of the model proposed here is the sensing of environmental and social factors such as temperature, nutrition, pheromones, and worker behavior (Penick & Liebig, 2012; Wheeler, 1986; Wheeler & Nijhout, 1984). The nutritional regulation of castes is predominantly used by more derived species where adult workers regurgitate food to immobile larvae (known as trophallaxis) and thus control larval nutritional levels (Cassill & Tschinkel, 1995; Wilson, 1971; Wilson & Holldobler, 2005b). In contrast, larvae from species belonging to the Poneroid clade are mobile and trophallaxis occurs much less frequently leaving adult workers with limited control over larval nutrition (Wilson & Holldobler, 2005b). In the Poneroid ant species *H. saltator* where trophallaxis is absent, Penick and Liebig (2012) demonstrated that behavioral regulation in the form of worker aggression is involved in caste determination. Specifically, JH-treated larvae that

become queen-destined are aggressed (through biting) by adult workers which leads to a reversion to worker development, possibly through inhibition of JH (Penick & Liebig, 2012). Therefore, we have included worker aggression in our model as one of the factors that may have regulated caste determination and wing polyphenism during the early evolution of ants.

These environmental and social cues are sensed by hormones such as IIS and JH. The absence of worker aggression may lead to an increase in JH synthesis (Fig. 8a), while presence of worker aggression might lead to JH reduction (Fig. 8b). High nutritional levels may also be directly sensed by JH and cause its increased synthesis (Fig. 8a). A study in the ant *Pogonomyrmex rugosus* has shown that IIS may be downstream of JH (Libbrecht et al., 2013), therefore, an increase in JH production may lead to an increase in IIS. Nutritional levels may also be directly sensed by IIS, which can in turn positively regulate JH as shown in *Drosophila* and honeybees (Mutti et al., 2011; Tatar et al., 2001; Tu et al., 2005). If future studies provide evidence for the latter, then a positive feedback mechanism between JH and IIS may exist in ants. Therefore, high nutritional levels, as well as the absence of worker aggression, lead to an increase in JH levels and consequently queen development (Fig. 8a). On the other hand, low nutrition and/or the presence of worker aggression result in a decrease in JH synthesis and the activation of worker development (Fig. 8b).

How might differential levels of JH between queen- and worker-destined larvae control wing polyphenism? We present two non-mutually exclusive possibilities. First, high levels of JH in queen-destined larvae induce wing growth through transcriptional regulation of the wing GRN (Fig. 8a). Conversely, in worker-destined larvae, low JH levels are unable to activate the wing GRN leading to interruption points and the degeneration of the rudimentary wing discs (Fig. 8b). This hormonal regulation of wing GRN may be mediated by different isoforms of dsx, as suggested by Klein et al. (2016). Second, JH may be influencing wing development through the modulation of other factors and hormones such as ecdysone. Ecdysone levels, contrary to JH, are high in worker-destined larvae compared with queen-destined larvae (Schwander et al., 2008; Suzzoni et al., 1980, 1983). It has been shown in the ant *P. rugosus*, as well as in *Drosophila*, that JH inhibits ecdysone production (Libbrecht et al., 2013; Mirth et al., 2014; Richard & Gilbert, 1991). Therefore, we propose that high JH in queen-destined larvae reduces

ecdysone production (Fig. 8a), whereas in worker-destined larvae, low JH levels are unable to suppress ecdysone and hence its increased levels (Fig. 8b). Ecdysone, in turn, may be regulating insulin signaling. Numerous studies in *Drosophila* have shown that ecdysone antagonizes insulin, whereas insulin can increase ecdysone production (Colombani et al., 2005; Orme & Leevers, 2005). This regulatory interaction between ecdysone and IIS remains to be tested in ants. However, if future studies show that ecdysone antagonizes IIS in ants, then it might reveal a positive feedback loop where ecdysone contributes to its own synthesis by inhibiting IIS and preventing JH increase (Fig. 8b). More importantly, ecdysone regulates dimorphic traits in insects through the induction of apoptosis (Kijimoto et al., 2010; Lobbia et al., 2003). Therefore, we propose that high levels of ecdysone in worker-destined larvae may be activating apoptosis that leads to the degeneration of the rudimentary wing discs (Sameshima et al., 2004; Shbailat et al., 2010). Alternatively, apoptosis may also be induced through an interruption in the wing GRN (Fig. 8b), as suggested by the spatiotemporal correlation between GRN interruption and apoptosis pattern in the ant *P. morrisi* (Shbailat et al., 2010).

1.7. Origin of wing polyphenism via genetic accommodation

Since the 1800s, and perhaps earlier, entomologists have tried to understand the evolutionary origin of wing polyphenism in ants. Early work, notably by Dewitz (1878) and Wheeler (1905, 1916), relied on morphological and developmental observations of the female caste to elucidate the origin of wing polyphenism in ants. Wheeler (1905) discovered rarely appearing anomalous workers with small vestigial wings (he termed 'pterergates') in a colony from the derived subfamily Myrmicinae (Fig. 5b-d). In addition to pterergates, the wide range of wing morphologies that have evolved in the queen caste across species (i.e., winged, short-winged, wingless) led Wheeler to dismiss the idea that wing polyphenism evolved in a single saltational step. Instead, Wheeler (1905, 1916) proposed that the ancestral ant lineage would have exhibited higher variation in the degree of wing development in adult workers, of which only the extremes (winged and wingless) survived in many taxa. Both Wheeler (1916) and Dewitz (1878) recognized the relative ease with which wing development can be altered in ants, leading Wheeler (1916) to propose that the winged queen and wingless worker caste evolved through the gradual suppression of intermediate forms. His theory (Wheeler 1916) was largely

based on those of Darwin (1859), Emery (1894), and later Wilson (1953) for how discrete worker subcastes, such as minor workers and soldiers (Fig. 1), in ants evolved through the gradual suppression of intermediate forms.

In her seminal book Developmental Plasticity and Evolution (West-Eberhard, 2003), Mary Jane West-Eberhard proposed that polyphenisms originate from an evolutionary process she called 'genetic accommodation.' Here, we propose a model for the origin of wing polyphenism in ants via genetic accommodation, which gave rise to two alternative phenotypes–winged queens and wingless workers–that are determined during development in response to specific environmental cues. Genetic accommodation starts with a novel phenotype that is recurrently induced by specific environmental conditions (Moczek et al., 2011; Suzuki & Nijhout, 2006; West-Eberhard, 2003). In ants, environmental induction of the novel wingless worker phenotype may have occurred in at least two alternative ways: (1) it may have been induced by a new or significant change in an existing environmental factor, such as poor larval nutrition, infections by parasites, or temperature fluctuations (Hunt, 1991; Wheeler, 1928a); or (2) it may have been induced by a mutation(s) in the genes that regulate the sensitivity of the hormones or wing GRN to the environmental cue (Billard et al., 2020; Sieriebriennikov et al., 2018). If the novel, environmentally induced, phenotype is adaptive, then selection will act on the genes that control its frequency and form of expression to genetically accommodate it into the population as an alternative, conditionally expressed, phenotype (Moczek et al., 2011; Suzuki & Nijhout, 2006; West-Eberhard, 2003). In ants, this would have led to the accommodation and incorporation of the wingless worker phenotype into the colony giving rise to the origin of wing polyphenism with two alternative phenotypes expressed under specific environmental conditions; a winged queen phenotype adapted for dispersal and a wingless worker phenotype adapted for performing tasks underground.

Previous studies have shown that the exposure to a new or to a significant change in an existing environment leads to the induction of a wide range of phenotypic variation (Gibson & Hogness, 1996; Rajakumar et al., 2012; Suzuki & Nijhout, 2006). For example, heat shocking larvae of the moth *Manduca sexta* gives rise to a wide range of color variants (Suzuki & Nijhout, 2006) and shocking fruit fly embryos with ether vapor gives rise to a wide range of variation in

the growth of the haltere (hindwing) (Gibson & Hogness, 1996). This means that exposure to discretely different environments alone is not sufficient to produce discrete alternative phenotypes. Therefore, at a mechanistic level, the origin of a polyphenic trait via genetic accommodation also requires the evolution of a developmental threshold. Polyphenic traits have an underlying continuously distributed character (such as a hormone gradient) that has a threshold, which can be defined as the point of discontinuity that results in the expression of discrete traits (Falconer, 1960). For instance, individuals above a certain threshold develop into one morph, while individuals below the threshold develop into an alternative morph. A developmental threshold therefore transforms an underlying continuous character into one with discontinuous phenotypic expression (Falconer, 1960). Hormones mediate environmental cues and affect gene expression, which in turn affects phenotype.

How is a concentration gradient of a hormone, or any other substance, translated into discrete alternative phenotypes without intermediates? Here, we propose two possible mechanisms: the first mechanism is based on a model by Lewis et al. (1977). Their mechanism provides an explanation for how discrete phenotypes develop through a sharp transition in gene activity. In this model, production of a gene product is controlled by a continuous substance (e.g., hormone gradient) in a sigmoidal fashion through positive feedback. As a result, changes in hormone levels lead to sharp transitions in the production of the gene product. Depending on the parameters of the model, the shape of the sigmoidal curve can change (Nijhout, 1991) – if the sigmoidal curve between the substance and the gene product has a gentle slope it may result in a broad threshold that gives rise to discrete phenotypes, including intermediates (Fig. 9a-a'). If, however, the sigmoidal curve has a steep slope, then it may result in a narrow threshold that gives rise to discrete phenotypes with no intermediates (Fig. 9b-b').

The second mechanism is based on a toggle binary switch, which is composed of two components that mutually inhibit each other (Gardner et al., 2000; Lugagne et al., 2017). These components can be genes, endocrine hormones, and developmental or physiological factors. For instance, a key gene in the wing GRN may be responsible for the activation of wing development, whereas another key gene may be responsible for the inhibition of wing

development. These two genes mutually suppress each other resulting in a discrete switch. Two genes from the wing GRN – *dpp* and *brk* – are promising candidates for these mutually inhibiting genes: *dpp* is essential for wing development and it downregulates *brk* expression; *brk* prevents wing disc growth, and although it does not suppress *dpp*, it suppresses the activity of its targets (Fig. 7a; Campbell & Tomlinson, 1999; Martin et al., 2004; Minami et al., 1999; Winter & Campbell, 2004). The induction of one of these genes over the other may be under the control of hormonal signals which mediate environmental cues (Shbailat et al., 2010).

Based on these two threshold mechanisms, we propose two of many possible models for the origin of wing polyphenism in ants, which we call the '*de novo* threshold model' and the 'cryptic threshold model.' In the *de novo* threshold model, the developmental threshold that facilitated the origin of wing polyphenism evolved *de novo* in the ancestral lineages leading to the ants. In contrast, in the cryptic threshold model, wing polyphenism in ants evolved from a cryptic developmental threshold that evolved originally to produce winged males and wingless females in their solitary wasp ancestors and was later co-opted to regulate winged queens and wingless workers.

In the *de novo* threshold model, we propose that the ancestral wasps did not have a threshold for wing development, instead, this threshold specifically evolved in early ants. In the ancestral wasps, we propose that expression levels of genes in the wing GRN were environmentally insensitive, and therefore, did not vary based on hormonal levels, which mediate environmental conditions (Fig. 9c). This robust gene expression to environmental variation would have produced the constitutive expression of a winged phenotype regardless of hormone levels (Fig. 9c'). Next, we propose that a mutation rendered gene expression levels sensitive to the environmental / hormone gradient resulting in a linear relationship between gene expression and hormonal levels (Fig. 9d). If we assume that gene expression levels also had a positive linear relationship with the trait that they control, then different gene expression levels (which are based on hormone gradient) would generate different phenotypes creating a continuous reaction norm (Fig. 9d'). All the phenotypes in the reaction norm may have been produced in the ancestral wasp lineage, or perhaps the hormone concentration always reached a level that induced sufficient gene expression to produce fully winged individuals. Regardless,

in the early ants, the hormone gradient may have evolved a threshold as a result of a mutation or genetic variation that caused one (or multiple) key wing genes to gain a positive feedback mechanism for the synthesis of their protein products. Based on Lewis et al.'s (1977), a sigmoidal relationship between the hormone gradient and a gene product is achieved through the gain of a positive feedback mechanism. Initially, this sigmoidal curve has a gentle slope, and therefore, in addition to producing discrete winged and wingless phenotypes, would also result in the production of individuals with a range of intermediate wing lengths (Fig. 9e'). Subsequently, a transition to a sigmoidal curve with a narrow slope evolves producing two discrete phenotypes with no intermediates (Fig. 9f-f'). The transition from a broad threshold boundary to a narrow one may have evolved very rapidly, reducing the likelihood that intermediate wing phenotypes were preserved in the fossil record.

This gradual transition from environmental insensitivity, to a linear reaction norm, to a sigmoidal curve with a broad threshold, and finally, to a sigmoidal curve with a narrow threshold is consistent with Wheeler's (1905; 1916) hypothesis that the origin of wing polyphenism (winged queens and wingless workers) evolved through the gradual suppression of intermediates. However, we cannot rule out that a de novo threshold originated in a single saltational step from a linear reaction norm (Fig. 9d-d') to a sigmoidal curve with a narrow threshold (Fig. 9f-f'). This can be achieved through Lewis et al. (1977) mechanism and also through the toggle binary switch mechanism.

The second model–the cryptic developmental threshold model–proposes that a cryptic threshold existed in the wasp lineages leading to ancestral lineages of ants. In the section titled 'Hymenopteran clues for the origin of wing polyphenism in Formicidae', we mapped on a phylogeny of the Hymenoptera the numerous wasp lineages that evolved a sexual wing dimorphism with winged males and wingless females. Here, we assume that this wing determination in wasps is controlled by a hormonal threshold, such that hormonal levels in male wasps pass the critical threshold resulting in wing development, whereas hormonal levels in female wasps fall below the threshold for wing development (Fig. 9g). Because sexual wing dimorphism is genetically determined, a likely genetic regulator of hormone levels may be the haplodiploidy sex-determination mechanism common to all Hymenoptera. In haplodiploidy, the

females develop from fertilized eggs and are therefore diploid, whereas the males develop from unfertilized eggs and are haploid (Grimaldi & Engel, 2005). Consequently, the prevalence of sexual wing dimorphism among the Hymenoptera (Fig. 2) raises the possibility that the ancestral lineage of ants or their wasp ancestors possessed a cryptic developmental potential to generate a sexual wing dimorphism (Fig. 9h). In other words, this ancestral lineage possessed a threshold for the production of winged and wingless phenotypes, but the genetic factors controlling morph development always produced winged phenotypes. In the early ants, the genetically regulated cryptic threshold may have become environmentally sensitive. This environmental sensitivity now results in hormonal levels to fall below or above the threshold for wing development, therefore releasing the wingless alternative phenotype (Fig. 9i).

Both models illustrate how the expression of two discrete phenotypes—winged queen and wingless workers—originated through the creation of a new or modification of an existing developmental threshold. Both of these models involve changes to the concentration of a hormone. However, polyphenic traits can also arise in several different ways by modifying hormone concentration, threshold, the timing of hormone secretion, or the timing of the hormone-sensitive period (Nijhout, 1999; Nijhout, 2003). All that is required for these modifications to control polyphenic development is for them to become sensitive to environmental variables. We, therefore, acknowledge that many other possible models exist and present these two models as a starting point for the exploration of the evolutionary steps into wing polyphenism. Regardless of which model is correct, the novel wingless worker phenotype, once environmentally induced, was genetically accommodated in a wing polyphenism in ants approximately 160 mya. Testing these models will be challenging, but not impossible. The evolution of a polyphenic trait via genetic accommodation has been demonstrated in lab settings and inferred from natural populations and several authors have provided frameworks for testing how developmental plasticity can result in evolutionary adaptation (Jones & Robinson, 2018; Levis & Pfennig, 2016; Moczek et al., 2011; Rajakumar et al., 2012; Schlichting & Wund, 2014; Suzuki & Nijhout, 2006).

1.8. The origin of wing polyphenism enabled subsequent morphological diversification in ants

In this section, we highlight how the origin of wing polyphenism in ants laid the foundations for subsequent morphological diversification within the worker caste and within the reproductive caste in ants. We first explore how the loss of wings in the worker caste allowed for subsequent modifications in thoracic architecture leading to the increased strength of individual worker ants. We then discuss how the origin of wing polyphenism facilitated the evolution of the novel soldier subcaste, and finally, we discuss the evolution of wing polyphenism/dimorphism in the reproductive castes and its possible association with the origin of queen-worker wing polyphenism in ants.

A universal feature of the thorax of the worker caste is an enlarged first thoracic segment (T1) and its associated muscles (Keller et al., 2014; Peeters et al., 2020). This is in contrast to queen ants where T1 and the third thoracic (T3) segments are reduced and the second thoracic (T2) segment is extensively enlarged. The reduction of T2 and enlargement of T1 in worker ants was enabled by the loss of wings from T2 and T3 segments in workers during the origin of wing polyphenism in ants. Indeed, this exact pattern is also observed in almost all wingless female wasps (Reid, 1941), and wingless queen ants (Keller et al. 2014). Keller et al. (2014) and Peeters et al. (2020) proposed that the enlargement of the T1 segment in workers provided added strength and mobility to the neck muscles that control head movements, thereby allowing ants to carry large objects and exploit various trophic resources. Therefore, the origin of wing polyphenism in ants enabled subsequent thoracic modifications in the worker caste of ants to evolve, and this modification has contributed to the ecological success of ants.

Another factor that contributed to the diversity and success of ants is the evolution of 'worker polymorphism,' which refers to colonies with a striking degree of variation in size and head-to-body allometry within the worker caste (Wilson, 1953). Worker polymorphism is largely generated and regulated by environment cues (Alvarado et al., 2015; Wills et al., 2018). Wilson (1953) organized the variation in worker polymorphism into four categories, one of which is called 'complete dimorphism.' In completely dimorphic species, such as in the ant genus *Pheidole*, the worker caste is divided into two morphologically distinct worker subcastes with no intermediates (Wilson, 2003). These two subcastes are called 'minor workers' and

'soldiers or majors' (Fig. 1). Soldiers have disproportionately large heads relative to their bodies as compared to minor workers (Rajakumar et al., 2018; Wilson, 2003). Although minor workers and soldiers are completely wingless as adults, the soldiers develop vestigial or rudimentary wing discs during larval development that degenerate by apoptosis before becoming adults (Sameshima et al., 2004; Shbailat et al., 2010). By contrast, the minor workers lack any visible wing rudiments during larval development (Abouheif & Wray, 2002; Sameshima et al., 2004; Wheeler & Nijhout, 1981a). A recent study by Rajakumar et al. (2018) showed that these rudimentary wing discs in *Pheidole* soldiers have been co-opted to function in generating their larger size and disproportionately large heads. Therefore, the origin of wing polyphenism released the constraint on the wing discs of having to produce functional wings in workers. This allowed wing discs to acquire novel functions that led to the evolution of further morphological diversification in the worker caste in ants, such as the big-headed soldier subcaste. Future studies should investigate whether the rudimentary wing discs in less-derived Poneroid species that evolved worker polymorphism, such as *Myopopone castanea* and *Amblyopone australis* (Ito, 2010; Peeters & Molet, 2010). This will reveal whether the role of the rudimentary wing discs in generating worker polymorphism is conserved across varied taxa or evolved only later during ant evolution in the more derived clades. Finally, the absence of wing polyphenism in other social insects (bees and wasps), may indeed be the reason why they never evolved dramatic worker polymorphism like that observed in the ants.

Following the origin of wing polyphenism, additional wing polyphenisms or dimorphisms evolved within the reproductive queen or male castes. The evolution of ergatoid (wingless) and brachyapterous (short-winged) queens evolved independently in more than 50 ant genera across all subfamilies (for a list of genera see: Heinze & Tsuji, 1995; Peeters, 2012). The evolution of wing polyphenism in the male caste, on the other hand, is less common than wing polyphenism in the female queen caste and has been observed in only half a dozen genera, most notably in the genus *Cardiocondyla* (Heinze, 2017; Heinze & Tsuji, 1995). Typically, ergatoids or brachyapterous queens, in addition to wingless males, occur together with winged queens and males, however, there are instances where some species have completely lost the production of winged queens and males (Heinze et al., 2005; Molet et al., 2007; Peeters, 2012).

Environmental conditions and social regulation play an instrumental role in wing morph determination in the reproductive caste (Cremer & Heinze, 2003; Ito, 1996; Schrempf & Heinze, 2006). However, a few studies have also found evidence for a strong genetic influence on the development of ergatoids (Buschinger & Schreiber, 2002; Fersch et al., 2000; Heinze & Buschinger, 1989). Fave et al. (2015) and Oettler et al. (2018) have explored the wing GRN and revealed interruption points in the wing GRN of wingless queens and males. Although these interruption points in the wingless reproductive caste occur further downstream relative to the interruption points found in wingless workers of the same species, they show that wing GRN interruption is a general mechanism that was co-opted to regulate polyphenic wing disc growth in reproductive queens and males. Collectively, these examples not only demonstrate the evolutionary role wing polyphenism played in the subsequent morphological diversification of ants, but they also pave the way for new avenues in ant research.

1.9. Conclusions

The goal of this review is to synthesis the ecological, developmental, and evolutionary factors that led to the origin of wing polyphenism in ants. Figure 10 briefly summarizes the multiple models and hypotheses proposed by this work. Briefly, we propose that there may have existed an ancestral developmental potential in Hymenoptera to generate sexual wing dimorphism (Fig. 10a). This potential – a preexisting cryptic threshold for wing development present in the ancestral wasps – may have facilitated the origin of wing polyphenism in ants (Fig. 10b). Alternatively, the wing polyphenism in ants may have originated by evolving a threshold *de novo* (Fig. 10c). The fossil record provides clear evidence that wing polyphenism and eusociality evolved together in early ants (Fig. 10d). However, it remains unclear whether wing polyphenism evolved prior to eusociality and contributed to its origin, or it originated after eusociality and contributed to its maintenance (Fig. 10e). Once wing polyphenism originated, interactions between the environment, hormones, wing GRN, and apoptosis were likely present and regulated the polyphenic switch between winged and wingless females in the early ants (Fig. 10f). Because wing polyphenism in ants evolved just once and is a universal feature of ants, these interactions evolved and contributed to the remarkable morphological diversity of ants, facilitating several morphological innovations: it enabled the evolution of thoracic modifications

that increased the strength of ants and enabled the evolution of novel worker and reproductive castes in ants, including the evolution of soldiers, wingless queens and males (Fig. 10g).

Undoubtedly, the factors that facilitated the evolution of wing polyphenism are complex and intricately linked. Much remains to be elucidated about how wing polyphenism in ants originated – from ecological factors to hormonal regulation. In Table 1, we have summarized some of the main outstanding questions to be investigated by future work. Since polyphenism is associated with several major evolutionary transitions in individuality, understanding the origin and elaboration of wing polyphenism in ants can help us understand how other major evolutionary transitions in individuality arose and diversified.

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1.11. References

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Table 1. A list of outstanding questions for investigating the evolution of wing polyphenism in ants.

What are the ancestral characteristics and phylogenetic correlations between wing development and life history traits?	Section 1.3
What is the developmental mechanism for sexual wing dimorphism in wasps?	Section 1.3
Is there an ancestral developmental potential for a sexual wing dimorphism in Hymenoptera?	Section 1.3, 1.7
To help understand the relationship between the origins of eusociality and wing polyphenism, what is the relationship between wing development and reproductive capacity?	Section 1.5
What is the function of the metapleural gland in early branching ant lineage?	Section 1.5
What are the biomechanical constraints placed on the metapleural gland by the presence of wings?	Section 1.5
Is there an interruption in the wing GRN in taxa from the Poneroid clade?	Section 1.6
Is apoptosis a conserved mechanism in wing disc degeneration in Formicidae?	Section 1.6
What is the functional role of *dsx* and sphingolipid synthesis genes in ant wing development?	Section 1.6
What are the roles of ecdysone and IIS in regulating wing polyphenism in ants, and are these roles conserved among taxa?	Section 1.6
Is there a positive feedback mechanism between JH and IIS?	Section 1.6
Does JH levels regulate gene transcription in the wing GRN? Precisely, are low JH levels in worker destined larvae unable to activate the wing GRN?	Section 1.6
Does JH control *dsx* expression and ecdysone levels similar to other insects with polyphenic traits?	Section 1.6
Does ecdysone induce apoptosis in the rudimentary wing discs?	Section 1.6
Did ants evolve a discrete winged and wingless phenotypes through gradual transitions or through a single saltational step?	Section 1.7
Did wing polyphenism in ants evolve via genetic accommodation?	Section 1.7
Did the developmental thresholds for wing polyphenism evolve *de novo* or through a cryptic developmental threshold that evolved from a sexually wing dimorphic wasp ancestor?	Section 1.7
Are there evidences for positive feedback or toggle mechanisms in the wing GRN?	Section 1.7
Do rudimentary wing discs facilitate polymorphic worker development in less-derived species?	Section 1.8
Is the evolution of wing polyphenism in queens and males attained through co-option of the mechanisms controlling queen-worker wing polyphenism?	Section 1.8

Figure 1.

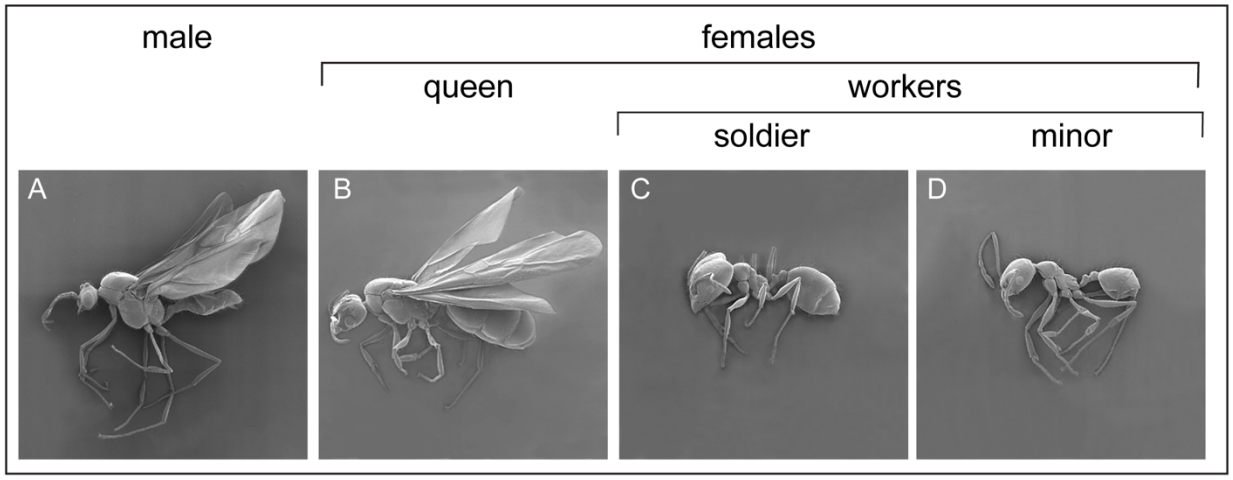

Figure 1. Wing polyphenism in the female caste of ants. Scanning electron micrographs of adult castes of *Pheidole morrisi*. Unfertilized eggs laid by the queen develop into winged males (a). Fertilized eggs laid by the queen develop into females (b to d). Female embryos develop either into winged queens (b) or wingless workers (c, d) in response to temperature and photoperiod. In a subset of taxa, worker larvae develop into either wingless soldiers (c) or wingless minor workers (d) in response to nutritional cues.

Figure 2.

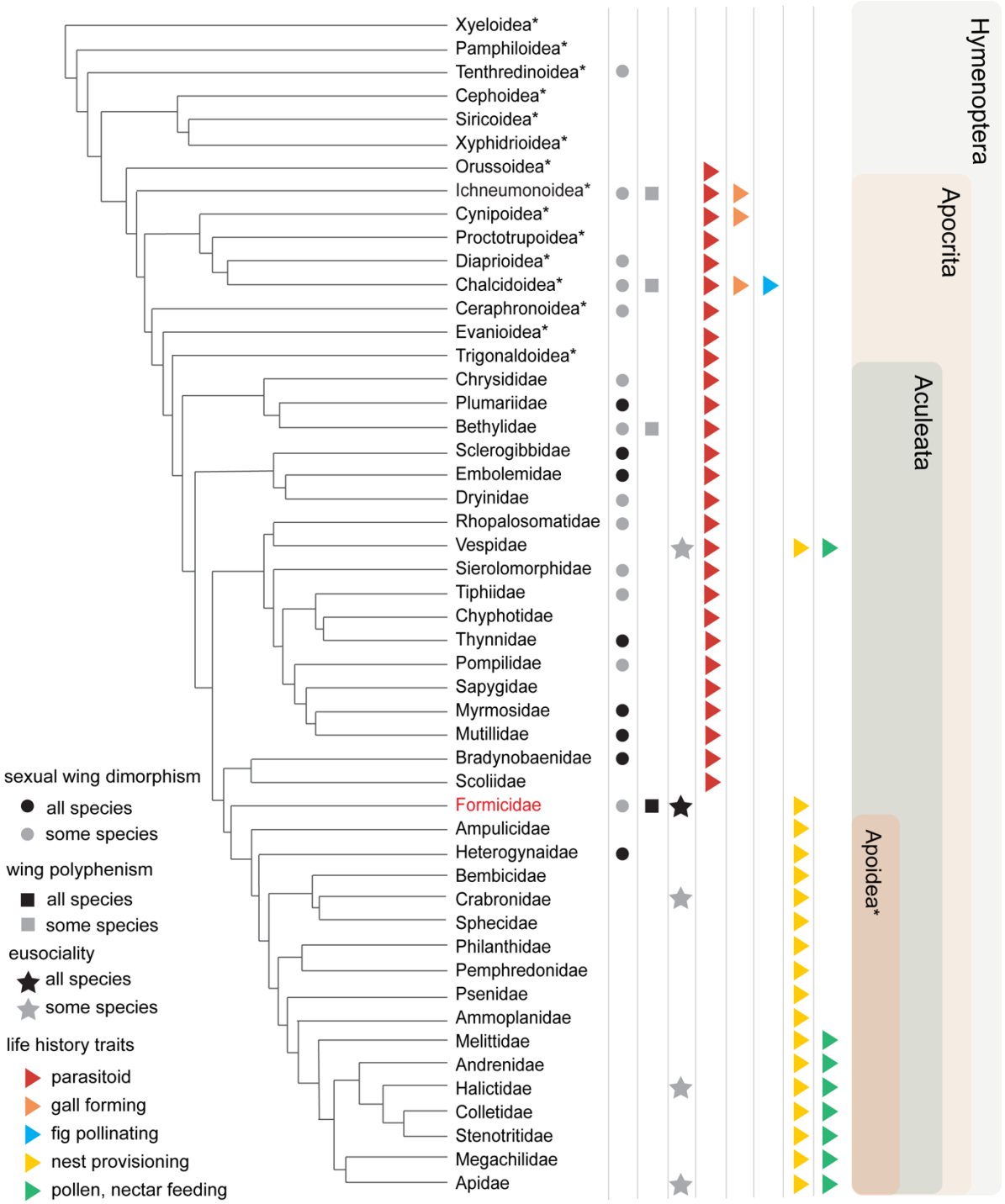
Xyeloidea*
Pamphiloidea*
Tenthredinoidea*
Cephoidea*
Siricoidea*
Xyphidrioidea*
Orussoidea*
Ichneumonoidea*
Cynipoidea*
Proctotrupoidea*
Diaprioidea*
Chalcidoidea*
Ceraphronoidea*
Evanioidea*
Trigonaldoidea*
Chrysididae
Plumariidae
Bethylidae
Sclerogibbidae
Embolemidae
Dryinidae
Rhopalosomatidae
Vespidae
Sierolomorphidae
Tiphiidae
Chyphotidae
Thynnidae
Pompilidae
Sapygidae
Myrmosidae
Mutillidae
Bradynobaenidae
Scoliidae
Formicidae
Ampulicidae
Heterogynaidae
Bembicidae
Crabronidae
Sphecidae
Philanthidae
Pemphredonidae
Psenidae
Ammoplanidae
Melittidae
Andrenidae
Halictidae
Colletidae
Stenotritidae
Megachilidae
Apidae
Hymenoptera
Apocrita
Aculeata
Apoidea*
sexual wing dimorphism
all species
some species
wing polyphenism
all species
some species
eusociality
all species
some species
life history traits
parasitoid
gall forming
fig pollinating
nest provisioning
pollen, nectar feeding

Figure 2. Major life history and morphological traits informally mapped on a phylogeny of the main hymenopteran families and superfamilies. The characteristics include the evolution of eusociality (black and grey stars), wing polyphenism (black and grey squares), sexual wing dimorphism (black and grey circles), and various life history traits (colored triangles; see key for details). Phylogenetic relationships based on Branstetter, Danforth, et al. (2017) and Sann et al. (2018). Character states of wing dimorphism and polyphenism for each family (or superfamily) is based on Goulet and Huber (1993) and Smith (1993), and character states for life history traits are based on Whitfield (1998). Branch lengths do not represent evolutionary time. Hymenoptera (order); Apocrita (suborder); Aculeata (subclade). Asterisks indicate superfamily level.

Figure 3.

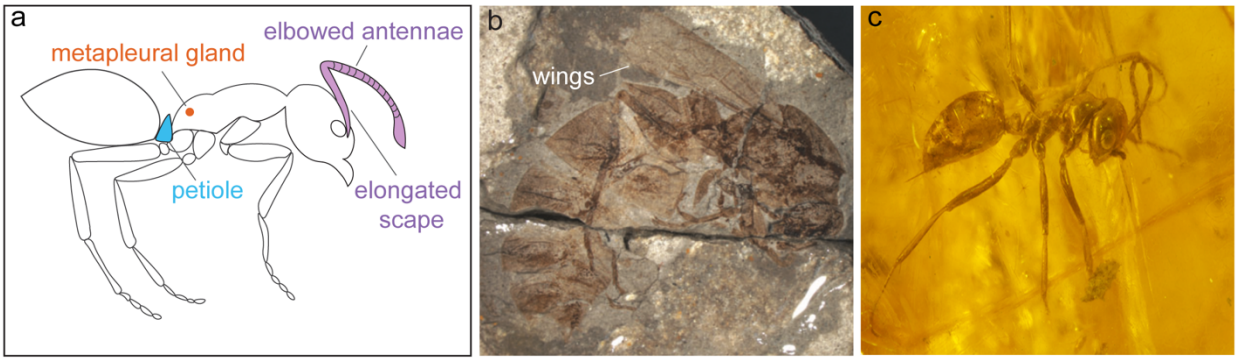

Figure 3. Ant synapomorphies and Early Cretaceous ant fossils. (a) an illustration of the four morphological synapomorphies used to distinguish ants: metapleural gland (orange circle), petiole (blue triangle), elbowed antennae and elongated scape (purple). (b) rock imprint fossil of Armaniidae (*Armania robusta*). Reprinted from LaPolla et al. (2013) with permission. (c) *Sphecomyrma freyi* (image by Frank M. Carpenter, courtesy of Edward O. Wilson).

Figure 4.

