THE DEVELOPMENTAL BASIS OF CASTE EVOLUTION IN ANTS

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DEDICATION

This thesis is dedicated to my best friend, wife and soul mate Marina as well as my parents, Joy and Chellappa, and my son Anthony

ABSTRACT

Phenotypic plasticity is the ability for a single genotype to give rise to alternative adaptive phenotypes in response to environmental conditions facilitating their survival. In some cases, environmental conditions can influence the course of development of an organism, leading to the induction of novel phenotypic variation, the raw materials for selection in evolution. Although this fact has the potential to unify the disparate fields of ecology, development and evolution, we have only begun to investigate the underlying molecular mechanisms that translate the environment into phenotypic diversity. Ants are highly plastic; during development a single genotype can give rise to an array of alternative phenotypes related to dramatic differences in morphology, longevity, reproduction and behavior. This environmental sensitivity is the basis for the diversity of complex ant caste systems. Here, I used ant development of the hyperdiverse genera *Pheidole* and *Camponotus* as models to investigate my major goal, which is to understand how ecology (environment) acts on development, generating morphological variation, which can then lead to morphological diversification and evolution. The first specific goal (Chapter 2) of my thesis is to investigate the hormonal and developmental genetic basis underlying the evolution of novel worker ant subcastes. Specifically, the genus *Pheidole* is composed of over 1000 species, all of which comprise a complex worker caste system of minor workers and soldiers. In a hand full of these species, there exists an additional novel worker subcaste, the supersoldier. Through phylogenetic and developmental genetic analysis, I determined that this subcaste has evolved in parallel in different species. I then discovered through field observations and hormonal manipulations that there exists an ancestral developmental potential in this group: all

Pheidole species have the hidden capacity to produce supersoldiers through environmental induction, the recurrence of which can lead to their evolution. The second specific goal of my thesis (Chapter 4) is to investigate the epigenetic mechanisms that translate environmental conditions into morphological variation within castes. Specifically, I investigated the involvement of DNA methylation in generating continuous sizing in the worker caste of the genus *Camponotus*. I discovered that DNA methylation is responsible for generating a continuous distribution of worker size and that one of its primary targets is the gene *Egfr*. Furthermore, the methylation level of *Egfr* is associated with quantitative variation in worker size and pharmacological inhibition of EGFR signaling demonstrated that this pathway is capable of generating the continuous distribution of size found within this caste. DNA methylation is an epigenetic mechanism that is known to cause transgenerational inheritance and therefore it can facilitate the evolution of environmentally generated quantitative variation. Collectively, the results of my thesis show how the environment acts on development through the integration of hormones, genes and epigenetic mechanisms to generate phenotypic variation for selection to act on. Perhaps we are coming closer to a point in time in evolutionary theory when we can say that the environment is as important in generating phenotypic variation as it is in the process of selection.

ABRÉGÉ

La plasticité phénotypique est l'habileté d'un génotype unique de produire des phénotypes adaptatifs alternes en réponse à des conditions environnementales facilitant leur survie. Dans certains cas, les conditions environnementales peuvent influencer le cours du développement d'un organisme, menant à l'induction d'une variation

phénotypique nouvelle, qui est la matière brute pour la sélection en évolution. Bien que ce fait ait le potentiel d'unifier les champs distincts de l'écologie, du développement et de l'évolution, on commence seulement à étudier les mécanismes moléculaires fondamentaux qui traduisent l'environnement en diversité phénotypique. Les fourmis démontrent une grande plasticité phénotypique; durant le développement, un génotype unique peut produire une diversité de phénotypes adaptatifs qui démontrent des différences dramatiques de morphologie, de longévité, de reproduction et de comportement. Cette sensibilité environnementale est à la base de la diversité des systèmes complexes de castes chez les fourmis. Ici, j'ai utilisé le développement des genres hyperdiversifiés *Pheidole* et *Camponotus* comme modèles pour investiguer mon but principal, qui est de comprendre comment l'écologie (l'environnement) agit sur le développement, en générant de la variation morphologique qui peut par la suite mener à une évolution morphologique. Le premier objectif spécifique de ma thèse (Chapitre 2) est d'investiguer les bases hormonales et du développement des nouvelles sous-castes ouvrières chez les fourmis. Plus spécifiquement, le genre *Pheidole* est composé de plus de 1000 espèces, toutes démontrant un système de castes ouvrières complexe comprenant des ouvrières mineurs et des soldates. Chez un petit groupe de ces espèces, il existe une caste ouvrière additionnelle, la supersoldate. En utilisant des analyses phylogénétiques et de génétique du développement, j'ai déterminé que cette sous-caste a évolué en parallèle chez les différentes espèces. J'ai par la suite découvert, par des observations sur le terrain et des manipulations hormonales, qu'il existe un potentiel ancestral de développement dans ce groupe: toutes les espèces de *Pheidole* ont une capacité cachée de produire des supersoldates par induction environnementale, cette récurrence pouvant mener à leur

évolution. Le second objectif spécifique de ma thèse (Chapitre 4) est d'investiguer les mécanismes épigénétiques qui traduisent les conditions environnementales en variation morphologique entre les castes. Plus spécifiquement, j'ai investigué le rôle de la méthylation de l'ADN dans l'élaboration d'une distribution de taille continue chez la caste ouvrière de *Camponotus*. J'ai découvert que la méthylation de l'ADN génère une distribution continue de taille chez la caste ouvrière et que l'une de ses cibles principales est le gène *Egfr*. D'ailleurs, le niveau de méthylation de *Egfr* est associé à une variation quantitative de la taille des ouvrières et une inhibition pharmacologique de la signalisation EGFR a démontré que cette voie de signalisation est capable de générer la distribution continue des tailles dans cette caste. La méthylation de l'ADN est un mécanisme épigénétique qui est connu pour causer une héritabilité transgénérationelle et donc, elle peut faciliter l'évolution d'une variation quantitative générée par l'environnement. Collectivement, les résultats de ma thèse montrent comment l'environnement agit sur le développement par l'intégration des hormones, des gènes et des mécanismes épigénétiques pour générer de la variation phénotypique sur laquelle la sélection naturelle peut agir par la suite. Peut-être que nous nous rapprochons d'un moment où la théorie de l'évolution peut proposer que l'environnement soit également important pour générer de la variation phénotypique qu'il peut l'être au cours du processus de sélection.

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PREFACE: Contribution of Authors

This manuscript-based thesis comprises my original work, Chapter 2 has been published, Chapter 3 is in the process of publication (in press) and Chapter 4 has been submitted for publicaiton. I am the primary author for all (co-primary author for Chapter 4) chapters. I conceived and performed the majority of the experiments; analyzed data generated and wrote the manuscripts. Ehab Abouheif mentored and supervised all aspects of the work. The details of the contributions of the co-authors for these works are detailed below:

Chapter 2

A version of chapter 2 has been published as: "Rajakumar, R., San, Mauro, D., Dijkstra, M.B., Huang, M.H., Wheeler, D.E., Hiou-Tim, F., Khila, A., Cournoyea, M., and Abouheif, E. (2012). Ancestral developmental potential facilitates parallel evolution in ants. *Science* 335, 79-82". The following authors helped with experiments: Dr. D. San Mauro, Dr. M.B. Dijkstra, Dr. M.H. Huang, F. Hiou-Tim, Dr. A. Khila and M. Cournoyea. I collected and reared the majority of the ants used in this study with the help of Dr. D. San Mauro, Dr. M.B. Dijkstra, Dr. M.B. Huang and M. Cournoyea. I designed experiments with the help of Dr. D. San Mauro, Dr. M.B. Dijkstra, Dr. M.H. Huang. I analyzed the data with the help of Dr. D. San Mauro, Dr. M.B. Dijkstra and Dr. M.H. Huang. I wrote the original manuscript and the other authors helped in revisions.

Chapter 3

A version of chapter 3 is currently in press: "Rajakumar, R., and E. Abouheif. Ancestral developmental potential: a new tool for animal breeding? *Proceeding of the 62nd Annual National Breeders Roundtable*". I conceived and wrote the manuscript.

Chapter 4

A version of chapter 4 is submitted for publication review: “Alvarado, S., Rajakumar, R., Abouheif, E., and Szyf, M. Epigenetic variation in the *Egfr* gene generates quantitative variation in a complex trait in ants”. Dr. S. Alvarado and myself are the co-primary authors of this work. I conceived this project and designed all experiments in collaboration with Dr. S. Alvarado. I carried out all pharmacological manipulations, ant collecting and rearing and sample preparations for epigenetic and molecular analysis while Dr. S. Alvarado conducted molecular assays and epigenetic assays with my help. I conducted all morphological and developmental descriptions and I carried out the analysis of the data with Dr. S. Alvarado. Dr. S. Alvarado and I wrote the original manuscript. Dr. Abouheif and Dr. Szyf both mentored and supervised this project.

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There are many people over the years that have helped me in various ways whether conceptually, in the lab or in the field. I would like to begin by thanking my parents who raised me to love science, discovery and the exploration of the unknown, ever since I was a little child. Specifically, I would like to thank my dad for introducing me to Ehab Abouheif in the most serendipitous of ways (both an orthopedic surgeon and a general practitioner, my dad was the family doctor of the Abouheif family). I would also like to thank my brothers for their encouragement over the years. Although I have an acknowledgements section specific to each of my empirical chapters (chapters 2 & 4), I would like to thank all former Abouheif lab alumni for endless hours of help and insight but I would also like to highlight the following individuals (in alphabetical order): Khashayar Afshar, Travis Chen, Michael Cournoyea, Mischa Dijkstra, Marie-Julie Favé, Benjamin Fung, Francois Hiou-Tim, Ana Sofia Ibarraran-Viniegra, Abdherrahman Khila, Maryna Lesoway, Evelyn Lo, Jason Lum, Kyle Martin, Matteen Rafiqi, Peter Refki, Diego San Mauro, Seba Shbailat, Steven Silvestrin, Benoit St.Halaire and Kevin Wei. I would like to especially thank Marie-Julie for translating my thesis abstract into French. Furthermore, I would like to thank Lloyd Davis, Walter Tschinkel, Josh King, Marc Seid, Bob Johnson and Ray “the Antman” Sanwald for all of their critical help in the field and great ideas. I would like to thank my longtime committee members Hans Larson, Laura Nilson and Francois Fagotto for all of their advice and guidance over the years while overseeing my doctoral work. I would like to thank Moshe Szyf for all of his critical comments and help for chapter 4 and for insights on epigenetics in general. I would like to give a very big thank you to Sebastian Alvarado who made the first step in the ant-

epigenetics collaboration by asking about what cool epigenetics studies can be done in ants. Sebastian and I were an incredible tag team, coming up with ingenious experimental ideas to test. The failures were fun and the ideas that were proven successful have been formulated into my chapter 4.

Of course, I would like to thank Marina Lagodich, my best friend of 12 years, who became my girlfriend along the way and then became my wife and soon-to-be mother of my child, for being so incredibly understanding, for putting up with me and encouraging me for all of these years. I really think that I would not have accomplished what I have, if it was not for her. She truly has inspired me to be the best at what I do and I love her very much for that.

Last, but certainly not least, I would like to thank Ehab Abouheif. Ehab taught me the many ways of generating questions in science. My favorite of all though, was that one could simply go into the wild (Indiana Jones-style), observe, and let natural phenomenon generate the questions for you. He opened my eyes to ecology, evolution, and development and made me realize that it is the path that strives to integrate these three that leads one to become a complete biologist. He has been one of my greatest role models, and has spent countless hours in both guiding me through the Pandora's box that is science and molding me into an aspiring researcher. I had the fortune of making a fundamental discovery with him but, before all of the success that followed, there were countless hours of unforgiving days and nights (some without sleep) of guidance in experimental design, training, endless discussion and manuscript revision. He taught me how to take a story about ants that spanned 8 years of hard work and crystallize it into 2500 perfectly orchestrated words. It was in this process that I truly understood the

biological question itself. The journey went from frustration, to progress, to lots and lots of laughs, to insight, and then… Eureka! For this rollercoaster ride, I thank you with all of my heart.

CHAPTER 1: INTRODUCTION

1.1 Genes and Environment

The phenotype of an organism is comprised of many traits, ranging from morphologies to behaviors. In order to understand how phenotypes evolve, one must understand how phenotypic variation originates and what biological mechanisms are involved. What determines a phenotype? One factor is the genotype (nature), i.e.: an organism's genome, which comprises the heritable genetic blueprint. Another, less explored factor is the environment (nurture) of an organism. Abiotic (temperature, photoperiod) and biotic (predation, nutrition) environmental factors can cause a single genotype to result in variation in behavior, physiology or morphology; this is called phenotypic plasticity (West-Eberhard, 1989). What is the importance of these factors (nature and nurture) in phenotypic diversity and the process of evolution?

The concepts of genotype and phenotype were defined separately over 100 years ago (Johannsen, 1911) based on the fact that one does not necessarily directly lead to the other. The reason for this is the contribution of environmental variation (Johannsen, 1926) which give rise to phenotypic plasticity. Nevertheless, few studies give proper credit to the contribution of the environment in the generation of trait variation as compared to the role of genetics (West-Eberhard, 2003). In fact, for a long time the environment was completely neglected. For example, Bradshaw (Bradshaw, 1965) notes that botanists studying adaptation had described trait variation due to environmental variation in experiments "usually as only an embarrassment" (further discussed by West-Eberhard, 1989). One quotation that sums up the disregard for the importance of the environment by both geneticists and evolutionary theorists (highlighted by West-Eberhard, 1989) is that of R.A. Fisher (one of the founding fathers of both modern

evolutionary theory and population genetics). In reference to the appearance of environmentally generated phenotypes in genetic studies, Fisher stated, “it is not surprising that such elaborate [genetic] machinery should sometimes go wrong.” In stark contrast, plasticity has been proposed by many to be instrumental in the generation of novelty, speciation and macroevolution (Pigliucci, 2001; West-Eberhard, 1989, 2003, 2005). Today, the importance of plasticity is becoming apparent and it is considered one of the key concepts in the formation of an “extended evolutionary synthesis” (Beldade et al., 2011; Muller, 2007; Pigliucci, 2007). Furthermore, plasticity has become the principle, which unifies the disparate field of developmental biology, ecology and evolution (Beldade et al., 2011). This integrative principle, which ties developmental plasticity with phenotypic evolution, is the conceptual basis of my thesis.

The major goal of my work is to understand the developmental mechanisms that regulate plasticity in order to understand how the environment can generate morphological novelty in evolution. Specifically, in my thesis I have used the complex caste system of ants as a model to characterize the epigenetic and hormonal basis of developmental plasticity, in order to ultimately understand how these caste systems have evolved.

Before describing what we know of the evolution and development of the complex caste system of ants, the following three sections (1.1.1, 1.1.2 and 1.1.3) are a brief introduction to our understanding of the contribution of genes and the environment in

generating phenotypic variation and what we know of how developmental processes can evolve and the role of the environment in this process.

1.1.1 Genetic Basis of phenotypic variation

After Gregor Mendel, geneticists developed a concrete understanding of the genetic basis of discrete characters. How is it though, that the binary rules of mendelian genetics can explain continuous traits and more importantly, how can mendelian genetics explain the gradual process of evolution proposed by Darwin? The combined efforts of Fisher, Haldane and Wright led to the formulation of quantitative genetics, which considers the action of many genes, their alleles and their interactions in generating continuous traits (Fisher, 1919; Haldane, 1932; Wright, 1921). Following this, there are several models that have emerged to explain phenotypic variation from a genetic perspective which can range dramatically from the infinitesimal model (Fisher, 1919; Rockman, 2012; Roff, 2007) to the polygenic model (Flint and Mackay, 2009; Mackay, 2001) to the exponential model (Farrall, 2004). The infinitesimal model postulates that quantitative phenotypic variation can be explained by the small effect of the alleles of innumerable genes with the notion that each are equal and mutations that generate variation is random. Alternatively, the polygenic model incorporates the idea of quantitative trait loci (QTLs). In this model, trait variation is mapped to specific genetic regions, each of which has a specific function, but there are many of small effect. The exponential model describes QTLs or genes of major effect underlying trait variation with the addition of several genes of small effect.

These models include other genetic components such as genetic recombination, where different alleles of genes can recombine in diverse ways to giving rise to different

phenotypic outcomes. Furthermore, whether many genes or few genes are the basis of phenotypic variation, genetic mutation is a major source of new genetic information for all models above. Finally, these models also take into consideration the interaction of different alleles of a single gene (dominance) or interactions between different genes (epistasis) as further genetic contributions to phenotypic variation. The idea that this genetic framework underlies the most important contribution to phenotypic change was the mainstream view during the Modern Synthesis of evolutionary theory, in defining how phenotypic evolution occurs while overlooking the importance of the environment (Schlichting and Pigliucci, 1998). This mainstream view has persisted even till today (Mackay et al., 2009; Rockman, 2012; Roff, 2007).

1.1.2 Phenotypic variation and the Environment

The relevance of the environment in generating phenotypic variation and phenotypic evolution is undergoing a renaissance. Some have even given the environment primacy as it has the capacity to both generate phenotypic variation and be the agent of selection in the evolutionary process (Gilbert and Epel, 2009; Pigliucci, 2001; Schlichting and Pigliucci, 1998; West-Eberhard, 2003). Phenotypic plasticity is widespread among plants and animals (West-Eberhard, 2003) and is key to the origins of biological diversity (Beldade et al., 2011; West-Eberhard, 1989, 2005). According to Schlichting and Pigliucci (1998), Plastic responses have four attributes: (1) amount, (2) pattern, (3) rapidity and (4) reversibility. Amount refers to the magnitude of the response to the change in environment, while pattern refers to the directionality of the response, i.e.: increase, decrease, non-linear. Rapidity simply refers to the speed at which the trait responds to the environmental perturbance for example, behavioral plasticity can be

almost immediate while morphological plasticity may take much longer. Finally, reversibility refers to a trait, which can change back and forth as a result of fluctuating environments. All 4 of these attributes for any given trait can evolve independently or together in varying combinations during the course of evolution (Schlichting and Pigliucci, 1998). Specifically, in some cases different environmental combinations or novel environments can give rise to novel phenotypes that can subsequently evolve. For many morphological traits, including the morphological variation found within the complex caste systems of ants, differences in the phenotype are the result of the environment influencing developmental pathways.

1.1.3 Evolution and development of morphological diversity

The key process, which sets the stage for an organism's genotype and the input of the environment in generating a trait like morphology, is development. Although the fields of embryology/developmental biology and evolutionary theory have both existed for over a century and a half, rather than integrating fully to merge the concept of pattern and process in biology, the fields have spent more time antagonizing one another (Wilkins, 2002). Fortunately, over the last 30 years (Haag and Lenski, 2011), the two fields have flourished as one in the form of evolutionary developmental biology or EvoDevo. One of the key findings that reunited these two classic disciplines was the discovery that there exists a set of developmental genes, which are critical for the formation of the adult body plan of all animals. These genes, best known as Hox genes were first discovered in the fruit fly *Drosophila melanogaster* (Kaufman et al., 1980; Lewis, 1978; Mcginnis et al., 1984; Scott and Weiner, 1984) and were subsequently identified in mammals (Carrasco et al., 1984). Following these discoveries, countless studies emerged that attempted to

understand the evolution of developmental pathways (Carroll et al., 2005). For instance, there are studies on developmental gene network evolution (Abouheif and Wray, 2002; Davidson et al., 2002), gene duplication (Holland et al., 1994) developmental gene regulation (Carroll, 2008; Wray, 2007; Wray et al., 2003), developmental homology (Abouheif et al., 1997; Hall, 2003) the evolution of appendages (Averof and Patel, 1997; Cohn and Tickle, 1999; Panganiban et al., 1997; Riddle et al., 1993; Shubin et al., 1997; Shubin and Alberch, 1986), embryological segmentation (Patel et al., 1989), Hox gene and body plan evolution (Carroll et al., 1995; Holland and GarciaFernandez, 1996), developmental plasticity (Brakefield et al., 1996; Moczek and Emlen, 1999; Moczek et al., 2011; Pfennig et al., 2010; Suzuki and Nijhout, 2006) and the developmental basis of life history evolution (Flatt and Heyland, 2011; Strathmann, 1985; Sultan, 2000). Most importantly, the process of development is now considered to be the playing field of evolution in which the environment and genome can interact to generate a landscape of phenotypes (West-Eberhard, 2003), or as Leigh Van Valen (Valen, 1973) said: “evolution is the control of development by ecology.” In order to gain further insight into the contribution of environmental factors in the generation of morphological diversity, it is necessary to find a model organism in which development is highly sensitive and responds to environmental perturbation with alternative phenotypes, i.e.: a model organism for developmental plasticity.

Developmental plasticity is the basis for the generation of different morphological castes in ants (Wheeler, 1991). Furthermore, ants are now considered one of the best models to study developmental plasticity (Jenner and Wills, 2007; Sommer, 2009). As stated earlier in order to address the general goal of my thesis I have focused on

determining what are the key hormonal and epigenetic mechanisms involved in the development and evolution of these complex ant caste systems. Therefore, the following section (1.2) introduces ant castes, on an evolutionary and developmental level and further specifies the particular genera that I have chosen to work with.

1.2 Castes in ants

The ecological success of ants has been credited to the evolution of eusociality (Hölldobler and Wilson, 1990), the origin of which is considered to be one of the major transitions in evolution (Maynard Smith and Szathmáry, 1995). Eusociality is the term that describes the formation of groups in which some individuals can reproduce (in this case, the winged queen caste) while most others do not and instead care for the brood generated (in this case, the wingless worker caste). Although debate still remains concerning at which level selection was acting during the evolution of eusociality, the general developmental mechanism that facilitates the production of reproductive and non-reproductive individuals is known. In the case of ants, winged queens and wingless workers develop from the exact same genotype. Specifically, during the course of development, external environmental factors like nutrition and temperature influence developing ant larvae to either become one caste or another; this is called caste polyphenism.

Throughout ant evolution, the wingless worker caste has expanded into a complex system of morphological subcastes facilitating division of labor in the colony (Hölldobler and Wilson, 1990; Wilson, 1953). Although this system has intrigued biologists for over a century (Darwin, 1859; Goetsch, 1937; Gregg, 1942; Hölldobler and Wilson, 2009; Wheeler, 1986; Wheeler, 1902; Wilson, 1953), little is known about the molecular

underpinnings involved in generating these subcastes. The following two sections (1.2.1 and 1.2.2) summarize what has been studied thus far in terms of development and evolution of castes in ants.

1.2.1 From Darwin to Wilson: 150 years of curiosity about ant castes

Ants have long been admired by society (Wheeler, 1911) for their work ethic, wisdom, sociality and life history. There are countless literary examples that have personified ants such as: Homer's Iliad, Egyptian hieroglyphics (the ant was used as a symbol to represent knowledge), the Old Testament, the Quran and Aesop's Fables. Darwin was no less awe struck. In fact, in his magnum opus, On the Origin of Species (in his chapter: Difficulties on Theory), Darwin mentions several examples of traits in animals that are difficult to explain with gradual evolution. When mentioning the worker caste of the ant, he described it as "one of the gravest" and stated that it will be an example treated in the next chapter (chapter 7: Instinct), most likely due to the fact that it required a much longer introduction and explanation. In his Instinct chapter, there are several passages, which make it very clear that he had long contemplated on the diversification of worker subcastes in ants:

> "*I will not here enter on these several cases, but will confine myself to one special difficulty, which at first appeared insuperable and fatal to my whole theory. I allude to the neuters or sterile females in insect communities.*"

The proximate explanation for this conundrum of Darwin is polyphenism, which as stated above, is a biological occurrence in which a single genotype may result into alternative phenotypes depending on the state of the environment during development. Nevertheless, an explanation for the proximate mechanism was not the true quest of

Darwin in this matter; he wanted to know how such a system could evolve. How could the traits of a sterile worker evolve, if by being sterile, it thus cannot propagate its traits through the process of inheritance? Darwin's stance on this was that the only way such a system could evolve would be through (family) group selection, a topic of which remains at the heart of a controversy in evolutionary theory. This was not the end of Darwin's difficulty:

> *"But we have not as yet touched on the climax of the difficulty; namely, the fact that the neuters of several ants differ, not only from the fertile males and females, but from each other, sometimes almost to an incredible degree, and thus are divided into two or even three castes… being as distinct from each other as are any two species of the same genus, or rather as any two genera of the same family."*

Darwin saved his theory with an incredible hypothesis: species of ants, which have various distinct sterile worker subcastes that have elaborate disproportional bodyplans or allometries, evolved from species that initially had all transitional forms between the castes. Subsequently, selection occurred on the extremes of the worker size distributions and eventually, the intermediate castes disappeared through processes like family (group) selection.

Following Darwin, there were several famous ant biologists (myrmecologists) whom continued the debate on the proximate causes for worker caste differences found in ant species. The two most eminent of the 19th century were Carlo Emery and August-Henri Forel. Emery was convinced that environmental factors dictated which caste would develop from an ant embryo while Forel was equally convinced that it was due to blastogenic, i.e.: genetic causes (Light, 1942).

The one who wrote about the feud between Emery and Forel, was William Morton Wheeler, a man who led myrmecology at the beginning of the 20th century. Wheeler is famous for proposing the "superorganism" concept by analogizing ant colonies as a unitary whole, similar to a cell or a person (Wheeler, 1911). In this context, individuals of an ant colony can develop into adults with highly variable phenotypes as compared to their parents due to the buffering, maternal-like, social structure of the colony. This maternal care allows extreme phenotypes to persist, so long as they can be nurtured and fed by their siblings (Wheeler, 1902) and therefore have a much better chance at survival and eventual selection. The idea that Wheeler had about the social structure of ants facilitating the production of extreme phenotypes began to address the details of the family (group) level evolutionary mechanisms of caste differences that Darwin was searching for.

Although Wheeler spent most of his life on the fence in the gene-environment debate (Light, 1942), he had early on been quoted as being slightly on the genetic side. Later, in his final literary effort: "Mosaics and Other Anomalies Among Ants" (Wheeler, 1937), Wheeler had come to the decision of fully espousing the genetic side. This was in spite of the fact that, through his own observations, there was evidence for the environment being the mediator of caste determination. He found evidence in a colony of leaf-cutter ants sent to him, which contained several anomalies including gynandromorphs (part male, part female) and several mosaic castes. He thought that the only way these individuals could have been generated would have been genetic.

Although he was correct about these specimens, as males and females are in fact of different genetic background (males are haploid, females are diploid), unfortunately he

wrongfully extrapolated this finding to all castes. Indeed, there are a few examples of ant species wherein castes are determined genetically (Schwander and Leimar, 2011; Smith et al., 2008) but this remains to be the exception as caste development of most ants has been demonstrated to be controlled by the environment (Hölldobler and Wilson, 2009; Hölldobler and Wilson, 1990; Wheeler, 1991). At the most, genetic variation can contribute to diversity within castes but the environment remains the key in dictating which developmental trajectory the embryo or larvae takes through hormone production, growth signaling pathway regulation, and epigenetic mechanisms (Simpson et al., 2011). Work by Goetsche, Gregg, Brian, Passera & Suzzoni, Wheeler (Diana) & Nijhout and many others solidified this environmental viewpoint (Brian, 1974; Brian, 1951, 1956; Colombel, 1978; Dartigues and Passera, 1979; Goetsch, 1937; Gosswald and Bier, 1954; Gregg, 1942; Ledoux and Dargagno.D, 1973; Passera, 1969; Passera and Suzzoni, 1979; Plateaux, 1970; Robeau and Vinson, 1976; Talbot, 1948; Terron, 1977) (Fowler, 1984; Nijhout and Wheeler, 1982; Passera, 1980; Wheeler, 1986, 1991; Wheeler and Nijhout, 1981c, d, 1983).

Following Wheeler came arguably the most important myrmecologist of all: E.O. Wilson. In several classic papers (Wilson, 1953; Wilson, 1968; Wilson, 1976, 1980b, 1984, 1985) and books (Hölldobler and Wilson, 2009; Hölldobler and Wilson, 1990; Oster and Wilson, 1978; Wilson, 1971, 2003) spanning over 50 years, Wilson set the groundwork for understanding caste behavior, ecology and evolution. Furthermore, Wilson was a strong advocate of caste determination having an environmental basis (Hölldobler and Wilson, 1990) and that the colony, which senses and regulates the environment as a single unit, can be viewed as a superorganism (Hölldobler and Wilson,

2009). Finally, he continued the idea initiated by Darwin concerning the possibility that a group, i.e.: the colony, can be a target for natural selection (Nowak et al., 2010; Wilson and Holldobler, 2005).

1.2.2 Development of ant castes

All of this pioneering work expanded our knowledge to a great degree concerning the physiology, behavior, ecology and evolution of ant castes. In contrast, very few studies (except Abouheif and Wray, 2002) have actually attempted to integrate molecular and developmental biology into this context. Ant colonies of advanced ant societies seem to behave as superorganisms, wherein pheromones and molecules can promote or inhibit the development and production of different castes. The following section will describe some of what we know about the embryological, hormonal, developmental and epigenetic basis of development in insects (and in some cases ants specifically), which pertains to the development of different ant castes.

1.2.2.1 Embryology

Holometabolous insects (which includes ants, bees, flies, beetles & butterflies) undergo three major phases of development before becoming an adult: embryogenesis, larval development and pupal development (Fig. 1, for an example of these stages for the ant *Camponotus floridanus*) (Gilbert, 2010). During embryogenesis (Fig. 1A), the embryo undergoes key cellularization, cell migration, determination and differentiation events including the establishment of segments of the eventual body plan and neurogenesis. Larvae have several stages (the number of which is species-specific) called larval instars (Fig. 1B to F). During larval development, populations of cells pattern and grow together and serve as precursors to the adult's tissues and organs. Finally, during

pupal development, the individual undergoes metamorphosis wherein all larval tissues are transfigured into corresponding adult tissues (Fig. 1G to H).

1.2.2.2 Developmental physiology: JH and Insulin signaling

JH signaling: There are two hormones that are essential for controlling the duration and timing of growth during larval development in holometabolous insects (Fig. 2): juvenile hormone (JH) and ecdysone (Nijhout, 1994). Changes in the presence or absence of these hormones as well as varying the concentrations of each, are physiological mechanisms that allow for control of developmental switches (Nijhout, 1999). JH is a non-polar terpenoid produced by the corpora allata (CA) and ecdysone is a steroid hormone produced by the prothoracic gland (PG) (Nijhout, 1994). Ecdysone controls both molting (going from one larval instar to the next) and the onset of metamorphosis in insects (Edgar, 2006; Nijhout, 1994). Prothoracicotropic hormone (PTTH) triggers the PG to secrete ecdysone (Edgar, 2006; Nijhout, 1994). JH can suppress and prevent PTTH from triggering the PG and thus has an antagonistic dynamic with ecdysone (Edgar, 2006). For metamorphosis to occur, JH levels drastically drop in order for a high level of ecdysone to be produced and cause the onset of metamorphosis (Edgar, 2006; Nijhout, 1994). Since the presence of JH prevents the larva from undergoing metamorphosis, it essentially allows for a longer duration of developing organs and appendages to grow (Edgar, 2006). Ecdysone levels which initiate the onset of metamorphosis directly interacts with developing organs and tissues through the regulation of their growth and patterning gene networks (Mirth et al., 2009). Although the hormone receptors, which interact with downstream gene regulation, are well understood for ecdysone (Riddiford et al., 2003),

we are only beginning to understand the actual molecular mechanisms underlying the mode of action of JH (Jindra et al., 2013).

In ants, the initiation of divergent developmental trajectories (caste determination) leading to different morphological castes is hormonally regulated by JH and Ecdysone. A good example of caste determination can be found in the ant genus *Pheidole* (Fig. 3). If a *Pheidole* queen (Fig. 3A) is exposed to optimal photoperiod and temperature, there will be an immediate, high level of endogenous production of JH which embryos are exposed to as they are released (Nijhout and Wheeler, 1982). If this occurs the embryos will be destined to become winged female reproductive larvae. If these optima are not met, the embryos go on to become worker larvae. This is the first developmental switch involved in caste determination in *Pheidole*. In addition, if larvae do not attain this early switch yet are given sufficient nutrition late in development, they produce a second pulse of JH that can trigger a late developmental switch. This is what leads to the production of soldiers (Fig. 1-3B). Any larvae that fail to undergo this second and final switch will become minor workers (Fig. 1-3C), which are significantly different in their form and function compared to soldiers.

Insulin signaling/TOR pathway: Another key developmental physiological pathway implicated in caste development in ants and other social insects is the insulin/TOR-signaling pathway (Fig. 2). The insulin signaling pathway, highly conserved among metazoans, has been extensively studied over the past century because of the critical role it plays in regulating development, growth and metabolism in various organisms (Baker et al., 1993; Banting and Best, 1922; Brogiolo et al., 2001; Colombani et al., 2003; Jones and Clemmons, 1995; Kimura et al., 1997; Oldham and Hafen, 2003; Saltiel and Kahn,

2001; Schlessinger, 2000). It has been demonstrated to crosstalk with JH/ecdysone signaling (Fig. 2) during development (Colombani et al., 2005; Flatt et al., 2005; Mutti et al., 2011a; Tu et al., 2005; Xu et al., 2013) and thus may be involved in developmental switches between different ant castes. The insulin signaling pathway acts in a systemic, nutrient-dependent and non-autonomous fashion making it an important mediator of the external environment and organismal development (Edgar, 2006; Emlen and Allen, 2003)

In vertebrates, insulin and insulin-like growth factors (IGFs) have been identified. They perform different functions: insulin mainly regulates metabolic processes while IGFs control proliferation and survival of cells (Geminard et al., 2006). Invertebrates have also been shown to have homologous molecules and associated signaling systems to that of insulin and IGFs, called insulin-like peptides (ILPs) (Chan and Steiner, 2000; Leroith et al., 1993). The first invertebrate ILPs were discovered in *Bombyx mori* (Kawakami et al., 1989; Nagasawa et al., 1986) and called bombyxins. Following this discovery, ILPs were found in the mollusc *Lymnaea stygnalis* (Smit et al., 1988), the orthopteran *Locusta migratoria* (Lagueux et al., 1990), the Lepidopteran *Agrius convoluvuli* (Iwami et al., 1996), the nematode *Caenorhabditis elegans* (Kawano et al., 2000) and the dipterans *Drosophila melanogaster* (Brogiolo et al., 2001) and *Anopheles gambiae* (Krieger et al., 2004). These ILPs were highly conserved with the insulin molecules of the vertebrates, so much so that bovine insulin supplemented to *Anopheles aegypti* ovaries stimulated ecdysteroid production in vitro (Riehle and Brown, 2003). Insect ILPs are produced by neurosecretory cells (NSCs or insulin secreting cells) located in the pars intercerebralis of the brain (Fig. 2). Interestingly, ILPs of invertebrates fulfill the roles of both insulin and IGFs by regulating metabolism and cellular growth. They are

critical in regulating the rate of cellular proliferation and growth of developing tissues and organs (Edgar, 2006).

ILPs and the genes of the insulin signaling pathway have been well characterized in terms of their structure, function and timing of expression during dipteran larval development (Brogiolo et al., 2001; Riehle et al., 2006). In *Drosophila melanogaster,* three *Drosophila* ILPs (Brogiolo et al., 2001) can be expressed by small clusters of neurosecretory cells in the brain and act non-autonomously by accessing larval tissue through the haemolymph. Furthermore, particular insulin signaling genes can also play a dual role in the fat body, acting as nutrient sensors affecting growth in a non-autonomous fashion (Colombani et al., 2003). Interestingly, particular DILPs can also be expressed locally in an intrinsic tissue-specific manner. In addition, the insulin signaling pathway that is activated by the binding of ILPs to the insulin receptor normally acts intrinsically within each cell (Edgar, 2006).

The insulin signaling pathway in insects consists of ILPs, an insulin receptor (InR), the insulin receptor substrate (Chico), the type IA phosphatidylinositol 3-kinase (PI3K), the lipid phosphatase PTEN, the protein kinase AKT and the transcriptional activator FOXO which activates a translational repressor named 4EBP (Nijhout, 2003). The signaling pathway is also highly integrated with the TOR pathway (often they are considered together), which is comprised of the GTPase RHEB that activates target of rapamycin (TOR, a conserved protein kinase), which promotes translational initiation, ribosome biogenesis, nutrient storage, autophagy and many other metabolic processes. These two pathways are highly sensitive to the environment as they are nutrient-dependent. High amino acid and glucose levels can lead to a block of insulin signaling

repressors such as the suppressors of RHEB, which are tumor suppressors named TSC1/2. High levels of nutrients can cause an increase production of ILPs leading to an upregulation of AKT, which not only represses FOXO but also represses TSC1/2.

In *Drosophila melanogaster,* when the insulin receptor is mutated early in development, developmental timing is altered causing a delay and elongation of each stage. When the insulin receptor is mutated later in development (during the last larval stage and pupation), wing cell size and the degree of proliferation is altered (Shingleton et al., 2005).

In the social bee *Apis mellifera*, the key insulin signaling genes, such as *Amtor*, *AmPTEN*, *AmIR-2*, *AmILP1, AmILP2* are differentially regulated and expressed in the queen and worker castes (Barchuk et al., 2007; Kucharski et al., 2008; Patel et al., 2007; Wheeler et al., 2006). Using reverse genetics and pharmacological techniques *Amtor* was shown to not only be involved in caste determination in *Apis mellifera* but also in specifying caste-specific tissues such as the pollen box which is a leg modification found in the workers (Patel et al., 2007). Subsequent studies have further demonstrated the involvement of different insulin/TOR signaling pathway components in caste determination in bees (Mutti et al., 2011a; Mutti et al., 2011b; Wolschin et al., 2011). Finally, a study simultaneously demonstrated in flies and bees that epidermal growth factor receptor (EGFR) signaling is a nutrition-dependent key upstream pathway that regulates insulin signaling to coordinate growth differences between castes (Kamakura, 2011).

