Year : 2022

Polyphenism in Pogonomyrmex ants: maternal effects and molecular mechanisms underlying caste determination

Genzoni Eléonore

Genzoni Eléonore, 2022, Polyphenism in Pogonomyrmex ants: maternal effects and molecular mechanisms underlying caste determination

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_FB45E1DEBEDB0

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département d'Écologie et d'Évolution

Polyphenism in *Pogonomyrmex* ants: maternal effects and molecular mechanisms underlying caste determination

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Eléonore GENZONI

Titulaire d'un Master of Science in Behavior, Evolution and Conservation
Université de Lausanne

Jury

Prof. Niko Geldner, Président
Prof. Laurent Keller, Directeur de thèse
Prof. Tanja Schwander, Co-directrice de thèse
Prof. Marc Robinson-Rechavi, Expert
Prof. Abderrahman Khila, Expert

Lausanne 2022

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département d'Écologie et d'Évolution

Polyphenism in *Pogonomyrmex* ants: maternal effects and molecular mechanisms underlying caste determination

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Eléonore GENZONI

Titulaire d'un Master of Science in Behavior, Evolution and Conservation
Université de Lausanne

Jury

Prof. Niko Geldner, Président
Prof. Laurent Keller, Directeur de thèse
Prof. Tanja Schwander, Co-directrice de thèse
Prof. Marc Robinson-Rechavi, Expert
Prof. Abderrahman Khila, Expert

Lausanne 2022

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale
Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Monsieur	Prof.	Niko	**Geldner**
Directeur·trice de thèse	Monsieur	Prof.	Laurent	**Keller**
Co-directeur·trice	Madame	Prof.	Tanja	**Schwander**
Expert·e·s	Monsieur	Prof.	Marc	**Robinson-Rechavi**
	Monsieur	Prof.	Abderrahman	**Khila**

le Conseil de Faculté autorise l'impression de la thèse de

Eléonore Genzoni

Maîtrise universitaire ès Sciences en comportement, évolution et conservation
Université de Lausanne

intitulée

Polyphenism in *Pogonomyrmex* ants: maternal effects and molecular mechanisms underlying caste determination

Lausanne, le 8 avril 2022

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Niko Geldner

REMERCIEMEMENTS

Je tiens à remercier **Laurent Keller** pour m'avoir donné la chance de faire une thèse dans son groupe, ainsi que de la liberté dans le choix de mes projets. Un immense merci à **Tanja Schwander** d'avoir accepté d'être ma co-directrice de thèse, merci pour ton exigence qui m'a poussée à donner le meilleur de moi-même. Tu as su trouver l'équilibre entre me laisser de l'espace pour évoluer à mon rythme et être présente (avec de vrais encouragements !) lorsque ce fut nécessaire. Ce fut un vrai plaisir et un honneur de travailler avec toi.

Je remercie **Marc Robinson-Rechavi**, **Abderrahman Khila** and **Niko Geldner** d'avoir accepté de faire partie de mon comité de thèse et pour la riche discussion lors de la défense privée.

Je souhaite remercier toutes les personnes qui m'ont apporté une aide concrète et/ou du soutien durant ma thèse: **Guillaume Lavanchy**, **Marjorie Labédan** et **William Toubiana** pour m'avoir supporté comme collègue de bureau malgré mes sautes d'humeurs et mes petites « sensibleries », **Claire Morandin**, **Hugo Darras**, **Jérôme Gippet**, **Jelisaveta Djorjevic, Sean McKenzie**, **Quentin Helleu**, **Miya Qiaowei Pan**, **Matthias Rüegg** et **Darren Parker** pour m'avoir aidée de diverses manières, **Laure Ménin** pour avoir finalement réussi à détecter l'hormone juvéniles dans mes œufs de fourmis, **Lena Kolecek** pour le soutien administratif et nos petites discussions, **Zoé Dumas**, **Céline Stoffel**, **Christine La Mendola**, **Françoise Dolivo**, **Loriane Savary** pour m'avoir formée, aidée, renseignée, dépannée au labo, **Falon Pasquier-Genoud** et **Alan Castico** pour votre aide très précieuse avec mes fourmis, **Franck Chalard** pour m'avoir prêté tes outils pour customiser des nids de fourmi et autres bricoles, **Yvan Marendaz** et **Thomas Sauteur** pour avoir, entre autre, géré d'une main de maître tous les problèmes techniques des salles fourmis, et finalement merci à **Amaranta Fontcuberta** pour nos discussions et escapades à la salle de grimpe.

Je remercie tout particulièrement **Jason Buser** entre autres pour tous les bons moments à refaire le monde dans les salles fourmis, **Guillaume Lavanchy** (hé coucou !), collègue et ami d'exception, **Catherine Berney** pour ton aide inestimable pour développer des protocoles et résoudre tous les petits problèmes de labo!

Une pensée à **Silvia Paolucci**, avec qui j'ai réalisé mon premier projet sur les fourmis *Messor* !

Merci à **Raphaële Beck**, **José Rüegger**, **Edith Schneiter**, **Morgane Dickler-Doukelsky** et **Marie Masnerova** pour m'avoir permis de garder un esprit sain dans un corps sain pendant ces 4 ans et demi.

Merci à **Laura Clément** et **Anne-Françoise Vuilleumier**, amies de longue date, pour m'avoir remis les idées en place lorsque c'était nécessaire, soutenue et encouragée !

Je remercie ma famille, et tout particulièrement **Marie-Claire**, ma maman, et mes sœurs **Salomé** et **Elsa**, pour leurs encouragements et soutien, surtout pendant mes interminables mois d'expériences.

Un gigantesque merci à **Sean** pour ton soutien inépuisable à tous les niveaux, tes encouragements et ton aide immense à la fin. Je ne serais pas arrivée aussi loin sans toi ! (P.S. je te revaudrai cela lorsque les rôles seront échangés !)

Et finalement, merci à mes chères ***Pogo*** qui se sont dévouées au nom de la science.

Eléonore

ABSTRACT

Phenotypic plasticity, the capacity of a single genome to produce different phenotypes in response to environmental cues, has been a topic of interest for the past decade. Polyphenism is a special case of phenotypic plasticity in which individuals with the same genome exhibit different phenotypes at the same developmental stage. With their reproductive queens and non-reproductive workers, eusocial insects provide one of the best models to study polyphenism, exhibiting a great diversity of morphology, physiology, behavior, and life history. The aim of my thesis is to contribute to the understanding of phenotypic plasticity by investigating two cases of polyphenism in *Pogonomyrmex* seed harvester ants, the first one on worker size, and the other on the female caste determination (the differentiation into queens and workers). In many ant species, polyphenism is not only found between reproductive queens and non-reproductive workers but also within the worker caste, with the first workers raised in newly founded colonies (nanitics) being much smaller in size than workers raised in mature colonies. In the first chapter, I test whether previously identified miRNAs are involved in the regulation of adult worker size, and more precisely in the development of the nanitic worker phenotype. I experimentally manipulated the levels of the four miRNAs in ant embryos, by injecting a synthetic miRNA, either a mimic or an inhibitor. No difference in size was observed between pupae from the treatment (injection of one synthetic miRNA) and those from the control (injection of water), which strongly suggest that the targeted miRNAs are not involved in worker size. Unexpectedly, I found that the inhibition of one miRNA (miRNA-1) increased the number of larvae developing into gynes. In chapter 2, I investigate the possibility that this miRNA might be involved in caste differentiation. In the following two chapters, I investigated two other instances of caste differentiation: an environmental caste determination using *P. rugosus* species (chapter 3) and a genetic caste determination using *Pogonomyrmex* J lineage (chapter 4). In the third chapter, I report evidence that trophic eggs are an evolved maternal adaptation in *P. rugosus* ants that may regulate caste differentiation. I demonstrate that first instar larvae that consume trophic eggs mostly develop into workers, whereas first instar larvae without trophic eggs mostly develop into gynes, which strongly suggests that trophic eggs influence the larval caste fate. Following this discovery, I investigated the molecular content of these trophic eggs to identify which molecules could induce worker larval development. I found that miRNAs are potential candidates. Finally, in the fourth chapter, I used transcriptomic analysis to investigate the molecular mechanisms underlying gyne and worker caste differentiation in early developmental stages in ants. I found caste-specific gene expression patterns in embryos as early as 24 hours after egg laying, which suggests that caste differentiation starts early in embryonic development in *Pogonomyrmex* J lineages. Moreover, my results support the idea that the queen phenotype is the default developmental pathway in ants and worker development is a derived pathway that need to be actively switched on. Altogether, this thesis proposes the hypothesis that miRNAs and trophic eggs are two novel factors influencing caste determination in *P. rugosus* and provides important insights into the understanding of the molecular mechanisms involved in genetic caste determination in ants.

RÉSUMÉ

La plasticité phénotypique, qui est la capacité d'un unique génome à produire différents phénotypes en réponse à des facteurs environnementaux, est un sujet d'intérêt depuis plusieurs décennies. Un cas particulier de plasticité phénotypique est le polyphénisme, dans lequel des individus avec un même génome présentent des phénotypes différents au même stade de développement. Avec leurs reines spécialisées dans la reproduction et leurs ouvrières non-reproductrices, les insectes eusociaux fournissent l'un des meilleurs modèles pour étudier le polyphénisme, grâce à leur grande diversité de morphologie, physiologie, comportement et histoire de vie. L'objectif de ma thèse est de contribuer à la compréhension de la plasticité phénotypique en étudiant deux cas de polyphénisme chez les fourmis moissonneuses du genre *Pogonomyrmex* ; le premier sur la taille des ouvrières, et l'autre sur la détermination de la caste femelle (i.e. le développement en reine ou en ouvrière). Chez de nombreuses espèces de fourmis, le polyphénisme est observé non seulement entre les castes reine et ouvrière, mais aussi au sein même de la caste ouvrière, les premières ouvrières qui émergent dans les colonies fondatrices (appelées nanitiques) étant beaucoup plus petite que les ouvrières élevées dans des colonies établies. Dans le premier chapitre, je teste l'hypothèse selon laquelle quatre microARNs (miARNs) suspectés d'être impliqués dans la taille des ouvrières, influencent réellement le développement du phénotype nanitiques. J'ai manipulé expérimentalement la concentration de ces quatre miARNs dans les œufs de fourmi en injectant un miARN synthétique, imitateur ou inhibiteur. Aucune différence de taille n'a pu être observée entre les nymphes (dernier stade de développement ayant déjà la morphologie adulte) du traitement (injection d'un miARN synthétique) et celles du contrôle (injection d'eau), ce qui suggère fortement que les miARNs ciblés ne sont pas impliqués dans la taille des ouvrières. De manière inattendue, j'ai découvert que l'inhibition d'un des miARN (miARN-1) semble influencer le ratio des castes reine-ouvrière en augmentant significativement le nombre de larves se développant en reine. Dans le chapitre 2, j'étudie la possibilité que ce miARN-1 puisse être impliqué dans la différenciation des castes reine-ouvrière. Dans les deux chapitres suivants, j'ai étudié deux autres cas de différenciation de la caste : une détermination environnementale de la caste avec l'espèce *P. rugosus* (chapitre 3) et une détermination génétique de la caste avec la lignée génétique J de *Pogonomyrmex* (chapitre 4). Dans le troisième chapitre, j'apporte des preuves à l'appui que les œufs trophiques sont une adaptation évolutive maternelle chez *P. rugosus* et qu'ils pourraient être impliqués dans la différenciation des castes. Je démontre que les larves du premier stade larvaire qui consomment des œufs trophiques se développent principalement en ouvrières, tandis que sans œufs trophiques elles se développent principalement en reines. Ceci suggère fortement que les œufs trophiques influencent le destin des larves vers la voie ouvrière. À la suite de cette découverte, j'ai étudié la composition des œufs trophiques pour identifier quelles molécules pourraient induire le développement des larves en ouvrières. Les miARNs apparaissent comme des candidats potentiels. Enfin, dans le quatrième chapitre, j'ai étudié un cas de détermination génétique de la caste. J'ai utilisé l'analyse transcriptomique pour étudier les mécanismes moléculaires sous-jacents à la différenciation des castes reine-ouvrière dans les premiers stades de développement des fourmis. J'ai trouvé des profils d'expression de gènes spécifiques à la caste dans les embryons dès 24 heures après la ponte, ce qui suggère que la différenciation des castes commence tôt dans le développement embryonnaire des lignées génétiques de *Pogonomyrmex*. De plus, nos résultats soutiennent l'idée que le phénotype de la reine est la voie de développement par défaut chez les fourmis et que la voie de développement en ouvrière doit être activée. En conclusion, cette thèse propose les miARNs et les œufs trophiques comme deux nouveaux facteurs potentiels influençant la détermination de la caste chez *P. rugosus* et apporte de nouveaux éléments pour la compréhension des mécanismes moléculaires impliqués dans la détermination génétique de la caste chez les fourmis.

TABLE OF CONTENTS

- GENERAL INTRODUCTION –

GENERAL INTRODUCTION

Phenotypic plasticity

There is an extraordinary phenotypic diversity between species, driven by the interactions between the genotype of an organism and its environment (Bradshaw 1965; Thompson 1991). Even within a species, or between close relatives, individuals can present extremely different phenotypes despite having similar genomes. This phenomenon is known as phenotypic plasticity, whereby different phenotypes can arise from a single genome under different environmental conditions (Pigliucci 2001; Moczek *et al.* 2011).

