The developmental basis of the head in ants: a hotspot for the evolution of ant castes

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April 2024

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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Dedication

Dedico esta tesis a Má, Pá y Lala,

siempre una inspiración, compañía y apoyo en mi vida.

.... A la Cochita y mimi, el fenotipo extendido.

Acknowledgments

First and foremost, I express my deepest gratitude to my family, whose invaluable support inspired me to pursue basic science in a country where there is no appreciation for the importance of studying the fundamental questions of life. Second, I extend my heartfelt thanks to my advisor, Dr. Ehab Abouheif, for welcoming me into his lab, supporting my ideas, providing me with new ones, and guiding me through the projects and experiments. Third, I am indebted to Bill Weislo for advocating for my career at McGill and to Callum Kingwell for imparting invaluable lessons on how to navigate the path of science, especially in the realm of insects.

Additionally, I wish to express my appreciation to Hermogenes Fernandez, whose shared passion for the ocelli in *Atta* soldiers sparked inspiration for much of this work. Fourth, I am immensely grateful to all my collaborators who embraced and bolstered these ideas, contributing with data, specimens, and insightful discussions. I extend my thanks to Dr. Arjuna Rajakumar for showing me the ethical dimensions of coauthorship in academia and for his invaluable input and discussions on my projects.

Special thanks also go to Erik Plante, Dominic Oulette, and Shahab Mohseni for their invaluable assistance with various aspects of the projects. I must also acknowledge Johanna Arnet, whose dedicated efforts as an undergraduate researcher significantly improved our understanding of ocelli and contributed numerous ideas to the paper. Finally, I am grateful to all the undergraduates who have cared for the ants and diligently maintained the experimental system, ensuring the success of our work.

To my friends who have supported me in all ways and whom I met during this path, Juan Oscar, Simon, Juan Carlos, Benita, and Victoria.

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Abstract

How novel traits originate and evolve is a fundamental question in evolutionary developmental biology. Ant colonies, with their caste determination and division of labour, provide a valuable system to study the developmental mechanisms of novelty. My thesis aims to investigate the processes driving the development and evolution of ant heads, focusing on caste-specific traits, particularly, between the queen andworker caste and within the worker caste. In the introductory chapter, I explore historical hypotheses and models addressing the emergence of novel castes and the role of the head as a phenotypic hotspot for novelty. In Chapter 1, I addressed the evolutionary pathways from simple to complex worker caste systems in ants. The evolution of morphological complexity of worker castes is reflected in head-to-body allometry, ranging from simple (monomorphic) worker castes, in which workers are relatively uniform in head and body size to complex worker castes, in which workers evolve inter-individual differences in size and head-to-body scaling. I used phylogenetic comparative methods to reconstruct the evolutionary pathways of the worker polymorphism in the leaf cutter ants. I first sampled workers across 18 species of the clade and used statistical models to classify the types of head-to-body allometry in each species. I then performed an ancestral state reconstruction to determine the evolutionary route through which complex worker caste systems evolved in this group. I discovered a novel transition between the types of worker polymorphism, including the punctuated transition from monomorphic to biphasic associated with the evolution of leaf cutting. Altogether, my thesis has taken steps toward understanding the ultimate and proximate mechanisms that contribute to the development of novel traits in castes in ants, by focusing on the head as a key driver of morphological change in the group. In Chapter 3, I tested the hypothesis that the appearance of queen-like traits in the worker caste arises from the expression of latent genetic programs and tested whether the maintenance of the developmental program respond to a novel function into the evolution of soldiers. For this, I reconstructed the evolutionary history of ocelli in Formicinae and showed that ocelli evolved within the worker caste multiple times within the subfamily and their evolution does not correlate with light-related ecological variables. I then determined the expression of their underlying GRN across castes in the species C. floridanus. To do that, first, I

established a developmental fate map detailing the regions responsible for head development in *Camponotus floridanus*, across larval stages and castes. I focused on the eve-antenna disc, which forms the adult head capsule, eyes, antennae, and maxillary palps. I used developmental genes from Drosophila melanogaster, to mark the region that gives rise to eyes (eyeless), head capsule paralog (orthodenticle-1), and antennae (Distal-less). Second, I determine the GRN of ocelli using the genes: orthodenticle-1, hedgehog, eyes absent, twin of eyeless and sine oculis. Although ocelli are not present in adult workers and soldiers in C. floridanus, I discovered the expression of the underlying ocelli GRN is conserved at 4th larvae instar, but the expressions of more downstream genes become interrupted just before larvae undergo pupation. This means that despite the organ being lost on these species, its development machinery remains latent across development. To test whether the latency responds to novel mechanisms of caste regulation instead of a consequence of pleiotropic effects. I conducted RNA interference experiments targeting the gene known to function in ocelli and head capsule development in Drosophila (otd-1). Preliminary results suggest that knockdown of orthodenticle did not penetrate the head capsule, and its knockdown in the brain does not affect head size and shape in soldiers. Instead, the knockdown of *otd-1* induces a primordium of an organ like ocelli. These preliminary results suggest that the *otd-1* function might have been co-opted to play a novel function across castes in ants.

Resumé

La façon dont les nouveaux traits se forment et évoluent est une question fondamentale en biologie évolutive du développement. Les colonies de fourmis, avec leur détermination de caste et leur division du travail, offrent un système pour étudier les mécanismes de développement de la nouveauté. Ma thèse vise à étudier les processus qui sous-tendent le développement et l'évolution des têtes de fourmis, en mettant l'accent sur les traits spécifiques aux castes, notamment entre reine-ouvrière et à l'intérieur de la caste ouvrière. Dans le chapitre introductif, j'explore les hypothèses historiques et les modèles traitant de l'émergence de nouvelles castes et du rôle de la tête en tant que point chaud phénotypique de la nouveauté. Dans le Chapitre 1, j'ai abordé les voies évolutives des systèmes de castes ouvrières simples à complexes chez les fourmis. L'évolution de la complexité morphologique des castes ouvrières se reflète dans l'allométrie tête-corps, allant des castes ouvrières simples (monomorphes), dans lesquelles les ouvrières sont relativement uniformes en taille de tête et de corps, aux castes ouvrières complexes, dans lesquelles les travailleurs développent des différences interindividuelles de taille et d'allométrie tête-corps. J'ai utilisé des méthodes comparatives phylogénétiques pour reconstruire les voies évolutives du polymorphisme ouvrier chez les fourmis coupeuses de feuilles. J'ai d'abord échantillonné des ouvriers dans 18 espèces du clade et utilisé des modèles statistiques pour classer les types d'allométrie tête-corps dans chaque espèce. J'ai ensuite réalisé une reconstruction de l'état ancestral pour déterminer la voie évolutive par laquelle les systèmes de castes ouvrières complexes ont évolué dans ce groupe. J'ai découvert une transition novatrice entre les types de polymorphisme ouvrier, y compris la transition ponctuée de monomorphe à biphasique associée à l'évolution de la coupe des feuilles. Dans l'ensemble, ma thèse a fait avancer la compréhension des mécanismes ultimes et proximaux qui contribuent au développement de nouveaux traits chez les castes de fourmis, en se concentrant sur la tête en tant que moteur clé du changement morphologique dans le groupe. Dans le chapitre 3, j'ai testé l'hypothèse selon laquelle l'apparition de traits de reine dans la caste ouvrière résulte de l'expression de programmes génétiques latents, et j'ai vérifié si le maintien du programme développemental répond à une nouvelle fonction dans l'évolution des soldats. Pour cela, j'ai reconstruit l'histoire évolutive des ocelles chez les Formicinae et montré que les ocelles ont

évolué plusieurs fois au sein de la caste ouvrière au sein de la sous-famille et leur évolution ne corrèle pas avec des variables écologiques liées à la lumière. J'ai ensuite déterminé l'expression de leur réseau génétique sous-jacent à travers les castes dans l'espèce C. floridanus. Pour ce faire, j'ai d'abord établi une carte de destinée développementale détaillant les régions responsables du développement de la tête chez Camponotus floridanus, à travers les stades larvaires et les castes. J'ai mis l'accent sur le disque œil-antenne, qui forme la capsule céphalique adulte, les yeux, les antennes et les palpes maxillaires. J'ai utilisé des gènes de développement de Drosophila *melanogaster*, pour marquer la région qui donne naissance aux yeux (*eyeless*), au paralogue de la capsule céphalique (orthodenticle-1) et aux antennes (Distal-less). Deuxièmement, j'ai déterminé le réseau génétique régissant les ocelles en utilisant les gènes : orthodenticle-1, hedgehog, eyes absent, twin of eyeless et sine oculis. Bien que les ocelles ne soient pas présents chez les ouvrières et les soldats adultes chez C. floridanus, j'ai découvert que l'expression du réseau génétique sous-jacent aux ocelles est conservée au 4e stade larvaire, mais que l'expression de gènes plus en aval est interrompue juste avant que les larves subissent la nymphose. Cela signifie que malgré la perte de l'organe chez ces espèces, son mécanisme de développement reste latent tout au long du développement. Pour vérifier si la latence répond à de nouveaux mécanismes de régulation des castes plutôt qu'à une conséquence d'effets pléiotropes, j'ai mené des expériences d'interférence par ARN ciblant le gène des ocelles et de la capsule céphalique chez Drosophila (otd-1). Les résultats préliminaires suggèrent que les réductions d'orthodenticle n'affectent pas la taille et la forme de la tête chez les soldats. Au lieu de cela, la réduction de otd-1 induit un primordium d'un organe semblable à des ocelles. Ces résultats préliminaires suggèrent que la fonction encore inconnue d'orthodenticle pourrait avoir évolué pour jouer une nouvelle fonction à travers les castes chez les fourmis.

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List of abbreviations

MYA	Millions of years ago
HW	Head width
WL	Weber length
AIC	Akaike information criterion
CV	Coefficient of variation
MCMC	Markov Chain Monte Carlo
JH	Juvenile hormone
SD	Standard deviation
GRN	gene regulatory network
HCR	Hybridization chain reaction
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
YFP	yellow fluorescent protein
ANOVA	Analysis of variance
РР	posterior probability
SEM	Scanning electron microscope

OTX	Orthodenticle
MET	major evolutionary transitions
SET	standard evolutionary synthesis
EAD	Eye-antenna imaginal disc

EAD

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Contribution to the Knowledge

All chapters of my thesis constitute original scholarship performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy at McGill University. This thesis is structured around two manuscripts and embodies my original contributions, supported by contributions from several collaborators. For the two Chapters, I am the sole first author. Both chapters are in the process of being submitted for publication.

Chapter 1. *Reconstructing evolutionary transitions to worker caste complexity in the attine ants: Wilson's Transitions Revisited and Revised*

Angelly Vasquez-Correa, Hermogenes Fernández, Shahab Mohseni, Jeffrey Sosa-Calvo, Erik Plante, Dominic Ouellette, Cameron Currie, William Wilson, Ehab Abouheif.

Angelly Vasquez-Correa and Ehab Abouheif conceived the project. Angelly Vasquez-Correa designed the experiments and conducted measurements and analysis. Shahab Mohseni conducted measurements. Hermogenes Fernández, Jeffrey Sosa-Calvo, Cameron Currie collected ants. Erik Plante and Dominic Ouellette took the image in Figure 2. William Weislo provided space and materials for collecting and conducting measurements in Panama. Angelly Vasquez-Correa and Ehab Abouheif wrote the manuscript.

Chapter2. Latent genetic potential underlies the evolutionary reversibility of ocelli in workers in *Formicinae*.

Angelly Vasquez-Correa, Johanna Arnet, Travis Chen, Ehab Abouheif

Angelly Vasquez-Correa and Ehab Abouheif conceived the project. Angelly Vasquez-Correa and Johanna Arnet gathered ocelli and ecological variables data. Angelly Vasquez-Correa conducted phylogenetic analysis. Angelly Vasquez-Correa and Travis Chen performed gene cloning and dsRNA construction, and Angelly Vasquez-Correa conducted the HCR and RNAi injections. Angelly Vasquez-Correa and Ehab Abouheif wrote the manuscript.

1. Introduction

This work has been developed under the framework of evolutionary developmental biology, a field of study that offers a comparative and integrative approach to address the most intricate questions on the origin of morphological complexity across the tree of life. Under this framework, the general goal of this thesis is to study the development and evolution of caste complexity in ants. Ants are eusocial organisms that have evolved the complexity of social living through the elaboration of distinct morphological castes. In the evolution of castes in ants, these organisms first evolved into queens and worker castes as mechanisms of reproductive division of labor. Second, through the elaboration of worker caste. The evolution of caste complexity has allowed ant colonies to allocate tasks efficiently and adapt to their environments. By investigating the evolutionary mechanisms underlying social complexity and how these processes may contribute to our overall understanding of the evolution of organismal complexity across different biological scales.

The overall thesis is centered on the evolution of worker caste complexity. Thus, each chapter of this thesis analyzes the complexity of worker castes, covering from the proximate to the ultimate steps that ant colonies have passed through to elaborate the diversity and complexity of the worker caste systems. The first chapter provides a literature review addressing the evolution and development of complex caste systems from the concept of novelty. This chapter explores the foundational theories and empirical studies that have shaped our understanding of how novel castes evolve in ants and the main hypothesis and predictions in the development of these morphological units. This chapter sets the stage for subsequent empirical investigations and research questions into the study of caste complexity in social organisms.

In the second chapter, the main objective is to investigate the evolutionary pathways leading to the development of complex caste systems in ants. Specifically, to test one of the most widely accepted hypotheses regarding caste evolution, proposed by Edward Wilson in 1953 (Wilson, 1953). Wilson's hypothesis suggested that the evolution of worker castes occurs through changes in the head-to-body allometry, using the allometric function $y = aX^b$. According to Wilson's hypothesis, shifts in the slope and intercept of the head-to-body regression drive the evolution of complex caste systems via transitional changes in allometry. Therefore, the evolution of more complex worker caste systems is preceded by simpler forms of worker caste variation explained by simpler allometry equations.

In this thesis, I test Wilson's hypothesis by using the group of leaf-cutter ants, a clade known for their highly complex worker caste systems. To validate or challenge Wilson's hypothesis, I reconstruct the head-to-body allometry of workers across the phylogeny of this group. This chapter is the first attempt in the field to show how allometric changes might influence the diversification of worker morphology within a colony, ultimately contributing to the evolution of more complex and hierarchical caste systems in ants. Therefore, this chapter offers valuable insights into the evolution of worker caste complexity and the broader implications for complex social systems. In this chapter, I reveal that the evolution of worker caste types does not follow a simple, stepwise pattern on the evolution of head-to-body allometry. Instead, the mechanisms driving caste polymorphism may be more unpredictable, occurring in punctuated steps. This chapter highlights the effectiveness of allometry analysis in explaining morphological variation and the intricate levels of complexity found in caste systems.

The third chapter of this thesis explores the developmental mechanisms that drive caste-specific traits in ants. This research reveals that the evolution of queen-like traits in worker ants can be attributed to developmental ancestral potentials, which means that the developmental program remains latent and can be reactivated under specific developmental and evolutionary conditions across castes in ants. Specifically, it focused on the ocelli, three simple eyes located on the dorsal side of the head, commonly found in most flying insects. In the evolutionary history of worker ants, ocelli have been repeatedly lost but also regained in specific lineages. The findings in this chapter show that within the subfamily Formicinae, one of the most hyper-diverse clades of ants, the ocelli evolved only once in this group. However, this trait has been independently lost several times in worker ants and reversed twice in the soldier caste of two species. The underlying developmental capacity remains latent in species where ocelli have yet to develop in workers. This phenomenon highlights the role of developmental ancestral potentials in facilitating the re-

evolution of lost traits and underscores how these latent mechanisms can contribute to evolutionary changes throughout the evolution of caste in ants.

Overall, this piece of work provides insights into the mechanisms that explain how colonies generate complexity through the elaboration of worker caste polymorphism. Although worker caste polymorphism has been extensively studied from behavioral and anatomical perspectives, the developmental mechanisms that colonies use to generate this complexity have remained largely unexplored. This work sheds light on these developmental processes, revealing how changes in growth patterns can lead to the emergence of diverse worker morphologies within a colony and how ancestral developmental potentials remain central to our understanding of developmental programs and the evolution of caste identity. By integrating developmental biology with evolutionary theory, this study deepens our comprehension of caste evolution and opens new avenues for research into the intricate dynamics of social organization in ants.

Chapter 1. Literature review

1.1 Novelty as a concept, has shaped the field of evolutionary developmental biology.

The question of how novelty evolves is essential to our understanding of the origins of morphological complexity in nature (West-Eberhard, 2003a). The origin of novel traits is an old and still unsolved question. Darwin himself acknowledged the importance of the evolutionary question of novel traits. However, he also recognized the challenges associated with defining them in the first place: "felt much difficulty in understanding the origin of simple parts" (Darwin, 1988), by which he opted to leave the explanation outside the scope of natural selection, instead choosing a more Lamarckian approach, when he proposed that evolutionary novelties are environmentally induced (Muller & Wagner, 1991; West-Eberhard, 2003a). The classic definition of novelty defines novel traits as such that are neither shared with the ancestor of the organisms (homologous) nor identical to any other trait within the same organism (serial homology) and play a novel functional/ecological role (Muller & Wagner, 1991; Pigliucci, 2008). Based on this definition, traits such as the turtle shell, the transition from fish fins to tetrapod limbs, the origin of neural crest, and the evolution of multicellularity have been broadly recognized as novelties (Gilbert et al., 2001; Hall & Kerney, 2012). Although this definition set the beginning of studying the origin of novel traits, it remained insufficient to explain the characters and the mechanisms that originate novelty in nature.

The concept of novelty is crucial for our understanding of the evolution of morphological diversity and organismal complexity. In evolutionary studies, the concept has gone through various interpretations and definitions as far as new paradigms emerge. Likewise, the interpretation of novelty relies on the biological scale at which it is intended to define a novel feature. For instance, the concept of novelty may encompass the molecular definition (Kapheim, 2016), phylogenetic (Hall, 2003), or a more mechanistic developmental perspective (Levis et al., 2018; Muller & Wagner, 1991; West-Eberhard, 2003a). The fact that novelty has been defined from different levels or organizations and approaches has brought disparities, and misinterpretations around the concept, especially in delineating the phylogenetic and genetic

scale at which a character, a developmental mechanism, or a gene should be considered to originate de "novo" (Brigandt & Love, 2012; Moczek, 2008; Peterson & Müller, 2013; West-Eberhard, 2003a).

Much of the limitations on defining novelty in the first place rely on adjusting the term under the framework of the modern synthesis or Standard Evolutionary Theory (SET) (Peterson & Müller, 2013). SET is generally defined asallelic changes underlying pre-existing continuous traits, caused primarily by mutation and driven by selection, genetic drift, or migration over time (Gould, 1979; Muller & Wagner, 1991). Under the SET, novelties are hard to track deep into their origins since the selection mechanisms are usually over existing variation, changes tend to be gradual at the population level, and the results in phenotypic changes are usually small (Pigliucci, 2008). Therefore, the only explanation left for the origin of novelties would be genetic mutations caused by de novo or larger-scale genomics events affecting developmental processes, such as gene duplication, horizontal transfer, and gene regulatory network modifications (Levis et al., 2018; Mayr, 1960; Müller, 2020; Simpson, 1953).

Many of the tenets of the modern synthesis of evolutionary theory have yet to be shown to explain all the evolutionary origins of new traits and species. For instance, the evolution of the phenotype is not always gradual, traits are not always adaptive, and developmental mechanisms account for most of the morphological variation instead of single genes (Müller, 2010). Thus, the field of evolutionary developmental biology (Evo-Devo) has reshaped the fundamental evolutionary questions and concepts around the origins of novelty, as this field provides a broader spectrum of mechanistic explanations that go from environmental, epigenetic, and developmental processes, and how those mechanisms individually or in concert, control the expression of genes and their regulatory networks bringing morphological novelties (Abouheif et al., 2014; Hall & Kerney, 2012; Moczek, 2008; Muller & Wagner, 1991; Zhang et al., 2010). Therefore, evo-devo provides a complete framework from the ultimate (evolutionary) consequences of the proximate (developmental) mechanism of phenotypic variation and novelty.

Evo-Devo has shown that the origin of novelty relies on different levels of the organisms at which a novel feature could emerge. Therefore, three main definitions of novelty have been established to provide a more pluralistic explanation and classification and allow more predictability within the context of the development and evolution of novel traits (Müller, 2010, 2020b; Muller & Wagner, 1991; Peterson & Müller, 2013). Thus, novelties Type 1, refers to a novel body plan, Type 2 a structural element with a non-homologous counterpart, more specifically a discrete trait added to an existing body plan, for instance, avian feathers, turtle shells, and Type 3, a unit of character that has been individualized due to variation in a new direction that was not possible before, this type of novelty is related more to specialized characters that originated from pre-existing body plans, such as beaks shapes in Darwin finches, horns in beetles (Peterson & Müller, 2013).