In contrast, till now only a few preliminary studies exist that has investigated the involvement of insulin/TOR signaling in caste determination in ants. An example is the

characterization of insulin receptor expression differences between developing queens and workers of the fire ant (*Solenopsis invicta*). Furthermore, expression differences have been measured between a couple of ILPs following simulated seasonal changes and JH application with developing *Solenopsis invicta* individuals (Libbrecht et al., 2013). As demonstrated in bees, insulin signaling can serve a major regulatory role in generating morphological differences between castes. In order to properly understand the role of hormones and physiology in caste development in ants our knowledge of the JH pathway's role in regulating caste differences must be further coupled with more functional insulin signaling studies.

1.2.2.3 Developmental epigenetics: DNA methylation and histone modification

At the physiological level, Juvenile hormone and insulin signaling are considered to be the primary regulators of caste development in social insects. These hormones have been long considered the means of translating environmental differences into gene expression differences (Barchuk et al., 2007; Oostra et al., 2011; Simpson et al., 2011). As mentioned above, the mode of action of JH at the molecular level is unclear. How do changes in physiology of developing ant larvae translate into gene expression differences leading to different phenotypes? One candidate mechanism is the possible interaction between JH and epigenetic mechanisms such as DNA methylation and histone modifications. These epigenetic mechanisms can facilitate one genotype to give rise to different phenotypes. A simple example of this is a study, which tracks monozygotic twins (genetically identical), during their lifetime. Although there are no appreciable differences between these twins early in life, older twins differ markedly in DNA

methylation and histone modification levels, which translate into gene expression differences (Fraga et al., 2005).

DNA methylation: In vertebrates, DNA methylation involves the non-genetic modification of DNA through the addition of methyl groups to cytosine of CG dinucleotides of DNA (Gold et al., 1963; Razin and Szyf, 1984). DNA methylation leads to the suppression of transcriptional expression (Bird, 2002; Jaenisch and Bird, 2003). In some cases it has been shown to regulate alternative splicing (Shukla et al., 2011) and is considered essential for: gene regulation and cellular differentiation during development (Bird, 2002; Futscher et al., 2002; Okano et al., 1999; Reik et al., 2001; Stancheva et al., 2002; Stancheva and Meehan, 2000), stress behavior and social interaction (Champagne et al., 2006; McGowan et al., 2009; Meaney and Szyf, 2005; Szyf et al., 2008; Weaver et al., 2004), cancer onset and metastasis (Esteller, 2007; Esteller and Herman, 2002; Herman and Baylin, 2003; Jones and Baylin, 2002, 2007; Jones and Laird, 1999), and transgenerational epigenetic inheritance (Bossdorf et al., 2008; Jablonka and Raz, 2009; Morgan et al., 1999; Rakyan et al., 2003).

Based on the fact that DNA methylation is pivotal in the differentiation of cells (Razin et al., 1984) and that it is associated with nutritional-dependent gene expression (Cropley et al., 2006; Van den Veyver, 2002; Waterland and Jirtle, 2003), the superorganism concept of William Morton Wheeler would suggest that: different ant castes, like cells, may be influenced by the external environment during development which can result in caste determination through hormonal and epigenetic mechanisms. Several examples have emerged over the last 6 years in social insect studies implicating DNA methylation with caste determination and differential caste behaviors.

In *Apis mellifera*, it was demonstrated that by knocking down the expression of a key DNA methylation enzyme (DNA methyltransferase3; Dnmt3), individuals that were destined to become a worker bee instead developed as a queen bee and exhibited queen gene-specific methylation levels (Kucharski et al., 2008). Following this work, it was demonstrated that caste-specific gene expression in the brain might be regulated by DNA methylation (Lyko et al., 2010). Furthermore, it was demonstrated that DNA methylation is also associated with alternative splicing as had been previously shown in vertebrates (Foret et al., 2012; Lyko et al., 2010). Subsequent genome-wide analysis gave rise to large-scale predictions for the relationship between castes in bees and transcriptional regulation (Elango et al., 2009; Foret et al., 2009) as well as several high-throughput methylomic analyses in bees (Foret et al., 2012; Foret et al., 2009; Herb et al., 2012; Shi et al., 2013) and one in ants (Bonasio et al., 2012).

What remains a mystery is: 1) how does the methylation control the expression levels of a specific gene in social insects; 2) which genes, associated with phenotypic differences between castes, are actually differentially expressed due to differential methylation (Weiner and Toth, 2012)? Unfortunately, the first question is currently being dealt with assumptions that methylation does the same thing in social insects as it does in vertebrates. Thus far, no empirical/manipulation work has molecularly and/or functionally demonstrated the effect of methylation of a gene on its transcriptional output in social insects (Weiner and Toth, 2012). Furthermore, in order to demonstrate that DNA methylation is in fact involved in integrating the environment/hormone interface with differential gene expression underlying caste polyphenisms we need to investigate, on a

DNA methylation level, genes that have previously been functionally implicated in caste differences in social insects.

Histone modification: In regards to histone modifications, there are many forms but the most predominant is histone acetylation/deacetylation/methylation (Allfrey et al., 1964; Rice and Allis, 2001). When histone tails are acetylated by histone acetyl transferases (HATs), chromatin decondenses and transcription factors gain access to genes (Jenuwein and Allis, 2001; Roth et al., 2001). In contrast, histone deacetylases (HDACs) remove the acetyl modification of histone tails leading to chromatin condensation and transcriptional suppression (De Ruijter et al., 2003; Jenuwein and Allis, 2001), which also occurs through chromatin methylation by histone methyltransferaes (Cao et al., 2002; Jenuwein and Allis, 2001). Histone modification, like DNA methylation, has also been implicated in translating external environmental signals into chromatin/gene expression states (Bastow et al., 2004; Sheldon et al., 1999) and is involved in behavior (Meaney and Szyf, 2005), Cancer (Esteller, 2007; Jones and Baylin, 2007) and development (Cao et al., 2002).

Currently, only a few studies have looked at histone modification in social insects. Royal jelly is a component of the food that is fed to developing queens in honeybee colonies. It was recently demonstrated that a compound within royal jelly (a fatty acid called (*E*)-10-hydroxy-2-decenoic acid) has HDACi properties (Spannhoff et al., 2011). In other words, royal jelly can inhibit histone deacetylation and in the process decondense chromatin to allow for gene transcription. Another study examined larvae and queen ovaries in bees and described the different histone modifications that occur (Dickman et al., 2013). Furthermore, in ants, adult castes have been compared for histone

modifications (Simola et al., 2013). Because DNA methylation and histone modification pathways undergo extensive crosstalk, there remains much promise in further characterization. Future studies must begin to characterize the functions of different histone modifications in caste development and determination.

Both hormonal and epigenetic mechanisms have the potential to regulate morphological variation within and between castes. The following (1.2.2.4) describes the actual developing embryonic and larval tissues that are targets of these regulatory pathways. These tissues differentially pattern and grow during development based on how hormonal and epigenetic machinery translate the environment. It is their differential development which gives rise to the morphological differences observed within and between the different ant castes.

1.2.2.4 Developmental genetics: Imaginal discs and gene networks

Beneath the hormonal and epigenetic levels, which are involved in translating the external environment into phenotypic differences, are networks of genes. Each organ, whether it is the developing leg, wing, eye or genitalia, has its own module of genes, which signal and interact with each other to pattern and regulate growth of the organ during development (Carroll et al., 2005; Cohen, 1993; Williams et al., 1994). The populations of cells, which develop together for each organ, are called imaginal discs. They form a monocellular epithelial layer that consists of undifferentiated, proliferating cells (Cohen, 1993). For example, in *Drosophila melanogaster*, wing imaginal disc precursor cells separate from the epidermis during embryogenesis and are first observed as discs during the first larval instar (Cohen, 1993). These wing imaginal discs begin with 20 cells and rapidly proliferate during larval instars and reach an approximate total of 50-

75,000 cells before undergoing metamorphosis (Bate and Arias, 1991; Garcia-bellido, 1966; Klein, 2001).

Gene network underlying wing polyphenism in ants: One of the best examples of a gene network being dynamically regulated to generate a plastic phenotype between ant castes is the wing patterning gene network. As stated in the hormonal section above, ant castes in the genus *Pheidole* are determined by environmental inputs during development. The outcomes are queens, soldiers and workers, which differ dramatically for several traits. One of the most striking differences between the castes is the presence of wings in queens versus the absence of wings in soldiers and workers. The development of fully functional wings is coordinated by three phases. In the first and second phase, a series of signaling molecules and transcription factors interact to specify wing primordia. In the third phase, a larger set of signalling molecules and transcription factors interact to give rise to fore- and hindwing imaginal discs, which will eventually evert and become wings. Although wingless worker castes in ants do not possess wings, they do have vestigial wing imaginal discs that are morphologically diverse in their shape and size in different species (Abouheif and Wray, 2002; Wheeler and Nijhout, 1981a).

In 2002, Abouheif and Wray found that the wing-patterning network of these vestigial wing imaginal discs is actually interrupted at different points in four different ant species. As an example, in the species *Crematogaster lineolata,* the interruption point of the wing-patterning network in the wingless caste was the upstream gene *engrailed,* which gave rise to small pad-like imaginal discs. In contrast, this gene is expressed in the posterior of the forewing disc in the wingless soldier caste in *Pheidole morrisi*, while it is off in the worker caste. In contrast, the gene *spalt*, which is typically expressed in the

hinge and medial area of the wing pouch of the queen wing imaginal discs to pattern the wing veins (Fig. 3D), is expressed only in the hinge of the soldier forewing disc (Fig. 3E). Meanwhile the expression of this downstream gene is completely absent in the worker caste (Fig. 3F). The forewing disc of the developing soldier larvae patterns and develops quite extensively but it eventually undergoes apoptosis, preventing soldiers from growing wings (Sameshima et al., 2004; Shbailat et al., 2010).

Taken together, not only is this gene network responding to hormonal fluctuations (which are translating the external environment and most likely acting through epigenetic mechanisms), through their differential expression, the plasticity of this gene network has evolved in different species (Abouheif and Wray, 2002).

1.2.3: My model system: Caste development in the hyperdiverse *Pheidole* and *Camponotus*

There are between 12,000-14,000 known species of ants (Pie and Tscha, 2009; Smith et al., 2011) from approximately 300 genera (Bolton et al., 2006; Moreau et al., 2006). Of these, approximately 1/6 of these species are from either the *Pheidole* or *Camponotus* genera alone (Moreau, 2008). Few genera, of any animal, are as species-rich as these two and thus they have been dubbed "hyperdiverse" (Wilson, 2003). The hallmark of these two genera is the existence of a complex system of morphological worker subcastes, which is a trait that is considered to have contributed to the species diversity of these groups (Hölldobler and Wilson, 1990).

1.2.3.1 *Camponotus*

In terms of division of labor, smaller workers (minor workers) tend to do more food collection compared to larger workers (major workers) that store food in an adipose

form in their abdomen and are involved in specialized collecting of other insect prey (Emery, 1898; Espadaler et al., 1990). Furthermore, larger workers in *Camponotus* tend to produce more alarm pheromone (Espadaler et al., 1990) and are known to be more aggressive for defensive purposes (Busher et al., 1985; Espadaler et al., 1990; Lamon and Topoff, 1981; Yamamoto and Del-Claro, 2008). Regarding plasticity in caste size, *Camponotus* species usually have a dramatic range in size that is typically continuous but in many cases there is allometry between the head size and the body (Baroni Urbani, 1976; Diniz-Filho et al., 1994; Espadaler et al., 1990; Gibson, 1989; Wilson, 1953; Wilson, 1971). Thus far, very few studies have been done examining what environmental factors contribute to the production of the different worker subcastes in *Camponotus*. It was demonstrated that administering synthetic JH to field colonies of *Camponotus pennsylvanicus* caused an increase in major worker production indicating that there are environmental cues that specify worker subcaste development (Fowler, 1984).

1.2.3.2 *Pheidole*

Minor workers nurse the developing brood (embryos and larvae) of the colony as well as forage for food (Mertl and Traniello, 2009; Wilson, 1971, 2003) while the major workers (also called soldiers) tend to process specialized food as well as defend the nest against predators (Wilson, 1971, 2003). In contrast to *Camponotus*, minor workers and soldiers of *Pheidole* typically form a discontinuous bimodal distribution in size frequency where there are no intermediates and there is a strong allometry between the head and the body (Wheeler and Nijhout, 1981d, 1983; Wilson, 1953, 1984; Wilson, 2003). Furthermore, there exist rare additional subcastes called supermajors (supersoldiers) in approximately 8 *Pheidole* species (Huang, 2010; Huang and Wheeler, 2011; Moreau,

2008; Wilson, 1953; Wilson, 2003). As mentioned earlier, castes in *Pheidole* are determined by JH, where high levels of JH initiate soldier development during larval development due to optimum nutrition levels (Hölldobler and Wilson, 1990; Wheeler and Nijhout, 1981d, 1983). It is very possible that *Camponotus* and *Pheidole* may have independently evolved developmental JH thresholds to regulate the production of worker subcastes of different size in response to specific environmental conditions.

Since these two genera display such morphological diversity within and between their worker subcastes, I have chosen them as models for my thesis in order to specifically elucidate the hormonal and epigenetic basis of plasticity in caste development and evolution.

1.3 Figures

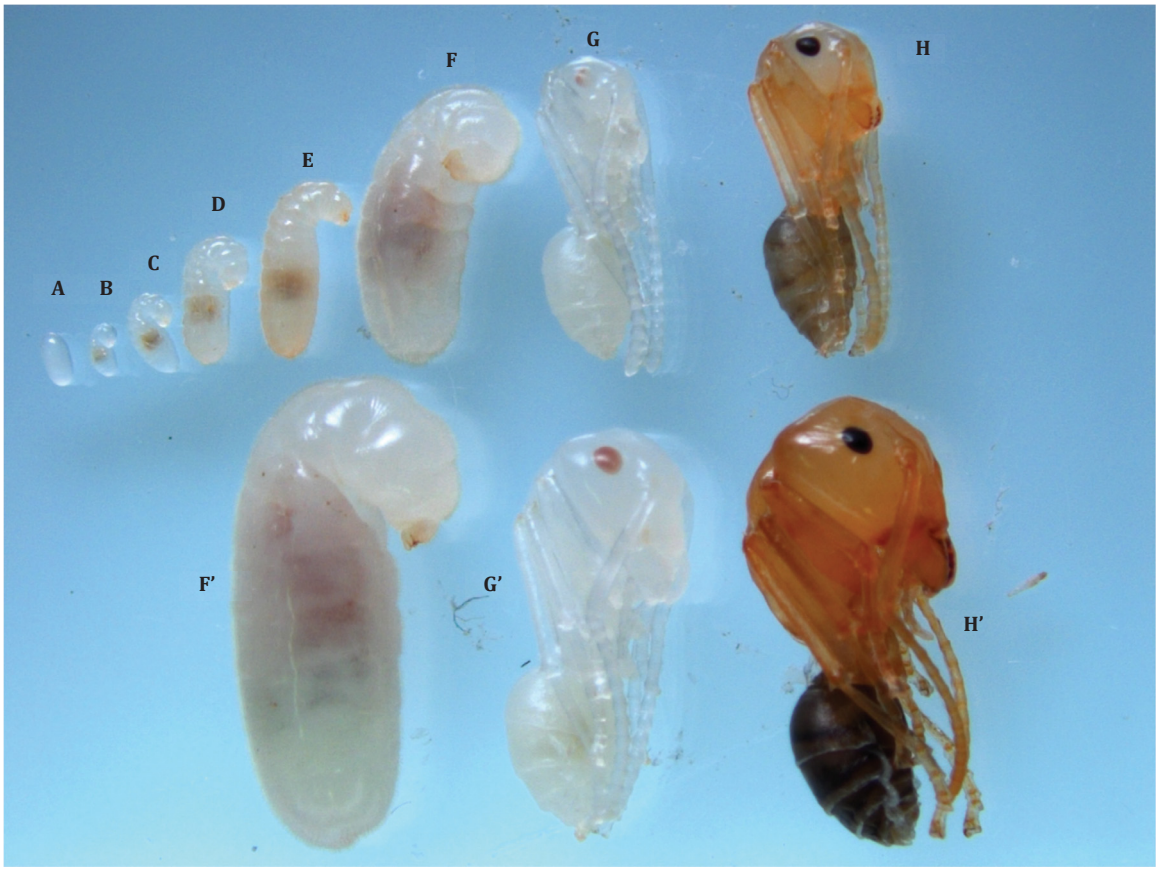


Figure 1: Developmental stages of *C.floridanus* workers. (A) embryo; (B) 1st instar; (C) 2nd larval instar; (D) 3rd larval instar; (E) early 4th intar; (F) minor worker 4th instar; (F') major worker 4th instar; (G) minor worker young pupae; (G') major worker young puape; (H) minor worker old pupae; (H') major worker old pupae.

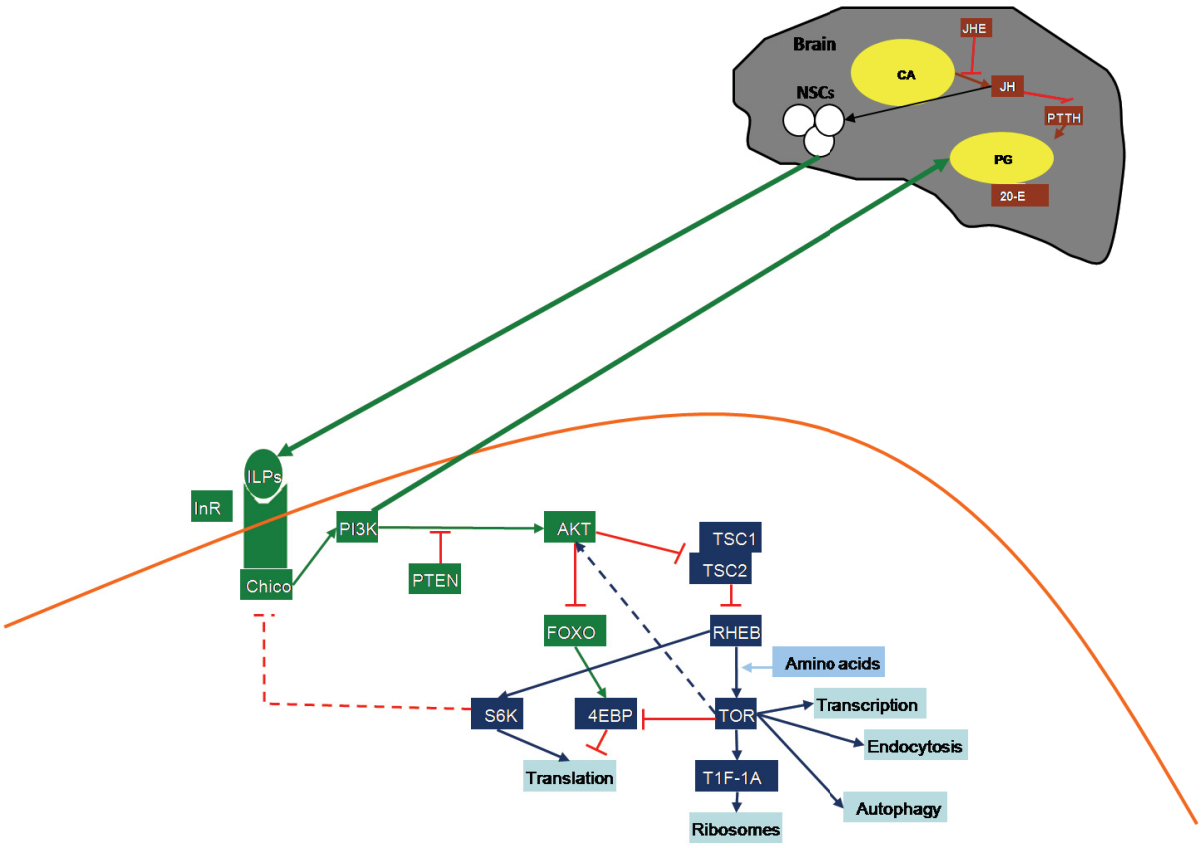
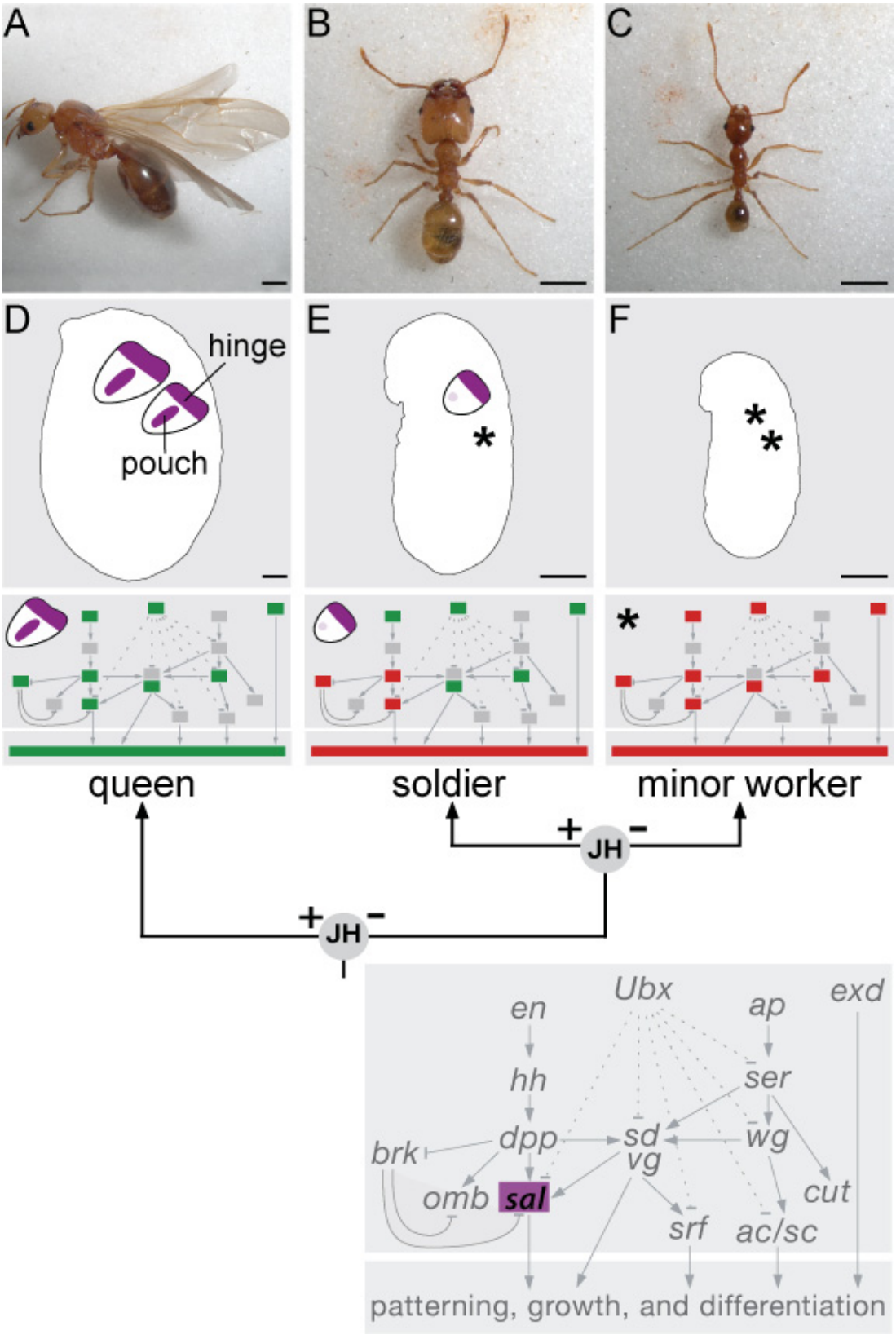

Brain
JHE
CA
JH
NSCs
PTTH
PG
20-E
ILPs
InR
Chico
PI3K
PTEN
AKT
TSC1
TSC2
FOXO
RHEB
Amino acids
S6K
4EBP
TOR
Transcription
Endocytosis
Translation
T1F-1A
Autophagy
Ribosomes

Figure 2: A simplification of the juvenile/ecdysone hormone and insulin/TOR-signaling pathway and the interactions between them (Colombani et al., 2005; Edgar, 2006).


A
B
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soldier
minor worker
+
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en
ap
exd
hh
ser
dpp
sd
wg
brk
vg
omb
sal
cut
srf
ac/sc
patterning, growth, and differentiation

Figure 3: The multiple developmental switch points that occur during caste determination in *Pheidole morissi*. (A) Adult queen; (B) soldier; (C) worker. *spalt* pattern and gene network expression of: (D) queen wing imaginal dics; (E) soldier; (F) worker. *spalt* expression in purple, active gene in green and interrupted gene in red. Hormonal switches based on Wheeler and Nijhout, 1982 and Abouheif and Wray, 2002. Gene network expression in wing imaginal discs, (based on Abouheif and Wray, 2002).

Link between Chapter 1 & 2

In Chapter 1, I discussed the general involvement of genes and the environment in generating phenotypic variation during development and the consequences for the evolution of morphological diversity. I then introduced the complex castes of ants as a model for the study of these interactions. I discussed a brief history of investigations into the evolution of ant castes and then described our current knowledge of how castes develop at an embryological, physiological, epigenetic and genetic level. I then finished by proposing two specific groups of ants, which contain complex morphological castes as my specific models for my thesis: *Pheidole* and *Camponotus*. With these models I have attempted to elucidate the hormonal and epigenetic basis of plasticity in caste development and evolution in order to address the major goal of my thesis: understanding how the environment acts on development, generating morphological variation, which can than lead to morphological diversification and evolution.

Chapter 2 addresses the hormonal aspects of ant caste development and evolution. Specifically, using a phylogenetic comparative context, I characterized how the soldier and novel supersoldier subcastes develop in *Pheidole* at the embryological and hormonal level in order to further understand how the novel supersoldier subcaste has evolved.

Chapter 2: Ancestral developmental potential facilitates parallel evolution in ants

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2.1 Abstract

Complex worker caste systems have contributed to the evolutionary success of advanced ant societies); however, little is known about the developmental processes underlying their origin and evolution. We combined hormonal manipulation, gene expression, and phylogenetic analyses with field observations to understand how novel worker subcastes evolve. We uncovered an ancestral developmental potential to produce a "supersoldier" subcaste that has been actualized at least two times independently in the hyperdiverse ant genus *Pheidole*. This potential has been retained and can be environmentally induced throughout the genus. Therefore, the retention and induction of this potential have facilitated the parallel evolution of supersoldiers through a process known as genetic accommodation. The recurrent induction of ancestral developmental potential may facilitate the adaptive and parallel evolution of phenotypes.

2.2 Main Text

The wingless worker caste, a universal feature of ants (Abouheif and Wray, 2002; Hölldobler and Wilson, 1990), has repeatedly expanded into a complex system of morphological and behavioral subcastes. The existence of these subcastes has long fascinated biologists (Darwin, 1859; Goetsch, 1937; Gregg, 1942; Hölldobler and Wilson, 2009; Wheeler, 1902; Wilson, 1953), yet little is known about their developmental and evolutionary origin (Wheeler, 1986; Wilson, 1953). The ant genus *Pheidole* is one of the most species-rich genera, with 1100 species worldwide (Moreau, 2008; Wilson, 2003). All *Pheidole* species have two worker subcastes: minor workers (Fig. 1C) that perform most tasks in the nest and forage and soldiers (Fig. 1B) that defend the nest and process food (Wilson, 2003). This complex worker caste system is thought to have promoted the remarkable diversification of *Pheidole* by enhancing the division of labor (Wilson, 2003).

In a wild *P. morrisi* colony, we discovered several anomalous soldier-like individuals. These individuals are anomolous because they are significantly larger than normal soldiers (Fig. 2, A and B, and fig. S1), and unlike normal soldiers, they have mesothoracic wing vestiges (Fig. 2, C and D) and rarely occur in nature. These anomalous soldiers are similar to a supersoldier subcaste, which is known to be continually produced in eight *Pheidole species* (fig. S2) (Huang and Wheeler, 2011; Moreau, 2008; Wilson, 2003). These species co-occur with army ants and live exclusively in the deserts of the American southwest and northern Mexico (Wilson, 2003). In one of these species, *P. obtusospinosa* (Fig. 2, E and I), a major function of the

supersoldier subcaste is to block the nest entrance with their extra-large heads and engage in combat to defend against army ant raids (Huang, 2010).

The similarity between the supersoldier-like anomalies in *P. morrisi* and the supersoldier subcaste suggests that they share a developmental origin. Normal soldier development in *Pheidole* may provide insight into how supersoldier-like anomalies may have originated. The soldier subcaste is determined late in larval development at a soldier–minor worker switch point (Fig. 1), which is largely controlled by nutrition (Goetsch, 1937) and mediated by juvenile hormone (JH) (Wheeler and Nijhout, 1981c, 1983). Soldier development is defined by two features: (i) Soldier-determined larvae grow larger than minor worker larvae; and (ii) they develop a pair of vestigial forewing discs in their mesothoracic segment (Fig. 1, E and F) (Wheeler and Nijhout, 1981a, c, 1983). These discs show a soldier-specific expression of *spalt* (*sal*) (Fig. 1E) (Abouheif and Wray, 2002), a key gene in the network underlying wing polyphenism in *Pheidole*. *Sal* is a key gene because its expression is spatiotemporally associated with the induction of apoptosis in these vestigial forewing discs (Sameshima et al., 2004; Shbailat et al., 2010). Therefore, the supersoldier-like anomalies we found in *P. morrisi* were likely to have originated from the abnormal growth of soldier larvae and their vestigial wing discs. Based on this insight, we predicted that the evolution of the supersoldier subcaste in *Pheidole* occurred through developmental changes that elaborated these two features.

We tested this prediction in *P. obtusospinosa* and *P. rhea*, two species that have a supersoldier subcaste (Wilson, 2003). As predicted, their supersoldier larvae grow larger (Fig. 2, F and J, and fig. S3, B and F) and develop two pairs of large vestigial wing discs relative to their soldier larvae (Fig. 2, G and K, and fig. S3, C and G). Furthermore,

vestigial wing discs in supersoldier larvae show an elaborated pattern of *sal* expression in the wing pouch relative to those of soldier larvae (Fig. 2, H and L, and fig S3, D and H, black arrows). We then resolved the evolutionary history of their supersoldier subcaste by reconstructing a phylogeny of 11 *Pheidole* species (fig. S5). Of these, only *P. obtusospinosa* and *P. rhea* have a supersoldier subcaste. Our phylogenetic analysis suggests that the supersoldier subcaste has evolved independently, because *P. rhea* is one of the most basal species of this genus, whereas *P. obtusospinosa* is derived (fig. S5) (Moreau, 2008). Therefore, the supersoldier subcaste has evolved in parallel, because similar developmental changes underlie its independent evolution in *P. obtusospinosa* and *P. rhea*. Furthermore, our phylogenetic analysis suggests that, relative to *P. obtusospinosa*, there are six basal and four derived species (fig. S5). We found that soldier larvae of these basal and derived species differ in their vestigial wing disc number and wing pouch expression of *sal* (fig. S6). This indicates that the supersoldier subcaste has evolved in parallel despite the evolutionary divergence of soldier development.

Application of methoprene (a JH analog) to *Pheidole* larvae has been shown to induce the development of unusually large soldier pupae (Wheeler and Nijhout, 1983). In *P. morrisi*, we found that methoprene can induce the development of larvae and adults that mimic the anomalous supersoldier-like individuals of *P. morrisi* and the supersoldiers of *P. obtusospinosa* and *P. rhea*. First, induced supersoldier larvae (Fig. 3G) and adults (Fig. 3B) are significantly larger than untreated controls (Fig. 3, D and A, and fig. S7), and several of the induced adult supersoldiers have mesothoracic wing vestiges (Fig. 3C). Second, the relative size ranges of induced supersoldiers overlap with those of anomalous and naturally produced supersoldiers (fig. S8). Finally, we found

vestigial wing discs of induced supersoldier larvae (Fig. 3, H and I, and fig. S9, B to D and F to H) that mimic those of supersoldier larvae in *P. obtusospinosa* (Fig. 2, K and L) and *P. rhea* (fig. S3, G and H). Therefore, although *P. morrisi* lacks a supersoldier subcaste, there is a developmental potential to produce supersoldiers that can be induced through JH. Furthermore, the occurrence of supersoldier-like anomalies in *P. morrisi* (Fig. 2A) and other *Pheidole* species (Wheeler and Nijhout, 1981a) suggests that this potential is recurrently induced in nature. This recurrent induction, which is probably mediated by JH, may be caused by nutrition, because it has been shown that environmental variation in nutrition (Wheeler, 1902) and experimentally increasing nutrition (Goetsch, 1937) produces supersoldier-like anomalies in *Pheidole* colonies.

We discovered that this developmental potential to produce supersoldiers can be induced by methoprene in other derived (*P. hyatti*) and basal (*P. spadonia*) *Pheidole* species that lack a supersoldier subcaste. As in *P. morrisi*, we found vestigial wing discs of induced supersoldier larvae in *P. hyatti* (fig. S9, J and N) and *P. spadonia* (fig. S9, R and V) that mimic those of supersoldier larvae in *P. obtusospinosa* (Fig. 2, K and L) and *P. rhea* (fig. S3, G and H). Therefore, the developmental potential to produce supersoldiers has been retained and was probably present in the common ancestor of all *Pheidole* (Fig. 4). Without a priori knowledge of this ancestral developmental potential, we would have inferred that the supersoldier subcaste has evolved de novo: once in *P. rhea* and once in *P. obtusospinosa* (fig. S5) (Moreau, 2008). However, our results support an alternative explanation for the parallel evolution of supersoldiers: The developmental potential and phenotypic expression of a novel supersoldier subcaste originated in the common ancestor of all *Pheidole* (Fig. 4, section i); the phenotypic expression of

supersoldiers was subsequently lost, but the ancestral potential to produce them was retained (Fig. 4, section ii); and this potential was then actualized in *P. obtusospinosa*, leading to the re-evolution of a supersoldier subcaste (Fig. 4, section iii).

Finally, we showed that this ancestral potential was actualized in *P. obtusospinosa* through the re-evolution of a second JH-sensitive period mediating a soldier-supersoldier switch point (fig. S10). We applied methoprene to larvae that had passed the soldier–minor worker switch point but whose caste fate as either soldiers or supersoldiers was still undetermined. We found that applying methoprene to these larvae induced the development of a significantly greater proportion of supersoldiers (fig. S11). Collectively, our results indicate that the supersoldier subcaste in *P. obtusospinosa* re-evolved through genetic accommodation. This process occurs when: (i) a novel phenotype is induced and (ii) this phenotype is incorporated into the population through selection on genes that control its frequency and form of expression (Suzuki and Nijhout, 2006; West-Eberhard, 2003). Environmental induction of the ancestral potential may have recurrently produced supersoldier-like anomalies in *P. obtusospinosa*. These anomalies would persist, because colonies of *Pheidole* generally care for and buffer anomalies against purifying selection (Wheeler, 1902). Selection on *P. obtusospinosa* colonies may have incorporated induced supersoldier-like anomalies by increasing their frequency through modification of the JH system (fig. S12) and by inhibiting the formation of any wing vestiges (fig. S13 and S14). Army ant raids may have been a selective pressure that incorporated these anomalies, because *P. obtusospinosa* supersoldiers currently use their extra-large heads to defend against these raids (Huang, 2010).

Selection for re-evolving supersoldiers may generally be reduced, because almost all *Pheidole* species lack a supersoldier subcaste (Moreau, 2008; Wilson, 2003). *P. hyatti* provides insight into how this selective pressure can be reduced: Although *P. hyatti* retains the developmental potential (Fig. 4) and lives in an ecological environment similar to that of *P. obtusospinosa*, it has not re-evolved a supersoldier subcaste (Wilson, 2003). Instead, *P. hyatti* uses nest evacuation behavior when attacked by army ants (Droual, 1983). The retention of this potential in *P. hyatti* and other *Pheidole* species that lack a supersoldier subcaste may therefore be due to a clade-specific constraint (Stearns, 1994). This constraint may have arisen from having the same hormone (JH) mediate the determination of both soldiers and supersoldiers in the common ancestor of all *Pheidole*. Soldiers and supersoldiers are both defined by their larval size and the development of their vestigial wing discs, which indicates that their developmental programs share many modules. Therefore, the ancestral potential to produce supersoldiers cannot be lost without compromising the developmental program of soldiers.

Recurrent phenotypes reflecting ancestral potentials have long been recognized as widespread in plants and animals (Armbruster et al., 2009; Bely and Sikes, 2010; Darwin, 1859; Goetsch, 1939; Harris et al., 2006; Ledon-Rettig et al., 2008; Meyer, 1999; West-Eberhard, 2003). Because of the lack of empirical evidence, however, the evolutionary significance of these recurrent phenotypes has been underappreciated (Stiassny, 2003; West-Eberhard, 2003). We uncovered an ancestral developmental potential to produce a novel supersoldier subcaste that has been retained throughout a hyperdiverse ant genus that evolved ~35 to 60 million years ago (Moreau, 2008)(Fig. 4). Our results suggest that the recurrent induction of ancestral developmental potential is an important source of

adaptive variation for selection that facilitates the adaptive and parallel evolution of novel phenotypes.