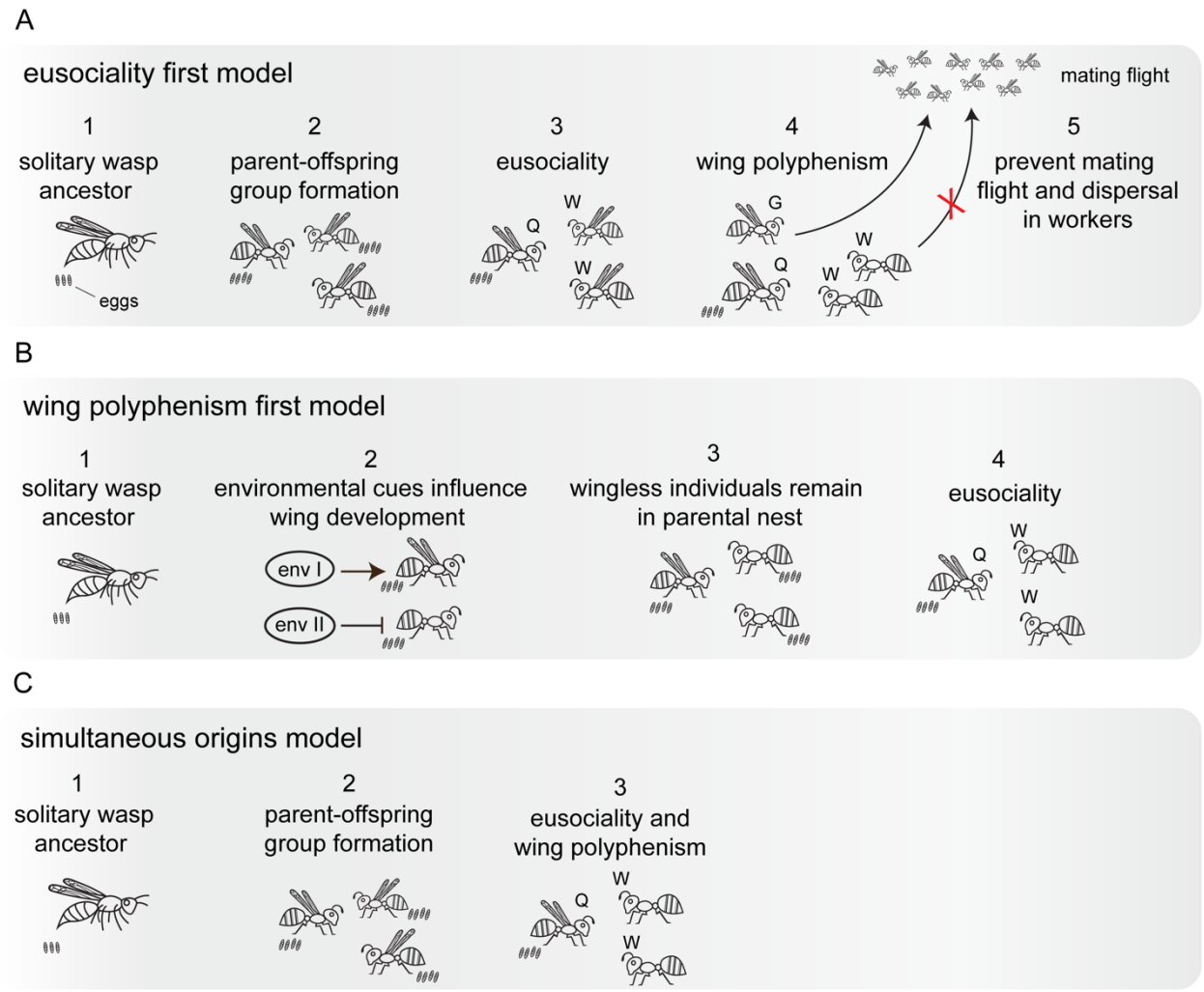
A
eusociality first model
mating flight
1
solitary wasp ancestor
eggs
2
parent-offspring group formation
3
eusociality
Q
W
W
4
wing polyphenism
G
Q
W
W
5
prevent mating flight and dispersal in workers
B
wing polyphenism first model
1
solitary wasp ancestor
2
environmental cues influence wing development
env I
env II
3
wingless individuals remain in parental nest
4
eusociality
Q
W
W
C
simultaneous origins model
1
solitary wasp ancestor
2
parent-offspring group formation
3
eusociality and wing polyphenism
Q
W
W

Figure 4. Hypothetical scenarios for the origin of wing polyphenism and eusociality in the ancestral ants. We propose three models: (a) eusociality first model, (b) wing polyphenism first model, and (c) simultaneous origins model. In all the models, ants evolved from ancestral wasps that constructed nests where they laid their eggs (step 1 in a-c). In the eusociality first model (a): (step 2) formation of parent-offspring groups that shared a nest site; (step 3) division of labor, brood care, and overlapping generations amongst group members led to the evolution of eusociality with reproductive queen (Q) and nonreproductive offspring (worker, W); (step 4) eusociality, among other factors, contributed to the evolution of wing polyphenism with wingless workers and winged gynes (G) that will become future queens; (step 5) wing polyphenism prevents wingless workers from participating in mating flights, while allowing winged gynes the same capability. In the wing polyphenism first model (b): (step 2) wing development evolved to response to different environmental conditions (env I and env II); (step 3) the wingless morphs remain in the parental nest (group formation); (step 4) group living advances to eusociality. In the simultaneous origins model (c): (step 2) formation of parent-offspring groups that shared a nest site; (step 3) various ecological and/or social factors led to the simultaneous origin of wing polyphenism and eusociality.

Figure 5.

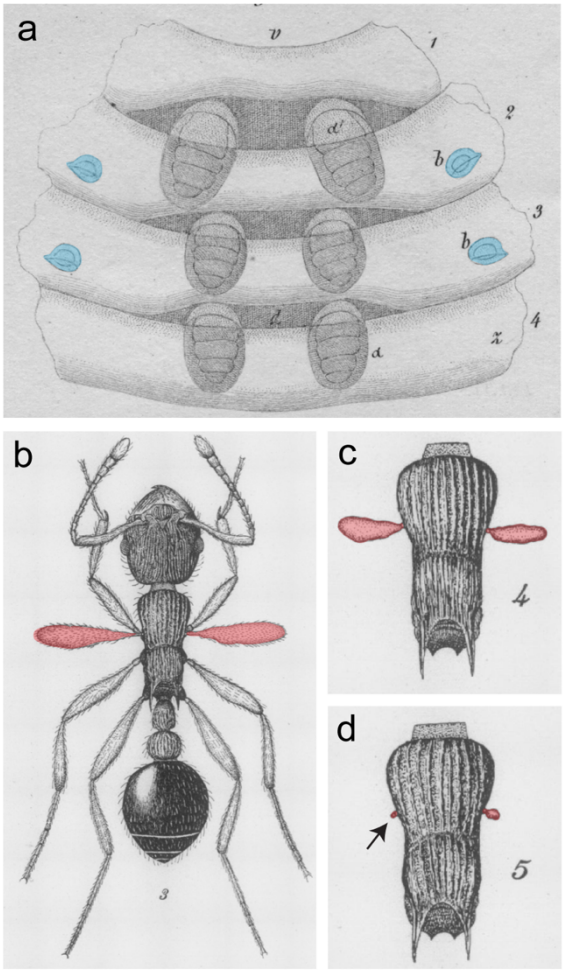

Figure 5. Rudimentary wing discs and pterergates. (a) ventral view of worker-destined larva of the species *Formica rufa* (subfamily: Formicinae) showing rudimentary fore- and hindwing discs (pseudo-colored in blue). Drawing from Dewitz (1887). (b-c) adult workers with vestigial forewings (pseudo-colored in red) known as 'pterergates' from the species *Myrmica rubra scabrinodis* (subfamily: Myrmicinae). Size as well as morphological features of the vestigial wings vary between workers. The left forewing in (d) is minute and nodule like (arrow). Drawings from Wheeler (1905). Small letters and numbers are from original images. See Dewitz (1887) and Wheeler (1905) for full descriptions.

Figure 6.

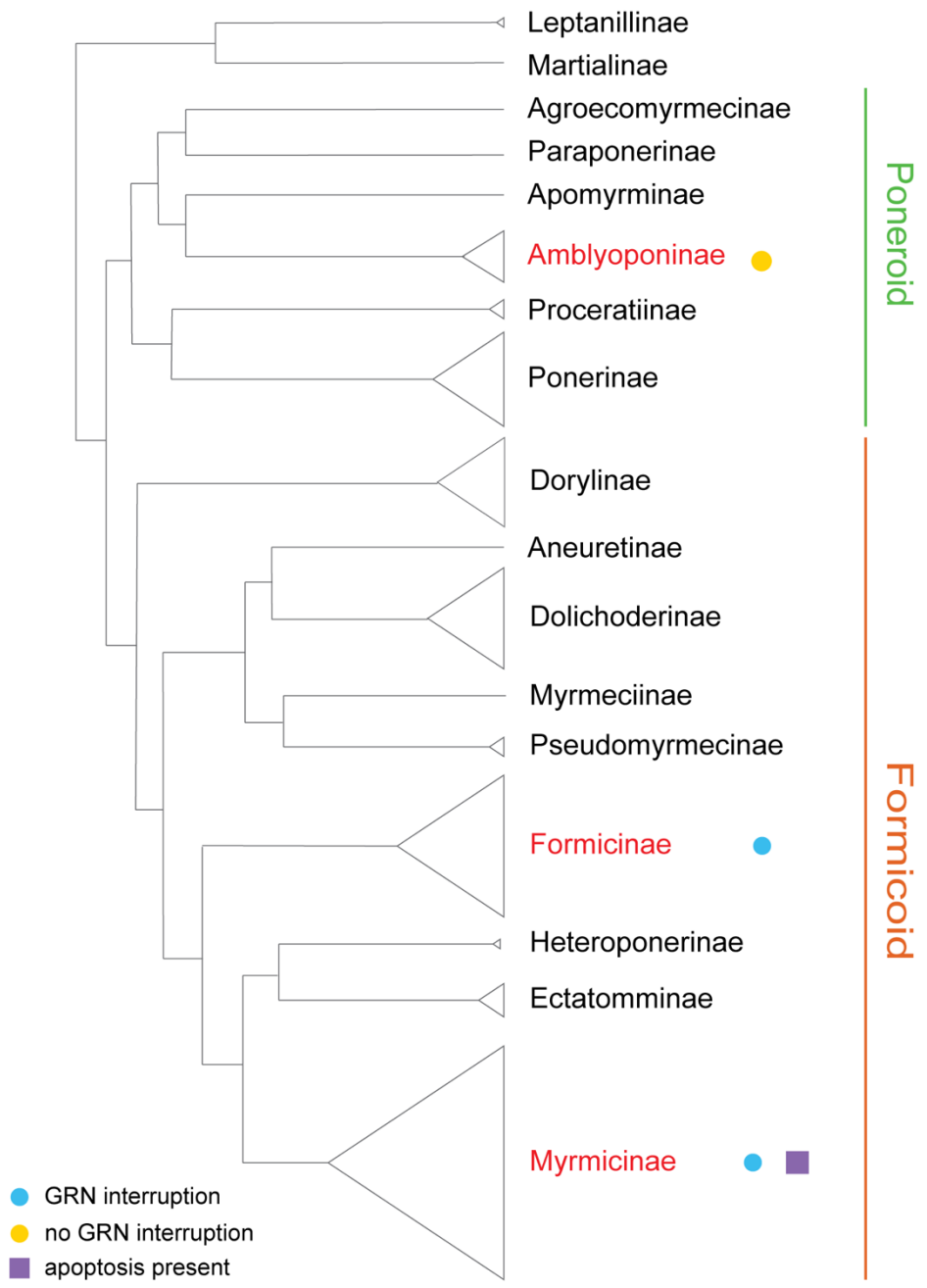
Leptanillinae
Martialinae
Agroecomyrmecinae
Paraponerinae
Apomyrminae
Amblyoponinae
Proceratiinae
Ponerinae
Dorylinae
Aneuretinae
Dolichoderinae
Myrmeciinae
Pseudomyrmecinae
Formicinae
Heteroponerinae
Ectatomminae
Myrmicinae
Poneroid
Formicoid
GRN interruption
no GRN interruption
apoptosis present

Figure 6. A phylogeny of the major extant ant subfamilies showing the relationship between the Poneroid and Formicoid clades. Subfamilies colored in red are those where developmental studies on ant wing polyphenism have been conducted. Relative size of triangles indicates size of subfamilies, after Moreau and Bell (2013). Phylogenetic relationships among subfamilies follow (Borowiec, 2019; Branstetter, Longino, et al., 2017). Blue circles indicate presence of GRN interruption; yellow circle indicates absence of GRN interruption; purple square indicates presence of apoptosis. Apoptosis has only been tested in species belonging to Myrmicinae subfamily.

Figure 7.

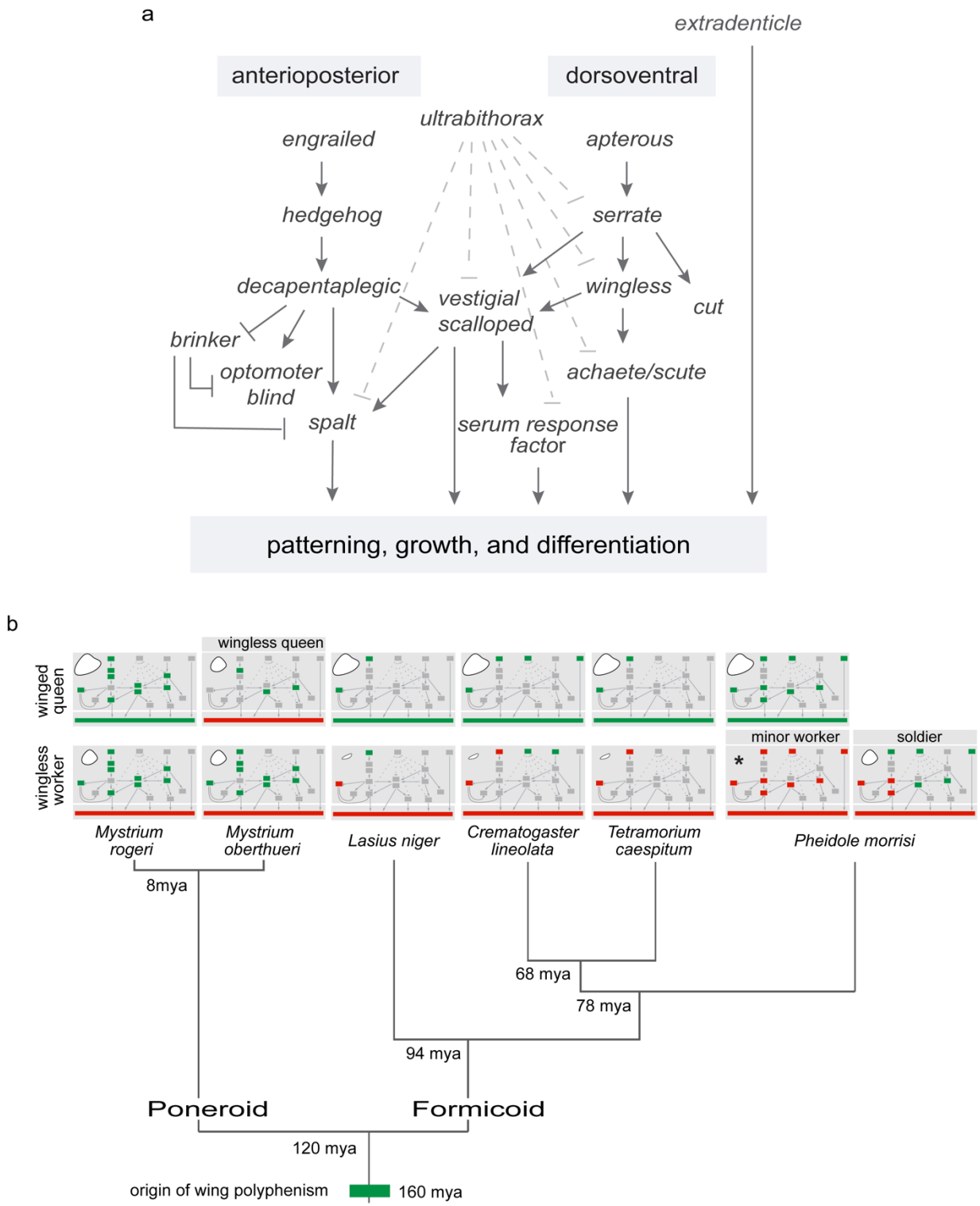
a
extradenticle
anterioposterior
dorsoventral
ultrabithorax
engrailed
apterous
hedgehog
serrate
decapentaplegic
vestigial
scalloped
wingless
cut
brinker
optomoter
blind
achaete/scute
spalt
serum response
factor
patterning, growth, and differentiation
b
wingless queen
winged
queen
wingless
worker
minor worker
soldier
*
Mystrium
rogeri
Mystrium
oberthueri
Lasius niger
Crematogaster
lineolata
Tetramorium
caespitum
Pheidole morrisi
8mya
68 mya
78 mya
94 mya
Poneroid
Formicoid
120 mya
origin of wing polyphenism
160 mya

Figure 7. Wing gene regulatory network in *Drosophila* and network interruption in ants. (a) the wing patterning network in *Drosophila melanogaster* during late larval development. Transcription factors and signaling molecules control the formation of the anterioposterior and dorsoventral compartments. Elements from both compartments regulate vestigial and scalloped to control the growth and differentiation of the wing discs. Arrows represent activation and perpendicular lines represent inhibition. Grey, dashed perpendicular lines are only active if ultrabithorax is expressed. Redrawn from Shbailat and Abouheif (2013) with permission. (b) a summary diagram re-drawn with some modifications from Behague et al. (2018) with permission, showing interruption points in the wing GRN of queens and workers belonging to Formicoid and Poneroid species. Workers belonging to Formicoid species exhibit interruptions in several wing genes (indicated by small, red squares), while workers from Poneroid species do not exhibit interruptions in any of the tested genes (indicated by small, green squares). Asterisk indicates absence of imaginal wing discs.

Figure 8.

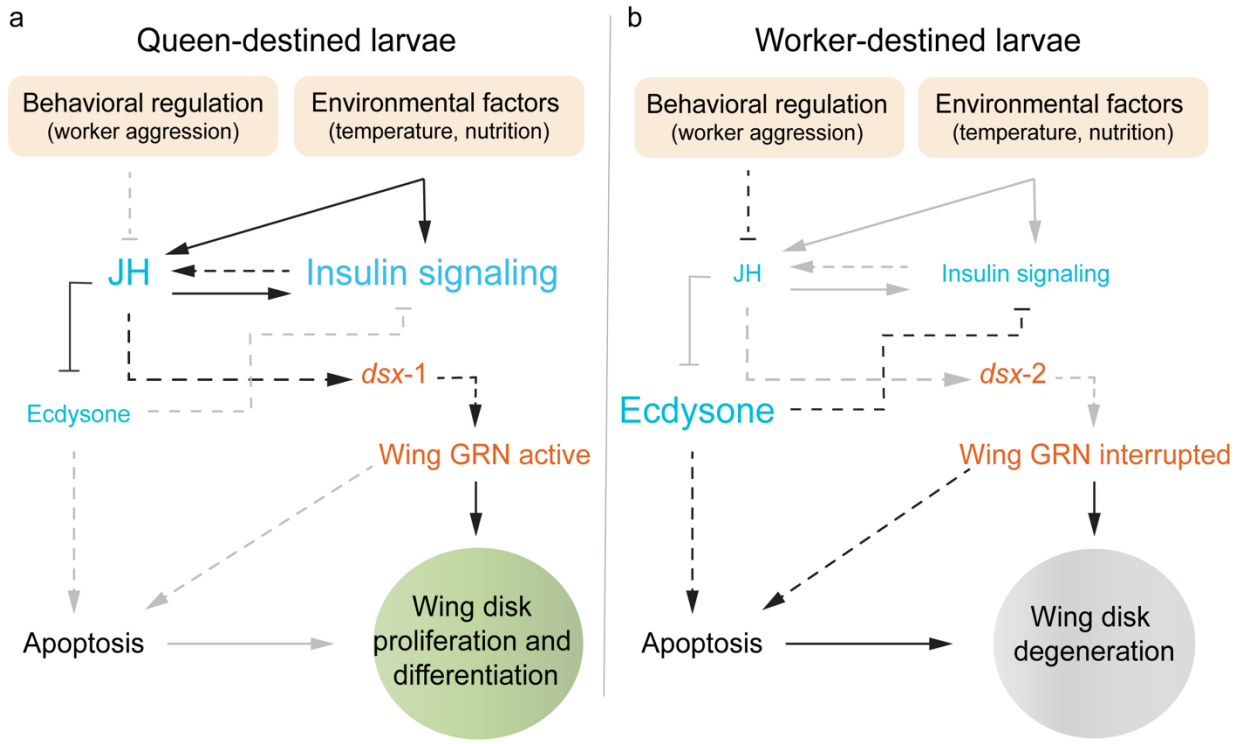

Figure 8. A proposed model for the interactions between the environment, endocrine hormones, wing GRN, *dsx*, and apoptosis in the development of wing polyphenism in ants. Pathways for the development of a winged queen (a) and a wingless worker (b). Environmental and social cues regulate JH and insulin signaling levels. In turn, JH regulates ecdysone levels and wing GRN activation. Ecdysone and the wing GRN regulate apoptosis. Endocrine hormones are labeled in blue and genes/GRN are labeled in orange. Font size of the endocrine hormones indicates their relative levels. Pathways highlighted in black are activated and those highlighted in grey are inactivated. Arrows represent activation and perpendicular lines represent inhibition. Solid arrows and perpendicular lines indicate activation/inhibition relationships that have been directly tested in ants. Dashed arrows and perpendicular lines indicate activation/inhibition relationships that have been tested in other insects and await future testing in ants.

Figure 9.

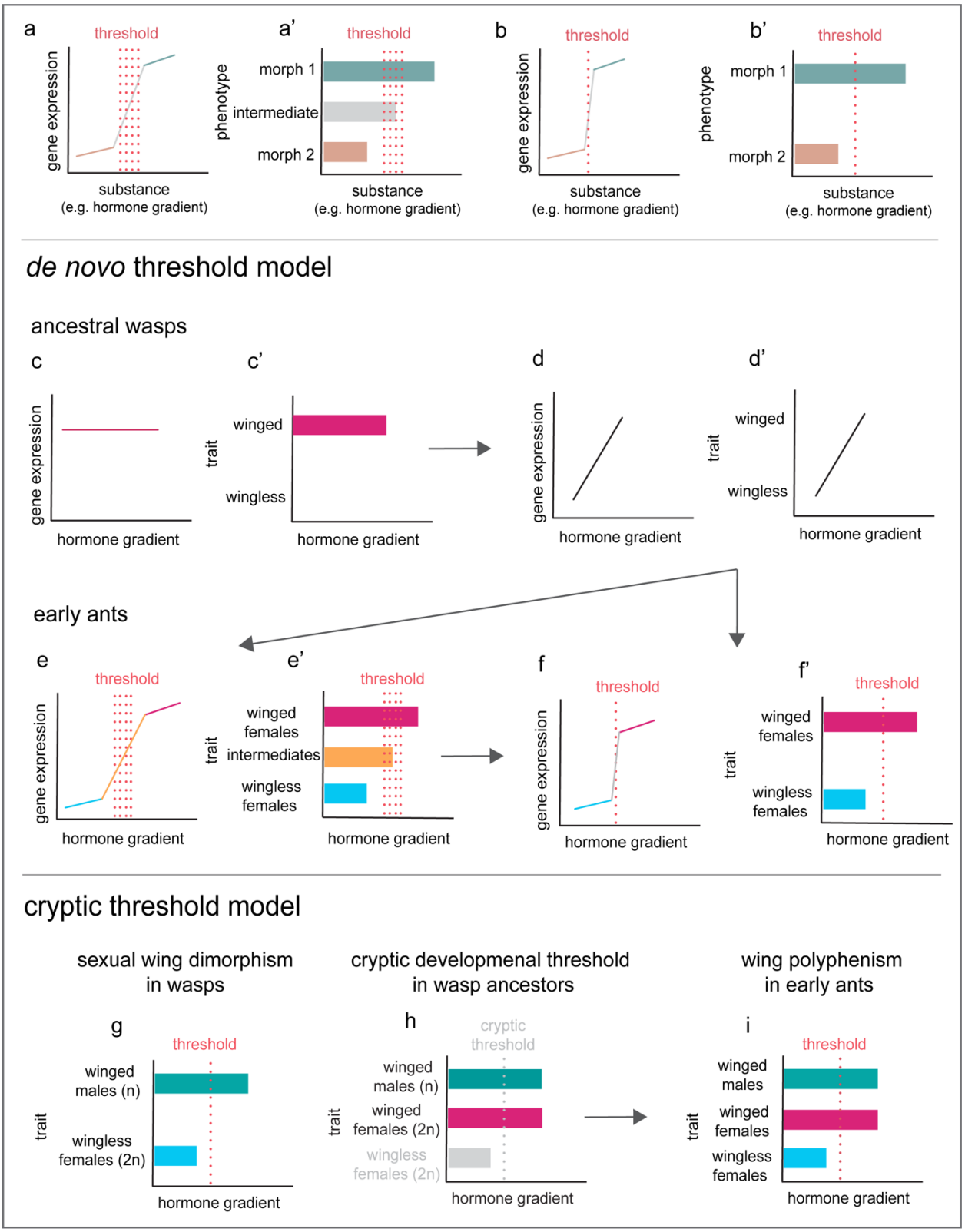
a
threshold
gene expression
substance
(e.g. hormone gradient)
a'
threshold
phenotype
morph 1
intermediate
morph 2
substance
(e.g. hormone gradient)
b
threshold
gene expression
substance
(e.g. hormone gradient)
b'
threshold
phenotype
morph 1
morph 2
substance
(e.g. hormone gradient)
de novo threshold model
ancestral wasps
c
gene expression
hormone gradient
c'
trait
winged
wingless
hormone gradient
d
gene expression
hormone gradient
d'
trait
winged
wingless
hormone gradient
early ants
e
threshold
gene expression
hormone gradient
e'
threshold
trait
winged females
intermediates
wingless females
hormone gradient
f
threshold
gene expression
hormone gradient
f'
threshold
trait
winged females
wingless females
hormone gradient
cryptic threshold model
sexual wing dimorphism in wasps
cryptic developmenal threshold in wasp ancestors
wing polyphenism in early ants
g
threshold
trait
winged males (n)
wingless females (2n)
hormone gradient
h
cryptic threshold
trait
winged males (n)
winged females (2n)
wingless females (2n)
hormone gradient
i
threshold
trait
winged males
winged females
wingless females
hormone gradient

Figure 9. A proposed conceptual model for the evolution of wing polyphenism via an environmentally regulated developmental switch. (a) a sigmoidal relationship between a continuous substance, such as a hormone gradient, and gene expression. A broad threshold (illustrated by multiple lines) forms when the sigmoidal curve has a gentle slope. The broad threshold results in the development of discrete phenotypes with intermediates (a'). (b) a sigmoidal curve with a steep slope gives rise to a narrow threshold that results in the development of discrete phenotypes with no intermediates (b'). (c-f') depict a scenario where a threshold evolved de novo in the early ant lineage. (c) in the ancestral wasps, gene expression levels were robust and did not respond to hormonal levels. (c') the robustness of gene expression resulted in winged phenotype regardless of hormonal levels. (d-d') a mutation caused gene expression, and the resulting trait, to become sensitive to hormonal levels resulting in a linear reaction norm. (e) in the early ant lineage, a mutation or genetic variation (perhaps gain of a positive feedback mechanism) changed the relationship between gene expression and hormonal levels from linear to sigmoidal. This sigmoidal relationship might have created a broad threshold boundary, indicated by multiple threshold lines, that allowed the development of intermediate phenotypes (e'). Gene expression color in (e) corresponds with phenotype color in (e'). Subsequently, a more narrow threshold, indicated by one threshold line, formed resulting in two discontinuous phenotypes (f-f'). The transition from a broad to a narrow threshold may have not occurred; instead a sigmoidal relationship with a narrow threshold (f-f') may have directly followed the linear reaction norm (d-d'). (g-i) depict a scenario where wing polyphenism evolved from a pre-existing threshold. (g) sexual wing dimorphism in wasps is controlled by a hormone gradient with a threshold. Genetic factors, such as ploidy levels, may control whether hormone levels fall above or below the threshold. (h) the developmental potential to generate a sexual wing dimorphism was retained but not realized in the wasp ancestors of ants. (i) during the early evolution of ants, the hormone levels of the females became environmentally-sensitive resulting in some individuals falling below or above the pre-existing threshold for wing development. For panels a-f', gene expression color corresponds with phenotype/trait colors.

Figure 10.

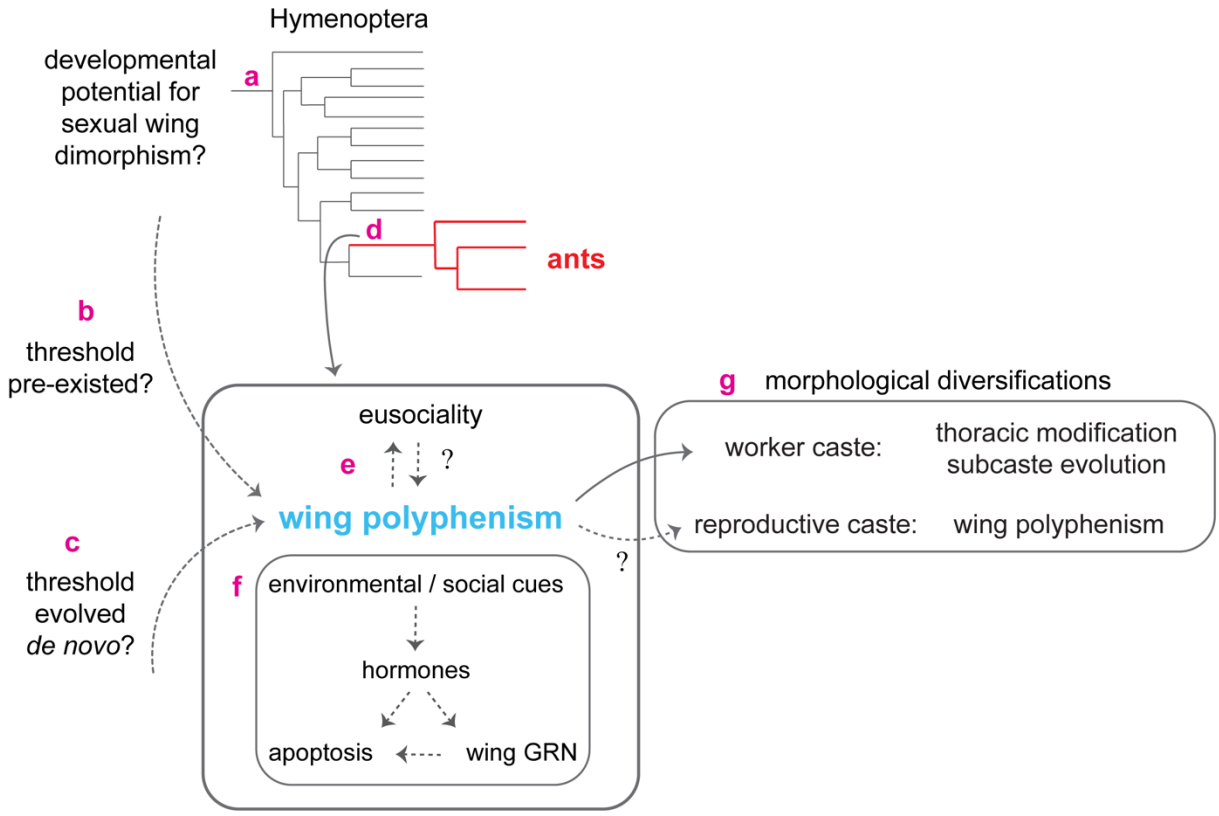

Figure 10. A visual illustration of the main models and hypotheses proposed in this work. (a) Hymenoptera may possess a developmental potential for the generation of sexual wing dimorphism. (b) this developmental potential (in the form of a threshold) may have led to the generation of wing polyphenism in ants; alternatively, the threshold may have evolved *de novo* (c). (d) based on fossil evidence, wing polyphenism and eusociality originated early during the evolution of ants. (e) wing polyphenism may have originated prior to and drove the evolution of eusociality, or wing polyphenism followed eusociality but led to its reinforcement. (f) a proposed model for the interactions between the environment, social cues, hormones, GRN interruption, and apoptosis to generate a wingless worker caste. (g) wing polyphenism facilitated morphological diversifications in the worker and reproductive caste.

CONNECTING STATEMENT BETWEEN CHAPTERS 1 & 2

In Chapter 1 of this thesis, I discuss the ecological, developmental, and evolutionary factors that contributed to the origin of wing polyphenism in ants. Regarding the developmental factors, I discuss how wings in ants arise and develop during the larval stages and synthesize the up-to-date studies investigating the developmental mechanisms that play a role in generating wing polyphenism between the winged queens and wingless workers. Some of these mechanisms include interruption in the wing gene regulatory network (section 1.6.1), apoptosis (1.6.2), differential gene expression outside the wing GRN (1.6.3), and juvenile hormone, ecdysone, and insulin signaling (1.6.4). After discussing the role of each of the above factors in insect polyphenisms and ant wing polyphenism, I constructed a model that hypothesizes how these factors could be regulating wing polyphenism and their potential interactions. Unlike many of the other factors, apoptosis has received little attention and only two studies to date have been conducted examining its role in wing polyphenism in ant species (Sameshima et al. 2004; Shbailat et al., 2010). Despite this, my literary search into the role of apoptosis (and programmed cell death in general) showed a plethora of examples where this process is found to play a role in generating morphological diversity in the form of polyphenic and dimorphic traits. These examples were not restricted to insects, but encompassed mammals, plants, and even protists. This raised many questions into the prevalence and precise function of programmed cell death in contributing to alternative phenotypes. In the next chapter, I start to unravel these questions by examining the deep origins of programmed cell death, its many conserved forms and genetic pathways, where in the tree of life does it contribute to the development of polyphenic and dimorphic traits, and finally examine under which conditions we can predict this mechanism to be utilized by development to generate alternative phenotypes.

Chapter 2: Deep conservation and co-option of programmed cell death facilitates evolution of alternative phenotypes at multiple biological levels

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2.1. Abstract

Alternative phenotypes, such as polyphenisms and sexual dimorphisms, are widespread in nature and appear at all levels of biological organization, from genes and cells to morphology and behavior. Yet, our understanding of the mechanisms through which alternative phenotypes develop and how they evolve remains understudied. In this review, we explore the association between alternative phenotypes and programmed cell death, a mechanism responsible for the elimination of superfluous cells during development. We discuss the ancient origins and deep conservation of programmed cell death (its function, forms and underlying core regulatory gene networks), and propose that it was co-opted repeatedly to generate alternative phenotypes at the level of cells, tissues, organs, external morphology, and even individuals. We review several examples from across the tree of life to explore the conditions under which programmed cell death is likely to facilitate the evolution of alternative phenotypes.

2.2. Introduction

Alternative phenotypes are discrete traits that are differentially expressed between individuals within the same population, such as between different morphs within the same sex or between different sexes, but also include discrete traits that are differentially expressed within individuals, such as different cell types within an organism (Moran, 1992; West-Eberhard, 1986, 2003). They are phylogenetically widespread and occur at all levels of biological organization (Gross, 1996; Mori et al., 2017; Nguyen et al., 2013). Alternative phenotypes are typically produced through differential gene expression that regulates cellular and developmental processes, potentially influencing organ development, morphology, color, size, and behavior (Gross, 1996; Mori et al., 2017; Nguyen et al., 2013). This differential gene expression underlying an alternative phenotype is regulated by one or more condition-sensitive developmental switches or thresholds, which in turn are regulated by interactions between genes and their environment (Emlen & Nijhout, 2000; Nijhout, 2003). However, the degree to which either genes or environment contributes or influences developmental thresholds varies from case to case (W. C. Clark, 1976; Evans & Wheeler, 2000; Gilbert & Epel, 2009; Schwander & Leimar, 2011; Yang & Andrew Pospisilik, 2019).

Alternative phenotypes can take the form of polyphenisms, which are discrete traits produced from the same genome during development in response to environmental cues (Mayr, 1963; Michener, 1961; Nijhout, 2003). Some examples of polyphenisms include caste polyphenism in eusocial insects (i.e., ants, honeybees, and termites), where the same genome can produce morphologically and behaviorally different females (workers and queens) in response to temperature and nutrition (Figure 1A), heterophylly in plants, where a plant develops different types of leaves, and predator induced polyphenism, where the presence of a predator induces the development of alternative forms in plants and animals (Agrawal et al., 1999; Simpson et al., 2011; Wells & Pigliucci, 2000). Although the term polyphenism is largely used to distinguish alternative phenotypes that are largely environmentally influenced from those that are genetically influenced (termed polymorphisms), both types are based on gene–environment interactions, where either genes or environmental influence dominates in one of the types (W. C. Clark, 1976; Yang & Andrew Pospisilik, 2019).

While polyphenisms and polymorphisms occur between same-sex individuals, alternative phenotypes can also evolve between the sexes, most often through sexual selection, and are called sexual dimorphisms (Darwin, 1871; Mori et al., 2017). Some examples of sexual dimorphisms include horns in beetles and somatic gonads in fruit flies (Figure 1B-C). Sexual dimorphisms are also influenced by gene–environment interactions, but again the degree of influence of each differs between species (Kopp, 2012; Post et al., 1999; Williams & Carroll, 2009). For example, in some species of turtles and fishes sexual dimorphism is environmentally-influenced (temperature, predation), while in others it is genetically-influenced (Ceballos & Valenzuela, 2011; Hassell et al., 2012; Williams & Carroll, 2009).

Alternative phenotypes can also be observed across multiple levels of biological organization – from genes and cells to morphology and behavior (West-Eberhard, 2003). While the examples listed above illustrate alternative phenotypes that are observed externally at the level of the whole organism, multicellular individuals also express alternative forms in their cell types and tissues that are not readily observed externally. Differentiated cell types are considered polyphenisms occurring at the cellular level because they arise from the same genome and are induced by cues from the internal environment (e.g., morphogens or signaling molecules) or the external environment (e.g. starvation). Examples include differences between blood cells and liver cells in animals as well as between phloem and xylem tissues in vascular plants (Figure 2A) (Arendt et al., 2016; West-Eberhard, 2003). Differentiated cell types can also occur in unicellular organisms that aggregate and display multicellular-like behavior, such as stalk cells and spore cells in slime molds (Figure 2B) (Raper & Fennell, 1952).

In the following sections, we explore the association between alternative phenotypes and programmed cell death (PCD) at multiple levels of biological organization and across unicellular and multicellular organisms. PCD is an adaptive process responsible for eliminating cells as well as for playing normative roles in development and immunity (Buss et al., 2006; Fuchs & Steller, 2011; Williams & Dickman, 2008). By reviewing several examples, we investigate whether the processes of programmed cell death were co-opted to play a role in the development and evolution of alternative phenotypes by differentially eliminating cells, organs, or entire structures in some individuals in the population but not others.

2.3. Programmed cell death: deep conservation of function, forms, and genes/protein domains

Here we use a mechanistic definition of 'programmed cell death' (PCD), which is the existence of genetic regulation and specialized cellular machinery responsible for the induction and control of cell destruction (Kroemer et al., 2009; Yan et al., 2020). PCD is activated in response to a variety of stimuli originating from within the cell or from the internal or external environment such as cellular stress, signals from neighboring cells, DNA damage, or pathogenic infections (Golstein & Kroemer, 2005). PCD also has many functions during normal development, which include homeostasis, morphogenesis, cell cycle regulation, reproduction, cell differentiation, and defense against pathogenic infections (Fuchs & Steller, 2011; Gonçalves et al., 2017; Vaux & Korsmeyer, 1999; Williams & Dickman, 2008).