The emergence of novel traits, as explored through the lens of Evo-Devo, highlights the intricate and interconnected processes that shape the evolutionary landscape. These novelties, whether at the level of entire body plans, structural elements, or specialized characters, exemplify the dynamic transitions throughout evolutionary history. Major evolutionary transitions, such as the shift from single-celled to multicellular organisms or the development of complex body structures, often parallel the emergence of these novelties. By understanding the mechanisms and definitions of novelty within Evo-Devo, we gain deeper insights into how these transitions drive the diversity of life and the continual adaptation of organisms in response to their environments.

1.2 Ants, the major transitions in the evolution of complexity and the emergence of novelty

1.2.1 Eusociality as a major transition in the evolution of complexity in nature

Evo-Devo aims to unravel the processes through which morphological diversity and complexity emerge, tracing their origins from individual cells to entire ecosystems. The evolutionary events in which most of the novelty arises comprise the major transitions in the evolution of complexity in nature. Maynard Smith and Szaathmary (1995) laid the foundational framework for delving into the study of the emergence of living complexity through their concept of major transitions in evolution (MET): "*If we see, even in rudimentary form, that originally independently reproducing units join, somehow use functional synergies among the units, and that there is some novelty in the inheritance system as well, then the population is on its way to a "major transition*"" (Szathmáry & Smith, 1995). Thus, MET explains the evolution of novelty, such as chromosomes from the integration of individual genes, multicellularity from the integration of unicellular organisms, obligate symbiosis from the integration of two distantly related organisms, and eusocial colonies from the integration of solitary organisms (Boomsma & Gawne, 2018).

One of the major transitions in the evolution of complexity is Eusociality, defined as the most complex way of social integration, representing an evolutionary transition from solitary to obligate group living. Eusociality is a group dynamic characterized by the reproductive division of labor, generational overlap, and adult care for the young. Eusociality has evolved independently in insects (Isoptera, Hemiptera, Coleoptera, Hymenoptera), crustaceans, and mammals (Wilson & Hölldobler, 2005). The evolution of Eusociality has resulted in a series of evolutionary novelties that have facilitated the complete integration and innovation of these systems, one of the most popular and major evolutionary novelties in these organisms comprises the evolution of reproductive labor, in which individuals from the same colony develop a differential reproductive capacity (Hölldobler et al., 1990)

Although the causality for the evolution of Eusociality, which occurred several times independently in insects and mammals, remains debated (Kramer & Meunier, 2016), it has been highly accepted that Eusociality in the Hymenoptera evolved through altruism, in which some individuals stop their reproductive capacity to help their siblings to reproduce (Hamilton, 1963). The evolution of reproductive division of labor contradicts the basis of the standard natural selection, where organisms are favoured to maximize their fitness through their reproductive success (Darwin, 1988; Huxley, 1888). However, the fact that in eusocial groups, some organisms give up their reproductive potential to work to maximize the reproductive potential of their counterparts, could be explained if selection is applied to the whole family. More precisely, if there is a degree of genetic linkage among individuals and small population size, just so natural selection could favor altruistic genes (Darwin, 1859, p.204)(Dugatkin, 2007; Haldane, 1932). This explanation was postulated as a kin-selection theory theoretical framework (Hamilton, 1963).

Under the framework of kin selection, the success of individuals that suppress reproduction in a population is given by the spread of their genetic pool in helping their relatives to rear their offspring and increase their fitness, so that individual fitness is the sum of their direct and indirect fitness components, what is called inclusive fitness (Hamilton, 1964). From this,

Hamilton's rules are expressed as C < rB, where C is the cost to the helper (the offspring not produced), B is the benefit to the recipient (number of offspring) and r is the relationship between the two. Under this theory, groups evolve and persist as cohesive organisms only if they are genetically related. Thus, social evolution does not maximize individual fitness when relatives are present, but inclusive fitness is (Hamilton, 1963, 1964).

Although kinship plays an essential role in the evolution of Eusociality, especially in hymenopterans, inclusive fitness might not account for the maintenance of Eusociality and all the emerging properties that arise because of group living (Korb & Heinze, 2004; Kramer & Meunier, 2016; Nowak et al., 2010, 2017). Thus, multilevel selection, the selection of group traits over individual traits (kin selection), could explain many of the social traits that emerge from Eusociality, especially existing traits that could contradict the kinship framework, such as the case of polygamy and intracolonial conflict, these specific cases led to a reduction of genetic similarity among sisters (Korb & Heinze, 2004). For instance, species that evolved Eusociality exhibit a range of evolutionary variations that might be explained by group adaptation or additive factors among individuals, such as the case of eusocial species that evolved polyandry, in which the queen reproduces multiple times with different males, reducing the relatedness among reproductive versus non-reproductive individuals. In other cases, workers in eusocial organisms have retained the capacity to reproduce and lay male eggs, which increases their relatedness with their sons rather than sisters. Therefore, based on Hamilton's equation, colony conflict increases among reproductive groups. However, through the multilevel selection framework, these traits could be favoured at the colony level if they benefit the entire unit (Korb & Heinze, 2004).

1.2.2 Ants evolved Eusociality once, followed by several novelties in the group

Originating during the early Cretaceous, ants are a monophyletic group of eusocial insects of Hymenoptera. This group comprises the wasps, ants, and bees (Wilson & Hölldobler, 2005). Ants can be divided into stem and crown ants; stem ants are the extinct lineages, whereas crown ants are extant lineages that belong to the family Formicidae. For both groups, there is evidence for the evolution of Eusociality, suggesting that Eusociality has evolved in the common ancestor of Formicidae (Boudinot, Khouri, et al., 2022; Boudinot, Richter, et al., 2022; Hanna & Abouheif, 2021). Ants originated from the Formicoidea group, known for their ground-hunting behaviors; the species belonging to this group exhibited a sub-social lifestyle, where

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reproductive females prolonged their parental care, resulting in an overlap of generations, which elevated their relatedness to the extent that it led to the evolution of their eusocial way of life. Likewise, the reproductive division of labor and the maintenance of monogamy seem to have played an essential role in the evolution of Eusociality in ants by maximizing the relatedness and minimizing the intracolonial conflict (Boomsma et al., 1999; Ward, 2014; Wilson & Hölldobler, 2005).

The major morphological and behavioral synapomorphies in ants originally came from their predatory behavior and ground lifestyle, possibly correlating with Eusociality's origin. Among the significant morphological novelty in ant evolution, the first is the wing removal from the queen once is fertilized and the loss of wings in non-reproductive workers (Hanna & Abouheif, 2021). Since all the sisters of the colony shared the same genome, these discrete differences between reproductive and no reproductive are a case of wing polyphenism, which is the ability of a single genome to produce two or more alternative morphologies in response to an environmental cue (Boudinot, Richter, et al., 2022; Hanna & Abouheif, 2021). Second, the metapleural gland is an organ that evolves to provide antibacterial protection, and third, the elongated and elbowed scape and a petiole (Boudinot, Khouri, et al., 2022). In addition to morphological novelties, ants have developed various ecological interactions contributing to their ecological success. They have formed symbiotic relationships with hundreds of organisms; some lineages have also evolved specific adaptive specializations, including cultivating fungi, harvesting seeds, communal nest weaving, social parasitism, mutualisms, and slave-making. (Davidson, 1978; Hölldobler et al., 1990; Mueller et al., 2005; Topoff, 1990; Ward, 2014). These diverse adaptations have played a crucial role in their ability to thrive in various ecological niches.

Crown ants represent one of the most ecologically successful organisms on the earth, with around ~15000 known ant species, and an estimated number of 20×1015 individuals (AntCat, 2023). The latter corresponds to a biomass of ~12 megatons of dry carbon (Schultheiss et al., 2022). Most crown ants (~90%) belong to the Formicoid clade (Moreau et al., 2006; Ward, 2014). This clade possesses species with the most novelties to ants to increase their complex social organization, species diversification, and geographical expansion. This clade represents species that have crossed the 'point of no return,' characterized by dramatic differential

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reproductive capacity and morphology between queen and worker castes, to the level where individual separation becomes an irreversible process (Abouheif, 2002; Hanna & Abouheif, 2021; Khila & Abouheif, 2010; C. Peeters, 2019). Likewise, species belonging to this group have evolved multiple times independently of worker caste polymorphism, in which non-reproductive individuals vary in their morphology, bringing high task specialization of the labor (Else J. Fjerdingstad, 2006; W. M. Wheeler, 1911; Wills et al., 2018; Wilson, 1953). These events in caste evolution have positioned ants within groups that showcase the most intricate caste systems among all eusocial insects.

1.3 The caste system in ants: novelty at the point of no return

1.3.1 The evolution of the caste systems in ants

Our understanding of the evolution of Eusociality and the emergence of complexity relies on deciphering the origin of caste in those systems. In ants, the caste system evolved at first through the reproductive division of labor. Second, colonies had the evolution of queen-worker polyphenism, in which discrete morphological features between the queen and workers facilitated the elaboration of specialization of labor and increased colony efficiency (C. Peeters, 2019; Wilson, 1953). Interestingly, the evolution of queen-worker polyphenism in ants seems to have evolved coordinately with the evolution of Eusociality, thus from the fossil record the first distinct castes comprise the winged reproductive and wingless non-reproductive (Barden & Grimaldi, 2016; Hanna & Abouheif, 2021), indicating that queen-worker wing polyphenism evolved once at the same time as Eusociality, which probably contribute to reinforce the group living and promote innovation of caste in these organisms (Hanna & Abouheif, 2021).

In the evolution of queen-worker polyphenism, reproductive queens typically live longer and possess wings, fully developed ovaries, and spermatheca. They also feature larger thoraxes with well-developed wing muscles, extensive metabolic reserves, bigger eyes, and additional ocelli: three simple eyes at the dorsal side of the head, that aid in flight stabilization. In contrast, subordinate workers lack wings and have degenerate or reduced ovaries and spermatheca. They also possess smaller thoraxes and eyes; in most species, workers lack ocelli. (Hölldobler et al., 1990; C. Peeters, 2019; C. Peeters & Molet, 2010). Besides the evolution of reproductive castes, ants evolved inter-individual variation within the non-reproductive caste, called worker caste

polymorphism (Wilson, 1953). Whether worker caste polymorphism has already evolved in stem ants remains unknown. However, the fossil record has shown some degree of size differences between wingless individuals in some of the fossil records (Cao et al., 2020). In crown ants, worker polymorphism evolved eight times in groups of ants and was estimated to occur in 13% of the approximately 337 ant genera (Blanchard & Moreau, 2017; La Richelière et al., 2022; Wilson, 1953). This evolutionary feature is thought to be facilitated by queen-worker polyphenism, wherein the selection pressure for reproduction is relaxed in non-reproductive. Therefore, workers face selection pressures corresponding to their specific roles within the colony (Wills et al., 2018)

The evolution of the division of labor among workers is highly constrained by colony size. Usually, bigger colonies harbor higher morphological variation among workers (Bourke, 1999; Ferguson-Gow et al., 2014, 2014; Lecocq de Pletincx et al., 2021). One hypothesis on the evolution of worker castes states that in large colonies, individual variation in size emerged primarily due to changes in overall body size. These alterations enabled individuals to specialize in tasks aligned with their size, enhancing their efficiency in performing these tasks, called the ergonomic framework (Wilson, 1968). Thus, natural selection would favor size variation as an optimization of the colony efficiency, which is explained by task partitioning at first with subsequent morphological specialization afterward, such as in the case of the evolution of soldiers, a highly specialized caste in conducting task more related with defense and food processing.

Unlike queen-worker polyphenism, which evolved once, and worker caste polymorphism that evolved multiple times across the phylogeny, ants have also evolved wingless reproductive queens, known as ergatoid in 55 genera and short-winged queens or brachypterous in 15 genera (C. Peeters, 2012), or workers with fully functional spermatheca, an organ for sperm storage, called gamergates, that evolved in about 200 species (Monnin & Peeters, 2008; C. P. Peeters, 1991). Rarely, colonies may develop anomalies, such as gynandromorphs; individuals with male and female traits, or intercastes; anomalous intermediates that develop traits in between winged queens and workers (Molet et al., 2012; Okada et al., 2013; C. P. Peeters, 1991; Yang & Abouheif, 2011). Although there have been several reports of those mosaics, their role in the colony and the evolutionary significance of these individuals has not been well established, however, although these mosaics are rare and, in many cases, non-adaptive at the colony level, their appearance has highlighted the plastic development of caste and opportunity for the evolution of novel castes. Therefore, these anomalies have set the basis for several hypotheses that have aimed for decades to elucidate how novel castes evolve and develop in the colony (Gregg, 1942; Metzl et al., 2018).

1.3.2 Development of castes in ants

Ants belong to the group of holometabolous insects, in those organisms, every organ develops from imaginal discs, a semi-independent cluster of cells (Beira & Paro, 2016; Held Jr, 2002). Imaginal disc development begins during embryogenesis, when clusters of cells set aside during larval development and form a 2D disc in later stages of larval development that are precursors of adult structures, such as the head and wing (Held Jr, 2002). They originated from the embryonic ectoderm, composed of two epithelial layers: the disc proper and a peripodial membrane that encases the disc. Given cell proliferation, each disc grows until pupation develops to form the imaginal disc, which invaginates and forms the body wall and appendages (Held Jr, 2002). For instance, there are 19 discs in *Drosophila melanogaster*: nine pairs form the head and thorax wing and a medial disc for genitalia. The imaginal disc identity and its developmental trajectory are regulated by highly conserved regulatory networks, morphogen gradients, and hormonal regulation, which ultimately will determine cell growth, proliferation, and tissue identity (Held Jr, 2002; Koch & Abouheif, 2019).

During development, the response of every imaginal disc to the environment is mediated by thresholds or reaction norms. Thresholds produce nonlinear phenotypic responses, whereas reaction norms produce linear phenotypic responses (Nilsson-Örtman & Rowe, 2021; Pigliucci et al., 1996). Thresholds and reaction norms play a crucial role in altering growth parameters and reprogramming of organ growth. These developmental changes ultimately influence the timing and critical size at which each caste develops, therefore, their morphological differences at the adult stage (Abouheif, 2021; D. E. Wheeler, 1991).

In ants, larvae undergo two main developmental mechanisms: caste determination and differentiation. Caste determination sets embryos or larvae down specific developmental pathways, which subsequently allows larvae to pass through growth and morphogenesis,

therefore, caste differentiation (Trible & Kronauer, 2017, 2020). In ants, caste determination is mainly driven by environmental cues that ultimately determine the larvae's fate (Lillico-Ouachour & Abouheif, 2017; Molet et al., 2012). However, several studies have already established that female eggs can have different propensities to develop into queen or worker caste, or minor worker and soldier caste. In the case of the reproductive caste, genetic mechanisms such as maternal effects can strongly determine larvae to undergo reproductive fate (Libbrecht et al., 2013). Likewise, genetic influence on the development of worker polymorphism has been reported in cases where several males inseminate the queen. Therefore, genetic differences can, in some species, exert a large influence on the morphological differences among workers' subcastes (Anderson et al., 2008; Boomsma et al., 1999; Libbrecht et al., 2016; Schwander et al., 2005; Trible et al., 2023).

Although genetic mechanisms can influence caste determination and differentiation in some instances, the concept of caste in ants has been widely recognized as a case of phenotypic plasticity. In this context, environmental and social factors influence the fate of ant larvae, such as nutrition, temperature, colony size, and workers' behavior, particularly how and how much they feed the brood (Alvarado et al., 2015; Lagos-Oviedo & Sarmiento, 2021; Linksvayer et al., 2012). Nutrition is known to be asymmetric in eusocial insects, in which queen-destined larvae are fed more than worker-destined, likewise, soldier-destined are fed more than worker-destined (D. E. Wheeler & Nijhout, 1981). Such is the case of the species of the genus *Pheidole*, a hyperdiverse genus of ants, in which worker caste polymorphism has evolved into two distinct morphological subcastes: workers and soldiers. In Pheidole species, the activation of the soldier or worker program is determined by a nutritional switch point or threshold that activates Juvenile Hormone (JH), a hormone involved in organ growth. Thus, high levels and nutritional quality activate the soldier programs, whereas a lower dose and lower quality maintain the worker program (Libbrecht et al., 2013; Nijhout & Wheeler, 1982; D. E. Wheeler & Nijhout, 1981). Besides nutritional inputs, caste development is influenced by the interaction with queen or worker pheromones, temperature, worker fluids, and policing, among others (Knaden, 2016; Psalti & Libbrecht, 2020).

1.4 An allometry framework for explaining the evolution and development of worker caste in ants

Although queen-worker polyphenism is common across ant species, worker polymorphism is less universal. Worker caste polymorphism has evolved independently at least 23 times, accounting for about 4,571 of the 15,518 ant species (La Richelière et al., 2022; Wilson, 1953). While worker caste polymorphism has developed in relatively few families, the species that possess worker caste polymorphism tend to be from species-rich genera (Economo et al., 2015; Frumhoff & Ward, 1992; La Richelière et al., 2022; Oviedo et al., 2003; Wcislo & West-Eberhard, 1995). The independent evolution of worker caste polymorphism in several ant lineages suggests that this colony trait is evolutionarily flexible and has contributed to increased social and morphological complexity within colonies (Blanchard & Moreau, 2017)

In 1953, E.O. Wilson proposed an allometry hypothesis to explain the development and evolution of worker subcastes. He suggested that worker polymorphism primarily evolved through changes in head-to-body allometry, where proportional growth of the head relative to body size varies among individuals within a colony. Wilson hypothesized that worker polymorphism began with size variation without differential head growth relative to the body (isometry), derived from an ancestral monomorphic state where individuals were morphologically similar. As worker size variation expanded, some individuals exhibited disproportionate head growth relative to body size, leading to changes in shape. Wilson described these allometric changes as biphasic, triphasic, and finally dimorphic, based on the number of groups that resulted from changes in the slope across the head-to-body regression. He also outlined the evolutionary transitions that might explain the evolution of the different types of worker caste polymorphism, suggesting, for instance, that species with diphasic allometry evolved from monophasic allometry and so on (Wilson, 1953).

In 1991, Diana Wheeler reinterpreted Wilson's allometry transitions through a developmental lens, suggesting that size variation and allometric changes are driven by three main developmental processes: (1) changes in critical size, (2) changes in growth parameters, and (3) organ reprogramming (Wheeler, 1991a). For species with linear isometry but increasing size variation, changes in critical size, i.e., the larval size at metamorphosis, which correlates with the adult size. This variation may result from colony regulation, where workers differentially feed larvae, or from nutrition availability, causing some individuals to metamorphose earlier than others and thus leading to size differences across individuals. These size changes also influence

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allometry parameters, altering intercepts and slopes of head-to-body relationships across colony members. Breakpoints in head-to-body regression occur only when organ growth is reprogrammed, leading to developmental switches where larvae follow different developmental pathways in response to external inputs. These nonlinear responses establish developmental thresholds that differentiate morphological subcastes. Although previous studies have demonstrated that worker polymorphism arises from nutritional changes and developmental thresholds (Nijhout & Wheeler, 1982; Rajakumar et al., 2018a; Wheeler & Nijhout, 1981), the evolutionary mechanisms behind these allometric changes remain hypothetical.

1.5 The endless debate on how novel caste originated and evolved in ants

The evolution of the caste system in ants remains ongoing research with several pluralistic frameworks that explain the proximate and ultimate mechanisms of caste morphological differentiation and novelty. The theoretical framework for understanding caste development and evolution remains contentious, marked mainly by three models. These three models propose a different mechanism for caste determination and subsequent differentiation, arguing whether novel castes are derived from existing caste programs or whether castes arise de novo through novel developmental mechanisms.

The "standard model", highlights the importance of developmental thresholds and the modularity of the developmental systems (Abouheif, 2021), suggesting that novel castes arise from preexisting developmental programs. Therefore, new variations arise as mechanisms acting upon the developmental thresholds of traits independently of size. During development, size and shape are decoupled, so new variants arise because of independent reprogramming events that determine organ growth and coordination and act semi-independently for each trait. (Boudinot et al., 2021; Rajakumar et al., 2018; D. E. Wheeler, 1986, 1991).

Two alternative models to the standard model are the "hourglass model" and the "Baroni and Urbani" model. The hourglass model proposes that novel caste is the result of linear responses or reaction norms of larvae size across development, under this model, size changes mainly drive the fate of the caste as a response to hormonal inputs across the reaction norm (Trible & Kronauer, 2020). More specifically, it proposes that caste determination is firstly caused by the factors that affect the size of the larvae, where small larvae develop workers-like traits, falling

onto one side of the spectrum. Larger-sized individuals fall on the other extreme and develop more queen-like traits. Therefore, small larvae fail to develop queen-like traits because they do not reach the size thresholds that allow queen traits to develop. Under this model, novel castes are explained as new variations that either fail in the worker or queen program. For instance, under this hypothesis, soldiers are the intermediates across the reaction norms. Therefore, soldiers are a mix of queen and worker-like traits (Trible & Kronauer, 2020).