2.3 Main Text Figures

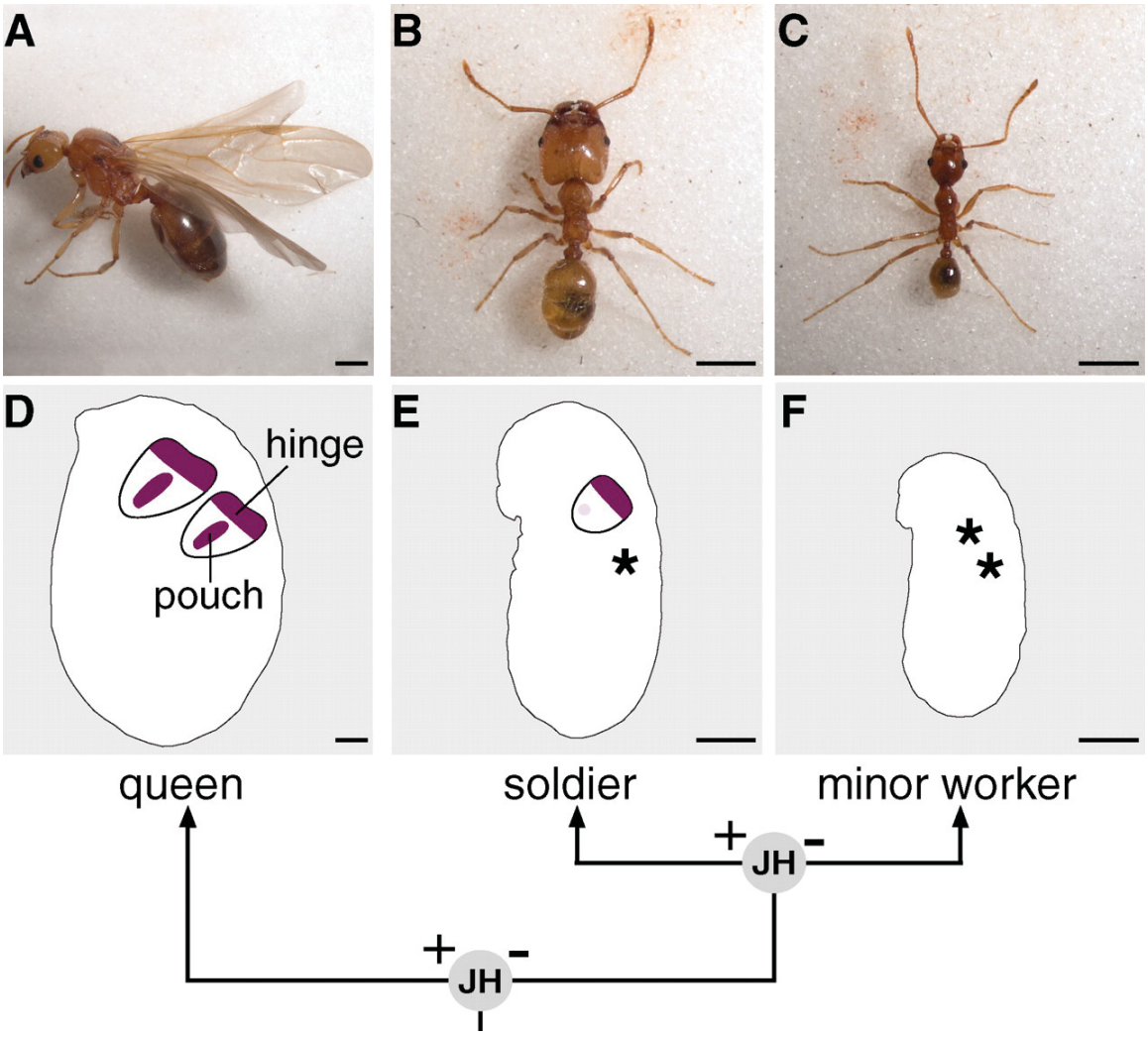


Figure 1: Wing polyphenism in *P. morrisi*: the ability of a single genome to produce (**A**) winged queens and wingless (**B**) soldiers and (**C**) minor workers (2). Caste determination occurs at two JH-mediated switch points in response to environmental cues (Abouheif and Wray, 2002; Passera and Suzzoni, 1979; Wheeler and Nijhout, 1983). (**D**) Wing discs in queen larvae showing conserved hinge and pouch expression of *sal*. (**E**) Vestigial wing discs in soldier larvae showing a soldier-specific pattern of *sal* expression, where it is conserved in the hinge but down-regulated in the pouch. Asterisks represent the absence of visible wing discs and *sa*l expression in **(E)** soldier and (**F**) minor worker larvae. Scale bars indicate the relative sizes of queen, soldier, and minor worker larvae and adults.

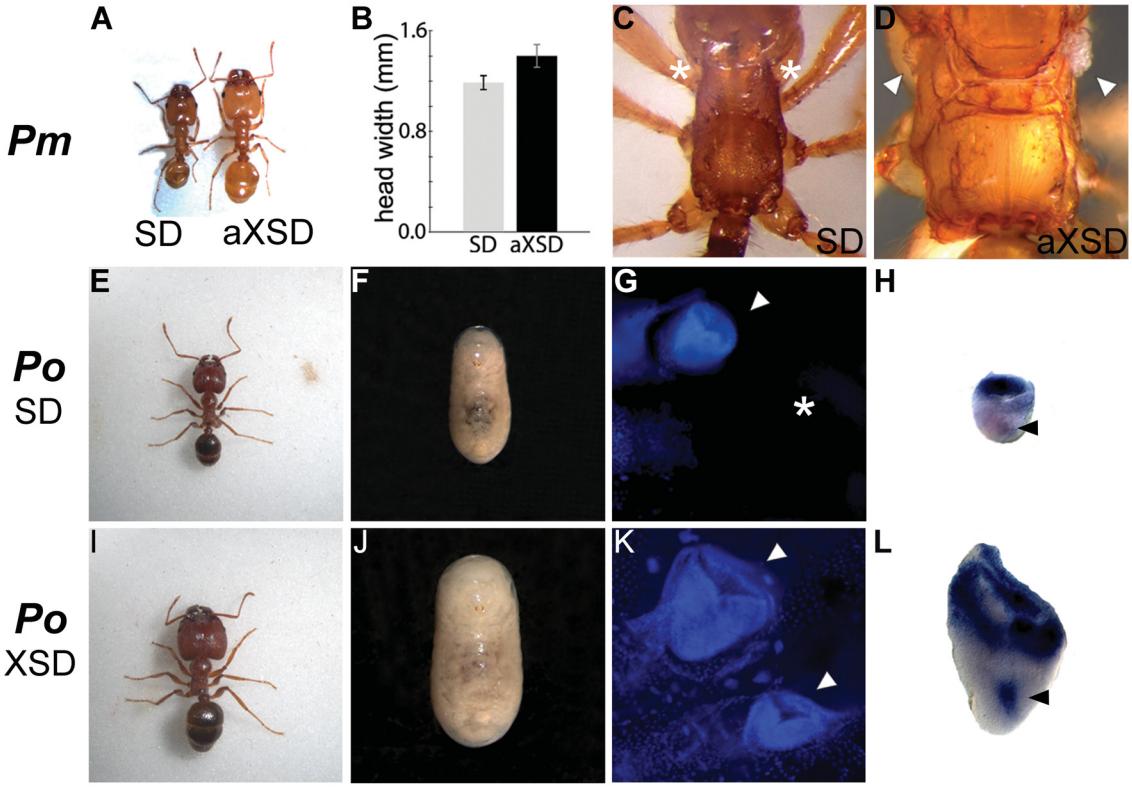

A
B
C
D
Pm
SD
aXSD
head width (mm)
1.6
0.8
0.0
SD
aXSD
SD
aXSD
E
F
G
H
Po
SD
I
J
K
L
Po
XSD

Figure 2: Comparison of *P. morrisi* (Pm) ants: (**A**, left) Normal adult soldier (SD) and (**A**, right) anomalous supersoldier (aXSD). (**B**) Mean and standard deviation of head width of normal SD (gray) and aXSD (black) (fig. S1), and thorax of (**C**) a normal SD and (**D**) an aXSD. Comparison of *P. obtusospinosa* (*Po*) ants: adults of (**E**) SD and (**I**) supersoldier (XSD); larvae of (**F**) SD and (**J**) XSD; and vestigial wing discs [stained with 4′,6′-diamidino-2-phenylindole (DAPI)] and *sal* expression in SD (**G** and **H** and fig. S4A) and XSD (**K** and **L** and fig. S4B). White arrowheads indicate the presence of mesothoracic vestigial wing buds or discs; asterisks denote their absence. Black arrowheads indicate *sal* expression in the wing pouch. Adult, larval, and vestigial wing disc images are all to scale. See fig. S3 for a comparison of *P. rhea* SD and XSD.

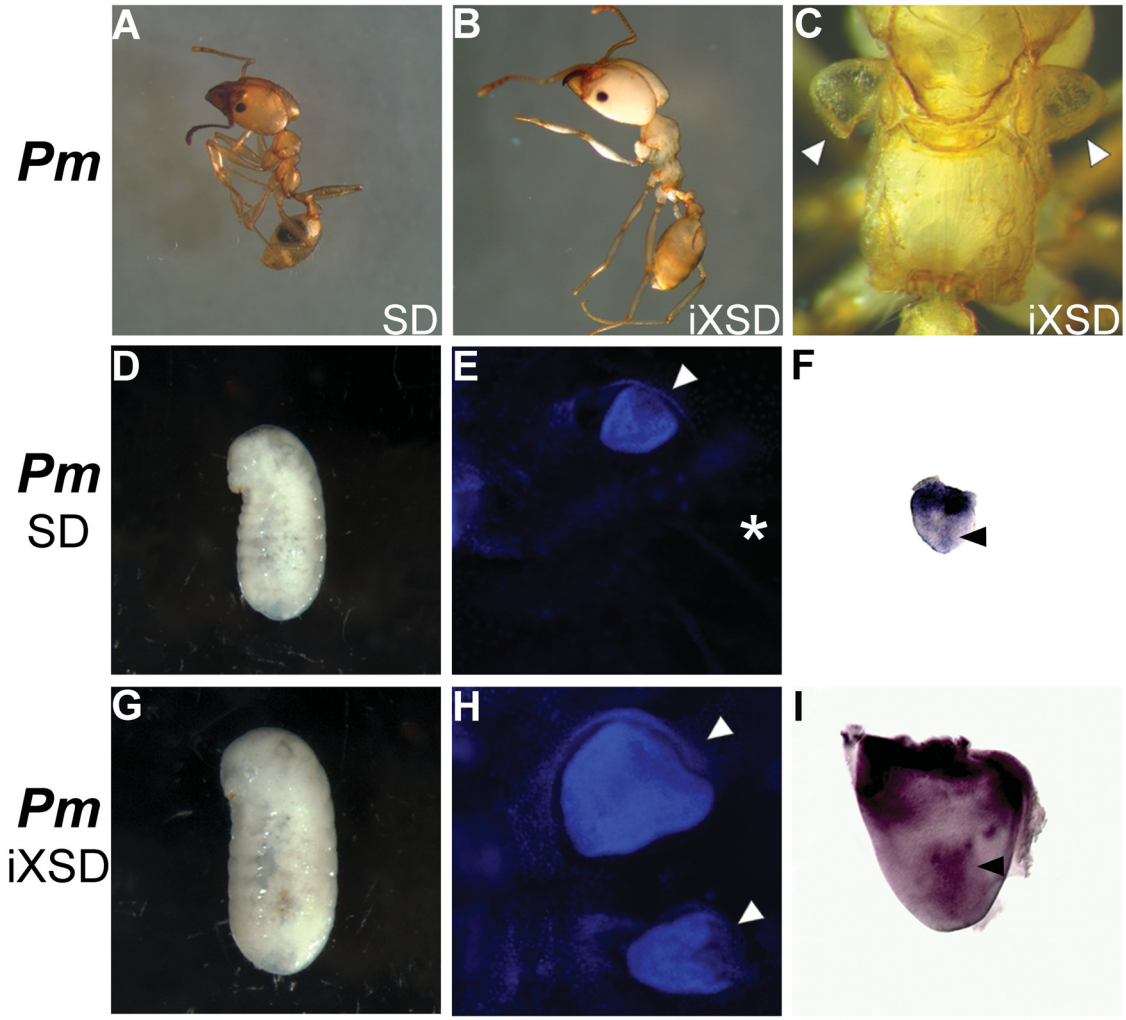

Pm
A
SD
B
iXSD
C
iXSD
Pm
SD
D
E
*
F
Pm
iXSD
G
H
I

Figure 3: Methoprene-induced supersoldiers (iXSD) in *P. morrisi* (*Pm*): comparison of (**A**) normal SD with (**B**) iXSD. (**C**) Thorax of an iXSD. Comparison of (**D**) SD and (**G**) iXSD larvae, and of vestigial wing discs (stained with DAPI) and *sa*l expression in (**E** and **F**) normal SD and (**H** and **I**) iXSD. White arrowheads indicate the presence of mesothoracic vestigial wing buds or discs; asterisks denote their absence. Black arrowheads indicate *sal* expression in the wing pouch. Adult, larval, and vestigial wing disc images are all to scale.

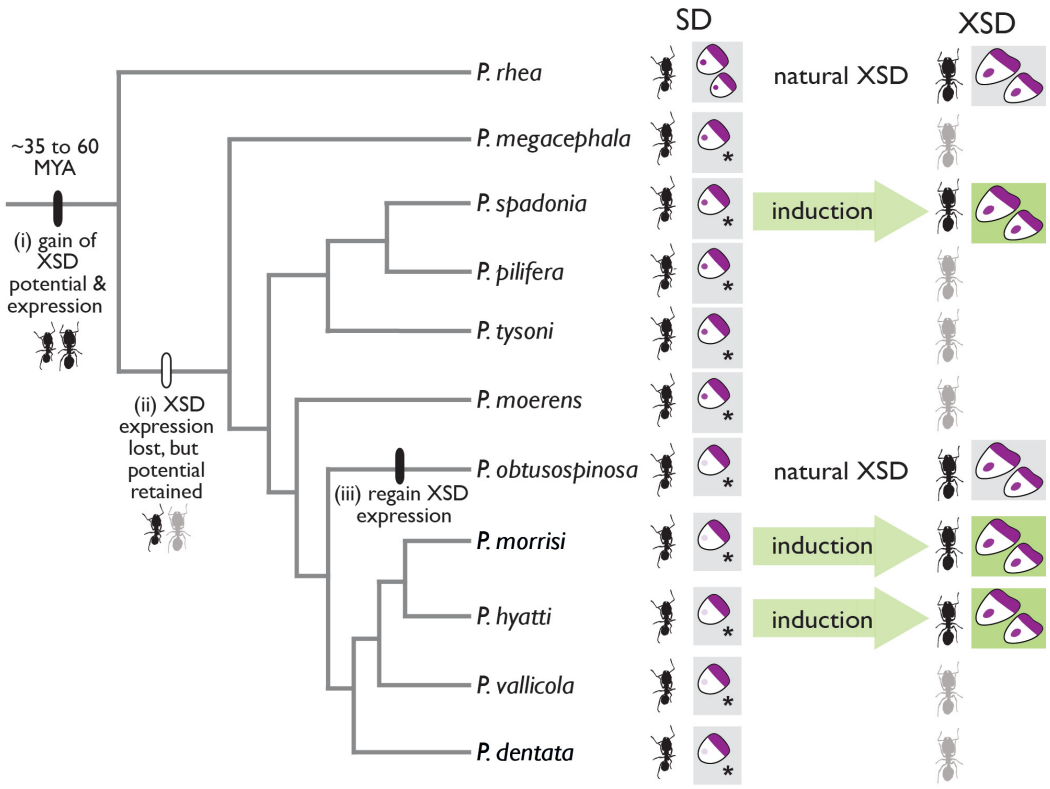

SD
XSD
P. rhea
P. megacephala
P. spadonia
P. pilifera
P. tysoni
P. moerens
P. obtusospinosa
P. morrisi
P. hyatti
P. vallicola
P. dentata
natural XSD
induction
induction
natural XSD
~35 to 60 MYA
(i) gain of XSD potential & expression
(ii) XSD expression lost, but potential retained
(iii) regain XSD expression

Figure 4: Evolutionary history of ancestral developmental potential and phenotypic expression of supersoldiers (XSDs). MYA, million years ago. Purple represents the pattern of *sal* expression; asterisks indicate the absence of vestigial wing discs and *sal* expression. Green arrows and boxes represent the induction of XSD potential.

2.4 Acknowledgements

We thank R. Sanwald, R. Johnson, S. Cover, M. Seid, W. Tschinkel, and J. King for help with ant collection or identification; Wellmark for methoprene; and T. Flatt, G. Wray, C. Metzl, S. Jolie, Abouheif lab members, and Konrad Lorenz Institute (KLI) fellows for comments. This work was supported by a Natural Sciences and Engineering Research Council grant and a KLI fellowship to E.A., as well as grants from NSF (0344946) and the Arizona Agricultural Experiment Station (ARZT 136321-H3106) to D.E.W. DNA sequences were deposited in GenBank (accession nos. JN205075 to JN205109).

2.5 Supplemental Material

2.5.1 Materials and Methods

Animal collection and culturing. We collected queen-right colonies of *P. morrisi*, *P. tysoni*, *P. pilifera* from Long Island, New York, USA, and *P. rhea*, *P. obtusospinosa*, *P. hyatti*, *P. spadonia*, and *P. vallicola* from the southwest of Arizona, USA, and *P. dentata*, *P. megacephala*, and *P. moerens*, from Tallahassee, Gainesville, and Fort Lauderdale, Florida, USA. Colonies were maintained in plastic boxes with glass test tubes filled with water constrained by cotton wool, and were fed a combination of mealworms, cockroaches, crickets, sunflower seed, and Bhatkar-Whitcomb diet (Bhatkar and Whitcomb, 1970). All colonies were maintained at 27 °C, 70% humidity, and 12 hour day:night cycle.

Isolation of sal homologues. We isolated fragments of *sal* from *P. morrisi* (Abouheif and Wray, 2002), as well as from *P. obtusospinosa* and *P. rhea* using PCR on cDNA that

was generated from reverse transcription of their larval mRNA. We cloned and sequenced these fragments to confirm their identity, and performed gene tree analyses to confirm their orthology with *Nasonia*, *Apis*, and *Drosophila sal*. PCR primers and conditions are described in (Abouheif and Wray, 2002). GenBank accession numbers are JN205075 for *P. obtusospinosa sal* and JN205076 for *P. rhea sal*.

Whole mount in situ hybridization and immunohistochemistry. We fixed and processed last instar larvae just prior to the prepupal stage (Patel, 1994). We carefully dissected larvae under a Zeiss Discovery V12 stereomicroscope to remove obstructive tissues surrounding imaginal discs. We then synthesized a digoxigenin-labelled riboprobe (Roche Diagnostics Canada) for *P. morrisi sal* (Tautz and Pfeifle, 1989). We used this *P. morrisi sal* probe for all *in situ* hybridizations because: (i) *sal* sequences in *P. morrisi*, *P. obtusospinosa*, and *P. rhea* are 96% similar to each other (fig. S15); and (ii) the *P. morrisi sal* probe cross-reacts and reveals a conserved pattern of *sal* expression in the wing discs of winged castes of the *Pheidole* species used in this study (fig. S16).

Phylogenetic analyses. To infer the relationships of the 11 *Pheidole* species included in our study, we performed a Bayesian analysis using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Following Moreau (Moreau, 2008), *Myrmica incompleta* was used as an outgroup. We isolated, cloned, and sequenced the following fragments using PCR (Table S1): (i) a ~616bp fragment of mitochondrial (mt) protein-coding *cox1*; (ii) a ~744bp fragment of mt protein-coding *cytb*; (iii) a ~535bp fragment of nuclear protein-coding *lwr*. GenBank accession numbers for these sequences are listed in Table S2. Additionally, we retrieved sequences from GenBank for: (i) a ~340bp fragment of nuclear protein-coding *H3*; (ii) a ~360bp fragment of mt ribosomal *12S*; and (iii) all

sequences for *Myrmica incompleta*. In total, we used approximately 2.6kb of concatenated sequence spanning 5 genes. We initially aligned these sequences using ClustalX v1.83.1 (Thompson et al., 1997), and subsequently adjusted these alignments by eye using McClade v4.06 (Maddison and Maddison, 2003). To determine the optimal model of sequence evolution for each gene, we used AIC criteria within ModelTest v3.7 (Posada and Buckley, 2004; Posada and Crandall, 1998). As a result, we used GTR+Γ+I for *cox1* and *cytb*, GTR+I for *H3*, and GTR+ Γ for *lwr* and 12S. Bayesian analysis involved two independent runs and used four Markov chains with an MCMC (Monte Carlo Markov Chain) length of 10,000,000 generations. The chains were sampled every 200 generations following a burn-in period of 1,000,000 generations. Data used for the analysis was partitioned by gene, each of which was assigned a separate substitution model. The analysis resulted in a sample of trees with a mean likelihood score of –lnL = 12156.91 for run 1 and –lnL=12156.95 for run 2. The average standard split frequencies of the chains after 10,000,000 generations was 0.002391, suggesting that the chains had reached convergence. The trees from both analyses were thus combined and summarized with a fully resolved majority-rule consensus tree.

Hormonal applications. We topically applied methoprene (Wellmark International), a juvenile hormone (JH) analogue, to the dorsal abdomens of *Pheidole* larvae. We applied methoprene to *P. morrisi larvae* (range of longest larval lengths was 0.9-1.8mm), *P. spadonia* larvae (1.5-2.7mm)*, and P. hyatti* larvae (1.0-1.5mm) prior to their determination as either minor workers or soldiers. For *P. obtusospinosa*, a species that naturally produces supersoldiers, the final size range (longest larval length) is 1.7-2.18mm for minor worker larvae, 3.3-3.6mm for soldier larvae, and 4.1-4.6mm for

supersoldier larvae. We therefore applied methoprene to *P. obtusospinosa* larvae that had already passed the soldier-minor worker switch point (2.2-3.3mm), but whose caste fate as either soldiers or supersoldiers had not yet been determined. We applied methoprene at a concentration of 5 mg/ml in acetone to *P. morrisi* larvae, 3 mg/ml in acetone to *P. hyatti* larvae, and both 3.5 mg/ml and 5 mg/ml in acetone to *P. spadonia* and *P. obtusospinosa* larvae. For *P. morrisi*, we followed Wheeler (*16*) and set-up multiple replicates of 40 methoprene-treated larvae raised by 40 workers and no soldiers. We fixed half of the surviving larvae just prior to the prepupal stage, and we let the other half of surviving larvae continue to develop. As controls, we set up multiple replicates in *P. morrisi* of 40 acetone-treated or untreated larvae raised by 40 workers and no soldiers. To confirm that methoprene can induce supersoldier larvae in other species that lack supersoldiers, we treated a large number of *P. spadonia* and *P. hyatti* larvae with methoprene and raised them in replicate boxes each of which contained 50-100 minor workers. To test whether a second JH-sensitive period mediates the switch between soldiers and supersoldiers larvae in *P. obtusospinosa*, we treated 200 larvae with methoprene and 200 larvae with acetone only. We raised these methoprene and acetone-treated larvae in replicate boxes, each of which contained 50-100 minor workers. For all experiments, we identified "supersoldier" larvae by finding the smallest larva (shortest larval length in mm) in the sample with two pairs of vestigial wing discs (a defining feature of supersoldier larvae). We then considered any larva longer than this as "supersoldier" larvae. Furthermore, adult *Pheidole* supersoldiers are defined as showing discrete differences from their soldiers in: (i) their head size and/or; (ii) the allometric scaling relation between the size of their heads and size of their bodies (Huang, 2010;

Huang and Wheeler, 2011). Naturally produced supersoldiers in *P. obtusospinosa* have significantly larger heads than their soldiers, whereas in *P. rhea* they have both significantly larger heads and their heads are smaller relative to their bodies than in soldiers (Huang, 2010; Huang and Wheeler, 2011). We therefore considered adults that were produced by methoprene treatment to be "induced supersoldiers" if their heads were significantly larger than the heads of untreated controls.

Statistical analyses and measurement. We used a Zeiss Discovery V12 stereomicroscope and Zeiss Axiovision software to measure the larval size (body length in mm) and adult size (head width in mm). We used an unequal variances t-test (Ruxton, 2006; Sokal and Rohlf, 1995) to determine whether in *P. morrisi* the mean size of: (i) adult minor workers from anomalous colony is equal to that of normal minor workers; (ii) adult anomalous supersoldiers is equal to that of normal soldiers; (iii) methoprene-treated larvae is equal to that of untreated controls; and (iv) adults resulting from methoprene-treated larvae is equal to that of untreated controls. We used a Fisher's exact test (Sokal and Rohlf, 1995) to determine whether in *P. obtusospinosa* the proportions of supersoldier relative to soldier larvae in methoprene treatments are equal to the proportions of acetone-treated controls. Finally, we calculated the coefficient of variation to compare the relative amounts of variation (Sokal and Rohlf, 1995) in the surface area of fore- and hindwing vestigial discs in normal and methoprene-induced supersoldier larvae in *P. obtusospinosa*, as well as induced supersoldier larvae in *P. morrisi*, *P. hyatti*, and *P. spadonia*.

2.5.2 Supplemental Figures and Tables

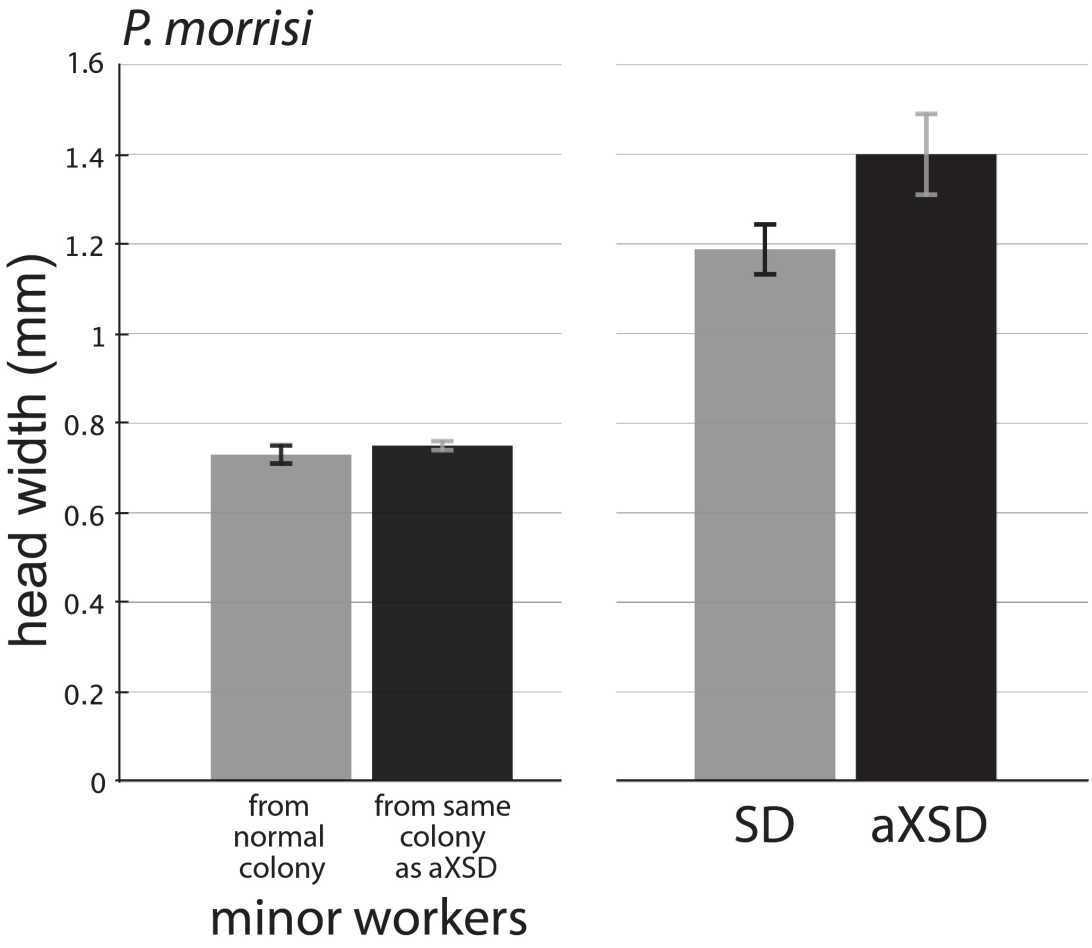


Figure S1: Anomalous *P. morrisi* supersoldiers are significantly larger than normal soldiers. X-axis showing the mean (bars) and standard deviation (error bars) for head widths (mm) of normal minor workers (gray bar, n= 20), minor workers from the anomalous colony (black bar, n = 5), normal soldiers (SD; gray bar, n = 20), and anomolous supersoldier-like individuals (aXSD; black bar, n = 8). An unequal variance t-test (two-tailed) between minor workers from the normal and from the anomalous colony shows that they are not significantly different from one another ($t = 2.083$, $df = 12$, $P = 0.06$). In contrast, an unequal variance t-test (two-tailed) between normal SD and aXSD shows that they are significantly different from one another ($t = 6.265$, $df = 9$, $P = 0.0002$). This shows that aXSD are significantly larger in size than normal soldiers.

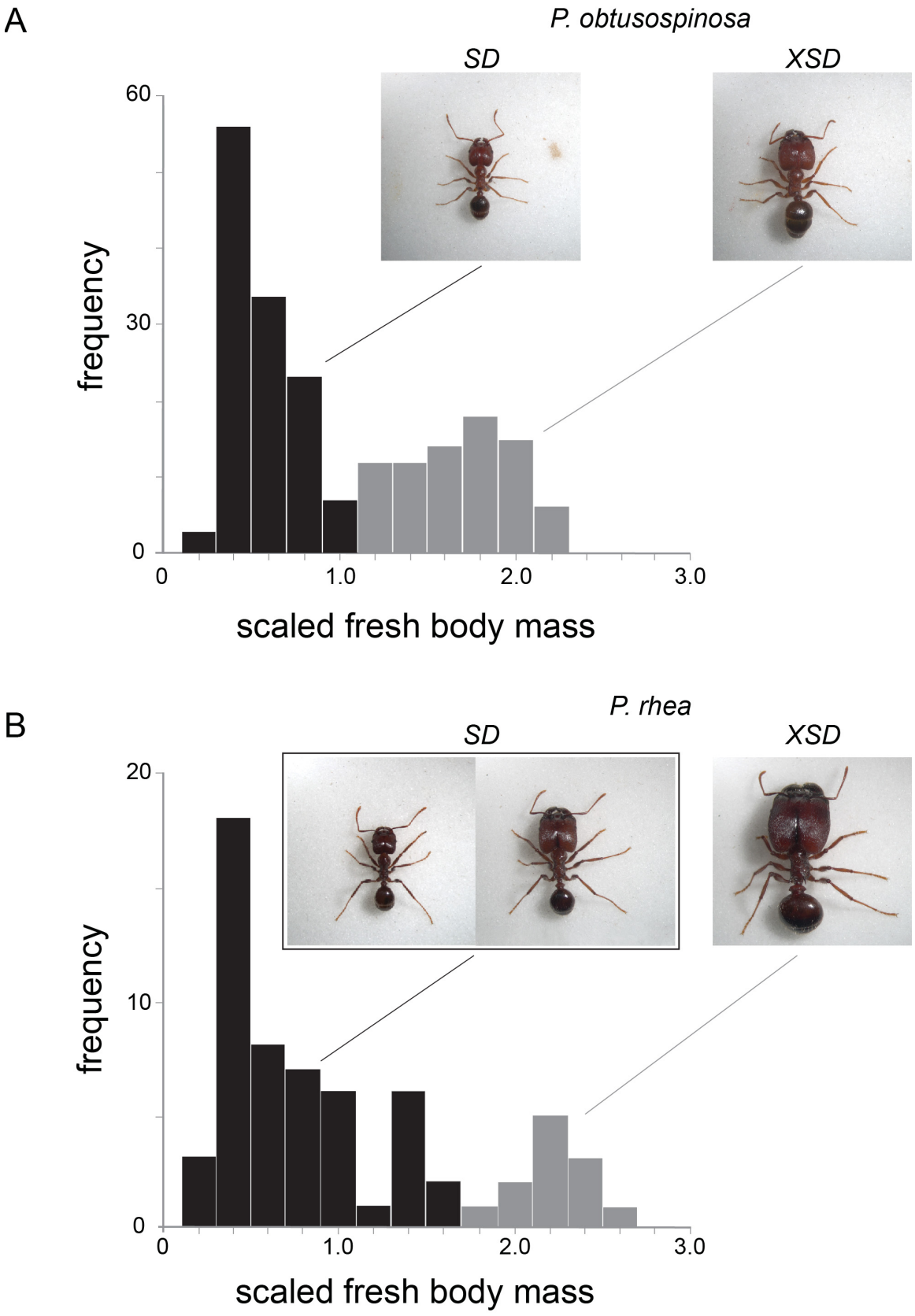

A
P. obtusospinosa
SD
XSD
60
30
0
frequency
0
1.0
2.0
3.0
scaled fresh body mass
B
P. rhea
SD
XSD
20
10
0
frequency
0
1.0
2.0
3.0
scaled fresh body mass

Figure S2: Definition and frequency distributions of the supersoldier subcaste in *P. obtusospinosa* and *P. rhea. Pheidole* supersoldiers (XSD) are defined as showing discrete differences from their soldiers (SD) in: (i) their head size and/or; (ii) the allometric scaling relation between their head and body size (Huang, 2010; Huang and Wheeler, 2011). In *P. obtusospinosa*, naturally produced XSD have significantly larger heads than their SD (compare SD to XSD in **A**), whereas in *P. rhea* XSD have both significantly larger heads and their heads are smaller relative to their bodies than in SD (compare SD to XSD in **B**) (Huang, 2010; Huang and Wheeler, 2011). Histograms showing the frequency distributions of soldiers (black bars) and supersoldiers (dark gray bars) based on total fresh body mass for **(A)** *P. obtusospinosa* and **(B)** *P. rhea*. Y-axis: indicates frequency of the soldier and supersoldier subcaste. X- axis: scaled fresh body mass. To facilitate comparing the distributions of *P. obtusospinosa* and *P. rhea*, we scaled fresh body masses of each species by dividing every fresh body mass (mg) by a mean fresh body mass (mg) that was calculated over all supersoldiers and soldiers. All images are to the same scale. These distributions are consistent with those of (Huang, 2010; Huang and Wheeler, 2011) in showing that the distribution of soldiers in *P. rhea* is much broader than that of *P. obtusospinosa*.

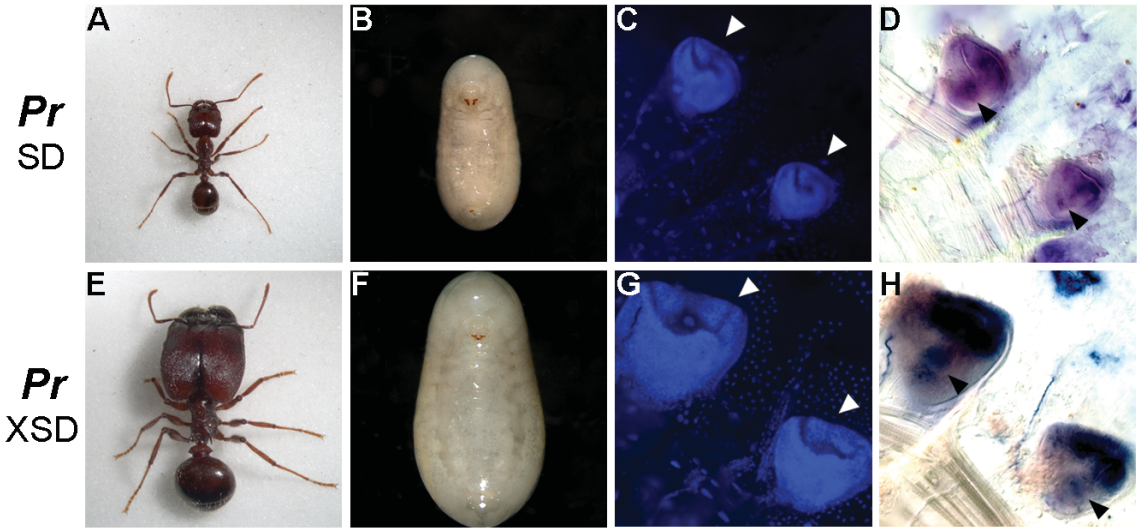

A
B
C
D
Pr
SD
E
F
G
H
Pr
XSD

Figure S3: Development of supersoldiers in *P. rhea*. Comparison of adults of (**A**) SD and (**E**) XSD as well as larvae of (**B)** SD and (**F)** XSD. Comparison of vestigial wing discs (DAPI) and *sal* mRNA expression (purple) of (**C** and **D)** SD and (**G** and **H)** XSD. White arrowheads indicate presence of mesothoracic wing vestiges or vestigial wing discs, whereas asterisks indicate their absence. Black arrowheads indicate *sal* expression in wing pouch. Adult, larval and vestigial wing disc images are all to scale.