Traditionally phenotypic plasticity was limited to the discussion of morphological traits (Woltereck 1909; Schlichting and Pigliucci 1998) triggered against a background of diverse environmental signals. For example the butterfly *Precis octavia* Cramer exhibits extreme seasonal polyphenism, with wing surfaces and colors being dramatically different between the wet and dry seasons (McLeod 2007). Morphological changes can also be triggered by local environmental stimuli, such as the shape of the food on which *Nemoria arizonaria* caterpillars feed, with spring brood resembling the oak catkins they consume, and summer brood feeding on oak leaves and resembling oak twigs (Whitman and Agrawal 2009). The presence or absence of predators can also induce phenotypic plasticity, such as in the case of *Daphnia lumholtzi* with individuals exposed to predators manifesting elongated heat and tail-spines to protect themselves (Engel *et al.* 2014).

However, it is now recognized that phenotypic plasticity is a much wider phenomenon and also incorporates plastic physiological and behavioural changes (Agrawal *et al.* 2007; Whitman and Agrawal 2009). In the case of clownfish, individuals can change sex once reaching a certain size, or in other species, after female disappearance (Casas *et al.* 2016). Similarly, sea-turtles exhibit temperature dependent sex determination (Woolgar *et al.* 2013), illustrating the complex physiological and developmental changes that can occur in response to environmental signals and cues. Behaviour is also included in the umbrella of phenotypic plasticity, with courtship behaviour differing between populations of oceanic sticklebacks, depending on whether group cannibalism is present or absent (Shaw *et al.* 2007). This last example also demonstrates the role of the social environment in phenotypic plasticity with an example of reciprocal phenotypic change between interacting individuals.

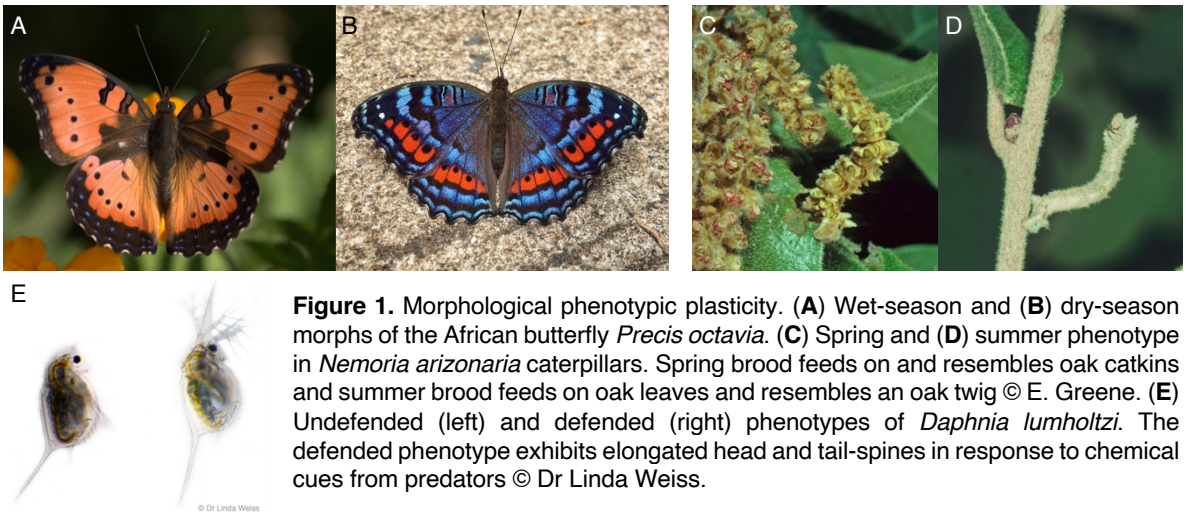


Figure 1. Morphological phenotypic plasticity. (**A**) Wet-season and (**B**) dry-season morphs of the African butterfly *Precis octavia*. (**C**) Spring and (**D**) summer phenotype in *Nemoria arizonaria* caterpillars. Spring brood feeds on and resembles oak catkins and summer brood feeds on oak leaves and resembles an oak twig © E. Greene. (**E**) Undefended (left) and defended (right) phenotypes of *Daphnia lumholtzi*. The defended phenotype exhibits elongated head and tail-spines in response to chemical cues from predators © Dr Linda Weiss.

Why is phenotypic plasticity so important to study?

Phenotypic plasticity is of immense importance to biology. While it is valuable in itself to study plastic responses to assess the potential adaptive advantages they may bring (Wilson and Franklin 2002; West-Eberhard 2003), it may be of even more fundamental importance to our understanding of evolution as phenotypic plasticity can give rise to novel phenotypes that precede genetic changes (West-Eberhard 1989; Whitman and Agrawal 2009; Laland *et al.* 2014). Subsequently the question of how phenotypic plasticity might expand our understanding of adaptation and speciation continues to generate substantial scientific debate (Laland *et al.* 2014). Consider for example the case of the fruit fly, *Rhagoletis pomonella*. Following the colonization of North America by Europeans, a number of non-native fruit tree species were introduced. Although *R. pomonellas* original host plant was the hawthorn tree, this species was subsequently documented as jumping from hawthorn to the newly introduced apple and cherry trees (Bush 1975; Prokopy *et al.* 1988; Feder *et al.* 1994). Hawthorn fruit flies prefer hawthorn fruits (Whitman and Agrawal 2009), however, in their absence, an adult will lay its brood on a novel host, and, when the brood emerge, they behaviourally imprint on this new host species, returning to it when they reach maturity and begin laying their own eggs (Prokopy *et al.* 1988). This behavioural phenotypic plasticity results in host-specific oviposition behaviour (Feder *et al.* 1994) and in turn appears to have become a target of selection resulting in sympatric host race formation. A number of studies have compared the “hawthorn” and “apple” races of *R. pomonella* and described a number of race-specific variants in life-history traits such as variability in the size of the ovipositor (Bush 1969), shifted diapause to match the phenology of host-tree fruit production (Dambroski and Feder 2007) and distinctive physiologies (Feder *et al.* 1997; Filchak *et al.* 2000). Although gene flow still exists between these races, it is low and appears to be decreasing (Ananthakrishnan and Whitman 2005) with a number of authors concluding that this is a case whereby behavioural phenotypic plasticity is driving rapid speciation (Feder *et al.* 1994; Dambroski *et al.* 2005; Bolnick and Fitzpatrick 2007; Whitman and Agrawal 2009).

Developmental plasticity in the eusocial insects

Eusocial insects feature extreme cases of polyphenism, which is a special case of phenotypic plasticity, where organisms express several discrete adaptive phenotypes at the same developmental stage (Moczek 2010), such as the distinct queen and worker phenotypes in ant and social bee and wasp colonies (Miura 2005). These hymenopteran species undergo complete metamorphosis, with a distinct transformation from a single pre-adult form (the larva) to an adult form. Only larvae can express developmental plasticity and alter their ultimate caste fate; once they reach the adult form, there is no more possibility for external morphological change, as adult do not molt any more. Developmental plasticity in social Hymenoptera is largely binary: individuals are either “totipotent”, which is to say that they can become any of the castes existing in that species, or they are “committed”, their caste fate is fixed and they have lost plasticity (Crespi and Yanega 1995; Boomsma and Gawne 2018). An extreme example of fixed castes is found in a few ant species with a genetic caste determination system (see section below; Helms Cahan *et al.* 2002; Julian *et al.* 2002; Volny and Gordon 2002; Helms Cahan and Keller 2003; Schwander *et al.* 2010). There are of course some exceptions, such as *Polistes* paper wasps, which retain their plasticity into adulthood (Jandt *et al.* 2014), and males for which caste fate is determined genetically in all species (i.e. non-fertilized eggs develop into males).

But largely, in the social Hymenoptera, an individual has the potential to become any caste until some point in development, after which it is developmentally committed to a specific caste.

Polyphenisms within worker caste

In many ant species, polyphenism is also observed within the worker caste with the subdivision of the workers into two or more groups of morphologically and/or behaviourally distinct individuals. Their task in the colony is normally allocated according to their specialised phenotypes (Wilson 1971; Oster and Wilson 1978; Scharf *et al.* 2007). For instance, *Pheidole* species have two distinct worker subcastes composed of minor and major workers, the latter exhibiting enlarged head and mandibles used for defense and food processing (Traniello 2010), or in the turtle ants *Cephalotes*, the soldier subcaste use their flat and oversized head as a door to block the nest entrance (Gordon *et al.* 2019). Many species also have distinct worker phenotypes in founding versus established colonies. In species with claustral colony foundation, the queen initiates the colony by herself after the mating flight and she uses her own fat reserves to feed the first developing larvae (Wheeler 1994). In these species, the first batch of workers is relatively small, called “minims” or “nanitics”, and they are normally miniature forms of the smallest workers found in an established colony (Wilson 1971; Porter and Tschinkel 1986; Hölldobler and Wilson 1990). This nanitic phenotype is believed to be an adaptation to maximize the survival of founding colonies (Hölldobler and Wilson 1990). Ant colony survival is strongly affected by the number of workers but as resources are limited at the founding stage, the queen, by producing minims, can divide the limited available resources into an optimum number of workers (Oster and Wilson 1978; Porter and Tschinkel 1986; Hölldobler and Wilson 1990). Therefore the nanitic phenotype may represent an optimal trade-off between number and size, in accordance with environmental factors, such as quantity of food provided to the larvae (Hasegawa and Imai 2012) or queen condition (Liu *et al.* 2001).

Because eusocial insects demonstrate this amazing variety of morphological, behavioural and physiological polyphenism, it has been suggested that the polyphenism they exhibit has heavily contributed to many species ecological dominance (Manfredini *et al.* 2019), and therefore understanding the proximate mechanisms by which eusocial polyphenism arises is critically important to ecology and evolution (Whitman and Agrawal 2009; Revely *et al.* 2021). Eventually, only the identification of the molecular mechanisms enabling plastic responses of the organism to the environment will pave the way for full acceptance of plasticity in evolution and its significance for evolutionary change (Sommer 2020). Such studies must also reveal that plasticity is subject to selection, ultimately resulting in adaptive phenotypes.

Female caste determination

It has been documented in different eusocial species that whether a female egg develops into a worker or into a gyne (virgin queen) is influenced by environmental, nutritional, social, hormonal, pheromonal, epigenetic and/or genetic factors (Haydak 1970; Wheeler 1986; Hölldobler and Wilson 1990; Richards and Packer 1994; Schwander *et al.* 2008; Smith *et al.* 2008; Bignell *et al.* 2010; Miura and Scharf 2011; Guo *et al.* 2013; Walker 2017; Taylor *et al.* 2019). In the honeybee *Apis mellifera*, the quantity and quality of food is known to be responsible for caste differentiation (Asencot and Lensky 1985; Leimar *et al.* 2012; Slater 2017; Slater *et al.* 2020; Vieira *et al.* 2021), as well as maternal effects (Wei et al. 2019), juvenile hormone (Barchuk et al. 2007),

and miRNAs (Guo *et al.* 2013; Shi *et al.* 2015; Ashby *et al.* 2016; Zhu *et al.* 2017; Vieira *et al.* 2021). Different genes and genetic pathways have also been linked to caste determination, TOR (target of rapamycin), hexamerin gene, insulin/insulin-like growth signaling, to cite only a few (Patel *et al.* 2007; Cameron *et al.* 2013; Corona *et al.* 2016). In ants, many factors have been found to influence caste development with variations between species: food, worker:egg ratio, queen hibernation, queen absence, pheromones, age or condition (Libbrecht *et al.* 2013). The genotype of the nurses taking care of the developing larvae can influence caste fate as well (Linksvayer 2006; Linksvayer *et al.* 2011; Teseo *et al.* 2014; Villalta *et al.* 2016). All factors are described as influencing the probability of gyne production, however it remains unknown whether one or several of these factors are necessary or sufficient for gyne development. Finally, in some species of the genus *Pogonomyrmex* (Helms Cahan *et al.* 2002; Julian *et al.* 2002; Schwander *et al.* 2010; Sirviö *et al.* 2011), *Solenopsis* (Hung and Vinson 1977; Helms Cahan and Vinson 2003) or *Messor* (Norman *et al.* 2016), the genotype of the individual is the main determinant of the caste, with queens and workers having different genomic compositions.

What is currently missing in the literature is studies investigating caste determination in early developmental stages before the appearance of morphological differences between queen and worker-destined individuals. Most studies so far have used already morphologically distinct individuals and therefore their comparison does not help in understanding caste determination but rather highlight caste differences in already differentiated individuals. However a major challenge to overcome before such research can be carried out is to find a means to distinguish individuals without morphological differences. Here species with a genetic caste determination system provide an interesting opportunity to study caste differentiation as queen and worker-destined individuals can be separated at any developmental stage through genotyping methods.

It is worth mentioning that while environmental caste determination falls within the generally accepted definition of polyphenism, genetic caste determination does not, as in the latter, the caste fate of the individuals is determined by their genotype and not (or at least not entirely) by environmental factors, therefore two individuals from a different caste do not have a similar genome. In this thesis I have explored both environmental (Chapter II and III) and genetic caste determination (Chapter IV) in *Pogonomyrmex* ants.