The evolution of caste systems in ants remains an understudied area, with ongoing research gradually uncovering the mechanisms behind caste differentiation and novelty. The standard model highlights the influence of pre-existing developmental programs and modularity in shaping new castes. In contrast, alternative models like the hourglass and Baroni and Urbani models provide different views on how size and hormonal factors affect caste outcomes. These diverse frameworks underscore the complex interplay between genetic, developmental, and environmental factors that shape the specialized roles within ant colonies. As research continues to explore the question of caste evolution, it is increasingly evident that the complexity of caste systems requires further investigation and integrating multiple theoretical approaches.

1.6 The standard model, caste-specific traits, and the role of ancestral developmental potentials on the origin of caste in ants

Mary Jane West-Eberhard stated, "In the origin of novel traits, the environment is a factor of change and the source of ancestral phenotype" (West-Eberhard, 2003a). The origin of novel traits has been explained by repurposing existing developmental and genetic mechanisms into new outcomes (Hall & Kerney, 2012; Moczek, 2008; West-Eberhard, 2003). Interestingly, although there is a remarkable diversity and phenotypic variation at intra and inter-specific scales, current data reveals that only a few genetic changes in highly conserved genes are needed to bring new morphological outcomes in the individual. These changes are thought to be less disruptive because of the modular nature of developmental mechanisms (West-Eberhard, 2003a)

In ants, castes are developed by the influence of the environmental and developmental mechanisms of imaginal disc growth and coordination which develops differentially across castes. Caste-specific traits in ants play a crucial role in the evolution of caste systems within their colonies. These traits, such as morphology, behavior, and reproductive roles, are deeply

intertwined with the division of labor that defines ant societies. For example, worker ants typically exhibit smaller body sizes and lack reproductive organs, wings, and ocelli. At the same time, queens are larger and develop wings and ocelli, ovaries fully develop, and spermatheca, traits that go along with the reproductive role. These traits are not merely the result of immediate environmental conditions but are shaped by evolutionary pressures that have led to the differentiation of castes over time.

In ants, caste identity is based on traits that develop in a caste-specific manner, giving morphological distinction to each caste within the colony. The evolution of caste-specific traits in ants is believed to be driven by natural selection and ancestral developmental potentials. Ancestral potentials refer to the inherent genetic and epigenetic frameworks inherited from an ant's evolutionary predecessors, which can influence how caste traits are expressed. The case of wings has remained a remarkable example of the interplay between developmental ancestral potentials and the origin of novel caste in ants. Wing polyphenism, which is the ability of embryos laid by the queen to develop into winged queens or wingless workers in response to environmental factors, is a nearly universal feature of ants (Abouheif & Wray, 2002; Nijhout & Wheeler, 1982a; Passera & Suzzoni, 1979; Ward, Brady, Fisher, & Schultz, 2015). In the Pheidole species, although wings are absent in adult minor workers and soldiers, pair-forewing rudiments develop in soldier larvae. In *Pheidole*, Rajakumar et al. (2018) knocked down the expression of precursor genes of wing development (*vestigial* (vg)) using RNA-mediated interference (RNAi) in soldier-destined larvae. Like Drosophila, vg RNAi significantly reduced the size of and induced apoptosis in rudimentary forewing discs. However, unlike Drosophila, reducing the size of the soldier's rudimentary forewing disc altered the head-body size and scaling by reducing the growth of the head relative to that of the body. These results show that the rudimentary forewing disc in soldiers regulates size, disproportionate head-to-body scaling, and soldier subcaste determination. Therefore, the mechanism represents a possible co-option of an ancestral developmental program leading to the independent origin of allometry regulation leading to worker caste polymorphism (Behague et al., 2018; Rajakumar et al., 2018).

Thus, the fact that the developmental potential to produce caste-like traits is present across all castes in the colony brings a new hypothesis and explanation of how caste develops and is established through the colony and how some of the traits that we have defined like queen-like
traits are not necessarily restricted to the reproductive caste. However, instead, they represent traits with labile or biased developmental trajectories that have facilitated the evolution of new castes in ants, either by co-opted mechanisms or by the simple reappearance of the still conserved developmental program, promoting the reversals of the ancestral traits (Rajakumar et al., 2012, 2018). Additionally, latent genetic and developmental architecture for unexpressed phenotypic traits is prevalent across castes in ants. These mechanisms can accelerate evolutionary changes and lead to the reappearance of traits or promote the emergence and evolution of new castes in ants, which represent an unexplored avenue of research in our understanding of the evolution of morphological complexity in ant colonies.

1.7 References

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Chapter 2.

Reconstructing evolutionary transitions to worker caste complexity in the attine ants: Wilson's Transitions Revisited and Revised

Chapter 2. Reconstructing evolutionary transitions to worker caste complexity in the Attine ants: Wilson's Transitions Revisited and Revised

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Keywords: Allometry, leaf-cutter ants, Ancestral reconstruction, worker caste polymorphism, evolution, complex systems, farming fungus.

2.1 Abstract

Among hymenopterans that evolved eusociality, ants are the only eusocial group that increased the complexity of the social organization through the evolution of worker caste polymorphism. Despite the importance of worker caste polymorphism in improving colony efficiency, therefore, colony success, and ecological dominance, the evolutionary pathways of these complex systems remain poorly understood. E.O. Wilson proposed a classification system for worker polymorphism, categorizing them based on head-to-body allometry. He identified four types: monomorphic, monophasic, biphasic, and triphasic, with predicted transitions between them. However, formal testing of these predictions has been lacking. Our study focuses on testing Wilson's hypotheses in leaf-cutter ants, a diverse group ranging from simple to complex worker caste systems. We categorized the head-to-body allometry across 18 species of farming fungus ants. Using a statistical model of regression, we classified each species into Wilson's types of worker polymorphism and conducted an ancestral state reconstruction to explore the evolutionary paths of the different allometries throughout the phylogeny of the group. We observed that the evolution of worker caste polymorphism did not evolve by a stepwise pattern of allometry evolution, instead, species evolved complex allometries as biphasic from monomorphic, without intermediate steps. This evolutionary jump from the absence of worker polymorphism to complex worker polymorphism correlates with stepped transitions in farming strategies and colony size. Therefore, our findings provide empirical support for Wilson's hypotheses regarding the evolution of worker polymorphism. Moreover, they shed light on the relationship between farming strategies and worker caste complexity in leaf-cutter ants. By understanding these evolutionary transitions, we gain insights into the mechanisms driving cast complexity and success of eusocial insects.

2.2 Introduction

A hallmark of eusociality in ants is the evolution and development of morphologically distinct caste systems. Most species of ants are typically composed of a queen and worker caste, which are determined during development in response to environmental cues, such as temperature, nutrition, and social interactions (Hölldobler et al., 1990; Rahman et al., 2022; Wheeler, 1991; Wills et al., 2018). In most ant species, the worker caste is largely uniform in size. However,

unlike eusocial wasps and bees, the worker caste has been elaborated into a complex system of morphologically differentiated individuals at least 22 times independently during ant's evolution (Hölldobler et al., 1990; La Richelière et al., 2022). For example, dramatic inter-individual differences in body and head size have evolved independently in the two most hyper diverse genera of ants, *Pheidole* (the big-headed ants) and *Camponotus* (the carpenter ants), resulting in small-headed workers and big-headed soldiers. Such dramatic morphological differentiation between workers within an ant colony is called 'worker polymorphism' and is thought to enhance the division of labor generally associated with hyper diversity and ecological dominance (Hölldobler et al., 1990; La Richelière et al., 2022; Powell, 2009; Wills et al., 2018; Wilson, 2003). Despite the important role that complex worker caste systems play in the remarkable success of ants, the evolutionary pathways through which they have evolved remain poorly understood.

In 1953, E.O. Wilson established a system to classify the different types of worker polymorphism observed in ants, from the morphologically simple to the most complex. To do this, he applied Huxley's ideas on allometric growth—how different body parts and organs grow relative to one another (Huxley, 1972; Wilson, 1953). Wilson (1953) classified types of worker polymorphism based on patterns of head-to-body allometry, which is how the head grows relative to the body in workers, because, more than any other trait, body and head size are the two most variable traits across ants (Boudinot et al., 2021; Holley et al., 2016; Lecocq de Pletincx et al., 2021; Lillico-Ouachour et al., 2018; Wilson, 1953). Wilson used Huxley's allometric equation $Y = aX^b$ to describe the relative growth of a worker's head relative to the rest of its body: such that Y represents head size and X represents overall body size, while a and b are allometric parameters, intercept, and slope respectively. In addition to head-to-body allometry, Wilson (1953) also added two other parameters to classify types of worker polymorphism by the overall size variation and the size-frequency distribution, which according to Wilson (1952) "The size-frequency distribution can be interpreted as the outcome of varios degrees of divergence of two larval groups past a critical point in larval development" (Wilson, 1954). Therefore, by considering all three parameters simultaneously: allometry, size variation, and the size-frequency distribution–Wilson integrated both development and evolutionary factors to identify 4 distinct types of worker polymorphism and transitions between them (Wilson, 1953, 1954, 1980).

Most ant species are "monomorphic", which means there is no polymorphism (no differences in size or head-to-body allometry) between individual workers. In worker caste systems that are monomorphic, size variation between individuals is limited and appears uniform in size, growth is 'isometric' (meaning the regression line or slope between head size and body size is linear), and the size-frequency distribution is unimodal (Fig 1A.). Wheeler (1991) further translated Wilson's (1953) classification system into developmental terms. She focused on two parameters that govern the development of holometabolous insects: (1) 'growth rules' that determine the rate at which the head grows relative to the body during larval and prepupal development; and (2) 'critical size', which determines the size at which individuals stop growing, complete larval and prepupal development, and metamorphose into their final adult size and form(Wheeler, 1991). Therefore, in monomorphic worker caste systems, growth of the head relative to the body is constant throughout development, resulting in adult workers with a linear relationship between head and body size, and all individuals metamorphose at a similar critical size resulting in adult workers with limited size variation (Fig.1A).

The first type of worker polymorphism is called "monophasic" allometry where individuals within the worker caste show substantial variation in size between individuals, the slope between head size and body size is linear, and the size-frequency distribution tends toward being bimodal (Fig.1B). In monophasic worker caste systems, the 'growth rules' determining the rate of head growth relative to the rate of body growth is constant throughout the development of all individuals in the worker caste, but the critical size at which a particular larva will metamorphose is different between individuals producing a wide size range. The second type of worker polymorphism is called "biphasic allometry", which is characterized by a large variation in size between individuals, a curved non-linear slope between head and body size with a single breakpoint, and the size-frequency distribution is distinctly bimodal (Fig 1C). In biphasic worker caste systems, the non-linearity in the slope results from a reprogramming of the growth rules that results in a change in the rate of head growth relative to body growth at a specific point during larval development. Furthermore, inter-individual variation in the critical size results in the large size variation between adult workers that are visibly divided into smaller 'minor' workers and larger 'major' workers. The third type of worker polymorphism is called "triphasic allometry" and is characterized by a large size variation between individuals, a sigmoidal (S-

shaped) curve or three-segmented lines along the regression between head and body size with two distinct breakpoints, and a wide bimodal size-frequency distribution (Fig 1D). In triphasic worker caste systems, there is a single reprogramming of the relative growth rate between head and body size, while inter-individual variation in the critical size results in a large size variation between workers (Fig.1C, D). This results in two kinds of adult workers, small head minor workers and large head majors or soldiers. Finally, the fourth type of worker polymorphism is called 'dimorphism,' which is characterized by two independent regression lines. This gives rise to two discrete morphological subcastes: minors and soldiers in which the regression lines may differ in slope and /or intercept (Fig.1E).

Wilson (1953) proposed a set of transitions between the 4 types of worker polymorphism, which were largely theoretical and based on how development and evolution could potentially modify the head-to-body allometry to transform one type of worker polymorphism into another. For example, he predicted that a monophasic worker caste system gradually evolved from a simpler, monomorphic one. Most transitions are predicted to occur in a gradual, stepwise manner. However, in some cases, a transition is predicted to be "punctuated," which means that a transition is jumping one or more steps. For example, he predicted that a biphasic worker caste system transition to a completely dimorphic one in one single step, thereby bypassing the transition to triphasic allometry, by suppressing the development of intermediately sized individuals (Fig 1). Despite the importance of these transitions for understanding the origin and elaboration of complex worker caste systems in ants, there has been no formal test of Wilson's predicted evolutionary and developmental transitions.

Therefore, the major goal of this study is to test Wilson (1953)'s predicted transitions between the 4 types of worker polymorphism in the leaf-cutter ants, an ecological dominant clade of ants with species that range from the simplest to the complex worker caste systems. Leaf-cutting ants are part of the farming fungus ants, a new world monophyletic group comprising 250 species (Branstetter et al., 2017; Mueller et al., 2001; Schultz & Meier, 1995). The group represents an evolutionary transition in ants from the life of hunters to farmers living in a symbiotic relationship with a fungus (Currie, 2003; Mehdiabadi & Schultz, 2009; Mueller et al., 2001; Schultz & Meier, 1995). The species of farming fungus ants are divided mainly into three agricultural groups, which represent major transitions in the evolution of fungal domestication: (1) lower attine including yeast and coral agriculture (2) Higher agriculture, and (3) Leaf-cutter agriculture (Mehdiabadi & Schultz, 2009; Mueller et al., 2005; Schultz & Brady, 2008). In farming fungus ants, the evolution of farming strategies correlates with increases in colony size. For instance, colony sizes range from a few hundred individuals in lower and higher agriculture to thousands and millions of individuals in colonies of leaf-cutter ants (Ferguson-Gow et al., 2014; Mehdiabadi & Schultz, 2009). Therefore, we also test whether transitions in worker polymorphism coincide with the evolution of lower and higher leaf-cutting agriculture and ask whether transitions between types of worker polymorphism evolved either gradually (step by step) or in a punctuated manner (jumping one or more steps). To formally test Wilson's transitions, we collected, mounted, and measured head and body size from 3600 individuals corresponding to 18 species across farming fungus ants. We generated the worker caste head-tosize allometry for each species and then classified each into one of Wilson's types of worker polymorphism by testing several linear and non-linear (polynomial and breakpoint) models to determine which model best fits the data (Fig.4-8, Table 3,4). We then used ancestral state reconstruction analysis to determine the transitions between types of worker polymorphism within in leaf-cutter ants.

2.3 Methods

Species collection and preparation. We selected phylogenetically key species of leafcutter, higher, coral, yeast, and lower farming fungus, for each group of the farming fungus clade (Table1.). All species were collected in Gamboa-Panamá (9°7'40.65" N-79°43'4.80" W) and Parque Nacional La Selva-Costa Rica (10°5'6'', W-84° 0'13''). Each species came from a single mature colony and each colony was identified by the number of entrances and the number of foragers carrying material to the nest. The final number of workers collected per species was based on the colony size reported in the literature, in which we aim to have a representative sample for each one (Table 1) (Ferguson-Gow et al., 2014; Mehdiabadi & Schultz, 2009).

Allometry characterization and statistical analyses (model fitting). To characterize the allometry of every species included in this study, we use head width and Weber length: "the diagonal length of the mesosoma in profile from the point at which the pronotum meets the cervical shield to the posterior basal angle of the metapleuron" (from antwiki.com). We

detached the ant head from the thorax and photographed the head in frontal view and thorax inside view, using Olympus Szx10 and Zeiss SE64 scopes. Head width was measured as the maximum width above the eyes in a face view (HW), and thorax length was measured as the Mesosoma (Weber) length (WL); which is defined as the diagonal length of the mesosoma from the point at which the pronotum meets the cervical shield to the posterior basal angle of the metapleuron. We measured the pictures using the software Zeiss Axiovision SE64 software and ImageJ (Schneider et *al.*,2012).

We characterized the pattern of head-to-allometry based on the power law $Y = Xa^b$, where Y represents the weber length (WL), X represents head width (HW), and a and b are the intercept and slope that result from the head-to-weber relationship. First, WL was plotted against HW, and a linear regression was tested for all species, for those that did not follow a strict linear regression, we followed the procedure suggested by Knell (2009). Thus, we tested three models over data: linear, two, third and fourth-degree polynomial, and breakpoint regression line, where a breakpoint is defined as an inflection point on the set of data where the slopes of the regression line are significantly different. The goodness of fit was estimated using the Akaike information criterion, where the model with the lowest AIC score is the best fit for the data (Table 3.). When the best model was the breakpoint model, we used the Davies test to detect statistically the presence of 'breakpoints or significant changes in the slope across the regression (Davies, 2002) (Table. 4). All statistical analyses were conducted in R software 3.6.3, and the breakpoint regression analysis and Davies test were developed using the package *segmented* in R (Muggeo, 2008). Our linear regressions were followed by the measure of the coefficient of variation (CV) in head width and Weber length independently as the measure of size variation among species, and for the frequency distribution we used the head width (Table. 5). Based on the three parameters (1) model of regressions, (2) size variation and (3) frequency distribution, we were able to determine which type of allometry characterizes worker polymorphism in farming fungus ants based on E.O Wilson's classification system and assigned a model for each category previously discussed, to obtain a discrete "character" of Wilson allometry for each species (Table. 6.).

Phylogenetic comparative methods. Once the model that best fits the head-to-body allometry was established for all the species, we assigned it as monomorphic, or a type of worker

polymorphism based on the criteria listed in Table 2. After that, we conducted an ancestral state reconstruction on the different types of worker polymorphism and analyzed the transition between them. We used the phylogenetic relationships and branch length information Branstetter et al. (2017) and Economo et al., (2015) and excluded species *Paratrachyrmex* since we do not have phylogenetic information for this species. Ancestral state reconstruction was conducted using the Markov chain in Revbayes v1.2.1 (Höhna et al., 2017), under a single model of equal rates (Lewis, 2001) given the fact that the tree is a subset of the overall phylogeny of the group. The MCMC chain ran for 500000 generations for each model, with sampling every 200 generations.

2.4 Results

Using all three parameters to classify the type of worker polymorphism—size variation, sizefrequency distribution, and head-to-body allometry, we first investigated *Mycocephurus smithii*, a species from the most basal group of farming fungus ants typically called the 'lower attines' or 'lower farming fungus ants ', in which colonies are small and the symbiosis with the fungus is not obligate (Mueller et al., 2001). We then investigated *Apteriostigma collare* and *Apteriostigma dentigerum*, species belonging to the coral fungus farming group as well as *Cyphomyrmex cornotus*, which belongs to a sister group with yeast agriculture. Our analyses show that the worker caste systems in these species is monomorphic because they exhibit limited size variation (CV ranging from 2.0% to 4.0%) (Table 5.), and they have a linear slope and unimodal distribution (Table. 2, Fig. 4,5).

We then classified *Sericomyrmex amabilis*, *Trachymyrmex* species and *Paratrachymyrmex sp*, which belong to the group typically called the 'higher attines' or 'higher agricultural ants.' These species have larger colony sizes ranging from 50 to 500 individuals as compared to the lower fungus-farming ants (Mehdiabadi & Schultz, 2009). Our analyses, however, still classified the worker caste systems in these species as monomorphic because they had a linear slope, unimodal frequency distribution and limited size variation (CV ranges from 2.0% to 7.0%) (Fig.4,6, Table 5)

The most derived group of farming fungus ants are called the leaf-cutting ants, which are the only attine group to have evolved worker polymorphism, yet the types of worker polymorphism observed within this group are poorly characterized. Our results show that *Acromyrmex coronatus* and *Acromyrmex laticeps* are monophasic because they have more than 2-3 times the size variation as compared to species within the lower and higher agriculture groups (CV from 20% to 35%) (Table 4), and have a linear allometry, and their frequency distribution is tending to be bimodal (Fig 4,7).

Although the other two species in the genus *Acromyrmex–Acromyrmex echinator* and *Acromyrmex octosospinosus*–show substantial size variation (CV head from 33% to 35%) (Table. 5) with a tendency towards a bimodal frequency distribution (Fig. 4), they exhibit a non-linear and more curvilinear relationship fitting a (polynomial) allometry, (Fig 7. Table 4.). These species are therefore classified as biphasic.

The most ecologically successful of the leaf-cutting ant species belong to the genus *Atta* where worker caste systems are complex with wide size variation in the workers and colony sizes reaching approximately 8 million individuals (Table 1.). In this study, we investigated five leaf-cutting species from this genus: *Atta texana*, *Atta sexdens*, *Atta colombica* and *Atta cephalotes*. Our analyses show that *Atta sexdens*, *Atta texana* and *Atta colombica* are biphasic with a non-linear or single breakpoint in the allometry and marked bimodal size-frequency distribution, especially for *Atta texana* and *Atta colombica*, while *Atta cephalotes* are triphasic with 2 breakpoints in the allometry and a distinctly wide bimodal size-frequency distribution (Fig.4.)