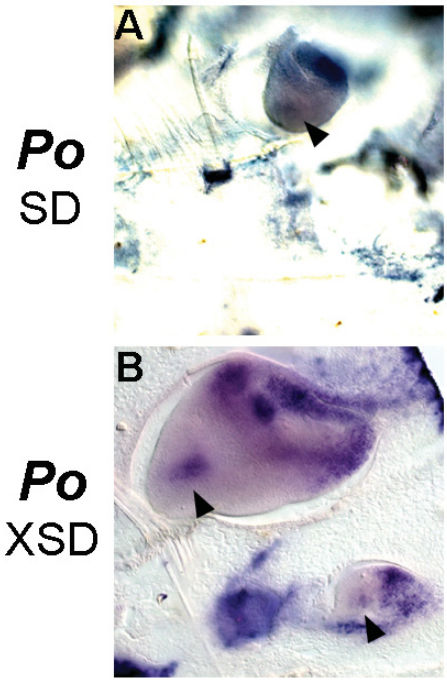
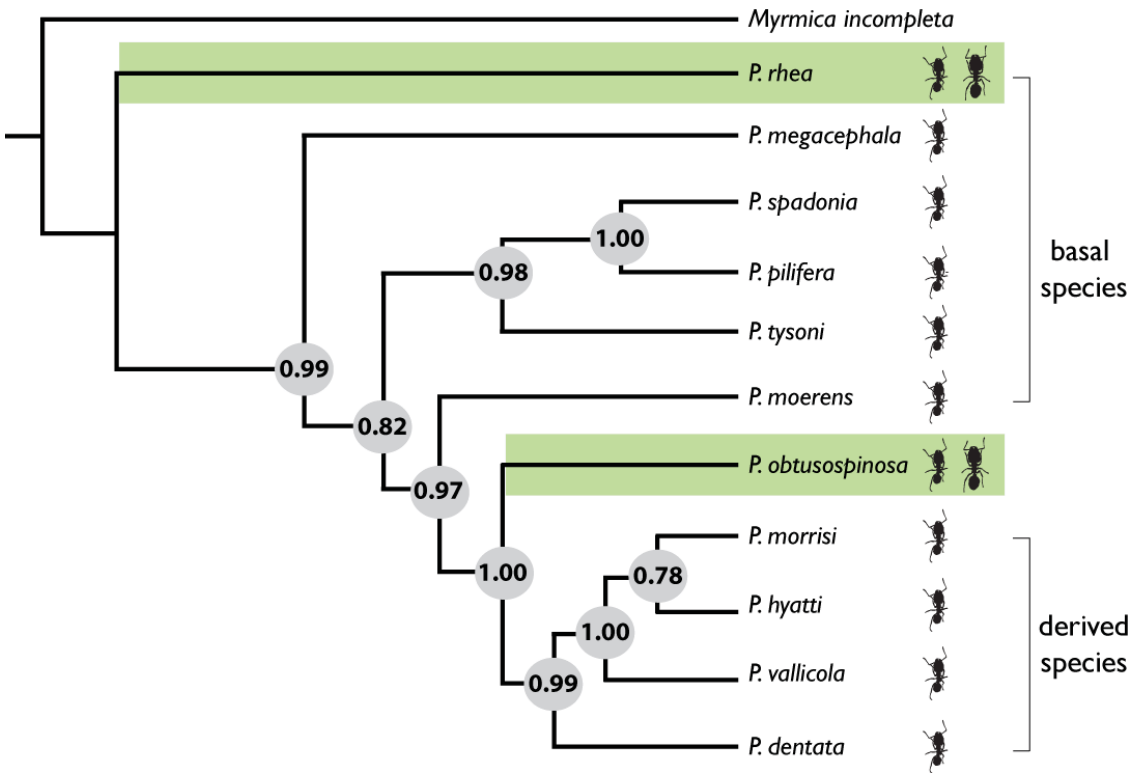

A
Po
SD
B
Po
XSD

Figure S4: Expression of *sal* in vestigial wing discs of soldier and supersoldier larvae in *P. obtusospinosa*. *sal* mRNA expression (purple) in the vestigial wing discs of *Pheidole obtusospinosa* (*Po*): (**A**) soldiers (SD) and (**B**) supersoldiers (XSD). Black arrowheads indicate wing pouch expression of *sal*. Note that, as in *P. morrisi*, *Po* SDs have (**A**) a single pair of vestigial forewing discs in which *sal* expression is conserved in the hinge region, but is highly downregulated in the wing pouch. (**B**) *Po* XSDs have both fore- and hindwing discs, in which *sal* expression is conserved in the hinge and elaborated in the wing pouch relative to the expression of *sal* in SD. Both images are to scale.


Myrmica incompleta
P. rhea
P. megacephala
P. spadonia
P. pilifera
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P. obtusospinosa
P. morrisi
P. hyatti
P. vallicola
P. dentata
1.00
0.98
0.99
0.82
0.97
1.00
0.78
1.00
0.99
basal species
derived species

Figure S5: Bayesian phylogenetic analysis of the 11 *Pheidole* species we used in this study confirms the independent evolution of supersoldiers of *P. rhea* and *P. obtusospinosa* and provides strong support for their inferred relationships. Although Moreau (Moreau, 2008) reconstructed a genus-level phylogeny using 142 *Pheidole* species, the phylogenetic relationships of the 11 species that we used in this study remained largely unresolved. This greatly decreases our ability to reconstruct the evolutionary history of supersoldiers. We therefore performed a detailed Bayesian phylogenetic analysis of all 11 species to further resolve their relationships and confirm the independent evolution of supersoldiers in *P. rhea* and *P. obtusospinosa*. Our analysis yielded high posterior probabilities for almost all nodes (gray circles) in the tree, which provides substantial confidence in the inferred phylogenetic relationships. Our results are consistent with (Moreau, 2008) in that *P. rhea* is supported as a sister taxon of almost all *Pheidole* species as well as one of the most basal species in the genus. Our phylogenetic analysis supports *P. obtusospinosa* as a derived species confirming the independent evolution of supersoldier subcastes (green lines) (Moreau, 2008). Our analysis also shows that there are six species that are “basal” and four species that are “derived” relative to *P. obtusospinosa*.

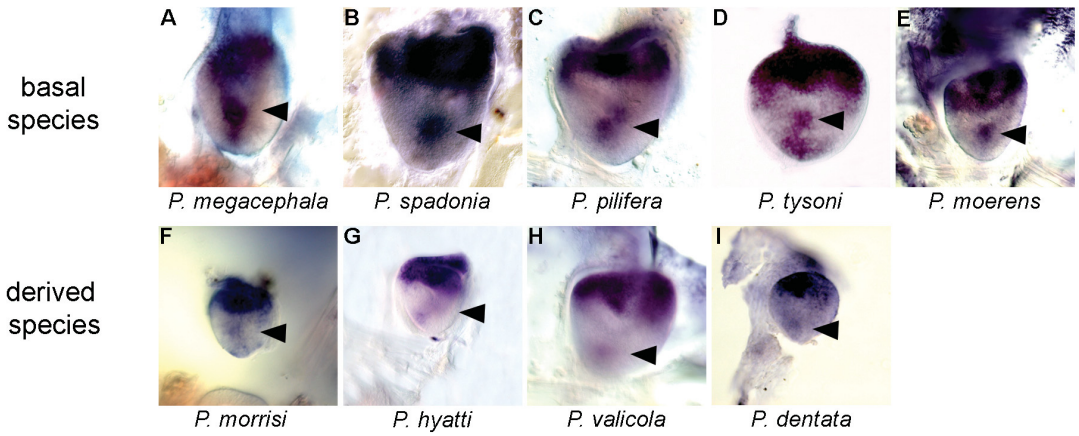

basal species
A
B
C
D
E
P. megacephala
P. spadonia
P. pilifera
P. tysoni
P. moerens
derived species
F
G
H
I
P. morrisi
P. hyatti
P. valicola
P. dentata

Figure S6: Interruption of *sal* expression in soldier forewing discs differs between basal and derived *Pheidole* species. (**A** to **I**), all images are to scale and represent vestigial forewing discs from late final instar soldier larvae. (**A** to **E**)**,** *sal* mRNA expression (purple) in vestigial soldier forewing discs of basal *Pheidole* species (fig S5): relative to its expression in the hinge**,** black arrows indicate *sal* is expressed as a well- defined patch of expression in the wing pouch (see fig S3D for *P. rhea*). (**F** to **I**) *sal* expression in vestigial soldier forewing discs of derived *Pheidole* species (fig S5): relative to its expression in the hinge, black arrows indicate *sal* is expressed as a barely visible and diffuse patch of expression in the wing pouch (see figS4A for *P. obtusospinosa*). These differences in *sal* expression, which occur regardless of the size of vestigial forewing discs, reflect genetically fixed differences that have evolved between these basal and derived *Pheidole* species.

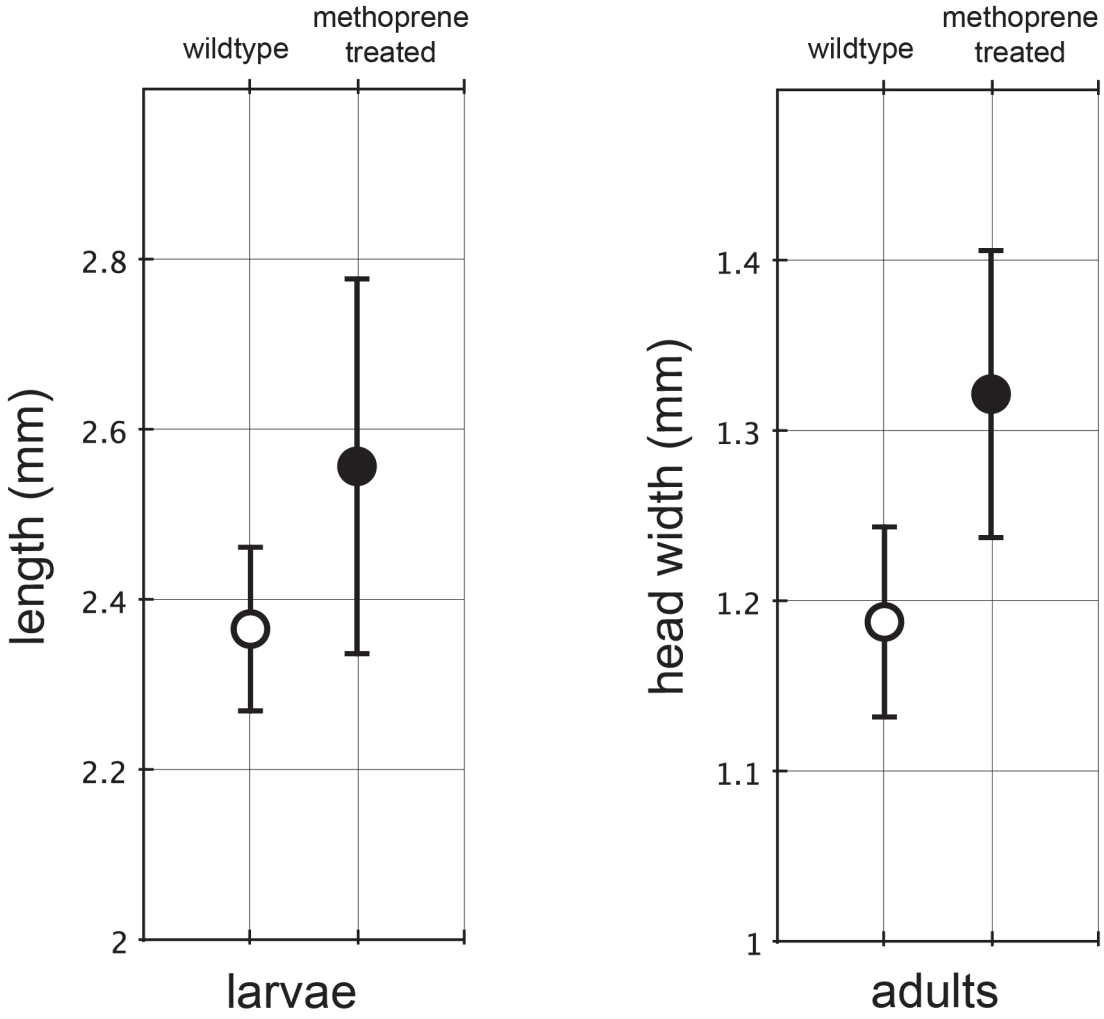

wildtype
methoprene treated
length (mm)
2.8
2.6
2.4
2.2
2
larvae
wildtype
methoprene treated
head width (mm)
1.4
1.3
1.2
1.1
1
adults

Figure S7: Application of methoprene induces the development of supersoldier larvae and adults in *P. morrisi*. Graph on left shows mean (circles) and standard deviation (error bars) for larval length (mm) of untreated (white circle; n = 50) and methoprene- treated (black circle; n = 36) larvae. The mean for methoprene-treated larvae represents larvae from all replicates. An unequal variances t-test (one-tailed) shows that the mean of methoprene-treated larvae is significantly larger than that of untreated larvae ($t = 4.887$, $df = 44$, $P < 0.0001$). All larvae that survived the acetone-treated controls (n = 24; from all replicates) developed into minor worker larvae. Graph on right shows mean and standard deviation for adult head width (mm) of adults that developed from untreated larvae (white circle; n = 30) and adults that developed from methoprene-treated larvae (black circle; n = 7). The mean for adults that developed from methoprene-treated larvae represents adults from all replicates. An unequal variance t-test (one-tailed) shows that the mean of adults that developed from methoprene-treated larvae is significantly larger than those that were untreated ($t = 3.742$, $df = 7$, $P = 0.0036$). All adults that survived the acetone-treated controls (n = 42 from all replicates) developed into adult minor workers.

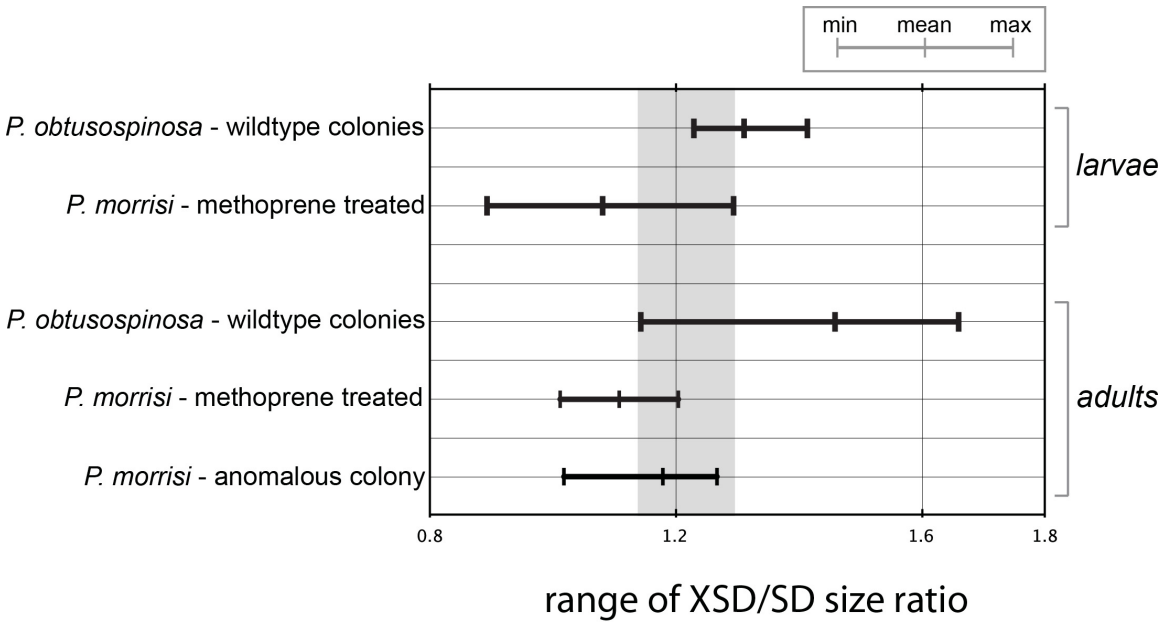

min
mean
max
P. obtusospinosa - wildtype colonies
P. morrisi - methoprene treated
P. obtusospinosa - wildtype colonies
P. morrisi - methoprene treated
P. morrisi - anomalous colony
larvae
adults
0.8
1.2
1.6
1.8
range of XSD/SD size ratio

Figure S8: The relative size ranges of methoprene induced supersoldiers overlap with those of anomalous and naturally produced supersoldiers. The x-axis shows the relative size range of supersoldiers as indicated by horizontal black bars, which was calculated in three steps: (i) the mean supersoldier (XSD)/soldier (SD) size ratio was taken by dividing the mean size (head width in mm) of either induced, anomalous, or evolved XSD adults or larvae by the mean size (head width in mm) of their respective normal SD adults or larvae; (ii) the maximum XSD/SD size ratio was calculated by dividing the largest induced, anomalous, or naturally produced XSD adult or larva in the sample by the mean size of their respective normal SD adults or larvae; and (iii) the minimum XSD/SD size ratio was calculated by dividing the smallest induced, anomalous, or naturally produced XSD adult or larva in the sample by the mean size of their respective normal SD adults or larvae. The vertical gray bar denotes the region of overlap between the relative size ranges of methoprene induced supersoldier adults and larvae and those of anomalous and naturally produced supersoldiers.

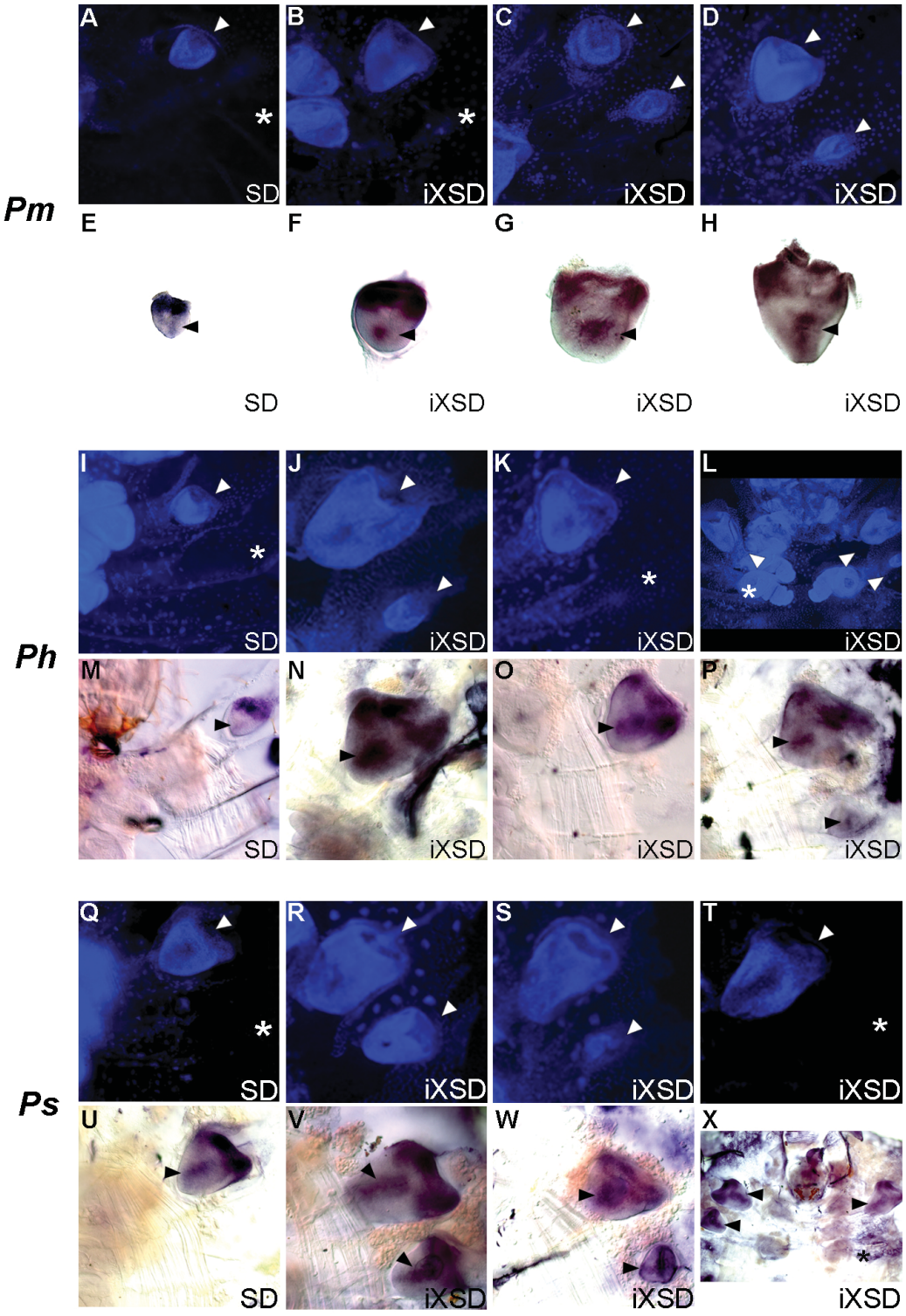

Pm
A
B
C
D
SD
iXSD
iXSD
iXSD
E
F
G
H
SD
iXSD
iXSD
iXSD
Ph
I
J
K
L
SD
iXSD
iXSD
iXSD
M
N
O
P
SD
iXSD
iXSD
iXSD
Ps
Q
R
S
T
SD
iXSD
iXSD
iXSD
U
V
W
X
SD
iXSD
iXSD
iXSD

Figure S9: Variation in the number, size, and expression of *sal* in vestigial wing discs of normal soldier (SD) and induced supersoldier (iXSD) larvae in *P. morrisi* (Pm), *P. hyatti* (Ph), and *P. spadonia* (*Ps*). (**A** to **X**), asterisks indicate absence of visible wing primordia, while white arrows indicate variation in the number of vestigial wing discs stained with DAPI. Black arrows indicate variation in wing pouch expression of *sal*. (**A** to **X**), images are to scale, with the exception of (**L**) and (**X**) that were taken at lower magnification to show asymmetry in the number of vestigial wing discs between the left and right sides of an induced supersoldier larva. Note that this induced variation in iXSD larvae is not due to the incomplete induction of JH, but rather is due to the release of hidden variation that has not yet been subject to selection. We detected variants (**B** and **K** and **T**) that we consider to be iXSD larvae, but which have only one pair of visible extra- large vestigial wing discs. This is because we define iXSD larvae by finding the smallest larva (shortest larval length in mm) in our sample that had two pairs of vestigial wing discs, and then considered any larva longer than this as iXSD larvae. That these are iXSD larvae rather than incompletely induced larvae is further supported by the existence of asymmetrical iXSD larvae: some iXSD have supersoldier-like vestigial fore- and hindwing discs on one side but only a forewing disc on the other side (**L** and **X**).

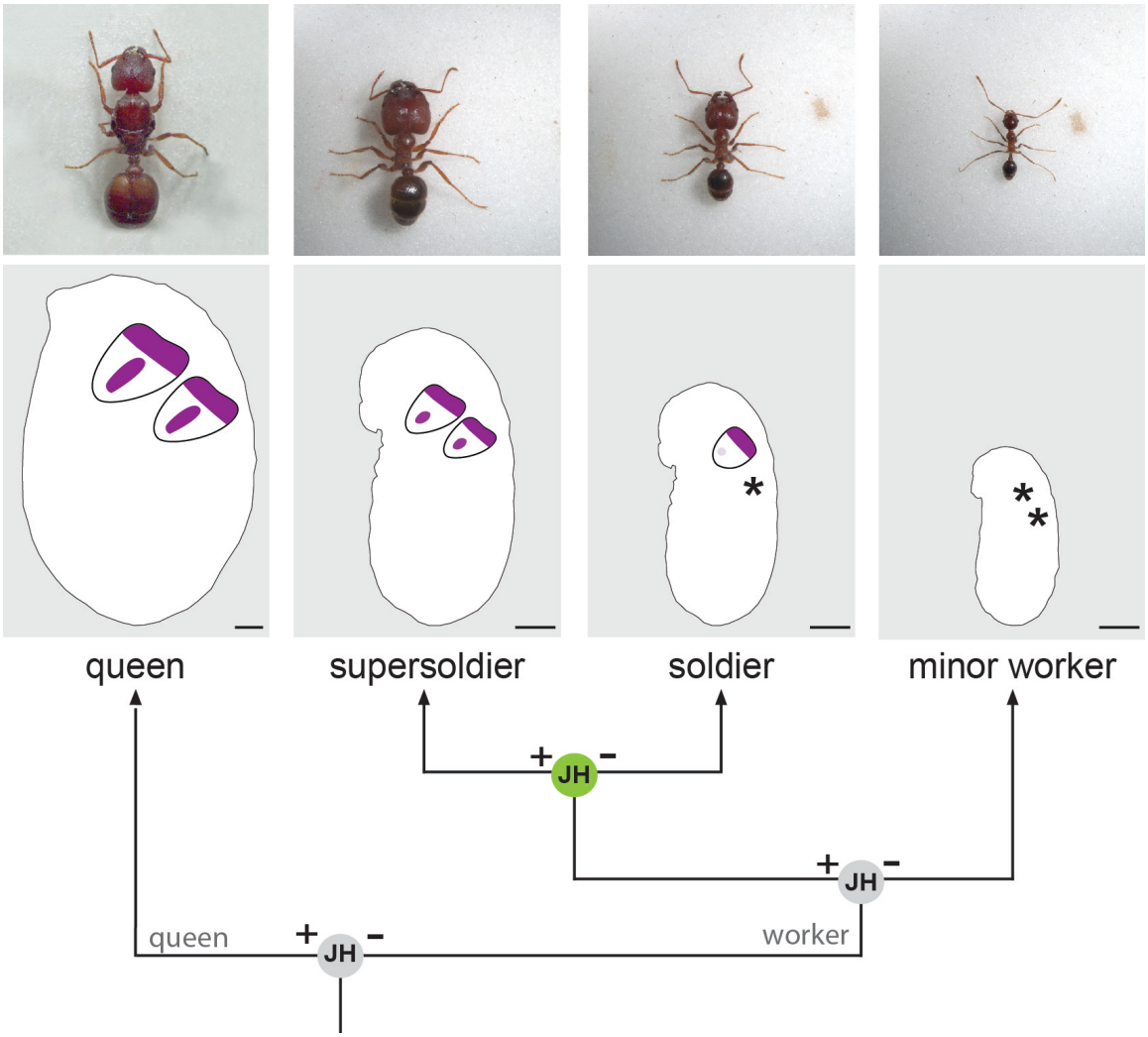
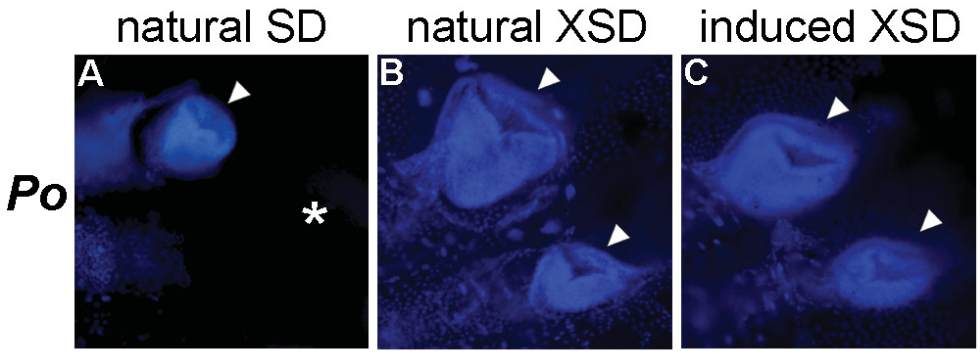

queen
supersoldier
soldier
minor worker
+
JH
-
+
JH
-
queen
+
JH
-
worker

Figure S10: Actualization of supersoldiers in *P. obtusospinosa* through the re-evolution of a second JH-sensitive period that mediates the switch (green) between soldiers and supersoldiers. See fig. S11 for data supporting this conclusion and fig. S12 for models that may explain the mechanisms through which this second switch point evolved**.** Scale bars indicate the relative size of queen, soldier, and minor worker larvae. All adult images are to scale. Purple represents *sal* expression, whereas asterisks indicate absence of visible wing primordia and *sal* expression.


natural SD
natural XSD
induced XSD
A
B
C
Po
*

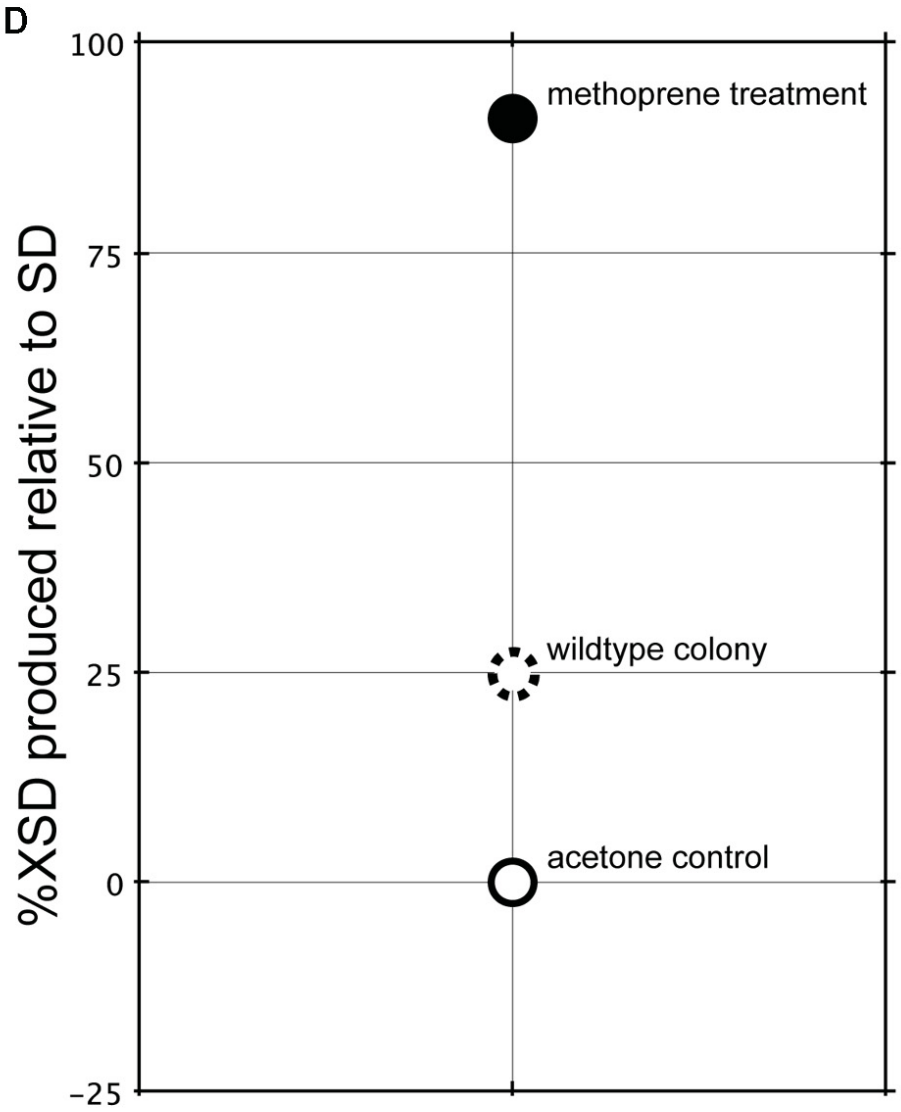
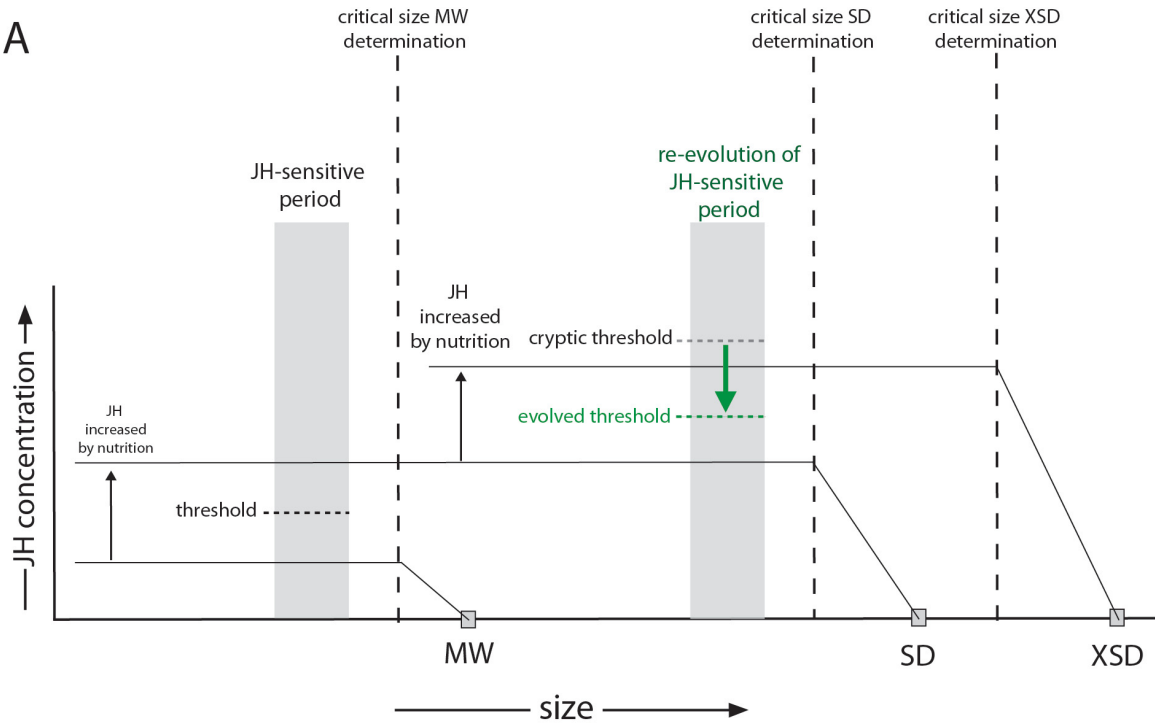

D
100
75
50
25
0
-25
%XSD produced relative to SD
methoprene treatment
wildtype colony
acetone control

Figure S11: A second JH-sensitive period mediates the switch between the soldier and the supersoldier subcaste in *P. obtusospinosa*. (**A** to **C**) All images are to scale. Asterisks indicate absence of visible wing primordia, whereas white arrows indicate their presence. In *P. obtusospinosa* (*Po*), DAPI staining (blue) shows that, unlike (**A**) soldiers (SD) that have a single pair of vestigial forewing discs, (**B**) supersoldiers (XSD) and (**C**), methoprene-induced supersoldiers (iXSD) have two pairs of large vestigial wing discs. Furthermore, (**D**) y-axis shows the percent of XSD produced relative to SD in methoprene-treated larvae (black circle), acetone-treated control larvae (white circle), and in wild *P. obtusospinosa* colonies (Huang, 2010; Huang and Wheeler, 2011) (dotted circle). A Fisher's exact test (one- tailed) shows that methoprene treatment produced a significantly greater proportion of XSD larvae than in acetone treated controls ($P < 0.0001$). Methoprene treatment also produced a greater proportion of XSD larvae than that normally observed in wild colonies. This indicates that the development of SD and XSD subcastes in *P. obtusospinosa* are determined through a second JH-sensitive period that mediates the switch between SD and XSD. Methoprene- or acetone-treated larvae that did not develop into XSD went on to develop as either SD or minor workers.


A
critical size MW determination
critical size SD determination
critical size XSD determination
JH-sensitive period
re-evolution of JH-sensitive period
JH increased by nutrition
cryptic threshold
evolved threshold
JH increased by nutrition
threshold
JH concentration
MW
SD
XSD
size

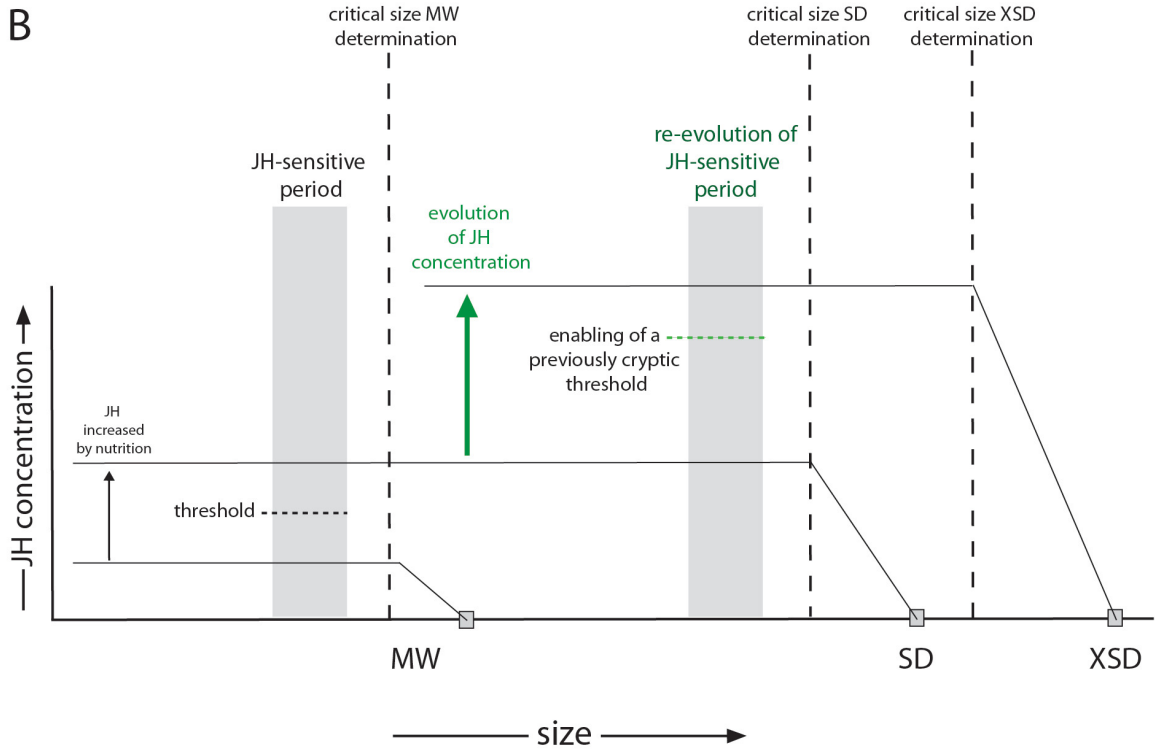
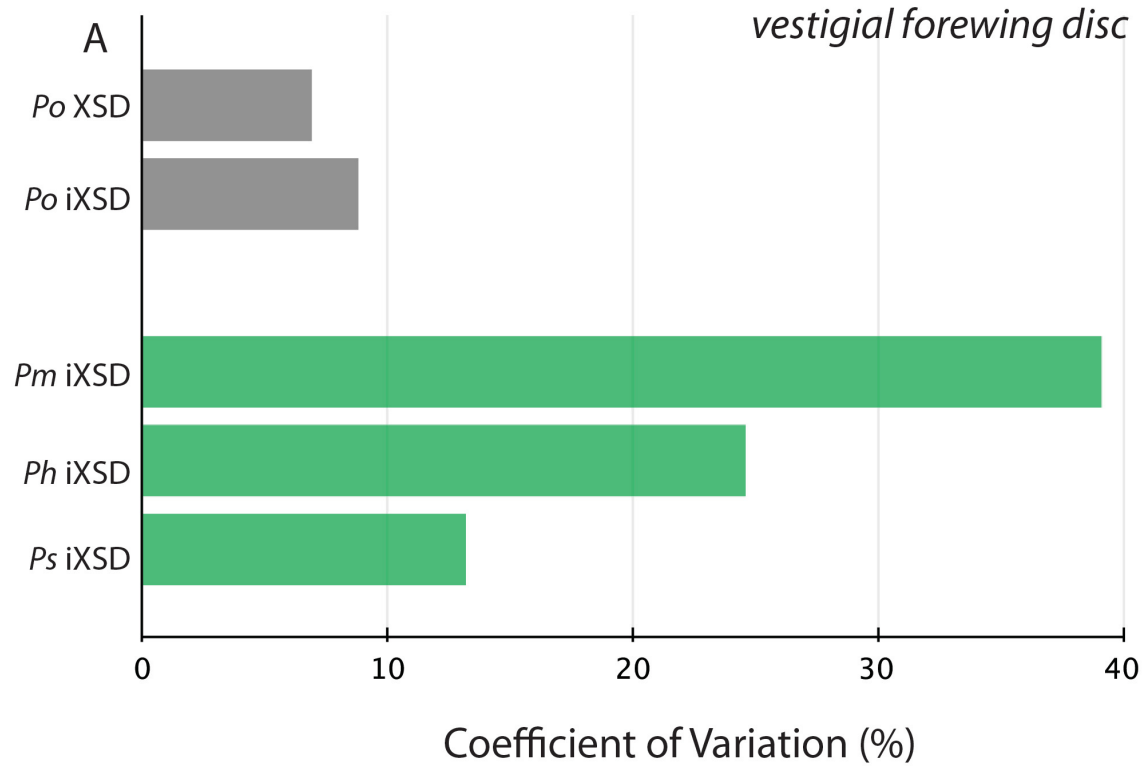

B
critical size MW determination
critical size SD determination
critical size XSD determination
JH-sensitive period
re-evolution of JH-sensitive period
evolution of JH concentration
enabling of a previously cryptic threshold
JH increased by nutrition
threshold
JH concentration
MW
SD
XSD
size

Figure S12: Two heuristic models showing how natural selection on the JH system may lead to the re-evolution of a continually produced supersoldier subcaste in *P. obtusospinosa*. The re-evolution of a supersoldier subcaste may have occurred either by a decrease in threshold sensitivity (as indicated by the green arrow in model **A**) or increase in JH level (as indicated by the green arrow in model **B**)**.** In both models, the switch between minor workers (MW) and soldiers (SD), as well as between SD and supersoldiers (XSD), requires an intact JH-sensitive period. The JH-sensitive period is the period during which all of the key elements, including JH level and threshold, are present for the transduction of an environmental cue into a binary developmental decision (Nijhout, 1999). The threshold is made up of tissue responsiveness via a receptor complex, whereas JH level is determined by a balance of synthesis and degradation (Nijhout, 1994). Note that in both models the threshold and JH level is shown as static for simplicity – the threshold can be modulated, for example, by proportion of adult soldiers in the colony (Wheeler and Nijhout, 1984), whereas JH levels can be modulated, for example, in response to variation in environmental cues (Nijhout, 1999). In both models, if JH levels are below threshold during the JH-sensitive period, larvae initiate metamorphosis upon reaching the critical size for minor workers (Wheeler and Nijhout, 1981b). If JH levels are above threshold, larvae continue to grow, their vestigial forewing imaginal discs appear, and the critical size is set (Wheeler and Nijhout, 1981b). Metamorphosis at the soldier critical size is controlled by a sensitive period that precedes it (Wheeler and Nijhout, 1981b). We propose that in the vast majority of *Pheidole* species there is a cryptic threshold that is set higher than the JH level, and therefore, all individuals initiate metamorphosis only at the soldier critical size (SD). In the case of

anomalous supersoldier-like individuals, interaction between the ancestral developmental potential and induction by the environment causes the larva to surpass this cryptic threshold by triggering the setting of a larger critical size (XSD). The re-evolution of supersoldiers in *P. obtuspspinosa* involves the actualization of the induced ancestral developmental potential such that a regular proportion of larvae, which surpassed both the minor worker and soldier threshold, are determined to be supersoldiers. The actualization can be achieved by natural selection on an evolved decrease in threshold sensitivity in model **A** or by natural selection on an increase in JH level as in model **B**.