Genetic caste determination in *Pogonomyrmex*

P. rugosus overlaps part of its range with another ecologically similar species, *P. barbatus*. Historical hybridization occurred in the overlap region between *P. rugosus* and *P. barbatus* both with an environmental caste determination (ECD), which gave rise to several co-dependent lineages with genetic caste determination (GCD; Helms Cahan *et al.* 2002; Julian *et al.* 2002; Volny and Gordon 2002; Helms Cahan and Keller 2003). These lineages are reproductively isolated from their parental species (Helms Cahan *et al.* 2006; Schwander *et al.* 2007) and characterized by an unusual reproductive system, called social hybridogenesis (Figure 2; Helms Cahan *et al.* 2002; Julian *et al.* 2002), in which the lineages are completely dependent on one another to produce the workers. In this system, the virgin queen needs to mate with at least two males during the mating flight to be able to develop her colony, one from her own lineage and one from the other lineage. Within lineage fertilization gives rise to gyne-destined eggs, whereas eggs fertilized by sperm from male of the other

lineage develop into workers (Helms Cahan *et al.* 2002; Julian *et al.* 2002; Volny and Gordon 2002). Therefore the male genetic material is only used in the alternative lineage to produce nonreproductive individual (i.e. workers) that contribute to colony growth, and is excluded from the reproductive line (i.e. gynes; Helms Cahan *et al.* 2002; Julian *et al.* 2002). Males are haploid and develop from unfertilized eggs laid by queens from both lineages.

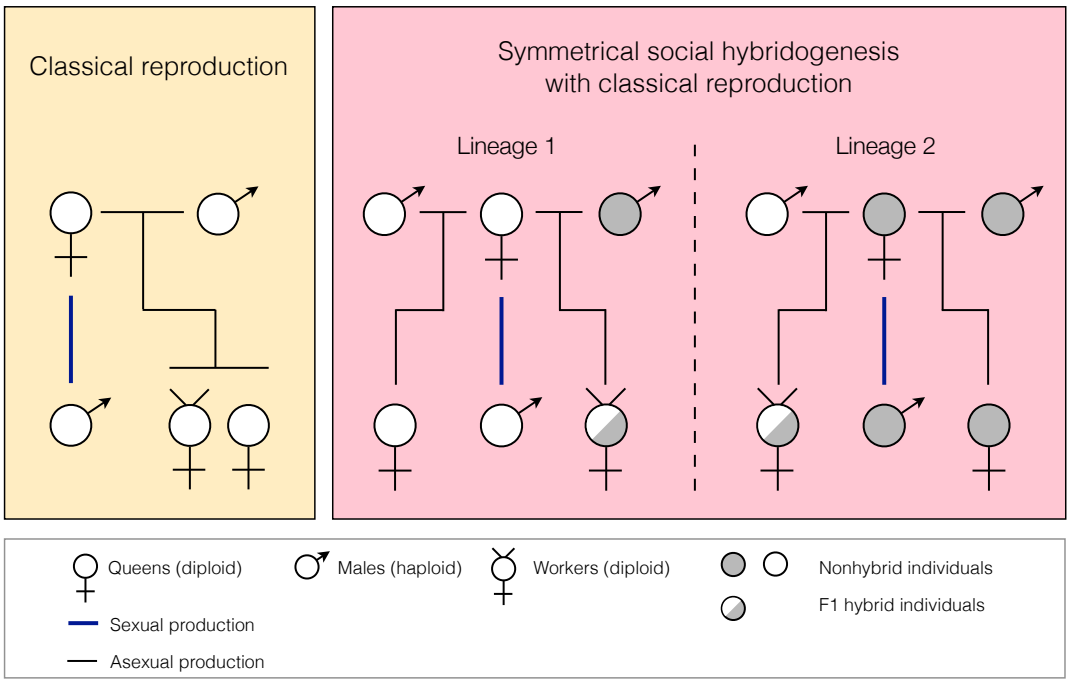


Figure 2. Schematic representation of two reproductive systems in ants, classical reproduction and social hybridogenesis. In the classical reproduction, which is the most widespread reproduction mode in ants, queens mate with one or several conspecific males and use the sperm to produce female offspring (queens and workers). Females are produced by sexual reproduction, they develop from fertilized eggs and are diploid, whereas males are produced by parthenogenesis (arrhenotoky), they arise from unfertilized eggs and are haploid. Some species have evolved unusual reproductive systems (i.e. symmetrical social hybridogenesis) where queens mate with at least one male from their own lineage to produce pure lineage gynes and from the other lineage than their own to produce inter-lineage workers (figure adapted from Darras *et al.* 2019).

MicroRNAs and microinjections

Several chapters of this thesis describe and use microRNAs (miRNAs), that are small non-coding RNAs of 20-24 nucleotides, which main function is the regulation of gene expression at the post-transcriptional level by targeting sites within messenger RNA. In insects, miRNAs have been shown to regulate a variety of physiological processes throughout insect development, including molting, metamorphosis, oogenesis, embryogenesis, behavior and host-pathogen interactions (Asgari 2013; Puthiyakunnon e*t al.* 2013). In addition, miRNAs have many features that make them useful tools for gene expression regulation, such as their small size and an easy targeting design based on sequence complementary. We designed synthetic miRNAs to manipulate the level of endogenous miRNA in ant embryos; miRNAs mimics were designed to mimic endogenous miRNAs, whereas miRNA inhibitors specifically bound to and inhibit endogenous miRNAs.

To address whether and how microRNAs (miRNAs) regulate worker size (chapter I & II), we have developed a microinjection method to deliver synthetic miRNAs into young embryos during their syncytial stage (i.e. when nuclei divide without cytokinesis; Pontieri *et al.* 2020), 8 to 16 hours after egg laying, which is the ideal time for genetic manipulation by microinjection (Yan *et al.* 2017; Sieber *et al.* 2021). Our microinjection method was adapted for *Pogonomyrmex* eggs from methods used in other insects (*Drosophila*, mosquito; Mollinari and González 1998; Déjardin and Cavalli 2004; Gompel and Schröder 2015).

Study species - *Pogonomyrmex* genus

Pogonomyrmex (Hymenoptera: Formicidae: Myrmicinae) is the most abundant genus of harvester ants living in the deserts and grasslands of the south-western North America and Northern Mexico (cf. map), and as their name suggests, they mainly forage for seeds.

Pogonomyrmex rugosus lives underground in nests that can reach a couple of meters under the surface. Nests consist of 12 to 15 broad interconnected galleries radiating out from a central tunnel (Whitford *et al.* 1976). Colonies maintained in the laboratory are generally initiated from newly mated queens collected just after the mating flight, before they start digging their nest, as digging out an established colonies takes 60-90 hours (MacKay 1981), with no guaranty to find the queen.

Like most of the species of this genus, *P. rugosus* is strictly monogynous (i.e. one queen per colony; MacKay 1981). Mating flights takes place between July and August and queens mate on average with 4 to 5 males (Hölldobler 1976). The newly mated queen will then remove her wings and look for a place to start her nest. *P. rugosus* uses a fully claustral colony-founding strategy, meaning that the queen seal herself in the nest after mating and raise the first clutch of workers entirely from body reserves (Hölldobler and Wilson 1990; Hahn *et al.* 2004). Fully claustral colony founding is thought to be a major innovation in ants because it eliminates the need for queens to leave the nest to forage, reducing their exposure to predators, desiccation, and other sources of mortality (Hölldobler and Wilson 1990; Brown and Bonhoeffer 2003). Colonies grow several thousands of workers and produce sexual brood only after several years of initial growth.

The development is separated in three stages: the egg stage which lasts 10 days for both female castes, the larval stage with includes 3 instars whose length vary between castes (approximately 18 days for workers and 30-35 days for gynes, pers. obs.), the pupal stage where there is a complete metamorphosis (approximately 12 days for workers and 14-16 days for gynes, pers. obs.) and finally the adult stage. The entire developmental time from eggs to adult lasts approximately 40 days for workers and 56-59 days for gynes.

In this thesis, I worked with the species *P. rugosus* ECD (environmental caste determination) and with the J1-J2 linages with genetic caste determination (GCD). *P. rugosus* ECD, and GCD J linage cannot be differentiated by their morphology, but can be by color: *P. rugosus* ECD is dark-brown to black, whereas *Pogonomyrmex* J lineages are red (Figure 3). Both present three castes: queens, specialised in reproduction, males whose role is limited to mating with the queens, and monomorphic workers, who accomplish all the other tasks in the colony, such as brood care, foraging, defending the nest, and digging new colony chambers and galleries (Hölldobler and Wilson 1990). In both *P. rugosus* ECD and GCD J lineages, males arise from unfertilized eggs.

Fertilized eggs develop into females in all cases, with the fundamental difference that in *P. rugosus* ECD, all females (i.e. queen and worker) arise from a single genome, their caste fate being determined by environmental factors, whereas in GCD J lineages, the female caste fate is genetically determined, meaning that queen and worker do not share the same genome. Queens develop from pure lineage fertilization (J1J1 or J2J2) whereas workers are interlineage (i.e. hybrid J1J2).

Colonies of *P. rugosus* and GCD J lineages are easy to raise in captivity, providing controlled temperature of 30°C on average and 60% humidity, as well as grass seeds, dead flies, and honey-water for food.

Figure 3. (**A**) *Pogonomyrmex rugosus* workers, (**B**) workers and (**C**) gyne from *Pogonomyrmex* J lineages, (**D**) an egg and freshly hatched larvae of *P. rugosus*. © Images from Dr Elizabeth Cash (www.flickr.com/photos/elizabethcash) and Eléonore Genzoni.

Thesis outline

The aim of this thesis was to provide a better understanding of the proximate mechanisms involved in polyphenism using *Pogonomyrmex* ants as a study species. In chapter 1, I investigated maternal effects on worker size at two different colony life cycle stages. In the following two chapters, I then investigated female caste determination in *P. rugosus* with environmental caste determination. In the last chapter, I worked with genetic lineages of *Pogonomyrmex* ants to investigate a case of genetic caste determination.

Recently, different microRNAs (miRNAs) levels have been found between eggs laid by founding and established queens of *P. rugosus* ants, which suggests that miRNAs are involved in worker size. In **Chapter 1**, I aimed at proving the influence of the previously identified miRNAs on the development of the nanitic worker phenotype (i.e. small workers developing in newly founded colonies). I experimentally manipulated the levels of four miRNAs in ant eggs, by injecting either a synthetic miRNA or a miRNA inhibitor. I injected the miRNAs in eggs laid by established queens to recreate the environment of an egg laid by a founding queen, and I therefore expected to obtain the development of smaller workers (i.e. nanitic phenotype) compared to the control ones. I did not find a difference in size between pupae from the treatment (injection of one synthetic miRNA) and those from the control (injection of water), which strongly suggest that the targeted miRNAs are not involved in worker size. However, instead of a difference in worker size, I found that the inhibition of one miRNA (i.e. miRNA-1) influenced the gyne:worker caste ratio. I found a significantly higher proportion of gynes developing from eggs treated with the miRNA inhibitor compared to eggs subjected to a control treatment, with 50% and 22% of gynes, respectively. This study provides evidence that the inhibition of miRNA-1 is associated with caste differentiation, by triggering gyne development. This unexpected result is presented in **Chapter 2**.

Since the beginning of my PhD, all the different projects or investigations I carried out involved ant eggs, which led to the discovery of trophic eggs in *P. rugosus* (ECD) as well as in *Pogonomyrmex* J lineages. I reviewed the literature on trophic eggs, especially within eusocial insects, and found relatively little information, which left most of following questions unanswered: By which caste, queen and/or worker are trophic eggs laid? Do season or time of the year (before-after hibernation) influence the number or proportion of trophic egg? Are trophic eggs laid at any colony life cycle stages (colony founding versus established stage)? What do they contain? What is their function? To answer some of these questions, I decided to investigate trophic eggs in *P. rugosus* ants and the results are presented in **Chapter 3**. I first characterized *P. rugosus* queen's egg laying behaviour as well as trophic and reproductive eggs. I found that only the queen lays both reproductive and trophic eggs, whereas workers, which lay eggs in queenless colonies, only produce reproductive eggs (that can develop into males). Trophic eggs differ morphologically and quantitatively from reproductive eggs, with less protein, triglycerides, glycogen, sugar and small RNAs. I then found empirical evidence supporting a role of trophic eggs on female caste determination in *P. rugosus*. I showed that first instar larvae that consume trophic eggs mostly developed into workers, whereas larvae without trophic eggs mostly developed into gynes, which strongly suggests that trophic eggs influence the larval caste fate. Following this discovery, I investigated trophic egg content to identify which molecules could influence larval development in the direction of the worker caste. Comparison of miRNAs fragment size distribution suggests differences in the composition of these miRNAs between the two egg types, which reveals miRNAs as potential candidates to influence larval caste

fate. These results raise the possibility that trophic eggs are an evolved maternal adaptation involved in caste determination in ants.