Finally, we used ancestral state reconstruction to test Wilson's predicted transitions between types of worker polymorphism in the leaf-cutting ants, under a model of equal rates evolution. Surprisingly, our reconstruction reveals a first punctuated transition from monomorphic species within the lower and higher farming fungus ants to biphasic species within the leaf-cutting ants approximately ~9 million years ago (MYA) (Fig.9). This punctuated transition was unpredicted by Wilson (1953) and suggests that evolution can simultaneously reprogram critical size and growth rules to transition from simple to complex worker caste systems. Furthermore, this punctuated transition is phylogenetically correlated to a transition in farming strategies; from

hunter-gatherer to cutting fresh vegetation, as well as a transition from small colonies sizes to very large colonies ranging from 10 thousand to 8 million individuals (Fig 9, Table 1).

Our reconstruction reveals a second transition approximately ~4 MYA from within the genus Accromyrmex from a biphasic worker caste system in Acromyrmex octinospinosis and Acromyrmex echinatior to a monophasic worker caste system in Acromyrmex coronatus and Accromyrmex laticeps (Fig.9). This transition, although gradual, was unpredicted because a transition from biphasic to monophasic worker caste system means a loss of a developmental reprogramming of growth rules and therefore, a step backward from a more complex to a simple worker caste system. Finally, our reconstruction reveals a third and final transition approximately ~3 MYA within the genus Atta, from a biphasic worker caste system in Atta texana, Atta sexdens, and Atta colombica to a triphasic caste system in Atta cephalotes (Fig.9). Although biphasic and triphasic allometry are produced from a single reprograming event in the growth rate of the head relative to the body, the size-frequency distribution of *Atta cephalotes* is wider and distinctly bimodal, suggesting faster growth of the head relative to the body after the reprogramming event. To test whether the breakpoints coincide with reprogramming events in Atta cephalotes, we mapped the presence of ocelli, which are simple eyes at the dorsal head of insects that were lost in the worker's castes but not in reproductive. We discovered that the number of ocelli is correlated with the breakpoints in the regression lines, such that minor workers have 0 ocelli, individuals close to the threshold (between the first and second breakpoint) have 1, and those above the breakpoint have 2 ocelli. (Fig.10)

2.5 Discussion

E.O Wilson's worker caste classification and evolution are based on transitions on the head-tobody allometry, which he summarizes as a "phylogenetic extension colony size, followed by variability that comes with changes in the head-to-body allometry" (Wilson, 1953, 1954). Based on our allometry characterization, worker polymorphism evolved by increases in size variation that cause inflection points across the head-to-body regression. However, our allometry characterization, followed by the ancestral state reconstruction, shows that the increase in size and changes on the slope across the regression happened at the same node, the node from which leaf-cutter ants evolved, and therefore, worker caste polymorphism in the group of farming fungus ants. Thus, our results show that size increase is a necessary condition for allometry changes in head-to-body allometry. However, on an evolutionary scale, these changes could occur simultaneously without a stepwise condition.

Head-to-body allometry changes that characterize worker caste polymorphism in ants are mainly given the fact that the head changes at a different rate than the body, though how those changes occur in an evolutionary context has been poorly studied. However, it has been well-known that changes in head-to-body allometry are associated with task partitioning (Hölldobler et al., 1990). Several studies have found, for instance, that the size of the organs belonging to the head, such as eye, antenna, mandible, and head capsule, vary among castes according to external or internal colony tasks, for instance, workers that work outside the colony develop differential visual systems and defense than smaller workers that work inside the colony, and those traits are generally associated with head and body size (Wilson, 1980). For instance, species like Pheidole evolved a dimorphic worker caste, composed of minor workers and soldiers. In this species, soldiers are distinguished by their big heads and mandibles, small eyes, and antennae. Therefore, head changes in terms of worker polymorphism in Pheidole, are related to mandibular specialization, mainly for defense (Gibson et al., 2018; Klunk et al., 2021; Lillico-Ouachour et al., 2018), whereas minor workers maintain the proportion of this trait related to size (Boudinot et al., 2021; Klunk et al., 2021; Lillico-Ouachour et al., 2018). In the case of farming fungus ants, our allometry analysis reflects the same pattern as in *Pheidole*, except for the lack of a complete dimorphism in which intermediates individuals persist in the colonies, although in low frequency once the species reached a bimodal frequency (Fig.4). However, the fact that the slope and intercept changes across the regression line, highlights the major adaptive relevance of head modifications in the evolution of worker polymorphism in the group. For instance, in leaf-cutter ants, the head has been an important organ in the division of labour among workers, as they performance a novel task as is the leaf-cutting behaviour (Camargo et al., 2015; Püffel et al., 2021; Wilson, 1984). Therefore, the shifts in head-to-body scaling could indicate differences in selection pressures, such as a strong selection upon the head given the ecological and social demands of the colony. Although the confidence of our results relied on our broad sampling, we recognize the limitation of our dataset to conduct a more robust evolutionary analysis that supports these hypotheses. Further studies, including more species along the phylogeny and robust statistical methods on regression modeling, will strengthen these results. Likewise,

evolutionary correlations with colony life traits and tasks will complete our understanding of the evolution of complex morphological castes in this group.

Changes in head-to-body allometry have been suggested to happen during development, when there is a developmental threshold that causes an organ to be reprogrammed, in which the head undergoes different growth rules and morphological changes independent of size. Therefore, individuals that reach the developmental thresholds will undergo soldier development, whereas the individuals that did not reach the thresholds will undergo minor worker development, a case of polyphenism in which same genome produce two difference outcomes across individuals (Wheeler, 1991). Reprogramming events have been reported to happen in ants. In *Pheidole* species, mechanisms of organ reprogramming have been revealed. In those species, soldiers develop through the growth of a rudimentary wing disc, in which once Juvenile hormone (JH), a hormone involved in organ growth, increases its levels, it activates the growth of the wing imaginal disc at prepupal stages of development once the wing disc growth is activated by juvenile hormone, the larvae undergo big-headed soldiers. Experiments inhibiting the development of wing imaginal disc in *Pheidole* using RNAi on the precursor gene for wing development (*vestigial*), were able to produce intermediates individuals between minor workers and soldiers, which does not occur in nature for these species. These findings showed that soldier development is determined through a developmental threshold, regulated by JH and the rudimentary wing disc (Metzl et al., 2018; Nijhout & Wheeler, 1982; Rajakumar et al., 2012, 2018; Wheeler, 1986; Wheeler & Frederik Nijhout, 1983). In terms of Wilson transitions, the wing imaginal disc in soldiers represents a developmental mechanism in promoting transitions from triphasic to dimorphic allometry. Our evolutionary results support this hypothesis by showing that intermediates are less frequent than minors and soldiers; therefore, in the evolutionary steps on the evolution of soldiers, it happens through the complete deletion of the intermediates mediated by the evolution of developmental thresholds. Further work on the role of the imaginal wing disc in regulation head-to-body allometry will strengthen what has been shown on *Pheidole* and whether this reprogramming mechanism is conserved across species in ants, facilitating the evolution of novel castes in ants.

Although in our study we did not conduct developmental experiments to show organ reprogramming, we did find that in species of *Atta cephalotes*, the two breakpoints in which

slopes change along the regression correspond not only to changes in head growth in relationship with the body, but also the appearance of the ocelli, a queen-related trait that initially was lost in workers, given they lost the capacity to fly. Ocelli is a visual trait that evolved in flying insects and comprises three simple eyes at the dorsal region of the head. In soldiers of *Atta cephalotes*, ocelli develop asymmetrically according to the head size of the soldier; thus, individuals that fall into the second segment after the first breakpoint develop mostly one single ocelli, whereas the individuals that fall into the third segment develop two and three ocelli (Fig. 10). These findings of ocelli presence and number of ocelli correlated with head size and therefore changes in head-to-body allometry, provide an indirect clue of a developmental switch during the development which brings ancestral traits to appear given head size-related mechanisms or simply by pleiotropic effects.

This study is the first attempt to provide a formal evolutionary analysis of Wilson's predictions in head-to-body allometry and the evolution of worker caste polymorphism in leaf-cutter ants. More specifically, our findings show how allometry changes did not follow stepwise rules, but instead correlated with major transitions in colony size and novel of novel farming strategies. Therefore, these results are the first analysis that aimed to explain E.O Wilson's hypothesis of worker caste polymorphism, using linear regressions and phylogenetic comparative methods. Further studies including more species with worker caste polymorphisms, will provide valuable insights into which are the developmental processes that shape the allometries and promote the generation of morphological variation in ants' colonies. Especially centring on the importance of colony life traits and ecological factors in the evolution of caste complexity.

2.6 Acknowledgments

We thank the Smithsonian Tropical Research Institute in Panama and the Insectarium in Montreal for providing us with the space and specimens to work on this project. We thank Frederik Nijhout for their comments and suggestions about the allometry analysis. This work was supported by the Doctoral fellowship BESS-CREATE by the Natural Sciences and Engineering Research Council of Canada (NSERC) to A.V-C. and NSERC Discovery Grant to E.A.

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2.8 Figures and Tables.

Figure 1. Wilson's 1953 Hypothesis for the origin and development of worker caste

(modified). The head-to-body allometry changes begin with monomorphic (Isometry) to slight changes in the slope and intercept of the regression line, which are given by changes in trait variance (Monophasic). Those changes transition from isometry (Monomorphic and Monophasic) to Biphasic allometry with one break point to triphasic allometry with two break points and the most derived case Dimorphism as two independent regression lines. In the allometry transitions, E.O. Wilsons proposes transitions from complex allometries to simpler ones indicated by segmented lines.

Figure 2. Species of farming fungus ants show an increase in worker caste complexity from most basal species to derived ones. Basal to most derives species from top to bottom. *Mycocepurus smithi, Cyphomyrmex cornutus, Apteriostigma dentigerun,Trachymyrmex cornetzi,Trachymyrmex intermedius, Acromyrmex coronatus,Acromyrmex echinatior, Atta cephalotes.* Photos taken by Erik Plante and Dominic Oulette

Figure 3. Head to body allometry for (A) *Mycocephurus smithii* (B) *Apteriostigma collare* (C) *Apteriostigma dentigerun* (D) *Cyphomyrmex cornutus* (E) *Sericomyrmex amabilis* (F) *Trachymyrmex zeteki* (G) *Trachymyrmex cornetzi* (H) *Paratrachymyrmex sp.* (I) *Trachymyrmex intermedius* (J) *Trachymyrmex septentrionalis* (K)*Acromyrmex coronatus* (L) *Acromyrmex laticeps* (M) *Acromyrmex echinatior* (N) *Acromyrmex octospinosus.* (O) *Atta Texana* (P) *Atta mexicana* (Q) *Atta sexdens* (R)*Attacolombica* (S) *Atta cephalotes*

Figure 4. Density distribution of the head width for species of farming fungus ants. (A) *Mycocephurus smithii* (B) *Apteriostigma collare* (C) *Apteriostigma dentigerun* (D)

Cyphomyrmex cornutus (E) Sericomyrmex amabilis (F) Trachymyrmex zeteki (G) Paratrachymyrmex sp. (H)Trachymyrmex intermedius (I) Trachymyrmex cornetzi (J) Trachymyrmex septentrionalis (K)Acromyrmex coronatus (L) Acromyrmex laticeps (M)Acromyrmex echinatior (N) Acromyrmex octospinosus. (O) Atta sexdens (P) Atta texana (Q) (R) Atta mexicana (S) Atta cephalotes

Figure 5. Linear regression to head width and Weber length for species of lower **attine**. (A) *Mycocephurus smithii* (B) *Apteriostigma collare* (C) *Apteriostigma dentigerun* (D) *Cyphomyrmex cornotus*

Figure 6. Linear regression of head width and Weber length for species of Higher attine. (A) *Sericomyrmex amabilis* (B) *Trachymyrmex zeteki* (C) *Trachymyrmex cornetzi* (D) *Paratrachymyrmex sp.* (E) *Trachymyrmex intermedius* (F) *Trachymyrmex septentrionalis*

Figure 7. Linear and Polynomial regression of head width and Weber length for Leaf-cutter ants. Linear (A)*Acromyrmex coronatus* (B) *Acromyrmex laticeps*. **Polynomial 3:** (C)*Acromyrmex echinatior*, **Polynomial 2:** (D) *Acromyrmex octospinosus*.

Figure 8. Breakpoint and polynomial regression for species of Atta: one breakpoint: (A)*Atta sexdens* (B) *Atta texana* (C) *Atta mexicana* (D)*Atta colombica*. Two breakpoints (E) *Atta cephalotes*

Figure 9. Wilson's head-to-body allometry does not occur by stepwise in the evolution of worker polymorphism.

Figure 10. Pattern of Ocelli development in soldiers in *Atta cephalotes* in a sample of 1000 individuals. In *A. cephalotes*, ocelli develop according to the allometry breakpoints, whereas in *A. coronates* their development happens in bigger adults, but not in all individuals that fall into the same size.

Tables.

Table 1. Colony size reported in the literature versus sampled individuals for each species in this study.

Table 2. E.O. Wilson's categories of head-to-body allometry and the statistical model that corresponds to each category are defined in this study.

Table 3. Coefficient of variation of head width and body size for the species of this study

Table 4. Model fitting based on Akaike values for species of leaf-cutter ants follows a non-linear regression and Wilson's category that corresponds to each statistical model.

Table 4. Number of breakpoints and *P-values* from the David test for species in which head-tobody allometry fitted a model of breakpoint regression.

Table 5. Coefficient of variation of head and body size

Table 6. Wilson categories were assigned to each species in this study according to the three

 variables studied. Type of allometry, frequency distribution and variation in size.

2.9 Supplementary Figures and Tables.

Figure 1. Log-log plots of Head to body allometry. Linear allometry for (A) *Mycocephurus* smithii (B) Apteriostigma collare (C) Apteriostigma dentigerun (D) Cyphomyrmex cornutus (E) Sericomyrmex amabilis (F) Trachymyrmex zeteki (G) Trachymyrmex cornetzi (H) Paratrachymyrmex sp. (I) Trachymyrmex intermedius (J) Trachymyrmex septentrionalis (K)Acromyrmex coronatus (L) Acromyrmex laticeps. Nonlinear allometry:(M) Acromyrmex echinatior (polynomial-2) (N) Acromyrmex octospinosus (Polynomial 3) (O) Atta texana (Polynomial 3) (P) Atta mexicana (1-breakpoint) (Q) Atta sexdens (polynomial-3) (R) Atta colombica (1-breakpoint) (S) Atta cephalotes (2-breakpoints)

Supplementary Table 1. Model fitting for the log-log plots, based on Akaike values for species of leaf-cutter ants, follows a non-linear regression and Wilson's category that corresponds to each statistical model.

Supplementary Table 2. Number of breakpoints and *P-values* from the David test for species in which the log-log plots of head-to-body allometry fitted a model of breakpoint regression.

Supplementary Table 3. General Summary of statistics for head and body regressions.





Figure 2.


Figure 3.







Head width (mm)





Weber length (mm)

Figure 6.











Weber length (mm)

Figure 9.



Age (Ma)

Figure10.



Tables.

Table 1.

Species	Reported range of colony	Collected individuals
	size for the genus	
Mycocepurus smithi	50-100	24
Apteriostigma collare	10-100	24
Apteriostigma dentigerun	10-100	11
Cyphomyrmex cornutus	25-500	37
Trachymyrmex zeteki	100-500	125
Sericomyrmex amabilis	100-1000	214
Trachymyrmex cornetzi	100-1000	80
Paratrachymyrmex	100-1000	30
Trachymyrmex intermedius	100-1000	150
Trachymyrmex septentrionalis	100-1000	70
Acromyrmex coronatus	17000-270000	150
Acromyrmex laticeps	17000-270000	70
Acromyrmex echinator	17000-270000	500
Acromyrmex octospinous	17000-270000	300

Atta Texana	Up 8000000	500
Atta sexdens	Up 8000000	700
Atta colombica	Up 8000000	200
Atta cephalotes	Up8000000	1000

Table 2.

Type of worker	Size-Frequency	Size variation	Head to body regression
polymorphism	Distribution		
Monomorphism	Unimodal	Limited size variation (CV between 1 to 10%)	Linear
Monophasic allometry	Tendency to bimodality	Wide size variation (CV more than 20%)	Linear
Diphasic allometry	Bimodal	Wide size variation (CV more than 20%)	Polynomial or one breakpoint
Triphasic allometry	Bimodality in frequency curve becomes sharply marked, with medias	Wide size variation (CV more than 20%)	Two Break Points
Complete dimorphism	bimodal with discrete separation in size between minor workers and soldiers	Wide size variation (CV more than 20%)	Two independent regression lines

Table 3.

SPECIES	MODEL	R-square	AIC	AICc
Acromyrmex echinatour	Linear	0.9938	-1785.609	88.684336
	Polynomial 2	0.9946	-1853.778	20.515571
	Polynomial 3	0.9948	-1867.052	7.240884
Acromyrmex octospinosus	Linear	0.9922	-1023.681	76.84095
	Polynomial 2	0.9938	-1089.802	10.7194
	Polynomial 3	0.9938	-1087.854	12.66767
Atta texana	Linear	0.9655	-743.00	89.61
	Polynomial 2	0.969	-825.20	7.4
	Polynomial 3	0.969	-832.61	0
	Breakpoint	0.967	-825.96	6.65
Atta colombica	Linear	0.9866	-385.3496	126.59835

	Polynomial 2	0.9911	-469.2142	42.73369
	Polynomial 3	0.992	-492.5631	19.38481
	Breakpoint	0.9928	-511.9479	0
Atta sexdens	Linear	0.9538	-353.5944	98.9834
	Polynomial 2	0.9599	-452.5778	0
	Polynomial 3	0.9599	-450.5782	1.999543
Atta cephalotes	Linear	0.9655	680.96057	1100.7413
	Polynomial 2	0.9835	-34.24969	385.531
	Polynomial 3	0.9836	-38.13387	381.6469
	Polynomial 4	0.9872	-280.83622	138.9445
	Breakpoint	0.9889	-419.78072	0

Table 4.

	Species N	Number of breakpoints	Breakpoint 1 at X	Breakpoint 2 at X	P-value
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Atta texana	1	2.81 (st 0.12)		4.22E-04
Atta colombica	1	2.525 (st 0.083)		7.35E-06
Atta cephalotes	2	3.471 (st 0.199)	4.087 (st 0.187)	3.22E-05

Table 5.

Species	CV-Head width	CV-Weber length
Cyphomyrmex cornutus	2.168948185	2.813271435
Mycocepurus smithii	2.51455912	3.24280989
Paratrachymyrmex sp	3.472454371	2.924586982
Trachymyrmex intermedius	3.88679403	4.151534326
Apterostigma dentigerum	4.157139929	4.067940314
Apterostigma collare	4.184381499	2.586298391
Trachymyrmex cornetzi	4.261466549	3.930282684
Trachymyrmex septentrionalis	4.698097201	4.531411797
Trachymyrmex zeteki	5.027477403	4.561641271
Sericomyrmex amabilis	7.408202204	7.000229975
Acromyrmex coronatus	23.14123133	24.32300521
Acromyrmex laticeps	31.58599824	27.76196261

Acromyrmex echinatior	33.08770092	35.07174932
Atta texana	34.18887192	29.08735632
Acromyrmex octospinosus	35.72294556	37.82990934
Atta colombica	47.84492429	42.13201043
Atta sexdens	51.93451236	42.08514079
Atta cephalotes	63.25419743	47.77057351

Table 6.

Type of worker	Size-	Size variation	Head to body	Species
polymorphism	Frequency		regression	
	Distribution			
Monomorphism	Unimodal	Limited size variation	Linear	Lower Attine-
		(CV between 1 to 10%)		Higher Attine
Monophasic	Tendency to	Wide size variation (CV	Linear	
allometry	bimodality	more than 20%)		
Diphasic	Bimodal	Wide size variation (CV	Polynomial or	Acromyrmex
allometry		more than 20%)	one breakpoint	group and Atta
				texa, Atta
				Colombica and
				Atta sexdens

Triphasic	Bimodality in	Wide size variation (CV	Two Break	Atta cephalotes
allometry	frequency	more than 20%)	Points	
	curve			
	becomes			
	sharply			
	marked, with			
	medias			
Complete	bimodal with	Wide size variation (CV	Two	
dimorphism	discrete	more than 20%)	independent	
	separation in		regression lines	
	size between			
	minor workers			
	and soldiers			

Supplementary Figure 1.



Supplementary table 1.