A
vestigial forewing disc
Po XSD
Po iXSD
Pm iXSD
Ph iXSD
Ps iXSD
0
10
20
30
40
Coefficient of Variation (%)

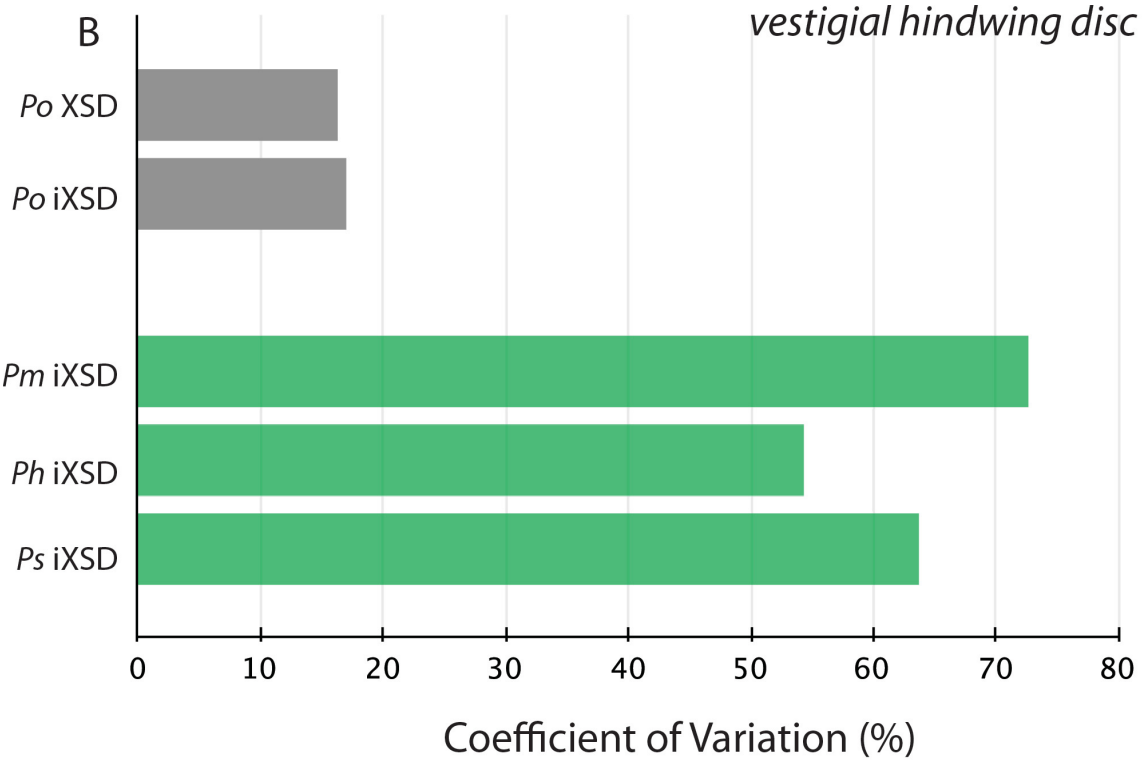

B
vestigial hindwing disc
Po XSD
Po iXSD
Pm iXSD
Ph iXSD
Ps iXSD
0
10
20
30
40
50
60
70
80
Coefficient of Variation (%)

Figure S13: A signature of natural selection on the vestigial wing discs in naturally produced supersoldiers in *P. obtusospinosa*. The naturally produced supersoldiers (XSD) and methoprene induced supersoldiers (iXSD) in *P. obtusospinosa* (*Po*) (fig. S11) generally show less variation in their size of (**A**) forewing and (**B**) hindwing vestigial discs than that of (**A**) forewing and (**B**) hindwing vestigial discs of the iXSD larvae of *P. morrisi* (*Pm*), *P. hyatti* (*Ph*), and *P. spadonia* (*Ps*), all of which lack a XSD subcaste. Furthermore, in *Pm*, *Ph*, and *Ps*, we also observed variation in the number of vestigial wing discs, asymmetry of vestigial wing discs as well as variation in the expression of *sal* (fig. S9). Greater variation is expected in an induced response that has not yet been subject to selection, which suggests that the developmental response of vestigial wing discs to JH has been canalized by selection in *P. obtusospinosa*, but not in *Pheidole* species that lack XSD.

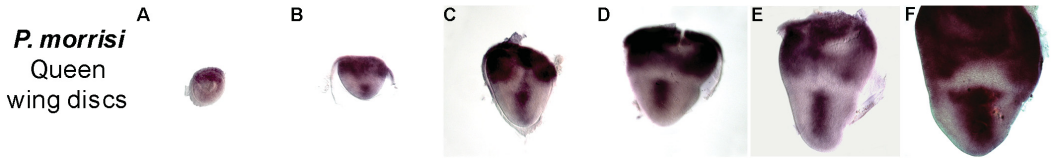

A
B
C
D
E
F
P. morrisi
Queen
wing discs

Figure S14: Dynamic expression of *sal* during wing disc development in *P. morrisi* queens suggests that the winglessness of adult supersoldiers (XSD) in *P. obtusospinosa* evolved through selection on environmentally induced supersoldier variants with novel interruption of *sal* expression. (**A** to **F**) All images are to scale. The wing discs of queens appear and begin to grow early in development during the first larval instar. *sal* mRNA expression (purple) in these discs remains constant in the hinge, but is dynamic in the wing pouch where it increases in length as development of the disc progresses. In normal soldier (SD) forewing discs, interruption of *sal* expression (Fig. 1) is similar to (**A**) *sal* expression at the earliest stages of wing disc development in queens, which results in the development of completely wingless soldiers (Fig. 1). In contrast to normal SD, vestigial wing discs in methoprene induced supersoldiers (iXSD) are larger in size and their *sal* expression (fig. S9) is interrupted at a later time, similar to that of (**B** to **D)** queen wing disc development. These results, together with the fact that wing vestiges appear on the mesothorax of iXSD (Fig. 3C) and anomalous XSD adults (Fig. 2D), suggest that wing development has been reactivated in iXSD and anomalous XSD. Wing development, however, is not completed because unlike queen wing discs, SD wing discs only appear in last instar SD larvae and their growth is prematurely halted as they have to undergo metamorphosis sooner than queen wing discs. Collectively, these observations suggest that the evolution of wingless adult XSD in *P. obtusospinosa* (Fig. 2I) evolved through selection on environmentally induced supersoldier variants with a novel interruption of *sal* such that any maladaptive development of wings is completely halted.

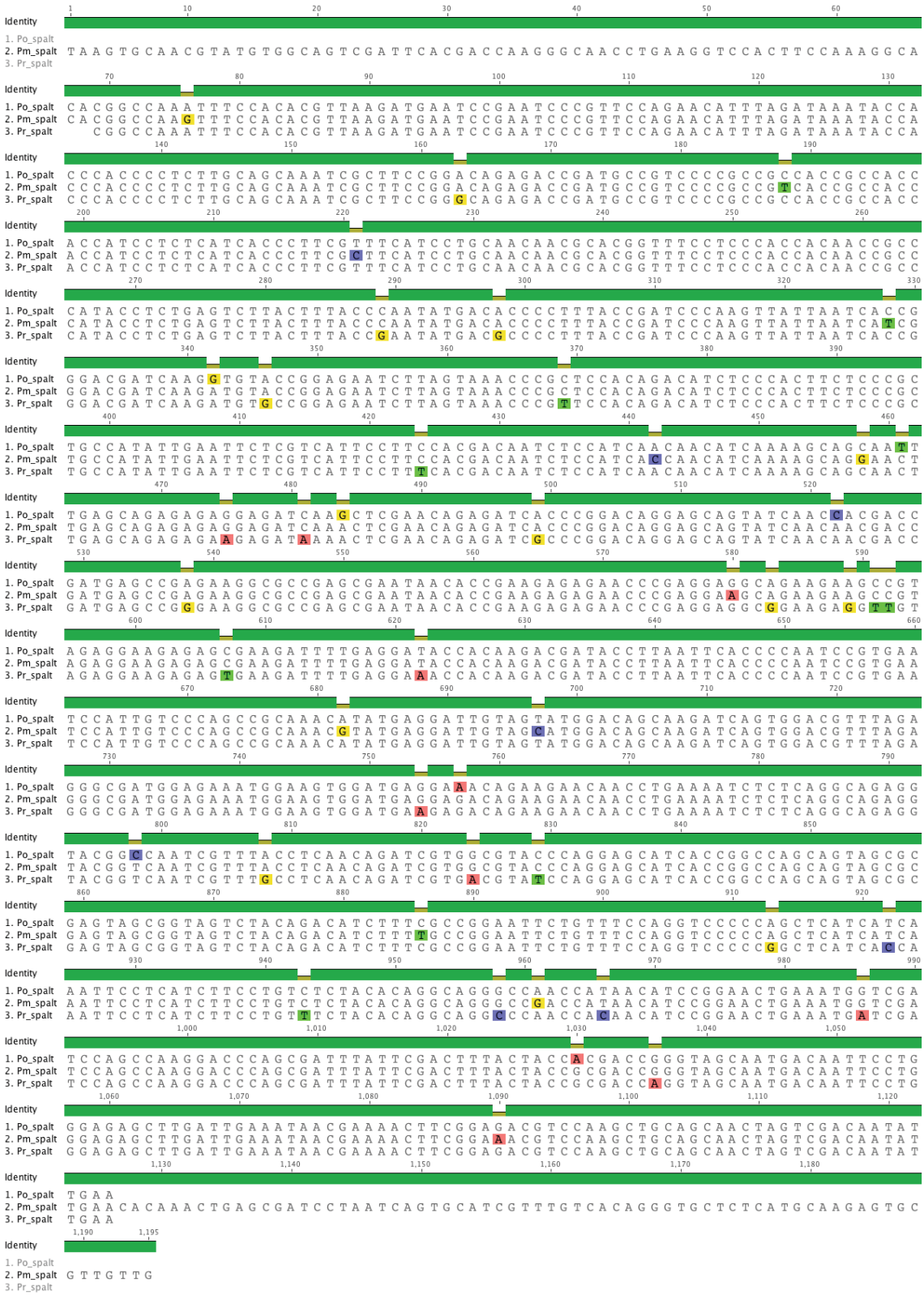

Identity
1. Po_spalt
2. Pm_spalt T A A G T G C A A C G T A T G T G G C A G T C G A T T C A C G A C C A A G G G C A A C C T G A A G G T C C A C T T C C A A A G G C A
3. Pr_spalt
1. Po_spalt C A C G G C C A A A T T T C C A C A C G T T A A G A T G A A T C C G A A T C C C G T T C C A G A A C A T T T A G A T A A A T A C C A
2. Pm_spalt C A C G G C C A A G T T T C C A C A C G T T A A G A T G A A T C C G A A T C C C G T T C C A G A A C A T T T A G A T A A A T A C C A
3. Pr_spalt C G G C C A A A T T T C C A C A C G T T A A G A T G A A T C C G A A T C C C G T T C C A G A A C A T T T A G A T A A A T A C C A
1. Po_spalt C C C A C C C C T C T T G C A G C A A A T C G C T T C C G G A C A G A G A C C G A T G C C G T C C C C G C C G C C A C C G C C A C C
2. Pm_spalt C C C A C C C C T C T T G C A G C A A A T C G C T T C C G G A C A G A G A C C G A T G C C G T C C C C G C C G T C A C C G C C A C C
3. Pr_spalt C C C A C C C C T C T T G C A G C A A A T C G C T T C C G G G C A G A G A C C G A T G C C G T C C C C G C C G C C A C C G C C A C C
1. Po_spalt A C C A T C C T C T C A T C A C C C T T C G T T T C A T C C T G C A A C A A C G C A C G G T T T C C T C C C A C C A C A A C C G C C
2. Pm_spalt A C C A T C C T C T C A T C A C C C T T C G C T T C A T C C T G C A A C A A C G C A C G G T T T C C T C C C A C C A C A A C C G C C
3. Pr_spalt A C C A T C C T C T C A T C A C C C T T C G T T T C A T C C T G C A A C A A C G C A C G G T T T C C T C C C A C C A C A A C C G C C
1. Po_spalt C A T A C C T C T G A G T C T T A C T T T A C C C A A T A T G A C A C C C C T T T A C C G A T C C C A A G T T A T T A A T C A C C G
2. Pm_spalt C A T A C C T C T G A G T C T T A C T T T A C C C A A T A T G A C A C C C C T T T A C C G A T C C C A A G T T A T T A A T C A T C G
3. Pr_spalt C A T A C C T C T G A G T C T T A C T T T A C C G A A T A T G A C G C C C C T T T A C C G A T C C C A A G T T A T T A A T C A C C G
1. Po_spalt G G A C G A T C A A G G T G T A C C G G A G A A T C T T A G T A A A C C C G C T C C A C A G A C A T C T C C C A C T T C T C C C G C
2. Pm_spalt G G A C G A T C A A G A T G T A C C G G A G A A T C T T A G T A A A C C C G C T C C A C A G A C A T C T C C C A C T T C T C C C G C
3. Pr_spalt G G A C G A T C A A G A T G T G C C G G A G A A T C T T A G T A A A C C C G T T C C A C A G A C A T C T C C C A C T T C T C C C G C
1. Po_spalt T G C C A T A T T G A A T T C T C G T C A T T C C T T C C A C G A C A A T C T C C A T C A A C A A C A T C A A A A G C A G C A A T T
2. Pm_spalt T G C C A T A T T G A A T T C T C G T C A T T C C T T C C A C G A C A A T C T C C A T C A C C A A C A T C A A A A G C A G G A A C T
3. Pr_spalt T G C C A T A T T G A A T T C T C G T C A T T C C T T T C A C G A C A A T C T C C A T C A A C A A C A T C A A A A G C A G C A A C T
1. Po_spalt T G A G C A G A G A G A G G A G A T C A A G C T C G A A C A G A G A T C A C C C G G A C A G G A G C A G T A T C A A C C A C G A C C
2. Pm_spalt T G A G C A G A G A G A G G A G A T C A A A C T C G A A C A G A G A T C A C C C G G A C A G G A G C A G T A T C A A C A A C G A C C
3. Pr_spalt T G A G C A G A G A G A A G A G A T A A A A C T C G A A C A G A G A T C G C C C G G A C A G G A G C A G T A T C A A C A A C G A C C
1. Po_spalt G A T G A G C C G A G A A G G C G C C G A G C G A A T A A C A C C G A A G A G A G A A C C C G A G G A G G C A G A A G A A G C C G T
2. Pm_spalt G A T G A G C C G A G A A G G C G C C G A G C G A A T A A C A C C G A A G A G A G A A C C C G A G G A A G C A G A A G A A G C C G T
3. Pr_spalt G A T G A G C C G G G A A G G C G C C G A G C G A A T A A C A C C G A A G A G A G A A C C C G A G G A G G C G G A A G A G G T T G T
1. Po_spalt A G A G G A A G A G A G C G A A G A T T T T G A G G A T A C C A C A A G A C G A T A C C T T A A T T C A C C C C A A T C C G T G A A
2. Pm_spalt A G A G G A A G A G A G C G A A G A T T T T G A G G A T A C C A C A A G A C G A T A C C T T A A T T C A C C C C A A T C C G T G A A
3. Pr_spalt A G A G G A A G A G A G T G A A G A T T T T G A G G A A A C C A C A A G A C G A T A C C T T A A T T C A C C C C A A T C C G T G A A
1. Po_spalt T C C A T T G T C C C A G C C G C A A A C A T A T G A G G A T T G T A G T A T G G A C A G C A A G A T C A G T G G A C G T T T A G A
2. Pm_spalt T C C A T T G T C C C A G C C G C A A A C G T A T G A G G A T T G T A G C A T G G A C A G C A A G A T C A G T G G A C G T T T A G A
3. Pr_spalt T C C A T T G T C C C A G C C G C A A A C A T A T G A G G A T T G T A G T A T G G A C A G C A A G A T C A G T G G A C G T T T A G A
1. Po_spalt G G G C G A T G G A G A A A T G G A A G T G G A T G A G G A A A C A G A A G A A C A A C C T G A A A A T C T C T C A G G C A G A G G
2. Pm_spalt G G G C G A T G G A G A A A T G G A A G T G G A T G A G G A G A C A G A A G A A C A A C C T G A A A A T C T C T C A G G C A G A G G
3. Pr_spalt G G G C G A T G G A G A A A T G G A A G T G G A T G A A G A G A C A G A A G A A C A A C C T G A A A A T C T C T C A G G C A G A G G
1. Po_spalt T A C G G C C A A T C G T T T A C C T C A A C A G A T C G T G G C G T A C C C A G G A G C A T C A C C G G C C A G C A G T A G C G C
2. Pm_spalt T A C G G T C A A T C G T T T A C C T C A A C A G A T C G T G G C G T A C C C A G G A G C A T C A C C G G C C A G C A G T A G C G C
3. Pr_spalt T A C G G T C A A T C G T T T G C C T C A A C A G A T C G T G A C G T A T C C A G G A G C A T C A C C G G C C A G C A G T A G C G C
1. Po_spalt G A G T A G C G G T A G T C T A C A G A C A T C T T T C G C C G G A A T T C T G T T T C C A G G T C C C C C A G C T C A T C A T C A
2. Pm_spalt G A G T A G C G G T A G T C T A C A G A C A T C T T T T G C C G G A A T T C T G T T T C C A G G T C C C C C A G C T C A T C A T C A
3. Pr_spalt G A G T A G C G G T A G T C T A C A G A C A T C T T T C G C C G G A A T T C T G T T T C C A G G T C C C C C G G C T C A T C A C C A
1. Po_spalt A A T T C C T C A T C T T C C T G T C T C T A C A C A G G C A G G G C C A A C C A T A A C A T C C G G A A C T G A A A T G G T C G A
2. Pm_spalt A A T T C C T C A T C T T C C T G T C T C T A C A C A G G C A G G G C C G A C C A T A A C A T C C G G A A C T G A A A T G G T C G A
3. Pr_spalt A A T T C C T C A T C T T C C T G T T T C T A C A C A G G C A G G C C C A A C C A C A A C A T C C G G A A C T G A A A T G A T C G A
1. Po_spalt T C C A G C C A A G G A C C C A G C G A T T T A T T C G A C T T T A C T A C C A C G A C C G G G G T A G C A A T G A C A A T T C C T G
2. Pm_spalt T C C A G C C A A G G A C C C A G C G A T T T A T T C G A C T T T A C T A C C G C G A C C G G G G T A G C A A T G A C A A T T C C T G
3. Pr_spalt T C C A G C C A A G G A C C C A G C G A T T T A T T C G A C T T T A C T A C C G C G A C C A G G G T A G C A A T G A C A A T T C C T G
1. Po_spalt G G A G A G C T T G A T T G A A A T A A C G A A A A C T T C G G A G A C G T C C A A G C T G C A G C A A C T A G T C G A C A A T A T
2. Pm_spalt G G A G A G C T T G A T T G A A A T A A C G A A A A C T T C G G A A A C G T C C A A G C T G C A G C A A C T A G T C G A C A A T A T
3. Pr_spalt G G A G A G C T T G A T T G A A A T A A C G A A A A C T T C G G A G A C G T C C A A G C T G C A G C A A C T A G T C G A C A A T A T
1. Po_spalt T G A A
2. Pm_spalt T G A A C A C A A A C T G A G C G A T C C T A A T C A G T G C A T C G T T T G T C A C A G G G T G C T C T C A T G C A A G A G T G C
3. Pr_spalt T G A A
Identity
1. Po_spalt
2. Pm_spalt G T T G T T G
3. Pr_spalt

Figure S15: Sequence alignment of *sal* orthologs in *P. morrisi*, *P. rhea*, and *P. obtusospinosa* shows a 96% sequence similarity between their sequences. Sequence identity (dark green bar) is indicated for each nucleotide position above sequence alignment. 1078 nucleotides are conserved between the sequences of all three species, and there are only 46 unique sequence differences, which are marked by different colors (unique changes to G are in yellow, C in purple, T in light green, A in red).

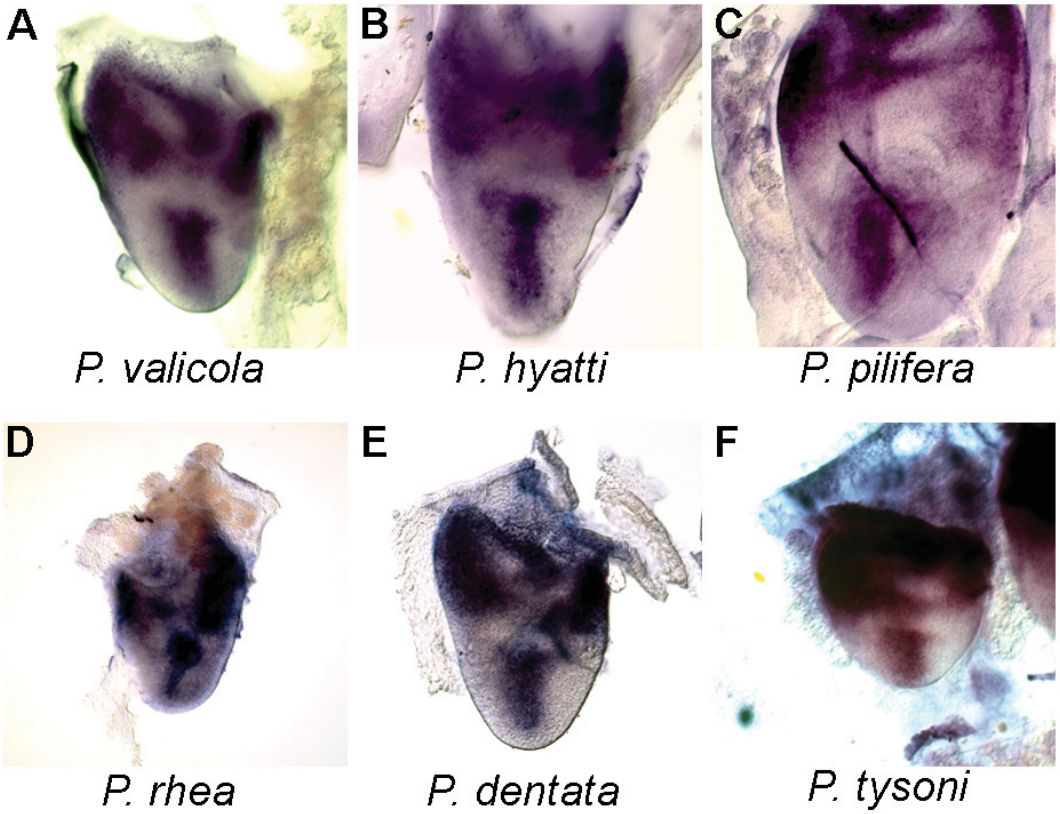

A
B
C
P. valicola
P. hyatti
P. pilifera
D
E
F
P. rhea
P. dentata
P. tysoni

Figure S16: *In situ* hybridization using a *P. morrisi sal* antisense probe detects a conserved expression pattern of *sal* in the wing discs of winged castes in six different *Pheidole* species. (**A** to **F**) *sal* mRNA expression (purple) in the wing discs of winged castes showing a conserved pattern of expression in the hinge and wing pouch (Fig. 1)(Abouheif and Wray, 2002) in both basal and derived *Pheidole* species (fig. S5).

Table S1: PCR primers used for genes in phylogenetic analysis of 11 *Pheidole* species.

Gene	Primer	Sequence	Reference
cox1	Forward: LCO1490	5'- GGTCAACAAATCATAAAGATATTGG - 3'	(Folmer et al., 1994)
cox1	Reverse: HCO2198	5'- TAAACTTCAGGGTGACCAAAAAATCA - 3'	(Folmer et al., 1994)
cytb	Forward: CB-J-10933	5'- TATGTACTACCATGAGGACAAATATC - 3'	(Simon et al., 1994)
cytb	Reverse: TS1-N-11683	5'- TATTTCTTTATTATGTTTTCAAAAC - 3'	(Simon et al., 1994)
lwr	Forward: LR143F	5'- GACAAAGTKCCACCRGARATGCT - 3'	(Ward and Downie, 2005)
lwr	Reverse: LR639ER	5'- YTTACCGRTTCCATCCCRAACA - 3'	(Ward and Downie, 2005)

Table S2: Genbank accession numbers for genes sequenced for phylogenetic analysis of 11 *Pheidole* species.

Species	GenBank Accession # for mtDNA COxI	GenBank Accession # for mtDNA cytb	GenBank Accession # for nDNA LW-Rh
Pheidole megacephala	JN205088	JN205099	JN205077
Pheidole moerens	JN205089	JN205100	JN205078
Pheidole spadonia	JN205090	JN205101	JN205079
Pheidole tysoni	JN205091	JN205102	JN205080
Pheidole rhea	JN205092	JN205103	JN205081
Pheidole obtusospinosa	JN205093	JN205104	JN205082
Pheidole hyatti	JN205094	JN205105	JN205083
Pheidole vallicola	JN205095	JN205106	JN205084
Pheidole pilifera	JN205096	JN205107	JN205085
Pheidole morrisi	JN205097	JN205108	JN205086
Pheidole dentata	JN205098	JN205109	JN205087

Link between Chapter 2 & 3

Since Darwin the occurrence of ancestral reversions has been much discussed. As in Chapter 2, various studies report the sporadic occurrence of ancestral reversions in natural populations, artificially in the lab (with developmental genetic manipulations) or observed in the re-evolution of ancestral phenotypes. In Chapter 3, we have reviewed some of this literature with our findings in Chapter 2 to understand further the occurrence of reversions that result specifically following the process of poultry breeding and artificial selection of poultry in domestication. Furthermore, we describe how a good understanding of ancestral developmental potentials of poultry can reveal what types of phenotypes can be induced environmentally or genetically and most importantly what potential maladaptive side-effects can result as a byproduct. Finally, Chapter 3 ends with a protocol, which suggests new strategies for poultry breeding in regards to activating ancestral developmental potentials while simultaneously selecting against the secondary production of maladaptive phenotypes. In general, Chapter 3 can be viewed as a translation of Chapter 2 to the field of animal breeding. The hope is that this manuscript may provide new artificial selection strategies, which can improve animal breeding methods for selecting for favorable traits and against maladaptive traits such as disease.

CHAPTER 3: Ancestral developmental potential: a new tool for animal breeding?

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Proceeding of the 62nd Annual National Breeders Roundtable (*in Press*)

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"... I wish to guard the reader against supposing that reversion is due to some rare or accidental combination of circumstances. When a character, lost during hundreds of generations, suddenly reappears, no doubt some combination must occur; but reversions may constantly be observed, at least to the immediately preceding generations, in the offspring of most unions ... Reversion is most likely the rule, as Mr Sedgwick has shown, with certain diseases..." (Darwin, 1868)

3.1 Introduction

This insightful quote from Darwin's classic *The Variation of Animals and Plants under Domestication* illustrates that although a trait is lost during the evolution of a lineage, the potential to produce that trait is retained, such that it may reappear in individuals in modern populations (Darwin, 1868). Whales and dolphins, for example, are closely related to hoofed animals but lost their hind limbs ~34-41 million years ago when they re-entered water (Thewissen et al., 2006). Today, several anomalous individuals of this group have been discovered with partial hind limbs indicating that hind limbs have "reappeared" in these individuals (Hall, 2003; Tomic and Meyer-Rochow, 2011). The sudden reappearance of such ancestral traits in these anomalous individuals is most often called "atavism," which is derived from the word "atavus" or ancestor (Darwin, 1868). Atavistic traits can suddenly reappear in individuals even though they had been lost for hundreds, thousands, and even millions of years (Collin and Miglietta, 2008; Wiens, 2011).

Unfortunately, modern evolutionary biologists appear to have undervalued the true significance of atavisms for evolutionary theory. The consensus view is that atavisms are rare mistakes in the developmental system that only provides evidence of ancestry (Levinton, 1986). Apparently, as stated above in his quote, Darwin failed in "*guarding the reader against supposing that reversion is due to some rare or accidental*

combination of circumstances" and as a consequence, atavisms are currently viewed as rare "freaks of nature" that contribute little to the raw material that natural selection can act upon (Blumberg, 2009). We argue in the following paragraphs that Darwin was right about his observation that reversions/atavisms are a common occurrence in nature and that this type of variation is likely to be "the rule" and not the exception. This is especially true for poultry, where ancestral traits frequently reappear in individuals in modern populations and can also be induced experimentally (Darwin, 1868; Hampe, 1959; Harris et al., 2006; Muller, 1989). Furthermore, we summarize evidence from our recent study (Rajakumar et al., 2012) showing that reversions/atavisms reflect "ancestral developmental potentials" that, when induced, provide raw material that natural selection can act upon to facilitate adaptive evolution. Giving reversions/atavisms their proper place in evolutionary theory (Stiassny, 2003; West-Eberhard, 2003), brings forth entirely new perspectives on how "ancestral developmental potentials" can be used to improve animal breeding and understand complex disease, especially with respect to poultry. We will finish by briefly outlining these perspectives.

3.2 Discussion

3.2.1 Reversions/Atavisms occur frequently in poultry

The field of experimental embryology provided some of the first evidence that atavisms in poultry can be experimentally induced. First, Hampe (Hampe, 1959) and Müller (Muller, 1989) inserted a physical barrier within a specific region of the chicken hindlimb during its development. The outcome of this experiment was startling – the adult bone and musculature now resembled that of reptiles. Because birds descended

from reptiles (Hugall et al., 2007; Lee, 2001), this classic experiment demonstrates that it is possible to revert the hindlimb in chickens to its reptilian state. Another celebrated example of atavism in poultry is the induction of teeth in *talpid2* mutant chickens. Although birds lost teeth approximately 70-80 million years ago, Harris et al. (2006) discovered that teeth had reappeared (or at least had initiated development) in *talpid2* mutant chickens (Harris et al., 2006). In both these examples, the atavistic traits had been lost for millions of years, showing that ancestral developmental potentials can be retained for vast periods of time. Darwin, however, shows how these potentials can be frequently induced by natural means in populations after the trait has been lost for generations (Darwin, 1868). Darwin states: "*The best yet simplest characters lying dormant are, perhaps, those previously given, in which chickens and young pigeons, raised from a cross between differentially colored birds, are at first of one color, but in a year or two acquire feathers of the color of the other parent; for in this case the tendency to a change in plumage is clearly latent in the young bird.*" These examples of atavism illustrate that ancestral developmental potentials are retained for variable lengths of time and in many traits. Indeed, we have just scratched the surface in terms of surveying the ancestral potentials that exist in poultry.

3.2.2 "Supersoldier ants" show that reversions/atavisms reflect ancestral developmental potential that can facilitate adaptive evolution

Most new perspectives in science come from unexpected sources and in roundabout ways. In our case, we provide a new perspective on the biological significance of atavistic traits by studying the evolutionary developmental biology of ants. One of the most exciting discoveries to emerge from the field of evolutionary

developmental biology (also known as EvoDevo) is the deep conservation, over hundreds of millions of years of evolution, of the genes that regulate development of an organism (Carroll, 2005; Carroll et al., 2005). For example, in all animals, including, ants, chickens, and humans, the developmental regulatory gene *hedgehog* is essential in the formation of limbs (Ingham and McMahon, 2001; Riddle et al., 1993), and the gene *Pax6* functions to specify where an eye will develop (Gehring and Ikeo, 1999). A team led by Walter Gehring conducted an experiment that beautifully illustrates the functional conservation of these developmental regulatory genes: they genetically inserted and expressed the mouse *Pax 6* gene in the developing wing or leg of a fruit fly, and demonstrated that adult compound eyes (resembling those of a fruit fly) appear on wings or legs in the adult fly (Halder et al., 1995). How is it that these developmental regulatory genes, which are so conserved in their function and expression, can account for the amazing morphological diversity in the animal kingdom? The Abouheif Lab's approach to answering this fundamental question has been to study how these genes interact with their environment in the context of the complex societies of ants.

In the most advanced ant societies, the non-reproductive worker caste is very large, and can be composed of thousands and even millions of individuals (Hölldobler and Wilson, 2009; Hölldobler and Wilson, 1990; Wilson, 2003). The individual workers in these highly advanced societies are morphologically differentiated into several subcastes such that they can efficiently divide labor in the colony (Hölldobler and Wilson, 1990). In the genus *Pheidole*, which is one of the most evolutionarily diverse genera of ants with over 1000 species, the worker caste is divided into "minor workers" and "soldiers" (Moreau, 2008; Wilson, 2003). Minor workers forage and nurse the young,

whereas soldiers defend the nest and help process food. In 8 of the ~1000 species in this genus a third "supersoldier" subcaste has evolved (Moreau, 2008). This supersoldier subcaste has a massive head which functions to block the nest entrance when attacked by army ants (Huang, 2010).

In our recent study (Rajakumar et al., 2012), we found that the ancestral species of all *Pheidole* had a supersoldier subcaste that was subsequently lost in most of the ~1000 species for ~35 to 60 million years. Supersoldiers then re-evolved multiple times independently in the genus. By applying a key growth hormone, called Juvenile Hormone, to larvae at a very specific time in development, we discovered that supersoldiers can be experimentally induced in species that had lost the supersoldier subcaste for over 35 to 60 million years (see Fig. 3 & fig. S9 in Rajakumar et al. 2012). This means that although the supersoldier phenotype had been lost for millions of years, all *Pheidole* species retain an ancestral developmental potential to produce supersoldiers. We demonstrated that experimentally induced supersoldiers are produced through the same developmental pathway as naturally evolved supersoldiers, and as adults, both experimentally induced and naturally evolved supersoldiers are significantly larger than regular soldiers. The only distinct morphological difference we observed between experimentally induced and naturally evolved supersoldiers was the appearance of tiny wing vestiges on the thorax of those that were experimentally induced (See Fig. 3 in Rajakumar et al 2012). These wing vestiges are thought to be detrimental; they do not allow individuals to efficiently maneuver underground, and therefore, are a negative side consequence of experimentally inducing supersoldiers. These tiny wing vestiges are important for understanding how ancestral developmental potential relate to complex

disease, as we will soon describe below.

We also discovered in wild colonies of one *Pheidole* species, which does not have a supersoldier subcaste, several anomalous individuals that looked very similar to supersoldiers but also had tiny wing vestiges on the thorax (see Fig. 2 Rajakumar et al. 2012). Furthermore, anomalous supersoldier-like individuals were also found in wild colonies by other researchers in different species (Goetsch, 1937; Wheeler, 1902), meaning that this ancestral developmental potential is being induced in nature all the time and is therefore a source of raw material for natural selection to act upon. In addition, these researchers found that it was changes in nutrition that likely caused the induction of supersoldier-like anomalies in wild colonies (Goetsch, 1937; Gregg, 1942; Wheeler, 1902). As mentioned above, both experimentally induced and anomalous supersoldier-like individuals exhibit a potentially adaptive trait (large size) but also a maladaptive trait (tiny wing vestiges). One possible explanation is that not enough time has passed for the re-evolution of mechanisms that can suppress these wing vestiges during the development of induced or anomalous supersoldiers. Therefore, in order for such individuals to evolve as a functional subcaste of the colony, there must be selection on large size and on elimination of wing vestiges.