In contrast to the many identified environmental factors affecting queen vs worker production, little is known about the molecular mechanisms regulating queen vs worker development. Several genomic studies have identified genes differentially expressed between castes, at the adult stage in different social insects (Sumner *et al.* 2006; Gräff *et al.* 2007; Grozinger *et al.* 2007; Hoffman and Goodisman 2007; Toth *et al.* 2007; Hunt *et al.* 2010; Colgan *et al.* 2011; Ometto *et al.* 2011). However, adults are morphologically and physiologically differentiated and therefore do not provide information on caste determination. Investigations at early developmental stages have thus far only been done in bees where individual caste fates are known (Pereboom *et al.* 2005; Cameron *et al.* 2013) as a result of distinctive differences between worker and queen cells (Shi *et al.* 2011). In **Chapter 4**, I investigated the molecular mechanisms underlying gyne versus worker caste differentiation in early developmental stages using the J lineages of *Pogonomyrmex* ants. Thanks to their genetic caste-determination system, I could determine the caste-fate of an individual at any developmental stage via genotyping approaches. I used transcriptomic analysis to identify caste-biased genes and profile gene expression dynamics at five early developmental stages: in 24-hour old, 6-days old and 9-days old eggs, and in first and second instar larvae. I found caste specific gene expression patterns in embryos as early as 24 hours after egg laying, and two developmental stages show a burst of caste-biased genes, in 6 day old eggs and in the first instar larvae, which suggests that gyne and worker caste differentiation starts early in embryonic development in *Pogonomyrmex* J lineages. There were more worker-biased than gyne-biased genes in all five developmental stages, which supports the idea that the queen phenotype is the default developmental pathway in ants and worker developmental paths are actively switched on.

The concluding chapter highlights some ongoing work and potential future projects on caste determination in *Pogonomyrmex* ants.

Glossary

Adult The final stage after the last ecdysis.

Caste Social insects are usually divided in three castes, each having a specialized function in the colony and characterized by anatomical, morphological, and behavioural differences: reproductive females (queens), non-reproductive females (workers) and males. Some species have a subdivision of the worker caste in several groups characterized by different morphologies, size, and behaviour, such as the soldier, forager, and nurse; they are called worker castes or subcastes.

Caste determination In social insects, whether a female egg or larva develops into a worker or into a queen is influenced by environmental, nutritional, social, hormonal, pheromonal, epigenetic and/or genetic factors. Most ant species have an environmental caste determination system, and a few species have a genetic caste determination system, which means that the castes are not determined by environmental factors but by the genotype of the individual.

Established stage Life cycle stage where the colony has reached a critical size (usually several years after colony foundation) and starts producing gynes and males.

Founding stage First colony life cycle stage. After mating, the queen(s) starts a new nest and raises her first worker offspring.

Gyne Virgin female which lives in the colony until the mating flight ,after which she is referred to as a queen and start her own colony. In most ant species, gynes have wings that they use for the mating flight and remove soon after.

Larva Juvenile form, between the egg and pupa developmental stages. Composed of several instars each separated by a molt allowing larval growth. Social insect larvae are non-motile and therefore dependent on workers and/or queen (in the founding stage) to be fed.

Males Winged individuals, which are generally present within a colony for only a short time each year and typically live a few weeks, dying very soon after mating.

miRNA (microRNA) Small single-stranded non-coding RNA molecule containing between 20 to 24 nucleotides found in plants, animals, and some viruses, which regulates gene expression at the post-transcriptional level.

miRNA inhibitor Synthetic miRNA designed to specifically bind to and inhibit endogenous miRNAs, resulting in up-regulation of target mRNA translation.

miRNA mimic Synthetic miRNA designed to mimic endogenous miRNAs, resulting in down-regulation of target mRNA translation due to mRNA sequestration or degradation.

Nanitic worker First workers that develop in a founding colony which are characterized by a relatively smaller size than workers developing in an established colony.

Pupa Last developmental stage before adulthood that undergo complete metamorphosis.

Queen Reproductive female which, after having mated with one or several males, will normally remove her wings and start her own colony.

Reproductive egg Egg which contains an embryo developing into a female (worker or queen) if fertilized, or into a male if not fertilized.

Trophallaxis Transfer of fluids between colony members through mouth-to-mouth or anus-to-mouth, which can contain nutrients, molecules such as hormones or pheromones, symbionts, etc.

Trophic egg Egg which does not contain an embryo, in species where recorded, differ from reproductive eggs in morphology and size, and are usually eaten by larvae and other colony members.

Worker Non-reproductive females, always wingless, they do all tasks in the colony apart from reproduction and typically live from several months to a few years. Depending on the species, workers can lay unfertilized eggs that develop into males, trophic eggs, or may lack ovaries completely.

CHAPTER 1

Maternal effect on worker size

E. Genzoni, S. Paolucci, L. Keller and T. Schwander

Author Notes

This chapter is dedicated to the memory of Dr. Silvia Paolucci.

Author contributions: EG and TS designed the study. EG performed the experiment and analysed the data. EG wrote the manuscript, with input from TS and LK.

Abstract

In many eusocial Hymenopteran species, polyphenism is not only found between reproductive queens and non-reproductive workers but also within the worker caste. In newly founded ant colonies, the first workers to eclose (nanitics) are much smaller in size than workers in mature colonies. Recently, different levels of miRNAs have been found between eggs laid by founding and established queens of the ant species *Pogonomyrmex rugosus*, which suggests that miRNAs are involved in worker size. In order to investigate the potential role of miRNAs in worker size, we experimentally manipulated the levels of four miRNAs in ant eggs, by injecting either a synthetic miRNA or a miRNA inhibitor. Injections were performed on eggs laid by established queens, to recreate the miRNA environment of an egg laid by a founding queen. We did not detect a difference in size between pupae that developed from eggs injected with a miRNA compared to those injected with water as a control. Our results strongly suggest that the targeted miRNAs are not involved in worker size. It is highly likely that they are involved in another developmental or physiological pathway which differs between colony life cycle stages, such as miRNA-1 which has been found to influence caste ratio (see Chapter 2).

Keywords: phenotypic plasticity, miRNA, colony life cycle stages, ants, *Pogonomyrmex rugosus*.

Introduction

In many eusocial species, polyphenism is not only found between reproductive queens and non-reproductive workers but also within the worker caste. Workers can exhibit extreme differences in their morphologies that are related to their specific roles in the colony (Oster and Wilson 1978; Hölldobler and Wilson 1990). Many species also have distinct worker phenotypes in founding versus established colonies. In species with claustral colony foundation, the queen initiates the colony by herself after the mating flight and she uses her own fat reserves to feed the first developing larvae (Wheeler 1994). The first few workers are relatively small, called "minims" or "nanitics", and they are either miniature forms of the smallest workers found in an established colony (Wilson 1971; Hölldobler and Wilson 1990), or constitute a distinct caste from the other workers, as concluded by Watanabe *et al.* (2017), in the ant *Camponotus obscuripes*. This worker polyphenism across the colony life cycle is believed to be an adaptation to optimize the survival of founding colonies (Hölldobler and Wilson 1990). Colony survival is strongly positively affected by the number of workers. Therefore, by producing minims, the queen divides the limited available resources into an optimum number of workers (Oster and Wilson 1978), which is determined by the balance of the advantages of a larger initial worker force and the disadvantages of smaller body size (Porter and Tschinkel 1986; Hölldobler and Wilson 1990). Therefore in ants, the size of the first workers is determined by the trade-off between number and size (Watanabe *et al.* 2017), which remain flexible and change in accordance with environmental factors (Liu *et al.* 2001; Hasegawa and Imai 2012). Porter and Tschinkel (1986) showed that in *Solenopsis invicta*, the numerous nanitic workers, although less efficient on an individual basis to rear brood than minor workers, are more efficient as a group of equal combined weight.

The nanitic size of founding colony workers is generally believed to stem from the limited nutritional resources available during larval development (Hasegawa and Imai 2012). However, a cross-fostering experiment in *P. rugosus* ants showed that worker size was also constrained by maternal effects (Paolucci *et al.* unpublished data). Maternal effects occur when the phenotype of the mother or the environment she experiences influence the phenotype of her offspring over and above the direct effect of transmitted genes (Marshall and Uller 2007). For the cross-fostering experiment, eggs laid by founding and established queens were collected. These eggs were reared in recipient colonies of workers from established colonies. The brood was collected at the pupal stage for size measurements. The results showed that, when reared under the same conditions, pupae originating from eggs laid by established queens were on average 11% larger than pupae originating from eggs laid by founding queens (Paolucci *et al.* unpublished data). This indicates that there is a maternal effect on worker size and suggests that queens can influence the development of their offspring through manipulating egg composition. In addition to the cross-fostering experiment, microRNAs (miRNAs) were isolated from eggs from founding and established queens. MiRNAs are small non protein coding RNAs of 18 to 24 nucleotides in length, produced by animals, plants and viruses (Jones-Rhoades and Bartel 2004; Wienholds *et al.* 2005; Hussain *et al.* 2008). They modulate gene expression at the post-transcriptional level in numerous important biological processes, such as development, differentiation, apoptosis and immunity (Lee *et al.* 1993; Bushati and Cohen 2007). MiRNA typically binds complementary target sites within messenger RNAs (mRNAs), resulting in either translational inhibition or mRNA degradation (Bartel 2004).

Paolucci *et al.* (unpublished data) found differences in the quantity of miRNAs in eggs laid by founding and by established queens. They identified 237 miRNAs, including 80 conserved miRNAs described in *A. mellifera* and 157 novel miRNAs that have not previously been reported in any other species. Twelve of the 237 miRNAs had significantly different concentration in eggs laid by founding queens compared to established queens, of which eight were conserved miRNAs (four at low concentration and four at high concentration in founding queen's eggs) and four were novel miRNAs (three at low concentration and one at a high concentration in founding queen's eggs). They suggested that those miRNAs could be involved in worker size, and more precisely in the development of the nanitic phenotype.

To assess the involvement of miRNAs on worker size, we designed an experiment aiming at recreating the nanitic phenotype. For this, we experimentally manipulated the levels of four miRNAs in eggs by injecting synthetic miRNAs and miRNA inhibitors. The injections were designed to change miRNA levels in eggs from established queens to miRNA levels in eggs from founding queens. Therefore, we expected workers developing from established queens' eggs that were injected with a miRNA to have a smaller size than workers developing from control eggs.

Materials and methods

The aim of this study was to influence embryonic development using synthetic miRNAs. Among the 12 miRNAs whose concentration differed between eggs from founding vs established queens (Paolucci *et al*. unpublished data), we selected the 4 miRNAs with the highest consistent difference in concentration between replicates. Two had a low concentration in eggs from founding queens (miRNA-1 and miRNA-2) and the two others had a high concentration in eggs from founding queens (miRNA-3 and miRNA-4). The bee homologue of miRNA-2 has been described as associated with lipid loss in *A. mellifera*, involved in regulation of behavioral maturation and was up-regulated in 4-day-old queen larvae (Chen *et al.* 2017). The miRNA-4 has also been identified in *A. mellifera* but its function remains unknown. MiRNA-1 and miRNA-3 are novel miRNA.

The four selected miRNAs were used as a template for the creation of synthetic miRNAs (done by Qiagen). MiRug-u3 and -u4 were created to mimic the naturally occurring miRNAs found in founding queen's embryo (miRNA-3 and miRNA-4 respectively), whereas miRug-d1 and -d2 were created to act as inhibitors of miRNA-1 and miRNA-2 respectively.

Two experiments were carried out using *Pogonomyrmex rugosus* colonies. The first one took place after hibernation (January to March 2020; hereafter referred to as Exp. A) and the second one before hibernation (July to September 2020; hereafter Exp. B). Each colony was initiated by one queen collected after the mating flight in 2008 (Bowie, Arizona, USA) and 2013 (Bowie and Florence, Arizona, USA). They were maintained in the laboratory at 28°C with 60% humidity, with a 12-h/12-h light:dark cycle and fed once a week with grass seeds and flies, and 20% honey water every 3 days.

For Exp. A, 12 and 18 colonies were used for miRug-d2 and miRug-u3 groups, respectively. An unexpectedly high proportion of gynes developed in both the treatment and control recipient colonies, decreasing substantially the number of worker pupae required for size comparison. The fact that the experiment was carried out after hibernation could explain the high proportion of gynes, which develop after hibernation in wild colonies. We therefore stopped the experiment prior to the injections of the two other miRNAs (miRug-d1 and miRug-u4). For Exp. B, 10 to 13 colonies were used for each of the four miRNAs injected individually as well as for all together (five different treatments).

The experimental design was the same for both experiments. As the egg laying rate decreases after 24 hours of isolation (pers. obs), we isolated the queens for 16 hours for egg laying, with two egg collections (every eight hours). The queens were then put back in their native colony for eight hours until the next isolation session. After three days under this isolation schedule, the queens were returned to their native colonies for an uninterrupted 24-hour break.

After the egg collection, reproductive eggs were kept in a clean and humid petri dish for eight hours until the injection. Eggs ranged from eight hours old (if laid at the end of the isolation period) to a maximum of 16 hours (if laid at the start of the isolation period) at the injection time. Reproductive eggs laid within the same eight hours were randomly separated into two groups; half were injected with ~7nl of 10pmol/μl miRNA and half

injected with Milli-Q® ultrapure water as a control. For the treatment with all miRNAs together, the concentration of each miRNA was the same as when injected individually (i.e. 10pmol/μl).