Remarkably, PCD is a deeply conserved and ubiquitous process present in all multicellular (animals, fungi, and plants) and unicellular (eukaryotes and prokaryotes) organisms (Figure 3, blue stars) (Ameisen, 2002; Bidle et al., 2010; Chaloupka & Vinter, 1996; Fuchs & Steller, 2011; Kaczanowski et al., 2011; Reape & McCabe, 2010; Sharon et al., 2009). PCD occurs through multiple forms across taxa, each of which is classified by how cells are dismantled and how their components are eliminated (D'Arcy, 2019; Yan et al., 2020). Some forms of PCD, such as apoptosis and autophagic cell death, occur across animals, plants, fungi and protists, while other forms are unique to specific lineages, such as the hypersensitive response found only in plants, as well as heterokaryon incompatibility found only in fungi (Figure 3, circles) (Gonçalves et al., 2017; Michaeli et al., 2016; Morel & Dangl, 1997; Reape & McCabe, 2010; Sharon et al., 2009).

We focus on both apoptosis and autophagic cell death because their role in alternative phenotypes has been documented across a broad range of taxa, allowing us to compare them in a phylogenetic framework. Apoptosis, which is by far the most well studied form of PCD, is characterized by cell shrinkage, condensation of nuclei and organelles, fragmentation of DNA, and disintegration of the cell into apoptotic bodies that are then engulfed (phagocytosed) by neighboring cells (Elmore, 2007). In contrast, autophagic cell death is characterized by the

accumulation of autophagic vacuoles (termed autophagosomes) inside the cell that deliver cell content to the lysosome for digestion (Parzych & Klionsky, 2014).

Although apoptosis and autophagic cell death are highly conserved across taxa (Figure 3, orange and purple circles), the degree of conservation of their underlying genetic pathways remains understudied. To examine the degree to which the underlying regulators of apoptosis are conserved across animals, we first describe the genetic pathway underlying apoptosis in mammals and then compare it to those in the model fruit fly (*Drosophila melanogaster*) and nematode worm (*Caenorhabditis elegans*). Apoptosis in mammals occurs via two pathways: the intrinsic pathway (also known as the mitochondrial pathway) and the extrinsic pathway (also known as the death receptor pathway) (D'Arcy, 2019; Wang & Youle, 2009). The intrinsic pathway is activated in response to stimuli received from within the cell and is dependent on the mitochondrial release of pro-apoptotic proteins (Estaquier J et al., 2012). This is achieved when cell death signals are sensed by the Bcl-2 family of proteins, which promotes the release of Cytochrome c and Smac/DIABLO from the mitochondria (Wang & Youle, 2009). Cytochrome c interacts with apoptosis protease-activating factor 1 (Apaf-1) to activate protease caspase-9. At the same time, Smac/DIABLO antagonize the inhibitor of apoptosis proteins to release their inhibition of caspase 9 (Estaquier J et al., 2012; Fuchs & Steller, 2011). Activated caspase-9 then activates the executioner caspases (caspase-3 and 7), which in turn carry out the dismantling of the cell. The extrinsic pathway of apoptosis in mammals occurs when death receptors on the cell are activated by the immune system (D'Arcy, 2019). Activation of death receptors via the binding of death ligands produced by immune cells triggers the activation of caspase-8. This in turn activates the executioner caspase-3 that dismantles the cell (D'Arcy, 2019).

Many of the genes in the mammalian apoptosis pathway we describe above are conserved in *D. melanogaster* and *C. elegans*, particularly caspase activation (Fuchs & Steller, 2011; Li & Yuan, 2008; Ryoo & Baehrecke, 2010). For example, apoptosis is dependent on the activity of caspases, where the caspase Dronc in *D. melanogaster* and the caspase CED-3 in C. elegans are homologs of caspase-9 in mammals (Fuchs & Steller, 2011; Ryoo & Baehrecke, 2010). Furthermore, a comparison of apoptosis protein domains amongst animals has revealed that many of these domains present in mammals are also present in invertebrates and early

branching animals (Zmasek & Godzik, 2013). This indicates that these protein domains were present in the last common ancestor of metazoans, but subsequently diverged in specific lineages, such as in *C. elegans*, where there have been notable losses (Zmasek & Godzik, 2013). Therefore, there exist both similarities and differences in the protein domains, genes, and regulatory linkages in the pathway underlying apoptosis within animals. For example, different lineages have evolved differences in the function of caspases—in *C. elegans*, CED-3 functions in cell destruction, while in mammals and *D. melanogaster*, caspase-9 and Dronc, respectively, are initiator caspases that activate downstream executioner caspases (D'Arcy, 2019; Fuchs & Steller, 2011; Ryoo & Baehrecke, 2010). Inhibitors of apoptosis proteins are another example—they are present in mammals and fruit flies but not in nematodes (Fuchs & Steller, 2011; Lettre & Hengartner, 2006). However, in *D. melanogaster*, inhibition of the inhibitors of apoptosis proteins by the genes *Reaper*, *Hid*, and *Grim* is sufficient to induce caspase activation, whereas in mammals the activation of caspases requires both the release of Cytochrome c and the inhibition of inhibitors of apoptosis proteins (Estaquier J et al., 2012; Ryoo & Baehrecke, 2010).

Furthermore, the hallmark features of animal apoptosis, such as DNA fragmentation and nuclear condensation, are also present in plants, fungi, and protists (Kaczanowski et al., 2011; Mpoke & Wolfe, 1996; Reape & McCabe, 2010; Sharon et al., 2009). The time scale through which these lineages have diverged from their last common ancestor is over a billion years, and therefore, it would be expected that these lineages have evolved unique features and genetic regulators underlying apoptosis (Koonin, 2010; Wray et al., 2003). It is therefore surprising given this time of divergence, that several of the apoptosis regulators, which include proteases (caspases in animals and metacaspases in plants, fungi, and protists), DNases, inhibitors of apoptosis proteins, Ap-GTPase, and apoptosis inducing factors, are all present in multicellular and unicellular eukaryotes (Aravind et al., 2001; Kaczanowski et al., 2011; Klim et al., 2018; Koonin & Aravind, 2002). This suggests that these regulators and protein domains make up an apoptosis core network that was present before the divergence of eukaryotic lineages (Figure 3, purple rectangle) (Klim et al., 2018). Remarkably, these hallmark features of apoptosis and some of its core genes are even found in prokaryotes. Caspase-homologs are widespread in archaea and have been identified in some, but not all, of the bacteria studied to date (Aravind

et al., 1999, 2001; Bidle et al., 2010; Chaloupka & Vinter, 1996; La et al., 2022; Lewis, 2000; Uren et al., 2000). Bacteria also have several of the eukaryotic apoptotic protein domains, but PCD varies and exhibits different events and morphological features from eukaryotic apoptosis. In Archaea, caspase activity is associated with death-related function, but it is currently unknown whether these caspase-homologs and protein domains also function in bacterial PCD (Aravind et al., 1999, 2001; Bayles, 2003; Bidle et al., 2010; Uren et al., 2000).

We use the hierarchical approach of Abouheif (1997), Wray and Abouheif (1998), and Abouheif (1999) to informally map similarities and differences at three different biological levels: (1) PCD function; (2) apoptosis as a form of PCD; and (3) the genetic pathways underlying apoptosis across the tree of life. Our informal mapping suggests that apoptosis (as a form of PCD) and its underlying core regulatory network are homologous across animals, fungi, plants, and protists because they were acquired before the divergence of eukaryotes (Figure 3, purple circles and rectangles) (Klim et al., 2018). Subsequently, novel genes and regulatory linkages were added to this apoptosis core network independently in different eukaryotic lineages (Figure 3, half grey / half purple rectangles) (Aravind et al., 2001; Zmasek & Godzik, 2013). This suggests that the networks underlying apoptosis across eukaryotes are ‘partially homologous’, a term used by Abouheif (1999) to describe a type of gene regulatory network evolution where the core part of the network is homologous across the taxa being compared, but the novel genes and regulatory linkages that were added independently since the divergence from the last common ancestor are not.

A similar pattern can be inferred for the evolution of autophagic cell death (Figure 3, orange circle and rectangles). In animals, autophagic cell death occurs through the formation of an autophagosome, which is a vacuole that envelopes contents of the cell and delivers them to the lysosome for digestion (Kroemer et al., 2009; Yuan & Kroemer, 2010). In mammals, these processes require the interactions of several complexes including ULK1, P13K, and a complex composed of ATG (autophagy-related) genes (Allen & Baehrecke, 2020; D'Arcy, 2019). The autophagosome, a hallmark feature of autophagic cell death, as well as homologs of the ATG genes underlying autophagic cell death are conserved across animals, plants, fungi, and protists (Michaeli et al., 2016; Pollack et al., 2009; Zhang et al., 2021). In prokaryotes, although an

autophagy pathway is absent, several remote homologs of the ATG-related genes have been identified in both bacteria and archaea (Zhang et al., 2021). Like apoptosis, this suggests that autophagic cell death (as a form) and its underlying core network were present in the last eukaryotic common ancestor and are therefore homologous (Figure 3, orange circle and rectangle). Notably, some of the ATG-related genes were already present before the divergence of eukaryotes and prokaryotes and were recruited into the autophagy pathway during the evolution of eukaryotes (Figure 3, light orange rectangle) (Zhang et al., 2021). Subsequent duplications and losses of ATG genes occurred independently in different eukaryotic lineages suggesting the gene regulatory networks underlying autophagic cell death are also partially homologous (Figure 3, half grey / half orange rectangles) (Zhang et al., 2021). Although we informally mapped apoptosis and autophagic cell death independently on the tree of life, the relationship between these forms is complex. In some cases, apoptosis and autophagic cell death pathways are mutually exclusive, while in others there is significant cross talk between them, such that they either activate or inhibit one another (Maiuri et al., 2007; Rubinstein & Kimchi, 2012). The complex interactions between apoptosis and autophagic cell death may explain the similarity in their patterns of evolution across the tree of life.

Current evidence suggests that some components of the PCD genetic toolkit (apoptosis protein domains, caspase-related homologs, and ATG-related proteins) are present in all domains of life (La et al., 2022; Zhang et al., 2021). If this is true, then it is reasonable to infer that these components first evolved in the last universal common ancestor (Figure 3: LUCA, light purple, brown, and orange rectangles) (La et al., 2022; Zhang et al., 2021). However, because genes and proteins in the PCD toolkit have several functions, including cell survival and cell death, it remains unclear what role these components of the PCD toolkit were playing in the LUCA. It is currently thought that the original role of these genes and proteins in the LUCA was in cell survival and was subsequently co-opted to play a role in cell death in bacteria, archaea and eukaryotes (Figure 3; blue stars) (Ameisen, 2002; La et al., 2022).

Furthermore, the incongruency between species trees and gene trees for several caspase-homologs, as well as the crucial role of the mitochondria in cell death, led researchers to propose a bacterial origin for eukaryotic PCD—eukaryotes acquired the apoptosis genetic

toolkit from bacteria via horizontal gene transfer and/or during the domestication of the proto-mitochondrial endosymbiont (Figure 3; solid and dotted arrows) (Klim et al., 2018; Koonin & Aravind, 2002; La et al., 2022). This horizontal gene transfer event is thought to have occurred through the proto-mitochondria, which might have evolved cell death genes to kill their host cells under unhospitable conditions. Once these cell death genes evolved, they would have been subsequently acquired by the eukaryotic host to form the apoptosis genetic toolkit (Figure 3; solid and dotted arrows) (Frade & Michaelidis, 1997; Klim et al., 2018; Kroemer, 1997).

In the following section, we argue that the deep evolutionary history and ubiquity of PCD has facilitated the evolution of alternative phenotypes at multiple biological levels and throughout the tree of life. We propose co-option, *sensu* True and Carroll (2002), as a key process through which PCD facilitated the evolution of alternative phenotypes—PCD acquired new roles in the development of alternative phenotypes from its ancestral functions, forms, and underlying core regulators. At the dawn of multicellularity, PCD evolved a dual function: one in orchestrating the destruction of cells and one in immunity and development, including, but not limited to, the regulation of the cell cycle, morphogenesis, maintaining homeostasis, sculpture of organs and structures, protection against the spread of pathogens, elimination of larval tissue during insect metamorphosis, and prevention of genetic incompatibility during mating of filamentous fungi (Fuchs & Steller, 2011; Gonçalves et al., 2017; Vaux & Korsmeyer, 1999; Williams & Dickman, 2008). Therefore, it is the deep conservation of PCD combined with the sheer ubiquity of its roles that makes its co-option for the evolution of alternative phenotypes highly probable. However, we propose the alternative hypothesis of convergent evolution in cases where PCD was co-opted to function in the alternative phenotypes being compared, but through completely different forms and underlying pathways in different lineages (Abouheif, 1999, 2008).

2.4. Programmed cell death regulates alternative phenotypes at multiple levels of biological organization

We begin this section by proposing a heuristic model, which we see as a starting point for thinking about how PCD links the evolution of alternative phenotypes at different levels of biological organization—from the cellular and organ to the whole-organism level. At the

cellular level (Figure 4A), regulators of PCD are recruited to eliminate components of a cell (not the entire cell) to produce different cell types within an organ of an individual. In this case, it is not the entire process of PCD that is involved since the cells themselves are not eliminated. Instead, it is the PCD genetic toolkit that functions to differentiate cells through the elimination of specific organelles or other components within one cell type and not the other. At the organ level (Figure 4B), PCD eliminates some (Figure 4B, top row) or all (Figure 4B, bottom row) of the cells that make up a certain organ or morphological trait in one sex or morph to generate polyphenisms or sexual dimorphisms observed in internal organs or external structures. Finally, at the whole-organism level (Figure 4C), PCD modifies entire individuals (via elimination of cells throughout the organism) to produce viable and non-viable individuals in a population (see Table 1 for examples of cases 1, 2, and 3). Each level is distinguished by the degree of cell destruction (organelles vs entire cell) and which/how many cells are eliminated. We therefore propose that alternative phenotypes on the cellular or organismal levels stem from a similar mechanism (PCD) operating on different levels. In other words, PCD links all these cases of alternative phenotypes to each other since they are all manifestations of the similar processes occurring on different scales. In the following sections, we review in detail some of the examples in Table 1 to demonstrate PCD's role in the development and evolution of alternative phenotypes at each of these three levels (Figure 4).

2.4.1. Case 1: Differentiation of cells within organs of an individual

Distinct cell types are the building blocks of multicellular organisms and represent an example of alternative phenotypes (polyphenism) on the cellular level within an organism (Bonner 1999; Bonner 2003). Differentiation of one cell type from another is achieved via condition-sensitive thresholds that activate different gene expression programs (Arendt et al., 2016). Here we explore the role that PCD plays in cellular level alternative phenotypes. Expression of some PCD toolkit genes during cell differentiation results in the fragmentation of DNA and elimination of organelles, but not the complete disintegration of the cell (Figure 4A) (Dahm, 1999; Turner et al., 2007; Whittingham & Raper, 1960). Consequently, these cells are hollow, yet intact, and serve important functions in the organism. Below we will discuss

examples where the activity of caspases and/or other PCD regulators facilitates cellular differentiation via inducing morphological features of PCD (such as elimination of organelles).

We illustrate Case 1 (Figure 4A) with two vertebrate examples of cellular differentiation that involve PCD toolkit genes: lens fiber cell differentiation and erythroid cell differentiation. During vertebrate eye development, lens fiber cell differentiation undergoes similar events to those that occur during apoptosis – namely DNA fragmentation and the degradation of all organelles and nuclei (Bassnett & Beebe, 1992; Dahm, 1999). This results in an alternative cell-type that is transparent due to its lack of organelles and nuclei. Many apoptosis regulators, such as the expression of Bcl-2 protein family, expression of caspases, increased concentration of Ca2+, and expression of cyclins are involved in this process (Dahm, 1999). This role of apoptosis regulators in differentiating lens fiber cells is conserved among vertebrates (Bassnett, 1997; Dahm et al., 1998; Ishizaki et al., 1998). Similarly, during erythroid (red blood cells) differentiation, the organelles and nuclei are eliminated by PCD genetic toolkit but the cells remain intact (Morioka et al., 1998). This results in an alternative blood cell type that functions to transport oxygen and carbon dioxide. Differentiation of erythroid cells involves several mechanisms and phenotypes characteristic of apoptotic cell death such as activation of caspase activity and DNA and nuclear fragmentation (Testa, 2004).

The tracheal elements of vascular plants serve as another example of Case 1 (Figure 4A; Figure 2A). They are the water-conducting cells that compose the xylem. These specialized tissues arise from procambial cells via four general steps: cell origination, elongation, secondary cell wall deposition, and autolysis of cell content (Figure 2A) (Fukuda, 1996; Turner et al., 2007). During the last step, the collapse of the large central vacuole, which is a striking feature of tracheal element differentiation, activates various enzymes including proteases and DNases that lead to the degradation of cell content thereby producing dead hollow cells that function as conduits for the transport of water (Turner et al., 2007). The PCD of tracheal elements does not exhibit any of the hallmark features of animal apoptosis nor those of plant hypersensitive response (Groover et al., 1997). Moreover, none of the plant autophagy genes are involved in tracheal element differentiation (Turner et al., 2007). This indicates that PCD of tracheal elements is distinct from apoptosis, autophagy, and hypersensitive response. More studies of

the genetic and molecular mechanisms that induce and regulate PCD of tracheal elements may help in identifying the PCD form that is involved.

Finally, although cellular differentiation is generally a characteristic of multicellular organisms, it can also occur among single celled organisms. When the single-celled slime mold *Dictyostelium* are starved, they aggregate and form a multicellular mass (Bonner, 1999, 2003; de Chastellier & Ryter, 1977). Aggregating *Dictyostelium* cells break symmetry and differentiate into two cell types, the stalk cells and spore cells (Bonner, 1999, 2003; Maeda & Takeuchi, 1969). The function of the stalk cells is to elevate the spore cells into the air where they release their spores (Figure 2B) (Kimmel & Firtel, 2004; Loomis, 1982; Raper & Fennell, 1952). Stalk cells are considered dead because they are vacuolized and non-growing (George et al., 1972; Whittingham & Raper, 1960). Several studies have shown that the differentiation process begins with starvation signals that induce autophagy and the subsequent expression of the differentiation-inducing factor (DIF-1) promotes stalk cell differentiation through autophagic cell death, where atg-1 has been shown to be essential for cell vacuolization (Giusti et al., 2009; Kosta et al., 2004; Morris et al., 1987; Tresse et al., 2008). These studies therefore provide evidence for the role of autophagy and autophagic cell death in cell differentiation of *Dictyostelium*. This example illustrates that autophagic cell death and at least some of its core regulators (ATG genes) are present in *Dictyostelium* and are facilitating the development of alternative phenotypes in this organism.

Altogether, the function of PCD machinery in cellular differentiation across vertebrates, plants, and slime molds, is similar in that they eliminate components of the cell to produce hollow, yet intact, cells that serve a structural or visual function for the organism (Figure 4A). Within vertebrates the elimination of the organelles is achieved through similar apoptosis genes and features. This suggests that the same PCD form and its core network were co-opted to facilitate the independent evolution of non-homologous alternative cell types within vertebrates (figure 5, purple triangles). By contrast, the autophagic cell death pathway was co-opted to facilitate cellular differentiation of spore and stalk cells in *Dictyostelium* (figure 5, orange triangle). In plants, it is yet unknown which PCD form is operating in tracheal element differentiation (figure 5, open triangle). Altogether, there is evidence for co-option of apoptosis

and its core regulators to facilitate the evolution of alternative cell types within several groups of vertebrates, but at the level of eukaryotes, we propose that the alternative cell types we discussed here evolved convergently through different forms of PCD in animals, plants, and protists.

2.4.2. Case 2: Differentiation of organs/structures between individuals

In addition to differentiating cell types within an organ of an individual (Case 1), PCD also contributes to the differentiation of organs, external morphologies, or behaviors between individuals. In this section, we discuss several examples to show how PCD eliminates some or all of the cells of an organ and/or external structure between individuals in one sex or morph to produce polyphenisms and sexual dimorphisms (Figure 4B).

Ant are eusocial insects whose colonies are typically composed of a reproductive queen caste and a nonreproductive worker caste (Holldobler & Wilson, 1990). Ants are considered a classic example of polyphenism because these morphologically distinct female castes are most often determined by environmental cues through an environmentally sensitive threshold during development (Holldobler & Wilson, 1990). In addition, the worker caste in some ant species is further subdivided into two or more subcastes with distinct differences in size and allometry, like the minor workers and soldiers in the ant genus *Pheidole* (Holldobler & Wilson, 1990; Wilson, 1953). Such worker subcastes are also determined by an environmentally sensitive threshold during development. Therefore, alternative phenotypes in the form of polyphenism between the queen and worker castes (and in some cases between the minor worker and soldier subcastes) is a central characteristic of eusocial colonies (Figure 1A) (Simpson et al., 2011; Wheeler, 1986).

During the origin of ants, females evolved a wing polyphenism, where queens are winged and workers are wingless (Figure 1A) (Hanna & Abouheif, 2021). Although wingless as adults, the workers of many species transiently develop rudimentary wing imaginal discs during the larval stage (Abouheif & Wray, 2002; Dewitz, 1878; Wheeler & Nijhout, 1981). In the species *Pheidole megacephala* and *Pheidole morrisi*, rudimentary wing discs first appear during the final stage of soldier larval development and grow significantly in size only to degenerate later during the prepupal stage (Sameshima et al., 2004; Shbailat et al., 2010). These

rudimentary wing discs degenerate through apoptosis as inferred from TUNEL, an assay that detects apoptotic DNA fragmentation (Sameshima et al., 2004). Furthermore, the timing of activation of PCD in the rudimentary wing discs is critical to the development of the soldier subcaste. Rajakumar et al. (2018) showed that the growth of rudimentary wing discs in last stage of soldier larvae is necessary for development of the big-headed soldiers. Therefore, late activation of apoptosis during prepupal development allows the disc to grow but prevents it from producing a wing bud on the adult (Sameshima et al., 2004; Shbailat et al., 2010).

Similar to ants, honeybees are also eusocial insects whose colonies have morphologically distinct female castes. One of the hallmarks of eusociality is the reproductive polyphenism and division of labour between the queen and worker castes. In response to environmental cues during larval development, honeybee queens develop large ovaries composed of hundreds of ovarioles, while workers develop small ovaries with few ovarioles that are typically inactive. Early during larval development in honeybees, ovary size and number of ovarioles do not differ between queens and worker larvae, but at the end of larval development after the worker caste has been determined, high levels of PCD are activated in worker ovarioles (Hartfelder et al., 1995; Hartfelder & Steinbruck, 1997). This PCD in worker ovarioles occurs in the form of apoptosis, which involves DNA degradation and the differential expression of pro- and anti-apoptotic genes (Capella & Hartfelder, 1998; Dallacqua & Bitondi, 2014). Similar to ant wings, the reproductive polyphenism occurs within same-sex individuals and in a caste-specific manner.

PCD also plays a role in regulating polyphenism in termites, which are also eusocial insects but belong to a different insect Order (Blattodea) than ants, bees and wasps (Hymenoptera). In termites, the soldier caste is a nearly universal and homologous trait in this group that evolved just once in the ancestor (Noirot & Pasteels, 1987). The soldier caste in termites is specialized for defense and in early-branching families develops exaggerated mandibles that are remarkably larger than those found in other castes (Koshikawa et al., 2003). However, the soldier caste in phylogenetically derived termite genera have evolved reduced mandibles that are significantly smaller (and perhaps functionless) as compared to other castes. Therefore, the size of the mandibles is an alternative phenotype within the colony (Emerson,

1961). Toga et al. (2011) used TUNEL to infer that PCD via apoptosis functions to reduce the size of the soldier mandible through the partial elimination of cells. Furthermore, the reduction of soldier mandibles is thought to have, as a side consequence, released a physical constraint on space, thereby allowing for the evolution of the nasute, a horn-like head gland on these soldiers that shoots toxic substances (Toga et al., 2011).

Sexual dimorphism in wing development is a common phenomenon in insects (Allen et al., 2011; Stubblefield & Seger, 1994). Particularly, winglessness in female Lepidoptera (butterflies and moths) has been associated with a trade-off between flight and egg production (Kimura & Masaki, 1977). In two species of tussock moths, *Orgyia recens* and *Orgyia leucostigma*, adult females develop vestigial wings compared to their winged males, whereas in the winter moth *Nyssiodes lefuarius* the females are completely wingless as compared to their winged males (Lobbia et al., 2003; Nardi et al., 1991; Niitsu, 2001). Studies in these moths have found that the wings of males and females undergo similar development up to the pupal stage, but in females only, the wing primordia subsequently undergo massive cell death resulting in vestigial wings or complete winglessness (Lobbia et al., 2003; Nardi et al., 1991; Niitsu, 2001). Signs of both apoptosis (via TUNEL assay) and autophagic cell death (via formation of autophagic vacuole) were detected in these moths (figure 5, purple and orange hexagon) (Lobbia et al., 2003; Niitsu et al., 2014). Moreover, experimental manipulations showed that the ecdysone hormone 20-hydroxyecdysone (20E) triggers PCD in the female wings (Lobbia et al., 2003; Niitsu et al., 2014). Here, PCD is eliminating most, but not all, of the cells that make up the female wing primordia in the female tussock moths resulting in vestigial wings in adult females, whereas it is eliminating all of the cells in the wing primordia of the female winter moths resulting in completely wingless adult females.

Scarab beetles, which include stag, dung and rhinoceros beetles, among others, produce horns on their heads and/or thorax. In species that have evolved a sexual dimorphism in the development of horns, adult males typically have horns whereas females are hornless. Even in those few species where the females do develop horns, they are typically smaller than those of males. In the genus *Onthophagus*, although horn development is sexually dimorphic, both sexes transiently develop horns during larval development to crack the cuticle open in order to

undergo the molt from larva to pupa (Figure 1B) (Moczek et al., 2006). Subsequently, the prepupal horns are resorped in females through the activation of PCD, leading to their reduction or absence in adult females (Figure 1B) (Kijimoto et al. (2010). PCD in these horns is induced by ecdysone and involves caspase activation and DNA fragmentation, suggesting that this process is achieved through apoptosis form of PCD (Kijimoto et al., 2010).

In the fruit fly *D. melanogaster*, PCD plays a key role in the development of sexual dimorphism of the somatic gonads. Both male and female gonads are made of somatic gonadal precursors as well as germ cells (DeFalco et al., 2003). The male gonads, however, are much larger because they develop additional male-specific somatic gonadal precursors (DeFalco et al., 2003). DeFalco et al. (2003) found that females actually develop these male-specific somatic gonadal precursors during the initial stages of female gonad development but are subsequently eliminated via PCD in females but not in males (Figure 1C). PCD in female gonads is caspase-dependent and requires the activity of hid, one of the antagonists of the inhibitors of apoptosis proteins (DeFalco et al., 2003). This provides another example where apoptosis plays a key role in the development of sexual dimorphism by eliminating male-specific cells during female gonad development in fruit flies.

PCD also regulates sexual dimorphism in zebrafish gonads. In contrast to the fruit fly example above, PCD functions in the development of gonadal sexual dimorphism in zebrafish through its role in testes differentiation in males. During the juvenile stage, all individuals are hermaphrodites and have undifferentiated ovary-like gonads, but during the period of sex differentiation, oocytes in male, but not female, gonads undergo PCD in the form of apoptosis as inferred by TUNEL (Uchida et al., 2002). Following apoptosis in male oocytes, male gonads begin to differentiate into spermatocytes (Uchida et al., 2002). This example illustrates how apoptosis facilitates gonadal sexual dimorphism in both invertebrates and vertebrates, but in different ways.

Finally, PCD plays a role in the sexual differentiation of the nervous system in several invertebrate and vertebrate species, which can result in behavioral differences between the sexes (see Forger (2006) and Forger and de Vries (2010) for reviews). Here, PCD eliminates neurons during development of one sex resulting in sexual dimorphism in neuronal numbers.

For example, in adult zebra finches, males have larger and more neurons in brain areas that regulate singing behavior as compared to those of females. This sexual dimorphism in adult neurons is a result of both neurogenesis in males and PCD in females during development (Kirn & DeVoogd, 1989; Konishi & Akutagawa, 1985). These dying neuronal cells exhibit intense nuclear condensation, which suggests apoptosis is involved in the neuronal degeneration in female zebra finches (Kirn & DeVoogd, 1989), but further work using other known markers of apoptosis is required to confirm this possibility (Diez et al., 2021). Another example can be found in adult male frogs (*Xenopus laevis*), which have a greater number of neurons than females in brain areas associated with vocalization. Unlike zebra finches, however, neurogenesis does not occur in males and the neuronal sexual dimorphism is primarily a result of higher rates of PCD in female neurons as compared to males (Kay et al., 1999). This PCD in female neurons most likely occurs through apoptosis because both nuclear condensation and positive TUNEL signal have been detected (Kay et al., 1999). PCD in the sexually dimorphic neurons of zebra finches, frogs, as well as mammals, is also under hormonal control, suggesting that hormones play a key role in regulating alternative phenotypes through PCD (Kay et al., 1999; Konishi & Akutagawa, 1987; Waters & Simerly, 2009). In summary, differences in neuronal numbers between sexes provides us with yet another example of how PCD eliminates entire cells within an organ to produce a sexual dimorphism between the brains of males and females. This can also lead to sexual differences in specific behaviors, such as vocalization. See Table 1 for additional examples of PCD regulation of sexual neuronal dimorphism.

Altogether, the invertebrate and vertebrate examples we discuss in this section paint a larger picture for the role of PCD in generating alternative phenotypes through the elimination of cells within organs or external morphologies between individuals (sex or morph). In the majority of these examples, current evidence suggests that PCD is achieved through apoptosis, although our knowledge of the apoptosis genetic toolkit in most of these examples remains limited. Given this current evidence and the fact that the apoptosis genetic toolkit is highly conserved across animals (Zmasek & Godzik, 2013), it is reasonable to propose that a homologous (or partially homologous) apoptosis toolkit was co-opted to facilitate the

independent evolution of the alternative phenotypes we discuss this section (Figure 5, hexagons).

These examples also raise the possibility that hormones were co-opted to play a key role in regulating PCD during the development of these alternative phenotypes. In all insects, ecdysone is a central regulator of molting and metamorphosis, where it mediates the destruction of larval tissues through genes in the apoptosis and autophagy pathways, such as *hid*, *reaper*, *dronc*, and *atg1* (Nicolson et al., 2015; Tettamanti & Casartelli, 2019; Xu et al., 2020). In the examples we discuss above, ecdysone mediates PCD in a sex-specific manner to facilitate the development of sexual dimorphism in moths and beetles (Kijimoto et al., 2010; Lobbia et al., 2003; Niitsu et al., 2014). This raises the possibility that, in insects, ecdysone's ancestral role in regulating PCD during molting and metamorphosis was co-opted to function in generating alternative phenotypes. Furthermore, PCD in vertebrates is also under hormonal regulation. In the examples above, PCD is regulated by testosterone and estrogen to facilitate the development of neuronal sexual dimorphism in mammals, zebra finches, and frogs (Kay et al., 1999; Konishi & Akutagawa, 1987; Waters & Simerly, 2009). Although the examples we use here focus on the link between hormones and PCD in generating sexual dimorphisms, we predict that hormonal regulation of PCD was also co-opted to aid in generating polyphenisms.

2.4.3. Case 3: Differentiation of individuals in a population

Another level of biological organization where PCD regulates alternative phenotypes is at the level of the whole individual, where some individuals in the population become non-viable through arrested development or death (Figure 4C). This is distinct from cell death caused by organismal senescence or other external influences, like pathogens, because it is programmed, utilizes PCD regulators and phenotypes, and provides the remaining living individuals with some form of fitness benefit. Examples of PCD operating on the level of the individual can be observed in the production of trophic embryos in annelids and gastropods and the production of autolytic non-sporulating cells in bacteria.

Diverse animal groups produce trophic embryos that undergo developmental arrest and provide a food source to their developing siblings (Perry & Roitberg, 2006). In the slipper snail *Crepidula navicella* (Gastropoda), trophic embryos occur in large numbers and display two

distinct phenotypes suggesting that trophic embryos are an example of adaptive, alternative phenotypes (Lesoway et al., 2014, 2016). The mechanism for the development of trophic embryos has been investigated in the spinoid polychate worm *Polydora cornuta* as well as in the snail *C. navicella*. In both species, trophic and viable embryos are morphologically similar at the early stages of development but some embryos subsequently undergo apoptosis and differentiate into trophic embryos (Gibson et al., 2012; Lesoway et al., 2016). Specifically, in the worm *P. cornuta*, trophic embryos activate caspases and undergo DNA fragmentation and vesiculation, which is the breakup of trophic embryos into smaller units that are digested by the viable embryos (Gibson et al., 2012). In the snail *C. navicella*, trophic embryos express high levels of apoptosis-related transcripts relative to viable embryos and this is thought to maintain trophic embryos in a suspended 'zombie-like' state for viable embryos to consume (Lesoway et al., 2016). Therefore, these studies indicate that the production of two types of individuals in a population - trophic and viable embryos – is in part achieved via deploying PCD that functions to either arrest the development of the embryos or to break them up into digestible units to serve as a nutritive source for the viable embryos.

Some bacteria, under stressful conditions, aggregate and cooperate to form fruiting bodies that propagate the population via spore production. In *Myxococcus xanthus* and the filamentous genus *Streptomyces*, low nutrition triggers fruiting body formation. A small portion of the population within the fruiting body differentiates into sporulating cells, while the remaining non-sporulating individuals undergo autolysis and release nutrients to aid the completion of the sporulation process (Chaloupka & Vinter, 1996; Lewis, 2000). This is strikingly similar to fruiting body formation in slime molds under starvation conditions (see section 3.1). However, a key difference between slime molds and bacteria is the role that PCD plays in differentiating cell types. In slime molds PCD produces non-growing, vacuolized stalk cells that provide structural support to the spore cells, whereas in these bacteria it eliminates non-sporulating individuals to release their cellular content for the remaining cells to consume as nutrition. This process is analogous to producing trophic embryos in snails, in that entire individuals are eliminated from the population via PCD.

Similar to the other examples of alternative phenotypes in Cases 1 and 2, we propose that PCD (function, form, and underlying core regulators) was independently co-opted to function in the differentiation of viable and trophic embryos in eukaryotes and the differentiation of sporulating and non-sporulating bacteria in prokaryotes. In eukaryotes (annelids and gastropods), the function of PCD in alternative phenotypes is achieved via apoptosis with conservation of some of its underlying core regulators (Figure 5, purple squares). In contrast, it has been shown that, during sporulation in *Myxococcus* bacteria, there is resemblance between the genetic regulation of autolysis and PCD in eukaryotes (Nariya & Inouye, 2008). Currently, we cannot distinguish whether this resemblance is due to convergent evolution or to the fact that eukaryotic PCD originated from bacteria.

2.5. Conclusions

PCD—it's function, forms, and underlying core regulators—originated billions of years ago and is conserved across deep evolutionary timescales from prokaryotes (both bacteria and archaea) to eukaryotes. PCD is therefore a deeply homologous process that serves critical functions throughout both unicellular and multicellular life (Ameisen, 2002). We propose here that this ancient and deeply conserved process has been co-opted repeatedly across the tree of life to facilitate the generation of alternative phenotypes at multiple levels of biological organization. The examples reviewed here reveal that this repeated co-option of PCD repurposed its original function in new contexts to help generate alternative phenotypes by eliminating organelles, cells, tissues, organs, external phenotypes, and even whole individuals in one of the morphs or sexes but not the other.

Undoubtedly, not all alternative phenotypes will involve PCD. Therefore, it is reasonable to ask under what conditions would we predict that PCD was co-opted during evolution to play a role in producing alternative phenotypes? We suggest that one of these conditions occurs when organs/structures have functions in all individuals during an early life stage but are no longer needed in later ones. For example, the rudimentary wing discs of ants and the pupal horns of beetles discussed above have transient functions that are important during juvenile development, but these structures are not needed by all individuals in the adult stage. Therefore, these structures are eliminated via PCD in some individuals but not others resulting

in alternative phenotypes in the adults. Another condition occurs when it is less costly to develop cells and organs in all individuals and later eliminate them in one morph or sex using PCD versus having to *de novo* evolve two alternative developmental programs to produce dimorphic structures from the outset. Potential examples of this condition include: (1) the development of unspecialized cells in plants before tracheal element differentiation takes place; (2) the development of ovaries in all honeybee larvae before the onset of caste differentiation (queen vs. worker); and (3) the development of monomorphic gonads and neurons in various vertebrate and invertebrate animals before sex differentiation takes place. Finally, a condition with potentially high impacts on the evolutionary process is the major evolutionary transitions in individuality, which occur when solitary individuals integrate to form a single replicating organism (Maynard-Smith & Szathmary, 1997). Some examples of major transitions include the emergence of eukaryotic cells, multicellularity, and eusocial insect societies (Maynard-Smith & Szathmary, 1997). A key feature of such major transitions is the division of labor between reproductive (germline) and non-reproductive (soma) between the constituent individuals. In this context, PCD has been co-opted to play key role in generating reproductive and non-reproductive alternative phenotypes during transitions to multicellularity in *Dictyostelium* and *Volvox* (colonial multicellular algae) and in the ovary polyphenism in honeybee societies (discussed in section 3.2) (Durand et al., 2019). Collectively, we predict that PCD will be co-opted to play a role in the generation of alternative phenotypes when dimorphic/polyphenic cells and organs are initially produced in all individuals because: (1) they perform a function during an early stage of development but not later; (2) it is less costly to eliminate them using PCD in one morph or sex than to *de novo* evolve alternative developmental programs from the outset; and (3) a division of labour between germline and soma evolves during major transitions of individuality.