We showed that an evolutionary process known as genetic accommodation was responsible for the re-evolution of supersoldiers in *Pheidole*. This process occurs when an environmentally induced phenotype becomes fixed in populations through natural selection (West-Eberhard, 2003). Specifically, genetic accommodation of phenotypes occurs by selection on genes responsible for increasing the frequency and adjusting the form of a trait. In the case of the re-evolution of *Pheidole* supersoldiers: first, to increase

frequency the supersoldier-like anomalies in the colony, selection occurred on genes that increase the environmental sensitivity for producing supersoldiers, such that supersoldiers become regularly induced by recurrent variation in nutrition or other environmental cues and their frequency increases to approximately 4% of the colony; and second, to adjust the form of supersoldier-like anomalies, selection occurred on genes that eliminate the production of wing vestiges. To summarize, Rajakumar et al. 2012 shows that the induction of ancestral developmental potentials, like that which produces supersoldiers, occurs frequently in natural populations and that they are neither hopeless monsters nor freaks of nature. On the contrary, they are raw materials for natural selection to act upon. This conclusion has important consequences for animal breeding and understanding complex disease as we outline in the following sections.

3.2.3 The association between ancestral developmental potential and complex disease in poultry

Inducing the ancestral developmental potential for supersoldiers not only illuminates the fact that ancestral potentials offer a rich source of raw material for natural selection to act upon, but has also illuminated another important fact – that inducing ancestral potentials in natural populations is often accompanied with the induction of detrimental or negative side consequences (Rajakumar et al., 2012; West-Eberhard, 2003). As we discussed above, we were never able to induce supersoldiers in species that normally lack them without also inducing the appearance of the tiny, but detrimental, wing vestiges (Rajakumar et al., 2012). This observation appears to be generally applicable to other species, including poultry. In the quote at the very beginning of this article, Darwin's (1868) remark that "*Reversion is most likely the rule, as Mr Sedgwick*

has shown, with certain diseases..." indicates that he was well aware of the association between the induction of reversions/atavisms and the appearance of disease. Darwin follows this remark with an example in poultry describing the association between the induction of ancestral potentials and ovarian cancer (Darwin, 1868): "*I will here add a somewhat different case, as it connects in a striking manner latent characters of two classes. Mr. Hewitt possessed an excellent Seabright gold-laced hen bantam, which, as she became old, grew diseased in her ovaria, and assumed male characters. In this breed the males resemble the females in all respects, except in their combs, wattles, spurs, and instincts; hence it would have been expected that the diseased hen would have assumed only those masculine characters which are proper to the breed, but she acquired, in addition well-arched tail sickle-feathers quite a foot in length, saddle-feathers on the loins, and hackles on the neck,— ornaments, which, as Mr. Hewitt remarks "would be held abominable in this breed." The Seabright bantam is known to have originated about the year 1800 from a cross between a common bantam and a Polish fowl, recrossed en-tailed bantam, and carefully selected; hence there can be hardly a doubt that the sickle-feathers and hackles which appeared in the old hen were derived from the Polish fowl or common bantam; and we thus see that not only certain masculine characters proper to the Seabright bantam, but other masculine characters derived from the first progenitors of the breed, removed by a period of above sixty years, were lying latent in this hen-bird ready to be evolved as soon as her ovaria became diseased."* This intricate association between the induction of ancestral developmental potential and negative side consequences leading to disease is likely to be the rule and not the exception in poultry.

3.2.4 How to use ancestral developmental potentials to select for desirable features in poultry

In the previous section, we briefly described the negative role that ancestral developmental potentials can play in animal breeding through its association with negative side consequences leading to complex disease. In this section, we show how ancestral potentials can also play a positive role in animal breeding. After the publication of our article on the role of ancestral developmental potential in the origin and evolution of supersoldiers (Rajakumar et al., 2012), we received a storm of media attention (all you have to do is type "supersoldier ants" into Google for this to become immediately obvious). One of the media posts by Iain Thompson in *The Register* entitled "Boffins hack evolution, create SUPERSOLDIER ANTS: Genetic prestidigitation could engineer new species" makes the following statements about the implications of our findings: "*For example, the aurochs – the massive ancestor to modern cattle that was hunted to extinction by the 1600s – may be recreatable by examining a cow's genome and finding a way to activate the processes that would cause the much larger and more aggressive aurochs to develop. In the plant world too, crops could be subjected to environmental and chemical stressing to see if the dormant genotypes could be activated. This could usher in new crops that can better deal with current conditions – not to mention changing conditions as climate change wreaks its havoc.*" Could this really be possible? We argue that it is, so long as the focus is on resurrecting specific desirable traits and not whole species. This means that researchers and animal breeders would need to be very familiar with the ancestral traits in the group of interest. The challenge, as we discussed above, will be to find a way to eliminate any negative side consequences that may also be

induced. In the following sections we briefly outline two ways that ancestral developmental potentials could be induced in poultry for further artificial selection:

3.2.5 Crossing as a tool to release ancestral developmental potential for artificial selection

Once again we return to Darwin's ingenious observations in 1868 in *The Variation of Animals and Plants under Domestication*. He recognized that crossing different lineages or species generally induced the appearance of atavistic traits in animals and plants (Darwin, 1868): "*...When two races or species are crossed there is strongest tendency to the reappearance in the offspring of long lost characters, possessed by neither parent nor immediate progenitor."* He then goes on to give a remarkable example of his observation in poultry: "*I raised several chickens from a Polish hen by a Spanish cock, –breeds which do not incubate, –and none of the young hens at first recovered the instinct, and this appeared to afford a well-marked exception to the foregoing rule; but one of these hens, the only one which was preserved, in the third year sat well on her eggs and reared a brood of chickens. So that here we have the appearance with advancing age of a primitive instinct, in the same manner as we have seen that the red plumage of the Gallus bankiva is sometimes reacquired by crossed and purely-bred fowls of various kinds as they grow old.*" This example beautifully shows that crossing is not only a useful tool for inducing the reappearance of lost physical traits, like plumage, but can also be used as a tool for inducing the reappearance of lost behavioral traits. Indeed, ancestral developmental potential is already being considered key in the generation of behavioral variation and the re-evolution of complex behavioral traits (Foster, 2013).

3.2.6 Environmental stress as a tool of releasing ancestral developmental potential for artificial selection

The induction of ancestral developmental potential in supersoldier ants shows that environmental factors, such as nutrition, hormones, temperature, and even particular chemicals, can potentially be used as tools to induce ancestral developmental potentials in animals and plants for artificial selection. William Morton Wheeler and Goetsch showed that increased nutrition in colonies could give rise to supersoldier-like anomalies in species that normally lack supersoldiers (Goetsch, 1937; Wheeler, 1902). Because nutrition is so closely linked to levels of particular hormones, it becomes clear why high levels of juvenile hormone could induce the development of supersoldiers in species that lack them. Therefore, it is entirely possible that in poultry, ancestral developmental potential could be induced by alternating sudden increases in nutrition (or hormones) with regular amounts of nutrition in the feed. Temperature and chemical shocks could also be used (Waddington, 1953, 1956). For instance, Waddington performed a classic experiment in fruit flies, where he applied chemical (ether) to developing fruit fly embryos (Waddington, 1956). Flies by definition only have one pair of wings, whereas all other insects have two pairs. This means that flies lost the second pair of wings during their evolution. The environmental shocks that Waddington applied induced the reappearance of hindwings, producing adult flies with four wings (Waddington, 1956). Therefore, a range of environmental shocks can be used during poultry development to induce ancestral developmental potentials for artificial selection.

3.3 Protocol: Fixation of induced ancestral developmental potentials and suppression of negative side consequences through artificial selection

Although supersoldiers with no wing vestiges took millions of years to evolve, ancestral developmental potentials in poultry can be induced and desirable traits can be fixed through artificial selection in just a few generations. Classic experiments by Suzuki and Nijhout (2006) and Waddington (1956) in insects have demonstrated that ancestral traits induced by temperature or chemical shock, such as pigmentation in caterpillars or the presence of hindwings in fruit flies, can be fixed through artificial selection in as little as 7 generations (Suzuki and Nijhout, 2006; Waddington, 1956). Darwin also acknowledged the speed with which induced ancestral potentials can be fixed by artifical selection: *"By the aid of a little selection, carried on during a few generations, most of our cultivated plants could probably be brought back, without any great change in their conditions of life, to a wild or nearly wild condition"* (Darwin, 1868). Therefore, to select for desirable ancestral traits in poultry, breeders should induce ancestral potentials by crossing or by administering environmental shocks each generation, followed by artificial selection. Furthermore, several studies suggest that there are two ways to repress maladaptive traits of induced ancestral potentials: first, studies in chickens suggest that diet regulation, such as calorie-restriction and content, can be manipulated to repress ovarian cancer in chickens (Ansenberger et al., 2010; Carver et al., 2011), and second, studies in insects suggest that artificial selection can be used to simultaneously select positively for desirable ancestral traits while selecting against the appearance of any maladaptive traits (Suzuki and Nijhout, 2008). In summary, to use ancestral developmental potentials to enhance genetic lines, poultry breeders should: (1) induce ancestral developmental potentials by crossing different breeds or species of poultry and/or by exposing individuals to environmental shock each generation; (2) artificially

select on the induced trait to increase its frequency; and finally (3) repress the development of maladaptive traits by manipulating diet or selecting against negative maladaptive traits while selecting on positive ancestral traits.

3.4 Conclusion

We are grateful for the opportunity to have exposed our basic research on the evolution and development of complex societies in ants. We hope to have convinced you or at least provoked discussion on the possibility that ancestral developmental potential is a powerful tool for improving breeding and understanding complex disease in poultry. We maintain that basic research is the fuel of innovation, and that insights gained from the highly organized societies of ants may actually serve as good models for understanding the mechanisms underlying the positive and negative roles of inducing ancestral potentials for animal breeding.

3.5 Questions & Answers

Question: Dr. Jiuzhou Song

After induction by the hormone did you check other genes' behavior?

Answer:

We focused on the expresson of the *spalt* gene as a "read out" of the gene network responsible for wing development because it's position is relatively far downstream in the network and has a unique pattern of expression in the vestigial wing imaginal discs (precursor cells that will develop into the adult wing) of soldier larvae relative to those

queens and minor workers. More importantly, *spalt* expression is associated with apoptosis in the vestigial wing imaginal discs of soldiers, which means that *spalt* may play an important role in eliminating wing vestiges before metamorphosis is complete. We therefore considered *spalt* to be a key gene, which not only serves as a "read out" for the whole network, but also serves to characterize the similarities and differences between castes, including the naturally evolved and induced supersoldiers.

Question: Dr. Jiuzhou Song

Is it a single gene or one of gene family?

Answer:

spalt itself is a single gene, but it is part of the gene network responsible for wing development. Once again, *spalt* expression can be used as a proxy to indicate the overall expression of this network. Future work will have to formally characterize the expression of other genes in this network before and after induction by the hormone.

Question: Dr. Sue Lamont

Following on your thought of the positive aspects of "release of genetic variation" by uncovering ancestral potential, what would you speculate as good nutritional manipulations? Methyl donors, to enhance epigenetic changes?

Answer:

Methyl donors would be a very interesting group of molecules to manipulate. There is a possibility that supplementing methyl donors in food given to the animals may lead to

effects on epigenetic pathways. Since epigenetic pathways (ex: DNA methylation) use folate as a substrate and, along with hormonal pathways, work hand in hand to translate environmental status (ex: nutrition) into effects on development. Therefore, perturbing epigenetic pathways may be as efficient or even more so than perturbing nutrition. On the other hand, perturbing environmental factors like nutrition can induce ancestral developmental potentials, like in the case of ants, where protein has been proposed to be a potential candidate that might specifically be involved in the induction of supersoldiers. That being said, many different nutrients might be involved, and to determine which nutrients are worth testing, we recommend close examination of the diet and natural history of the ancestors of the poultry line of interest.

Question: Dr. Frank Siewerdt

Where should we look for clues to explore specific ancestral developmental potential? For example, how could we find out if chickens may lay 3 eggs a day, or sows developing 40 functional teats will one day become a possibility?

Answer:

The first question one should ask is “what are their ancestors like?” If their (remote or recent) ancestors exhibited a particular trait, then there is a good chance that the potential to produce that trait is laying dormant in the genome of the contemporary domesticated animal that you are working with. It is also important to note that when ancestral potentials are induced and variation is released, new and different combinations of ancestral traits can arise and be artificially selected for. This process, which is called “developmental recombination” (West-Eberhard, 2003), makes it possible to create new

combinations from ancient ancestral traits. So in theory, chickens that lay 3 eggs a day, or sows developing 40 functional teats may one day become a possibility using ancestral developmental potentials, although it may not be easy. Starting to explore the deep and recent evolutionary history, ecology, development, physiology, and life history of domesticated animals and plants is of primary importance for using ancestral developmental potential to improve animal breeding.

Question: Dr. Gerald Herbert

Your talk may infer that "long-term" epigenetic effects and "long-term" multi generational imprinting may play a major role in evolution. This contrasts with the concept of mutations in DNA increasing and decreasing in frequency, (changes in gene frequency) as the mechanism underlying evolution. Evolution may change partially due to activation and inactivation of genes by environmental influences.

Answer:

Your last sentence elegantly summarizes the general implications of our work. However, it is currently thought that although it is indeed the perturbation of hormonal pathways or epigenetic mechanisms may facilitate the initial emergence of an ancestral phenotype following an environmental induction, it is the fixation of standing genetic variation or *de novo* genetic mutation that preserves the induced phenotype across generations. However, it is entirely possible that trans-generational epigenetic imprinting may be a mechanism, which permits the environment to have a persistent effect across generations, which is only subsequently followed by the "genetic fixation" of the initial imprint. Clearly, much research remains to be done in this new and exciting area of research.

Link between chapters 2, 3 & 4

Chapter 2 focused on the hormonal mechanisms involved in the development of worker subcastes of *Pheidole* and how these mechanisms can evolve to generate novel worker subcastes. Chapter 3 is an attempt to synthesize the findings of Chapter 2 with literature of the occurrence of ancestral reversion in crossing and artificial selection, in order to translate the findings of Chapter 2 into an applied protocol for animal (or plant) breeding. Furthermore, questions asked in Chapter 3 (section 3.5) relate to how epigenetic mechanisms play a role in Chapters 2 & 3. While Chapter 2 focused on how novel subcastes develop and evolve, Chapter 4 focuses on how variation within a worker subcaste is developmentally regulated. The JH pathway regulates the production of adaptive discrete alternative worker subcastes but the pathways that regulate the generation of adaptive morphological variation within a worker subcaste is unknown. Chapter 4 focuses on the role of developmental epigenetics (DNA methylation) in generating morphological variation within subcastes of the hyperdiverse ant genus *Camponotus*. Specifically, Chapter 4 investigates how DNA methylation functions during worker development on a global genomic-level and on a local gene-specific level. We have demonstrated that DNA methylation is a key developmental mechanism (not addressed in chapter 2), which translates environmental variation into adaptive intra-caste morphological variation through the quantitative regulation of the epidermal growth factor receptor (*Egfr*). Cumulatively, Chapter 2 and 4 attempt to bridge our understandings of the environment, developmental hormonal signaling, epigenetics and genes in contributing to phenotypic diversity of worker morphology in ants.

Chapter 4: Epigenetic variation in the *Egfr* gene generates quantitative variation in a complex trait in ants

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4.1 Abstract

Complex quantitative traits, like size and behavior, are a pervasive feature of natural populations. Understanding how variation in quantitative traits is generated is important for mapping the genetic basis of disease, improving plant and animal breeding, and predicting evolutionary changes (Falconer and Mackay, 1996; Lynch and Walsh, 1998). Quantitative trait variation is the product of both genetic and environmental factors (Falconer and Mackay, 1996; Flint and Mackay, 2009; Idaghdour and Awadalla, 2012; Lynch and Walsh, 1998; Vieira et al., 2000), yet the mechanisms through which their interaction generates this variation is poorly understood. Epigenetic processes, such as DNA methylation, can mediate gene-by-environment interactions during development (Jaenisch and Bird, 2003; Schmitz et al., 2008), and can generate discrete phenotypic variation (Kucharski et al., 2008; Weaver et al., 2004). We therefore investigated how these processes generate quantitative trait variation by determining the role of DNA methylation in generating continuous size variation of workers in an ant colony, a key trait associated with division of labor (Hölldobler and Wilson, 1990; Oster and Wilson, 1978; Wilson, 1953). Here we show that in the carpenter ant *Camponotus floridanus*, global (genome-wide) DNA methylation indirectly regulates methylation of the conserved cell-signaling gene *Epidermal Growth Factor Receptor* (*Egfr*) to generate continuous size variation of workers. Pharmacological manipulation that increases or decreases global DNA methylation dramatically affects mean size of workers as well as *Egfr* expression and methylation. Furthermore, the pharmacological inhibition of EGFR also dramatically affects the mean size of workers. DNA methylation directly represses *Egfr* expression *in vitro* and the level of developmental methylation of *Egfr* is inversely

correlated to the final size of workers. Our results reveal a mechanism, where DNA methylation generates quantitative variation in a complex trait through the graded transcription of a specific gene, and in this case, continuous size variation is generated through epigenetic variation in *Egfr*. This mechanism may underlie associations found between epigenetic variation and disease susceptibility in humans (Toperoff et al., 2012) as well as quantitative traits in plants (Johannes et al., 2009), and more generally, may enhance the phenotypic possibilities for a given locus for generating quantitative trait variation in natural populations.

4.2 Main Text

In ant societies, variation in the size of individuals in the worker caste is considered to be a key trait associated with the division of labor, where differently sized workers can specialize in tasks such as excavation, brood transport, and foraging (Busher et al., 1985; Hölldobler and Wilson, 2009; Hölldobler and Wilson, 1990; Oster and Wilson, 1978; Wilson, 1953, 1980a). The final size of a worker in an ant colony is established during development and is polyphenic, which means that the same genome can produce a spectrum of final adult sizes in response to environmental factors (Abouheif and Wray, 2002; Wilson, 1953). Studies in the wild and in the lab spanning more than 100 years have identified nutrition levels and social interactions as critical environmental factors that influence the final size of workers during development (Brian, 1956; Goetsch, 1937; Gregg, 1942; Rajakumar et al., 2012; Wheeler, 1986; Wheeler and Nijhout, 1984; Wheeler, 1902). Here, we chose to study the species *Camponotus floridanus* because worker size variation is continuous and in the genus *Camponotu*s worker size is affected by nutrition in the form of protein, vitamins, and minerals (Smith, 1942, 1944). Furthermore, relatedness between individuals in a colony is high (on average 75%)(Gadau et al., 1996) and extensive sequencing efforts have confirmed that within a colony there is no allelic bias between differently sized workers (Bonasio et al., 2012). This indicates that genetic variation alone cannot fully explain the diversity of worker size (Bonasio et al., 2012). Finally, the genome of *C. floridanus* is sequenced and has a DNA methylation system (Bonasio et al., 2012; Bonasio et al., 2010), which functions to repress gene expression through the covalent modification of cytosine residues via methylation (Razin and Riggs, 1980; Razin and Szyf, 1984). Therefore, *C.*

floridanus is a good model for understanding how gene-by-environment interactions generate quantitative trait variation in natural populations.

We first tested if differential methylation at the genomic and gene levels during development may be involved in generating the continuous distribution of worker size in *C. floridanus*. We characterized the size distribution of adult workers in *C. floridanus* as continuous with two peaks in frequency that represent minor and major workers (Wilson, 1953) (Fig. 1A). Although these two worker subcastes form a continuous size distribution, they are distinguished by head allometry – the size of their heads relative to their bodies (Fig. S1). To identify the developmental stage where this variation in worker size is established, we first characterized this trait in a number of larval instars. We found that there are a total of 4 larval instars, where most of the growth differences in size between workers are established during the 4th (final) instar (Fig. 1B). The early 4th instar larvae have not yet experienced this rapid growth, whereas late 4th instar larvae undergo a burst of growth producing a continuous range of final larval sizes, the extremes of which will develop into minor and major workers, (Fig. 1A). Before examining the entire size continuum, we first used the extreme ends of the distribution to screen for dynamic changes in global DNA methylation that are associated with the early and late phases of larval growth during the 4th instar. We found that early 4th instar larvae have significantly lower levels of global methylation relative to late 4th instar larvae (Fig. 1C) and 4th instar minor worker larvae were significantly hypermethylated compared to 4th instar major worker larvae (Fig. 1C). We next asked whether any of the key enzymatic regulators of DNA methylation (*Dnmt1*, *Dnmt3*, *Tet2*, *Mbd* and *Mecp2*) and histone modification (*Hdac1*, *Hdac3*, *Hat* and *Lsd1*) are associated with this pattern of global

DNA methylation. We found that gene expression levels of *Dnmt1*, *Dnmt3*, *Tet2*, *Mbd* and *Mecp2* (Fig. 1D and E and Fig. S2A-C) as well as *Hdac1* and *Hat* (Fig. S2D-G) are consistent with global methylation levels during the 4th instar, indicating that these specific epigenetic regulators may be involved in the dynamic changes of global methylation during the 4th instar. These results are consistent with a link between global DNA methylation and the regulation of the continuous worker size variation during the fourth instar in *C. floridanus*.

We therefore manipulated levels of global methylation during the early 4th instar to determine whether global DNA methylation plays a functional role in regulating the continuous size variation of workers. We used a hypermethylating agent, a methyl donor S-Adenosyl Methionine (SAM), (van der Westhuyzen, 1985) and a hypomethylating agent, an inhibitor of DNA methyltransferases 5-AZA-dCytidine, (5-AZA-dC) (Wilson et al., 1983). Relative to controls, we discovered that SAM shifts the entire continuous size distribution by significantly decreasing the mean size of adult workers (Fig. 2A,C and D, and fig. S3A-C). The effect of SAM treatment was so dramatic that it produced workers that were smaller than any worker observed in wild type colonies (Fig. 1A and Fig. 2A). In contrast, we found that 5-AZA- dC shifts the entire continuous size distribution by significantly increasing the mean size of adult workers relative to controls (Fig. 2B, E and F, and Fig. S3D-F). While SAM has many biological functions (van der Westhuyzen, 1985), its role mediating global DNA methylation and sizing is reinforced by the contrasting effect on phenotype caused by 5-aza-dC, a well-known hypomethylating agent (Wilson et al., 1983). These results demonstrate that global DNA methylation

functions during larval development to regulate continuous worker size distribution in *C. floridanus*.

In honeybees, EGFR signaling has been shown to respond to dietary cues (royal jelly) to regulate discrete differences in size and developmental timing between queen and worker bees (Kamakura, 2011). Furthermore, in fruit flies, mapping loci that underlie quantitative traits (Quantitative Trait Loci or QTLs) has identified *Egfr* as a genetic locus with a major effect on variation in quantitative sizing (Turner et al., 2011). Indeed, among several genes of pathways known to play critical roles in regulating discrete size differences in social insects35,(Mutti et al., 2011b; Patel et al., 2007; Wolschin et al., 2011) (Fig. S4), we found that *Egfr* shows the most dramatic differences in expression during the 4th instar (Fig. 3A and S4). To determine the role of EGFR in generating the continuous variation in worker size in *C. floridanus*, we inhibited EGFR signaling during the 4th instar using a pharmacological inhibitor (PD 153035) highly specific to EGFR (Bos et al., 1997; Fry et al., 1994). We discovered that, relative to controls, inhibition of EGFR shifts the entire continuous size distribution by significantly increasing the mean size of adult workers (Fig. 3C, D and E; Fig. S3G-I). This dramatic effect of EGFR indicates that it can regulate both discrete and continuous variation in size and may be positioned as a key regulator of other pathways involved in generating the continuous worker size distribution in *C. floridanus*.

Altogether, our results suggest that global DNA methylation may regulate continuous size distribution in workers through EFGR. Indeed, increasing global methylation by SAM treatment during the 4th larval instar results in decreased methylation and increased expression of *Egfr*, whereas decreasing global methylation by

5-AZA-dC treatment results in increased methylation and decreased expression of *Egfr* (Fig. S6). The inverse relationship between levels of global DNA methylation and *Egfr* expression and methylation indicates it is mediated by other intermediate genes or processes downstream to global changes in DNA methylation state (Broday et al., 1999; Lubin et al., 2008). Finally, DNA methylation and EGFR signaling may regulate worker size differences by affecting developmental timing because the duration of development was shortened following SAM treatment, whereas it was extended after EGFR inhibition (Fig. S7). Therefore global DNA methylation indirectly regulates the expression and methylation levels of *Egfr* to regulate the continuous size distribution of workers.

We then discovered that DNA methylation through the specific methylation of *Egfr* not only shifts, but generates the continuous worker size distribution. We found that quantitative differences in DNA methylation of *Egfr* are correlated to the final size of individual workers. In the *C. floridanus* genome, CpG dinucleotide methylation is primarily concentrated at the beginning of the protein-coding region of all genes (Bonasio et al., 2012). We therefore screened the first 225bp of *Egfr* and identified all CpG dinucleotides. We then determined the % methylation for each of these CpG dinucleotides in 50 individual late 4th instar larvae that represent the entire size continuum of workers. Of all the CpG dinucleotides screened, we discovered 4 sites for which the level of DNA methylation is significantly correlated to final size of late 4th instar larvae (Fig. 3B, 3F and Fig. S5). Two of these sites remained statistically significant after Bonferroni correction for multiple comparisons (Fig. S5 and Table S2). To determine whether these CpG dinucleotides, when methylated, affect the transcription of *Egfr* we cloned this sequence into a CpG-free luciferase construct. We show that DNA

methylation can indeed repress *Egfr*'s ability to drive luciferase expression *in vitro* (Fig. 3G). Collectively, our results provide clear evidence that DNA methylation regulates *Egfr* during larval development to generate the continuous worker size distribution.

Generation of the continuous worker size distribution in *C. floridanus* by the epigenetic control of *Egfr* can be hypothesized as follows (Fig. 4): variation in environmental factors leads to the hyper-methylation (increase) or hypo-methylation (decrease) of global DNA methylation levels of developing 4th instar *C. floridanus* larvae. Although our study did not determine the specific environmental factors that cause natural variation in DNA methylation, previous studies in ants, including other species in the genus *Camponotus*, have established both nutritional variation and social interactions as causes for variations in worker size (Brian, 1956; Goetsch, 1937; Gregg, 1942; Rajakumar et al., 2012; Wheeler, 1986; Wheeler and Nijhout, 1984; Wheeler, 1902). Hyper- or Hypo-methylation in global methylation levels then translates into the differential quantitative methylation of *Egfr* through intermediary genes. This results in quantitative inter-individual differences in the transcription of *Egfr* in the colony. In honeybees, diet activates EGFR to regulate several important downstream pathways including the juvenile hormone, TOR and insulin signaling pathways (Kamakura, 2011). Therefore, *Egfr* in *C. floridanus* may be a key regulator of these important pathways to generate the differential growth attained by 4th instar larvae leading to a continuous distribution of final adult sizes. In other animals, EGFR signaling is known to play a role in regulating DNA methylation through downstream (Rouleau et al., 1995; Sontag and Weber, 2012) activation of DNMTs (Samudio-Ruiz and Hudson, 2012) resulting in altered cellular growth. This raises the possibility that in *C. floridanus*, *Egfr* may not only

be a target of DNA methylation but may in turn also regulate DNA methylation. DNA methylation and pathways like EGFR are highly conserved, and DNA methylation is known to cause transgenerational inheritance (Bossdorf et al., 2008; Jaenisch and Bird, 2003; Johannes et al., 2009). The quantitative methylation of specific loci, like *Egfr*, may therefore represent a more general mechanism for the generation and evolution of quantitative trait variation. Indeed, in vertebrates, quantitative differences in methylation of an inserted retroviral element in front of the Agouti gene in mouse defines differences in coat color (Waterland and Jirtle, 2003), and the distribution of coat colors could be shifted by altering the methyl content in maternal diet (Waterland and Jirtle, 2003). It remains to be shown, however, whether natural inter-individual epigenetic variation of this inserted retroviral element generates variation in coat color in natural populations. This mechanism may also underlie associations found between epigenetic variation and disease susceptibility, like that found for Type-II diabetes in humans (Toperoff et al., 2012), as well as quantitative traits, like that found for flowering time and height in plants (Johannes et al., 2009).

Finally, our results hold important implications for quantitative genetics. The unification of Mendelian inheritance with Darwin's theory of natural selection eventually led to the infinitesimal model (Provine, 2001), which assumes that quantitative trait variation is generated by the action of an infinite number of loci that have small and equal effects on the phenotype (Rockman, 2012; Roff, 2007). The evolution of quantitative traits is therefore thought to occur through random mutations across these loci (Rockman, 2012; Roff, 2007). In contrast, the empirical search for QTLs has revealed that trait variation often maps to specific genetic regions, either of small or large effect, with

specific functions (Farrall, 2004; Flint and Mackay, 2009; Mackay, 2001). Countless studies, however, have demonstrated that QTLs cannot in themselves explain all heritable variation underlying quantitative traits (Eichler et al., 2010), such as growth or size in humans (Yang et al., 2010), *Arabidopsis* (Kroymann and Mitchell-Olds, 2005) and yeast (Steinmetz et al., 2002). This difficulty, as well as the apparent gap between the results of QTL analyses and the assumptions of the infinitesimal model, underscores the many challenges that remain in understanding the genetic basis of quantitative trait variation (Eichler et al., 2010; Roff, 2007; Slate, 2013). Our findings therefore suggest that, in addition to genetic variation, quantitative DNA methylation can enhance the phenotypic possibilities of a genetic locus of small or large effect and may help resolve these outstanding challenges in the field of quantitative genetics.

4.3 Acknowledgements

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4.4 Main Text Figures

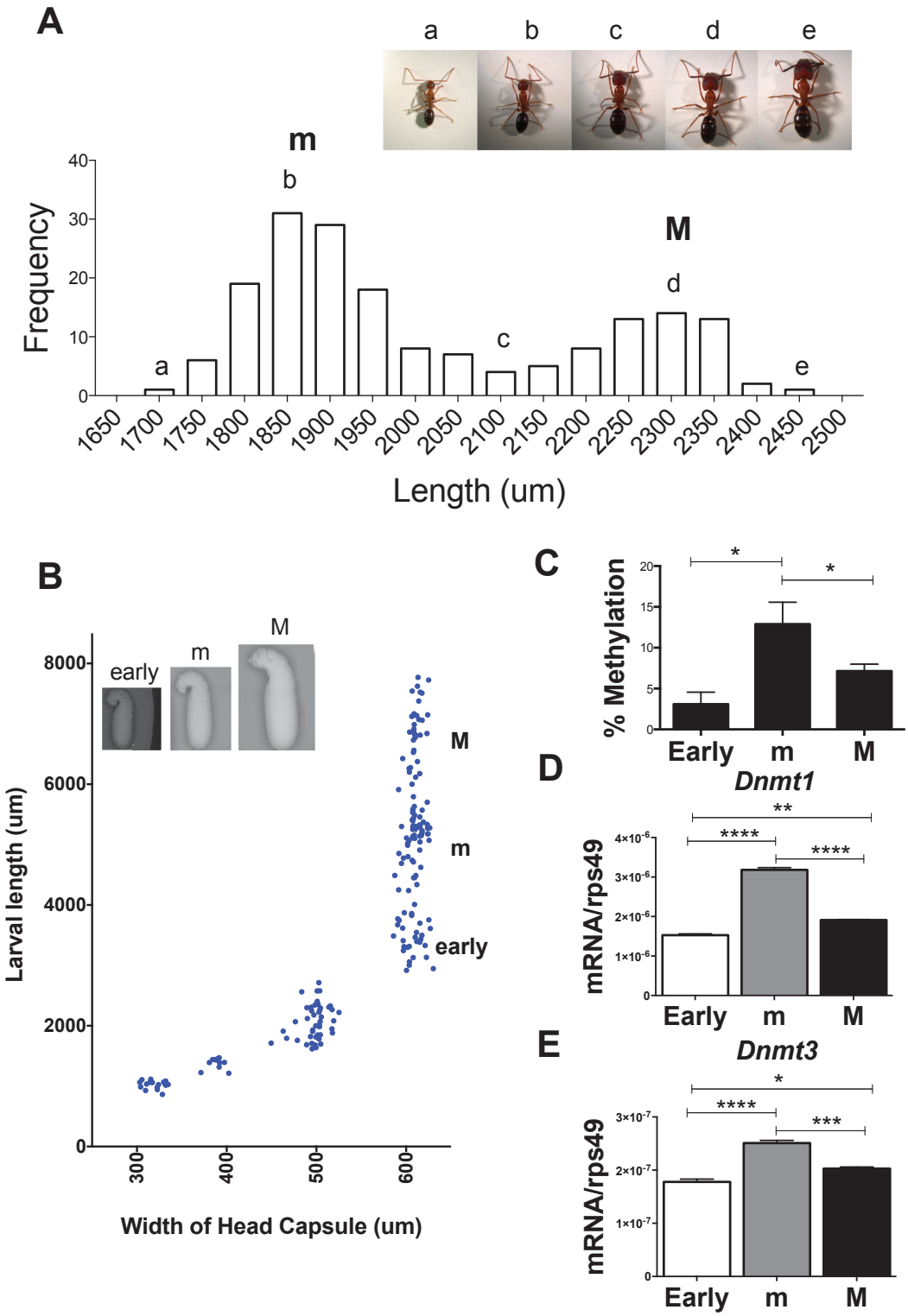

Figure 1: Continuous size distribution of workers in *C. floridanus*: development and methylation patterns. (A) Continuous distribution of adult size of *C. floridanus* workers (n=179). This distribution is based on scape length (Fig S1), a common proxy for body size (see methods). **(B)** Developmental stages and establishment of final worker sizes (n=197) **(C)** Genomic methylation in early 4th instar, as well as minor and major larvae in late 4th instar. Transcription level of **(D)** *dnmt1* and **(E)** *dnmt3* in early 4th instar larvae (white bar), late minor worker larvae (grey bar) and late major worker larvae (black bar). Bars indicate mean and error bars indicate ± s.e.m. Statistical significance values for Student's t-test are as follows: *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$. The head/body allometry of a minor worker is indicated by **m** and major workers is indicated by **M**.

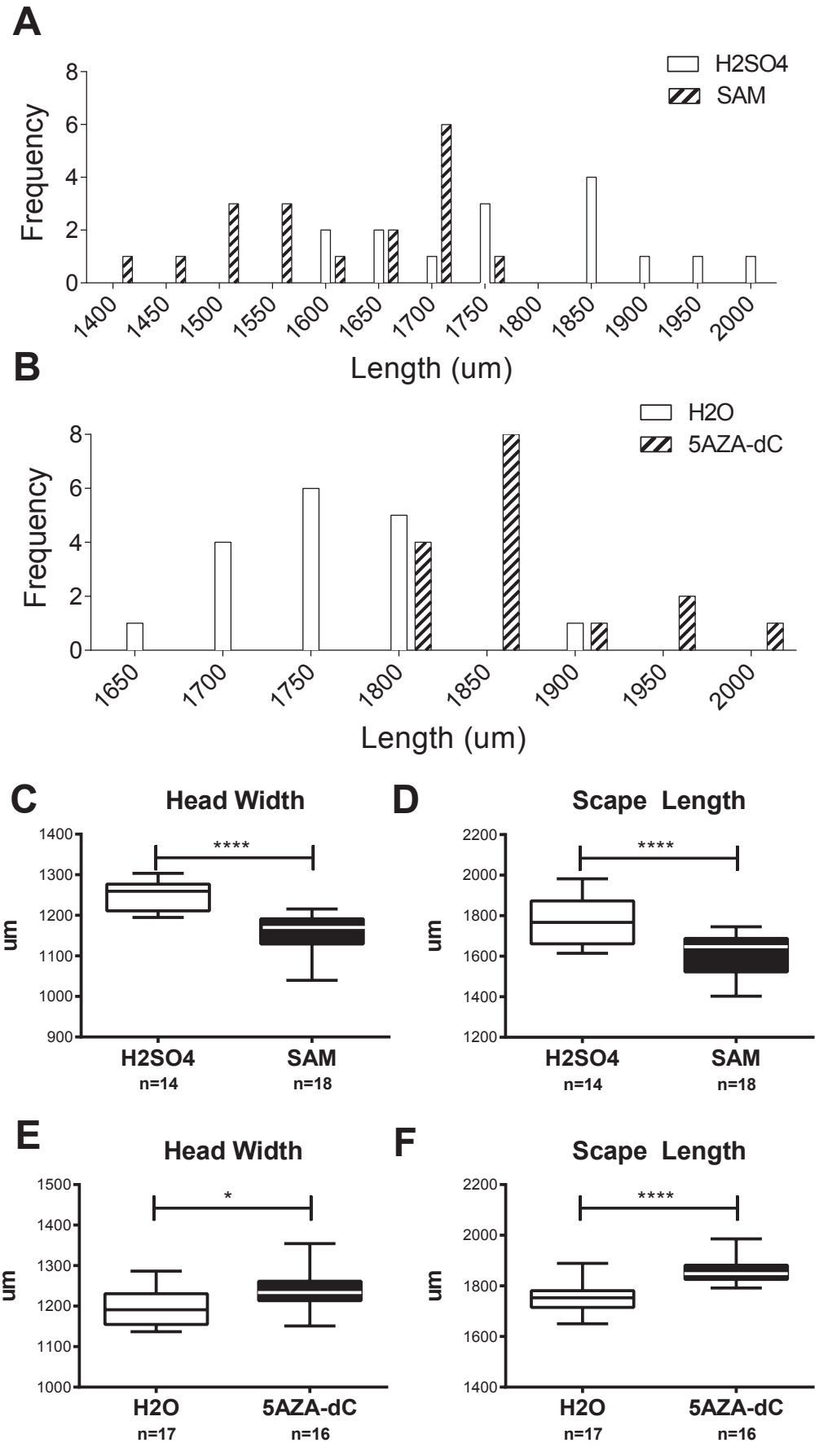
A
H2SO4
SAM
Frequency
Length (um)
B
H2O
5AZA-dC
Frequency
Length (um)
C
Head Width
um
****
H2SO4
n=14
SAM
n=18
D
Scape Length
um
****
H2SO4
n=14
SAM
n=18
E
Head Width
um
*
H2O
n=17
5AZA-dC
n=16
F
Scape Length
um
****
H2O
n=17
5AZA-dC
n=16

Figure 2: Pharmacological manipulation of global (genome-wide) DNA methylation shifts mean of continuous worker size distribution. Hatched bars indicate effect of **(A)** SAM and **(B)** 5-AZA-dC administration at the early 4th instar, whereas white bars indicate controls (H2SO4 for SAM and H2O for 5-AZA-dC). SAM treated animals (black box) compared to control (white box) for **(C)** head width and **(D)** scape length. 5-AZA-dC treated (black box) animals compared to control (white box) for **(E)** head width and **(F)** scape length. Boxplot whiskers indicate min and max. Box defined by 25th percentile, mode and 75th percentile. Statistical significance values for Student's t-test are as follows: *= $p<0.05$, ****=$p<0.0001$. Note that all scape length and head width measurement data are normally distributed (tested with Shapiro-Wilk's test). For all remaining measurements on treated and control larvae refer to Fig S3.