For the injection, the eggs were placed on a 600μm nylon mesh, to prevent them from moving during the injection. Injections were performed manually using a FemtoJet ® 4x micro-injector (Eppendorf) with glass needles made from pulled capillary tubes using a P-97 Pipette Puller. The exact settings were adjusted for every needle separately to have an identical volume injected in the eggs for every injection session. Eggs stayed in the mesh, untouched, for two hours to recover from the injection. They were then removed carefully from the mesh and placed into a petri dish containing a water reservoir for humidity where they developed for 10 days. Larvae were collected right after hatching (petri dishes monitored every four hours) and placed into recipient colonies as they need care and food to develop until pupation. We created three recipient colonies for the control and three for the treatment to take into account the box effect of the recipient colonies on the developing larvae. In addition, for miRug-u4 treatment in Exp. B, when each larva was placed in a recipient colony, we also transferred three trophic eggs, to increase larval developmental success (pers. observation). A recipient colony contained 20 foragers taken from the foraging arena and 20 nurses taken from inside the nest. There was no crossfostering between the origin of the eggs and of the workers, so they both came from the same native colony. When pupae developed black pigmentation in the eyes, individuals were collected, pictures were taken using a stereo microscope and the head width and tibia length were measured using Zen light software (Carl Zeiss Microscopy).

We compared pupal head width and tibia length between the treatment and control group, within caste and native colony. We used Rstudio (RStudio Team 2015) and the package *lme4* (Bates *et al.* 2015) to perform a linear mixed effects analysis, with head width as response and treatment type as explanatory variables. We used recipient colonies nested in native colony as random effects. Recipient colonies missing pupae in the treatment or control group were excluded from the analysis as average worker size varies between native colonies and therefore requires data from both control and treatment to make a meaningful comparison within native colonies.

To test whether the treatment might influence the survival (from egg to larvae and from larvae to pupae), we performed a linear mixed effects analysis with survival as response variable, treatment type as an explanatory variable, and colony as random effect.

Results

For Exp. A, a total of 589 and 1204 reproductive eggs were collected for miRug-d2 and miRug-u3 groups, respectively, and a total of 85 and 385 pupae (details in Table 1) developed from those eggs, which represent a developmental success of 14.4% and 32.0%, respectively. Pupae developed in both the treatment and control groups of 7 colonies (out of 12) for miRug-d2 and in 15 colonies (out of 18) for miRug-u3.

For Exp. B, a total of 1100, 612, 851, 396 and 756 reproductive eggs were collected for the groups miRug-d1, -d2, -u3, -u4 and all miRNAs together, respectively, and a total of 177, 170, 297, 159 and 117 pupae (details in Table 1) developed from those eggs, which represents a developmental success from egg to pupae of 16.1%, 27.8%, 34.9%, 40.2% and 15.5% respectively. In Exp. B, pupae were found in both the treatment and control groups in all 10 colonies for miRug-d1, in 10 out of 11 for miRug-d2, in all 13 colonies for miRug-u3, in all 9 colonies for miRug-u4 and in 9 out of 10 colonies for all miRNAs injected together (details in Table 1).

Table 1. Number of eggs injected, number of pupae collected, developmental success from egg to pupae for Exp. A and B, per miRNA groups, for treatment and control. The small numbers on the right in each column show the total number of eggs, the total number of pupae and the average between miRNA treatment and control, respectively. The last column gives the number of colonies in which pupae developed in both the treatment and control groups.

			Number of eggs injected		Number of pupae		Developmental success		Number of colonies producing pupae
Exp. A	miRug-d2	Treatment	295	589	48	85	16.3%	14.4%	7 out of 12
		Control	294		37		12.6%		
	miRug-u3	Treatment	605	1204	200	385	33.1%	32%	15 out of 18
		Control	599		185		30.9%		
Exp. B	miRug-d1	Treatment	553	1100	80	177	14.5%	16.1%	10 out of 10
		Control	547		97		17.7%		
	miRug-d2	Treatment	309	612	87	170	28.2%	27.8%	10 out of 11
		Control	303		83		27.4%		
	miRug-u3	Treatment	424	851	162	297	38.2%	34.9%	12 out of 13
		Control	427		135		31.6%		
	miRug-u4	Treatment	201	396	85	159	42.3%	40.2%	9 out of 9
		Control	195		74		37.9%		
	All miRNAs	Treatment	377	756	64	117	17.0%	15.5%	8 out of 10
		Control	379		53		14.0%		

In Exp. A, there was no significant difference in worker or gyne size (head width or tibia length) between treated and control pupae, neither for miRug-d2 (Figure S1) nor miRug-u3 groups (Figure 1A; details per colony on Figure S2). In Exp. B, there was no significant difference in worker size between miRug-u3 treated and control pupae (Figure 1B, details per colony on Figure S5). However, gyne pupae treated with miRug-u3 were significantly smaller than the control ($\beta = -0.11$, $SE = 0.03$, $t_{(23)} = -3.29$, $p = 0.003$; Figure 1B). No difference in size between treated and control pupae was found among workers or gynes in all the other miRNA groups (miRug-d1, miRug-d2, miRug-u4 and all miRNAs together; Figure S3-S4,S6-S7 respectively).

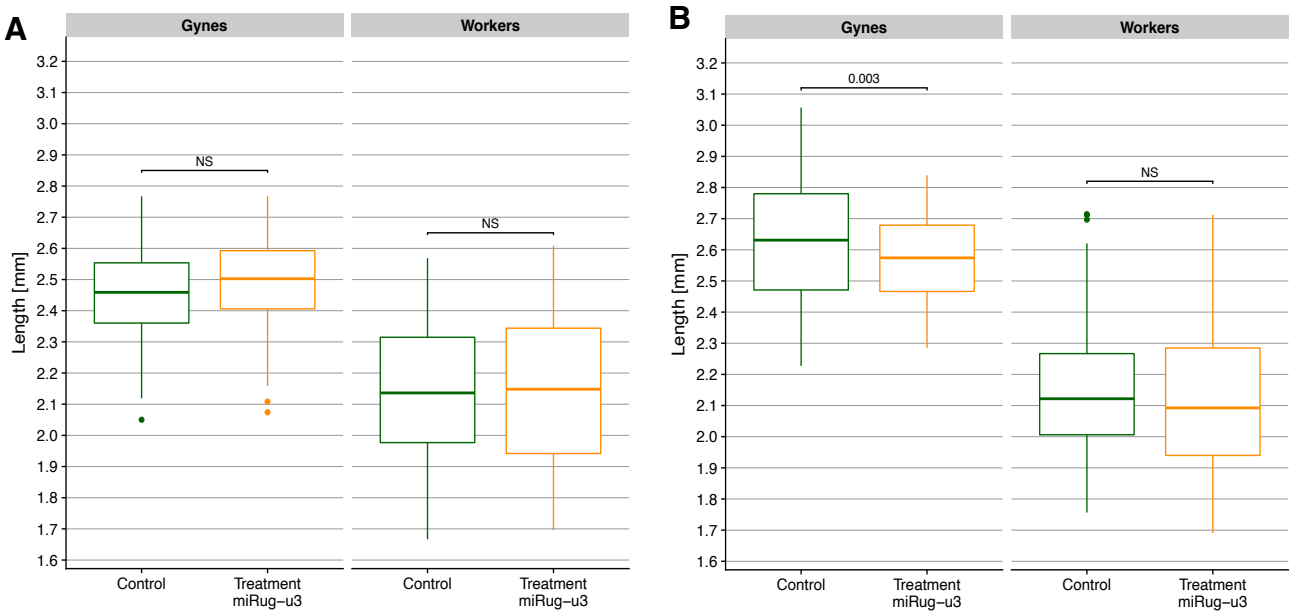


Figure 1. Workers and gynes head width from miRug-u3 and control groups, for Exp. **A** and Exp. **B**.

In Exp. A, there was no significant effect of the injection on survival from egg to larvae nor larvae to pupae neither for miRug-d2 nor miRug-u3 (Table 2). In Exp. B, there were significantly more larvae that hatched from eggs injected with miRug-d2 (55.6±5.5% mean ± SE) than from control eggs (41.9±7.5%; β = 13.97, SE = 4.41, t(9) = 3.17, p = 0.011). There was no significant difference in survival for the three other miRNAs in any transitions (egg to larvae and larvae to pupae; Table 2).

Table 2. Percentage of survival at two developmental stages: egg to larvae and larvae to pupae, for Exp. A and Exp. B (average ±SE). ♣ Each larva from miRug-u4 group (both treatment and control) was placed in its respective recipient colony with 3 trophic eggs.

Percentage of survival (average ±SE)		Exp. A		Exp. B	
		Egg to larva	Larvae to pupa	Egg to larva	Larvae to pupa
miRug-d1	Treatment	-	-	47.2 ±7.3%	19.8 ±5.0%
	Control	-	-	42.9 ±7.7%	21.8 ±4.5%
miRug-d2	Treatment	46.3 ±5.6%	41.2 ±10.6%	55.6 ±5.5% *	30.3 ±9.6%
	Control	38.9 ±5.4%	44.1 ±10.2%	41.9 ±7.5% *	29.4 ±8.8%
miRug-u3	Treatment	54.8 ±2.8%	39.5 ±3.8%	57.9 ±5.0%	32.5 ±5.8%
	Control	58.3 ±1.9%	36.1 ±3.1%	52.4 ±4.4%	35.4 ±8.0%
miRug-u4	Treatment	-	-	64.9 ±7.9%	27.0 ±3.9% ♣
	Control	-	-	53.6 ±8.5%	37.4 ±6.8% ♣
All miRug injected together	Treatment	-	-	43.0 ±6.6%	26.5 ±11.0%
	Control	-	-	41.3 ±5.5%	21.5 ±7.1%

Discussion

Our results do not support the hypothesis that either of the four tested miRNAs play a role in worker size. While gyne pupae treated with the miRNA mimic miRug-u3 had a significantly smaller head width than the control ones in Exp. B, this result was not reproducible, and in all other experiments there was no statistically significant effect of any of the miRNAs tested.

A negative result often requires a higher degree of confidence, especially in an emerging field of science where there are a number of technical challenges. The lack of an effect of the miRNAs on worker size could arise from erroneous timing of the injection or the amount of miRNA injected. However, in our study, eggs were injected at the syncytial stage (i.e. when nuclei divide without cytokinesis; Pontieri *et al.* 2020), 8 to 16 hours after egg laying, which is the ideal time for genetic manipulation by microinjection (Yan *et al.* 2017; Sieber *et al.* 2021). The quantity of miRNAs injected in zygote, embryo or larva of different organisms varies substantially between studies, ranging from $1.5*10^{-7}$ to 0.5 nmol (see Table S1). Here, we injected $7*10^{-5}$ nmol per egg, which falls in the range of previous studies, but is about 7 times higher than the amount recommended by Mollinari and González (1998) for *Drosophila*. In addition, in chapter 2 we found that one synthetic miRNA, miRug-d1, influenced the caste ratio by significantly increasing the proportion of gynes. This strongly supports the fact that the miRNAs were injected at the appropriate time and concentration to influence the embryo's development.

Another consideration which might explain the negative result of our miRNA injections is redundancy. A single miRNA can target a large number of mRNAs and a single mRNA can be targeted by several miRNAs (Palanichamy and Rao 2014; Fischer *et al.* 2015; Guo and Wang 2019). This is of relevance for our two synthetic miRNA inhibitors miRug-d1 and miRug-d2. Despite their inhibiting action on miRNA-1 and miRNA-2 respectively, other miRNAs might have substituted their action, and therefore the miRNA inhibition would have been counteracted by the action of redundant, non-inhibited, miRNAs. However, miRug-d1 did have a highly significant effect on development (see Chapter 3) in that it influenced caste ratio. This suggests that redundancy is unlikely to have affected the outcome reported here, as this does not reconcile with the effect on development reported in Chapter 3. Therefore, we conclude that redundancy, whilst an issue that needs further consideration for miRug-d2, did not affect the outcome of the miRug-d1 injection experiment.

Finally, we have not tested whether the synthetic miRNAs acted as intended, either as a miRNA mimic or miRNA inhibitor. Several studies have demonstrated that synthetic miRNAs sometimes, and for unknown reasons, do not act as designed to and specifically activated targeted genes instead of acting as posttranscriptional gene silencers and inducing mRNA transcript degradation (Li *et al.* 2006; Vasudevan *et al.* 2007; Turner *et al.* 2014). If this happened in our experiments, it would mean that the injections did not mimic the environment of an egg laid by a founding queen and therefore did not produce the expected phenotype (i.e. nanitic workers). For instance, miRug-u3 and miRug-u4 were designed to mimic miRNA-3 and miRNA-4, and therefore aimed at inhibiting their respective mRNA targets. If they did not mimic but rather inhibited their respective miRNA, we would be unable to detect a decrease in size among the pupae. Further study should demonstrate that the synthetic miRNAs do influence the concentration of the targeted miRNAs, by performing

Northern blot analysis or quantitative real-time PCR (qPCR) using total RNA, and probes or primers specific for a given miRNA and mRNA target. This would allow to rule out any problem of accuracy and specificity of the designed miRNAs.