SPECIES	MODEL	R2	AIC	AICc
Acromyrmex echinatour	Linear	0.9946	-2048.3265	87.8366449
	Polynomial 2	0.9954	-2122.9939	13.1692728
	Polynomial 3	0.9954	-2121.9084	14.2547649
	Break-point			
Acromyrmex octospinosus	Linear	0.9922	-1200.3736	38.5091495
	Polynomial 2	0.9929	-1229.0162	9.86657415
	Polynomial 3	0.9932	-1237.5843	1.29845636
	Break-point			
Atta texana	Linear	0.9625	-1299.3424	67.2268043
	Polynomial 2	0.9664	-1352.8312	13.7379798
	Polynomial 3	0.967	-1361.0097	5.55954641
	Break-point	0.9673	-1364.0653	2.50393112
Atta mexicana	Linear	0.9897	-1639.4425	218.211882
	Polynomial 2	0.9933	-1854.6618	2.99260057
	Polynomial 3	0.9934	-1856.9875	0.66682155
	Break-point	0.993	-1829.7518	27.9025867
Atta colombica	Linear	0.9797	-2335.8529	187.655744
	Polynomial 2	0.9834	-2484.1687	39.3399039
	Polynomial 3	0.9834	-2485.7384	37.7702796
	Break-point	0.9841	-2516.2314	7.27724585
Atta sexdens	Linear	0.9817	-1519.675	197.77767
	Polynomial 2	0.9852	-1654.669	62.78378
	Polynomial 3	0.9863	-1702.3	15.15271
	Break-point	0.9861	-1695.268	22.18484
Atta cephalotes	Linear	0.984	-2125.4737	1104.62065
	Polynomial 2	0.9925	-2857.5584	372.535941
	Polynomial 3	0.9933	-2969.525	260.569413
	Break-point	0.9949	-3230.0944	0

Supplementary table 2.

Species	P- value	Breakpoint 1 at	Breakpint 2 at X	
		X		
Atta colombica	9.45E-06	0.8765973		
Atta mexicana	1.09E-04	0.7893797		
Atta cephalotes	1.45E-05	0.7867889	1.3345622	

Supplementary table 3.

Species	Median	Mean	SD head	Median	Mean	SD weber
	head	head	width	weber	weber	length
	width	width		length	length	
Cyphomyrmex cornutus	0.796	0.794	0.017	1.348	1.345	0.038
Apterostigma collare	0.789	0.789	0.033	1.832	1.833	0.047
Mycocepurus smithii	1.326	1.320	0.033	1.961	1.964	0.064
Apterostigma dentigerum	0.855	0.849	0.035	1.918	1.894	0.077
Trachymyrmex cornetzi	0.911	0.910	0.039	1.293	1.290	0.051
Paratrachymyrmex sp	1.248	1.248	0.043	1.773	1.769	0.052

Trachymyrmex intermedius	1.126	1.123	0.044	1.623	1.618	0.067
Trachymyrmex septentrionalis	0.957	0.950	0.045	1.303	1.301	0.059
Trachymyrmex zeteki	1.113	1.105	0.056	1.712	1.712	0.078
Sericomyrmex amabilis	1.207	1.226	0.091	1.508	1.521	0.107
Acromyrmex coronatus	1.293	1.262	0.292	1.865	1.855	0.451
Acromyrmex laticeps	1.025	1.163	0.367	1.419	1.561	0.433
Acromyrmex echinatior	1.496	1.402	0.464	2.097	1.966	0.690
Acromyrmex octospinosus	1.178	1.345	0.480	1.589	1.850	0.700
Atta texana	1.692	1.722	0.589	2.128	2.138	0.622
Atta colombica	1.566	1.720	0.823	2.247	2.336	0.984
Atta sexdens	1.512	1.680	0.872	2.030	2.179	0.917
Atta cephalotes	2.213	2.932	1.855	2.897	3.322	1.587

Connecting statement Chapter 1 to Chapter 2

In Chapter 1, I use an evolutionary approach to test Wilson's hypothesis on the evolution of worker caste polymorphism, particularly focusing on changes in head-to-body scaling across the phylogeny of farming fungus ants and their role in producing allometric variations. This chapter concludes by highlighting how alterations in this scaling relationship can resurrect traits lost in worker castes, such as the reappearance of ocelli, a trait that develops only in reproductive but has been regained several times in workers and soldiers across ants. Through my allometric analyses, I showed how the appearance of ocelli occurs by shifts in head-to-body scaling. This resurrection of a lost trait prompts crucial inquiries into the latent developmental mechanisms underlying trait expression and its correlation with novel castes and changes in head scaling. I address this question in Chapter 2, by examining the evolutionary history of ocelli in workers and the developmental program of ocelli across castes. I analyzed this using a different clade the Formicinae subfamily. Despite the change in ant families, both chapters converge on the evolutionary trajectory of soldier caste development and the intriguing phenomenon of ocelli evolution and reversal across workers and soldiers. Consequently, these chapters collectively contribute significant insights into the evolutionary developmental dynamics of the origin of castes and caste-related traits.

Chapter 3

Latent genetic potential underlies the evolutionary reversibility of ocelli in workers in Formicinae

Chapter 3. Latent genetic potential underlies the evolutionary reversibility of ocelli in workers in Formicinae.

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Keywords: ancestral developmental potential, ocelli, orthodenticle, ants, eye-antenna, reversals, Formicine, ancestral state reconstruction, rebates, gene regulatory network.

3.1 Abstract

Reversals of complex morphological traits challenge the notion of irreversibility in evolution, as proposed by Dollo's Law. Despite numerous examples across the Tree of Life indicating the reevolution of lost traits, the underlying evolutionary and developmental mechanisms remain elusive. In this study, we investigate the developmental and evolutionary history of ocelli, a caste reproductive trait that has been lost in workers but has reversed multiple times across the phylogeny of the group. Here, we first characterized this pattern of reversed evolution in the worker caste across one of the largest subfamilies of ants (Formicinae) and then asked whether the evolution responds to ecological variables or responds to neutral evolution. Second, we asked whether the species that lost the capacity of the trait lost the developmental potential to produce either by complete deletion of the developmental pathway or by redeployment of the precursor gene otd-1 in soldier development. We found that the evolution of ocelli in workers occurred once in Formicinae and underwent several losses and two reversals in soldiers in the group. We discovered that in species where the worker caste lacks ocelli, they retain the expression of the ocelli gene regulatory network (GRN). We show despite lacking ocelli in C. floridanus workers and soldiers, the expression of genes in the ocelli GRN is conserved at the latest stage of larvae development, but two of the most downstream genes become interrupted at the end of it. Knockdowns of the precursor gene of ocelli and head capsule in Drosophila and-1 through RNAi show the potential induction of the ocelli in soldiers in C. floridanus with no effect in shape or size. Therefore, these findings provide valuable insights into the developmental processes underlying caste-specific traits and their evolutionary significance, suggesting that there is a conservation of the genetic and developmental architecture for unexpressed phenotypic traits across castes.

3.2 Introduction

Reversals of complex morphological traits are a poorly understood feature of development and evolution. Despite Dollo's infamous law, which states that the evolutionary loss of complex traits is irreversible (Dollo, 1893; Gould, 1970; Marshall et al., 1994), there is abundant evidence across the Tree of Life that traits, once lost, can re-evolve. Many examples of reversals abound, including ocelli in cave crickets (Desutter-Grandcolas, 1993), super-soldiers in the worker caste

of *Pheidole* ants (Rajakumar et al., 2012), wings in water spiders (Andersen, 1997), digits in lizards of genus Bachia (Kohlsdorf & Wagner, 2006), and actinomorphy (radial symmetry) in plants (Donoghue et al., 1998). However, the evolutionary and developmental mechanisms promoting the reversal of complex traits remain poorly understood.

To address this question, we investigate the evolutionary and developmental factors underlying a complex morphological trait in ants, which has been gained and lost multiple times along the phylogeny. Because ant colonies are superorganisms, we can gain insight into how complex characters develop and evolve within solitary organisms. Like solitary organisms, superorganisms have a division of labour between reproductive (male and queen) castes, which are like the germline, and the non-reproductive (worker) castes, which are like the soma. This means that caste types in superorganisms (queens, males, and workers) are analogous to different cell types in a solitary organism (Hölldobler et al., 1990; W. M. Wheeler, 1911). Like cell types, the determination and differentiation of the queen and worker castes occur during development and are typically polyphenic, which means that a single genome (egg or larvae) has the potential to develop into a queen or worker in response to environmental cues, such as temperature, nutrition, and social interactions (Lillico-Ouachour & Abouheif, 2017; West-Eberhard, 2003; D. E. Wheeler, 1986).

Here, we focus on the development and evolution of the ocelli in the worker caste of ants, which have been gained and lost multiple times during the evolution of ants. In insects, there are typically three ocelli, each of which are simple eyes composed of a single lens on the head's dorsal side (Krapp, 2009). In addition to this single lens. Unlike the two large compound eyes in insects, which comprise hundreds to thousands of ommatidia, each ommatidium is made up of a lens, crystalline cone, and photoreceptors; each ocellus contains lens-secreting cone cells, an iris, a vitreous body, a cornea, a dorsal retina, and an ocellar neuropil (Buschbeck & Bok, 2023; Ribi & Zeil, 2018) (Narendra et al., 2016; Narendra & Ribi, 2017). The ocelli complement the function of the compound eye in mediating orientation by using polarized light and in the synchronization of daily activity (Buschbeck & Bok, 2023; Krapp, 2009). Ocelli have primarily evolved in flying insects because they generally function as sensors of light changes. In ants, ocelli are typically present in the queen and male castes because they develop wings to fly and engage in mating flights. Therefore, in these castes' ocelli function in flight stabilization and

orientation (Fig.1) (Moser et al., 2004; Narendra et al., 2016). By contrast, the ocelli are typically absent in individuals within the worker caste, like in *Camponotus floridanus*, because they lack wings and do not engage in mating flights. However, ocelli have repeatedly evolved in the worker caste in different ant species (Fig.1), and for some of the species that evolved ocelli in workers such as *Cataglyphis bicolor*, *Myrmecia pyriformis*, and *Melophorus bagoti*, studies have shown that ocelli function in light sensing and navigation, acting as a celestial compass that provides crucial directional information (Fent & Wehner, 1985; Johnson & Rutowski, 2022; Narendra & Ribi, 2017; Schwarz et al., 2011). In some species, like *Lasius niger*, the ocelli are present in workers but are highly reduced (Fig.1). In contrast, in others, such as in *Camponotus gigas*, the ocelli are present in the large-headed individuals (called soldiers), but not in the smallheaded minor workers (Fig.1). Therefore, the multiple losses and gains of ocelli in the wingless worker caste across ants raise the possibility that ocelli reversed evolution in ants.

Ants are part of the group of holometabolous insects in which adult body parts develop through imaginal discs, semi-independent clusters of the epithelial layer of cells (Held Jr, 2002; Koch & Abouheif, 2019). In *Drosophila melanogaster*, most of the head organs derive from the eyeantenna disc, that gives rise to the head capsule, eye, ocelli, antenna, and maxillary palps (Haynie & Bryant, 1986) (Fig.2A). In *Drosophila melanogaster*, the development of the three ocelli (two lateral and one media) begins with the activation of gene *orthodenticle (otd)* in the inter-ocellar domain, located at the ventral region of the head capsule (Fig.2A), *otd* regulates the expression of the *defective proventriculus (dve)*, *hedgehog (hh)* and downstream genes such us, *eyes absent (eya)*, *twin of eyeless (toy)*, *Sine oculis (so)* and *Atonal (ato)*(Jean-Guillaume & Kumar, 2022; Sabat et al., 2017) (Fig. 2B).

Orthodenticle (otd), the precursor gene known to be responsible for the development of ocelli in *Drosophila melanogaster*, has been duplicated in many lineages of insects(Finkelstein et al., 1990; Lynch et al., 2006; Schröder, 2003; Wilson & Dearden, 2011). Although *otd* in *D. melanogaster* has not duplicated and is orthologous to *otd-1* in the insect lineages where *otd* has undergone duplication (Wieschaus et al., 1992). In most insects, *otd-1* is expressed at and plays a role in specifying anterior identity to the embryo (Gao et al., 1996; Kotkamp et al., 2010; Lynch et al., 2006). However, during larval stages, *otd-1* expression and developmental role can vary across species. For instance, in *Drosophila, otd-1* expression is localized within the region that

will give rise to the head capsule and ocelli (Fig.2A) and mutations in *otd-1* cause the loss of ocellar structures and loss of medial part of the head capsule, without affecting it's lateral regions (Royet & Finkelstein, n.d.). However, in beetles, knockdown of *otd-1* expression results in different phenotypic effects in three species: in the horned beetle *Onthophagus taurus*, *otd-1* knockdown results in horn formation on the posterior side of the head in males while inducing the formation of horns in the anterior region in males and females, whereas in a closely related horned beetle species *Onthophagus sagittarius*, knockdown of *otd-1* induces ectopic eyes on the posterior regions. In contrast, the knockdown of *otd-1* in another beetle species *Tribolium castaneum* results in no observable phenotypic change in the head (Zattara et al., 2017a, 2017b). Hence, during larval development, *otd-1* has been co-opted to play different functional roles in the head of holometabolous insects. Here, we first determine whether the eye-antennae disc in ants is conserved and gives rise to the head, antennae, eyes, and ocelli and investigate the functional role *otd-1* in the larval disc(s) that give(s) rise to the head, antennae, eyes, and ocelli in minor- and soldier-destined larvae in the Florida carpenter ants, *Camponotus floridanus* (Fig.1).

In this study, we therefore explore the evolutionary history and developmental basis of ocelli in workers of a hyper-diverse clade of ants; the subfamily Formicinae. This subfamily is the most species-rich among the ants, with around 3000 species described (Blaimer et al., 2015; Ward, 2014). We first used ancestral state reconstruction to infer the gains, losses, and reversals of these traits in workers and soldiers across the clade. To gain insight into whether the presence or absence of ocelli may reflect adaptations to the ecological traits related to light conditions or colony traits, we performed the Pagel correlation method (Pagel, 1999), We test if gains or losses correlate with worker polymorphism, foraging substrate, period of activity, or a geographic distribution (latitude and longitude). Finally, to test whether the gains or reversals have been facilitated by the developmental gene regulatory network (GRN) underlying ocelli development in workers and soldiers of the ant subfamily formicine, we first determine and establish the regional identity of s, head capsule, antennae and ocelli during larval development using conserved genes of region identity; eyes; *eyeless (ey)*, antenna *distal-les (dll)* and head capsule and ocelli *otd-1*. Second, we determine whether the expression of ocelli GRN is interrupted or conserved in the Florida carpenter ant *C. floridanus* whose workers and soldiers completely

lacking ocelli. Finally, we knock down the expression of *otd-1* using RNA interference (RNAi), to test whether it plays a role in ocelli development or plays a different role in adult soldiers. Altogether, we hope this study will shed light on the developmental and evolutionary scenarios under which reversals of complex traits occur in the context of the evolution of novelty in ants.

3.3 Methods

Evolution of Ocelli in the Formicine Clade and the Correlation with Life History Traits

We examined the evolution of ocelli on workers across the subfamily Formicinae, using ancestral reconstruction for discrete traits under the Markov chain Monte Carlo (MCMC) incorporated on RevBayes v1.2.1(Höhna et al., 2017). Our analysis was based on the published phylogeny by (Economo et al., 2018; Ward et al., 2016). To determine the presence or absence of ocelli in workers, we used photographs of the studied species from the ant web (AntWeb.Version 8.106.1). We contrasted the observations with published information and reported ocelli in the studied species (SupTable 2). We classified ocelli as present in the worker caste of a species if individuals exhibit any one of the 3 ocelli (2 lateral and 1 medial ocelli). In the case of the presence of worker polymorphism, we classified the ocelli as present if any one of the 3 ocelli is present within any of the subcastes. Whereas the absence was the complete lack of ocelli.

We tested four separate models for each character: the Mk model (Lewis, 2001), with equal transition rates, the freeK model (Höhna et al., 2017), which assumes independent transition rate that are, in this study, exponentially distributed; the irreversible model based on the reversible jump models that establish the probability of gains losses being larger than zero. We selected the best model using marginal likelihoods: the probability of the data for a specific model integrated over all possible parameter values. The marginal likelihoods were compared with Bayes factors and model probabilities using stepping-stone sampling to approximate the marginal likelihoods (Höhna et al., 2017) (Table 1.). The MCMC chain ran for 900000 generations for each model, with sampling every 1000 generations. We used Tracer v.1.6 to assess parameter mixing and stationarity and employed the effective sampling size (ESS) criterion to ensure all parameters had ESS values above 200.

We determine if the presence of ocelli was correlated with colony life history traits such as worker polymorphism, period of activity and foraging strategies. We relied on a phylogenetic maximum likelihood correlation test based on Pagel's method (Pagel, 1999), which tests hypotheses of correlated evolution between two binary traits. The phylogenetic correlation test was conducted using corHMM v.1 implemented in R (Beaulieu et al., 2013). To establish the evolutionary correlation between geographical distribution and the presence /absence of ocelli in workers, we calculated the latitude and longitude centroid for each species and conducted phylogenetic ANOVA implemented in the R package *Geiger v 2.0.11*(Pennell et al., 2014) . Data of worker polymorphism and latitude and longitude were taken from the database produced in (La Richelière et al., 2022).

Ant maintenance and collection

Colonies of *Camponotus floridanus* are maintained in plastic boxes with glass test tubes filled with water-constrained cotton wool. Ants are fed with mealworms and agar. Colonies are maintained at 25C with 60% humidity in complete darkness.

Larvae fixation and in-situ HCR

Larvae at 4the 4th stage, which will give rise to males, soldiers, and minor workers, were selected in the *Camponotus floridanus* species. Larvae were fixed in a Pem 4% formaldehyde solution for 2 hrs at room temperature. Then, we progressively dehydrated in methanol baths (25%, 50%, 75% methanol for 15 min each, and 100% overnight at 4°C) and stored in 100% methanol at -30°C until use.

Gene expression was done by in situ, hybridization chain reaction experiments (HCR) by following the protocol for HCR (v3.0 protocol) (Schwarzkopf et al., 2021; Molecular Instruments). To carry on the fate map, we used the probes corresponding to *Orthodenticle-1* (*otd-1*), *Eyeless (ey)*, *Distal-less (dll)*. For *Otd*, we found two variants of the *orthodenticle* genes, a likely case of gene duplication across ants. Therefore, we obtained the two sequences using BLAST against *Drosophila melanogaster*, *Apis mellifera*, *Nasonia vitripennis*, *Ontophagus taurus* and established a gene tree to determine the paralog of each in *Camponotus floridanus* (supp Fig.1). To characterized the gene regulatory network for ocelli, we used probes

corresponding to *Heghegod*, (*hh*)(<u>XM_011262474.3</u>), the paralogs *orthodenticle-1* (*otd-1*) (<u>XM_025415315</u>), *eye absent* (*eya*)(<u>XM_025414466</u>), *sine oculis* (*so*) (<u>XM_011252868.3</u>) and *twin of eyeless* (*toy*) (<u>XM_011268499.3</u>). We conducted triplets using the fluorescence Hairpins (B1 546, B2 488, B3 647) synthesized by MOLECULAR INSTRUMENTS.

Larvae were dissected from methanol and tissues were washed in PBS-methanol washes and permeabilization in detergent for 2 hrs. After the tissue was pre-hybridized in a prewarmed Probe Hybridization Buffer (Molecular instruments) for 30 minutes at 37 C and incubated with HCR probes in a Probe Hybridization Buffer overnight at 37C. Tissues were washed the next day in a prewarmed Probe Wash Buffer four times, 15 minutes each and washed in 5X SSCT (UltraPure 20XSSC Buffer, Invitrogen, diluted in water) three times, 5 minutes at room temperature. Tissues were pre-amplified in Amplification Buffer (Molecular Instruments) for 30 minutes at room temperature and incubated with snap-cooled HCR hairpins in Amplification Buffer overnight at room temperature, Tissues were then washed with 5X SSCT at room temperature twice for 5 minutes, twice for 30 minutes, and once for 5 minutes before being mounted on glycerol-DAPI 80%.

Gene Cloning and Sequencing

Gene sequences were obtained from the NCBI GenBank database using genome BLAST against the assembled *C. floridanus* genome for *otd-1* <u>XM 025415315</u> and *otd-2* <u>XM 011264041.3</u>. RNA was isolated using Trizol (Invitrogen) from a pool of embryos and larvae of different developmental stages. RNA was then reverse transcribed to synthesize a cDNA library. We cloned fragments of the genes from cDNA libraries into a pGemT-easy vector (Promega), and subsequently sequenced using Sanger sequencing at the Genome Quebec Innovation Centre. The primers used for *otd-1* Forward 5'- CGTGTTGGAGGGGGCTATTCA- 3' and reversed 5-'CGAATCTGGAGTGCGTCTGT-3' and *Orthodenticle*-2 Forward 5'-TGCGTGAGGAAGTAGCGATG-3', reverse 5'-TTCGTCTCTCGTTTTGCTCCA-3'respectively. DNA templates of *otd-1*, and *otd-2* were used to generate dsRNA using T7 RNA polymerase. We conducted a PCR with M13 forward universal primer and M13 reverse universal primer containing a T7 promoter overhang on plasmids containing cloned gene fragments as templates. For controls, dsRNA was generated using a plasmid containing cloned 720 bp of the YFP coding sequence using the same method.