2.6. Future directions

For the examples we present here showing a relationship between PCD and the development of alternative phenotypes, there currently exists limited information on the PCD forms and underlying genetic regulators involved. In the majority of cases, the TUNEL assay has been used to infer the role of PCD in alternative phenotypes (Kay et al., 1999; Lobbia et al.,

2003; Sameshima et al., 2004; Toga et al., 2011; Uchida et al., 2002). While this assay marks the presence of DNA degradation (a feature of apoptosis), it does not reveal which genetic regulators from the apoptosis pathway are involved, and furthermore, if other PCD forms are also at play (Grasl-Kraupp et al., 1995; Kyrylkova et al., 2012). Therefore, future work should focus on uncovering, in a more precise way, the PCD forms and genetic networks underlying alternative phenotypes. Given that PCD is manifested via many forms, of which only a few have been well studied (such as apoptosis and autophagic cell death), we predict that a greater diversity of PCD forms have been co-opted to play a role in alternative phenotypes than is currently known. Furthermore, uncovering other deeply conserved components of the PCD genetic toolkit will help reveal to what extent they have been repeatedly co-opted to play a role in the development of alternative phenotypes across the tree of life. More generally, we predict that the co-option of PCD during the development and evolution of alternative phenotypes may be more widespread than the examples known so far, majority of which we have presented here.

Finally, in this work we propose at least three different ways in which PCD was co-opted to play a role in generating alternative phenotypes across different levels of biological organization, and argue that PCD links these levels by eliminating components of cells, entire cells or organs, or entire individuals (Figure 4). The development and evolution of alternative phenotypes at these different biological levels are typically treated as being unconnected both in terms of theory and mechanism. For example, there is little connection between theories on the origin and evolution of alternative (novel) cell types (cellular polyphenism), which largely focus on cellular stress, and theories on the origin and evolution of alternative (novel) phenotypes (morphological and behavioral polyphenism), which largely focus on the role of developmental plasticity (Abouheif et al., 2014; Arendt et al., 2016; Moczek et al., 2011; Pfennig, 2021; Wagner et al., 2019; West-Eberhard, 2003; Wund, 2012). Therefore, one important avenue for future research is to link mechanisms and theories for the development of alternative phenotypes at the cellular and phenotypic levels. We hope that this work will promote further investigations of the role and prevalence of PCD in alternative phenotypes across different biological scales.

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Table 1. A list of alternative phenotypes regulated by programmed cell death.

Alternative Phenotype	Example	Organism/Group	PCD form	Reference
Case 1: Differentiation of cells within an organism	Lens fiber cell differentiation	Vertebrates	Apoptosis	Bassnett and Beebe (1992); Dahm (1999)
	Erythroid cell differentiation	Vertebrates	Apoptosis	Testa (2004)
	Tracheal element differentiation	Vascular plants	?	reviewed in Turner et al. (2007)
	Stalk cell differentiation	Slime mold	Autophagic cell death	Arnoult et al. (2001); Luciani et al. (2009)
Case 2: Differentiation of organs/structures between individuals	Wing polyphenism: elimination of worker wings	Ants	Apoptosis	Sameshima et al. (2004); Shbailat et al. (2010)
	Ovary polyphenism: reduction of worker ovaries	Honeybees	Apoptosis	Hartfelder and Steinbruck (1997)
	Mandible polyphenism: reduction of soldier mandible size	Termites	Apoptosis	Toga et al. (2011)
	Wing sexual dimorphism: reduction or elimination of female wings	Moths	Apoptosis and autophagic cell death	Lobbia et al. (2003); Nardi et al. (1991); Niitsu (2001)
	Horn sexual dimorphism: elimination of male or female head horns	Dung beetles	Apoptosis	Kijimoto et al. (2010)
	Somatic gonad sexual dimorphism: elimination of male-specific cells in females	Fruit flies	Apoptosis	DeFalco et al. (2003)
	Gonad sexual dimorphism: elimination of ovary-like tissue in males	Zebrafish	Apoptosis	Uchida et al. (2002)
	Reproductive tract sexual dimorphism: regression of male Mullerian duct	Mammals	Apoptosis	Roberts et al. (1999)

	Flower sexual dimorphism: elimination of male organs in female flowers and elimination of female organs in male flowers	Various angiosperms	?	Calderon-Urrea and Dellaporta (1999); Caporali et al. (2003)
	Neuronal sexual dimorphism: elimination of different neurons in males and females	Moths	?	Thorn and Truman (1994)
	Neuronal sexual dimorphism: elimination of female neurons	Fruit flies	Apoptosis	reviewed in Kimura (2011)
	Neuronal sexual dimorphism: elimination of Hermaphrodite-specific neurons in males and male-specific neurons in hermaphrodites	Nematodes	Apoptosis	Conradt and Horvitz (1999); Sulston et al. (1983)
	Neuronal sexual dimorphism: elimination of male-specific neurons in females	Zebra finches, frogs	Apoptosis	Kay et al. (1999); Kirn and DeVoogd (1989)
	Neuronal sexual dimorphism: elimination of different neurons in males and females	Mice, rats	Apoptosis	Reviewed in Forger (2006)
Case 3: Differentiation of individuals within a population	Death or arrested development of trophic embryo	Gastropods, annelids	Apoptosis	Gibson et al. (2012); Lesoway et al. (2016)
	Autolysis of non-sporulating cells	Bacteria	Autolysis?	Chaloupka and Vinter (1996); Lewis (2000)

Figure 1.

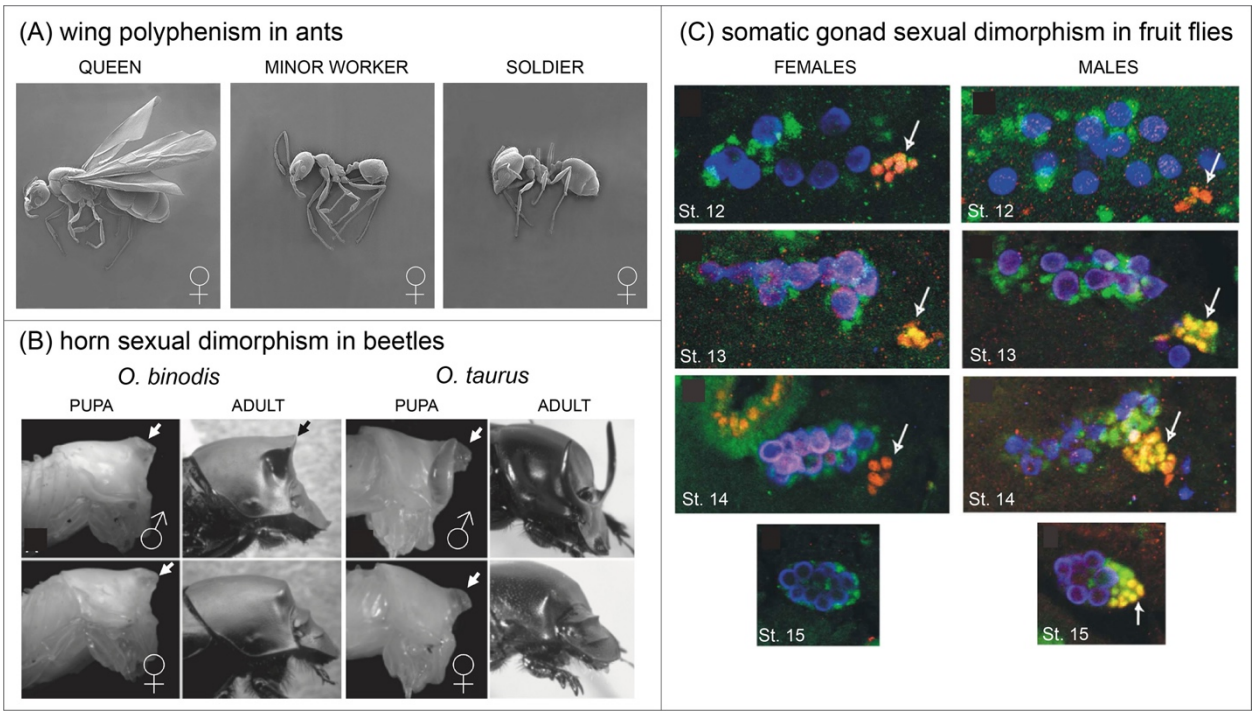

Figure 1. Examples of PCD's role in polyphenism and sexual dimorphism. (A) wing polyphenism in females from the ant genus *Pheidole*: winged queen caste (left) and wingless worker caste (middle and right). In *Pheidole*, the worker caste is typically composed of two worker subcastes: minor workers (middle) and soldiers (right). (B) horn sexual dimorphism in two species of dung beetles: *Onthophagus binodis* and *Onthophagus taurus*. Both sexes develop head horns during pupal development (white arrows) but these horns degenerate via PCD before the adult stage in a sex and species-specific manner. (C) Development of somatic gonad sexual dimorphism in *Drosophila melanogaster*: males and females develop similar somatic gonads initially (white arrows in Stage 12 [St. 12]), but as development progresses from stage 12 to 15 the cells that compose the male somatic gonadal precursors (white arrows) are eliminated in the females via PCD (left) but remain in the males (right). Immunofluorescence of Vasa (blue), Sox100B (red), and Eyes absent (green). (A) adapted from Hanna and Abouheif (2021), (B) from Kijimoto et al. (2010), and (C) from DeFalco et al. (2003), all reprinted with permission.

Figure 2.

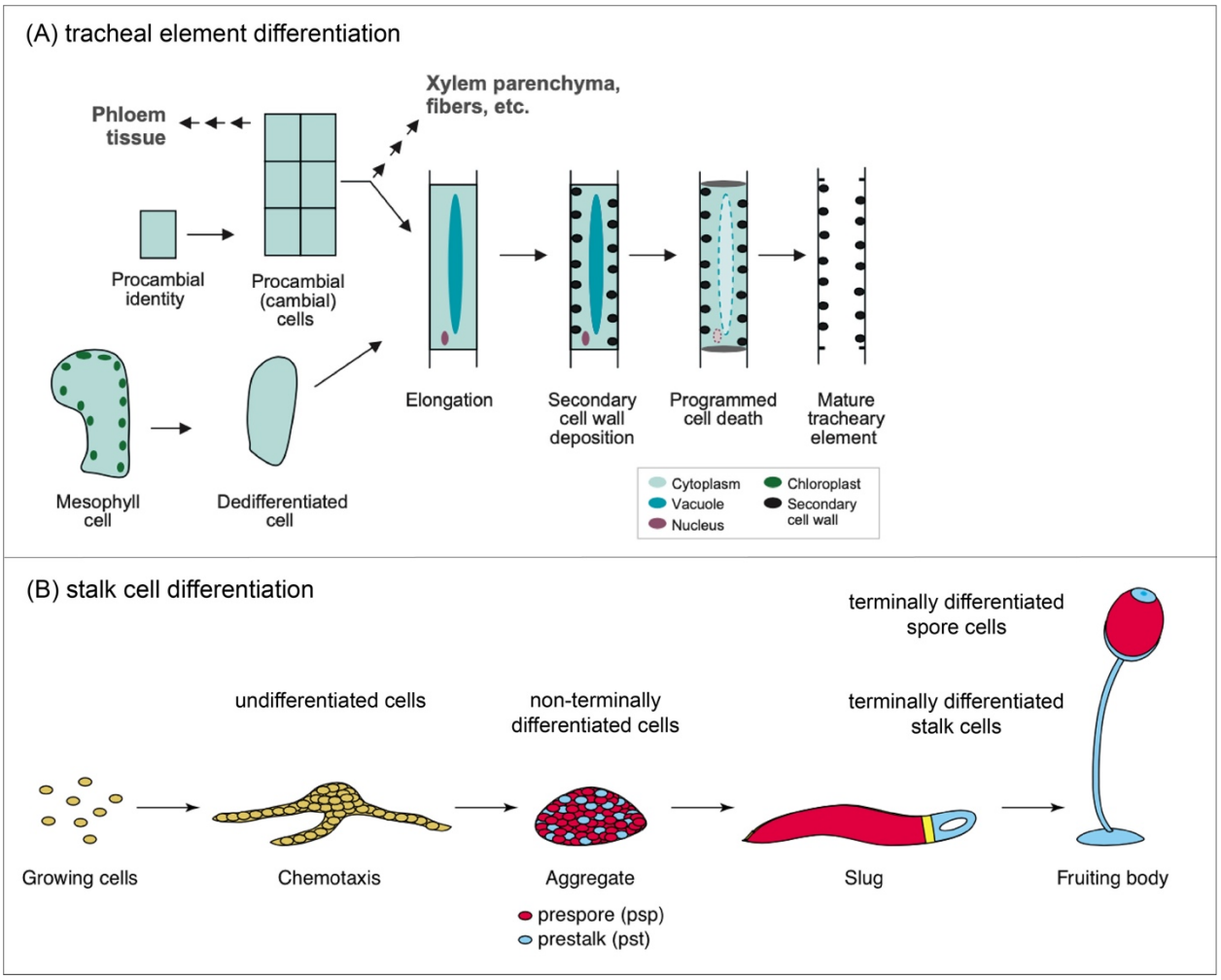

Figure 2. Examples of PCD's role in cellular differentiation. (A) differentiation of tracheal element cells of vascular plants requires PCD. Tracheal element cells originate from procambial cells or dedifferentiated cells, where they undergo elongation, secondary cell wall disposition, and PCD to develop and differentiate into mature tracheary elements that serve as hollow conduits for water transport. However, given appropriate internal cues, procambial cells alternatively differentiate into other tissue types such as phloem and xylem parenchyma. (B) differentiation of Dictyostelium cells requires PCD. Under starvation, cells aggregate to form a cluster of undifferentiated cells which subsequently breaks symmetry and differentiates into pre-spore (red) and pre-stalk (blue) cells. Pre-stalk cells undergo PCD to terminally differentiate into non-growing and vacuolized mature stalk cells. (A) from Turner et al. (2007) and (B) from Kimmel and Firtel (2004), all reprinted with permission.

Figure 3.

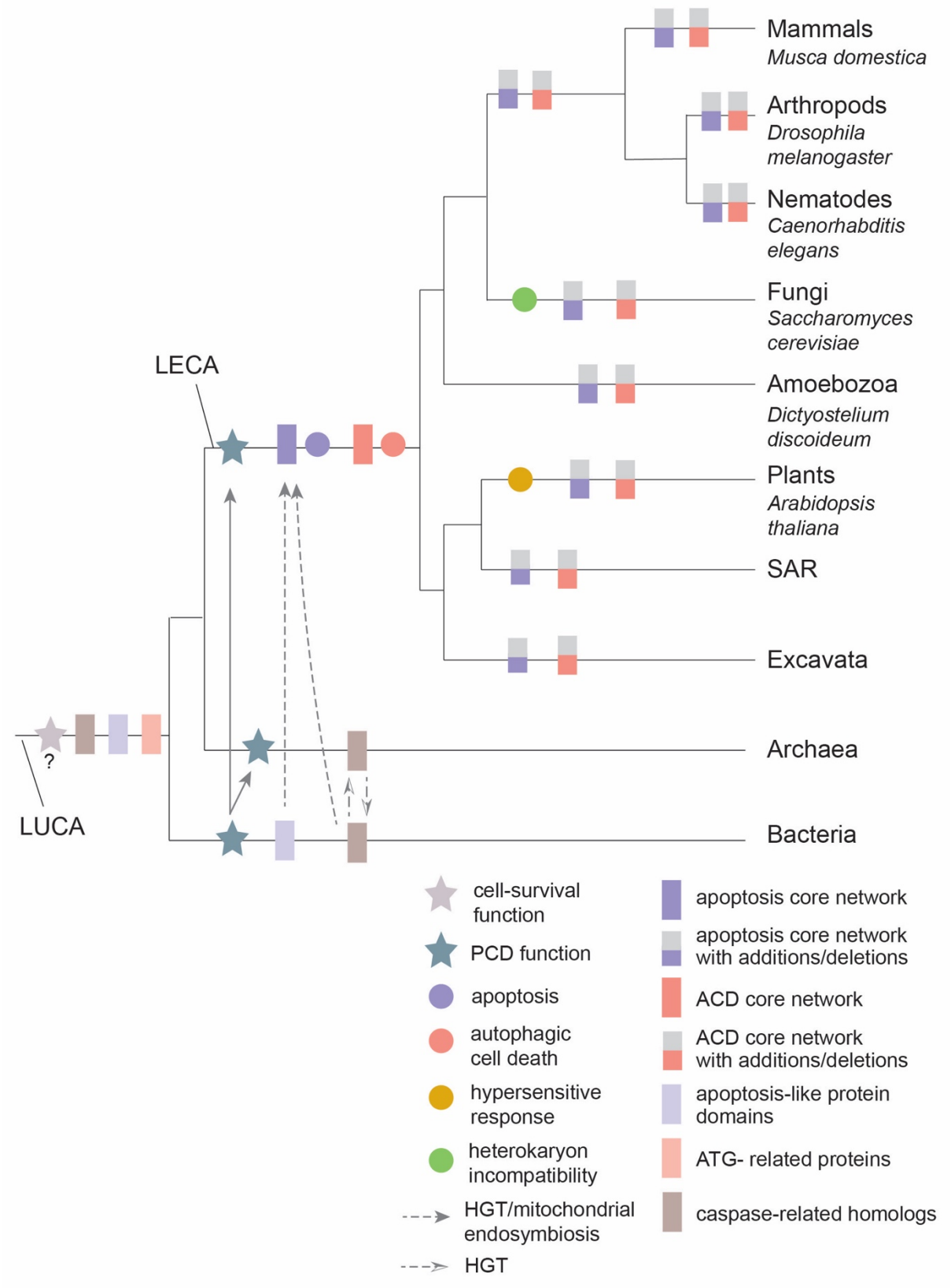
Mammals
Musca domestica
Arthropods
Drosophila melanogaster
Nematodes
Caenorhabditis elegans
Fungi
Saccharomyces cerevisiae
Amoebozoa
Dictyostelium discoideum
Plants
Arabidopsis thaliana
SAR
Excavata
Archaea
Bacteria
LECA
LUCA
?
cell-survival function
PCD function
apoptosis
autophagic cell death
hypersensitive response
heterokaryon incompatibility
HGT/mitochondrial endosymbiosis
HGT
apoptosis core network
apoptosis core network with additions/deletions
ACD core network
ACD core network with additions/deletions
apoptosis-like protein domains
ATG- related proteins
caspase-related homologs

Figure 3. Deep conservation and homology of PCD: function, representative forms and their underlying core regulators. Phylogenetic tree representing tree of life, where relationships are based on Keeling et al. (2009). Group and species names are indicated to the right of the tree. We informally map (using parsimony criteria) three PCD characters (function, form, and underlying core regulators), which are represented as different symbols, and their associated character states, which are represented as different colors on the phylogenetic tree of life. The grey star (with the question mark underneath) indicates the possibility that caspase-related homologs (brown rectangle), apoptosis-like domains (light purple rectangle), and ATG-related proteins (light orange rectangle) have initial cell-survival functions, whereas the blue stars indicate subsequent evolution of PCD function. PCD forms are indicated by circles, where the purple circle indicates apoptosis, the orange circle indicates autophagic cell death, the yellow circle indicates hypersensitive response, and the green circle indicates heterokaryon incompatibility. Core regulators underlying a PCD form are indicated by a rectangle, where the dark purple rectangle indicates apoptosis core regulators and dark orange rectangle indicates autophagic cell death (ACD) core regulators. Half purple/half grey or half orange/half grey rectangles represent partially homologous networks (with the purple or orange half representing conserved core network and grey half representing additions or deletions of genes and associated regulatory linkages). Solid arrows indicate bacterial origins for eukaryotic and archaeal PCD. Dotted arrows indicate transfer of caspase-related homologs and apoptosis-like protein domains from bacteria to archaea and eukaryotes via horizontal gene transfer and/or mitochondrial endosymbiotic event. ACD, autophagic cell death; HGT, horizontal gene transfer; LECA, last eukaryotic common ancestor; LUCA, last universal common ancestor.

Figure 4.

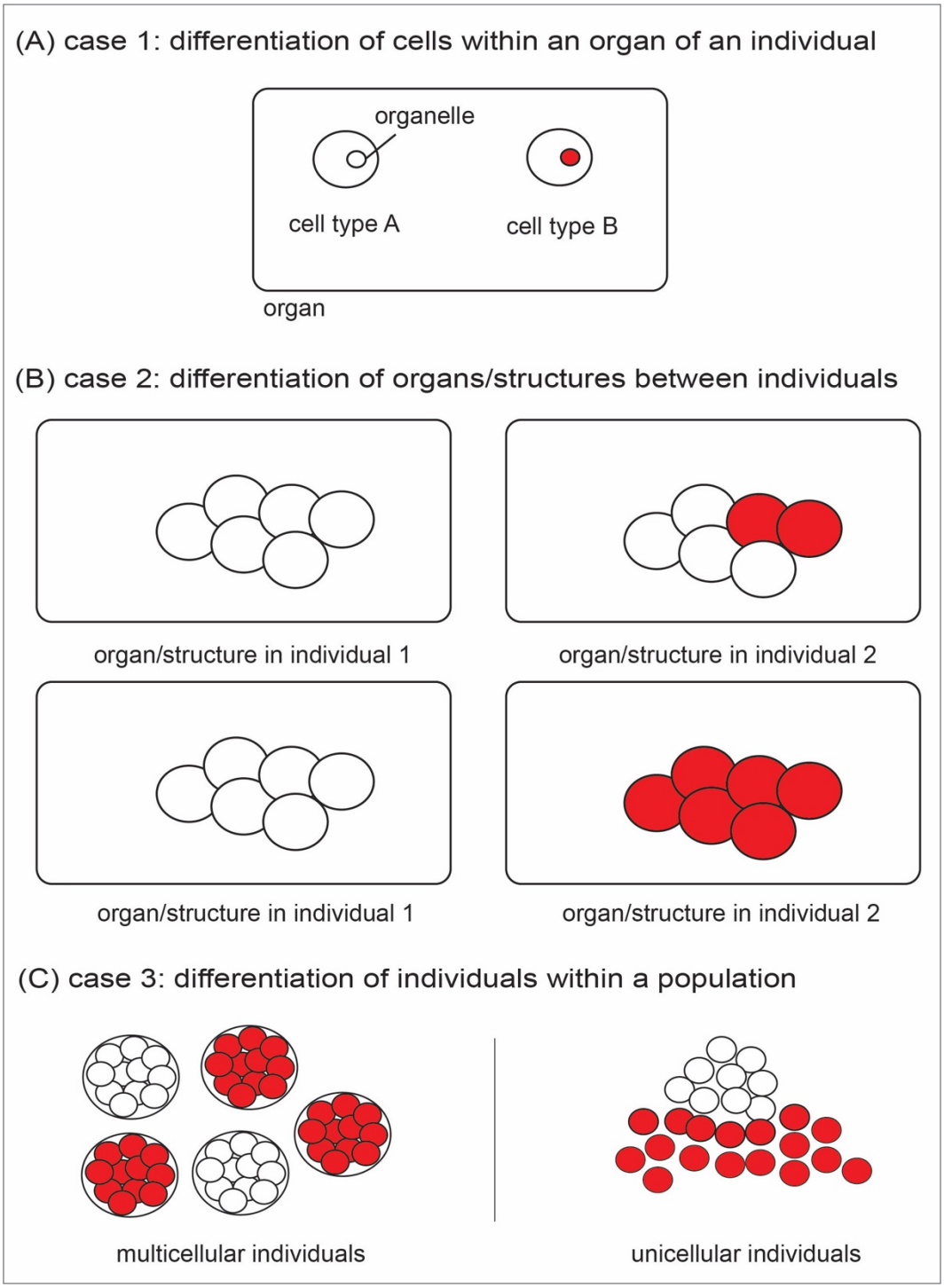
(A) case 1: differentiation of cells within an organ of an individual
organelle
cell type A
cell type B
organ
(B) case 2: differentiation of organs/structures between individuals
organ/structure in individual 1
organ/structure in individual 2
organ/structure in individual 1
organ/structure in individual 2
(C) case 3: differentiation of individuals within a population
multicellular individuals
unicellular individuals

Figure 4. Role of PCD in the development of alternative phenotypes at three levels of biological organization. In all panels, open circles represent viable cells and red colored circles represent PCD in cells. (A) case 1 indicates differentiation of cells within an organ of an individual: PCD eliminates organelles within cells (red circle in cell type B) during differentiation. (B) case 2 indicates differentiation of organs/structures between individuals: PCD eliminates some cells (top row) or all cells (bottom row) that compose an organ/structure during differentiation of internal organs or external structure to produce polyphenisms or sexual dimorphisms. (C) case 3 indicates differentiation of individuals within a population: PCD eliminates entire cell(s) that compose an individual to produce a population of viable and non-viable individuals.

Figure 5.

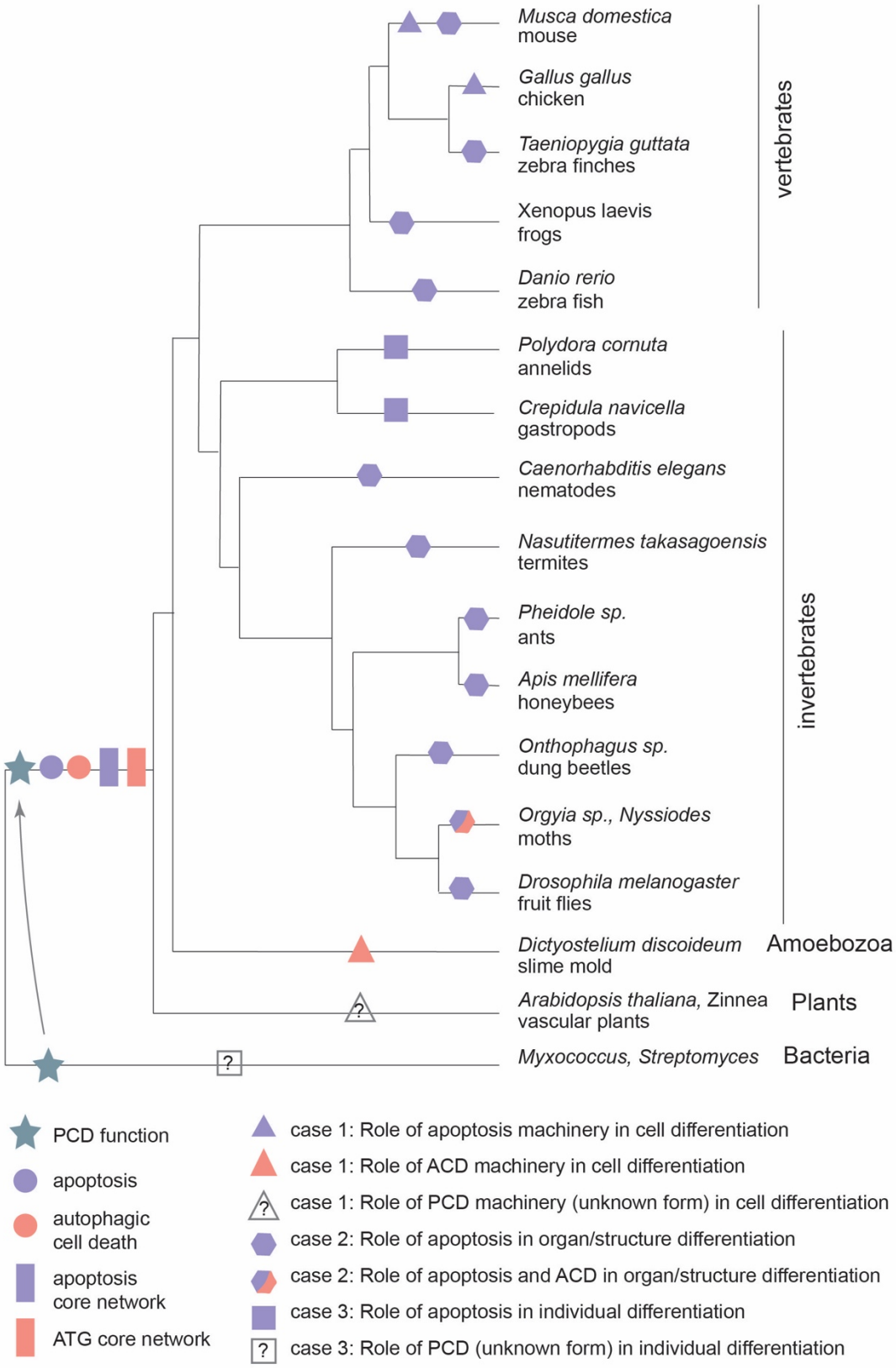
Musca domestica
mouse
Gallus gallus
chicken
Taeniopygia guttata
zebra finches
Xenopus laevis
frogs
Danio rerio
zebra fish
vertebrates
Polydora cornuta
annelids
Crepidula navicella
gastropods
Caenorhabditis elegans
nematodes
Nasutitermes takasagoensis
termites
Pheidole sp.
ants
Apis mellifera
honeybees
Onthophagus sp.
dung beetles
Orgyia sp., Nyssiodes
moths
Drosophila melanogaster
fruit flies
invertebrates
Dictyostelium discoideum
slime mold
Amoebozoa
Arabidopsis thaliana, Zinnea
vascular plants
Plants
Myxococcus, Streptomyces
Bacteria
?
?
PCD function
apoptosis
autophagic
cell death
apoptosis
core network
ATG core network
case 1: Role of apoptosis machinery in cell differentiation
case 1: Role of ACD machinery in cell differentiation
case 1: Role of PCD machinery (unknown form) in cell differentiation
case 2: Role of apoptosis in organ/structure differentiation
case 2: Role of apoptosis and ACD in organ/structure differentiation
case 3: Role of apoptosis in individual differentiation
case 3: Role of PCD (unknown form) in individual differentiation

Figure 5. The repeated use of deeply conserved PCD forms and core regulators in the independent evolution of alternative phenotypes. Phylogenetic tree (based on Adoutte et al. (2000), Blair and Hedges (2005), and Misof et al. (2014)) representing the relationship between species where PCD is known to play a role in alternative phenotypes (see Table 1). Species or group names are on the right of the phylogeny. PCD function is represented by a blue star. PCD forms are represented by circles: apoptosis (purple circle) and autophagic cell death (ACD; orange circle), while their underlying core regulators are represented by rectangles: apoptosis (purple rectangle) and autophagic cell death (ACD; orange rectangle). PCD function, forms, and underlying core regulators are ancestral to all eukaryotes. Arrow indicates the acquisition of eukaryotic PCD function from bacteria. The three cases of alternative phenotypes are mapped on the phylogeny using the following characters: cell differentiation (triangles), organ/structure differentiation (hexagons), and individual differentiation (squares). Colors of triangles, squares, and hexagons correspond with the form of PCD involved: apoptosis (purple) and autophagic cell death (ACD; orange). Open characters with question marks indicate unknown form of PCD.

CONNECTING STATEMENT BETWEEN CHAPTERS 2 & 3

In Chapter 2, I conducted a literary analysis into the role of programmed cell death (PCD) in alternative phenotypes. Before examining that role, I address the ubiquity and deep origins of this process, its many forms and their underlying genetic pathways. The informal mapping of these characteristics leads us to propose a partial homology where the core parts of the networks to be homologous whereas novel additions are not. Furthermore, due to its deep origins and conservation, we propose that PCD function in cell elimination was co-opted and utilized to generate alternative phenotypes. In Chapter 3, I specifically test the role PCD is playing in ant wing polyphenism during terminal larval development. I find that PCD, specifically the apoptosis pathway, is indeed activated in the rudimentary wing discs of workers starting around the mid-late terminal larval stage. I find this pattern in 15 out of 16 species I examined from across the ant phylogeny, encompassing species with ancestral-like features and species with more socially derived features. Furthermore, ancestral state reconstruction predicted the presence of apoptosis in the last common ancestor of all extant ants, further suggesting that apoptosis may have been a mechanism operating at the origin of the trait. The relationship between apoptosis and gene regulatory network interruptions that also occur in the rudimentary wing discs is discussed. Finally, we make interesting links between the origin of wing polyphenism and eusociality and discuss how the role of PCD in the generation of a wingless worker caste may have contributed to limiting dispersal and promoted the major evolutionary transition to eusociality.

Chapter 3: Inferring a role for programmed cell death during the origin and evolution of wing polyphenism in ants

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3.1. Abstract

Major evolutionary transitions in individuality, such as the evolution of multicellularity, occur when solitary individuals unite to form a single replicating organism with a division of labor between constituent individuals. Programmed Cell Death (PCD) has been proposed to play an important role during the origin and evolution of major transitions. Yet, it remains unclear to what extent PCD was involved in the major evolutionary transition to eusociality in ants, where solitary individuals united to form eusocial colonies with a division of labour between morphologically distinct queen and worker castes. Wing polyphenism is the ability of an egg (same genome) to develop either into a queen with wings or into a worker that is completely wingless in response to environmental cues. Because wing polyphenism and eusociality both evolved once and are a general feature of ants they were likely intimately linked and influenced each other during the origin of this group, . We therefore tested whether PCD plays a role in wing polyphenism in species from across the ant phylogeny encompassing taxa with both ancestral-like and derived characteristics. We show that PCD, mediated by the apoptosis pathway, is present in the rudimentary wing discs of worker larvae in 15 out of the 16 species tested. Using ancestral state reconstruction, we infer the presence of PCD in the last common ancestor of the poneroid and formicoid clades as well as all extant ants. Based on these results, our analysis predicts a role for PCD in regulating wing polyphenism during the evolutionary origin of ants. Implications for the role PCD in major evolutionary transitions to individuality are discussed.

3.2. Introduction

Major evolutionary transitions in individuality occur when solitary individuals unite to form a single replicating individual. During the history of life, such transitions include the evolution of eukaryotic cells from the integration of solitary prokaryotic cells, the integration of single-celled organisms to form multicellular organisms, the integration of solitary organisms to form eusocial colonies, and the integration of distantly-related organisms to form obligate endosymbioses (Buss, 2014; Maynard-Smith & Szathmary, 1997; West et al., 2015). Despite great advances made in understanding the evolutionary mechanisms underlying these major transitions, the developmental mechanisms that facilitated these events remain poorly explored.

Recent advances in uncovering the molecular and developmental processes during major evolutionary transitions to multicellularity, has been proposed that programmed cell death (PCD) is a mechanism that plays an essential and general role in the emergence of major evolutionary transitions (Durand et al., 2019; Huettenbrenner et al., 2003). Unlike other modes of cell death, PCD is a genetically regulated process that occurs under specific physiological conditions (Galluzzi et al., 2018). PCD has ancient origins, and in addition to multicellular life, is found in unicellular eukaryotes and prokaryotes (Ameisen, 2002). PCD is thought to play a role in facilitating major transitions by enhancing cooperation, formation of a morphological division of labor, and mediation of communication and conflict (Huettenbrenner et al., 2003; Libby & Ratcliff, 2014). For example, in some unicellular clonal or aggregate colonies, some individuals undergo PCD under stress conditions to provide nutrition to other members of the group hence enhancing the survival and growth of the population and thereby promoting group living – a precursor to multicellularity (Durand et al., 2014; Durand et al., 2011; Orellana et al., 2013). Moreover, experimental studies inducing multicellularity in yeast showed that the multicellular clusters fracture (via some cells undergoing PCD) allowing them to propagate into daughter cells (Ratcliff et al., 2012). Finally, in the eusocial honeybees, PCD functions to degenerate ovaries in worker-destined larvae but not in queen-destined larvae (Durand et al., 2019; Hartfelder & Steinbruck, 1997). This led Durand et al. (2019) to propose the hypothesis that

PCD may have played a role in the origin of reproductive division of labor in bees, and therefore, in one of the major transitions to eusociality.

Here, we test the hypothesis that PCD contributed to the major evolutionary transition to eusociality in ants (family Formicidae) (Boudinot, Richter, et al. 2022; Holldobler & Wilson, 1990). Wing polyphenism, the ability of an egg to develop either into a winged queen or wingless worker in response to environmental cues, such as nutrition and temperature (Hanna & Abouheif, 2021; Penick et al., 2012; Wheeler, 1986) (Fig. 1a), is a universal trait in ants (excepting for secondary losses) (Boudinot, Khouri, et al., 2022; Boudinot, Richter, et al., 2022; Hanna & Abouheif, 2021; Peeters, 2012; Perrichot et al., 2008). Typically, winged queens and males fly to participate in nuptial flights, while the wingless worker caste engages in foraging and brood care on or underground. At approximately the same time period that ants evolved eusociality, ants also evolved a wing polyphenism, and therefore, the evolution of eusociality and wing polyphenism may have been intimately linked. The origin of eusociality may have preceded and facilitated the origin of wing polyphenism, or alternatively, the origin of wing polyphenism may have preceded and facilitated the origin of eusociality (Hanna and Abouheif 2021). In the early lineages of ants, where queens and workers can mate, store sperm, and have similar reproductive capacities, reproductive division of labor primarily occurred through behavioral regulation (Peeters, 1991). Therefore, the early evolution of wingless workers may have played an important role in the evolution of eusociality by limiting worker's participation in nuptial flight and dispersal, which in turn limited the ability of workers to mate and reproduce while also facilitating cooperation in brood rearing and foraging. Here, we ask if PCD contributed to a major evolutionary transition in eusociality by playing a key role in wing polyphenism in ants by degenerating the wings in the worker caste thereby limiting worker dispersal.