With regards to the Paolucci *et al.* findings, that reported miRNAs associated with worker size, and provided the basis for this chapter, two elements potentially explain why we did not confirm their results. First, the difference in miRNAs expression between eggs laid by founding and established queens (Paolucci *et al.* unpublished) could have been a false positive, which would explain why manipulating the titres of the four miRNAs in the eggs did not influence worker size. The second potential explanation is linked to the miRNA quantification done by Paolucci *et al.*, which may have been biased by the presence of trophic eggs among their samples. When they collected the eggs for miRNA expression investigation, trophic eggs had not been identified in this species yet. Therefore, their samples contained a mix of reproductive and trophic eggs of unknown ratio, which, if different between founding and established queens, may have biased the miRNA titres and furthermore, the miRNA expression difference between the two queen's groups. Trophic eggs have been identified afterwards in established colonies, with a range between 30 - 50% of all eggs laid recorded as trophic (pers. obs, see chapter 3). We assume that there was a similar percentage among the established colonies at the time of Paolucci *et al.*'s sampling. However, the percentage of trophic eggs laid by founding queens of this species is currently unknown. In *Solenopsis invicta* and *Atta sexdens*, the percentage of trophic eggs laid by founding queens was 46.8% ± 4.5% (average ± SE; Glancey *et al.* 1973) and 90% (average; Huber 1907), respectively, and for established queens it was 9.6% ± 6.6% (Glancey *et al.* 1973) and 7.7% (Della Lucia *et al.* 1990; Augustin *et al.* 2011), respectively. If *P. rugosus* follow the same pattern as *S. invicta* and *A. sexdens*, then it is likely that the percentage of trophic eggs laid by *P. rugosus* founding queens was potentially much higher at this early stage in comparison to the established stage. It is currently unknown what compounds are present in trophic eggs, but if those miRNAs are not present or present but at different concentrations, this becomes of extreme relevance as then the titres collected by Paolucci *et al.* are likely to have been diluted. This would mean that the concentration of miRNAs is inversely proportional to the proportion of trophic eggs in the sample, and that the difference in miRNA titres, between eggs laid by founding and established queens, identified by Paolucci *et al.*, is different than the reality. Therefore, the synthetic miRNA injected could not have had the expected effect, which would explain why we did not detect a difference in worker size between treated and control groups. This second explanation could be tested by evaluating the percentage of trophic eggs laid by founding queens, as well as by determining whether the four targeted miRNAs are presents in trophic eggs and in which concentration (i.e. identical or not to that in the reproductive eggs).

Conclusion

To conclude, the four synthetic miRNAs did not significantly influence worker size. Further investigation should consider the use of a wider range of miRNAs as it is likely that many miRNAs influence size. Alternatively, a complementary experiment could be done whereby the environment of founding queens' eggs could be manipulated, to see if the findings of Paolucci *et al.* is confirmed when scrutinized in the alternative direction. In this case we would predict that the manipulated eggs of founding queens would develop into normal sized

worker or larger than the average sized nanitic workers. In addition, this is the first time a method has been developed for the microinjection in *P. rugosus* eggs and we firmly believe that such techniques could be used to further investigate miRNAs functions in development, and can additionally be used to manipulate egg content in a wider range of species, for a variety of measurable traits.

Acknowledgments

We thank J. Buser, F. Pasquier Genoud and S. Vogel for ants' care and assistance for the experiments. We are grateful to S. McGregor for his helpful comments on the manuscript. This work was supported by an ERC grant and the Swiss NSF (LK).

References

Augustin, J. O., J. F. Santos, and S. L. Elliot. 2011. A behavioral repertoire of Atta sexdens (Hymenoptera, Formicidae) queens during the claustral founding and ergonomic stages. *Insectes Soc.* **58**:197–206.

Bartel, D. P. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism and Function. *Cell.* **116**:281–297.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**:1–48.

Bushati, N., and S. M. Cohen. 2007. MicroRNA functions. *Annu. Rev. Cell Dev. Biol.* **23**:175–205.

Chen, X., C. Ma, C. Chen, Q. Lu, W. Shi, Z. Liu, H. Wang, and H. Guo. 2017. Integration of lncRNA-miRNA-mRNA reveals novel insights into oviposition regulation in honey bees. *PeerJ.* **5**:1–28.

Della Lucia, T. M. C., E. F. Vilela, D. D. O. Moreira, J. M. S. Bento, and N. Dos Anjos. 1990. Egg-laying in Atta sexdens rubropilosa, under laboratory conditions. Pp. 173–179 in R. K. Vander Meer, K. Jaffé, and A. Cedeno, eds. Applied Myrmecology: A World Perspective. Westview Press, New York.

Fischer, S., R. Handrick, A. Aschrafi, and K. Otte. 2015. Unveiling the principle of microRNA-mediated redundancy in cellular pathway regulation. *RNA Biol.* **12**:238–247.

Glancey, B. M., C. E. Stringer, and B. P. M. 1973. Trophic egg production in the imported fire ant, *Solenopsis invicta*. *Georg. Entomol. Soc.* **8**:217–220.

Guo, W. T., and Y. Wang. 2019. Dgcr8 knockout approaches to understand microRNA functions in vitro and in vivo. *Cell. Mol. Life Sci.* **76**:1697–1711.

Hasegawa, E., and S. Imai. 2012. A trade-off between number and size within the first workers of the ant Camponotus japonicus. *J. Ethol.* **30**:201–204.

Hölldobler, B., and E. O. Wilson. 1990. The Ants. Harvard Un. Harvard University Press, Harvard USA.

Huber, J. 1907. The founding of colonies of Atta sexdens.

Hussain, M., R. J. Taft, and S. Asgari. 2008. An Insect Virus-Encoded MicroRNA Regulates Viral Replication. *J. Virol.* **82**:9164–9170.

Jones-Rhoades, M. W., and D. P. Bartel. 2004. Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. *Mol. Cell.* **14**:787–799.

Joo, J. Y., J. Lee, H. Y. Ko, Y. S. Lee, D. H. Lim, E. Y. Kim, S. Cho, K. S. Hong, J. J. Ko, S. Lee, Y. S. Lee, Y. Choi, K. A. Lee, and S. Kim. 2014. Microinjection free delivery of miRNA inhibitor into zygotes. *Sci. Rep.* **4**:8–13.

Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. The C. elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. *Cell.* **75**:843–854.

Li, L.-C., S. T. Okino, H. Zhao, D. Pookot, R. F. Place, S. Urakami, H. Enokida, and R. Dahiya. 2006. Small dsRNAs induce transcriptional activation in human cells. *Proc. Natl. Acad. Sci. USA.* **103**:17337–17342.

Liu, Z., S. Yamane, J. Kojima, Q. Wang, and S. Tanaka. 2001. Flexibility of first brood production in a claustral ant, *Camponotus japonicus* (Hymenoptera: Formicidae). *J. Ethol.* **19**:87–91.

Marshall, D. J., and T. Uller. 2007. When is a maternal effect adaptive? *Oikos.* **116**:1957–1963.

Mollinari, C., and C. González. 1998. Microinjection of Drosophila Eggs. Microinjection Transgenes. 587–603.

Oster, G. F., and E. O. Wilson. 1978. Caste and Ecology in the Social Insects. Princeton University Press, Princeton USA.

Palanichamy, J. K., and D. S. Rao. 2014. miRNA dysregulation in cancer: Towards a mechanistic understanding. *Front. Genet*. **5**:1–10.

Pontieri, L., A. Rajakumar, A. M. Rafiqi, R. S. Larsen, E. Abouheif, and G. Zhang. 2020. From egg to adult: a developmental table of the ant *Monomorium pharaonis*. bioRxiv 2020.12.22.423970.

Porter, S. D., and W. R. Tschinkel. 1986. Adaptive Value of Nanitic Workers in Newly Founded Red Imported Fire Ant Colonies (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am*. **79**:723–726.

Puthiyakunnon, S., Y. Yao, Y. Li, J. Gu, H. Peng, and X. Chen. 2013. Functional characterization of three MicroRNAs of the Asian Tiger Mosquito, *Aedes albopictus*. *Parasites and Vectors.* **6**:1–10.

Ridlo, M. R., E. H. Kim, and G. A. Kim. 2021. MicroRNA-210 regulates endoplasmic reticulum stress and apoptosis in porcine embryos. *Animals.* **11**:1–14.

RStudio Team. 2015. RStudio: Integrated Development Environment for R. Boston, MA.

Sieber, K. R., M. Saar, C. Opachaloemphan, M. Gallitto, H. Yang, and H. Yan. 2021. Embryo injections for Crispr-mediated mutagenesis in the ant *Harpegnathos saltator*. *J. Vis. Exp*. **168**:1–14.

Turner, M. J., A. L. Jiao, and F. J. Slack. 2014. Autoregulation of lin-4 microRNA transcription by RNA activation (RNAa) in *C. elegans*. *Cell Cycle***.** **13**:772–781.

Vasudevan, S., Y. Tong, and J. A. Steitz. 2007. Switching from Repression to Activation: MicroRNAs Can Up-Regulate Translation. *Science*. **318**:1931–1934.

Watanabe, S., M. Terayama, R. Kawauchiya, N. Ogusu, Y. Fujita, S. Mikami, Y. Murakami, and E. Hasegawa. 2017. The First Workers of the Ant Camponotus obscuripes Are a Different Allometric Morph with Relatively Long Antennae to Communicate with Other Larger Colony Members. *Psyche (London)*. **2017**:1–6.

Wheeler, D. E. 1994. Nourishment in ants: patterns in individuals and societies. Pp. 245–278 in J. H. Hunt and C. A. Nalepa, eds. Nourishment and Evolution in Insect Societies. Westview Press, Boulder.

Wienholds, E., W. P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H. R. Horvitz, S. Kauppinen, and R. H. A. Plasterk. 2005. MicroRNA expression in zebrafish embryonic development. *Science*. **309**:310–1.

Wilson, E. O. 1971. The insect societies. Harvard University Press, Princeton.

Yan, H., C. Opachaloemphan, G. Mancini, H. Yang, M. Gallitto, J. Mlejnek, A. Leibholz, K. Haight, M. Ghaninia, L. Huo, M. Perry, J. Slone, X. Zhou, M. Traficante, C. A. Penick, K. Dolezal, K. Gokhale, K. Stevens, I. Fetter-Pruneda, R. Bonasio, L. J. Zwiebel, S. L. Berger, J. Liebig, D. Reinberg, and C. Desplan. 2017. An Engineered orco Mutation Produces Aberrant Social Behavior and Defective Neural Development in Ants. *Cell*. **170**:736-747.e9.

Zhang, J., Y. Wang, X. Liu, S. Jiang, C. Zhao, R. Shen, X. Guo, X. Ling, and C. Liu. 2015. Expression and potential role of microRNA-29b in mouse early embryo development. *Cell. Physiol. Biochem*. **35**:1178–1187.

Supplementary material

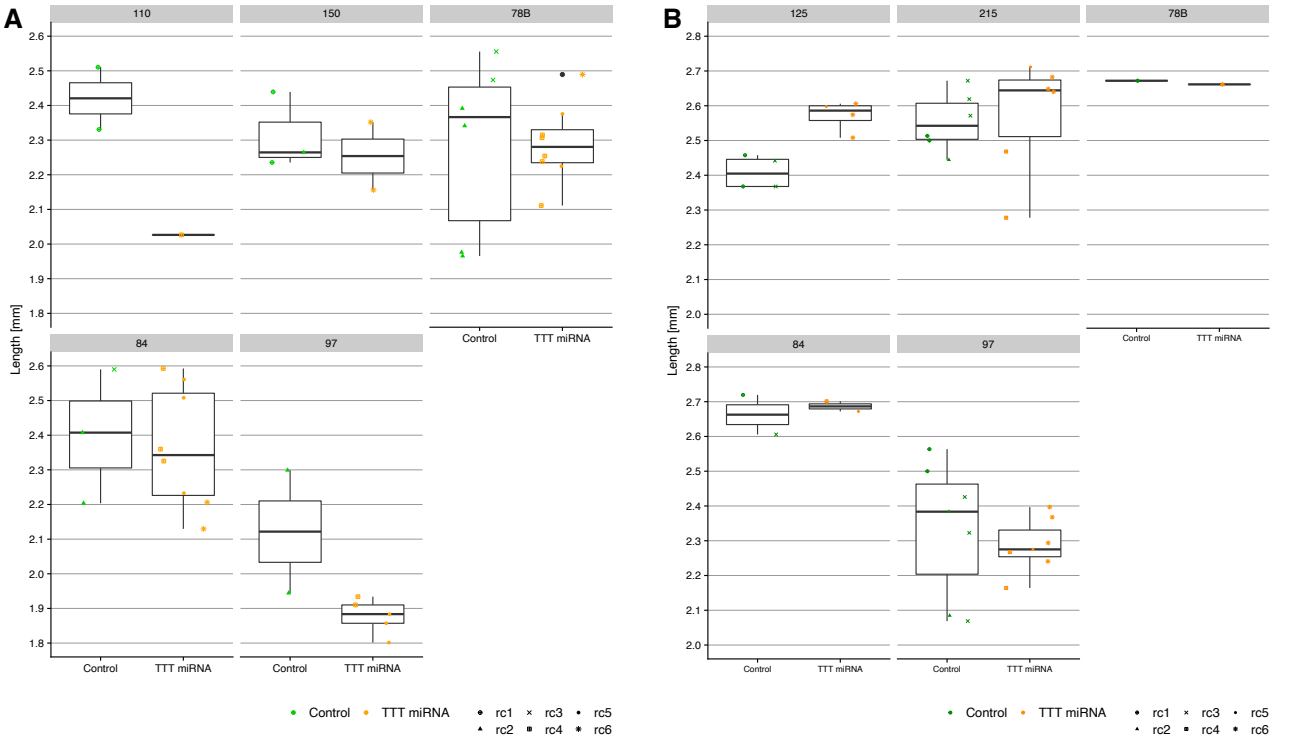


Figure S1. Exp. A - Workers (**A**) and gynes (**B**) head width, treated with miRug-d2 (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