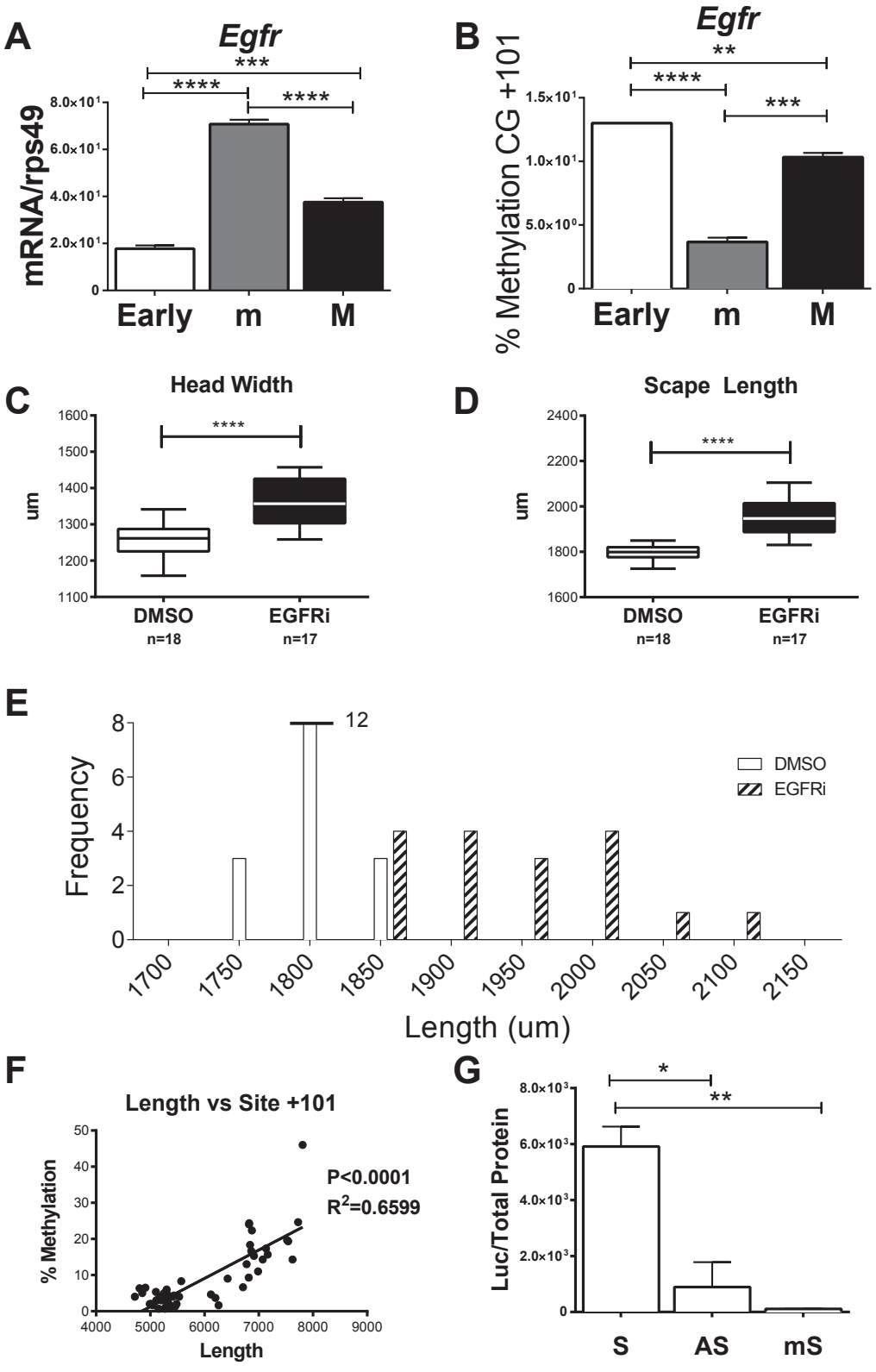
A
Egfr
mRNA/rps49
Early
m
M
B
Egfr
% Methylation CG +101
Early
m
M
C
Head Width
um
DMSO
n=18
EGFRi
n=17
D
Scape Length
um
DMSO
n=18
EGFRi
n=17
E
Frequency
DMSO
EGFRi
Length (um)
F
Length vs Site +101
% Methylation
Length
P<0.0001
R2=0.6599
G
Luc/Total Protein
S
AS
mS

Figure 3: The role and regulation of *Egfr* in generating quantitative variation in worker size. (A) *Egfr* expression in early 4th (white bar), late 4th instar minor (grey bar) and major larvae (black bar). **(B)** Percent (%) methylation of site CpG +101 in *Egfr* for early 4th instar (white bar), late 4th instar minor (grey bar) and major larvae (black bar). EGFR inhibition of early 4th instar worker larvae (black box) compared to control DMSO (white box) for **(C)** Head width and **(D)** scape length. Scape and head width measurement data are normally distributed (tested with Shapiro-Wilk's test). **(E)** EGFR inhibition at the early 4th instar and its effect on sizing distribution (hatched bars) compared to control DMSO (white bars). **(F)** Statistical relationship between percent (%) methylation of *Egfr* site +101 (y-axis) and final larval length (x-axis) across a spectrum of larval sizes ($n = 50$, $R2=0.6599$, $p<0.0001$). **(G)** *Egfr* promoter cloned into luciferase reporter pCpGl in sense (S), antisense (AS) and methylated sense (mS). Bars indicate mean and error bars indicate ± s.e.m. Boxplot whiskers indicate min and max. Box defined by 25th percentile, mode, and 75th percentile. Statistical significance values for Student's t-test are as follows: *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$. For all remaining measurements on treated and control larvae refer to Fig S3.

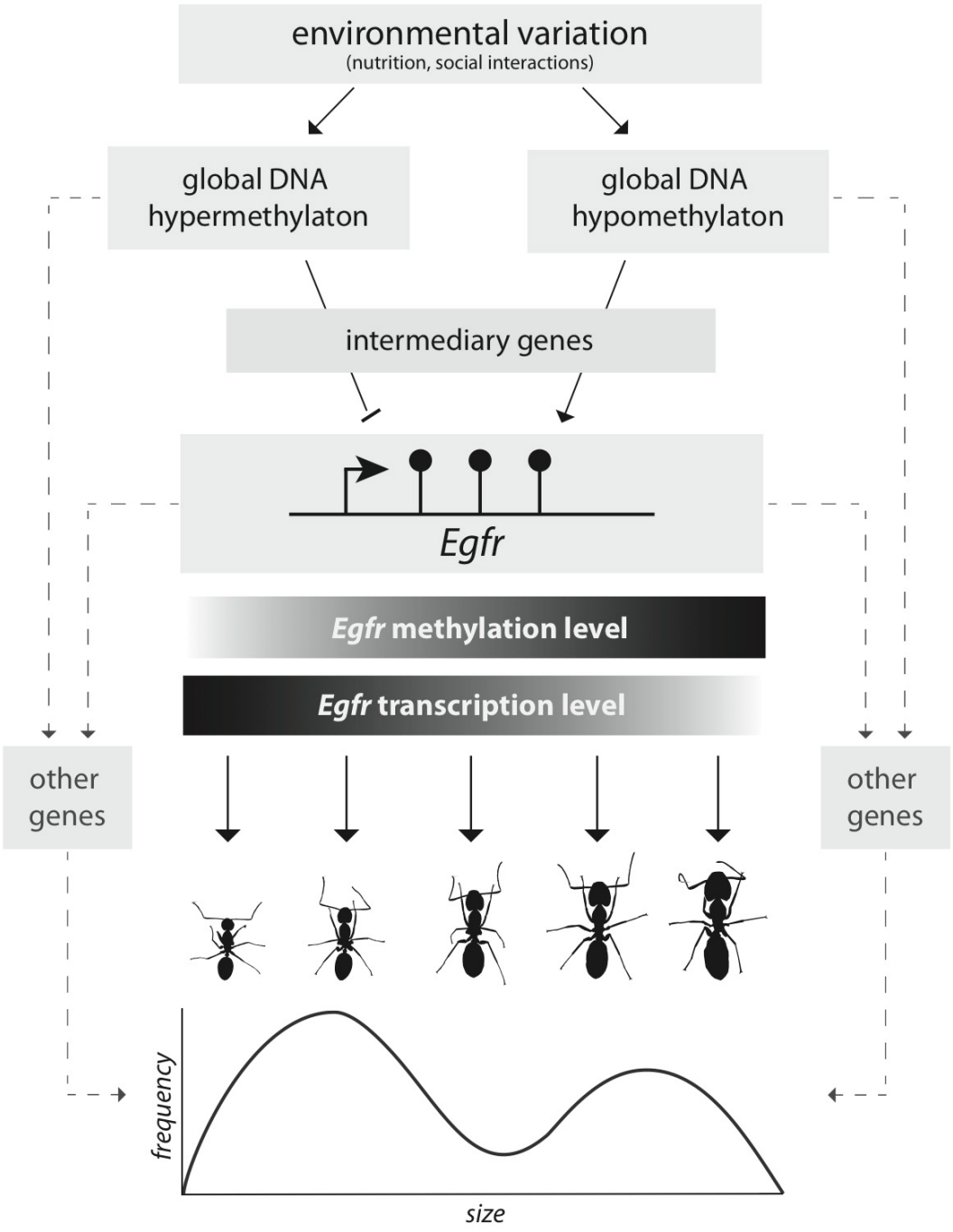
environmental variation
(nutrition, social interactions)
global DNA hypermethylaton
global DNA hypomethylaton
intermediary genes
Egfr
Egfr methylation level
Egfr transcription level
other genes
other genes
frequency
size

Figure 4: Model for the translation of a variable environment into quantitative changes in global (genome-wide) DNA methylation leading to quantitative changes in *Egfr* methylation in generating a continuous size distribution of workers in *C.floridanus*. Environmental variation leads to (black arrows) either global DNA hyper- or hypomethylation. Intermediary genes quantitatively modulate this global DNA methylation state (repression indicated by line with perpendicular tip and activation indicated by arrowhead) into a specific level of *Egfr* methylation (indicated by black lollipops and box with increasing gradient from left to right, where an increase in black corresponds to increasing levels of methylation), which results in a specific level of *Egfr* transcription level (box with decreasing gradient from left to right, where a decrease in black corresponds to decrease in *Egfr* transcription levels). This level of *Egfr* transciption then translates to quantitative size variation in the worker caste of *C.floridanus* (indicated by black arrows leading to differently sized cartoons of worker ants that represent the continuous size distribution of workers). Dashed arrows indicate the potential involvement of other loci that generate quantitative size variation.

4.5 Supplemental Material

4.5.1 Materials & Methods

Collection Of Samples. We collected mated queens from Tallahassee, Florida, USA. Mature colonies originating from single queens were maintained in plastic boxes with glass test tubes filled with water constrained by cotton wool, and were fed a combination of mealworms, crickets, fruit flies and Bhatkar-Whitcomb diet (Bhatkar and Whitcomb, 1970). All colonies were maintained at 27°C, 70% humidity, and 12 hour day:night cycle. Larval and adult sampling as well as experiments began after the colonies had matured for approximately 4 years.

Determination of the number of instars for worker larvae. In order to describe the epigenetic status of specific developmental stages of larval development, the number of larval instars was determined. Larvae were taken from colonies that were not in the process of producing reproductives (males or queens). This is important because larvae of *Camponotus* species that become reproductive have been suggested to have a different number of instars compared to worker larvae (Dartigues and Passera, 1979). In order to discriminate between instars, widths of the head capsules of larvae were measured as previously described (Solis et al., 2010).

Culturing of pharmacologically treated larvae. In order determine the role of methylation during worker larval development, early 4th instar (the last instar based on Fig. 1B) larvae were selected for methylome and EGFR signaling manipulation. S-adenosyl methionine (SAM; B9003S, New England Biolabs), a methyl donor (van der

Westhuyzen, 1985), was applied at a concentration of 32mM. 5-aza-deoxycytidine (5-AZA-dC; A3656, Sigma- Aldrich), a nucleoside analog inhibitor of DNA methyltransferases (Wilson et al., 1983), was applied at a concentration of 10mM and the highly specific and potent quinazolone EGFR inhibitor, PD153035 which acts through the suppression of epidermal growth factor-dependent EGFR phosphorylation (Bos et al., 1997; Fry et al., 1994) (234491, EMD Millipore), was applied at a concentration of 10uM. Larvae were isolated and placed laterally on a petridish with the aid of a microscope. 4ul of solution was then applied topically allowing for absorption and larval feeding. After treatment, larvae were setup in plastic boxes and fed in the same manner as colonies. For each treatment, 40 larvae were treated and 80 adult minor workers were supplemented to the box to care for them. Half of the larvae were collected at the end of larval development for further quantitative gene expression as well as pyrosequencing, while the other half were left to develop. Timing of eclosion (the beginning of adulthood) was monitored for the remaining larvae of each treatment. Newly emerged adults were subsequently measured to detect any morphological effects of the drug treatments.

Measuring of adult workers. Workers were measured for several parameters for the purpose of identifying any shifts in size that might have been caused by the manipulation of larval methylation or EGFR signaling inhibition. In particular, we measured scape length, head width, thorax length, thorax width, and mandible length as in Diniz-Filho (1994). In *Camponotus*, Diniz-Filho (1994) found that head width exhibited positive allometry (Fig. S1), while scape length was isometric and further suggested that for a bivariate analysis of allometry, scape length could be used as an independent variable

(due to its isometry) (Diniz-Filho et al., 1994). Isometric measures, like scape length, are highly correlated and proportional to body size (Diniz-Filho et al., 1994) and therefore serve as independent and accurate proxies for body size to examine the size frequency of individuals of a colony. In a similar manner, head width and scape length were used in another *Camponotus* species (Fraser et al., 2000) as well as other polymorphic ant species (Wilson, 1953). An added advantage for measuring the length of the scape is its simplicity and therefore it reduces any technical variation in measurements between individuals. Therefore to determine the relative distribution of sizes (Fig. 1A), we therefore used scape length as a proxy for body size. By characterizing the allometry found within the continuous size distribution of workers we could classify minor and major workers (Fig. S1).

Microscopy. We used a Zeiss Discovery V12 stereomicroscope and Zeiss Axiovision software to measure the larvae (in um) and adults (in um). For larval imaging we used an Olympus TM3000 tabletop SEM.

DNA and RNA extraction. Larvae were collected and pooled (n=20) for each instar and immediately frozen at -80°C. Since our trait of interest, size, is a variable specific to a large proportion of cells within the animal, we assumed that heterogeneous tissues across individual larvae would still provide data relevant to size as a trait. DNA and RNA were both extracted using Allprep DNA/RNA extraction kits (80204, Qiagen) as instructed for animal tissues. Homogenization of samples was achieved using RLT+ provided with pestle grinding. Individual larvae selected at the fourth instar were also processed in a

similar fashion. A DNAse on-column step was done in all samples during RNA extraction. All samples were quantified using Nanodrop 1000 (Thermo Scientific).

Luminometric Methylation Assay (LUMA). LUMA is a high throughput assay used to determine global (genome-wide) DNA methylation. The LUMA method used in our study is a modification described by Karimi et al. (Karimi et al., 2006a; Karimi et al., 2006b). LUMA involves the digestion of genomic DNA by a methylation sensitive (HPAII) or insensitive (MSPI) restriction enzymes in combination with an internal control restriction enzyme (EcoRI) to normalize the DNA input. EcorI (FD0274), HpaII (FD0514) and MspI (FD0541) were all purchased from Thermo Scientific.
Both HpaII and MspI restriction enzymes recognize and cleave 5'-CCGG-3' sequences producing 5'-CG overhangs, whereas EcoRI recognizes and cleaves 5'- GAATTC-3' sequences and produces 5'-AATT overhangs. The extent of cleavage is determined by a bioluminetric polymerase extension assay based on a four-step pyrosequencing reaction. Samples were incubated (37°C, 4h) and then heat inactivated (80°C, 20min). Digested genomic DNA (15µl) was mixed with pyrosequencing annealing buffer (15µl; Qiagen, Toronto, ON, Canada). Samples were transferred to 24-well pyrosequencing plates for sequencing (PyroMark 24; Biotage, Uppsala, Sweden). The nucleotide dispensation order used was based on Pilsner et al. (Pilsner et al., 2010). Peak heights for C and A represent the HpaII and MspI cuts (methylation) and EcoRI (input DNA), respectively. The formula to calculate % genomic methylation is: 1-[(HpaII (C)/ EcoRI (A) / MspI (C)/ EcoRI (A)] x 100. All samples were run in triplicate.

Bisulfite mapping and expression analyses. DNA was treated with sodium bisulfite and primers (Table S1) were designed for converted products of the beginning (based on Kamakura, 2011) of C. floridanus Egfr (scaffold 550: bp113394-bp113619; located using GBrowse of the Hymenoptera Genome Database (Munoz-Torres et al., 2011). Bisulfite conversion was done with Epitect Bisulfite Conversion kit (Qiagen). Bisulfite PCRs were amplified using two rounds of PCR using outer and nested primers (see Table S1). Cycling conditions involved an initial step of 5 minutes at 95°C followed by 35 cycles of [95°C for 1 minute, Tm for 2.5 minutes, 72°C 1 minute] Followed by 5 minutes of 72°C. PCR products were sequenced using the Biotage Pyrosequencer according to the manufacturer's protocol (Colella et al., 2003).

For all samples, 500ng of RNA was subjected to RT-PCR according to manufacturer's protocols (Roche) and quantified using quantitative PCR on the Lightcycler 480 (Roche). Primers for all genes (Table S1) were created across exon boundaries. As a housekeeping gene we used RP49 for normalization as it was previously shown to have stable expression both across larval development and following juvenile hormone manipulation in Apis mellifera (de Azevedo and Hartfelder, 2008; Lourenco, 2008; Mackert et al., 2008). Quantitative PCR was amplified with a pre- incubation at 95°C for 10 minutes followed by 45 cycles of [95°C for 10 seconds, 60°C for 10 seconds, 72°C for 10 sec] followed by 10 minutes of 72°C.

In vitro Luciferase Assay. The beginning of Egfr corresponding to that described in A. mellifera (Kamakura, 2011) was amplified using the "Luciferase construct PCR primers" (Table S1) generating a 378 bp fragment of the beginning of the C.floridanus Egfr gene

(scaffold 550: bp113394-bp113772; located using GBrowse of the Hymenoptera Genome Database14). BamHI and HindIII restriction sites were incorporated into primers in order to clone into the CpG-less pCpGl (Klug and Rehli, 2006) and cloned in 5' to 3' (sense) or 3' to 5' (antisense) orientation, respectively. Since the vector does not contain CpG sites all methylated sites are contained in the EGFR promoter sequences. The constructs were methylated in vitro with SssI CpG DNA methyltransferases (M0226L, New England Biolabs) as recommended by the manufacturer. Transfections were performed using calcium phosphate precipitation as described previously (Champagne et al., 2006) into HEK293 cells (CRL-1573, ATCC). Cells were harvested 48 hours after transfection and luciferase activity was assayed using the Luciferase Assay System (Promega).

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM), except developmental time is expressed as standard deviation (SD) and morphometrics in the form of box-and-whisker plots. Comparisons between groups were performed using 2-tailed, unpaired student's t-test, except in the case of unequal variance for which the Mann-Whitney U test was performed. MANOVA performed considered all morphometric traits, significance was set at $P<0.05$ and Pillai's Trace was used. Percent methylation with terminal larval size was calculated using linear regression and significance was initially set at $P<0.05$ followed by Bonferroni correction for multiple tests, implemented to correct for multiple comparisons. Statistical analysis was undertaken using Prism (GraphPad Software Inc, San Diego, California) except for MANOVA, which was performed in R.

4.5.2 Supplemental Figures and Tables

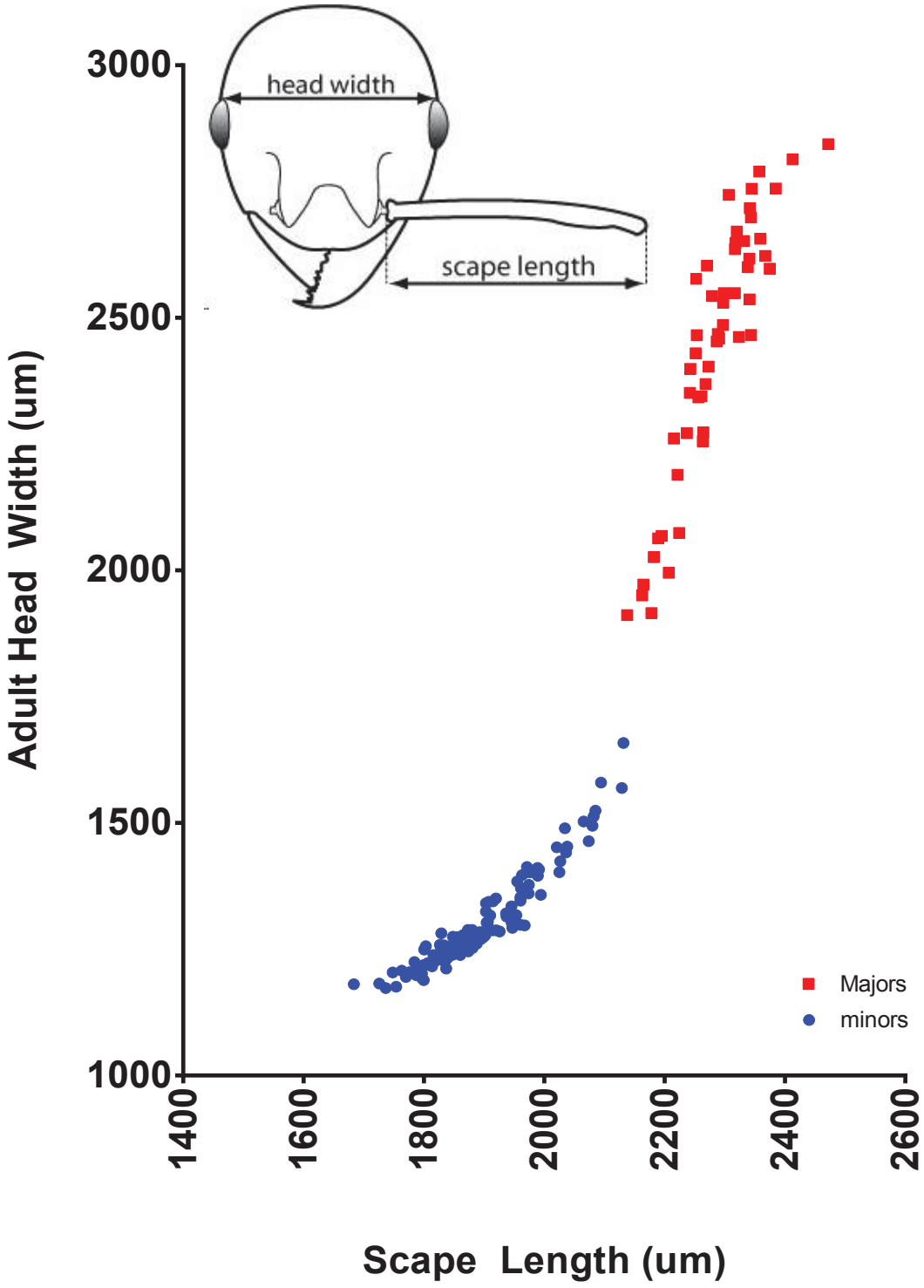

Figure S1: Allometric classification of adult *C.floridanus* workers into major and minor workers. Although size variation in workers in *C. floridanus* is continuous, there is an allometric relationship between the size of the head and size of the body, which means that the head is disproportionately larger than adult size). This has traditionally been used to classify minor and major workers. Adult head width (y-axis) was plotted against adult scape length (x-axis: is the first segment of the antennae and is a common proxy for body size). Minor and major adult workers are distinguished by a break in the allometry curve. Blue circles indicate minor workers and red squares indicate major workers.

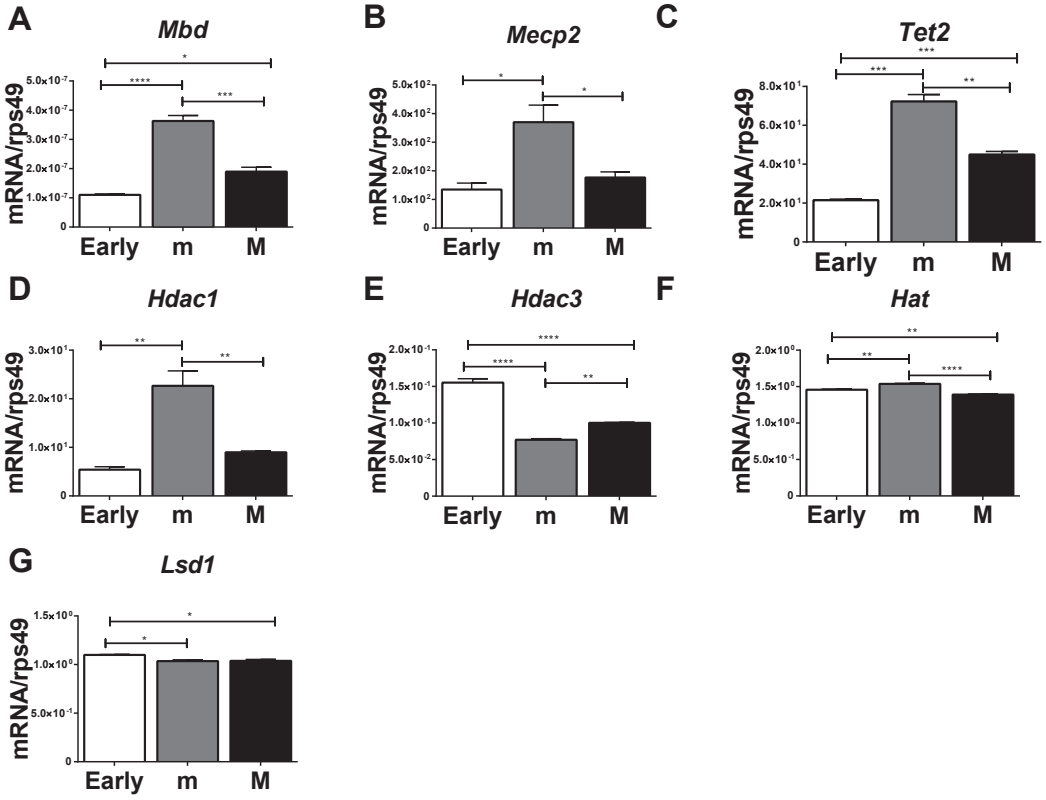
A
Mbd
mRNA/rps49
Early
m
M
B
Mecp2
C
Tet2
D
Hdac1
E
Hdac3
F
Hat
G
Lsd1

Figure S2: Quantitative gene expression of additional DNA methylation and histone modification regulators. Expression of **(A)** *Mbd*, **(B)** *Mecp2*, **(C)** *Tet2* **(D)** *Hdac1*, **(E)** *Hdac3*, **(F)** *Hat*, **(G)** *Lsd1* in early 4th instar larvae (white bar), late 4th instar minor worker larvae (grey bar) and late 4th instar major worker larvae (black bar). *Mbd*, *Mecp2*, *Tet2*, *Hdac1* and *Hat* are expressed in a pattern similar to *Dnmt1* and *Dnmt3* (see Fig. 1) reflecting an overall pattern of DNA methylation that is coordinated to some degree with histone modification regulators. Bars indicate mean and error bars indicate ± s.e.m. Statistical significance values for Student's t-test are as follows: *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

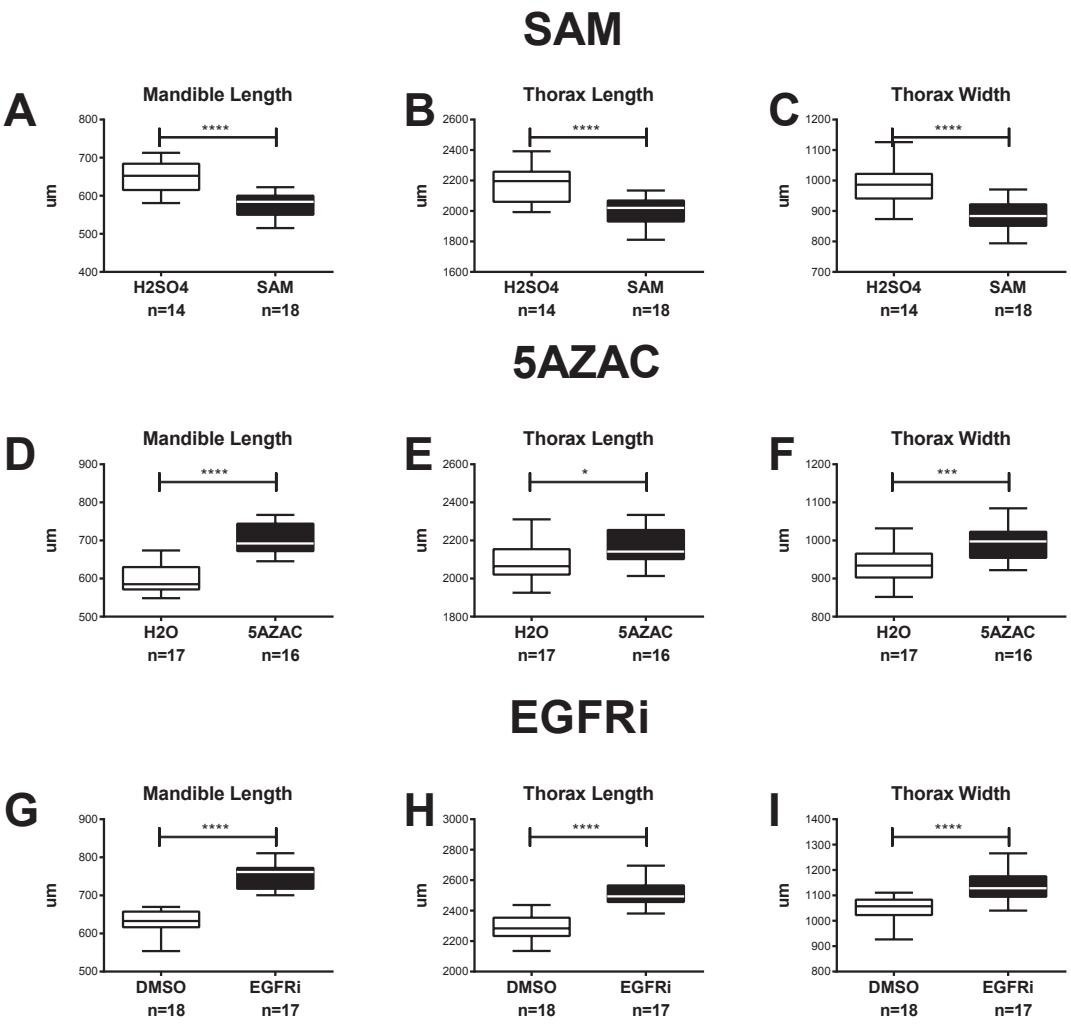
SAM
A
Mandible Length
um
****
H2SO4
n=14
SAM
n=18
B
Thorax Length
um
****
H2SO4
n=14
SAM
n=18
C
Thorax Width
um
****
H2SO4
n=14
SAM
n=18
5AZAC
D
Mandible Length
um
****
H2O
n=17
5AZAC
n=16
E
Thorax Length
um
*
H2O
n=17
5AZAC
n=16
F
Thorax Width
um
***
H2O
n=17
5AZAC
n=16
EGFRi
G
Mandible Length
um
****
DMSO
n=18
EGFRi
n=17
H
Thorax Length
um
****
DMSO
n=18
EGFRi
n=17
I
Thorax Width
um
****
DMSO
n=18
EGFRi
n=17

Figure S3: Additional sizing morphometrics of SAM, 5-AZA-dC and EGFRi treatments. (A) Mandible length **(B)** Thorax length and **(C)** Thorax width of SAM treated animals (black box) and controls (white box; H2SO4). **(D)** Mandible length, **(E)** Thorax length and **(F)** Thorax width in 5-AZA-dC treated animals (black box) and controls (white box; H2O). **(G)** Mandible length, **(H)** Thorax length and **(I)** Thorax width in EGFR inhibited animals (black box) and controls (white box; DMSO). Boxplot whiskers indicate min and max. Box defined by 25th percentile, mode, and 75th percentile. Statistical significance values for Student's t-test are as follows: *= $p<0.05$, ***=$p<0.001$, ****=$p<0.0001$. All measurement data are normally distributed (tested with Shapiro-Wilk's test). MANOVA analysis done on all 5 measurements to statistically test for differences of treatments with controls across the animal (including scape length and head width of Fig.2 and Fig. 3). MANOVA statistics are as follows: SAM (pillai trace=0.76571, F= 17.649, p=3.045e-09); 5-AZA-dC (pillai trace=0.81913, F= 24.456, p=3.045e-09); EGFRi (pillai trace= 0.85263, F= 33.556, p=3.293e-11).

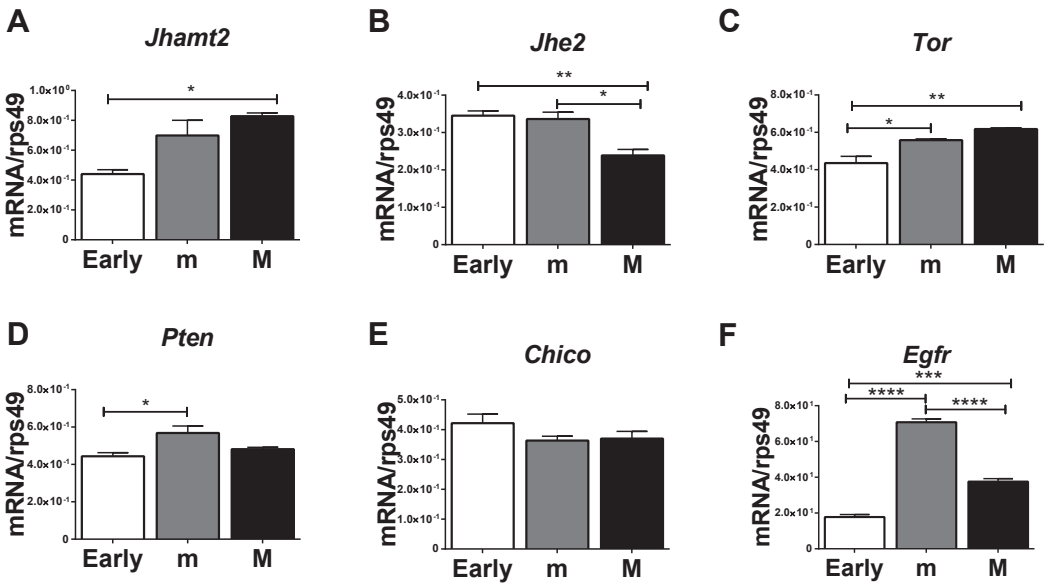
A
Jhamt2
mRNA/rps49
Early
m
M
B
Jhe2
C
Tor
D
Pten
E
Chico
F
Egfr

Figure S4: Quantitative gene expression of key regulators of growth. **(A)** JHAMT2, **(B)** JHE2, **(C)** TOR, **(D)** PTEN, **(E)** CHICO, **(F)** EGFR in early 4th instar larvae (white bar), late 4th instar minor worker larvae (grey bar) and late 4th instar major worker larvae (black bar). Bars indicate mean and error bars indicate ± s.e.m. Statistical significance values for Student's t-test are as follows: *= $p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$. While our screen identified statistically significant differences between major and minor worker-destined larvae for *Jhe2*, we chose to investigate *Egfr* because it showed the greatest differences.