Individuals in the worker caste of ants, although completely wingless as adults, transiently develop wing discs during larval development (Abouheif & Wray, 2002; Dewitz, 1878; Rajakumar et al., 2018; Wheeler & Nijhout, 1981) (Fig. 1b–d, blue arrowheads). These wing discs are therefore considered as rudimentary organs, which Hall (2003) defines as 'an embryonic primordium of a more fully developed feature (the insect wing) found in an ancestor

(a winged solitary wasp)'. Examining the highly conserved wing GRN in queen wing discs and worker rudimentary wing discs in derived ant species has revealed that the wing GRN is conserved between winged reproductive ants and other winged insects, but is interrupted in the wingless worker castes of different ant species (Abouheif & Wray, 2002; Shbailat & Abouheif, 2013; Shbailat et al., 2010). However, in two Amblyoponine species from the genus *Mystrium*, which are thought to reflect the ancestral characteristics of ants during the origin of eusociality, the wing GRN was conserved in the winged queens but no interruptions were detected in the wingless workers (Behague et al., 2018). This finding led Behague et al (2018) to propose that apoptosis (the most common genetic pathway for PCD) was co-opted to eliminate wing discs in the wingless worker caste during the origin of eusociality in ants. Although in two derived ant species from the genus *Pheidole*, apoptosis has been previously detected in the worker rudimentary wing discs (Sameshima et al., 2004; Shbailat et al., 2010), however, its presence across the ants and at the origin of wing polyphenism has yet to be tested.

In this study, we therefore tested for the role of PCD in wing polyphenism across the ant phylogeny by focusing on the two major clades of ants: the 'poneroid' and 'formicoid' clades. Species in the poneroid clade, like in the genus *Mystrium*, are of key importance to understanding the mechanism that may have been present in the early history of wing polyphenism since these species exhibit several developmental and behavioral characteristics that are similar to those found in early and extinct groups (Burchill & Moreau, 2016; Keller & Peeters, 2022; Peeters, 1997; Wilson & Hölldobler, 2005). In contrast, species in the formicoid clade largely exhibit derived social characteristics. First, we test for the presence of apoptosis markers in a focal species, *Harpegnathos saltator*, across a series of developmental stages, and second, across the ant phylogeny in an additional 15 species belonging to the poneroid and formicoid clades. We conduct an ancestral state reconstruction analysis to predict the presence of PCD in wing polyphenism at ancestral nodes and at the base of all extant ants. Finally, we test if only PCD is operating in the rudimentary wing discs of poneroid species since previous examination of two poneroid species did not reveal any GRN interruptions (Behague et al., 2018). Studying the mechanism(s) operating during the origin of wing polyphenism, and therefore, during or near the evolution of eusociality, provides us with insight on molecular /

developmental mechanisms contributing to the emergence of a major evolutionary transitions during evolutionary history.

3.3. Results

In *H. saltator*, a species with ancestral characteristics, we tested for the presence of PCD in the wing discs of males as a control, and in the rudimentary wing discs of worker-destined larvae using the TUNEL assay, a marker of apoptotic cell death that detects DNA fragmentation (Kyrylkova et al., 2012). We focused on the last larval instar (terminal stage) beginning from the early-mid to the larval-pupal transition (Fig. 2). In the wing discs of male-destined larvae, which will develop into adult males with fully functional wings we found that during the early and mid-period, the amount and intensity of the TUNEL signal in the wing discs in male-destined larvae is much lower as compared to that in the rudimentary wing discs of worker-destined larvae at similar stages (Fig. 2a'–d' white arrowheads, compare with Fig. 2f'–i'). Moreover, during the larval-pupal transition stage, where TUNEL expression is at its highest in the worker rudimentary wing discs (Fig. 2j'), we find no signs of DNA degradation in the male wing discs (Fig. 2e'). In the rudimentary wing discs of female larvae, we found small amounts of DNA degradation at the early and mid-periods in both the rudimentary wing pouch and hinge (Fig. 2f', g' white arrowheads; Fig. 1b). In contrast with male wing discs, the amount of DNA degradation in the rudimentary wing discs of workers increases substantially during the mid to late terminal larval development (Fig. 2h', i') and by the larval-pupal transition, TUNEL signal is present at high levels throughout the entire rudimentary wing disc (Fig. 2j'). Together, these results suggest that the strong levels of DNA degradation in the worker rudimentary wing discs relative to male wing discs is responsible for degrading the left-over rudiment after growth has ceased, leading to the emergence of wingless worker adults. In contrast, the presence of some DNA degradation in the male wing discs suggest that it may play a role in shaping the final adult wings in males.

We next tested for PCD (TUNEL) in the rudimentary wing discs of worker-destined larvae across 15 additional species from five major subfamilies of ants (Fig. 3; Supp. 1). We then conducted an ancestral state estimation analysis using data from all species tested to infer whether PCD was present or absent in the rudimentary wing discs of workers in the last

common ancestor of ants. Like in *H. saltator*, we found positive TUNEL signal in the rudimentary wing discs during late larval development in two other species belonging to the poneroid clade: *Odontomachus brunneus* (Ponerinae) and *Stigmatomma pallipes* (Amblyoponinae) (Fig. 3a–b'). We also find, positive TUNEL signal in species from the formicoid clade particularly during the larval-pupal transition (Fig. 3c-e', g-k'; Supp. 1). *Tetramorium immigrans* (Myrmicinae), which possesses very small rudimentary wing discs, is the only species where we do not observe TUNEL signal at any stage examined (Fig. 3f–f'). Our ancestral state estimation (under the equal rates model)) using data from all species tested strongly supports the presence of PCD (>95% confidence) as being ancestral to all living ants, including the poneroformicine clade (= poneroids + formicoids) (Supp. 2). Our inference remains robust after accounting for: (1) uncertainty in the presence or absence of PCD in species from missing ant subfamilies; (2) the absence of data for *Martialis* and Leptanillinae (the sister group to all other Formicidae); and (3) different taxon sampling regimes (Supp. 2). Altogether, our experimental data and ancestral state estimation analyses infer role for PCD in the regulation of wing polyphenism in the last common ancestor of all extant ants.

Next, we tested which specific forms of PCD are active in the rudimentary wing discs of workers in different ant species. Although the TUNEL assay is a general marker for apoptotic cell death, we confirmed the presence of apoptosis in the wing disc by testing for the presence of the active form of caspase 3, the human ortholog of the *Drosophila* ICE (Drice) protein, an effector caspase that carries out apoptosis (Xu et al., 2009). We find strong cleaved-caspase 3 expression in the rudimentary wing discs in workers from three different subfamilies. *H. saltator* (poneroid: Ponerinae) and *Camponotus floridanus* (formicoid: Formicinae) and *Pheidole dentata* (formicoid: Myrmicinae). In worker larvae of *H. saltator* and *C. floridanus*, cleaved-caspase 3 expression in the rudimentary wing disc starts around mid-terminal larval development and spreads throughout the remaining larval stage (Fig. 4a-a', c–c'). In *P. dentata*, we observe cleaved-caspase 3 expression only towards the late stages of terminal development (Fig. 4d–d'). To test for the presence of autophagy, which is a type of PCD characterized by the presence of vesicles (Yan et al., 2020), we used two different markers: *atg8* and LC3A/B. The rudimentary wing discs of *H. saltator* and *P. dentata* workers show no detectable signs of

autophagy (Fig.4 b–b', e–e'). These experiments therefore confirm that PCD in the rudimentary wing discs of workers across the phylogeny is carried out via the apoptosis pathway.

Finally, we tested for the presence of interruption points in five genes located upstream (*vestigial*, *vg*) and downstream (*optomoter blind*, *omb*; *spalt, sal; serum response factor, srf; achaete/scute, acsc*) in the wing GRN in the poneroid species *H. saltator* (Fig. 5a green and red). *vg*, which plays a critical role in wing growth (Halder et al., 1998), shows a similar expression pattern concentrated around the distal portion of the wing pouch in both winged males and wingless workers (Fig. 5b–b'). *omb*, which is responsible for the development of the central region of the wing (del Alamo Rodriguez et al., 2004), is expressed in the central domain of the wing in both males and workers (Fig. 5c–c'). *sal*, which controls notum and hinge development as well as vein positioning (de Celis & Barrio, 2000; Grieder et al., 2009), is expressed in male wing discs around the hinge area as well as a circular expression in the center of the wing pouch (Fig. 5d). Later in development, the circular expression in the male wing disc expands distally (Supp. 3b', c' arrowhead). In the worker rudimentary wing disc, however, *sal* is found in the hinge, but the circular expression in the wing pouch is not detectable at any stage (Fig. 5d'; Supp. 3a–d). Therefore, sal is interrupted in wingless workers. Finally, the expression of *srf*, which regulates the formation of intervein tissue, and *acsc*, which regulates bristle formation (Montagne et al., 1996; Skeath & Carroll, 1991), is different between winged males and wingless workers. In the male wing disc, *srf* is expressed in numerous patches indicating the location of the intervein regions, however, in the workers the expression is restricted to only one-two central patches (Fig. 5e–e' arrowheads). Similarly, in the male wing discs, *acsc* is expressed in multiple areas indicating the locations of bristle formation (Fig. 5f arrowheads), whereas in the workers, *acsc* expression is completely missing in the wing discs during a similar developmental stage (Fig. 5f'). Overall, we observed similar patterns and timing of expression in *vg* and *omb* in the wing discs of males and workers. However, the genes *sal*, *srf* and *acsc* show either reduced or completely missing expression in the worker rudimentary wing disc compared with that of the males, showing that these downstream genes are interrupted.

3.4. Discussion

PCD is a process that has been repeatedly co-opted to play a role in the regulation of polyphenisms and dimorphisms across the tree of Life (Hanna & Abouheif, 2023). Here we show that PCD contributes to the regulation of wing polyphenism in ants, a nearly universal trait in the group. We demonstrate that this process, carried out by the apoptosis pathway, is strongly expressed in late-stage rudimentary wing discs of workers from several subfamilies belonging to the two major clades of extant ants (the poneroid and formicoid clades) (Fig. 6 green circles). The presence of apoptosis in the rudimentary wing discs of 15 out of 16 species we sampled across the poneroid and formicoid clades strongly suggest that this process is conserved in ants and was involved in the regulation of wing polyphenism in the last common ancestor of these two clades, near the origin of ants. Indeed, formal ancestral state estimation using the experimental data generated in the present study strongly supports PCD as ancestral to the poneroformicines (= poneroid + formicoid clades) and the extant Formicidae as a whole (Fig. 6 green circles). We therefore predict that PCD is also operating in the 'basal-most' subfamilies (Leptanillinae and Martialinae; see, e.g., Romiguier et al. (2022), for which wing polyphenism in females is also known to occur (Borowiec et al., 2011; Chen et al., 2017; Hsu et al., 2017). In this context, rediscovery of Martialinae will be of particular value, given that only two workers and a few males have been collected to date (Boudinot, 2015; Rabeling et al., 2008). Furthermore, the occurrence of wing polyphenism in females has been recently confirmed to be present among the extinct stem lineages of ants (Boudinot et al., 2022(a)), which supports PCD as a derived condition for all Formicidae, extant and extinct (Fig. 6 dotted green circle). Future developmental studies focusing on species in the Leptanillinae will be able to resolve with strong experimental evidence whether PCD was present during the split of all extant ants. Whether this pattern is also true for other Hymenoptera with wing polyphenism in females (e.g., some Ichneumonidae, Cynipidae, Chalcidoidea, and Bethylidae) remains to be demonstrated.

PCD has contributed to the emergence of major evolutionary transitions in Earth's history by playing key roles in communication, conflict mediation, division of labor, and cooperation (Durand et al., 2019). Here we show that PCD was very likely operating in the origin

of wing polyphenism in ants, and therefore, in the major evolutionary transition to eusociality in ant. By generating a wingless worker caste, PCD may have been critical for limiting worker dispersal and mating thereby reinforcing the reproductive division of labor in ancestral ant societies. Multiple ecological, evolutionary, and developmental factors influenced the evolution of eusociality in ants and in other eusocial organisms. Here, we show that PCD may have been one of these factors during the origin of eusociality in ants.

In this study we show that PCD is present across the phylogeny, and moreover, we show that a poneroid species, *H. saltator*, also exhibits GRN interruptions, albeit downstream in the network (Fig. 6 purple boxes). This contrasts with GRN interruptions located upstream in previously examined formicoid species (Fig. 6 orange boxes) (Abouheif & Wray, 2002). The presence of both PCD and wing GRN interruptions, in addition to a correlation between the location of gene interruption and apoptosis signal in the ant *Pheidole morrisi* (Shbailat et al., 2010), suggests the presence of causal relationship between the two mechanisms. However, whether GRN interruption leads to PCD activation or vice-versa remains unknown in ants. Previous studies in the fruit fly *Drosophila melanogaster* have shown that mutations in various wing genes lead to tissue death (Giraldez & Cohen, 2003; James & Bryant, 1981; Sedlak et al., 1984). Therefore, it is likely that in ants, wing GRN interruptions may be also activating the apoptosis pathway. It is also possible, however, that the degeneration of the rudimentary wing disc tissue caused by apoptosis may be leading to interruptions in the wing GRN. For example, the reduction of *srf* expression pattern we observe in the rudimentary wing discs of *H. saltator* workers as compared with males might be due to the reduction of wing tissue thereby not allowing full gene expression, especially since this gene is activated in a late stage after the onset of cell death. However, we do not predict this to be the case in species where GRN interruptions occur in very upstream genes, and at a stage before apoptosis onset. Finally, the two mechanisms may operate independently of one another and do not interact, although this is less likely given the intimate relationship between the wing GRN and apoptosis network known from work in *Drosophila*. Future work in this area is required to determine the precise relationship that exists between the two processes underlying ant wing polyphenism, and how their interactions and co-regulation might have changed across the evolutionary history of ants.

Finally, many wasp taxa that are closely related to ants possess a sexual wing dimorphism where the females are wingless or short winged and the males are fully winged, leading to the possibility that wing polyphenism in ants may have arisen from a latent potential for sexual wing dimorphism (Hanna & Abouheif, 2021). Given the overwhelming role of PCD in sexual dimorphisms across the tree of life (Hanna & Abouheif, 2023), we predict that it may be operating in wasp wing development to generate differences between the sexes. If PCD is indeed regulating sexual wing dimorphism in wasps then it is an intriguing possibility that this role was deeply homologous and was retained in ants and subsequently elaborated to generate a wing polyphenism within the female castes, a defining characteristic of the entire group.

3.5. Materials and methods

Ant collection and colony care

Ant colonies were housed in plastic boxes lined with either fluon or talc powder. Artificial nests were constructed using glass test tubes half-filled with water and plugged with cotton. All colonies were maintained at 25°C, 70% humidity and a 12h day:night cycle. Ants were fed a combination of mealworms, crickets, fruit flies, fruits, and Bhatkar-Whitcomb diet (Bhatkar & Whitcomb, 1970). *Harpegnathos saltator* colonies were housed in plaster nests and only fed live crickets 2-3 times per week.

Ant colonies were obtained from the following locals. *Stigmatomma pallipes* were collected from McGill Gault Nature Reserve (Quebec, Canada). *Lasius niger*, *Tetramorium immigrans*, *Aphaenogaster rudis*, and *Aphaenogaster picea* were collected from Mont Royal Park and McGill Gault Nature Reserve (Quebec, Canada). *Odontomachus brunneus* and *Camponotus floridanus* were collected from Gainesville (Florida, USA). *Pheidole dentata* were collected from Gainesville (Florida, USA) and Austin (Texas, USA). *Pheidole hyatti* were collected from Tempe (Arizona, USA). *Pheidole pallidula* were collected from Lyon (France). *Pheidole noda*, *Tetraponera rufonigra*, and *Polyrhachis rastellata* were collected from Mae Tang (Chiang Mai, Thailand). *Atta colombica* and *Acromyrmex echinatior* were collected from Gamboa (Panama). *Harpegnathos saltator* colonies were propagated in the laboratory since the original collection in 1999 from different locations in India.

Immunohistochemistry, hybridization chain reaction, and TUNEL stainings

Fixation and Dissection

Terminal stage larvae were collected and fixed as previously described by Shbailat and Abouheif (2013). Fixed larvae were dissected using a Zeiss Discovery V12 stereomicroscope to expose the wing discs and remove any obstructing fat tissue.

Immunohistochemistry

The following primary antibodies were used for immunohistochemistry: anti-cleaved caspase-3 (1:100-1:200, Cell Signaling Technology, #9661) and anti-LC3A/B (1:100, Cell Signaling Technology, #4108). For all immunohistochemistry assays, fluorescent secondary anti-rabbit polyclonal Alexa Fluor-555 (AbCam) antibody was used at 1:500 dilution to detect the primary antibody, according to Khila and Abouheif (2008).

Hybridization Chain Reaction

To detect mRNA expression of *H. saltator atg8*, *vg*, *sal*, *omb*, *srf*, and *ac/sc* we utilized the hybridization chain reaction (HCR) methodology (Choi et al., 2018). Probe sets, amplifiers, and buffers were purchased from Molecular Instruments, Inc. Procedure followed based on the Protocols for HCRTM RNA-FISH (v3.0) (generic sample in solution) acquired from molecularinstruments.com. Prior to procedure, samples were fixed and dissected as described above, rehydrated in 25%, 50%, 75% and 100% PTw (1X PBS; 0.3% Tween 20) and permeabilized in PTw and 2% Triton X-100 for 1 hour. Larvae were post-fixed in 4% formaldehyde in SSCT (5X sodium chloride sodium citrate; 0.1% Tween 20) for 2 hours and then washed 3 times with SSCT before moving into glycerol.

TUNEL

To detect programmed cell death in the wing discs of all species, we used the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. We used the *In Situ* Cell Death Detection Kit, TMR red (Roche). Larval samples were fixed, dissected and stored in 100% methanol in -30°C prior to procedure. Dissected larvae were then rehydrated in 25%, 50%, 75%, and 100% PTw (1X PBS; 0.1% Tween 20). Samples were then washed 3 times (10 min each) in freshly made PBT (1X PBS; 0.1% Triton; 0.1% BSA) and permeabilized in PTw and 2% Triton X-

100 for 1 hour, followed by 5 min washes in PBT. Positive and negative controls were incubated in DNase buffer at 37°C for 10 minutes and subsequently washed with PBT for 5 min (Supp. 4). Enzyme mix and label solution were added to the samples and the positive controls (only label solution was added to negative controls) and incubated in the dark at 37°C for 1 hour followed by 3 washes in PTw 5 min each. Samples were stained with DAPI (1:1000) for 1 hour to overnight and followed by gradual moving into glycerol. Samples were stored in final concentration of 85% glycerol/DAPI before mounting and imaging.

Imaging

Fluorescent images of larval rudimentary wing discs of all species utilized in this study were taken using a Zeiss AxioImager Z1 microscope.

Ancestral state estimation

To reconstruct the ancestral conditions of PCD, we conducted a series of ancestral state estimation (ASEs) analyses given prior and new experimental data. To ensure that our estimates were based on well-supported phylogenetic results, we used the favored phylogram (P) and chronogram (C) of Romiguier et al. (2022), particularly as a recent simulation study has demonstrated the importance of branch lengths for ancestral state modeling (Wilson et al., 2022). We pruned the two trees (P, C) to three alternative taxon sets: (P1, C1) the first included only those terminals that match species with experimental data (17 taxa), (P2, C2) the second included experimental terminals plus Martialis and a leptanilline (22 taxa), and (P3, C3) the third included all ant terminals from the Romiguier et al. (2022) dataset in order to account for phylogenetic uncertainty (74 taxa). Because we had surplus experimental sampling of Aphaenogaster and Pheidole with identical results, we included only one species of the former and two species of the latter, as this matched the phylogenomic sampling of Romiguier et al. (2022). We also excluded non-ant outgroups as there is no applicable experimental data, thus insufficient information to parameterize a phylogenetic model that includes these taxa. As the 'all rates different' model overfit the data, resulting in complete statistical separation, we only report results under the 'equal rates' model.

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Figure 1.

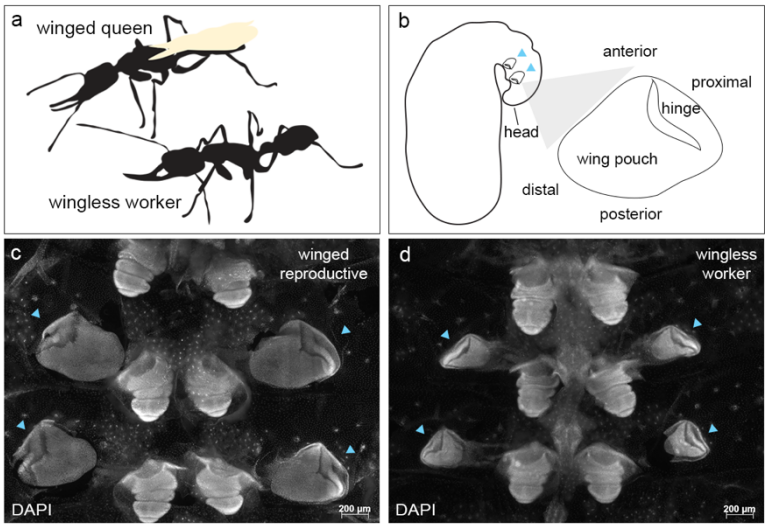


Figure 1. Wing polyphenism in the ponerine ant *H. saltator*: (a) adult queens are winged (wings indicated in yellow) while workers and reproductive workers 'gamergates' are wingless. (b) a cartoon illustration of *H. saltator* larva during terminal development indicating the location of the wing discs (blue arrowheads) and the general axes and regions of the wing disc in ants. Dissected terminal stage larvae in *H. saltator* stained with DAPI showing: (c) wing discs in winged males and (d) rudimentary wing discs in wingless female workers (blue arrowheads).

Figure 2.

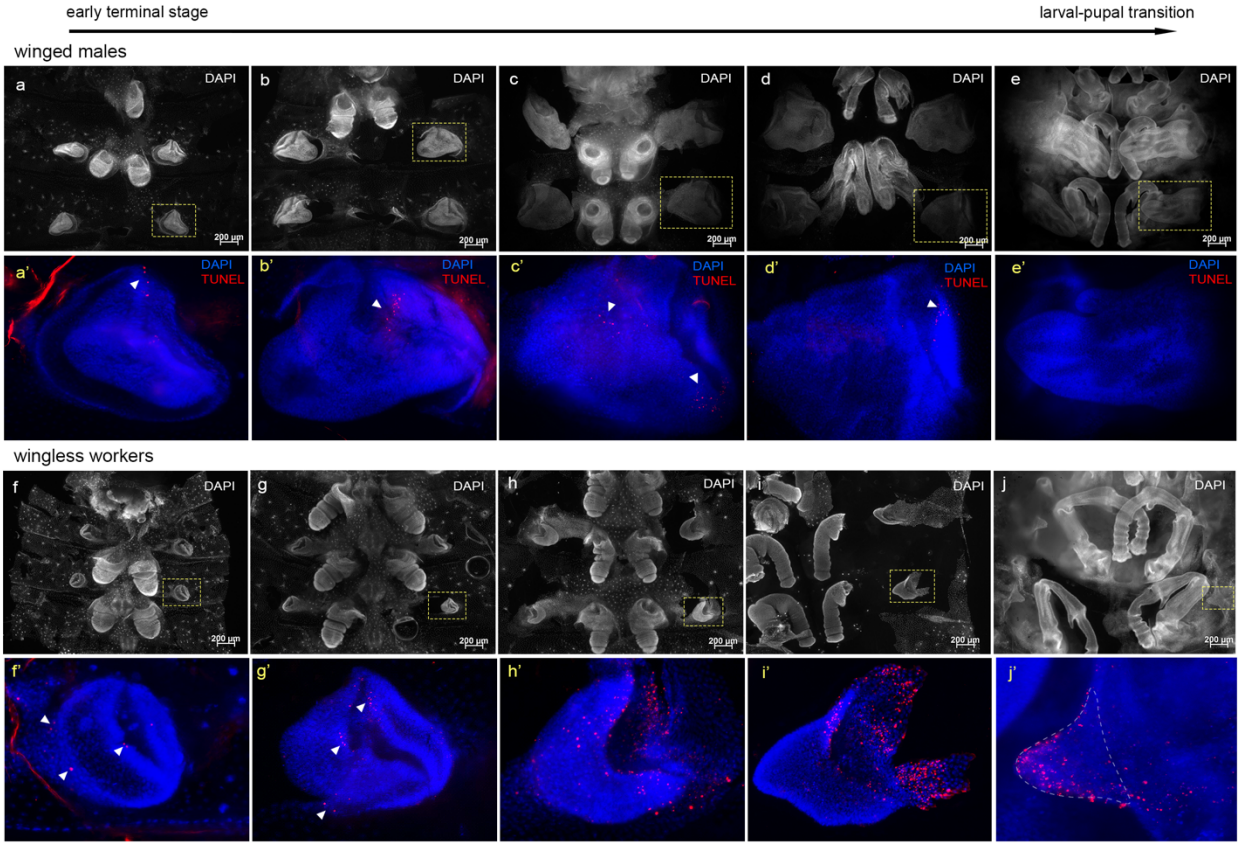


Figure 2. Programmed cell death in the wing discs of wingless workers and winged male larvae of *H. saltator*. (a–e) Larval developmental series of winged males from early/mid terminal stage to larval-pupal transition stained with DAPI. (a'–e') a magnified view of a wing disc from the larva in the panel directly above showing TUNEL signal (red) and DAPI (blue). White arrowheads in (a'-d') indicate area with TUNEL expression. (f–j) DAPI staining of a larval developmental series of wingless workers during terminal larval development corresponding in stage to those shown in the males. (f'–j') a magnified view of a wing disc from the larva in the panel directly above showing TUNEL signal (red) and DAPI (blue). White arrowheads in (f', g') indicate region where TUNEL is expressed. TUNEL expression in (h'-j') is widespread. Dashed line in (j') outlines the wing disc area. Yellow dashed boxes in (a–e) and (f–j) indicate the wing disc magnified in the panels below. Images in panels (a–e) and (f–j) are to scale. Images in panels (a'–e') and (f'–j') are not to scale. All wing discs are oriented with distal axis on left and proximal axis on right (see Figure 1b).

Figure 3.

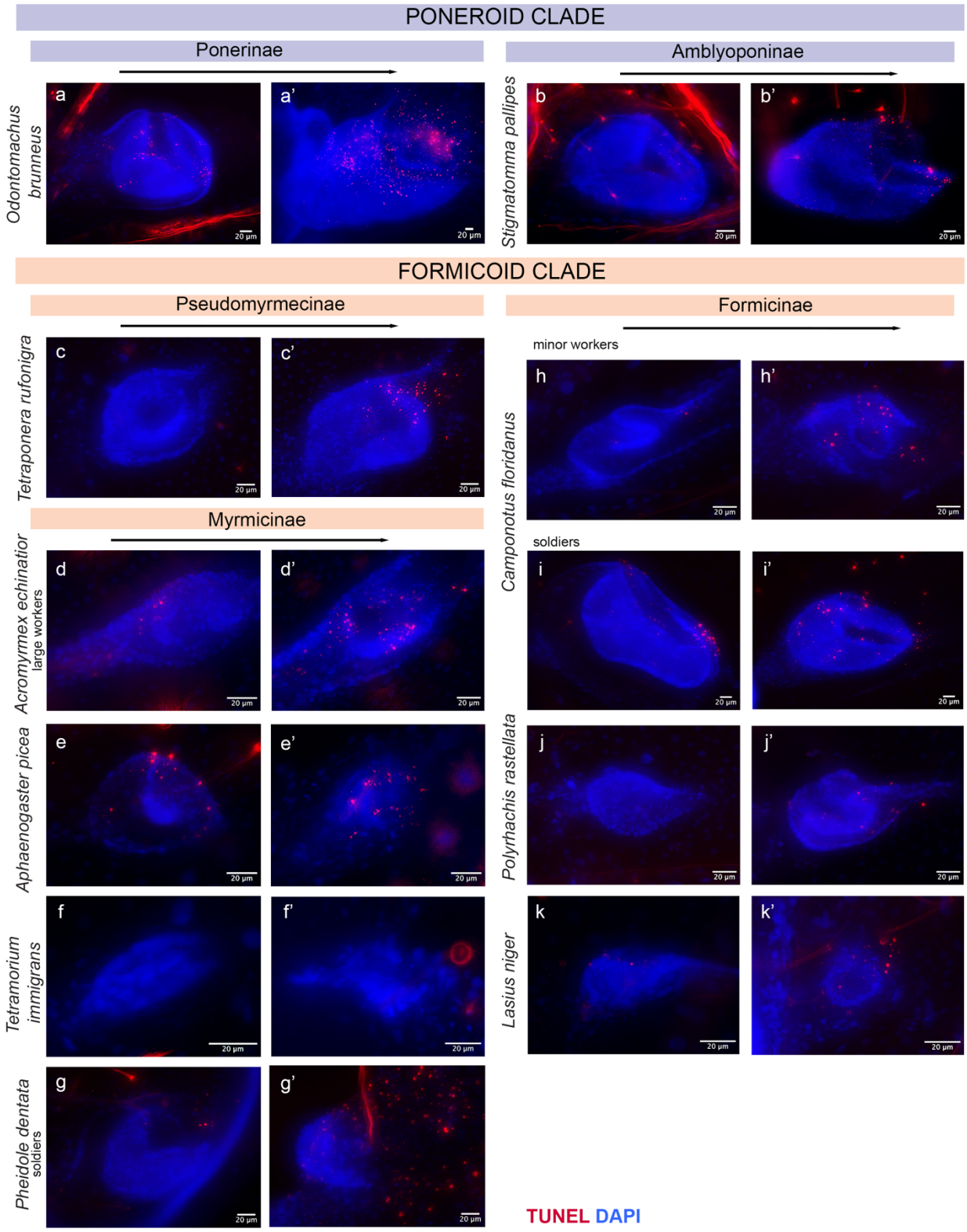

PONEROID CLADE
Ponerinae
Amblyoponinae
Odontomachus brunneus
Stigmatomma pallipes
a
a'
b
b'
FORMICOID CLADE
Pseudomyrmecinae
Formicinae
Tetraponera rufonigra
c
c'
minor workers
h
h'
Camponotus floridanus
soldiers
i
i'
Myrmicinae
Acromyrmex echinatior
large workers
d
d'
Aphaenogaster picea
e
e'
Polyrhachis rastellata
j
j'
Tetramorium immigrans
f
f'
Lasius niger
k
k'
Pheidole dentata
soldiers
g
g'
20 µm
TUNEL DAPI

Figure 3. Programmed cell death in two stages of rudimentary wing disc development in larvae of wingless workers across the ant phylogeny. DAPI (blue) and TUNEL (red) signal in rudimentary wing discs of worker larvae from two species in the poneroid clade (a-b') and eight species in the formicoid clade (c-k'). For each species, a rudimentary wing disc from the mid-terminal stage (left) and a rudimentary wing disc from the larval-prepupal transition stage (right) are shown. Black arrows indicate developmental time. Subfamily labels are marked on the top of the panels and species names are marked on the left of the panels. All wing discs are oriented with distal axis on the left and proximal axis on the right (see Figure 1b).

Figure 4.

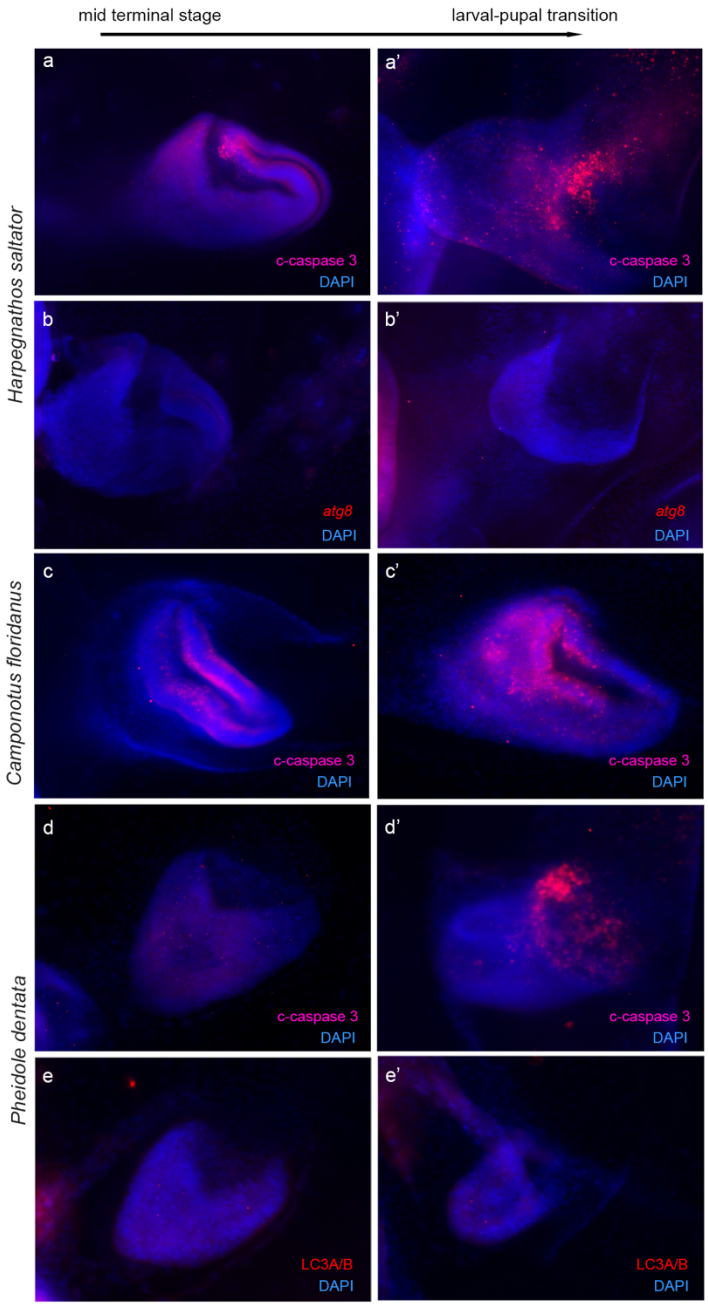


Figure 4. Programmed cell death is carried out via the apoptosis pathway. Expression of DAPI (blue) and cleaved-caspase 3 (red) during mid (left) and late terminal larval (right) stages in *H. saltator* (a–a'), and soldier larvae of *C. floridanus* (c–c') and *P. dentata* (d–d'). Expression of DAPI (blue) and autophagy marker *atg8* or LC3A/B (red) in rudimentary wing discs of *H. saltator* (b–b') and soldier larvae of *P. dentata* (e–e').

Figure 5.

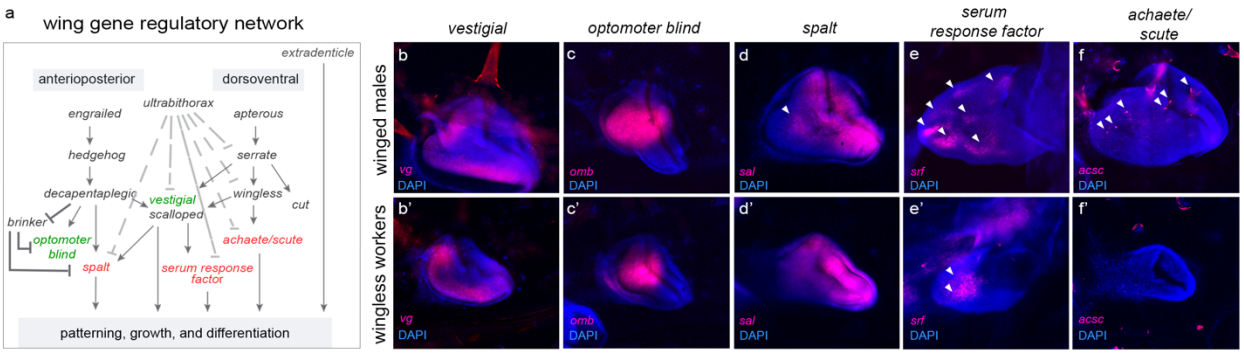


Figure 5. Wing GRN interruptions in *H. saltator*. (a) diagram of the conserved wing GRN in insects indicating in red or green color genes examined in this study. DAPI (blue) and *vestigial* expression (pink) in winged males (b) and wingless workers (b'). DAPI (blue) and *optomoter blind* expression (pink) in males (c) and workers (c'). DAPI (blue) and *spalt* expression (pink) in winged males (d) and wingless workers (d'). White arrowheads in (d) indicates circular expression in the male wing discs that is missing in the worker. DAPI (blue) and *serum response factor* expression (pink) in male wing discs (e) and worker wing discs (e'). Compare white arrowheads in wing discs between winged males (e) and wingless workers (e'). DAPI (blue) and *achaete/scute* expression (pink) in in male wing disc (f) and worker wing discs (f'). White arrowheads in (f) indicates regions of expression in the male wing discs that are missing in the worker. Green color in (a) indicates expression of genes in the wing GRN that are conserved, while red color in (a) indicates expression of genes in the wing GRN that are interrupted. Images of male and worker wing discs are to scale for each gene and stained at a similar developmental stage.

Figure 6.