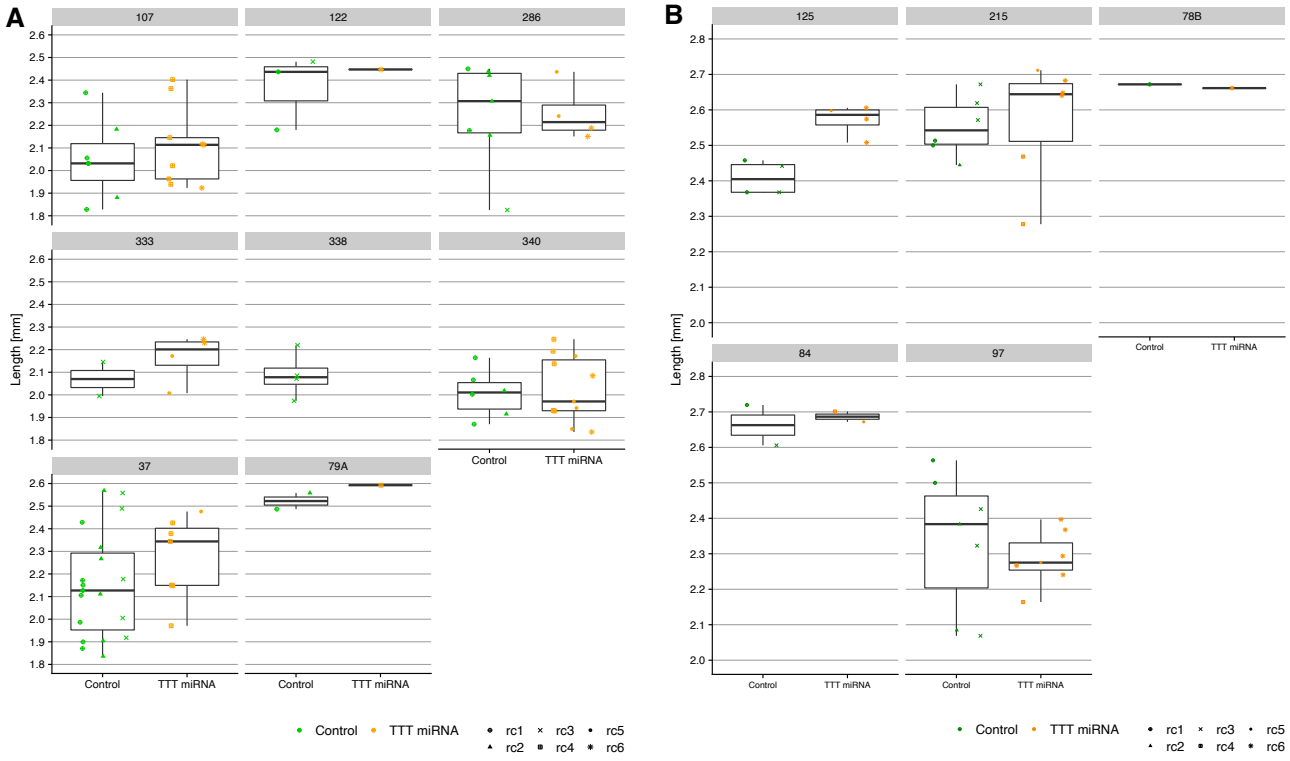


Figure S2. Exp. A - Workers (**A**) and gynes (**B**) head width, treated with miRug-u3 (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

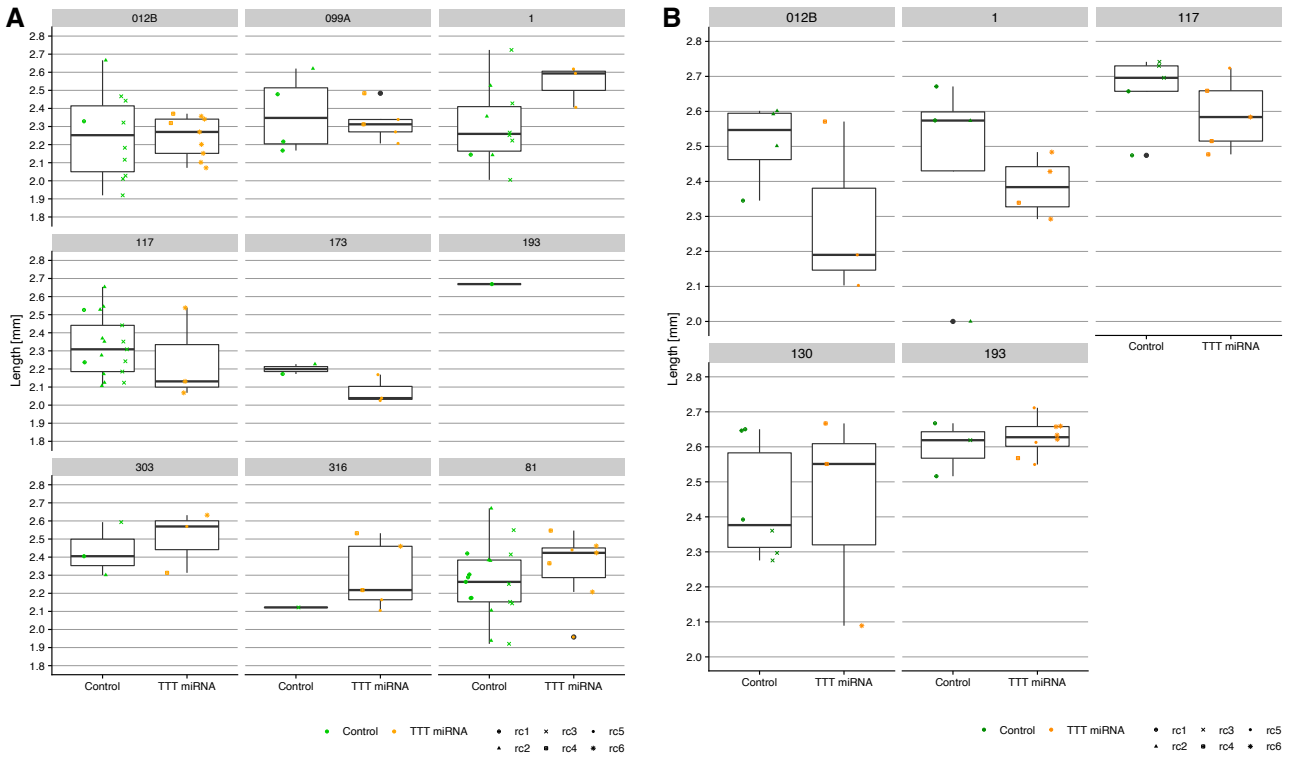


Figure S3. Exp. B - Workers (**A**) and gynes (**B**) head width, treated with miRug-d1 (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

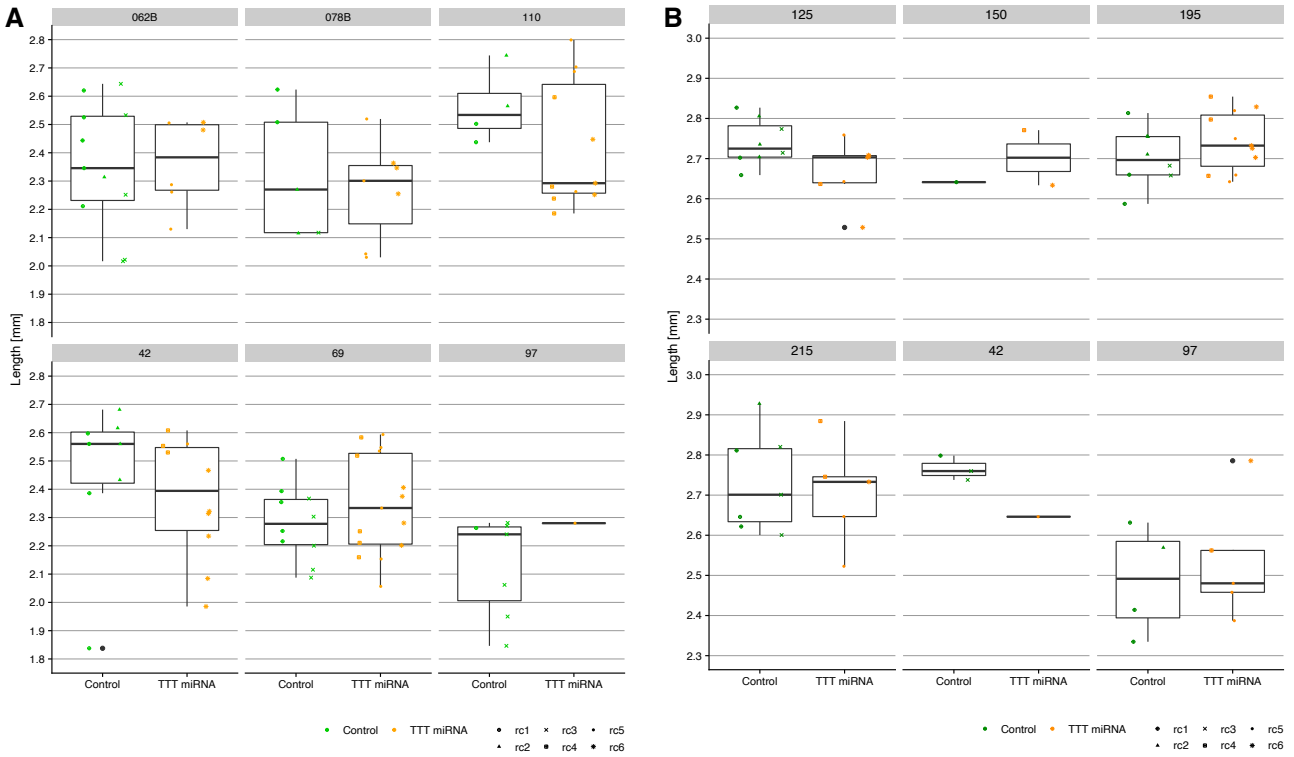


Figure S4. Exp. B - Workers (**A**) and gynes (**B**) head width, treated with miRug-d2 (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

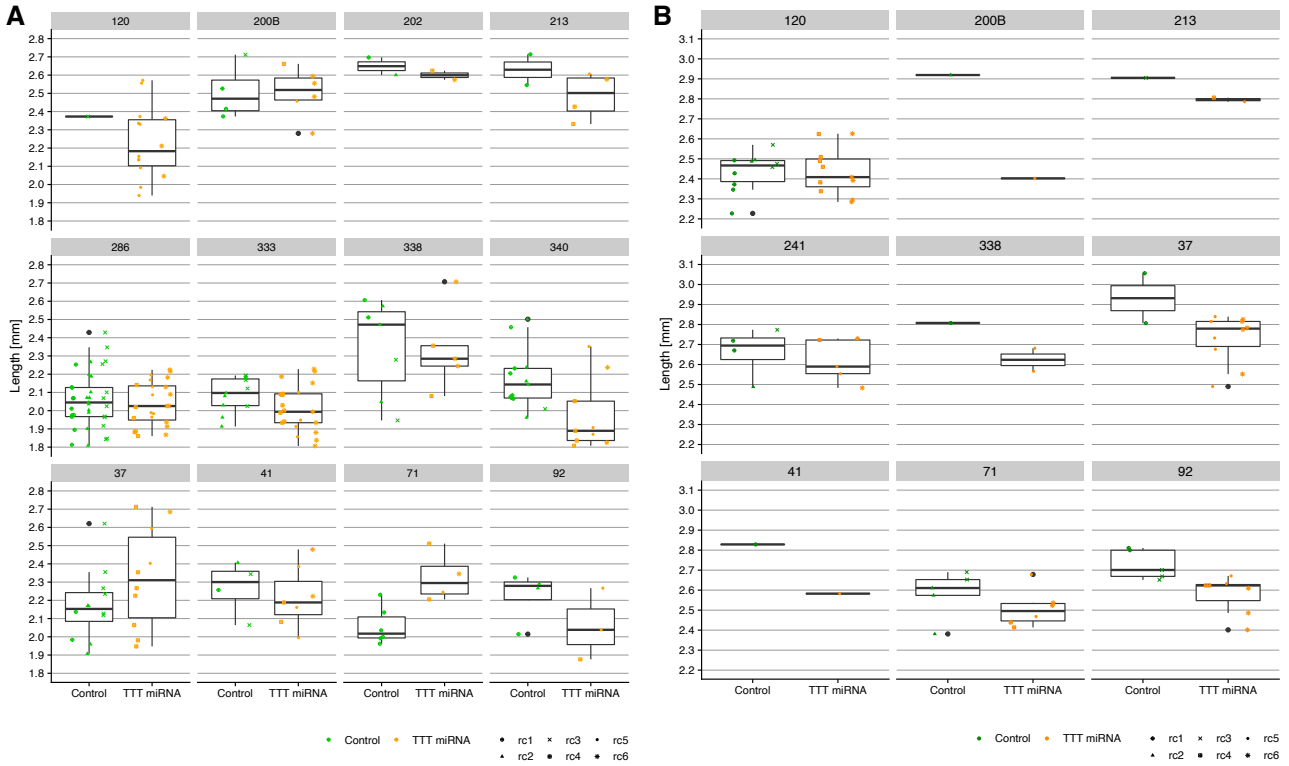


Figure S5. Exp. B - Workers (**A**) and gynes (**B**) head width, treated with miRug-u3 (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