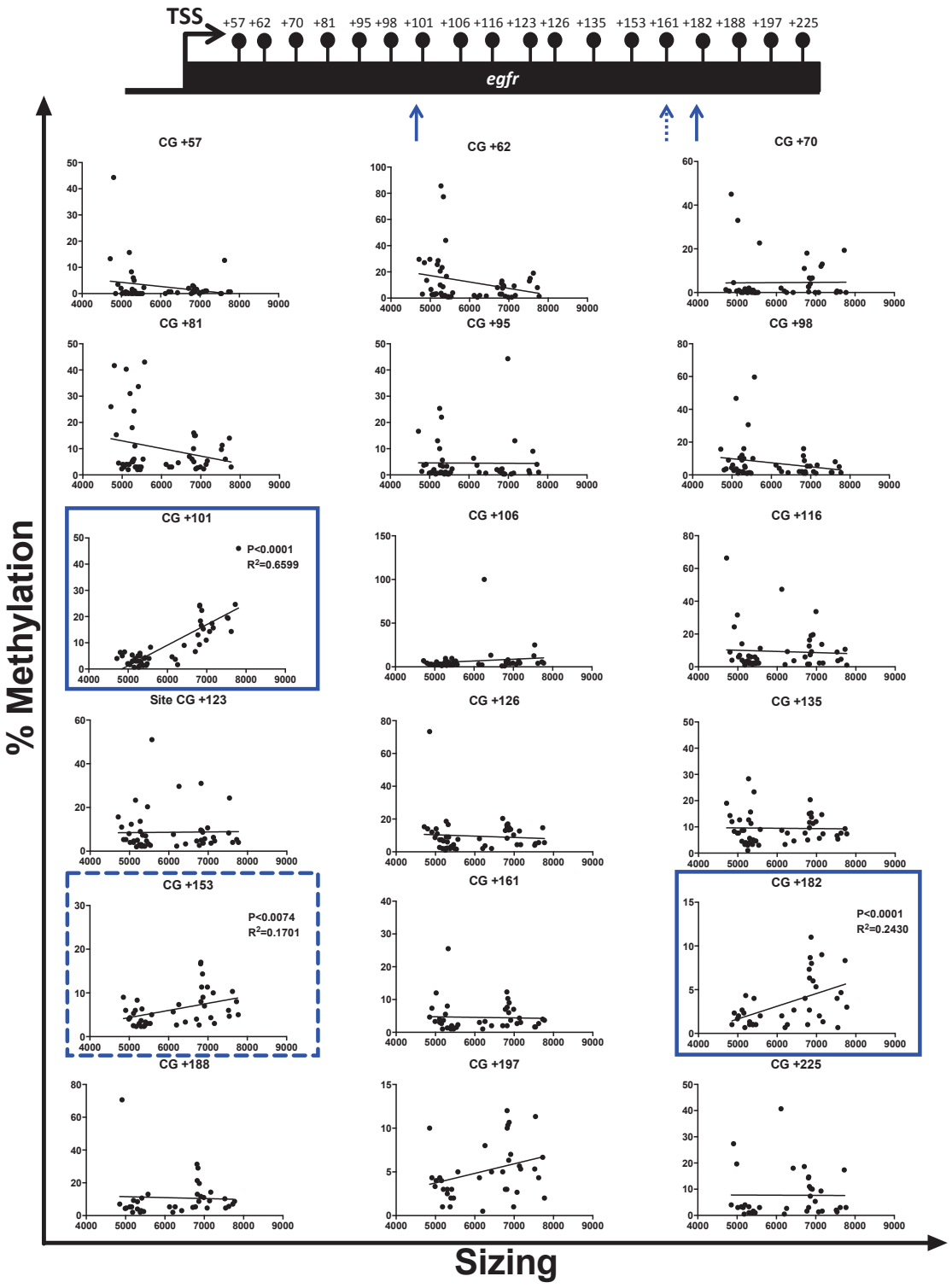
TSS
+57 +62 +70 +81 +95 +98 +101 +106 +116 +123 +126 +135 +153 +161 +182 +188 +197 +225
egfr
% Methylation
Sizing
CG +57
CG +62
CG +70
CG +81
CG +95
CG +98
CG +101
P<0.0001
R2=0.6599
CG +106
CG +116
Site CG +123
CG +126
CG +135
CG +153
P<0.0074
R2=0.1701
CG +161
CG +182
P<0.0001
R2=0.2430
CG +188
CG +197
CG +225

Fig. S5: Association of percent (%) methylation of *Egfr* CpG sites and final size of workers. Map of CpG dinucleotides at top (methylated sites indicated by black lollipops). Regression of percent (%) methylation (y-axis) versus terminal larval length (x-axis) for all 18 CpG dinucleotides occurring in the first 225bp of *Egfr.* Regressions of CpG sites +101, +153, +182 and +197 with larval size were significant (Table S2). All significant sites are indicated by blue box and arrows: following Bonferroni correction for multiple comparisons, sites +101 and +182 remain significant while site +153 is marginally insignificant (indicated by dashed box and arrow). Refer to table S2 for all statistical information).

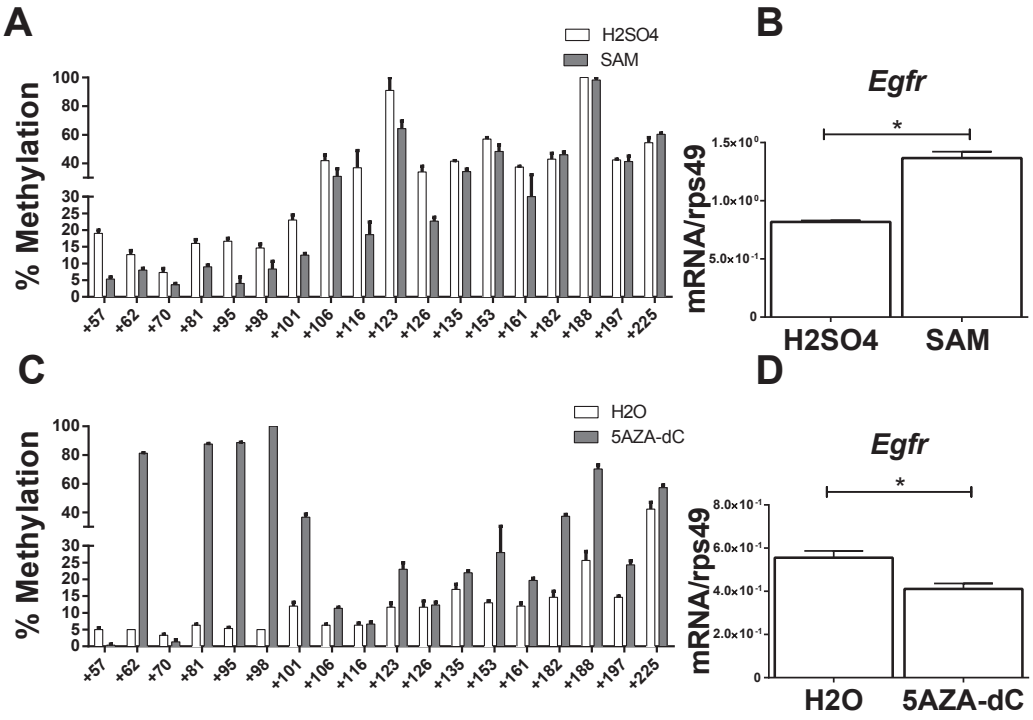
A
H2SO4
SAM
% Methylation
100
80
60
40
30
25
20
15
10
5
0
+57
+62
+70
+81
+95
+98
+101
+106
+116
+123
+126
+135
+153
+161
+182
+188
+197
+225
B
Egfr
*
mRNA/rps49
1.5×10⁰
1.0×10⁰
5.0×10⁻¹
0
H2SO4
SAM
C
H2O
5AZA-dC
% Methylation
D
Egfr
*
mRNA/rps49
8.0×10⁻¹
6.0×10⁻¹
4.0×10⁻¹
2.0×10⁻¹
0
H2O
5AZA-dC

Figure S6: Methylation and quantitative expression of *Egfr* following SAM and 5-AZA-dC administration. **(A)** Bisulfite map showing percent (%) methylation for *Egfr* (grey bars) and control (H2O; white bars) for each of the 18 sites within the first 225bp and **(B)** expression of *Egfr* following SAM administration relative to control (H2SO4). **(C)** Bisulfite map showing percent (%) methylation for 5AZA-dC (grey bars) and control (H2O; white bars) for each of the 18 sites within the first 225bp **(D)** expression of *Egfr* following 5-AZA-dC administration relative to control (H2O). An inverse effect of manipulating global DNA methylation levels of 4th instar larvae on *Egfr* methylation levels indicate that there are possibly intermediary genes regulating this inverse relationship. Furthermore, increase or decrease in *Egfr* methylation results in decrease or increase of *Egfr* transcriptional expression levels, respectively. Bars indicate mean and error bars indicate ± s.e.m. Statistical significance values for Student's t-test are as follows: *= $p<0.05$.

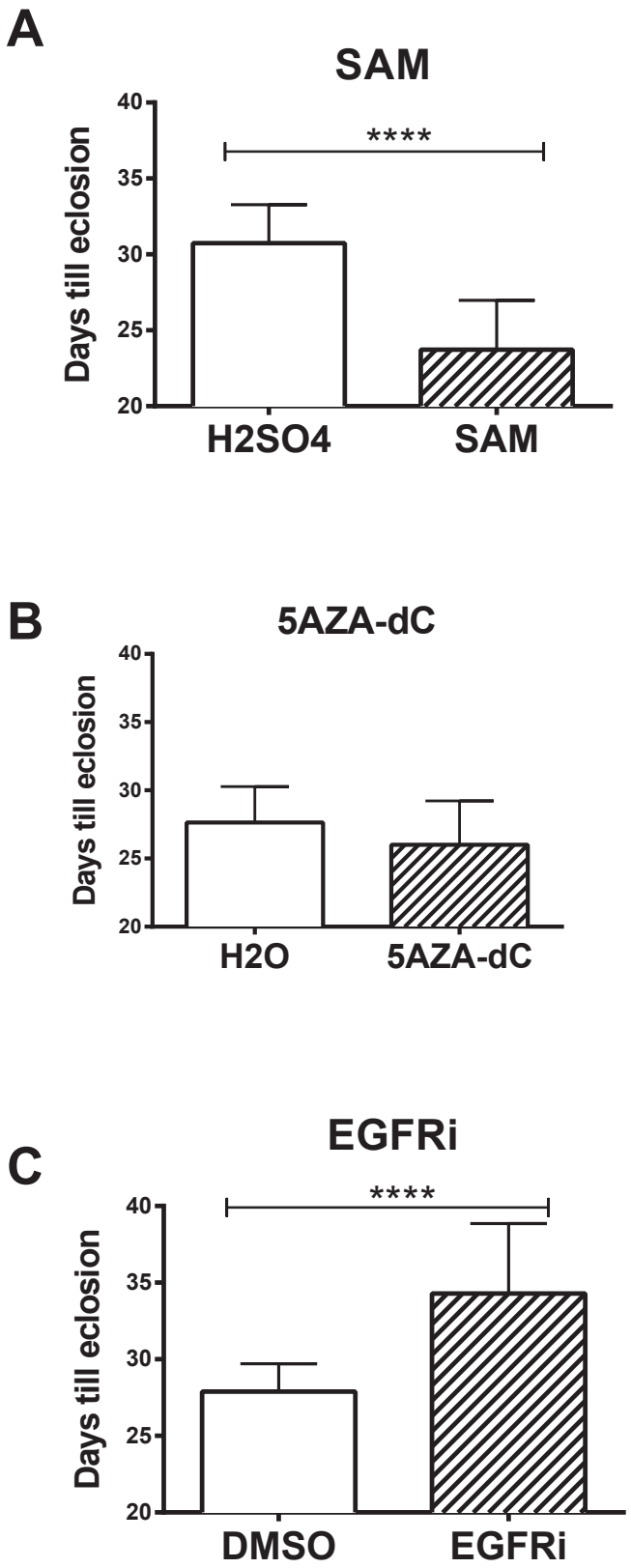
A
SAM
Days till eclosion
40
35
30
25
20
****
H2SO4
SAM
B
5AZA-dC
Days till eclosion
40
35
30
25
20
H2O
5AZA-dC
C
EGFRi
Days till eclosion
40
35
30
25
20
****
DMSO
EGFRi

Figure S7: Developmental time is affected by Global (genome-wide) DNA methylation and EGFR signaling. Days till eclosion (termination of development) for worker larvae after **(A)** SAM administration (hatched bar) relative to control (H2SO4; white bar), **(B)** 5-AZA-dC administration (hatched bar) relative to control (H2O; white bar), and **(C)** EGFR inhibition (hatched bar) relative to control (DMSO; white bar). Hypermethylating developing larvae with SAM results in a statistically significant decrease in developmental time to eclosion of workers while EGFR inhibition results in a statistically significant increase in developmental time to eclosion of workers. This effect on developmental timing may be one of the mechanisms for which worker larvae terminate development at different sizes. By increasing the duration of development the larvae have more time to grow which may result in larger adult workers and if the duration of development is decreased the larvae have less time to grow and this may result in smaller adult workers. 5AZA-dC did not affect developmental timing, possibly because higher concentration may be required to elicit such a response or because 5AZA-dC effects on sizing may operate through a developmental timing-independent mechanism. Frequency of eclosion timing was not always normally distributed (tested with Shapiro-Wilk's test). Therefore the Mann-Whitney U test was used to compare controls with respective pharmacological manipulation. Bars indicate mean and error bars indicate standard deviation. Statistical significance values for Student's t-test are as follows: ****=$p<0.0001$.

Table S1: Primers used in this study

Bisuflite PCR Primers	**Sequence**
Egfr (region 1) forward	TTTTAGTGTTGGTAGGGTAGGTGTT
Egfr (region 1) reverse	AAAAATTCTTACAAACAAAACAATC
Egfr (region 2) forward	TGGGTATGTATAATAATTATAATTTTTGTT
Egfr (region 2) reverse	ACACCTACCCTACCAACACTAAAAC
Pyrosequencing Primers	
r1s1 *Egfr*	GGTAGGGTAGGTGTTT
r2s1 *Egfr*	AATTGGGAGGAAATAATTA
Luciferase Primers	
Pcpgl *Egfr* forward (sense)	GGATCCTATA CCTCTGCCATATCAAGACGA
Pcpgl *Egfr* reverse (sense)	AAGCTTTATA GCAAAAAGACAGAAGAATCGTG
Pcpgl *Egfr* forward (antisense)	AAGCTTTATA CCTCTGCCATATCAAGACGA
Pcpgl *Egfr* reverse (antisense)	GGATCCTATA GCAAAAAGACAGAAGAATCGTG

(Table S1 cont'd)

Expression Primers	**Sequence**	**Expression Primers**	**Sequence**
chico	AGTCACGGGTGCGACTGT	*Mecp2*	TCATGCATCTCGCTCAAAAC
chico	CTGAGTCCGACGAGCACAT	*Mecp2*	AACGGCACCATCCGTAGTAG
Tor	TGCATTAAAGGTAGCAACGGTA	*Hdac1*	AATTCCTGAGGATGGTGCTG
Tor	TATCCGGATCTCCCAACAAG	*Hdac1*	TCCTGACCTTTTTCCAAACG
Pten	GGTCAAGCATGTCTGCGTTA	*Hdac3*	GAGTAAAGTCTGGCGCGAAG
Pten	TTCCGAACCTCGTAAACACC	*Hdac3*	ATCGACTTGGCTGCTTCAGT
Egfr	GCACGTACCAGAGGGATGTT	*Hat*	CACAAGGCGATTTGAGGTCT
Egfr	AAGCCGTATCCTGTGCACTT	*Hat*	AAAACCAATCGTCGCGTATC
JHAMT2	TCCAAATGCAGTAATAATGGGTA	*Lsd1*	TCGCCACATTTCGTAAATCA
JHAMT2	TTCGAATTTGAGCTGTTTCTCA	*Lsd1*	TCTTTTGGAACCGTTTGACC
JHE2	GCTGAATTCATCGCTGACAA	*Tet1*	AGATAGTTTGCCCGATGGTG
JHE2	GAAAATGCGGACCAAGAAGA	*Tet1*	TTTCGAGAGCTGTCATTCCA
Dnmt1	CTGTGTGCCTTTGACACTGG		
Dnmt1	TGGCCCCATATCTTTTGTTG		
Dnmt3	GACTGCTGCTTGAAGGAACC		
Dnmt3	TTTGAATGTAGTCGCGCATC		
MBD	GGAATGGATCTGCCAAAGAA		
MBD	CCGTTTTCGATCCTGTTTGT		

Table S2: Parameter estimates and Bonferroni-corrected statistical conclusions for regression analyses between percent methylation of 18 CpG sites within first 225bp of Egfr and final larval size (see Fig. S5)

CpG	R-squared	p-value	Bonferroni-corrected statistical conclusions (experiment-wide α value = 0.00278
+57	0.04525	0.1381	n.s.
+62	0.06923	0.0649	n.s.
+70	0.0001212	0.9395	n.s.
+81	0.05805	0.0919	n.s.
+95	0.0000637	0.9561	n.s.
+98	0.04392	0.1441	n.s.
+101	0.6599	<0.0001	significant
+106	0.01769	0.3572	n.s.
+116	0.002905	0.7107	n.s.
+123	0.0002577	0.9119	n.s.
+126	0.005211	0.6184	n.s.
+135	0.00005444	0.9603	n.s.
+153	0.1701	0.0074	n.s.
+161	0.003666	0.7069	n.s.
+182	0.243	0.0019	significant
+188	0.0002717	0.9229	n.s.
+197	0.1101	0.0449	n.s.
+225	0.001243	0.8359	n.s.

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Major findings and implications of Chapters 2 & 3

Chapter 2 advanced the first specific goal of my thesis, which was to understand the hormonal and developmental mechanisms involved in the evolution of castes in ants. Aside from this, its findings have potential societal applications discussed in Chapter 3, which led to the formulation of an applied protocol. Hopefully, this protocol will provide new artificial selection strategies, which can improve animal or plant breeding methods in the process of selection for favorable traits and against maladaptive traits such as disease.

Since the publication of this work (Chapter 2), its findings have already been applied and discussed within several research domains including: theory in neuroscience (LeBoeuf et al., 2013), behavioral evolution (Foster, 2013) parallel evolution (Forero et al., 2013; Prud'homme and Gompel, 2012) adaptation to climate change (Walters et al., 2012), aging theory and evolution of aging patterns (Trindade et al., 2013), and the role of mutation in the evolutionary process (Nei, 2013). Furthermore, based on the findings of Chapter 2 with the genus *Pheidole,* ants have been suggested as becoming an emerging model in evolutionary developmental biology (Sanger et al., 2013). The following are general implications of Chapter 2 and 3 for evolutionary theory.

5.1.1 Homology

The finding (Chapter 2) that a complex trait, like the supersoldier subcaste, can be preserved for ~35-60 millions years and can re-evolve has implications for our understanding of homology. This trait, which appears near the origin of the *Pheidole* group, was subsequently not phenotypically expressed (nor was its underlying developmental pathways) throughout the phylogeny (refer to Fig. 4 of Chapter 2).

According to current phylogenetic theory, it would be proposed that the trait has independently evolved in parallel in different *Pheidole* species representing a possible case of trait reversal, which would be considered a case of homoplasy (Mcshea, 1996; Wake et al., 2011). In contrast, our results demonstrated that this trait and its underlying developmental program could be environmentally reactivated throughout the phylogeny. This implies that this trait is actually evolutionarily homologous to the genus. Furthermore, the trait and its underlying developmental genetic, embryological and hormonal basis are homologous as well. Therefore, it may generally be that patterns of parallel evolution and evolutionary reversals of a trait which are thought to occur de novo and are thus considered homoplasy are actually in fact appearing due to the preservation of a dormant ancestral development potential to produce a homologous trait throughout a group. It has been proposed that rather than homology and homoplasy being dichotomous, they are in fact a continuum (Abouheif, 2008; Gould, 2002; Hall, 2007; Meyer, 1999; Wake, 1999).

5.1.2 Dollo's law challenged: Hormonal and genetic pleiotropy

McShea (1996) has stated that the reversal of an addition to a complex trait is relatively easy to occur. Specifically, imagine trait **A** is the ancestral condition, and trait **B** is added to make **AB**. McShea implies that losing **B**, resulting in exclusively **A**, is not difficult (**A→AB→A**). On the other hand, in regards to the re-evolution of a lost trait (**AB→A→AB**) he stated that it is "prohibitively improbable". This near certainty that it is impossible to regain a lost complex trait is based on acceptance of Dollo's law, which implies that it is impossible to redevelop or regain a complex trait that was evolutionarily lost (Dollo, 1893). On the other hand, our work demonstrates that at the developmental

level, a complex trait might not actually be lost. This raises the following question: how could it be that a trait could be retained even though it is not phenotypically expressed and subject to natural selection?

We propose that the occurrence of pleiotropy could be an explanation. Although supersoldiers are not made in most *Pheidole* species, the developmental pathways, which lead to their production, have been preserved. We demonstrated that the same, well-studied hormonal pathway that generates soldiers is also responsible for the production of supersoldiers. Therefore, mutations through genetic drift leading to the loss of the genetic capacity to produce supersoldiers cannot occur, as it would simultaneously lead to the loss of soldiers, a universal and necessary caste of all *Pheidole*. This concept of genes or biochemical pathways maintaining lost traits due to their pleiotropic nature is not new as it dates back to (at least) Sewall Wright, one of the fathers of the Modern Synthesis of evolutionary theory. Lande (Lande, 1978) summarizes Wright's view in that

> *"genes coding for a lost character could be maintained in the genome against the forces of mutation through the pleiotropic action of the genes on other characters which are of selective value to the organism(...) if the parts that affected pleiotropically are highly conservative during evolution, natural selection would tend to maintain the useless organ (or the genes for developing a lost organ) for a long time."*

Despite the proposal of this "pleiotropic preservation of ancestral traits", along with the numerous examples of reversals that directly or indirectly support it (Darwin, 1859, 1868; Hall, 2003; Lande, 1978; Tomic and Meyer-Rochow, 2011; Wiens, 2011), reversals are still viewed as "unlikely…rare at best" (Wake et al., 2011). This current mainstream view states that the developmental pathways underlying complex traits, that are not expressed,

erode over time. Wake et al. (2011) further go on to question recent examples of reversals such as the re-evolution of digits in vertebrates (Kohlsdorf and Wagner, 2006) with counterarguments (based on Galis et al., 2010), although there are well-constructed rebuttals to these counterarguments (Kohlsdorf et al., 2010). Interestingly, Wake et al., (2011) cite a "key" example of irreversibility, the loss of self-incompatibility (SI) in angiospersms (Goldberg and Igic, 2008), which has recently been overturned by the discovery of the re-evolution of SI in an angiosperm (Chantha et al., 2013). This debate will hopefully be resolved when traits and their underlying developmental pathways are traced together in evolutionary studies of reversals taking into account hormonal and genetic forms of pleiotropy.

5.1.3 Ancestral developmental potential: Recurrent induction

If pleiotropy can facilitate the conservation of genes and developmental pathways underlying complex traits which are not being expressed, what are the actual proximate and ultimate mechanisms involved in re-evolving the preserved trait? In Chapter 2, it was proposed that ancestral traits could be activated through the recurrent induction of environmental or genetic perturbations. For example, consider in the ancestor of a group there were traits **A** and **B** (both of which are regulated by a shared developmental pathway) and subsequent species have only **A** while some derived species have re-evolved the **AB** state. Further, consider that trait **B** in the ancestor is normally produced by the activation of a threshold-type pathway due to specific environmental conditions. It is possible that this preserved shared pathway can be activated if species that have only trait **A** are exposed to particular environmental conditions, resulting in **AB** individuals (refer to fig S12 of Chapter 2 for an example model of this). If these environmental

conditions are recurrent and there arises a stable selective pressure for **AB**, then **AB** can potentially evolve. This is essentially what we propose with Chapter 2 except in place of **AB** is soldier/supersoldier, the "specific environmental condition" is nutrition, the "threshold-type pathway" is the JH pathway and a candidate selective pressure is army ant attacks. This scenario represents the key elements of genetic accommodation wherein a trait is induced by environmental or genetic perturbations and natural selection (if the induction is recurrent) can subsequently select for the form and frequency of the trait, which can then be reproduced consistently by the same perturbation (West-Eberhard, 2003).

5.1.4 Ancestral developmental potential: alternative evolutionary strategies

One major concern with this model of ancestral developmental potentials evolving through genetic accommodation is the fact that, in many cases, species only have trait **A** even though they might have both equal exposure to specific environmental conditions that promote **AB** as well as exposure to the same selective conditions. Why have these species not evolved **AB**?

In the case of chapter 2, we demonstrated that larvae of *Pheidole hyatti* can be artificially induced to develop into supersoldiers even though they do not produce supersoldiers in the wild. In addition, *Pheidole hyatti* both live in the same geographic region and are subject to the same selective pressures (army ant predation) as that of species that do produce supersoldiers, like *Pheidole obtusospinosa*. In other words, *Pheidole hyatti* produces trait **A** (soldiers), has the ancestral developmental potential to produce trait **B** (supersoldiers), occurs in similar environment conditions that would

typically induce trait **B**, and is exposed to the same selective pressures (army ants) but, the species have not evolved trait **B**.

We proposed that for *Pheidole hyatti*, and perhaps many other *Pheidole* species, the evolution of supersoldiers was not necessary because they had instead evolved an alternative solution: a multi-stage nest evacuation strategy. It was documented that *Pheidole hyatti* colonies deal with army ants by the following strategy: (1) *Pheidole hyatti* workers, that detected army ant scouts, quickly entered their colony; (2) these workers than made physical contact with other workers causing a chain reaction leading to the awareness of the imminent attack throughout the colony; (3) workers began to immediately recruit one another in patterns that indicated that trail pheromones were laid down in a manner suggesting a distinct escape plan; (4) embryos, larvae and the queen were amassed; (5) evacuation ensued (Droual, 1983). Interestingly, this seemingly risky behavior wherein a whole colony of ants forego their home, is actually not very costly at all. It turns out that these ants are nomadic even without the provocation of an army ant attack (Droual and Topoff, 1981). After the *Pheidole hyatti* colony evacuates their home, they move to another colony that they previously constructed which is lying vacant for them (Droual and Topoff, 1981). In fact they have several vacant colonies that they can easily migrate to (Droual and Topoff, 1981). Therefore, rather than evolving a supersoldier caste to deal with army ants, they have evolved an advanced evacuation plan. In general, species may have an alternative evolutionary strategy to deal with the same selective pressures. Therefore trait **B** (supersoldiers) is not necessary to evolve even if trait **B** sporadically appears due its recurrent induction by environmental conditions that optimally induce trait **B**.

5.1.5 From spontaneous to taxic atavisms: The means to re-evolve

Atavisms, as mentioned in chapter 3, are the reappearance of ancestral traits. Based on Chapter 3, there are essentially 3 types of atavisms: (1) taxic, (2) spontaneous and (3) experimental. Taxic atavisms are synonymous with evolutionary reversals (Stiassny, 2003). There are numerous examples of taxic atavisms including the re-evolution of: wings in stick insects (Whiting et al., 2003), self-incopatibility in angiospersms (Chantha et al., 2013) teeth in amphibians (Wiens, 2011), larval stage in frogs (Wiens, 2011; Wiens et al., 2007), digit number in lizards (Brandley et al., 2008; Kohlsdorf et al., 2010; Kohlsdorf and Wagner, 2006; Siler and Brown, 2011), rotating sex combs in *Drosophila* (Seher et al., 2012), oviparity in sand boas (Lynch and Wagner, 2010), mode of development in marine snails (Collin, 2004; Collin et al., 2007), herbivore defense in plants (Armbruster et al., 2009) and even parasitism in dust mites (Klimov and Oconnor, 2013). Most of these studies were conducted in the past decade and certainly there will be many more to follow.

One way that complex traits can re-evolve in the form of taxic atavisms is through genetic accommodation. In the case of *Pheidole*, anomalous supersoldier-like individuals in species that are not known to produce supersoldiers can be found in the wild. These types of natural sporadic anomalies are also known as “spontaneous atavisms” (Darwin, 1868; West-Eberhard, 2003). These spontaneous atavisms can serve as an evolutionary stepping-stone or as raw materials for natural selection if they are recurrently induced. In the case of the social environment of an ant colony, the persistence of recurrently induced supersoldier-like individuals is facilitated by the maternal care of other workers. Natural selection can then select on the form of the trait (in this case, their large size) and the

frequency of its production. There are many examples of spontaneous atavisms in nature including whales and dolphins with hindlimbs, snakes with additional skeletal elements and humans with tails (Dubrow et al., 1988; Hall, 2003; Tomic and Meyer-Rochow, 2011). Unfortunately, this type of variation is considered to contribute little to the evolutionary process (Levinton, 1986; argued against by Stiassny, 2003). Based on our observations with *Pheidole*, spontaneous atavisms such as these may have led to the innumerable examples of taxic atavisms listed above and thus be of vital importance to their evolution.

Spontaneous atavisms can also contribute to the evolution of novelty through a process called developmental recombination (West-Eberhard, 2005), which was briefly mentioned in chapter 4. When an ancestral trait is induced in a derived species that is separated from the ancestral species by millions of years, it is very possible that cryptic genetic variation has accumulated. When the trait is reactivated, it might exhibit variation compared to the ancestral form, to varying degrees. Parts of the trait might be effectively recalled, other components missing and still others present but slightly different. Therefore, ancestral developmental potential is a means for ancestral traits to be preserved, developmental recombination is the process where this trait can recombine in different ways when activated and subsequently these phenotypic variants can then undergo selection for a specific form and frequency.

5.1.6 Experimental atavisms: The means to study re-evolution

Rather than contemplating over "exceptions" to Dollo's Law and upon further acceptance that trait reversals are more likely to be a general evolutionary pattern, we can focus on elucidating the molecular underpinnings of trait reversions in order to better understand

them. Chapter 2 and 3 suggests that the way to do this is to study experimental atavisms. Experimental atavisms can come in two forms, (1) an ancestral trait can be recalled through crossing and artificial selection or, (2) the result of developmental manipulation. Chapter 3 discussed at length observations of Darwin (1868), which described ancestral reversions caused by crossing in the process of domestication of plants and animals. For instance, artificial selection experiments used in caterpillars led to the discovery of the underlying developmental mechanisms of a polyphenism (Suzuki and Nijhout, 2006). In another study, *Drosophila melanogaster* lines were subjected to repeated environmental shocks, which induced the production of an ancestral insect 4-wing body plan (Waddington, 1956). These lines were then selected for 4-winged individuals over several generations until the environmental shock was no longer needed (Waddington, 1956). The key finding common to these two studies was that the induced ancestral trait could be selected for in as little as 7 generations. Work by Sewall Wright (1934), was also able to estimate the number of genetic and environmental factors involved in the threshold production of guinea pigs with additional toes using crossing methods (Wright, 1934a, b).

Another promising method of studying trait reversions is through developmental manipulations. There have been several incredible examples of ancestral traits being activated through genetic manipulation such as: chickens with teeth (Harris et al., 2006), freshwater stickleback fish with pelvic structures (Chan et al., 2010), flies with hindwings (Waddington, 1956, 1957; Weatherbee et al., 1998) teeth in fish (Jackman and Stock, 2006) and reversed *Drosophila* sex-combs (Seher et al., 2012). Unfortunately, as with spontaneous atavisms, experimental atavisms are given little weight in the study of the

evolutionary process and are typically called “hopeless monsters” (Levinton, 1986). As Chapter 2 demonstrates, experimental atavisms can be used to decipher the hormonal and developmental genetic basis of taxic atavisms. By determining what genes or pathways, upon manipulation, can lead to the development of ancestral traits, we can more properly understand the proximate mechanisms of how trait reversals arise and potentially how they evolve. As discussed in section 5.1.1, this can more generally apply to parallel evolution.

5.1.7 Ancestral developmental potential: from poultry and agriculture to medicine

Chapter 3 was an attempt to try to integrate the findings of Chapter 2 with the literature on atavisms and trait reversals in order to devise a protocol for breeders to use in order to select for traits of interest. With ancestral developmental potentials in mind, breeders could attempt to select for traits, which had previously been selectively advantageous in the past. This could be done with a combination of crossing, environmental perturbation and artificial selection procedures. Furthermore, we highlighted the importance of understanding the degree of pleiotropy involved in a trait that is being artificially selected for in order to predict the occurrence of maladaptive traits and to devise strategies to artificially select them away. The immediate audience of chapter 3 is the poultry research domain but much of the methods could be applied to the agricultural industry as well. In addition, modeling the relationships between atavisms, environmental and genetic perturbations, and the induction of correlated traits due to pleiotropy can also apply to the study of complex diseases and medicine (Tomic and Meyer-Rochow, 2011).

5.2 Majors findings and implications of Chapter 4

Chapter 4 accomplished the second specific goal of my thesis, which was to further understand the developmental epigenetic mechanisms underlying the adaptive morphological variation within ant castes. In addition, novel molecular mechanisms proposed in Chapter 4 have implications concerning the general rules of epigenetic regulation in social insects, the epigenetic basis of trait variation in social insect evolution as well as a better understanding of the role epigenetic mechanisms can play in regulating the environment-dependent production of traits that range from a discontinuous to continuous nature.

5.2.1 DNA methylation and social insects: Role in development and evolution

Till now, there is very little known of the function of DNA methylation in social insects (Weiner and Toth, 2012). One of the key enzymes in implementing DNA methylation, DNMT3, was functionally tested for its role in caste determination in honeybees with RNAi. This resulted in worker-destined bees developing as queens (Kucharski et al., 2008). Although this demonstrated that DNA methylation is involved in caste determination and the generation of phenotypic variation in honeybees, there was no clear idea of what genes were being specifically targeted by the DNA methylation machinery. The results of Chapter 4 demonstrate that the EGFR pathway, which was already functionally implicated in caste development in honeybees, is a specific target of DNA methylation involved in the regulation of sizing in ants. It is likely that many genes that have previously been shown to be involved in phenotypic plasticity (Emlen et al., 2012; Patel et al., 2007) are also regulated by epigenetic mechanisms like DNA methylation.

Sizing variation, whether within castes, between castes, between populations or between species is extraordinarily diverse in ants (Hölldobler and Wilson, 1990; Wilson, 1953). It is very possible that in tandem with hormonal signaling, epigenetic processes like DNA methylation may be important developmental mechanisms involved in generating this size diversity. Molecular details of the thresholds underlying hormonal signaling can evolve through genetic modifications and fixation (West-Eberhard, 2003). In addition, the methylation of DNA can be maintained across several generations (Bossdorf et al., 2008; Jablonka and Raz, 2009; Morgan et al., 1999; Rakyan et al., 2003). Potentially, methylation of key hormonal signaling system genes as well as the genes of other signaling pathways (like EGFR or Insulin signaling) implicated in sizing and caste diversity in ants may be epigenetically inherited. Therefore, this epigenetic process may be involved in the evolution of caste and species-specific sizing in ants.

5.2.2 DNA methylation: from continuous to discontinuous traits

Epigenetic mechanisms, such as DNA methylation, are known to be involved in the regulation of discrete plastic phenotypes, ranging from castes in social insects (Kucharski et al., 2008) to behavior in mammals (Weaver et al., 2004). Findings of chapter 4 demonstrate that continuous variation of a trait can be regulated by quantitative DNA methylation of a particular locus (*Egfr*). It is also possible that DNA methylation can quantitatively regulate the expression of several genes underlying a trait simultaneously. This possibility suggests that epigenetic mechanisms may be contributing together with genetic variation, giving rise to the generation of continuous distributions of quantitative traits. Further integrating epigenetic studies, with that of the myriad of quantitative genetic studies of continuous traits will hopefully provide a step towards understanding

the contributions of genes, the environment and the mechanisms mediating their interactions in generating quantitative traits.

5.3 Concluding thoughts and future research

There are many new questions that are raised from the findings in my thesis such as: what are the precise hormonal and genetic mechanisms that preserve ancestral developmental potentials for millions of years? Recently, two articles came out simultaneously (Colombani et al., 2012; Garelli et al., 2012) which discovered that developing imaginal discs secrete their own unique insulin-like peptide (Dilp8) in *Drosophila melanogaster*. Dilp8 acts as a relay signal to the larval brain, indicating whether the imaginal discs require more time to grow or do not (Hariharan, 2012). If they have terminated growth than metamorphosis ensues, if they require more time to grow, larval development is extended accordingly (Hariharan, 2012). This was demonstrated in the context of overgrowth mutant and regeneration- induced imaginal discs. Overgrowth imaginal disc mutants produce a lot of Dilp8 from their imaginal discs, extending development (Hariharan, 2012), as do regeneration-induced imaginal discs (Hariharan, 2012; Worley et al., 2012).

When developing *Pheidole* larvae initiate caste determination due to a spike in JH, the duration of the final larval instar is extended (Wheeler and Nijhout, 1981c) and their vestigial forewing imaginal discs begin to grow quite rapidly (Wheeler and Nijhout, 1981a). Furthermore, supersoldiers, which have an additional JH pulse, develop much larger and have an additional pair of wing imaginal discs (the hind-pair). We therefore hypothesize that when these imaginal discs begin to proliferate, they may release the ant homolog of Dilp8 causing the extension of growth or the promotion of soldier

development. It is possible that the vestigial wing discs, whose original function is to develop into wings in the queen and male caste, has been evolutionarily co-opted to facilitate the growth of soldiers. Furthermore, it is possible that the reason why the ancestral developmental potential for supersoldier development has been preserved throughout the *Pheidole* group is because of the ability for the vestigial wing imaginal discs to proliferate and grow in the presence of JH, which has been maintained to facilitate soldier development. Therefore, if the right perturbation arises, the developing vestigial wing imaginal discs can further respond to the activation of a second cryptic JH threshold, leading to the production of supersoldiers in species that normally do not produce supersoldiers. This hypothesis would explain why these vestigial structures have persisted throughout ant evolution and develop in *Pheidole* soldier and supersoldier larvae in a specific and consistent way.

Several other questions emerge from the findings of my research: What is the role of epigenetics in the process of environmental induction of ancestral developmental potentials and in the subsequent process of fixation of revealed variation? What is the interplay between hormonal, genetic and epigenetic mechanisms in generating continuous and discontinuous variation in phenotypes in social insects and other organisms? What are the transcriptomic and methylomic differences between minor worker, soldier and supersoldier development, i.e.: how is the genome being dynamically regulated and expressed to give rise to such dramatically different alternative phenotypes? How do artificial supersoldiers that are induced in species that do not produce supersoldiers behave, and in what ways are their behaviors similar or different to species that have evolved supersoldiers? How pervasive are ancestral developmental potentials in the plant

and animal kingdom and are they involved in the various examples of parallel evolution and re-evolution of complex traits?

Based on the literature surveyed in my thesis and the results of chapter 2 and 4, I feel that my thesis work has made a small step towards understanding what are the hormonal and epigenetic mechanisms that can influence adaptive morphological variation within and between castes of ants and how these mechanisms have evolved. These types of developmental processes are conserved throughout plants and animals. Therefore it is possible that the findings made in my thesis can be applied more generally to our understanding of how the environment can influence development and can generate both adaptive plasticity and novel variation which can evolve.

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