RNAi

Double-stranded RNA injections for otd1+2 was conducted together on soldier-destined larvae, and male-destined larvae and otd-1 on soldier-destined larvae (Suppl. Fig.3). For soldiers, we selected larvae that ranged from 5.5 mm to 6.5 mm with brown gut colour as an indicator of timing before pupation and minor worker larvae from 4.0 to 5.0 mm (Chen *et.al.* In prep). For males, we selected larvae that ranged from 4.0 to 5.0 mm; in the case of males, the size selection was based on gut color. At the time of the injection, we made size matches to keep consistency among control and treatment. The larvae were placed on a petri dish covered by parafilm, and dsRNA was injected into the dorsal side of the larvae's head. For the injections with both paralogs, we used a concentration of 2000 ng/µl in soldiers (1000 *otd-1*+ 1000 *otd-2*) and maledestined larvae. For *otd-1* injections we used soldiers-destined larvae 2000 ng/µl of dsRNA of *otd-1* and YFP and 2000 ng/µl dsRNA. Needles were made using a Sutter Instrument needle puller (Model P-97) and a Nari Shige microinjection apparatus attached to a Zeiss Discovery V8 dissection microscope fitted with a custom-made x–y movable platform was used for injections. After the larvae were injected, it was placed in replicates containing adults' minor workers 2:1 adult-larva ratio.

Quantitative polymerase chain reaction

Soldier-destined larvae were injected with 4000 ng/ul of *otd-1* and *otd-2* dssRNAi independently. After 6 days post-injection, larvae were collected, and brain and eye-antenna discs were dissected in RNA later. Tissues were prepared on the same day using the standard Trizol Method, and cDNA synthesis was done using Superscript-II reverse transcriptase (ThermoFisher). Housekeeping genes, target genes, and downstream genes used in this study are described in the Supplementary Table. Quantitative PCR was performed at the IRIC-Genomics Platform using qPCR Taqman method (Holland *et.al.*,1991)

Microscopy

We used confocal imaging, and we used a Leica SP8 confocal microscope. ImageJ2 to analyze the images. Scanning electron microscopy was done on a Hitachi TM3030 Scanning Electron Microscope

3.4 Results

Ocelli in workers have undergone 2 reversals within the ant subfamily Formicinae.

We first investigated if ocelli in workers violated Dollo's predictions of irreversibility within the worker caste of species in the ant subfamily Formicinae. To test for reversals across the phylogeny of the group, we performed an ancestral state reconstruction using the equal rates model, which was inferred as the best model for the evolution of ocelli in this group (Table.1, Fig.3). Our reconstruction shows that ocelli was first gained in Formicinae at the split of the most basal lineages within the Myrmelachistini (PP 0.62). After the gain of ocelli in workers, this trait undergoes multiple losses and 2 reversals. Considering only nodes with a posterior probability > 0.6, we inferred a single loss of ocelli in the clade Camponotini. Ocelli were also lost twice independently in the following genera: *Oecophilia*, and *Anoplolepis*. Subsequently, ocelli were re-gained through two reversals in the species *Camponotus gibbinotus* and *Camponotus gigas*. Altogether, our ancestral state reconstruction infers 1 gain, 3 losses, and 2 reversals of ocelli on workers throughout the Formicinae.

The evolution of ocelli in workers does not correlate with ecological and colony variables.

To gain insight into whether the evolution of ocelli in the worker caste across the Formicinae might be driven by natural selection, neutral evolution, or selection on a correlated trait (pleiotropy), we explored ocelli in workers on the Formicinae is phylogenetically associated with ecological factors related to light conditions or colony traits. To do this, we performed phylogenetic correlations using Pagel's (1999) method. More specifically, we tested whether the presence of ocelli correlates with the following ecological or colony-level factors: (1) foraging strategies (canopy or underground); (2) period of activity (nocturnal or diurnal); (3) worker polymorphism (presence or absence). Pagel's correlations show that for foraging strategies, period of activity, and worker polymorphism, the lowest AIC scores correspond to the independent model (Table 2.), which means that the evolution of ocelli is not phylogenetically

correlated with any of the three ecological or colony-level factors we tested. Furthermore, we conducted phylogenetic ANOVA analysis to estimate the correlation between the centroid of Latitude and Longitude and the presence of ocelli while controlling for phylogenetic relations of the species. Neither Longitude nor Latitude was significantly correlated with the presence of ocelli (phyloANOVA: N:70, *tlongitude*= 0.17091, *p-adjust*=0.7059, *tlatitude*=0.13401, *p-adjust*=0.3137) (Fig.4). Therefore, our analysis raises the possibility that the broad pattern of evolution of ocelli in workers is explained either by the response to an unknown variable not considered in this study or is evolving neutrally across the group.

The main regulatory genes of region identity are conserved in the eye-antenna disc in ants.

In ants, the development of ocelli is unexplored, mainly given the fact that the development of the head in ants has not been characterized yet. Therefore, we characterized the development eye-antenna disc in ants across larvae stages using the genetic toolkit of organ regionalization in Drosophila melanogaster eye-antenna disc (Fig.5). In Drosophila melanogaster, the eye-antenna disc segregates into various regions like the eye, antenna, ocellar, maxillary, and capsule regions and these regions are under the regulation of highly conserved genes. We selected the precursor gene of eye identity eyeless(ey), of antenna distal-less (dll) and head capsule and ocelli orthodenticle-1(otd-1). While maxillary palps development occurs primarily in the first larvae stages in Drosophila, regionalization occurs later in pre-pupal stages. Therefore, we excluded it from our study in ants. The expression patterns of genes like ey, dll, and otd-1 were found to be conserved across development stages between C. floridanus and Drosophila. During the first larval instar in Drosophila (L1), genes controlling eye and antenna development, especially ey and twin of eyeless (toy), are uniformly expressed across the eye-antenna disc (Fig.5). Conversely, in C. floridanus, the expression of ey and dll delineates the future eye and antenna regions from the early stage at L1. Likewise, Orthodenticle-1 expression begins to emerge, primarily around the midline (Fig.5A-E).

In the second larval instar of *Drosophila* (L2), regionalization within the eye-antenna disc initiates, with genes like *ey* becoming restricted to the eye region, *dll* to the antenna region, and *otd-1* expressed throughout the disc (Fig.5). In *C. floridanus*, similar patterns emerge, with *ey* and *dll* expressions confined to their respective regions, and *otd-1* predominantly expressed in

the developing head capsule, particularly in the medial region (Fig.5F-J). During the third larval instar in *Drosophila* (L3), distinct regions within the head capsule are regulated by *otd-1*. In *Camponotus floridanus*, larvae at this stage remain undifferentiated between soldier and worker castes, with consistent expression patterns of *ey*, *dll*, and *otd-1* across the eye, antenna, and head capsule regions (Fig.5K-O). Unlike *Drosophila*, *C. floridanus* exhibits a fourth larval instar (L4) known as the destined period, during which larvae commit to either soldier or worker castes. Expression patterns of *ey*, *dll*, and *otd-1* remain consistent, with *otd-1* showing stronger expression in the medial region of the head capsule compared to lateral regions. However, although expression patterns of conserved genes are consistent between workers and soldiers, at L4, soldier-destined larvae exhibit a higher area of the head capsule in comparison with minor workers, whereas eye and antenna regions remain similar between both (Fig.5P-Y)(Fig.6, N=4). These results show that genes are conserved between Drosophila and C. floridanus across stages. However, the timing of expression versus organ regionalization varies between the two, which explains the morphological divergence between the heads of both organisms. Likewise, across castes, there is a significant variation in head capsule development between workers and soldiers.

The retention of expression ocelli GRN underlies the reversibility of ocelli in workers in Formicinae.

To test whether genes within the ocelli GRN are expressed in species, where workers have lost ocelli in the Formicinae, we first determined the expression of the GRN of ocelli on *C*. *floridanus* (Fig.7-8), a species from the formicine tribe Camponotini. In this group, our ancestral reconstruction inferred a single loss of ocelli followed by two independent reversals in *Camponotus gigas* and *Camponotus gibbinotus* (Fig.3). Several species within the Camponotini have independently evolved worker polymorphism, in which the worker caste is subdivided into minor worker and soldier caste. We, therefore, determined the expression of the following genes in the ocelli GRN *otd-1*, *hh*, *eya*, *toy* and *so* in the eye-antennal disc in males, workers, and soldiers of *Camponotus floridanus* at early and late L4 larvae instar.

We found that the expression of all five genes within the ocelli GRN are conserved in the eyeantenna disc in males relative to their expression in the fruit fly *Drosophila melanogaster*. All five genes are expressed in the inter-ocellar and ocellar regions at the early and late L4 instar, establishing the identity of the ocellar region at the dorsal side of the eye-antenna disc (Fig.7,8). More specifically, *hh* expression is around the ocellar region at the dorsal region of the head capsule (Fig. 7AA'), while *Otd-1* expression goes across the head capsule primordia. However, *Otd-1* expression gets stronger on the ocellar region at the dorsal region of the eye-antenna disc, where it is strongly expressed demarking the regions that will give rise to the lateral and median ocelli (Fig.7BB'). In the ocellar region, genes like *toy* are expressed at both sides of the morphogenetic furrow that demarks the eye from the head capsule region, extending its expression at the antenna. At the ocellar region, *toy* is surrounding the region that will give rise to the two lateral ocelli (Fig.8 CC'). In the case of *eye*, this gene is expressed in the region where the two lateral and medial ocelli will develop, followed by such an expression, which overlaps with expression, demarking the two lateral and medial ocelli. (Fig 8.DD', EE'.)

In the minor workers and soldiers of *C. floridanus*, we discovered that in early L4, *hh*, *otd-1* are also expressed at the ocellar region. Although in males, *otd-1* is highly expressed in the regions that will give rise to the three ocelli, in soldiers *otd-1* is strongly expressed in the ocellar region, marking the three ocelli in males, *otd-1* is also expressed more strongly across the head capsule (Fig.7B). In minor workers *otd-1* expression is weaker that soldiers and males at the ocellar region and remain more homogeneous across the whole capsule (Fig.7B). Despite that *otd-1* remains expressed in the ocellar region, the expression of *otd-1* is subsequently decreased in worker at late L4, where it decreases in the ocellar region in comparison with males (Fig.7B').

In terms of the genes involved in the visual development of the ocelli, eye, so and toy are downregulated at the early L4 in minor workers (Fig.8 C-E), whereas, in soldiers, *toy* and *so* remained expressed like males (Fig.8C, E). Insofar as the larvae undergo late L4 instar in workers and soldiers, *so* and *toy* expression is completely absent, whereas e*ya* expression, which was very strong in soldiers and minor workers at the beginning of the L4, is already absent at this late stage, leaving a line of expression around the region where *eya* was initially expressed following the same pattern than males (Fig.8 C'-E'). These results indicate that the expression of genes within the ocelli GRN is latent across castes during most of *C. floridanus* development, but most downstream gene expression is absent at the stage before pupation. Minor workers showed earlier downregulation than soldiers.

Orthodenticle may repress the development of ocelli in soldiers independently of head and body size.

In ants, Otd-1 shows conserved expression in ocelli relative to Drosophila (Fig.2) (Jean-Guillaume & Kumar, 2022). But unlike in *Drosophila*, otd-1 marks the entire region of the head capsule, and this is the region that is correlated to the difference in size between minor workers and soldiers during the L4 (last) larval instar (Fig.6). We, therefore, expected that otd-1 would affect the ocelli and head size development in soldiers. Since otd has two paralogs (otd-1 and otd-2) in ants with partially overlapping in expression in the head capsule (Supp Fig.2), we first knocked down both otd-1 and otd-2. Surprisingly, in contrast to our expectations, we found no significant effect on ocelli or size effects in males (P>0.05)(supp Fig.3) and no significant differences in 3 head size metrics in soldiers, eye length, scape length (antennal size), and mesosoma length (body size) between otd-1 RNAi and the yfp control (N_{control}= 48, N_{otd1+2}=43, P>0.05) (Fig.9). Our qPCR results showed that RNAi did not penetrate the eye-antenna disc since there was no significant downregulation of either Otd paralog. However, both paralogs of Otd showed significant downregulation in the brain in the independent RNAi knockdowns (Supp Fig.). Interestingly, the qPCR results show that in *Otd-1* RNAi, *Otd-2* expression is not affected by the downregulation of the paralog, but, instead, in the Otd-2 RNAi knockdowns, Otd-1 show a significant downregulation, suggesting that Otd-2 regulates upstream Otd-1. Despite the significant results of the paralogs downregulation in the brain, there is no apparent effect on size and shape phenotypes in the adults. Therefore, *Otd* expression in the brain does not regulate size and shape in soldier castes in ants. Finally, RNAi knockdown of otd-1 expression caused ocelli development resulting in rudimentary lateral ocelli in 3 out 15 individuals (Analysis of contingency p=0.1121) (Fig.10) with no significant change in head, eye, or body size as compared to controls ($N_{control} = 11$, $N_{otd+1} = 11$, P>0.05) (Fig.11). Although the appearance of rudimentary ocelli is not statistically significant, it raises the possibility that *otd-1* may be repressing ocelli development in soldiers of C. floridanus independently of head and body size.

3.5 Discussion

Our ancestral state reconstruction inferred a single gain, 3 losses, and 2 reversals of ocelli in workers in the subfamily Formicinae. Furthermore, our phylogenetic correlation analyses

showed no significant evolutionary correlation between the presence or absence of ocelli and period of activity, foraging substrate, geographic distribution, or worker polymorphism. Finally, we show that in the ant species *C. floridanus*, which has completely lost ocelli, expression of ocelli GRN is conserved relative to winged reproductive early in development and only later during the final stages of larval development (end of metamorphosis), the most downstream genes within the ocelli GRN are interrupted. The species where the ocelli were reversed belong to the clade Camponotini. Our RNAi experiments in *otd-1*, the precursor gene of ocelli, show that an ocelli-like trait was rescued partially in soldiers of *C. floridanus*, a species that belongs to the Camponotini group. Altogether, our results show that the retention of conserved expression of genes within the ocelli GRN in a worker and soldier caste that completely lacks ocelli represents the presence of a latent genetic potential. Overall, our results suggest that ancestral potential remains latent across development, which may be available to re-activate the expression of ancestral traits and play novel functions in caste development.

In some ant species within the Formicinae, there is clear evidence that ocelli in workers are functional, and therefore, the evolution of the trait was the result of an adaptation. In species from the Formicinae clade; *Cataglyphis bicolor*, the correlation between ocelli in workers and their mode of locomotion in response to light clues is well documented (Fent & Wehner, 1985). However, our comparative phylogenetic analyses suggest that this may be unique to a few species that the function of ocelli has been reported within the Formicinae, in which ocelli is an adaptive trait. Our analyses suggest, instead, that once ocelli evolved, their loss was rare, and its presence does not correlate with ecological or colony-level variables. Therefore, the evolution of ocelli might not be the result of an adaptive response in this group. But, rather explained by pleiotropic mechanisms that can maintain the underlying components activated under specific selective pressures or developmental mechanisms that can subsequently allow the organ to get re-gained, either because the organisms are exposed to ancestral environmental contexts or simply by random effects that cause this trait to appear without any apparent function (Collin & Miglietta, 2008; Sadier et al., 2022; West-Eberhard, 2003). In the case of ocelli, the developmental architecture of the eye-antenna imaginal disc, in which a single disc produces the compound eyes, the ocelli, the antennae, and the maxillary palps may produce a constraint on the ocelli GRN in workers, retaining its expression. Genes within the ocelli GRN are highly
conserved and play multiple functions across the eye-antenna disc, such as the case of *otd-1*, which is a precursor gene of head capsule besides of ocelli, or *toy, eya* and *so*, which are genes involved in eye development, determining the development of structures such as, optic lobes, cone differentiation, rhabdomeres development (Blanco et al., 2009, 2010; Buschbeck & Bok, 2023b). Thus, the fact that those genes play a conserved role in the development of multiple roles in a single disc could constrain the disappearance of their expression in the ocellar region of the eye-antenna disc in workers. Future work on the functional role of ocelli across species will provide evidence of the evolutionary forces that promote the trait to evolve and be reversed in workers across the phylogeny of ants.

The evolutionary reversal of ocelli in soldiers of the species from tribe Camponotini, along with our preliminary finding that otd-1 can partially re-activate ocelli development in soldiers of *C*. *floridanus*, raises the possibility that Otd-1 is a key player in retaining the ancestral potential for ocelli development. Furthermore, the presence of two duplicates (Otd-1 and Otd 2) could indicate that one duplicate helps to canalize the developmental mechanisms that allow the maintenance of the latent expression during the loss of function of another duplicate. Alternatively, the two duplicated genes may have diverged with potentially antagonistic effect between them. Therefore, once Otd-1 is downregulated, Otd-2 loses the pressure by Otd-1 and induces the ocelli in individuals who do not develop the trait. When genes undergo duplication, any of the copies of the genes can undergo either neofunctionalization by purifying selection or sub functionalization by relaxing selection (Ohta, 1989; West-Eberhard, 2003).

In the case of beetles, *Otd-1* underwent neofunctionalization in horn development, whereas *Otd-2* did not show a functional role in the studied species (Zattara et al., 2016). However, in some cases, the two paralogs that result from a duplication event can act antagonistically, as in the case of the paralogs genes PICKLE (PKL) and PICKLE RELATED 2 (PKR2) in plants, in which both genes evolved antagonist roles affecting different seed development in plants. Thus, PKL knockouts lead to seed size increase with a decrease in fertility, whereas knockout of PKR2 rescues seed size and increases fertility (Carter et al., 2016; Dupouy et al., 2023). Our results show that double knockdowns of *Otd* (*Otd-1+2*) did not show any phenotype associated with shape and size in soldiers, but instead, the single knockdown of *otd-1* may have partially induced ocelli phenotypes because the gene is no longer suppressing ocelli. However, the qPCR results

show that in the brain, *Otd-2* downregulated *Otd-1* but not the opposite way, although this is a promising result in the regulation of duplicates, the interaction in the eye-antenna disc between paralogs could be different. Future work is needed to test these possibilities. First, *otd-1* and *otd-2* RNAi must be performed at higher concentrations in males, minor workers, and soldiers to test whether the frequency of reactivation of ocelli development is dependent of the dose of *otd-1* and/or *otd-2*. This could be followed up by transcriptomic analysis to identify downstream genes that would reveal the role of both genes on ocelli and caste development in ants.

Finally, the evolutionary appearance of queen-like traits in workers and/or soldiers has been a source of controversy for understanding the origin and evolution of novel castes in ants. The fact that soldiers and workers develop a queen-like trait (ocelli), that was originally lost in the worker caste has been explained by several competing models: the first is the 'standard model', which proposes that queen developmental modules, such as ocelli, can be induced during worker caste development. Expression of the queen module can then be constitutively expressed and become a normal part of the worker developmental program through an evolutionary process called genetic accommodation (Abouheif, 2021; Molet & Peeters, 2006; D. E. Wheeler, 1991). The second model is called the hourglass model, which proposes that body size causes the induction of queen-like traits as individuals move from a worker-like size to a queen-like size along what they call a sigmoid 'caste reaction norm'. Individuals who are intermediate in size will have a mix of queen and worker-like characters (Trible & Kronauer, 2020, 2021). In this context, our results provide evolutionary and developmental insights into the origin of novel castes in ants, by showing that first, ocelli across Formicinae evolved in several species with only a single worker caste and not a soldier caste. Therefore, ocelli have evolved equally in workers than soldiers independent of size; and second, the potential experimental induction of ocelli in soldiers did not imply a significant increase in the size of head shape as shown by the RNAi experiments (Fig.9, 11).

Collectively, our study is the first attempt to understand from an evolutionary and developmental approach how the ocelli, a trait that develops only in queens and males in ants, has evolved in workers and soldiers in ants. Further work focusing on the functional role of ocelli across workers and soldiers would strengthen our findings on the non-adaptive evolutionary history of this trait. Likewise, the role of latent developmental mechanisms involved in the reversal of

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complex traits in ants and the evolutionary scenarios that promote it. With these results, we provide further evidence that the latent developmental potentials may generally promote the irreversibility of traits that are common in nature. Likewise, how in the evolution of novel castes in ants, the repurpose of ancestral mechanisms into novel traits remains as the main explication of the evolution or morphological novelty in this group.