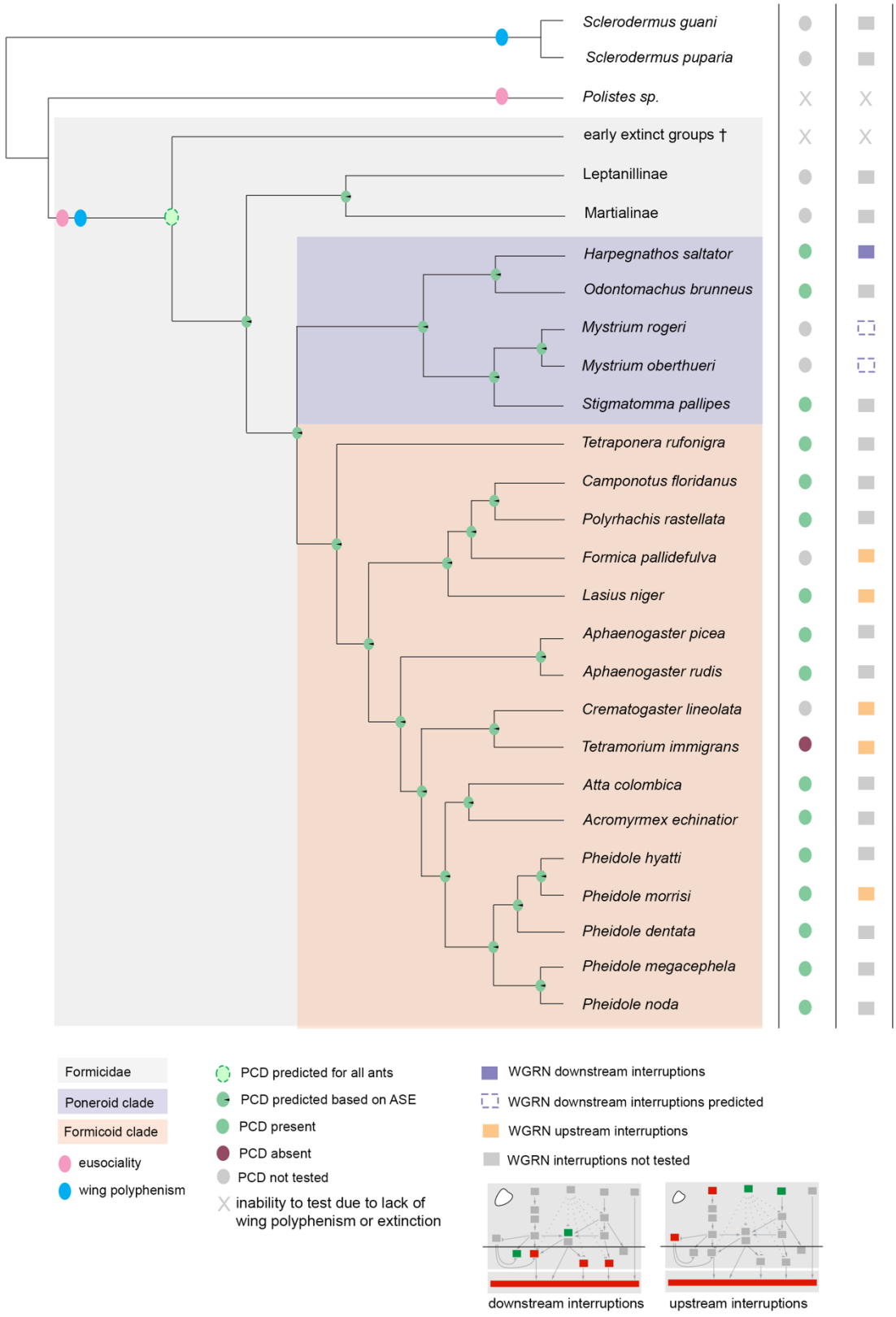
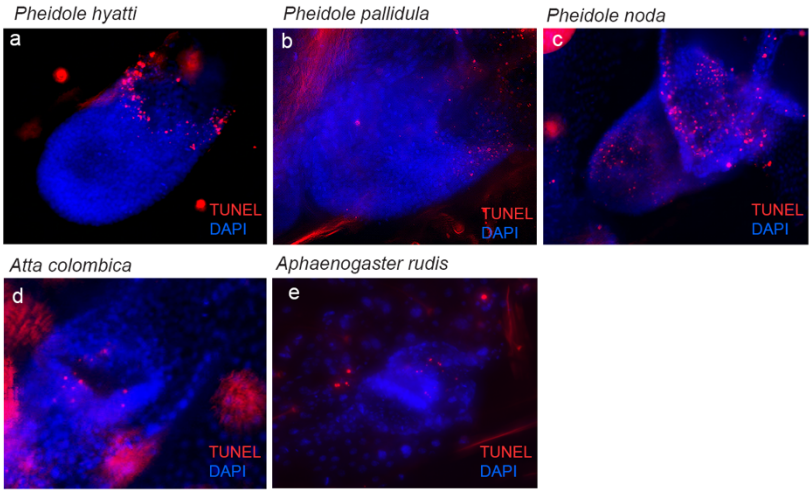

Sclerodermus guani
Sclerodermus puparia
Polistes sp.
early extinct groups †
Leptanillinae
Martialinae
Harpegnathos saltator
Odontomachus brunneus
Mystrium rogeri
Mystrium oberthueri
Stigmatomma pallipes
Tetraponera rufonigra
Camponotus floridanus
Polyrhachis rastellata
Formica pallidefulva
Lasius niger
Aphaenogaster picea
Aphaenogaster rudis
Crematogaster lineolata
Tetramorium immigrans
Atta colombica
Acromyrmex echinatior
Pheidole hyatti
Pheidole morrisi
Pheidole dentata
Pheidole megacephela
Pheidole noda
Formicidae
Poneroid clade
Formicoid clade
eusociality
wing polyphenism
PCD predicted for all ants
PCD predicted based on ASE
PCD present
PCD absent
PCD not tested
inability to test due to lack of wing polyphenism or extinction
WGRN downstream interruptions
WGRN downstream interruptions predicted
WGRN upstream interruptions
WGRN interruptions not tested
downstream interruptions
upstream interruptions

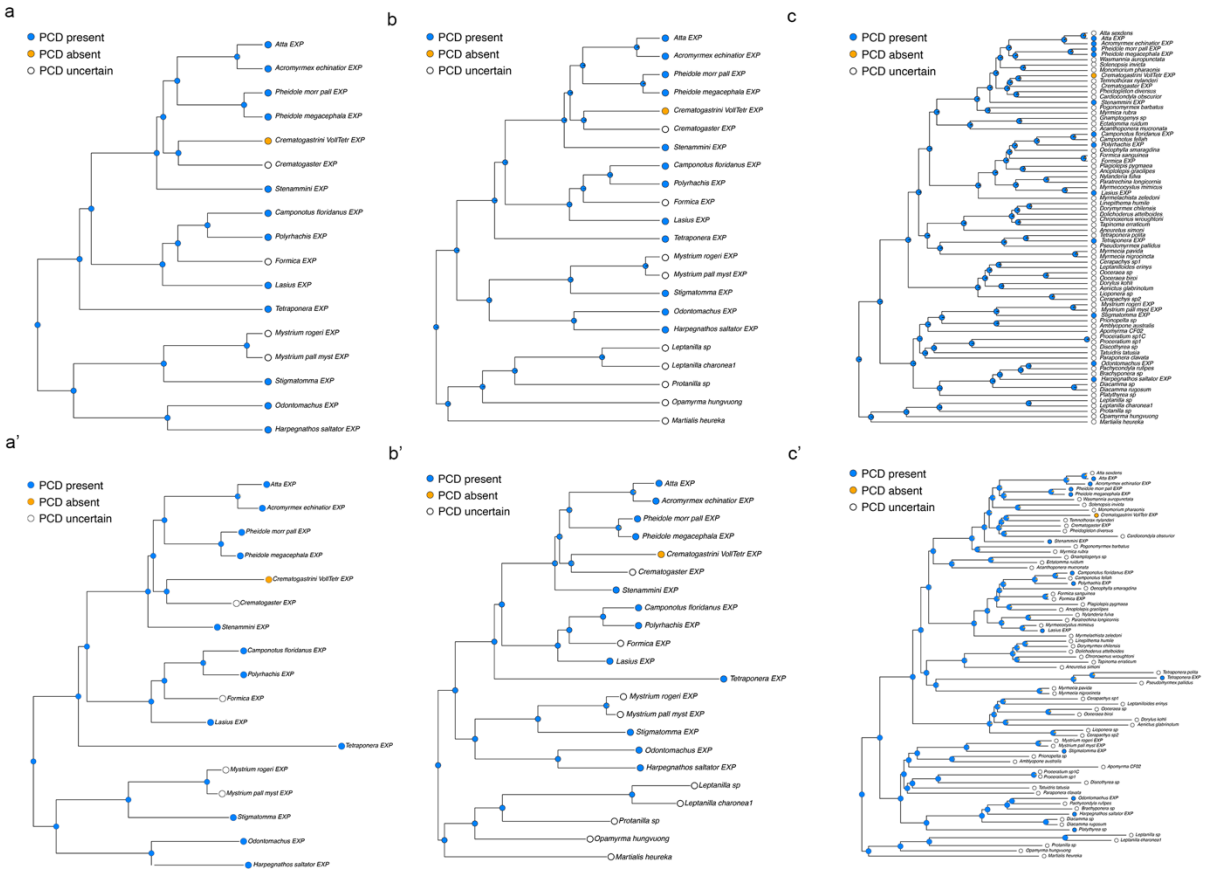
Figure 6. Programmed cell death and wing GRN interruptions in wing polyphenism across the ant phylogeny. Wing polyphenism (blue circle) and eusociality (pink circle) evolved concomitantly in ants (Formicidae, gray large box). Wing polyphenism and eusociality are found in some wasp species/groups. Outgroups for Formicidae are for demonstration purposes only and many branches in the Hymenoptera are excluded. Summary of PCD presence/absence as well as GRN interruptions results found by this study for poneroid species (purple box) and formicoid species (orange box) are listed on the right. GRN results summarized based on Abouheif and Wray (2002); Behague et al. (2018); and Shbailat et al. (2010). Characters indicate species with: presence of PCD (green circle), absence of PCD (red circle), PCD untested (gray circles), wing GRN with downstream interruptions (purple squares), wing GRN with downstream interruptions predicted (dashed purple squares), wing GRN with upstream interruptions (orange squares), wing GRN interruptions untested (gray squares). Gray X indicates species where testing is not possible. Examples of upstream and downstream interruptions are indicated. Genes above horizonal black line are considered upstream, genes below the black line are considered downstream. PCD regulation of wing polyphenism is predicted for all extant ants based on Ancestral State Estimations (ASE; green circles in nodes). ASE values for all tested models yielded over 95% confidence. PCD regulation of wing polyphenism at the origin of ants is proposed (dashed green circle). Branch length do not indicate evolutionary time. Phylogenetic relationships within the Formicidae are based on Boudinot et al. (2022)(b); Moreau (2008); and Ward et al. (2016).

Supplemental 1.



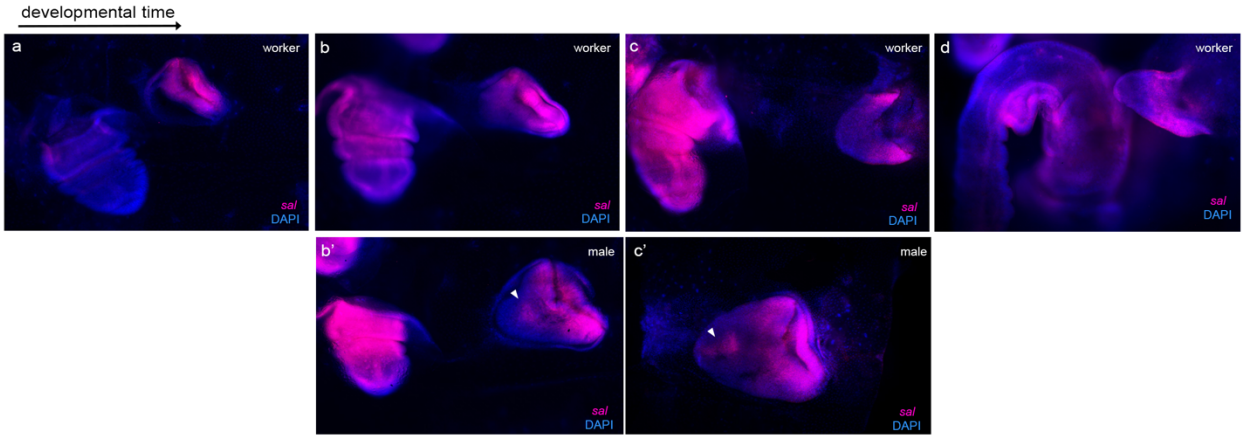
Supplemental 1. Programmed cell death in additional ant species from the subfamily Myrmicinae. For all panels, a rudimentary wing discs at the late terminal larval stage is shown stained in DAPI (blue) and TUNEL (red).

Supplemental 2.



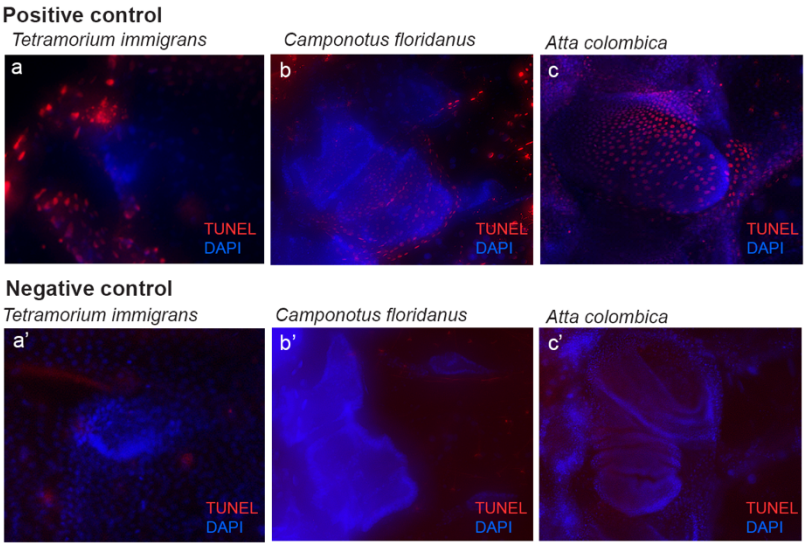
Supplemental 2. Ancestral State Estimations for presence and absence of PCD. Estimates based only on species where assays for PCD have been performed are shown using a phylogram (a) and chronogram (a') modeling. Estimates based on species where assays for PCD have been performed as well as the addition of basal outgroups *Martialis* and leptanilline are shown using a phylogram (b) and chronogram (b') modeling. Estimates based on 74 ant taxa including those without experimental data are shown using a phylogram (c) and chronogram (c') modeling. Filled blue circles indicate estimates for presence of PCD; open circles indicate estimates for absence of PCD, open circle indicate species where PCD has not been examined. All models yielded over 95% confidence.

Supplemental 3.



Supplemental 3. Detailed expression of *spalt* signal in worker and male wing discs in *H. saltator*. a–d) expression of DAPI (blue) and *spalt* (pink) in the rudimentary wing discs of worker larvae from early to very late terminal larval stage. b'–c') expression of DAPI (blue) and *spalt* (pink) in the male wing disc at stages similar to b and c, respectively. White arrowheads in b' and c' indicate area of circular expression and its expansion. Larvae in (b and b') and (c and c') are at a similar development stage. All images to scale.

Supplemental 4.



Supplemental 4. Positive and negative TUNEL controls shown for three ant species. DAPI (blue), TUNEL (red).

CONNECTING STATEMENT BETWEEN CHAPTERS 3 & 4

In Chapter 3, I show the presence of apoptosis in the worker rudimentary wing discs in various species across the ant phylogeny and predict its presence in the last common ancestor of extant ants. In Chapter 4, I specifically test the role that the apoptosis network is playing in worker polymorphism. Worker polymorphism is when the worker caste has further split into morphologically and behaviorally distinct groups, known as 'subcastes'. In the genus *Pheidole*, the worker caste is composed of minor workers and big-headed soldiers. A previous study showed that the rudimentary wing discs in soldiers regulate the head and body growth of this subcaste, thus giving it its subcaste identity (Rajakumar et al. 2018). In Chapter 3, I show that soldiers in the species *Pheidole dentata* activate apoptosis in their rudimentary wing discs during terminal larval development. Therefore, in Chapter 4, I test the prediction that activation of the apoptosis network and the degeneration of the rudimentary wing discs functions to limit the growth of the body since the wing discs are stopped from sending more signals to regulate head and body growth. I test this prediction by inhibiting apoptosis activation and measuring body and head size of the treated pupae. Surprisingly, I find that inhibiting apoptosis in the rudimentary wing discs leads to smaller pupae, suggesting that the apoptotic signaling and the death of the wing discs is necessary to release downstream signals responsible for body growth. Furthermore, I propose that one of these downstream players might be a stress-response mechanism that is typically activated under stress conditions by the apoptosis pathway. However, in *P. dentata* soldiers, this stress-response mechanism has been co-opted to be constitutively on and now part of normal development.

Chapter 4: Apoptosis signaling in a rudimentary organ is required for the development of a novel soldier subcaste in ants

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4.1. Abstract

Ant colonies are composed of reproductive castes (queen(s) and males) and a non-reproductive worker caste (females). Numerous species independently evolved a worker polymorphism in their worker caste where, in response to environmental cues, the workers develop into morphologically and behaviorally distinct subcastes. The genus *Pheidole* evolved one of the most striking worker polymorphism by generating a solider subcaste with distinctly large heads compared to their bodies. Previous work has shown that the rudimentary wing organs, that transiently develop and apoptose during larval development of workers, regulate the head and body growth of *Pheidole* soldiers. However, the signaling pathways released by the wing rudiments that control this function remain unknown. Here we investigate the role of the apoptosis signaling pathway in the rudimentary wing organs and its role on soldier development. Inhibition of apoptosis lead to larger rudimentary wing discs, however, contrary to expectation, inhibition of apoptosis signaling resulted in soldiers with smaller head and body scaling. Altoghether, we show that the expression of apoptosis and death of the wing organs is necessary for their function in regulating soldier head and body allometry. Furthermore, we show that the apoptosis pathway in the rudimentary wing organs of soldier larvae only constitutively activates JNK signaling - a stress-response mechanism. We propose a role for stress-induced response in evolutionary innovation and worker subcaste evolution in ants.

4.2. Introduction

Ants are an evolutionarily successful and ecologically dominant group of insects, with biomass exceeding that of wild birds and mammals combined (Schultheiss et al., 2022). This success is in large part due to their evolution of a remarkable morphological, behavioral, and reproductive division of labor between the winged queen caste and wingless worker caste (Holldobler & Wilson, 1990). Furthermore, an ant colony, as a whole, is considered to be a 'superorganism' where the reproductive queen serves as the germline and non-reproductive workers serving as the soma (Wheeler, 1911). During ant evolution, the worker caste of several ant species has independently evolved into a complex worker caste system composed of individuals showing differences in size and head-to-body allometry in a single colony, such as small-headed minor workers and big-headed soldiers in colonies of the genus *Pheidole* (Fig. 1a) (Lillico-Ouachour & Abouheif, 2017; Wilson, 1953). Complex worker castes have evolved at least 22 times independently and are thought to enhance the division of labour within the colony (Wills et al., 2018). Yet, the molecular and developmental mechanisms facilitating the evolution of complex worker caste systems in ants remain poorly understood.

The differences between individual workers in size and head-to-body allometry observed in complex worker caste systems are largely determined by environmental cues during development (Lillico-Ouachour & Abouheif, 2017; Wheeler & Nijhout, 1981, 1984). For example, in the hyperdiverse ant genus *Pheidole*, the switch between queens and workers is determined early during embryogenesis in response to environmental cues, such as temperature and photoperiod. The hormone called juvenile hormone (JH) mediates these environmental cues, and if the level of JH surpasses a threshold, embryos develop into queens, if not, they develop into workers (Supp. 1) (Wheeler, 1986). Later at the beginning of the last larval instar, the switch between small-headed minor workers and big-headed soldiers is determined in response to nutrition (Passera, 1974). This switch is also regulated by JH, and if the level of JH surpasses a threshold, larvae develop into the large big-headed soldiers, and if not, they develop into small small-headed minor workers (Supp. 1) (Wheeler & Nijhout, 1981).

Wing polyphenism is an alternative phenotype in ants where, in response to environmental cues, fully functional wings develop in queens whereas wing development in

workers is halted and are completely wingless as adults (Hanna & Abouheif, 2021). Rajakumar et al. (2018) showed that the rudimentary wing discs, which transiently develop in worker-destined larvae and disappear before the adult stage (Dewitz, 1878), have been repurposed during ant evolution to play a key role in the generation of the soldier subcaste in *Pheidole*. Genetically and physically disrupting the normal development of the rudimentary wing discs in *Pheidole hyatti* soldier-destined larvae led to a decrease in size and head-to-body allometry revealing a role for the rudimentary wing discs in regulating head and body growth, and specifically, the disproportionate head growth compared to the body (Fig. 1a) (Rajakumar et al., 2018). These findings indicate a deep connection between wing polyphenism and worker polymorphism, in the sense that the rudimentary wing discs, which are no longer producing functional wings in the worker caste, are being instead utilized by development to generate further morphological complexity in the form of distinct worker subcastes (Hanna & Abouheif, 2021).

Despite this critical link, the mechanism through which the rudimentary wing disc regulates size and head-to-body allometry of soldiers during development remains unknown. In a previous work, we have shown that the apoptosis pathway is activated in the rudimentary wing discs across ants and was likely activated in the ancestral lineages leading to the origin of wing polyphenism in ants (Chapter 3). Here, we ask how the activation of apoptosis in the rudimentary wing discs influences soldier subcaste development in the ant *Pheidole dentata*. Since apoptosis is a cell destruction mechanism that functions in degenerating the rudimentary wing discs (Chapter 3), we predicted that its activation will eliminate the rudimentary wing disc thereby reducing the signaling cascade released by the rudimentary wing discs that controls soldier growth. Specifically, we predicted that by inhibiting apoptosis activation and elimination of the rudimentary wing discs, the signals will continue to be excreted leading to larger head and body growth and a super-soldier-like adult (Fig. 1b), similar to those found in nature in some species, such as *Pheidole obtusospinosa* (Rajakumar et al., 2012).

We tested this prediction in the ant species *P. dentata* by first determining the pattern and timing of expression of key genes in the apoptosis pathway such as cleaved caspase 9, the mammalian homolog of *Drosophila* Dronc that initiates apoptosis, and cleaved caspases 3, and

7, the mammalian homologs of *Drosophila* DrICE, and Dcp-1 proteins, respectively, which execute apoptosis (Fig. 1c) (Fuchs & Steller, 2011). In *Drosophila*, caspases have functions in developmental cell death and stress-induced cell death, as well as several nonapoptotic functions relating to processes such as cell differentiation, innate immune response, and neural activation (Accorsi et al., 2015; Kilpatrick et al., 2005; Kuranaga, 2012; Lindblad et al., 2021; Waldhuber et al., 2005). We subsequently inhibited activation of the caspases using a pan-caspase inhibitor and examined the effect on the size of the rudimentary wing discs in larvae, and the head-to-body allometry of adults.

4.3. Results and Discussion

Previously, we have shown that cleaved caspase-3 is expressed in the wing hinge of *P. dentata* soldiers around late larval development (Chapter 3). We therefore wanted to confirm if this expression is consistent for the other caspases and, moreover, if the caspases are expressed in other regions outside of the rudimentary wing discs during larval development. We focused on three stages of terminal larval development: early-mid, late, and larval-pupal transition (Fig. 2a-c; Supp. 2a-c, d-f). We found that all three cleaved-caspases are expressed in the hinge area of the rudimentary wing disc around late larval development and their expression continues through larval-pupal transition and expands towards the wing pouch (Fig. 2b''-c''; Supp. 2b''-c'', e''-f''). We also find all three cleaved-caspases expressed throughout the head region of soldier larvae at all examined stages, which includes the head disc (eye, antennae, and head capsule) and the brain (Fig. 2a'-c'; Supp. 2a'-c', d'-f'). Since the rudimentary wing discs do not become wings in the adult, these results suggest that the expression of the caspases in the rudimentary wing discs is leading to apoptosis activation. Expression of caspases in the head may be related to their apoptotic function or to non-apoptotic functions, possibly functions such as neural differentiation (Accorsi et al., 2005).

To determine the function of these caspases in the development of the novel soldier caste, we inhibited the activation of apoptosis using a pan-caspase inhibitor. We applied this pan-caspase inhibitor to larvae shortly after acquiring a soldier fate and before caspases are expressed in the rudimentary wing discs (Supp. 1). As predicted, inhibiting caspase expression in soldier-destined larvae results in a larger rudimentary wing disc (Fig. 3a-b', c). Moreover, the

ratio of wing disc to leg disc area of caspase inhibited larvae is significantly larger compared with DMSO treated larvae, whereas the leg disc area between caspase inhibition and DMSO controls is not significantly affected (Fig. 3d-e). Furthermore, TUNEL signal, a marker for apoptosis, is also reduced in these enlarged rudimentary wing discs compared to DMSO treatment (Fig. 3a-b'). Surprisingly, however, instead of producing supersoldier-like individuals as predicted, larvae with enlarged rudimentary wing discs, resulting from caspase inhibition, developed into pupae with significantly smaller head width, body length, and head/body ratio compared with DSMO treated larvae (Fig. 3f-g, i-k). Moreover, the head-to-body slope is significantly different between caspase inhibition and the control (Fig. 3h). These results show that inhibiting or reducing the level of apoptotic cell death leads to larger rudimentary wing discs, but the head and body growth of the soldier subcaste is significantly reduced. Therefore, this suggests that activation of apoptotic signaling in the rudimentary wing discs is critical for their function in regulating soldier subcaste growth and allometry.

To rule out that the reduction of soldier head-to-body allometry after caspase inhibition is caused by caspase expression outside of the soldier rudimentary wing disc, we performed caspase inhibition experiment on minor worker larvae that do not develop any rudimentary wing discs, but express caspases in the head similar to soldier-destined larvae (Fig. 4a-c'). We found that caspase inhibition results in reduced amounts of TUNEL signal compared to controls (Supp. 3a-b'), but does not significantly affect head size, head/body ratio, and head-to-body slopes relative to controls (Fig. 4d-g, i). Caspase inhibition does make minor worker larvae significantly larger than controls, however (Fig. 4h), the opposite to what we observed in soldier pupae. These results confirm that the decrease in head and body size we observe in soldiers is specific to inhibition of caspase expression in the rudimentary wing disc.

We next asked if apoptotic signaling affecting size and head-to-body allometry is specific to a solider rudimentary wing disc or if it a general feature of all wing imaginal discs? To test for this possibility, we inhibited caspase activity in larvae destined to develop into male (reproductive) caste with two pairs of functional wing imaginal discs that develop into adult wings (Supp. 4a-c). We found caspase signal in the head region of males, similar to that in minor worker and soldier larvae (Supp. 4a'-c'). However, caspase signal in the wing discs is a different

pattern than that observed in soldiers (Supp. 4a''-c''). Like in minor workers, that have no visible rudimentary wing discs, caspase inhibition in male-destined larvae did not significantly affect head, body, head/body ratio, and head-to-body slopes relative to control treatments (Supp. 4d-i). Altogether, these results suggest that apoptosis signaling in the rudimentary wing discs of soldier larvae evolved a specific role in regulating changes in size and head-to-body allometry.

Since the apoptotic signal in the rudimentary wing discs of *P. dentata* soldiers is required for soldier growth, we next tested if the apoptosis pathway in the soldier wing discs is activating downstream signals known to function in organ proliferation and inter-tissue communication in *Drosophila*. Under stress-induced cell death (such as x-ray irradiation), cells undergoing apoptosis in *Drosophila melanogaster* wing imaginal discs activate a regenerative pathway that compensates for the dying cells by inducing proliferation and altering developmental timing, thus achieving tissue regeneration and homeostasis (Fan & Bergmann, 2008; Martin et al., 2009; Ryoo & Bergmann, 2012). This apoptosis induced proliferation is achieved by activating the JNK (Jun N-terminal kinase) pathway and its downstream cascade of genes (Pinal et al., 2019; Pinal et al., 2018). We therefore tested if the JNK pathway is active in the soldier rudimentary wing disc. We discovered that JNK is constitutively expressed in the rudimentary wing discs of wildtype soldier larvae with similar timing and pattern of the expression as the caspases (Fig.5b-c, b''-c'') (compare Fig. 5b''-c'' with Fig. 2b''-c''). We also found JNK expression in the head and central nervous system in all stages examined (Fig. 5a'-c'). This function maybe be related to roles of JNK in axon and dendrite pruning during larval development and metamorphosis (Zhu et al., 2019). In contrast, phosphorylated JNK, which is known to activate apoptotic cell death (Dhanasekaran & Reddy, 2017), has a ubiquitous expression in soldier-destined larvae and does not correspond to specific areas of caspase activation (Supp. 5). We next asked if the non-phosphorylated JNK signal in the rudimentary wing discs is activated by the apoptosis pathway, similar to *Drosophila* under stress conditions. We find that JNK expression is indeed reduced after caspase inhibition treatment compared to controls (Fig. 5d, e). These results show that, unlike other organisms where JNK is expressed in

response to stress conditions, JNK in soldier-destined ants is constitutively expressed in the rudimentary wing discs and in response to apoptosis signaling.

Finally, we asked if the constitutive JNK expression is specific to soldier rudimentary wing disc or is a feature of ant wing development? Therefore, we tested for JNK expression in *P. dentata* minor workers and winged males, and moreover, in the worker caste of another ant species, *Harpegnathos saltator*, that does not have worker polymorphism. JNK expression is present in the head and CNS of *P. dentata* minor workers and males, and *H. saltator* workers (Supp. 6a-b', c-d', e-f'). However, the wing discs of males and the rudimentary wing discs of *H. saltator* workers do not express JNK at any stage examined (Supp. 6c''-d'', e''-f''). These results show that constitutive JNK expression is specific to the rudimentary wing disc of the soldier subcaste in Pheidole.

4.4. Conclusions

In this study, we show that apoptosis is activated in the rudimentary wing discs of *P. dentata* soldiers. Inhibition or reduction of this apoptosis signal leads to enlarged rudimentary wing discs, but contrary to our initial predictions, reduction of apoptosis leads to smaller soldiers with a reduced head-to-body allometry. These results, therefore, suggest that activation of apoptosis in the larval rudimentary wing discs is necessary for the regulation of head and body size of the big-headed soldiers (Fig. 6a). This indicate that apoptosis signaling is part of, and may possibly be the main, signaling cascade released by the rudimentary wing discs to regulate the development of soldiers in *Pheidole* (Fig. 6a). Furthermore, we show that JNK signaling, which is typically only activated under stress conditions, is constitutively active only in the rudimentary wing disc of soldier larvae and under the control of the apoptosis pathway, suggesting that JNK might be part of the downstream network activated by rudimentary wing discs to regulate soldier growth and development.

In a previous work, we have shown that apoptosis signal is activated at the end of larval development in the rudimentary wing discs of workers from various species across the ant phylogeny (Chapter 3). This widespread and strong activation of apoptosis is likely the mechanism degenerating the worker rudimentary wing discs before the adult stage, and moreover, was possibly the mechanism operating in wing polyphenism at the origin of this trait

during the early stages of ant evolution (Chapter 3). Therefore, it is not surprising that when the rudimentary wing discs were utilized by development to facilitate the generation of a novel worker subcaste in *Pheidole*, this was done by co-opting signaling networks already present in the rudimentary wing discs. However, what is unexpected is that a degeneration signaling network such as apoptotic cell death is also playing a positive role that leads to the generation of morphological complexity.

The evolution of worker polymorphism occurred in only about 13% of all ant species (Wills et al., 2018). If apoptosis signal in the worker wing discs is widespread across species, why did only a few species co-opt this mechanism and evolve a polymorphic worker caste? One explanation might be that apoptosis regulation of worker size growth is achieved via the downstream activation of the JNK pathway. This aligns with our data thus far showing absence of JNK signal in the wing discs of male larvae and worker larvae from a monomorphic species. The specific activation of JNK by the apoptosis pathway in the soldier wing discs might be necessary to send signals to the body and head. JNK function in stress response is to activate a cascade of downstream genes, such as wingless and JAK/STAT, that function in tissue proliferation, and *Drosophila* insulin-like peptide 8 (Dilp8) that functions in inter-organ communication by blocking the hormone ecdysone and extending developmental time (Katsuyama et al., 2015; Ryoo & Bergmann, 2012) (Fig. 6b). Therefore, JNK and its downstream cascade of genes is a likely mechanism to be sending signals from the rudimentary wing discs to regulate overall head and body growth.

The majority of ant species that evolved a worker polymorphism are found in hot and arid regions (La Richeliere et al., 2022). Therefore, it is not unlikely that harsh environments and external stressors (e.g., heat) played a role in influencing the development of polymorphic worker subcastes. Indeed, co-option of stress response mechanisms have been implicated in the evolution of new cell types, whole tissues, and organs (Love & Wagner, 2021). Wagner et al. (2019) proposed a 'stress-induced evolutionary innovation' model outlining how stress-response mechanisms, originally under environmental control, become part of the normal physiology of the organism and lead to the emergence of a novel trait. Our data suggest the following model for the stress-induced evolution of *Pheidole* soldier subcaste: (1)

environmental stressors caused the already present developmental cell death in the rudimentary wing discs to activate JNK signaling (a stress-response mechanism) that led to the alteration of size proportions and development of specialized workers with access to new niches. (2) an anticipatory response evolves where JNK stress-response mechanism is activated by apoptosis prior to and in anticipation of the stressor. (3) JNK activation and soldier development become completely under the control of physiological signal, possibly the hormone JH, and are expressed constitutively in the species. Future work testing the role of JNK (and its downstream cascade of genes outlined in Fig. 6b) in soldier development have the potential to test this model and demonstrate how a stress-induced evolutionary innovation may have occurred in ants that lead to the evolution of a new worker subcaste.

4.5. Materials and methods

Ant collection and colony care

P. dentata colonies were collected at Gainesville (Florida, USA) and Austin (Texas, USA) from the years 2017 to 2023. All colonies were housed in plastic boxes lined with either fluon or talc powder. Artificial nests were constructed using glass test tubes filled with water and plugged with cotton. All colonies were maintained at 25°C, 70% humidity, and a 12h day:night cycle. Ants were fed a combination of mealworms, crickets, fruit flies, fruits, and Bhatkar-Whitcomb diet (Bhatkar & Whitcomb 1970). *H. saltator* colonies were propagated in the lab after their collection in several parts of India. Ants were housed in plaster nests and fed live crickets only 2-3 days a week.

Immunohistochemistry

Terminal stage larvae were collected and fixed as previously described (Abouheif & Wray, 2002). Fixed larvae were dissected using a Zeiss Discovery V12 stereomicroscope to expose the wing discs and remove any obstructing fat tissue. The following primary antibodies were used for immunohistochemistry: anti-cleaved caspase-3 (1:100-1:200, Cell Signaling Technology, #9661), anti-cleaved caspase-9 (1:100, Abclonal, #A22672), anti-cleaved caspase-7 (1:100, Abclonal, #A23154), anti-JNK 1/2/3 (1:100, Abclonal, #A4867), anti-phospho-JNK 1/2/3 T183/T183/T221 (1:100, Abclonal, #AP0631). For all immunohistochemistry assays, fluorescent

secondary anti-rabbit polyclonal Alexa Fluor-555 (AbCam) or Alexa Plus Fluor-647 (AbCam) antibody was used at 1:500 dilution to detect the primary antibody, according to a previous publication (Khila & Abouheif, 2008). Fluorescent images of larval rudimentary wing discs of all species utilized in this study were taken using a Zeiss AxioImager Z1 microscope.

TUNEL

To detect apoptosis, we used the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. We used the InSitu Cell Death Detection Kit, TMR red (Roche). Larval samples were fixed, dissected and stored in 100% methanol in -30°C prior to procedure. Dissected larvae were then rehydrated in 25%, 50%, 75%, and 100% PTw (1X PBS; 0.1% Tween 20). Samples were then washed 3 times (10 min each) in freshly made PBT (1X PBS; 0.1% Triton; 0.1% BSA) and permeabilized in PTw and 2% Triton X-100 for 1 hour, followed by 5 min washes in PBT. Enzyme mix and label solution were added to the samples and incubated in the dark at 37°C for 1 hour followed by 3 washes in PTw for 5 min each. Samples were stained with DAPI (1:1000) for 1 hour to overnight and followed by gradual moving into glycerol. Samples were stored in final concentration of 85% glycerol/DAPI before mounting and imaging.

Caspase inhibitor microinjections

To reduce or eliminate cell death in the wing discs of *P. dentata*, we used the InSolutionTM Caspase inhibitor VI (Millipore Sigma, #219011) diluted at a concentration of 100 μM in Spradling injection buffer (Spradling & Rubin 1982). Control samples were injected in DMSO diluted in injection buffer. Size-matched soldier-destined, minor worker-destined, and male larvae were injected with the caspase inhibitor solution or DMSO control. All injections were performed on the left side of the larvae near the wing disc. Soldier-destined larvae are identified as those ranging from 2050-2798.44μm, while minor worker-destined larvae range from 1400-2080μm (unpublished data). Soldier and minor worker-destined larvae were further subdivided by gut color (brown and black): brown indicating larvae during early and mid-development, while black gut color marks larvae near the end of larval development. We injected size-matched early soldier-destined larvae ranging from around 2100-2600μm and minor worker-destined larvae ranging from around 1400-1800μm with a brown gut to ensure

time for the caspase inhibitor to take effect. Male larvae were distinguished from minor worker- and soldier-destined larvae by their size and balloon-like body shape. Size-matched male larvae with a brown gut ranging from 1700-3300μm were injected. All injected larvae were placed in experimental boxes with about 20-30 adult workers and provided with water and food as above. A subset of larvae was selected and fixed 1-2 days after injections and used for TUNEL staining, while another subset was allowed to pupate and the pupae were subsequently measured for size.

Allometric measurements

Size measurements of the rudimentary wing discs and legs discs of dissected larvae was performed using Zeiss AxioVison software. Images of *P. dentata* pupae were performed using a Zeiss Discovery V12 stereomicroscope and size measurements of pupae and larvae were performed using the Zeiss AxioVision software. Body and head measurements of all pupae were performed on the dorsal side of the pupa with body length measured from the top of the head to the beginning of the petiole and head width was measured at the widest point of the head excluding the eyes. Pupae were used as proxy for adult size, since final adult size is set at end of the larval stage (Hanna et al., 2023).