3.6 Acknowledgments

We thank Hermogenes Fernandez, who shared his knowledge about ocelli in soldiers across the *Atta* species and contributed to inspiring work. We thank Dr.Lloyd Davis, Dr.Marc Seid, and Dr.Shelly Berger for helping us collect *Camponotus floridanus* colonies and Erik Plante and undergraduates for feeding and maintaining the *Camponotus floridanus* colonies. We thank Dr. Juan Carlos Penagos and Dr.Simon Joly for their suggestions on phylogenetic analysis. Dr.Arjuna Rajakumar for discussion and comments on the analysis and manuscript. We also thank McGill University's Integrated Quantitative Biology Initiative (IQBI) and Advanced Bioimaging Facility (ABIF) for imaging support. This work was supported by the Doctoral fellowship BESS-CREATE by the Natural Sciences and Engineering Research Council of Canada (NSERC) to A.V-C. and NSERC Discovery Grant to E.A.

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3.7 Figures Tables and Legends

Figure 1. Ocelli in queens and workers/soldiers across ants. Ocelli develops in queens and males across the phylogeny of ants. However, workers and soldiers evolved ocelli independently in ants (highlighted in red). Species *Camponotus gigas* showed ocelli in the soldier caste. *Camponotus floridanus* workers and soldiers did not evolve ocelli. *Lasius niger*, worker caste evolved but size is small that looks vestigial and *Tetraponera rufinogra* exhibit well-formed ocelli in the worker caste. Scale 1mm. *Photos*: antweb.com.

Figure 2. Eye-antenna development in Drosophila melanogaster and the main region of the

disc. A. Third larva instar of *Drosophila melanogaster* demarking the main regions: Antenna, Ocellar, Head capsule, and Eye. **B**. Schematic representation of the ocelli gene regulatory network at third larvae instar in *Drosophila melanogaster*. The genes used in this study are highlighted in magenta.

Figure 3. Bayesian ancestral estimation of 70 species of Formicinae and outgroups for presence and absence of ocelli. The reconstruction shows that ocelli were gained once in the group and underwent several loses and two reversals. Numbers on nodes represent Posterior Probabilities (PP), and the size of the circles varies according to the PP. The color in yellow is the presence of ocelli, dark blue color is the absence.

Figure 4. Species with ocelli did not show a correlation with geographic distribution. Latitude and longitude distribution of the species used in this study with the phylogenetic ANCOVA parameters.

Figure 5. Eye-antenna disc development across larvae stages and caste determination stages in *Camponotus floridanus* shows conservation of main regional identity genes identified in *Drosophila melanogaster:* First instar (A), second in star (F), third instar (K), worker-destined larvae (P) and soldier destined larvae (U.). Expression of studied genes on eyeantenna across stages and worker and soldier caste: *Distal-less* (green) (B, G,L,Q,V), *eyeless* (yellow) (C, H, M, R, W) and *orthodenticle-1* (*red*) (D, I, N, S,X).Merged images (E,J,O,T,Y). Fate map is followed by the *Drosophila melanogaster* eye-antenna disc development. Scale 100 um.

Figure 6. Head capsule has a higher area in soldiers than in minors' workers. Area of the antenna, eye, and head capsule in L4 and worker and soldier-destined larvae using 4 individuals per stage.

Figure 7. Inter-ocellar region specification in *Camponotus floridanus* **eye-antenna disc in conserved across castes in** *Camponotus floridanus*. Expression of *Hedgehog (hh)* and *orthodenticle-1(otd-1)* genes in the inter-ocellar region in males, workers, and soldiers at early (A, B) and late 4 (A', B') larvae instar. Hedgehog expression (A) divides the region that will give rise to the two lateral and medial ocelli in males, soldiers, and workers. *Orthodenticle-1* expression in males is across the head capsule. It marks the region where the two lateral and medial ocelli will develop in males at early and late stages of 4 instar, whereas gets weaker in soldiers and minor works at late 4 instars.

Figure 8. Ocelli specification in *Camponotus floridanus* is partially expressed across castes at early and late L4 instar. Expression of selected genes *Eyes absent (eya), twin of eyeless (toy)* and *Sine oculis (so)* in males, soldiers, and workers at early 4 stage and late 4 stages. At 4 larvae stages *eya-toy* and so are co-expressed at the ocellar region. In the late 4th instar, the expression of eye, toy, and *so* remains the same in males, whereas the expression is interrupted on workers and soldiers.

Figure 9. Double knockdowns of *otd-1+2 do* **not show a significant effect on the head and body in soldiers of** *Camponotus floridanus*. (A) Head above eyes, (B) Head below eyes, (C) Head across eyes, (D) Thorax length, (E) Eye length, (F) Scape length and (G) Head-to-body ratio.

Figure 10. The appearance of an ocelli-like structure in *otd-1* knockdowns in *Camponotus floridanus.* (A.) SEM of *Tetraponera rufonigra* pupae head, a species in which worker caste develops ocelli. (B) Dorsal view of the head of *Tetraponera* pupae showing the primordia of two

lateral ocellus (C) Control: View of the dorsal region o pupae head (C') Control: View in higher magnification of the homologous region where ocelli develop. (D) *Otd-1*: View of the dorsal region of pupae head (D') *Otd-1*: View in higher magnification of what looks like the primordia of a lateral ocellus

Figure 11. *Otd-1* **knockdowns do not show a significant effect in and body in soldiers of** *Camponotus Floridanus*. (A) Head above eyes, (B) head below eyes, (C) Head across eyes, (D) Thorax length, (E) Eye length, (F) Scape length, and (G) Head-to-body ratio.

Tables

Table 1. Model selection using marginal likelihoods compared with Bayes factors and model probabilities by stepping-stone sampling. Left column: models of evolution for discrete characters used in this study. Middle: marginal likelihood under stepping-stone sampling. Left: Bayes factors calculated as (Marginal lnL M₀- Marginal lnL M₁) where Model 0 is Mk and Model 1 either Freek model or Irreversible model.

Table 2. The evolution of ocelli does not show an evolutionary correlation with severalecological variables. Period of activity, Foraging substrate, and worker caste polymorphism.

Table 3. Geographic distribution does not correlate with the evolution of ocelli.Phylogenetic ANOVA for longitude and latitude and presence of ocelli

3.8 Supplementary Figures and Tables.

Figure 1. Simplified Gene tree based on the neighbor-joining tree showing relationships between *orthodenticle*/OTX paralogs in *Drosophila melanogaster*, *Apis mellifera*, *Nasonia vitripennis*, *Ontophagus Taurus*, *and Camponotus floridanus*.

Figure 2. *Orthodenticle* paralogs *otd-1* and *otd-2* expression in *Camponotus floridanus* In Soldiers and Males. Soldier Eye-antenna disc (A, A'), Males Eye-antenna disc (B, B')

Figure 3. Orthodenticle paralogs otd-1 and otd-2 expression in Camponotus floridanus brain (A, A')

Figure 3. *Otd-1* knockdowns do not show a significant effect on ocelli size and body of males of *Camponotus Floridanus*. (A) Head above eyes, (B) head below eyes, (C) Head across eyes, (D) Thorax length, (E) Eye length, (F) Scape length and (G) Media ocellus (H.) Lateral ocellus (R) (I) Lateral ocellus (L) (J) Adults of *otd 1+2* and control

Figure 4. Quantitative PCR (qPCR) for Brain and Eye-antenna disc (EAD) for *Otd* paralogs *Otd-1* and *Otd-2* RNAi Knockdowns independently. (A) *Otd-1* RNAi in the Brain (B) *Otd-1* RNAi in the EAD (C) *Otd-2* RNAi in the brain (D) *Otd-2* RNAi in the EAD

Figure 5. Ancestral state reconstruction using Unequal rates and Irreversible models.

Supplementary Table 1. Ocelli states for each species used in this study.

Supplementary Table 2. Database with the references used in this study for presence and absence of ocelli and each ecological variable used for the correlation analysis.

Supplementary Table 3. Primers for Housekeeping genes, target genes (otd-1 and otd-2) and downstrean genes used in this study.

Figures.

Figure 1.



Tetraponera rufinogra





В.



Figure 3.



Figure 4.











Figure.6







Figure 8.



Figure 9.



Control Otd-1+2

Figure 10.



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Figure 11.



Tables.

Table 1.

Model	ss. Marginal likelihood	Bayes Factors
Equal Rates -MMK	-43.59644	0
Unequal Rates-FreeK	-44.20686	0.61
irreversible -Irrev	-45.25448	1.047

Table2.

	Trait	Ind	ependent mo	odel	Hidden Markov independent model		Correlated model		Hidden Markov correlated model				
		Lnl	AIC	AICC c	Lnl	AIC	AICC c	Lnl	AIC	AICc	Lnl	AIC	AICC c
ocelli	Preiod of activity	-40.449	88.89725	90.3787	-39.1412	98.28245	108.7586	-38.75392	93.50784	99.76871	-38.58837	113.1767	165.7921
ocelli	Foraging substrate	-67.707	143.4141	144.183	-66.7935	153.587	158.3696	-66.77798	149.556	152.556	-65.0678	166.1358	184.1358
ocelli	Woker polymorphism	-81.133	170.2664	170.933	-79.3711	178.7421	182.8162	-79.04141	174.0828	176.6542	-78.51922	193.0384	207.908

Table 3.

Variable	df	F-value	P-value
Longitude	1	0.13401	0.7059
Latitude	1	0.17091	0.3137

Supplementary Figure 1.



0.03

Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.



Supplementary Figure 5.



Supplementary Figure 6.



Supplementary Table 1.

Species	Worker	Ocelli	Foraging	Foraging location
	Polymorphism		timing	(0=ground/underground/bush
	(0=monomorphic,	(0=absent,	(0=diurnal,	es, 1=arboreal/canopy)
	1=polymorphic)	1=present)	1=nocturnal)	
Acanthoponera minor	0	0	1	0
Acropyga acutiventris	0	0	Unknown	0
Aneuretus simoni	1	0	0	0
Anoplolepis custodiens	1	0	0	0
Anoplolepis gracilipes	0	0	1	0
Aphomomyrmex afer	1	1	Unknown	0

Bajcaridris theryi	0	1	Unknown	Unknown
Brachymyrmex depilis	0	0	Unknown	0
Calomyrmex albertisi	0	0	Unknown	0
Calomyrmex laevissimus	0	0	0	0
Camponotus claviscapus	1	0	Unknown	1
Camponotus conithorax	1	0	Unknown	Unknown
Camponotus gibbinotus	1	1	1	Unknown
Camponotus gigas	1	1	1	1
Camponotus hyatti	1	0	0	0
Camponotus maritimus	1	0	1	0
Camponotus saundersi	1	0	Unknown	1
Camponotus vitiensis	1	0	Unknown	0
Cataglyphis cursor	1	1	0	0
Cladomyrma petalae	1	0	0	1
Dolichoderus pustulatus	0	0	0	0
Echinopla australis	0	0	0	0
Euprenolepis procera	1	0	1	0
Formica moki	0	1	0	0
Formica neogagates	0	1	0	1
Gigantiops destructor	0	1	0	0

Iberoformica subrufa	0	1	0	0
Lasiophanes atriventris	0	1	Unknown	Unknown
Lasius californicus	0	0	Unknown	0
Lasius niger	0	1	0	0
Lepisiota canescens	0	1	Unknown	0
Manica bradleyi	0	0	Unknown	0
Myrmecia pyriformis	1	1	1	1
Myrmecocystus flaviceps	1	1	0	0
Myrmecorhynchus emeryi	1	1	Unknown	1
Myrmelachista flavocotea	0	0	Unknown	1
Myrmoteras iriodum	0	1	Unknown	0
Nothomyrmecia macrops	0	0	1	1
Notoncus capitatus	1	1	1	0
Notostigma carazzii	1	1	1	0
Nylanderia dodo	0	0	Unknown	0
Nylanderia hystrix	0	1	Unknown	0
Oecophylla longinoda	1	0	0	1
Oecophylla smaragdina	1	0	0	1
Opisthopsis respiciens	0	0	Unknown	1
Paraparatrechina	0	1	Unknown	0

glabra				
Paraparatrechina oceanica	0	0	Unknown	Unknown
ParatrechiUnknown	0	1	Unknown	0
antsingy				
ParatrechiUnknown	0	1	0	0
longicornis				
ParatrechiUnknown	0	1	Unknown	0
zanjensis				
Petalomyrmex phylax	0	1	0	1
Plagiolepis alluaudi	0	0	Unknown	1
Polyergus breviceps	0	1	0	0
Polyrhachis decumbens	0	0	Unknown	1
Prenolepis emmae	0	1	Unknown	Unknown
Prenolepis imparis	0	0	0	0
Proformica mongolica	1	1	Unknown	0
Prolasius convexus	0	1	Unknown	0
Pseudolasius australis	1	0	Unknown	0
Pseudonotoncus hirsutus	0	1	0	0
Rhytidoponera chalybaea	0	0	Unknown	Unknown
Rossomyrmex atolicus	0	1	Unknown	Unknown
Santschiella kohli	0	1	Unknown	1

Stimacros clivisp cf	0	0	Unknown	0
Teratomyrmex greavesi	0	1	Unknown	0
Tetraponera rufonigra	0	1	0	1
Zatania albimaculata	0	1	Unknown	Unknown

Supplementary Table 2.

Genus	Species	Worker	Ocelli	Period of	Forgaing
		Polymorphism		activity	Substrate
Acanthoponera	minor	Blanchard and Moreau (2017)	 Personal observations from AntWeb. Version 8.106.1. California Academy of Science images 	Antwiki.com	Antwiki.com
Acropyga	acutiventris	Blanchard and Moreau (2017)	Personal observations from AntWeb. Version 8.106.1. California Academy of Science images		Antwiki.com
Aneuretus	simoni	Wilson et al. (1956)	Personal observations from AntWeb. Version 8.106.1. California	Antwiki.com	Wilson et al. (1956)

			Academy of		
			Science images		
Anoplolepis	custodiens	Doering et al.	Personal	Lindsey and	Doering et al.
		(2018)	observations from	Skinner (2001)	(2018)
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Anoplolepis	gracilipes	Lee and Yang	Personal	Chong and Lee	O'Dowd et al.
		(2022) and La	observations from	(2009)	(2003)
		Richelière et al.	AntWeb. Version		
		(2022)	8.106.1.		
			California		
			Academy of		
			Science images		
Aphomomyrmex	afer	Snelling (1979)	Snelling (1979)		Gaume,
					Matile-Ferrero,
					and McKey
					(1999)
Bajcaridris	theryi	Greer and	Santschi (1936c)		
		Moreau (2021) -			
		for the genus			
Brachymyrmex	depilis	Blanchard and	Personal		Antwiki.com
		Moreau (2017)	observations from		
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		

Calomyrmex	albertisi	Blanchard and	Personal		Room (1975)
		Moreau (2017)	observations from		
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Calomyrmex	laevissimus	Antwiki.com	Personal	Antwiki.com-	Wilson (1959)
		(genus)	observations from	genus	
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Camponotus	bedoti cf	Yamane (2013)	Personal		
			observations from		
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Camponotus	claviscapus	Wheeler (1907)	Personal		DeRocha et al.
			observations from		(2015)
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Camponetus	aonithanar	MoArthur A. I	Emory (1014f)		
Camponolus	conunorax	(2007)	Emery (19141)		
		(2007)			
Camponotus	gibbinotus	McArthur, A. J. (2007) and Heterick (2021)	Personal observations from AntWeb. Version 8.106.1.	Heterick (2009)	
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			California Academy of Science images		
Camponotus	gigas	Pfeiffer and Linsenmair (2000) and La Richelière et al. (2022)	Personal observations from AntWeb. Version 8.106.1. California Academy of Science images	Orr and Charles (1993)	Orr and Charles (1993)
Camponotus	hyatti	Mackay and Mackay (2002)	Mackay and Mackay (2002)	Antwiki.com	Antwiki.com
Camponotus	maritimus	Blanchard and Moreau (2017)	Ward 2005	AntWeb. Version 8.106.1. California Academy of Science	AntWeb. Version 8.106.1. California Academy of Science
Camponotus	saundersi	Laciny et al. (2017)	Personal observations from AntWeb. Version 8.106.1. California Academy of Science images		Hashimoto (1997)
Camponotus	vitiensis	La Richelière et al. (2022)	Mann (1921)		Donisthorpe (1946)

Cataglyphis	cursor	La Richelière et	Personal	Thiélin-	Thiélin-
		al. (2022)	observations from	Bescond and	Bescond and
			AntWeb. Version	Beugnon	Beugnon
			8.106.1.	(2005)	(2005)
			California		
			Academy of		
			Science images		
Cladomyrma	petalae	Agosti (1991)	Agosti (1991) -	Moog (2009)	Moog et al.
			for the genus		(1997)
			D 1	A	4
Dolichoderus	pustulatus	Greer and	Personal	Antwiki.com -	Antwiki.com
		Moreau (2021) -	observations from	genus	
		for the genus	AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Fabinanla	augtuglig	Dispersion of	Damonol	Eal and (for	$T_{ovlow}(1002)$
Ecninopia	australis	Manager (2017)	Personal	Eol.org (lor	Taylor (1992)
		Moreau (2017)	AntWeb Version	Tamity)	
			Antweb. version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Echinopla	straita nr	La Richelière et	Personal		Xu and Zhou
_		al. (2022)	observations from		(2015)
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		

Euprenolepis	procera	LaPolla 2009	LaPolla 2009	Witte and	Witte and
				Maschwitz	Maschwitz
				(2008)	(2008)
Formica	moki	La Richelière et	Cole (1943)	MacArthur-	Whitman and
		al. (2022)		Waltz et al.	Gotelli (2011)
				(2021)	
Formica	neogagates	La Richelière et	Personal	Weseloh (2000)	Weseloh
		al. (2022)	observations from		(2001)
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Gigantiops	destructor	La Richelière et	Smith (1858)	Beugnon et al.	Wheeler
		al. (2022)		(2021)	(1922)
Iberoformica	subrufa	Greer and	Antwiki.com-	Cavia (1990)	Greer and
		Moreau (2021) -	genus		Moreau (2021)
		for the genus			- genus
Lasiophanes	atriventris	Greer and	Personal		
		Moreau (2021) -	observations from		
		for the genus	AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
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Lasius	californicus	Blanchard and	Personal		Greer and
		Moreau (2017)	observations from		Moreau (2021)
			AntWeb. Version		- genus
			8.106.1.		
			California		

			Academy of		
			Science images		
Lasius	niger	Okrutniak et al.	Personal	Jones et al.	Greer and
		(2020)	observations from	(2019)	Moreau (2021)
			AntWeb. Version		- genus
			8.106.1.		
			California		
			Academy of		
			Science images		
Lepisiota	canescens	Sorger et al.	Sharaf et al.		Sorger et al.
		(2016)	(2020)		(2016)
Manica	bradleyi	Greer and	Personal	Greer and	Greer and
		Moreau (2021) -	observations from	Moreau (2021)	Moreau (2021)
		for the genus	AntWeb. Version	- genus	- genus
			8.106.1.		
			California		
			Academy of		
			Science images		
Myrmecia	pyriformis	Dietemann et al.	Personal	Reid et al.	Narenda et al.
		(2014)	observations from	(2013)	(2016)
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Myrmecocystus	flaviceps	Blanchard and	Personal	Elst et al.	Antwiki.com
		Moreau (2017)	observations from	(2021)	
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		

Myrmecorhynchus	emeryi	La Richelière et	Wheeler (1917)		Shattuck
		al. (2022)			(2015)
Myrmelachista	flavocotea	Blanchard and	Personal		Longino
	5	Moreau (2017)	observations from		(2006)
			AntWeb Version		(2000)
			9 106 1		
			Galifarria		
			Academy of		
			Science images		
Myrmoteras	iriodum	Ito et al. (2017)	Personal		Greer and
			observations from		Moreau (2021)
			AntWeb. Version		- genus
			8.106.1.		
			California		
			Academy of		
			Science images		
Nothomyrmecia	macrops	Blanchard and	Personal	Taylor (1978)	Taylor (1978)
		Moreau (2017)	observations from		
			AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
			Science images		
Notoncus	capitatus	La Richelière et	Emery (1925) -	Brown (1955) -	AntWeb.
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		ui. (2022)	Sentas	Senus	8 106 1
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Notostigma	carazzii	Blanchard and	Emery (1920b)	Taylor (1992)	Taylor (1992)
Notostigma	carazzii	Blanchard and Moreau (2017)	Emery (1920b)	Taylor (1992)	Taylor (1992)

Nylanderia	dodo	Williams et al.	LaPolla (2011) -		Greer and
		(2020)	for the genus		Moreau (2021)
					- genus
Nylanderia	hystrix	Blanchard and	Kallal and		Greer and
		Moreau (2017)	LaPolla (2012)		Moreau (2021)
					- genus
Oecophylla	longinoda	La Richelière et	Personal	Dejean (1990)	Wikipedia
		al. (2022) and	observations from		
		Frumhoff and	AntWeb. Version		
		Ward (1992)	8.106.1.		
			California		
			Academy of		
			Science images		
Oecophylla	smaragdina	Blanchard and	Cole and Jones	Chong and Lee	Holldobler
		Moreau (2017)	(1948)	(2009)	(1983)
Onisthonsis		Dispersion d	Demonsl		Wheeler
Opisinopsis	respiciens	Maraan (2017)	Personal		
		Moreau (2017)	AntWah Varsion		(1918)
			Alltweb. Version		
			8.100.1.		
			California A codomy of		
			Academy of		
			Science images		
Paraparatrechina	glabra	Greer and	Personal		Lampasona
	0	Moreau (2021) -	observations from		(2015)
		for the genus	AntWeb. Version		()
			8.106.1.		
			California		
			Academy of		
			Science images		
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Paraparatrechina	oceanica	Greer and	Personal		
		Moreau (2021) -	observations from		
		for the genus	AntWeb. Version		
			8.106.1.		
			California		
			Academy of		
			Science images		
Paratrechina	antsingy	LaPolla and	LaPolla and		Greer and
		Fisher (2014) -	Fisher (2014)		Moreau (2021)
		for the genus			- genus
D (11			D 1	XZ C . 1	D 1 1
Paratrechina	longicornis	Blanchard and	Personal	Yusuf et al.	Brassard et al.
		Moreau (2017)	observations from	(2021)	(2021)
		and Kenne et al.	Antweb. Version		
		(2005)	8.106.1.		
			California		
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Paratrechina	zanjensis	LaPolla, Hawkes,	LaPolla, Hawkes,		Greer and
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Pseudolasius	australis	Blanchard and	Emery (1925)-		Greer and
		Moreau (2017)	genus		Moreau (2021)
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Supplementary Table 3.