Statistics

All data was log transformed before analysis and all statistical analyses were performed using Graphpad Prism v8. Wing and leg disc areas of each larva were averaged before being log transformed. In order to test whether head width, body length, head-to-body ratios, leg disc area, wing disc area, and wing disc to leg disc area between control and experimental groups were statistically significant, we used a parametric unpaired t-test. To test for significant differences in head-to-body scaling, each data set was fitted with simple regression line and the difference in their slopes and y-intercept was calculated using ANCOVA. For all statistical analyses, differences between experimental and control treatments were considered statistically significant at a P value < 0.05.

4.6. Acknowledgments

We thank the laboratory of Jürgen Liebig for providing us with *H. saltator* ants. This work was supported by a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant to E.A, and a Doctoral Fellowship from Fonds de Recherche du Quebec-Nature et Technologies (FRQNT) to L.H.

4.7. References

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Figure 1.

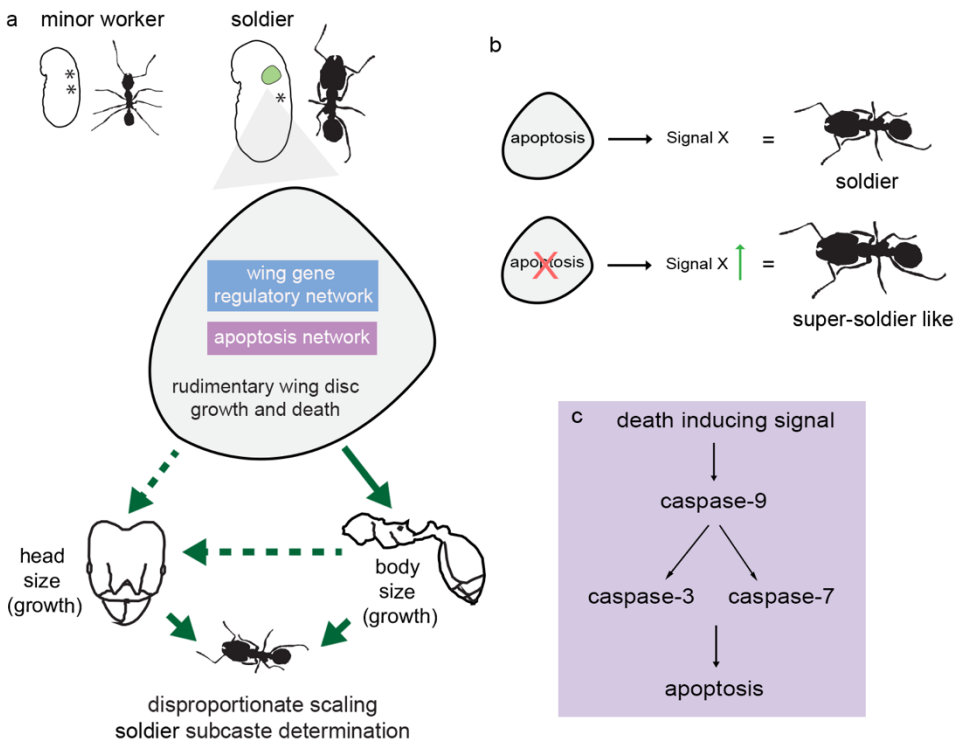


Figure 1. Worker polymorphism and experimental predictions. a) worker polymorphism in *P. dentata* is composed of a minor worker subcaste and a large-headed soldier subcaste. Minor worker larvae lack rudimentary wing discs, while soldier larvae develop one pair of rudimentary wing discs. The wing gene regulatory network and the apoptosis network are active in the soldier rudimentary wing discs. The rudimentary wing discs regulate the proportionate (solid arrows) growth of body size and the disproportionate (dashed arrows) growth of head size leading to disproportionate scaling between soldier head and body. Adapted from Rajakumar et al. (2018) with permission. b) experimental predictions where normal growth and apoptosis of rudimentary wing discs result in solider phenotype while inhibition of apoptotic signaling and wing discs death leads to super-soldier like phenotype. c) a simplified genetic cascade leading to apoptosis.

Figure 2.

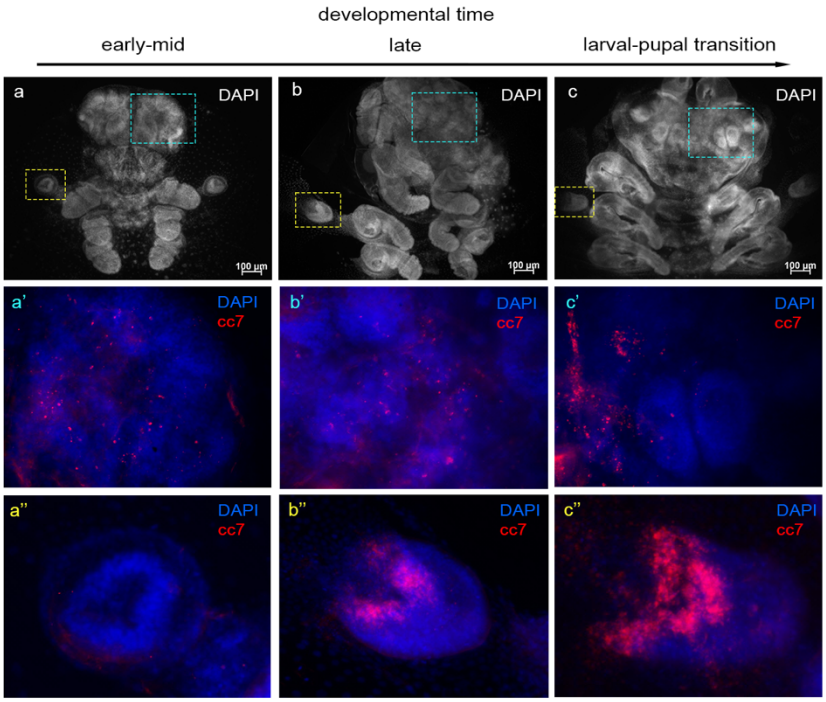


Figure 2. Expression of cleaved-caspase 7 in *P. dentata* soldier-destined larvae. Representative soldier-destined larvae during early-mid (a), mid-late (b), and larval-pupal transition (c) terminal development stained with DAPI (white). a'-c') expression of cleaved-caspase 7 (red) and DAPI (blue) in the head discs in areas corresponding to the location indicated by blue dashed boxes in a-c. a''-c'') expression of cleaved-caspase 7 (red) and DAPI (blue) in the rudimentary wing discs in areas corresponding to the location indicated by yellow dashed boxes in a-c. All image comparisons are to scale. cc7, cleaved-caspase 7. Arrow indicates developmental time.

Figure 3.

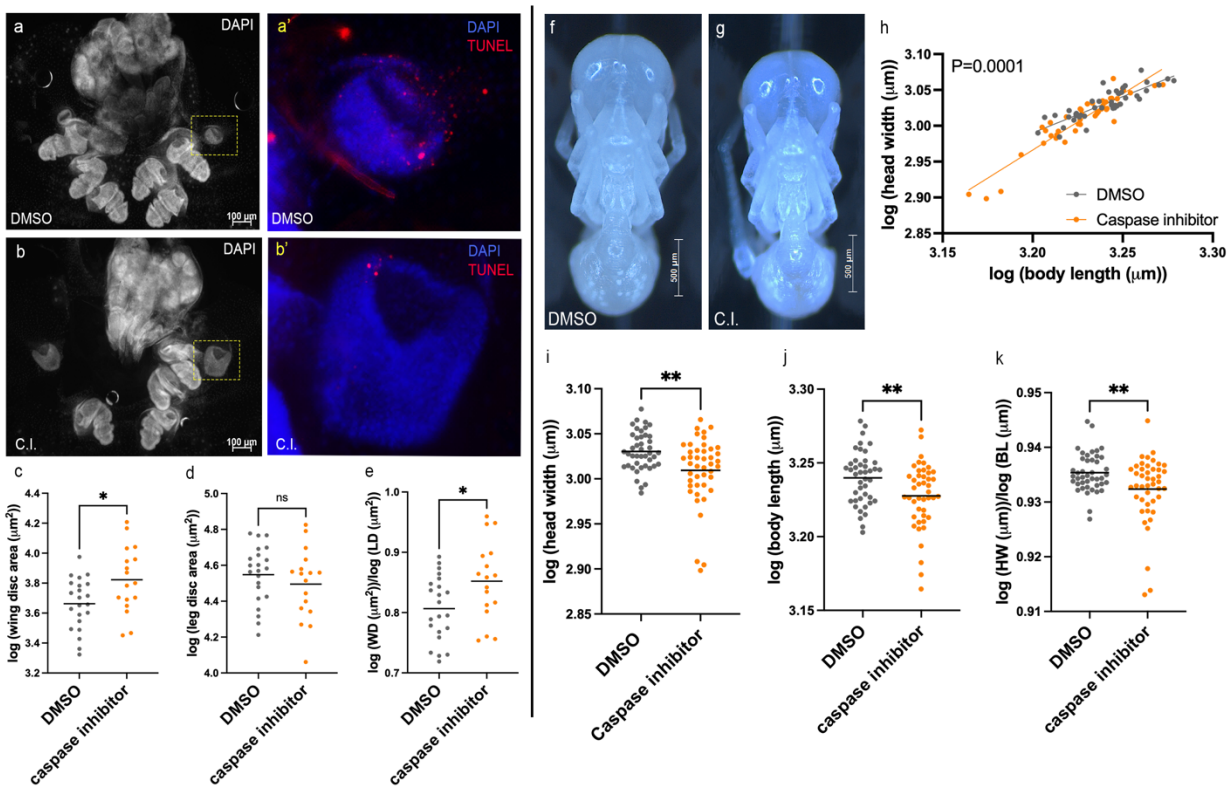


Figure 3. Caspase inhibitor experiments in soldier-destined larvae. Representative larva injected with DMSO (a) and caspase inhibitor (b) stained with DAPI (white). Expression of TUNEL (red) and DAPI (blue) in the rudimentary wing discs of DMSO (a') and caspase inhibitor (b') treated larva in the areas corresponding to the location indicated by the yellow dashed boxes in a-b. Comparison of logged wing disc area (c), leg disc area (d), and wing discs (WD) to leg disc (LD) area (e) between DMSO (gray dots; n=22) and caspase inhibitor treatment (orange dots; n=17). Representative soldier pupa treated with DMSO (f) and caspase inhibitor (g). h) comparing simple regression lines of logged body length and head width between DMSO (gray dots; n=43) and caspase inhibitor (orange dots; n=44) treatments. Comparing logged head width (i), body length (j), and head width (HW) to body length (BL) ratio (k) between DMSO (gray dots) and caspase inhibitor treatment (orange dots). C.I., caspase inhibitor. Horizontal black lines in c-e and i-k indicate mean. *P<0.05; **P<0.01**.**

Figure 4.

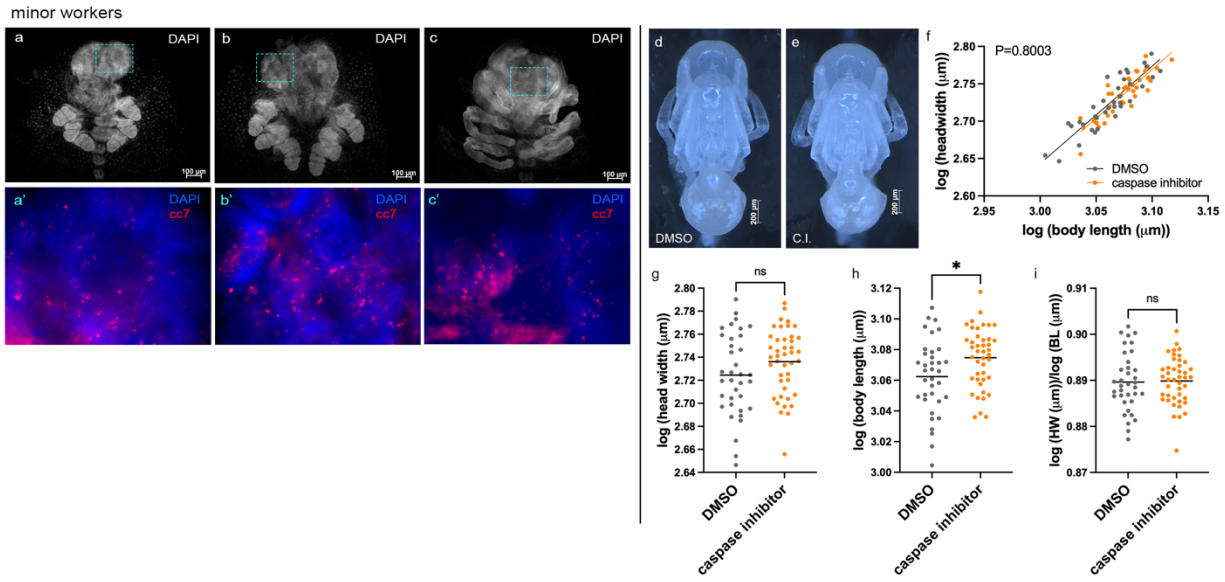


Figure 4. Cleaved-caspase 7 expression and caspase inhibition experiments in minor worker-destined larvae. Representative minor worker-destined larvae during early-mid (a), late (b), and larval-pupal transition (c) terminal development stained with DAPI (white). a'-c') expression of cleaved-caspase 7 (red) and DAPI (blue) in the head discs in areas corresponding to location indicated by blue dashed boxes in a-c. Representative minor worker pupa treated with DMSO (d) and caspase inhibitor (e). f) comparing simple regression lines of logged body length and head width between DMSO (gray dots; n=36) and caspase inhibitor (orange dots; n=43) treatments. Comparing logged head width (g), body length (h), and head width (HW) to body length (BL) ratio (i) between DMSO (gray dots) and caspase inhibitor treatment (orange dots). Horizontal black lines in g-i indicate mean. *P<0.05; ns=non-significance.

Figure 5.

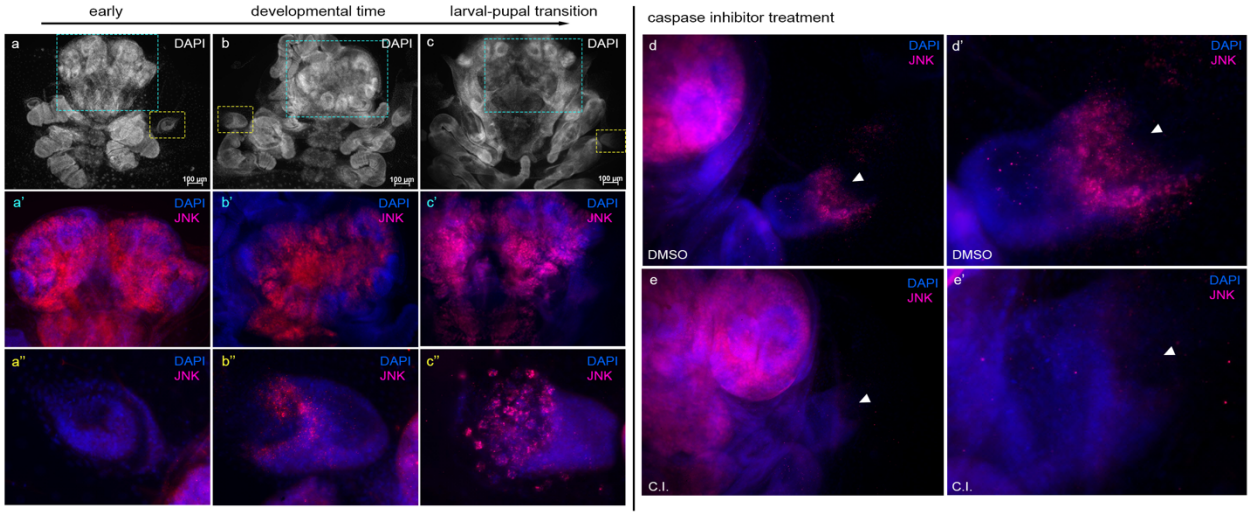


Figure 5. JNK expression in soldier-destined larvae in wildtype and under caspase inhibition conditions. Representative soldier-destined larvae during early-mid (a), late (b), and larval-pupal transition (c) terminal development stained with DAPI (white). a'-c') expression of JNK (pink) and DAPI (blue) in the head discs in areas corresponding to location indicated by blue dashed boxes in a-c. a''-c'') expression of JNK (pink) and DAPI (blue) in the rudimentary wing discs in areas corresponding to location indicated by yellow dashed boxes in a-c. For consistency, all rudimentary wing discs are oriented with proximal axis on the left. Expression of JNK in rudimentary wing disc (arrowhead) in late soldier-destined larvae treated with DMSO (d) and caspase inhibitor (e). d', e') magnified view of the rudimentary wing disc in (d) and (e), respectively. Shown rudimentary wing discs are on the same side of injection. All image comparisons are to scale.

Figure 6.

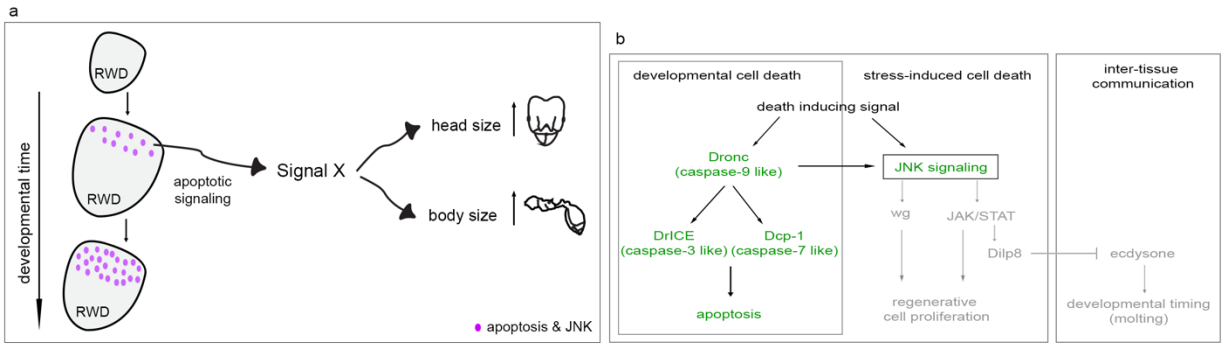
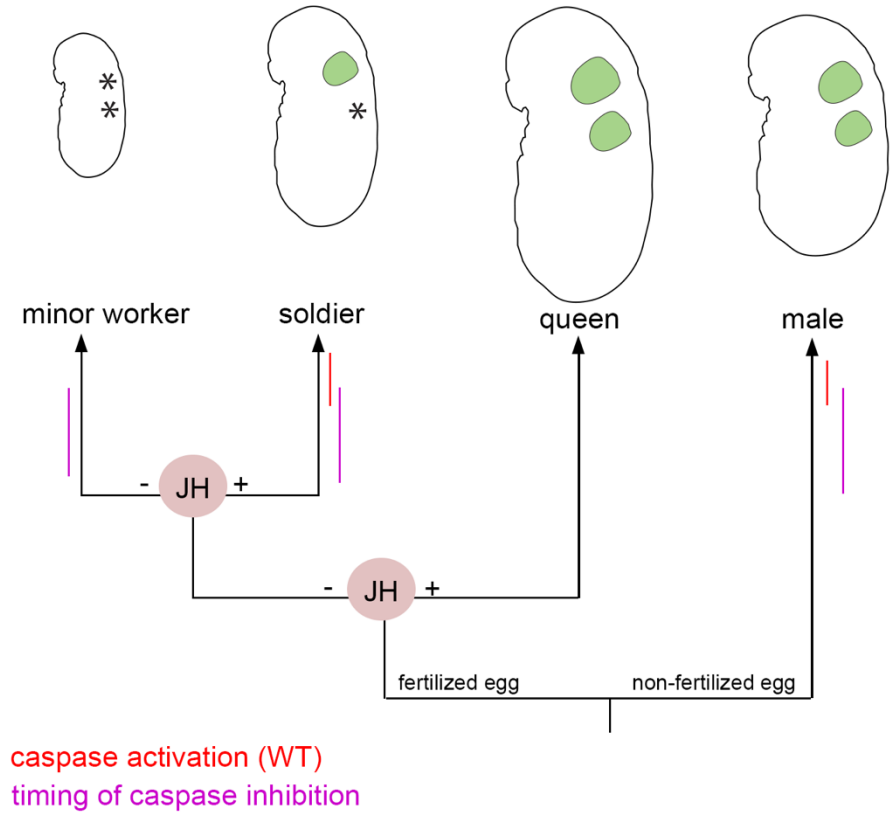


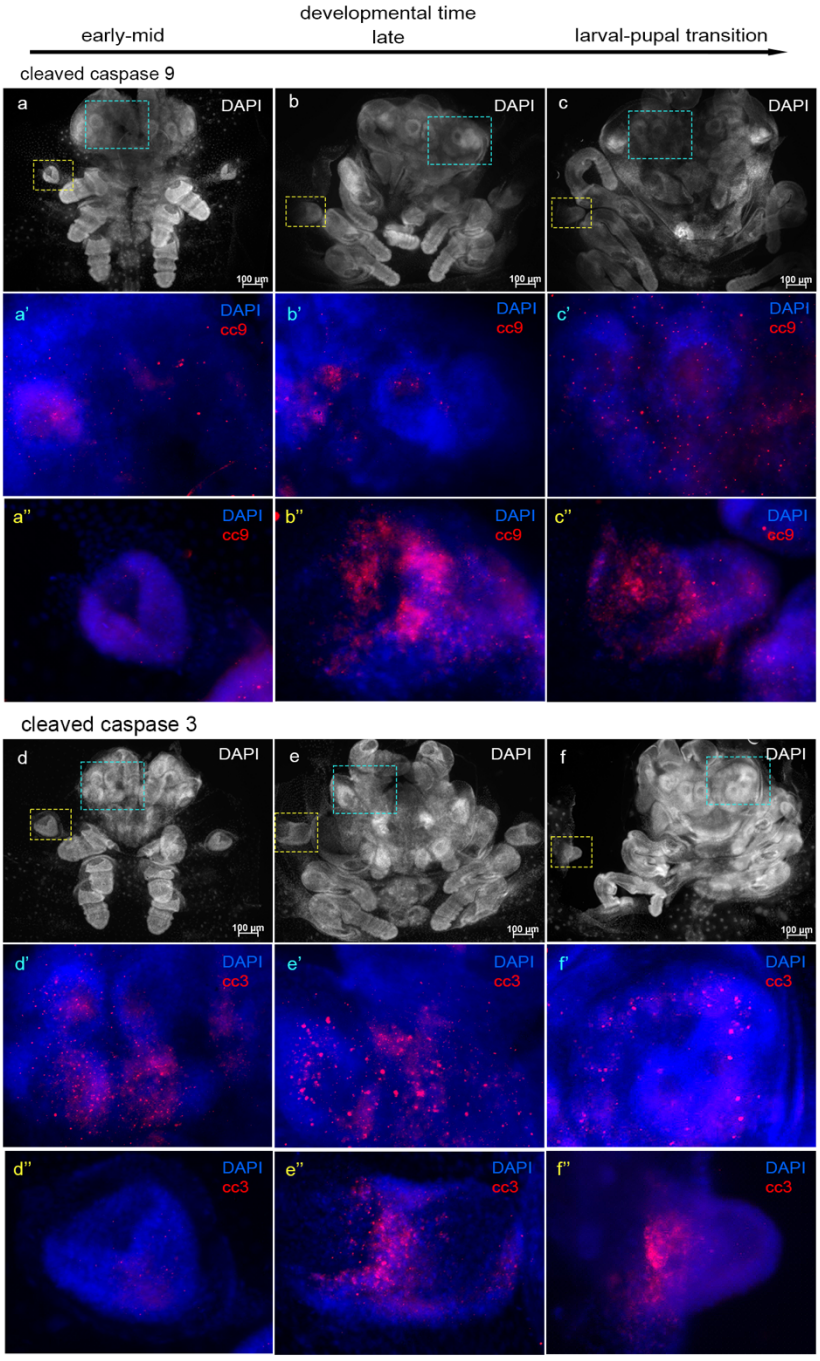
Figure 6. A model for the role of apoptosis signaling in soldier head and body growth. a) the rudimentary wing discs (RWD) grow and shrink during the terminal larval stage. Starting around the mid-late stage, apoptosis and JNK signaling (purple dots) are activated in the rudimentary wing discs. Apoptotic signaling is necessary to induce normal head and body growth in soldier larvae possibly via activation of downstream factors (signal X). b) developmental and stress-induced cell death pathways. Components of apoptotic cell death and stress-response pathway are active in the rudimentary wing discs of soldiers (green). Downstream factors of JNK that function in cell proliferation and developmental timing (gray) remain to be tested in ants.

Supplemental 1.



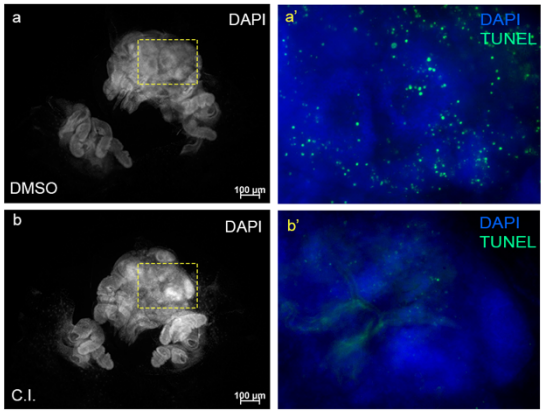
Supplemental 1. Development of *P. dentata* worker and reproductive cates. Non-fertilized eggs develop into males whereas fertilized eggs develop into females. Environmental cues mediated by juvenile hormone (JH) determine queen vs. worker development and minor worker vs. soldier development. Red lines indicate the estimated time range when caspases are expressed in the wing discs of soldier- and male-destined larvae. Pink lines indicate the estimated time range caspase inhibition injections were performed in minor worker-, soldier-. and male-destined larvae. Asterisks indicate absence of wing disc rudiment. Green oval indicates presence of wing disc rudiment.

Supplemental 2.



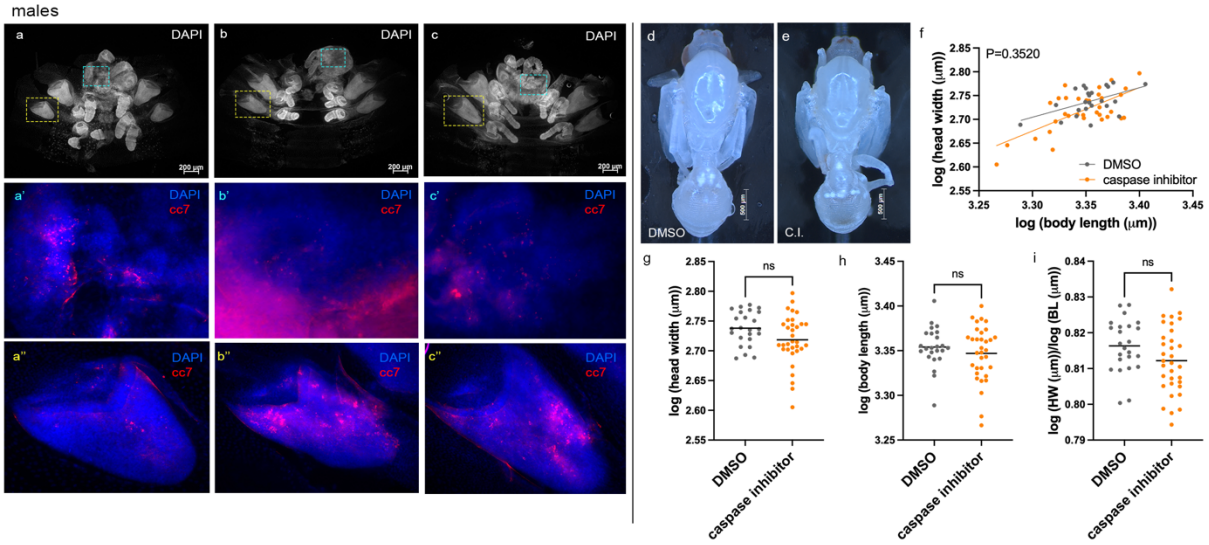
Supplemental 2. Expression of cleaved-caspase 9 and 3 in *P. dentata* soldier-destined larvae. Representative soldier-destined larvae during early-mid (a, d), mid-late (b, e), and larval-pupal transition (c, f) terminal development stained with DAPI (white). a'-c') expression of cleaved-caspase 9 (red) and DAPI (blue) in the head in areas corresponding to the location indicated by blue dashed boxes in a-c. a''-c'') expression of cleaved-caspase 9 (red) and DAPI (blue) in the rudimentary wing discs in areas corresponding to the location indicated by yellow dashed boxes in a-c. d'-f') expression of cleaved-caspase 3 (red) and DAPI (blue) in the head in areas corresponding to the location indicated by blue dashed boxes in d-f. d''-f'') expression of cleaved-caspase 3 (red) and DAPI (blue) in the rudimentary wing discs in areas corresponding to the location indicated by yellow dashed boxes in d-f. All image comparisons are to scale. cc9, cleaved-caspase 9; cc3, cleaved-caspase 3. Arrow indicates developmental time.

Supplemental 3.



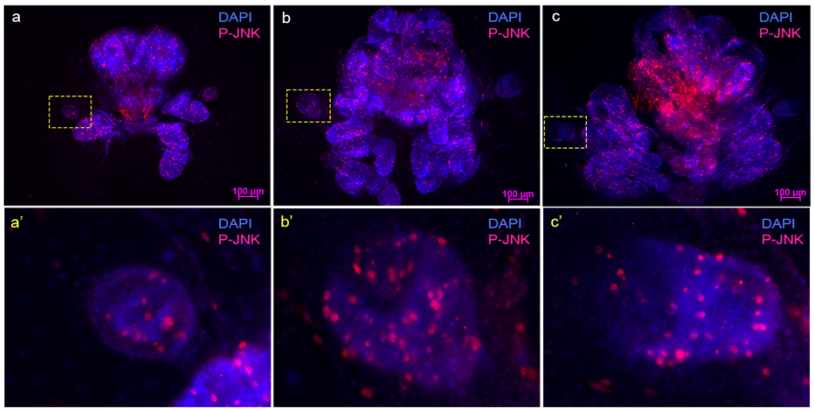
Supplemental 3. TUNEL signal in minor worker caspase inhibition experiments.
Representative larva injected with DMSO (a) and caspase inhibitor (b) stained with DAPI (white). Expression of TUNEL (green) and DAPI (blue) in the head region of DMSO (a') and caspase inhibitor (b') treated larva in the areas corresponding to the location indicated by the yellow dashed boxes in a-b.

Supplemental 4.



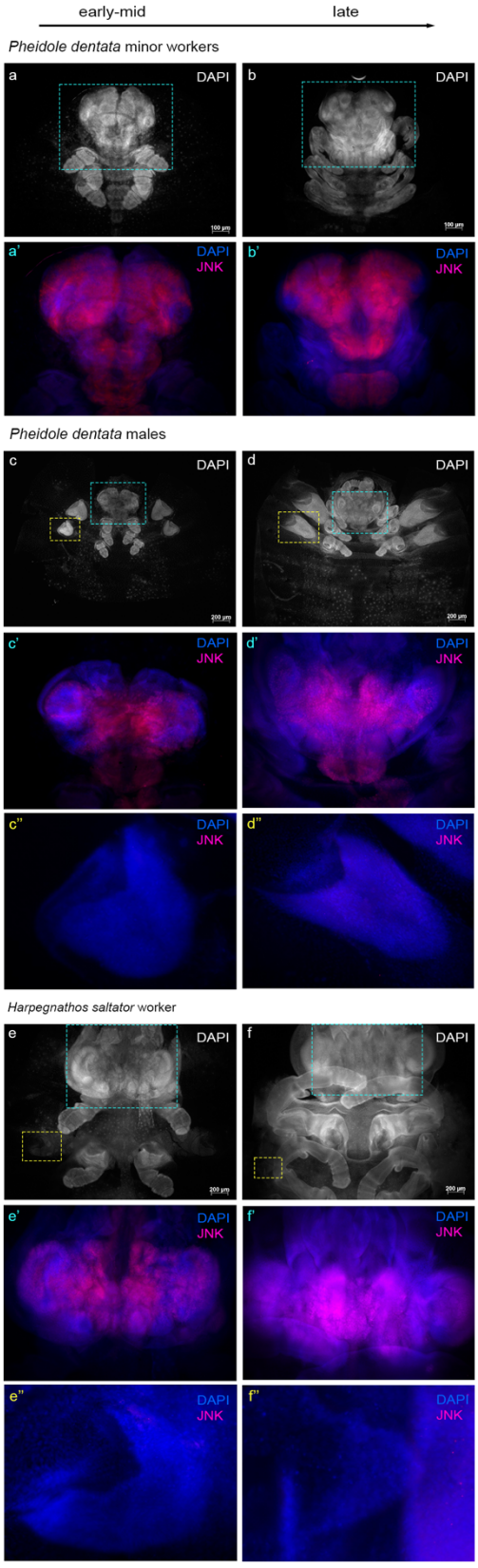
Supplemental 4. Cleaved-caspase 7 expression and caspase inhibition experiments in male-destined larvae. Representative male-destined larvae during early-mid (a), mi-late (b), and larval-pupal transition (c) terminal development stained with DAPI (white). a'-c') expression of cleaved-caspase 7 (red) and DAPI (blue) in the head in areas corresponding to location indicated by blue dashed boxes in a-c. a''-c'') expression of cleaved-caspase 7 (red) and DAPI (blue) in the wing discs in areas corresponding to the location indicated by yellow dashed boxes in a-c. Representative male pupa treated with DMSO (d) and caspase inhibitor (e). f) comparing simple regression lines of logged body length and head width between DMSO (gray dots; n=24) and caspase inhibitor (orange dots; n=33) treatments. Comparing logged head width (g), body length (h), and head width (HW) to body length (BL) ratio (i) between DMSO (gray dots) and caspase inhibitor treatment (orange dots). Horizontal black lines in g-i indicate mean. ns=non-significance.

Supplemental 5.



Supplemental 5. Expression of phosphorylated JNK (P-JNK) in P. dentata soldier-destined larvae. Representative larvae during early-mid (a), mid-late (b), and larval-pupal transition (c) terminal development stained with DAPI (blue) and P-JNK (pink). a'-c') expression of P-JNK (pink) and DAPI (blue) in the rudimentary wing discs in areas corresponding to location indicated by yellow dashed boxes in a-c.

Supplemental 6.



Supplemental 6. JNK expression in additional castes and species. Representative *P. dentata* minor worker-destined larvae during early-mid (a), late (b) terminal development stained with DAPI (white). a'-b') expression of JNK (pink) and DAPI (blue) in the head and CNS in areas corresponding to location indicated by blue dashed boxes in a-b. Representative male-destined larvae during early-mid (c), and late (d) terminal development stained with DAPI (white). c'-d') expression of JNK (pink) and DAPI (blue) in the head and CNS in areas corresponding to location indicated by blue dashed boxes in c-d. c''-d'') expression of JNK (pink) and DAPI (blue) in the wing discs in areas corresponding to location indicated by yellow dashed boxes in c-d. Representative *P. dentata* male-destined larvae during early-mid (c), and late (d) terminal development stained with DAPI (white). c'-d') expression of JNK (pink) and DAPI (blue) in the head and CNS in areas corresponding to location indicated by blue dashed boxes in c-d. c''-d'') expression of JNK (pink) and DAPI (blue) in the wing discs in areas corresponding to location indicated by yellow dashed boxes in c-d. Representative *H. saltator* worker-destined larvae during early-mid (e), and late (f) terminal development stained with DAPI (white). e'-f') expression of JNK (pink) and DAPI (blue) in the head in areas corresponding to location indicated by blue dashed boxes in e-f. e''-f'') expression of JNK (pink) and DAPI (blue) in the rudimentary wing discs in areas corresponding to location indicated by yellow dashed boxes in e-f.

CONNECTING STATEMNT BETWEEN CHAPTERS 4 & 5

In Chapter 4, I showed that the apoptosis signaling in the worker wing discs is necessary to induce head and body growth of the solider subcaste. In combination with other work, we now know that the rudimentary wing discs excrete signals that control size allometry in species with a worker polymorphism. In Chapter 5, I asked if the rudimentary wing discs of species without worker polymorphism (i.e., monomorphic worker caste) have a latent potential to regulate size. A recent fossil discovery found evidence for worker polymorphism in an early extinct species (Cao et al. 2020), suggesting that this trait evolved much earlier than previously thought. Therefore, in Chapter 5, I begin examining this possibility by first testing the function of the rudimentary wing discs in the species *Harpegnathos saltator* that have a monomorphic worker caste. To do this, I targeted the wing gene *vestigial (vg),* that is responsible for wing growth and development. The reduction of *vg* expression is known to hinder the growth of the wing discs thus enabling us to assess their functions. The results obtained on the effect of the wing discs on body proportions after reducing *vg* expression are thus far inconclusive due to low sample number. However, this study provides promising data suggesting that manipulating wing disc growth alters the head size but not the body size. Howerver, more robust sample size is needed to confidently confirm these results. Moreover, I find strong correlation between size and the presence of short-wings that workers of this species occasionally produce, suggesting that there is indeed a relationship between the wing discs and body growth. Further tests and more robust sample size should be able to confirm with certainty whether the rudimentary wing discs have a latent potential to regulate worker polymorphism.