Gene	Oligo FWD	Oligo REV
eIF3sE (eukaryotic translation initiation factor 3)	ACGCACATGGCTCATTCACT	GTGGCCGATAAAGAAACATCTCG
Cold Shock Domain Containing E1(CSDE1)	GGCAAGCCTATTGCAAGCAC	ACCAATGCATCGTTGATCACC
Squid (sqd)	ACGGGGAGATCGAGAGGTTT	GACATGATCACCGGCTGACA
SUMO-conjugating enzyme (UBC9-B)	ATGTCAGGCATAGCGATCGC	CATCCGGGTTTTTGGTTGGTC
THO complex subunit 1(THO)	CGGCACAGGATCTGTCAGAG	TCCTCTACCTCCACGTCGAC
Defective ventriculos (dve-1)	AGATGCCGTATCGCCAGAAG	GCCCTACGGTTCTTGAACCA
Orthodenticle -2 (Otd- 2)	AGCATGCCGTCAATAGATGCT	CGTTGCTTTCTCGGATTGCA
Orthodenticle-1 (Otd- 1)	CAGACGATCCTCGCCAGATT	GGCGTCGATAAGGGTGAGT
Eyeless (ey)	GCGCGTTTACGTCTGAAGAG	GTGCGTCCTCTCGAACTCTT

Discussion

4. Discussion

My thesis is built under the field of Evolutionary Developmental Biology (Evo-Devo), focusing on one of its core questions: the origin of new phenotypes. I have chosen ants as the focal organisms for investigating this question due to their extraordinary complexity, particularly in their caste systems. The system of castes in ants comprises the evolution of a reproductive male and queen caste and a non-reproductive worker caste within ant colonies. The worker caste was further elaborated several times independently during ant's evolution into a complex system of worker subcastes-minor workers and soldiers (Gregg, 1942; Hölldobler et al., 1990; Wilson, 1953). The evolution of a soldier caste in ants is an evolutionary novelty (Molet & Peeters, 2006). The primary morphological differences between soldiers and workers are predominantly explained by the head difference between the two. The large soldier head harbors a greater volume of muscle needed for a range of functions, including a strong bite for defense or seed processing; the morphological changes are explained by changes in head-to-body allometry (Casadei-Ferreira et al., n.d.; Huang, 2010; Klunk et al., 2021; Powell, 2009). The evolution of a minor worker and soldier's head is defined mostly by changes in head-to-body allometry, which arise from differences in the head's growth rate relative to the overall body (Huxley, 1972; Wheeler & Frederik Nijhout, 1983; Wilson, 1953)

Defining castes in ants poses a challenge due to the presence of conflicting hypotheses. The "hourglass model" views castes solely as variations in size, suggesting that colony individuals are part of uniform selection rules, with size differences reflecting responses to reaction norms. This model focuses on variation within the colony rather than the emergence of novel castes. In contrast, the "standard model", takes advantage of the superorganism concept of ants to propose that novel caste, like a multicellular organism, originated as independent traits (body parts), supported but the modular organization of the developmental programs, that allow modules to accommodate and re-evolved under different evolutionary rules. Under this framework, each caste is governed by its selective pressures, fostering the creation of novel castes. My work brings insights into the evolution and development of novel castes in ants through a comparative and integrative approach that focuses on the head as the major driver of morphological novelty. Thus, through the two chapters, I provide developmental and evolutionary evidence on the evolution of novel ty in ants; through first, I tested the evolution of the evolution of soldiers is

first, explained by the framework of allometry proposed by E.O Wilson on how from simpler allometry rules, we could get to the most complex allometries, therefore to explain the origin of morphological distinct castes, like soldiers and workers. In the second chapter, I explore the mechanisms of caste differentiation and how caste traits evolved and remained latent across development, maintaining an evolutionary opportunity to be repurposed into novel traits.

4.1 Major findings in Chapter 1.

In Chapter 1, I provide a formal test to the first formal hypothesis on the evolution of castes in ants through changes in head-to-body allometry, proposed by E.O. Wilson in 1953 (Wilson, 1953). Thus, the first work aims to test one of the classic and more broadly used qualitative and quantitative approaches to the categorization of worker castes in ants. However, examples of how head-to-body allometry explains the diversity of worker castes in ants abound. However, whether larger-headed individuals that are called soldiers constitute a discrete caste, or simply represent major workers along a continuum of size variation remains controversial. My study represents the first formal test of this hypothesis using the detailed characterization of the headto-body allometries throughout the group of farming fungus ants, one of the tribes where the most complex caste systems evolved: leaf-cutter ants. My research addresses this issue by first establishing a comprehensive characterization of allometry in worker polymorphism among farming fungus ants. Secondly, I explore the evolutionary trajectories of allometries within this group. I discovered that an increase in size variation within the group is a prerequisite for changes in allometric parameters along the regression line. Furthermore, through breakpoint analysis indicating shifts in head-to-body slope, I infer the evolution of soldiers in the group as a discrete morphological distinct caste.

One of the important results of this chapter is to challenge Wilson's 1953 predictions that the evolution of worker caste polymorphism is mostly gradual through transitional stages that respond to gradual changes in size variation (Wilson, 1953, 1954). More precisely, to evolve from monomorphic (only a single caste), represented by linear allometry with small variation in size, to worker polymorphism (several castes) could only happen with first an increase in size and afterwards a change in the head to regression without a rapid change in the linearity of the relationship, what he called monophasic. Instead, on the evolution of the head-to-body allometry

in farming fungus ants, I showed that the evolution of worker caste polymorphism does not require intermediate steps in allometric changes. Therefore, allometry shifts occur directly from species with low size variation (monomorphic) to those with biphasic allometry. This suggests that at the node where the leaf-cutter agriculture evolved, greater variation in size allowed the species to evolve biphasic allometry at the same time. My results align with what has been known in leaf-cutter ants, in which these evolutionary shifts in head-to-body allometry may have been driven by various innovations within the group, such as the transition from free-living cultivar to obligate symbiosis, leaf-cutter agriculture, in which species use fresh vegetation to farm their symbiont fungus and a significant increase in colony size (Branstetter et al., 2017; Mueller et al., 2005; Püffel et al., 2023; Wilson, 1980). The underlying cause-effect relationship among the novel features and which one catalyzes the emergence of the others remained unknown.

The head-to-body allometry conducted in this project counted with a big sample size per species, which allows us to capture the overall size variation plus allometry changes along the head-tobody regression. In total, around 4000 individuals were measured across 18 species. Previous studies have based their analysis on worker caste polymorphism only from a few individuals and relied only on the media of head-to-body size. With the data from this study, we were able to capture the variation in size along with the detection of slight changes in breakpoints in which the allometry parameters changed significantly. Therefore, the results of this work capture more precisely how the evolution of worker polymorphism is not just about the increase of a continuum of size variation but instead how the head growth gets readjusted to new parameters of organ growth and coordination. These changes produce variation needed for selection to act upon the mechanisms that promote it.

The choice of the system for this chapter was not accidental. Farming fungus ants have been categorized as one of the most complex societies after humans; they evolved in agriculture 50 MYA ago and are only distributed in America, restricted to tropical areas, from Texas, USA to Argentina (Branstetter et al., 2017; Mueller et al., 2005, 2018). Farming fungus ants evolved a complex symbiont relationship with the cultivar of the family *Leucocoprinus* and the bacteria that provide antibiotics to take care of the fungus (Currie, 2003). This tripartite symbiotic relationship has shaped the colony organization and, therefore, the evolution of caste complexity.

Generally, basal farming fungus species develop a facultative symbiosis with the cultivar in which the fungus has a free-living stage. However, towards more derived species, the symbiont relationship begins obligated. These transitions on the farming strategies are followed by the increase of colony size and agricultural strategies used by the species to maintain the fungus alive and healthy (Mueller et al., 2018). Leaf-cutter agriculture is derived from species of the clade; they evolved the obligate symbiosis, big colony size, and leaf-cutting behavior to farm their cultivar (Mueller et al., 2018; Shik et al., 2016). In these species, the slope and intercept changes across the regression line highlight the major adaptive relevance of head modifications in the evolution of worker polymorphism across leaf-cutter agriculture (Camargo et al., 2015; Püffel et al., 2021; Wilson, 1984). Therefore, the shifts in head-to-body scaling could indicate differences in selection pressures, such as a strong selection upon the head given the new colony and ecological roles that leaf-cutting agriculture demands. Further studies would support these results through a bigger sample across the group and conducting formal evolutionary correlations that establish which ecological, colony or external factors contributed to the evolution of worker caste polymorphism in a single node and the explosion of allometries afterward or whether several of these variables influenced in concerted the evolution of allometry and therefore worker caste polymorphism in the group.

In the studies of allometry in insects, the development explanation of how scaling differences between a specific organ and the overall body size occur are mainly explained by two possible scenarios. First, Organ re-programming; once there is a break in the slope changes, developmental mechanisms of organ growth and differentiation are driven by developmental thresholds, in which individuals below and above a "critical threshold" develop into different distinct morphologies (Abouheif, 2021; Moczek & Nijhout, 2002; Nilsson-Örtman & Rowe, 2021; Wheeler, 1986). Second, extreme positive allometries (when the trait has a higher growth rate than the body as a whole) until resources are exhausted (Tomkins et al., 2005). In this study, my data support the reprogramming event showing that on *Atta cephalotes*, one of the species that showed two breakpoints across the head-to-body regression develop medial ocellus at the first breakpoint and two and three ocelli after the second breakpoint. Therefore, the second hypothesis about positive allometries would not explain the emergence of losses that initially were lost and only remained in the reproductive caste, especially since the emergence of ocelli in

soldiers is rare. In contrast, size variation in workers and allometry changes occurred several times in ants, therefore, if the extreme positive allometries supported our results, we would expect a higher occurrence of ocelli in bigger or soldiers across the phylogeny.

Overall, this study is the first attempt to provide an evolutionary analysis of allometry to explain the evolution of worker caste polymorphism in ants. Here, we found evidence that head-to-body allometry changes correspond to the evolution of worker caste polymorphism in farming fungus ants. More specifically, our findings show how worker caste polymorphism correlates with an increase in colony complexity, including a stepped increase in colony size, along with the evolution of novel farming strategies. These results are the first analysis that aimed to explain E.O Wilson's hypothesis of worker caste polymorphism, using linear regressions and phylogenetic comparative methods. Further studies including more species with worker caste polymorphisms, will provide valuable insights into which are the developmental processes that shape the allometries and promote the generation of morphological variation in ants' colonies. Especially centring on the importance of colony life traits and ecological factors in ants' origin and evolution of social complexity.

This chapter aims to establish a standardized methodological and conceptual framework for defining castes within ant colonies. Despite ongoing efforts, the lack of consensus on the definition of worker castes and the identification of new castes in ants has persisted for years. While recent hypotheses and studies have advanced our understanding of caste polymorphism, the ambiguity remains unresolved and debated. Thus, I anticipate that this study will provide a foundational framework for researchers seeking to delineate ant castes, promoting greater methodological consistency and facilitating interdisciplinary dialogue. Ultimately, this will move the study of ant castes forward, enabling deeper insights into the evolution of complexity in these systems.

4.2 Major findings in Chapter 2.

In Chapter 2, I explore the evolution of ocelli in workers and soldiers and the implications of the appearance of a trait that was lost in workers and remained in reproductive (queen-related trait) in the evolution of novel castes in ants. Ocelli in the worker is a labile trait since this trait has

evolved several times across the evolution of ants. The fact that ocelli was lost in workers' and soldiers' castes but evolved afterward independently shows the ability of this trait to get reversed across the phylogeny. However, whether it is only a reversion of functional traits or represents a developmental ancestral potential playing a novel role in workers and soldiers remains unsolved; this study shed light on the evolutionary and developmental implications of the reversal of this trait.

To develop this chapter, we begin exploring the head in ants from a developmental approach. To begin with, we first develop a characterization of the eye-antenna disc in ants to establish the main region of organ development across larvae stages. The eye-antenna disc has not been broadly studied in holometabolous insects, one of the reasons for the lack of studies could be explained by the fact that the gene regulatory mechanism of organ regionalization and determination is highly conserved across developmental stages; therefore, the characterization in the model organisms like *Drosophila* is sufficient to extrapolate the main regulatory mechanisms of head regionalization and development across the group. I used highly conserved genes precursors of eye (eyeless), antenna (distal-less) and head capsule (orthodenticle)(Haynie & Bryant, 1986; Held Jr, 2002). Although I found that the three studied regions are conserved between C. floridanus and Drosophila melanogaster, C. floridanus shows an eye-antenna imaginal disc with a different reorganization than Drosophila. We found that whereas in *Drosophila*, the eye is the organ of a major area and develops from the early stages, in ants, the antenna and head capsule occupy big areas in comparison with the eye, and the development of the antenna and head capsule is established from the earlier stages of development what does not occur in *Drosophila*. Therefore, our results showed that despite ants and flies conserving the same regulatory genes in the regionalization of the eye-antenna imaginal disc, the organization of the regions within the imaginal disc is not conserved between them; these differences might account for the morphological disparities exhibited by the adults of these two species and across castes in ants, in which *Drosophila* head exhibits a bigger eye. In contrast, ants generally develop larger antennas and head capsule. Although along the phylogeny of ants, some nocturnal species of ants have developed bigger eyes such us Gigantiops destructor, making it the ant species with the largest eyes relative to body size, further studies developing a more comparative work of the

eye-antenna development in ants could explain at which extent the imaginal disc in conserved across species.

The first fate map constructed for ants in this study has provided valuable information on how the head develops in these organisms, likewise, which are the axes of major changes between workers and soldiers. We found that the head capsule covers a bigger area in soldiers than minor workers during development. These results complement what has been shown in terms of the head morphology of soldiers in ants. Studies have shown that the head morphology of adult soldiers arises mainly by an increase in muscle volume either for a stronger bite either for the defense of food processing or for colony protection by obstructing the nest entrance against intruders (Amador-Vargas, 2019; Huang, 2010; Klunk et al., 2021; Powell, 2009). For instance, in *Pheidole* species, the big head in soldiers is explained by the incremental muscle mass, capable of maintaining a stronger bite, that is made specifically for the colony defense (Casadei-Ferreira et al., n.d.; Huang, 2010; Klunk et al., 2021). In these species, the muscle in charge or closing the mandible- the cranio-mandibula's internus (0md1) -can occupy up to two-thirds of the overall head volume, which demands an elongation on the head capsule (Casadei-Ferreira et al., n.d.; Huang, 2010; Lillico-Ouachour et al., 2018). In contrast, relative to size, organs such as the brain, antenna and eye are smaller or remain equal in soldiers relative to workers.(Lillico-Ouachour et al., 2018; Tawdros et al., 2020; Tschinkel et al., 2003). Therefore, the head capsule plays an important role in soldier development, which is associated with the overgrowth of muscle development in these organisms. Further studies on muscle development in ants, which in Drosophila originates from mesodermal cells (Gunage et al., 2017), would elucidate the relevance of developing a bigger capsule and how that is orchestrated or coordinated but muscle development.

Big-headed soldiers are the result of big capsules. However, the gene precursor head capsule in Drosophila orthodenticle (*otd*), which showed expression across head capsules in ants and across castes, did not show to play a functional role in making big-headed soldiers in ants. In ants, wasps, bees and beetles, the gene previous studies have shown from other organisms that the gene *otd* is a gene that has played several roles in bringing novelties in insects, such as the case of beetles. In beetles, horns are considered an evolutionary novelty, a trait that is regulated by *otd-1* in the species of the genus *Onthophagus*. In *Onthophagus taurus*, RNAi experiments in

otd-1 reduced or caused complete deletion of large males' horns., *otd-1* RNAi induced horns in the females and small males, who usually are hornless. More surprising, in the sister group species, *Onthopagus Sagittarius otd-1* RNAi induced ectopic eyes in both males and female (Zattara et al., 2016, 2017). Although ants did not show effects on shape or size with *otd-1*, interestingly, we also found that three individuals from the RNAi treatments, develop two structures like the ocelli, a similar case that one reported in beetles. Therefore, compared with previous work in beetles, it showed that *otd-1* might have co-opted to play a role in big-headed soldiers, which was not captured in our RNAi experiments, but once the gene is silenced, an ancestral trait is recovered. Further work using gene expression of several genes downstream of *otd-1* will elucidate whether *otd-1* is playing a role in soldier development or whether it is co-opted to play a role in another developmental mechanism, likewise, to conduct more tissue-specific targets given the role that *otd-1* may play in the neural system, which make harder to take conclusion about its tissue-specific role.

In ants, ocelli development occurs only in the reproductive caste, although the trait has been lost in workers and soldiers. However, ocelli have evolved independently several times in workers and soldiers in ants. In most cases, ocelli develop completely and play a role in worker navigation; however, in other cases, ocelli develop as vestigial in workers or soldiers of several species. Our results support previous results that show that the potential to produce ocelli is latent throughout development. Therefore, this study complements previous findings in which targeting *orthodenticle*, *otd-1*, the precursor gene of the ocelli pathway, could produce the recovery of the potential that was lost, bringing back the ocelli in workers and soldiers. Although further work is needed to test whether the organ that develops from our mutants corresponds to the ocelli or simply a side effect of a latent mechanism that is activated and whether this effect is the result of *otd-1* and the *otd-2* role on head and ocelli development respectively, by which silenced of *otd-1* allow to *otd-2* to recover the ocelli by antagonistic effects.

These results are the primer for showing a developmental mechanism in which the genetic machinery of an ancient trait could be reversed, but the reversion could imply more than bringing an ancestral trait back; instead, it could mean the developmental reorganization of ancestral mechanisms into new outcomes. Consequently, in the studies of the reversibility of traits, understanding the developmental significance of the reversal is crucial since many of the traits

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that are reversed instead of being the reversal of a trait can carry a novel developmental function that is not always detected by modern evolutionary methods. Part of the results of this chapter remained preliminary. Therefore, further studies and experiments using different RNAi doses of *otd-1* and RNAi experiments targeting only the *otd* paralog *otd-2*, will provide information on whether the induction of ocelli is real with the silencing of *otd-1* and *otd-2* is playing antagonist effects on head and ocelli development, or *otd-2* play a more important role in soldier development instead of *otd-1*.

5. Conclusion

Much work is still needed to understand how novel phenotypes originate. However, studies under the framework of evolutionary developmental biology have provided valuable frameworks for understanding how novel traits originated and the importance of ancestral developmental programs on the emergence of novelties. In ants, the complex social organization that evolved in this system provides a good model for addressing questions regarding novel traits. These organisms exhibit a range of behaviors and morphological novelties that explain the nature of their social organization. In this work, we showed that the head is the hotspot of novelties and that part of the head, being the module of modularity, is explained by the general allometry rules of organ growth and scaling. In ants, caste development is by phenotypic plasticity. Therefore, novel castes are potentially induced by ancestral developmental potentials and regulated by developmental thresholds, in which some individuals cross the thresholds and activate ancestral mechanisms that play a novel role in regulating the novel features. The fact that the developmental potential to produce caste-like traits is present across all castes in the colony brings a new hypothesis and explanation of how caste develops and is established through the colony and how some of the traits that we have defined, like queen-like traits are not necessarily restricted to the reproductive caste. Still, instead, they represent traits with labile or biased developmental trajectories that have facilitated the evolution of new castes in ants, either by coopted mechanisms or by the simple reappearance of the still conserved developmental program, what is called atavisms. This study sets the basics on head development in ants and provides insights into which mechanisms might account for the development of novelty in castes.

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