Chapter 5: Exploring the functional role of rudimentary wing discs in a monomorphic ant species with ancestral characteristics

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5.1. Abstract

The evolution of morphologically distinct workers in some ant species (known as 'worker polymorphism') occurred sporadically across the ant phylogeny. The development of an elaborate worker subcaste (specifically big-headed soldiers) is, in part, regulated by the wing rudimentary organs that no longer give rise to functional adult wings in worker ants. Recent fossil discovery revealed the presence of worker polymorphism in early and extinct ant species, suggesting that worker polymorphism evolved much earlier during ant evolution than previously assumed. Here, we test if the wing rudimentary organs have a latent potential for regulating worker polymorphism in the Ponerine ant species *Harpegnathos saltator*, a monomorphic species that lacks worker polymorphism and displays numerous ancestral-like features. We experimentally manipulate the normal growth of the wing rudiments via RNAi and reveal an effect on head size, but not body size. This indicates that the rudimentary wing discs have a latent capacity for inducing morphological variation and disproportionate growth between head and body size in species lacking a worker polymorphism.

5.2. Introduction

Ants (Formicidae) are eusocial insects that live in a colony, cooperate, and display reproductive division of labor (Holldobler & Wilson, 1990). Eusociality evolved once in ants and is found in all species, except those that secondarily evolved a parasitic lifestyle (Fig. 1a green circle). In an ant colony, the females are morphologically differentiated into a queen caste (reproductive) and worker caste (non-reproductive). Concomitant with the evolution of eusociality, ants evolved a wing polyphenism where the queens (and males) produce wings and the worker females are wingless (Fig. 1a blue circle) (Hanna & Abouheif, 2021). In some species, the worker caste was further elaborated into several morphologically distinct castes, called 'subcastes'. This phenomenon is known as 'worker polymorphism' (Fig. 1a purple circles). Modern use of the term 'polymorphism', however, indicates a situation where morphologically distinct forms are generated due to genetic factors (Stearns, 1989). In social insects, and ants in particular, the term worker polymorphism is simply used in the traditional sense to indicate the presence of several alternative phenotypes in a colony. Current evidence supports that worker polyphenism is largely determined by environmental factors during development with some genetic influence (Alvarado et al., 2015; Lillico-Ouachour & Abouheif, 2017; Smith, 1944). Evolution of worker polymorphism in ants may have been driven by various ecological factors namely access to new foraging niches, competition and combat with other ant colonies, nest defense, and utilization of new food sources (Wills et al., 2018). Research on the developmental factors has shown the role of juvenile hormone, nutrition, social inhibitory pheromones, and epigenetics in driving worker polymorphism (Alvarado et al., 2015; Rajakumar et al., 2012; Wheeler, 1986; Wheeler & Nijhout, 1981, 1984). A recent study by Rajakumar et al. (2018) showed that the wing rudiments—organs that transiently develop in worker ant larvae and then degenerate before the adult stage—are necessary for generating size and head-to-body allometry of the big-headed soldier subcaste in the ant *Pheidole hyatti*. This finding supports the hypothesis that the origin of wing polyphenism paved the way for the origin of worker polymorphism by opening an opportunity to exploit and re-purpose the wing rudiment, which is free from the constraints of having to make an adult wing (Hanna & Abouheif, 2021; Rajakumar et al., 2018).

Worker polymorphism evolved at least 22 times independently during ant evolution (Wills et al., 2018). Most species with distinct worker subcastes (over 2,500 species) are found in the Myrmicinae and Formicinae subfamilies, belonging in the formicoid clade (Fig.1a purple circles) (La Richeliere et al., 2022). Unlike the formicoid clade, the evolution of worker polymorphism in the poneroid clade is rare (Fig. 1a purple circles) (La Richeliere et al., 2022; Wills et al., 2018). Species in the formicoid clade exhibit derived social and behavioral characteristics, whereas species from the poneroid clade are considered to possess many similarities with early and extinct groups (Keller & Peeters, 2022; Peeters, 1997). Worker polymorphism has therefore been regarded by many as an advanced eusocial trait (Peeters & Molet, 2010; Wilson & Holldobler, 2005). Surprisingly, a recent fossil finding revealed the presence of worker polymorphism in phylogenetically basal ant *Zigrasimecia ferox*, an extinct species estimated to have lived 99 million years ago (Fig. 1a) (Cao et al., 2020). Not only does this finding suggest that worker polymorphism evolved very early in the history of ants, but also that the developmental potential for its evolution may have existed as early as the evolution of the group itself (Fig. 1a, dashed purple circle). In a previous work, we discussed how the evolution of wing polyphenism (winged queens and wingless workers) at the origin of ant evolution (Fig. 1a green circle), may have facilitated the origin of worker polymorphism by facilitating co-option of the wing rudiment as a regulatory mechanism for generating differences in head-to-body allometry (Hanna & Abouheif, 2021; Rajakumar et al., 2018). Here, we test whether wing rudiments have a latent developmental capacity to induce worker polymorphism in a monomorphic ant species (i.e., lacking worker polymorphism) with ancestral-like characteristics. We used the Ponerine species *Harpegnathos saltator*, in which the females display very little dimorphism between the queen and workers, and where the workers are monomorphic (Fig. 1b). We targeted the wing-identity gene *vestigial* (*vg*; Fig. 1c), which is responsible for wing patterning and growth in *Drosophila* (Cohen, 1996). We reduced its expression using RNA interference in worker-destined larvae to halt the growth of the wing rudiments and ascertain its effect on size and head-to-body allometry.

5.3. Results

First, we traced the growth trajectory of the rudimentary wing discs in *H. saltator* worker-destined larvae and compared them to the wing disc growth of male-destined larvae. Male determination occurs during the egg stage, therefore, differences between male/worker are present during all of larval development. We measured the area of the wing discs at each of the four larval developmental stages as defined by Penick et al. (2012). During the 1st through 3rd instar, the growth of the worker wing discs is similar to that of males that develop fully functional wings as adults (Fig. 2a). Around the 4th instar, although the worker rudimentary wing discs continue to grow, their growth slows down and reaches a plateau. By the larval-pupal transition, they begin to shrink and degenerate (Fig. 2a).

Next, we tested if the rudimentary wing discs in *H. saltator* have a latent developmental capacity to affect size and head-to-body allometry. We hindered the growth of the wing discs by knocking down expression of *vg* using RNAi. Specifically, we targeted larvae around and before the early 4th instar stage since this is the stage before the rudimentary wing discs start to show an increase in growth before they begin to degenerate via apoptosis (Fig. 2a; Chapter 3). *vg* RNAi resulted in a significant difference in the head length-to-body slope relative to control (*yfp* RNAi; P=0.0228) (Fig. 2j). Moreover, the percent change of head length compared to body size is significantly different (P=0.0195) (Fig. 2k-l). *vg* RNAi also resulted in a similar difference in the head width-to-body slope relative to control, although not significantly so (*yfp* RNAi; P=0.0697) (Fig. 2e). Although the head width is changing more than the body (Fig. 2g), the effect is not significant (P=0.3652) (Fig. 2f). Finally, these differences were not accompanied by significant differences in body length, head width, head length, head width-to-body length ratio, and head length-to-body length ratio compared to *yfp* RNAi control (Fig. 2b-d, h, i). Altogether this indicates that, although body size is not changing significantly, the head size (especially length) is changing, revealing a disproportionate effect of *vg* RNAi on head vs body size.

Finally, despite the fact that the worker caste across all ants is wingless, some species, including *H. saltator*, seldomly produce adult workers with a short wing vestige (Wheeler, 1905, 1916). The appearance of these anomalies occurs rarely in nature and in lab colonies and are

termed ‘brachypterous workers’ (Fig. 3a, white arrow). Queens also seldomly emerge with a vestigial short wing. These individuals are identified as brachypterous workers due to the lack of flight muscles on their thorax that is characteristic of queens. If development of the wing rudiment in the worker caste is generally associated with changes in body size and allometry, then one would expect that workers that develop short wings should be larger than those workers that are completely wingless. We therefore measured the body and head size dimensions of wingless workers and brachypterous workers. We found that brachypterous workers were larger in head and body size compared to completely wingless workers (Fig. 3b-c).

5.4. Discussion

The discovery of worker polymorphism in a species that lived around a 100 million years ago drastically sets back estimates regarding when this trait first emerged during the evolutionary history of ants. Since fossils from this period are rare (LaPolla et al., 2013), it is not unlikely that other early extinct species also evolved increased complexity in their worker caste. Moreover, although generally less frequent than the formicoids, worker polymorphism did also evolve in numerous poneroid groups (Fig. 1) (La Richeliere et al., 2022). Altogether this indicates that the potential for evolving a complex worker caste existed early in ant evolution.

We find that in *H. saltator*, development of worker adult vestigial wing buds results in individuals that have generally bigger head and body size compared to workers that are completely wingless. This may be the result of static allometric growth where organ size and body size are coordinated (Shingleton et al., 2007; Stern & Emlen, 1999). We predict that the failure of the wing discs to degenerate during larval development in these individuals might be due to a disruption in the activation of the apoptosis cell death program (Chapter 3).

Finally, we show that developmentally manipulating the normal growth of the rudimentary wing discs influences the size of the head but not the size of the body. This suggests that, although wildtype individuals do not have disproportionate growth of the head compared to the body, manipulating the rudimentary wing discs releases a capacity to alter the proportionate growth. Disproportionate growth of head to body size is a characteristic feature of worker caste complexity in ants. In Chapter 4, we showed that the rudimentary wing discs

activate apoptotic cell death and JNK stress response pathway that influence the growth of the head in *P. dentata* soldier subcaste. We predict that the rudimentary wing discs of wildtype *H. saltator*, although activate apoptotic cell death, have not yet evolved a regulatory control of head size. Our manipulation of *vg* and the affects we observe on changing head size experimentally might be due to induction of this regulatory control, perhaps through activating the JNK stress response pathway that is normally not active in wildtype *H. saltator* (Chapter 4). Further investigation of JNK signal under *vg* RNAi conditions will confirm these predictions.

Altogether, our results thus far suggest a role for the rudimentary wing discs of a monomorphic species in altering the proportional growth of head and body size. This leads us to suggest that the rudimentary wing discs have the potential for inducing morphological variation in the worker caste of a monomorphic species, indicating a hidden latent potential for worker polymorphism evolution. A larger sample size showing a more robust change in head and body scaling will confirm our present conclusions. Moreover, further investigations of how this regulatory control of head size after *vg* RNAi manipulations is achieved, has the potential to further our understanding of how the rudimentary wing discs acquired their functions in elaborating growth and size in ants.

5.5. Materials and methods

Ant collection and colony care

H. saltator colonies were a generous gift from Juergen Leibig laboratory, where they were propagated since their original collection in 1999 from different locations in India. H. saltator colonies were housed in plaster nests with a water reservoir, fed live crickets 2-3 times per week, and maintained at 25 °C, 70% humidity, and a 12h day:night cycle. Fresh sawdust was added as necessary to provide a substrate for the larvae to spin a cocoon. One brachypterous worker was produced naturally in the laboratory. Additional brachypterous workers were previously produced in the Leibig laboratory and preserved in ethanol. Production of male larvae was induced by isolating newly emerged, previously unmated workers and allowed to lay unfertilized eggs.

RNAi injection

H. saltator vg sequence was obtained from NCBI (XM_011148267.2) and the following primers were used to clone the gene fragment (forward 5'-GAAGCGTCCCTCAAGGTCAA-3') (reverse 5'-CGAGTCTTACCGGAGAGGGG-3'). Cloning and RNAi synthesis was performed according to Rajakumar et al. (2018). Size matched *H. saltator* larvae between 4.2-5.2mm (early 4th instar) were selected and injected with *vg* and *yfp* dsRNA at a concentration of 1 µg/µl. Larvae were laid on their dorsal side and measurements were taken from the start to the end of the abdomen. The head and thorax were excluded since these regions are freely moved by the larvae. Larvae in this size range were targeted because the rudimentary wing discs are growing and expressing *vg* signal (Fig. 2a; Chapter 3: Fig. 5b-b'). It is also the stage before the onset of apoptosis (Chapter 3: Fig. 2). Larvae was placed dorsally on a double-sided tape and injected on their lateral side between the transition of thorax and abdomen. Aluminosilicate needles were pulled using a Sutter Instrument needle puller (Model P-97) attached to a Narishige microinjection apparatus attached to a Zeiss Discovery V8 dissection microscope. Larvae were then placed with established host colonies devoid of similar staged larvae and collected 1-2 weeks post cocoon formation for allometric measurements. Removal of the cocoon prior to pupation (< a week after cocoon formation) results in failure to pupate properly.

Allometric measurements

Images of dissected larvae were taken using Zeiss AxioImager Z1 microscope and area of wing and leg discs were measured using Zeiss AxioVision software. Since adult body size is determined at the end of larval development (Hanna et al., 2023), pupa measurements were taken to serve as proxy for adult size.i*vg* and *yfp* RNAi injected pupae were dissected out of the cocoon and imaged using Zeiss Discovery V12 stereomicroscope. Body length was measured from beginning of thorax to start of petiole while the pupa was placed laterally. For head measurements, pupa was placed dorsally between a ridge made from agar to provide stability and head width was measured at the widest region of the head excluding the eyes while head length was measured from top of the head to the start of the mouth parts. Since males and females are indistinguishable at the larval stages, injected male pupa were discarded and only worker pupae were measured. Images of adults were taken using Zeiss Discovery V12

stereomicroscope and body length was measured using mesosoma length and head width and length were measured as described above. Measurements for adult and pupa were performed using Zeiss AxioVision software.

Statistics

All data was log transformed before analysis and all statistical analyses were performed using Graphpad Prism v8. Wing and leg disc areas of each larva were averaged before being log transformed. Non-linear regression model was fit to the data. To test whether body length, head width, head length, head width-to-body ratio, and head length-to-body ratio between control and experimental groups were statistically significant, we used a parametric unpaired t-test. To test for significant differences in head-to-body scaling, each data set was fitted with a simple linear regression and the difference in their slopes and y-intercept was calculated using ANCOVA. To calculate percentage change in head length or width (µm) vs. percentage change in body length (µm) of *vg* RNAi compared to 1:1 line, we took the absolute value of ($X_{vg\ \text{RNAi}} - X_{yfp\ \text{RNAi average}}$)/$X_{yfp\ \text{RNAi average}}$)*100. For all statistical analyses, differences between experimental and control treatments were considered statistically significant at a P value < 0.05.

5.6. Acknowledgments

We thank the laboratory of Jürgen Liebig for providing us with *H. saltator* ants. This work was supported by a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant to E.A., and a Doctoral Fellowship from Fonds de Recherche du Quebec-Nature et Technologies (FRQNT) to L.H.

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Figure 1.

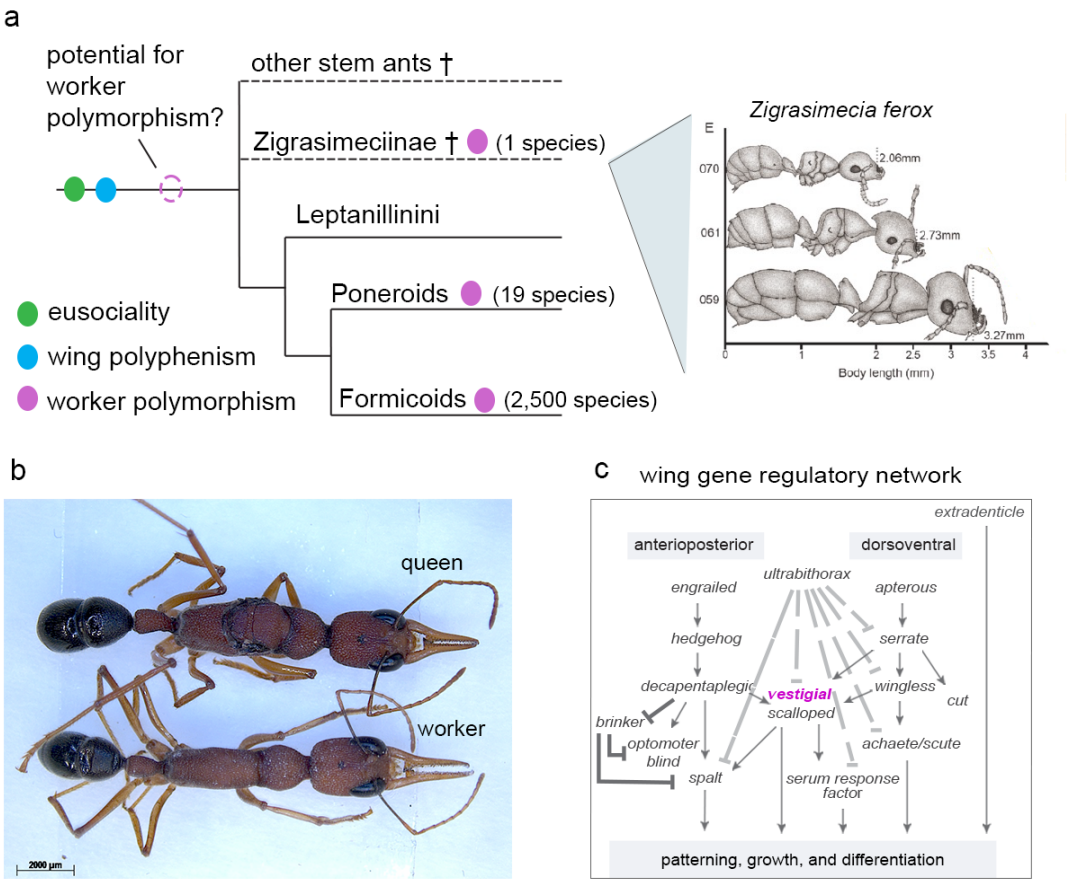

Figure 1. Worker polymorphism in ants. a) eusociality (green circle) and wing polyphenism (blue circle) evolved at the origin of ants. Worker polymorphism (purple circle) is found in about 2,500 formicoid species, 19 poneroid species, and 1 extinct early fossil. Diagram of *Zigrasimecia ferox* from Cao et al. 2020 with permission. b) queen and worker *H. saltator* showing little size dimorphism. c) the wing gene regulatory network of insects.

Figure 2.

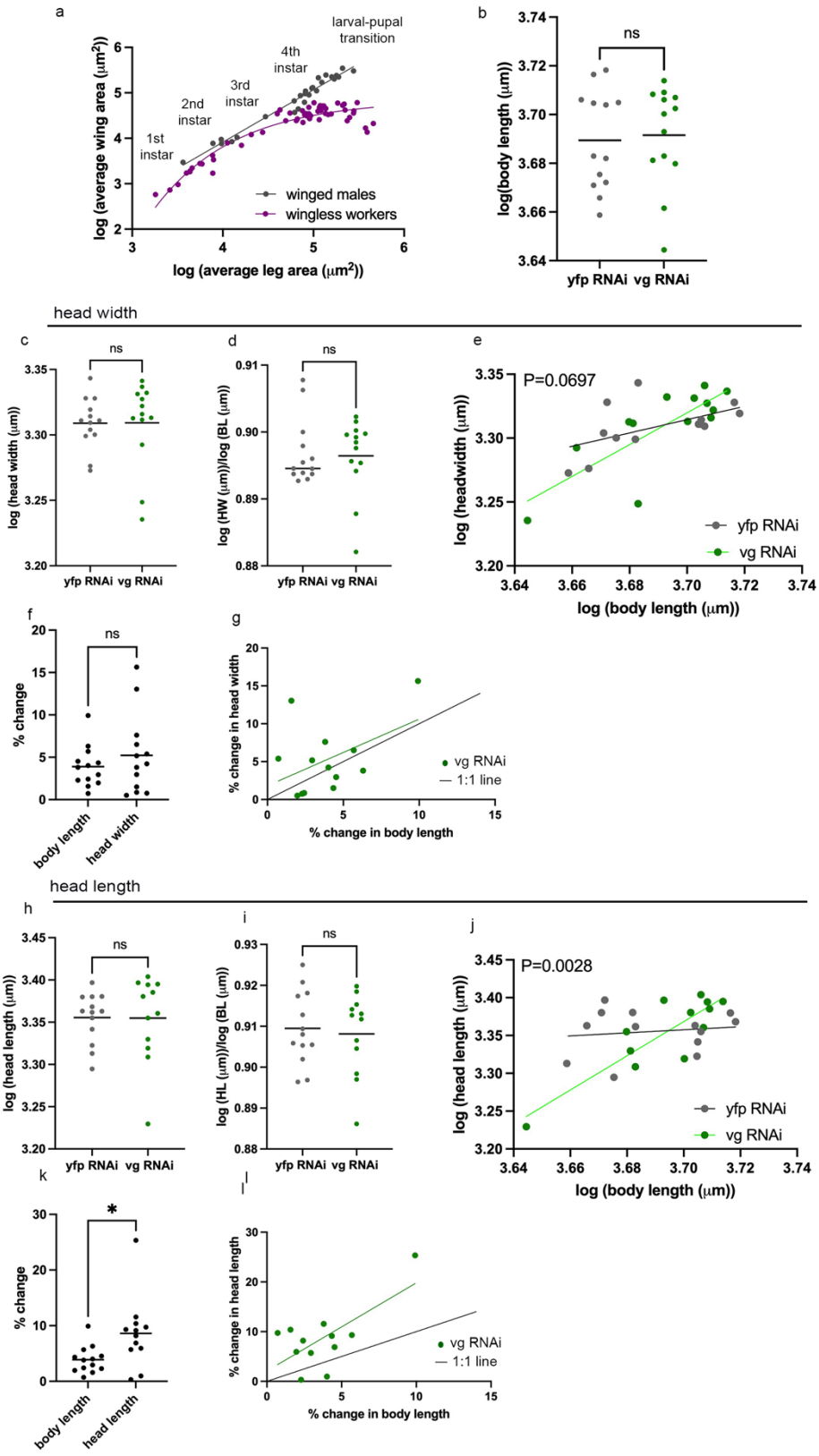
a
larval-pupal transition
4th instar
3rd instar
2nd instar
1st instar
winged males
wingless workers
log (average wing area (μm²))
log (average leg area (μm²))
b
ns
log(body length (μm))
yfp RNAi vg RNAi
head width
c
ns
log (head width (μm))
yfp RNAi vg RNAi
d
ns
log (HW (μm))/log (BL (μm))
yfp RNAi vg RNAi
e
P=0.0697
log (headwidth (μm))
yfp RNAi
vg RNAi
log (body length (μm))
f
ns
% change
body length
head width
g
% change in head width
vg RNAi
1:1 line
% change in body length
head length
h
ns
log (head length (μm))
yfp RNAi vg RNAi
i
ns
log (HL (μm))/log (BL (μm))
yfp RNAi vg RNAi
j
P=0.0028
log (head length (μm))
yfp RNAi
vg RNAi
log (body length (μm))
k
*
% change
body length
head length
l
% change in head length
vg RNAi
1:1 line
% change in body length

Figure 2. Function of rudimentary wing discs in body and head growth. a) non-linear regression of the growth of the wing discs in *H. saltator* male (n=27; gray dots) and worker (n=56; purple dots) larvae during the 4 larval instars and the larval-pupal transition stage. Comparing logged body length (b), head width (c), head width to body length ratio (d), head length (h), and head length to body length ratio (i) between *yfp* (n=13; gray dots) and *vg* (n=13; green dots) RNAi treatments. Comparing regression lines between logged body length and head width (e), and body length and head length (j) between *yfp* (n=13; gray dots) and *vg* (n=13; green dots) RNAi treatments. Comparing percentage change between body length and head width (f) and between body length and head length (k). Percentage change in body length and head width (g) and body length and head length (l) of *vg* RNAi compared to 1:1 line. Horizontal black lines in b-d, f, h-i, and k indicate mean.

Figure 3.

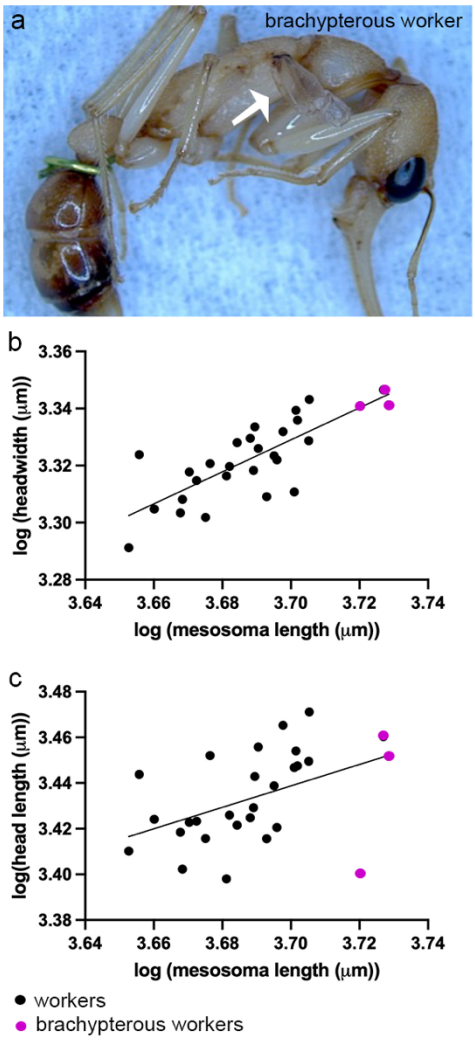

Figure 3. Correlation between body and head size and wing development. a) *H. saltator* worker with brachypterous wings (arrow). Simple linear regression of adult body size (mesosoma length) and (b) head width or (c) head length for wingless workers (n=28; black dots), and brachypterous workers (n=3; pink dots).

CONCLUDING REMARKS

With their intricate eusocial organization and complex array of morphological and behavioral diversity, ants have captured the awe and respect of many scientists and amateur hobbyists, myself included. My thesis work takes us a step closer to understanding just how these magnificent creatures became to be what they are. I am a strong proponent of the value of taking a holistic approach to understanding any aspect of biology – after all "*no [organism] is an island entire of itself*" (John Donne). As a consequence, I aimed to study the ants from a holistic approach that integrates ecology, evolution, and development, an emerging field known as eco-evo-devo. With this approach I aimed to answer the question of how ants evolved some of their most distinct morphological traits: wing polyphenism and worker polymorphism, that in turn influenced many of their complex behaviors.

In Chapter 1 of my thesis, I dedicated an extensive literature synthesis on the topic of wing polyphenism in ants. A subject, to my knowledge, not comprehensively reviewed previously. This work covered the topic of wing polyphenism truly from an eco-evo-devo perspective by covering the ecological, developmental, and evolutionary factors that led to the origin and elaboration of this trait. The goal of this work, in addition to providing all this information in one place, was to also provide testable models and hypotheses that might inspire and guide future work in this topic – mine included. How well these models and hypotheses will survive, time can only tell. For the present, the ideas presented in this work greatly influenced the experiments and implications of my proceeding work (Chapters 3-5).

Interested in the mechanism that regulates wing polyphenism during the origin and evolution of ants, writing the mechanistic section of Chapter 1 led me to the concept of programmed cell death and its multifaceted roles in development. In Chapter 2, I dove deeper into this topic and synthesized a work that outlined the ancient origins of programmed cell death, its conservation, and co-option in contributing to alternative phenotypes across animals, plants, fungi, and bacteria. This work did not aim to merely list and summarize the role of programmed cell death in dimorphic and polymorphic traits, but it sought to make a case for a repeated co-option of an ancient mechanism. Moreover, it aimed to link morphological diversity on the cellular levels with those on the organismal level by showing that the same

mechanism was operating in both. I hope this work paves the way for more interest in the varied roles programmed cell death plays in development and evolution. Roles previously not directly linked with a cell destruction process. Indeed, in my Chapters 3 and 4, I aim to show exactly this in ant morphological diversity.

The idea of major evolutionary transitions is key to how we think about ants. Ants being eusocial insects are a beautiful example of how a major evolutionary transition occurs – that is when solitary units integrate to form a higher-level organism (Szathmary & Smith, 1995). My motivation for Chapter 3 started out with wanting to test how wing polyphenism in ants was regulated at the origin, with programmed cell death being a primary candidate. For these reasons, I took a model clade approach that allowed me to compare closely and distantly related species and obtain representatives from across the major ant subfamilies. Using experimental data across the phylogeny, along with ancestral state reconstruction for ancestral nodes, I was able to predict that programmed cell death was indeed a mechanism operating at or near the origin of wing polyphenism in ants. The case for the connection between major evolutionary transitions and programmed cell death has previously been made (Durand et al., 2019; Huettenbrenner et al., 2003). Therefore, the connection between programmed cell death and the major evolutionary transition to eusociality in ants became obvious as well. Building on hypotheses made in Chapter 1 on the importance of wing polyphenism for eusociality in ants, the work in this chapter makes the case for the role that programmed cell death played in ant eusociality by degenerating wings in some individuals and thereby limiting their flight and dispersal away from the group.

Discovering that apoptosis was a conserved mechanism that is active in the rudimentary wing discs of workers across the ants naturally led to the topic of Chapter 4 of my thesis. Previous work has shown that the rudimentary wing discs have a function in generating worker polymorphism by influencing the growth of the big-headed soldier subcaste in *Pheidole* species (Rajakumar et al., 2018). The logical follow up question to ask was how the activation of the apoptosis pathway in the rudimentary wing discs is affecting the signaling mechanism released by the wing discs to influence head and body growth. Naturally, our prediction was that activation of apoptosis and the degeneration of the rudimentary wing discs stops additional

downstream signals from influencing soldier development. To our surprise, our results showed the opposite and we conclude that the apoptosis signaling in the wing discs is a necessary component of the signaling mechanism responsible for regulating soldier growth. In other words, the mechanism that is used to regulate wing polyphenism (i.e. programmed cell death) is also utilized to regulate worker polymorphism. This perhaps would not be surprising if it was any other typical pathway, however, being a mechanism for cell destruction, its role in generating morphological complexity was unexpected. The story does not end here, however. We also show that the apoptosis pathway specifically in the soldier wing discs is constitutively activating a stress-response mechanism. We predict that this stress-response mechanism might be part of the downstream signaling activated by the wing discs to regulate worker polymorphism. See below for how this environmentally activated stress-response mechanism might have been co-opted to become part of the normal physiology of soldier ants.

The last chapter of my thesis, although only providing preliminary results at this stage, holds the potential for very exciting discoveries. In this chapter, I examine if the rudimentary wing discs of a species with no worker polymorphism have the potential to regulate body and head size. In other words, I test if the rudimentary wing discs have a latent capacity for producing worker polymorphism. Functional experiments show that manipulating wing disc growth alters head size, but not body size. This suggests that the wing discs indeed do have a capacity for influencing head growth – disproportionate head size is a characteristic feature of many species with worker polymorphism. The results of this study, although promising, were not statistically robust, unfortunately due to small sample size. The species I chose to conduct this study in, *H. saltator*, although monomorphic and a beautiful study organism in many areas, has small colony size and long developmental time, making experimentation lengthy and challenging. I hope work on this question continues either in this organism or another with a similar potential for revealing a latent capacity for wing disc function.

Altogether, and simply put, my thesis makes two important contributions to understanding the developmental mechanisms contributing to how morphological diversity arose in ants. First, wing polyphenism is regulated, in part, by apoptosis and this process is conserved across the phylogeny and was likely the mechanism utilized by development at the

origin of the trait. Second, apoptosis activation in the rudimentary wing discs is necessary for worker polymorphism in *Pheidole* ants. The surprising beauty of the contribution of my thesis work is that the same mechanism (apoptotic cell death) is contributing to both forms of morphological complexity in ants. I sincerely hope that this work brings more attention and interest in the process of programmed cell death in all its forms and variations as an instrument utilized by development in unexpected ways to generate some of the diversity we observe around us.

Although I have answered many questions in my thesis, there are many that I did not have the opportunity to, and even more that naturally arose by the results of my work. Below I highlight some of these questions and topics that I think are worthy of further examination due to their potential and overall importance. I provide some possible answers and directions for tackling these outstanding questions.

Is programmed cell death involved in other transitions to eusociality in life's history?

Evolution of eusociality occurred many times independently in insects, including bees, wasps, ants, and termite, once in mammals (naked mole-rat), and at least four times in shrimp (Andersson, 1984; Chak & Rubenstein, 2019) However, communal and cooperative group living is found almost everywhere. Formation of a cooperative group is a step towards a major evolutionary transition (West et al., 2015). Programmed cell death has been identified in two traits important for eusociality: reproductive division of labor (honeybee ovaries), and limiting dispersal (ant wings) (Capella & Hartfelder, 1998) (Chapter 3). A promising avenue of research to answer the above question is to consider what other traits are important for the transition to eusociality and if programmed cell death is contributing to changing these traits in eusocial species or species that are part of a cooperative group. This opens up the opportunity to study programmed cell death in numerous traits and across many study groups and organisms.

Why was wing polyphenism important for eusociality in ants but not other social insects?

The only two completely eusocial groups of insects are ants and termites – that is, eusociality evolved once in these groups and is a universal trait. In contrasts, eusociality is not universal in bees and wasps since there are many solitary groups and groups with 'weaker' forms of social organization. Ants and termites have a wingless worker caste. Is that a coincidence or is there some evolutionary cause and effect between evolving wing polyphenism and a strong eusocial organization? The importance of wing polyphenism for limiting dispersal is undoubted. Forming a group of related or unrelated individuals is paramount for social organization which means that for group living to be maintained, the incentive or ability to disperse has to be curtailed. Since ants are ground foragers and the only function for wings is participating in mating flights (winged queens shed their wings after mating and never fly again), I predict that there was strong selection for the production of wingless workers. In species where flight is an important part of life history, such as in honeybees that fly and forage for nectar, selection for wingless forms would not be advantageous. Did this trade-off between flying to forage for food and dispersal away from the colony prevent honeybees and other social insects from becoming predominantly eusocial like the case in ants? It is certainly a

possibility. It is also important to note that wing polyphenism is not the only player that might have influenced the evolution of eusocial colony living. There are many others like nest sharing, reproductive division of labor, and relatedness, to name a few.

How are programmed cell death pathways and wing gene regulatory networks interacting with each other in ants to regulate wing polyphenism?

Results in my Chapter 3 along with numerous prior studies have shown that the rudimentary wing discs of worker ants exhibit an interruption in their gene network (Abouheif & Wray, 2002). An outstanding question that remains is whether these gene interruptions and the widespread activation of apoptosis in the rudimentary wing discs are related. Are the interruptions causing an activation of cell death? That is certainly possible considering many studies in *Drosophila* have shown such a relationship (Giraldez & Cohen, 2003; James & Bryant, 1981). Could the death of the wing tissue instead be causing a disruption in gene expression? That is also a possibility. I predict that a combination of the two might be occurring in different ant species. In species where interruptions occur in genes upstream in the network and prior to the activation of apoptosis, it's likely that this interruption is halting wing disc development and inducing apoptosis. In other species, like *H. saltator*, where the interruptions occur downstream in the network and in genes that are normally activated past the stage when apoptosis is turned on, the interruption might be caused by tissue death. This is a promising area of future research. Not only to figure out the regulatory relationship between the two mechanisms but to also see if there are any phylogenetic patterns that might reveal interesting evolutionary changes in the regulation of wing polyphenism in ants.

What is the role of the environment and particularly hormones in mediating wing disc growth and death?

An important player in insect development as a whole, and I predict also in wing polyphenism regulation, are hormones. In Chapter 1, I discuss and outline how hormones like JH, ecdysone, and insulin-like signaling might be regulating wing polyphenism. Unfortunately, I did not have the opportunity to explore this topic in my thesis. However, I believe it is a topic that can help us understand how the environment interacts with development. Part of my goal and the goal of my lab is to incorporate an eco-evo-devo approach. Environmental cues (like

nutrition and pheromones) influence development by altering hormone levels, therefore hormones act as mediators between the environment and development. We have a good understanding of how hormones, particularly JH, influences worker polymorphism (Rajakumar et al., 2012; Wheeler & Nijhout, 1981). However, the role of JH and the other hormones in wing polyphenism is an area wide open for exploration. Above I discussed how network interruptions and apoptosis might be interacting to regulate wing polyphenism. An important observation is that the rudimentary wing discs of workers are much smaller in size (compared with reproductive wing discs) even before any interruptions or apoptotic cell death occurs. What can account for this difference in initial size? I predict it to be due to lower levels of JH in the worker-destined larvae compared to queen-destined larvae. Lower levels of JH (possibly in response to lower nutrition and/or photoperiod) might cause stunted growth of the wing discs. Experiments in this area are exciting and have the opportunity to discover a missing part to the puzzle of wing polyphenism regulation, and as importantly, better understand how the environment is controlling development.

Did stress-induced evolutionary innovation play a part in worker polymorphism in ants?

An extremely interesting implication of Chapter 4 is the potential role of stress-response pathway in regulating worker polymorphic development in ants. The role of stress-response in inducing evolutionary innovation has previously been proposed in regards to cell types (Wagner et al., 2019). In many ways, worker ants are viewed as 'cell types' that are part of the larger superorganism since they specialize in performing certain functions for the colony. The findings of Chapter 4 regarding the role of the JNK stress-response pathway in regulating soldier subcaste growth remains to be explicitly tested. However, my data thus far shows that this mechanism that is typically only activated under stress conditions (Fan et al., 2014; Pinal et al., 2018), is continuously active in the rudimentary wing discs of soldier ants. This demonstrates a case where a temporary stress-response was re-wired by development to become a part of the normal physiology of the organism (Wagner et al., 2019). Recent findings by La Richeliere et al. (2022) show that worker polymorphism is highly concentrated in the tropics and areas of high temperatures and aridity. Could exposure to these extreme environments (a form of a stress) induce evolutionary innovation in ants by contributing to the development of worker subcastes

that were able to serve specialized roles in the colony? If our hypotheses regarding the role of JNK in regulating soldier growth is correct, then we can explain how (developmentally) hot and arid environments became hotspots for worker subcaste evolution.

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