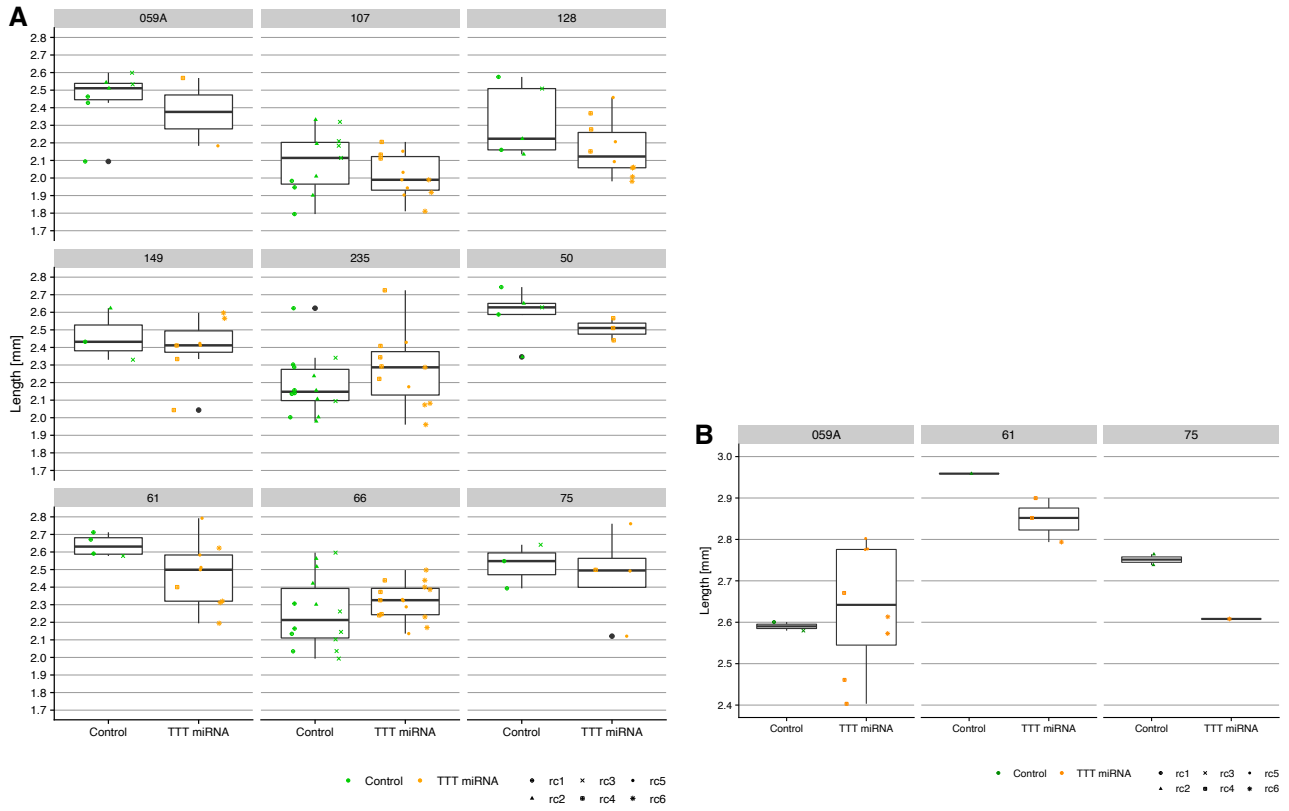


Figure S6. Exp. B - Workers (**A**) and gynes (**B**) head width, treated with miRug-u4 (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

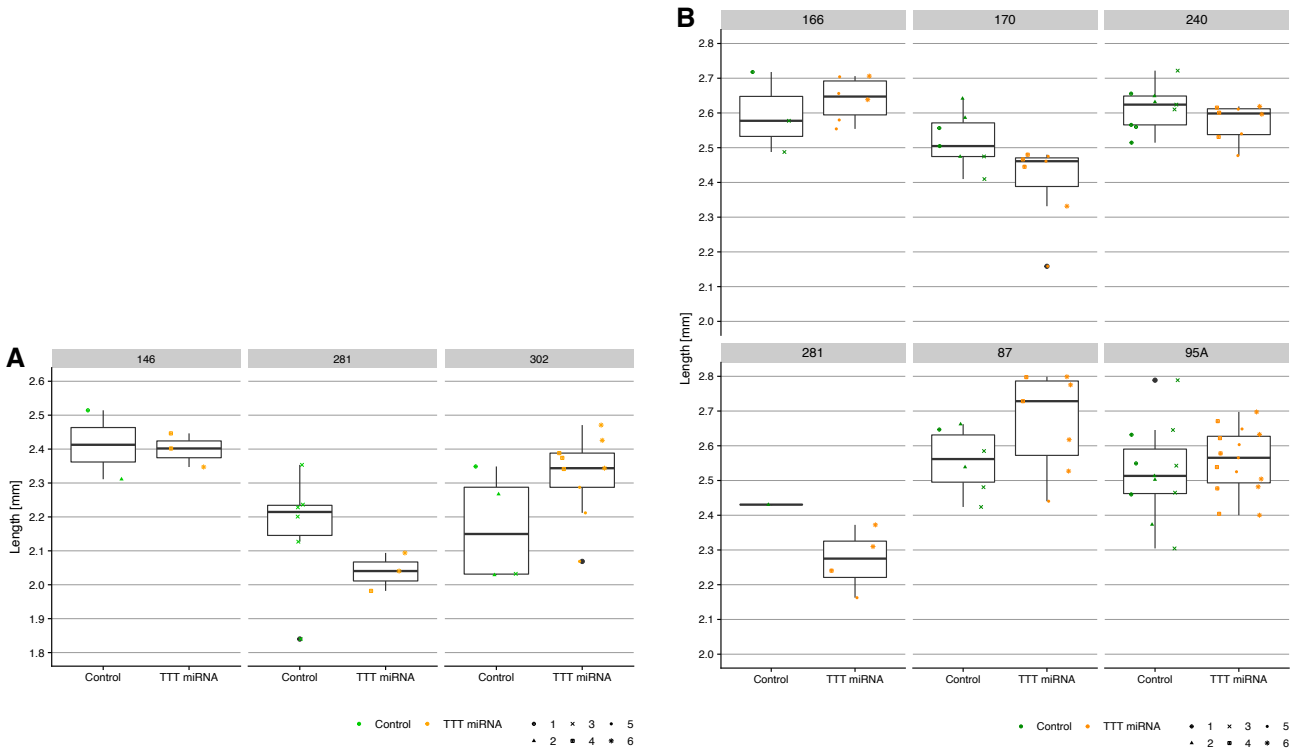


Figure S7. Exp. B - Workers (**A**) and gynes (**B**) head width, treated with all miRug injected together (orange) or with the control (green). The different point shapes show the data per recipient colonies (rc1-2-3 for the control and rc4-5-6 for the treatment).

Table S1. Amount of miRNA injected in different organism.

Organism	**Amount injected in nmol**	**Reference**
Mouse embryo	0.5 nmol	Joo *et al.* 2014
Mouse zygote	$5*10^{-4}$-$7*10^{-4}$ nmol	Zhang *et al.* 2015
Larvae and adult mosquitos	0.01 nmol	Puthiyakunnon *et al.* 2013
Porcine oocyte	$2*10^{-7}$ nmol	Ridlo *et al.* 2021
Drosophila embryo	$1.3*10^{-5}$ nmol	Mollinari and González 1998
Pogonomyrmex rugosus embryo	$7*10^{-5}$ nmol	This project

CHAPTER 2

MiRNA manipulation affects gyne development in *Pogonomyrmex rugosus*

E. Genzoni, L. Keller and T. Schwander

Author contributions: EG and TS designed the study. EG performed the experiment and analysed the data. EG wrote the manuscript, with input from all co-authors.

Abstract

In eusocial insects, queen and worker castes arise from a single genome and exhibit substantial morphological, behavioural and reproductive differences. It remains a fundamental challenge in biology to understand how this phenomenon occurs. While a number of factors have been implicated in caste determination, one that has thus far received little attention is the possible role of microRNAs (miRNAs), small noncoding RNAs. These play essential roles in embryonic development by regulating gene expression and several miRNAs have recently been described as involved in worker size regulation in the ant species *Pogonomyrmex rugosus*. In order to experimentally verify the influence of miRNAs on worker size, we manipulated the levels of four miRNAs in the egg, by injecting synthetic miRNAs and miRNA inhibitors. Instead of a difference in worker size, the results showed that the inhibition of one miRNA, miRNA-1, influenced caste differentiation. We found a significantly higher proportion of gynes developing from eggs treated with a miRNA inhibitor compared to eggs subjected to a control treatment, with 50% and 22% of gynes, respectively. Our study provides evidence that the inhibition of miRNA-1 is associated with caste differentiation, by triggering gyne development.

Keywords: microRNA, caste determination, gyne development, ants, *Pogonomyrmex rugosus*.

Introduction

There are a number of factors that influence caste development in ants with variations between species, such as food, worker:egg ratio, queen hibernation, presence/absence of the queen, hormones and pheromones, age and condition, and genotype (see Chapter 4; Haydak 1970; Wheeler 1986; Hölldobler and Wilson 1990; Richards and Packer 1994; Schwander *et al.* 2008; Smith *et al.* 2008; Bignell *et al.* 2010; Miura and Scharf 2011; Guo *et al.* 2013; Walker 2017; Taylor *et al.* 2019; Libbrecht *et al.* 2011). However, the mechanisms underlying caste determination in ants remain poorly understood and it is likely that several factors influencing caste fate have not yet been identified. Moreover, it is unknown whether one or several factors need to act jointly to regulate caste differentiation.

Among several experiments designed to investigate the potential effect of miRNAs on worker size (see Chapter 1), we serendipitously found that a high proportion of gynes emerged from eggs injected with miRug-d1. This happened twice, in two different experiments, which took place at different times of the year, (i.e. before and after hibernation). The production of gynes in the wild occurs after hibernation, in spring, in *Pogonomyrmex rugosus* (Smith and Tschinkel 2006), with the mating flight in the summer (Gordon 1995; Smith and Tschinkel 2006). It is rare to observe gyne development in colonies maintained in the lab, except after the queen's death (pers. obs) or after a treatment with methoprene (juvenile hormone analogue), which triggers gyne development (Libbrecht *et al.* 2013). This observation led us to investigate whether the miRNAs we were using as a treatment to influence worker size might influence the larval caste fate.

Materials and methods

The data used for the analyses of caste ratios comes from experiments designed to test whether four specific miRNAs influence worker size. 12 miRNAs have been found to be differentially expressed between eggs laid by founding and established queens (Paolucci *et al.*, unpublished data). MiRNA-1 was under-expressed in eggs from founding queens and seemed a potential candidate to be involved in influencing worker size. MiRNA-1 was used as a template to synthesise miRug-d1 (Qiagen), acting as an inhibitor of miRNA-1. The injections of miRug-d1 in eggs from established queens decreased miRNA-1 levels and mimicked its level in eggs from founding queens.

Here we focus on miRug-d1 which was the only synthetic miRNA found to influence gyne development; the three other synthetic miRNAs (miRug-d2, miRug-u3 and miRug-u4) are used as negative controls, to control for nucleotide toxicity (see Chapter 1 for the characterization of the synthetic miRNAs, and details on the method used for egg injection). Additionally, Milli-Q® ultrapure water (the solvent for mRNA dilution) was used as a control treatment for the effect of injection.

Two experiments were carried out, the first one (hereafter referred to as Exp. 1) started 15 days after the colonies came out of hibernation in March 2018 and the second one (hereafter Exp. 2) took place from July to August 2020. Nine and ten colonies of *Pogonomyrmex rugosus* were used for Exp. 1 and Exp. 2, respectively. Each colony was founded by a single queen collected after the mating flight in 2008 (Bowie, Arizona, USA) or 2013 (Bowie and Florence, Arizona, USA). They were maintained in the lab at 28°C with 60% humidity, with a 12-h/12-h light:dark cycle and fed once a week with grass seeds and flies, and 20% honey water every three days.

For Exp. 1, we isolated the queens for 48 hours. The eggs were collected every eight hours and sorted by type; trophic or reproductive (see Chapter 3), and trophic eggs were discarded. At the end of the 48h of isolation, the queens were put back in their colony. The procedure for the injection is the same as for Exp. 2 and is described below. During Exp. 1, we observed that the egg laying rate decreased after 24 hours of isolation, and so for Exp. 2 we isolated the queens only for 16 hours for egg laying, with an egg collection every eight hours. The queens were then put back in their colony for eight hours until the next isolation session. After three days under this isolation schedule, the queens were returned to their colonies for an uninterrupted 24-hour break.

After the collection, reproductive eggs were kept in a clean and humid petri dish for eight hours until the injection. Eggs from both Exp. 1 and exp. 2 ranged from eight hours old (if deposited immediately prior to collection) to a maximum of 16 hours (if deposited immediately post collection) at the injection time. Reproductive eggs laid within the same eight hours were randomly separated into two groups; half were injected with ~7nl of 10pmol/μl miRNA and half injected with Milli-Q® ultrapure water as a control.

For the injection, the eggs were put in a 600μm nylon mesh, to prevent them moving while injecting. Injections were performed manually using a FemtoJet ® 4x micro-injector (Eppendorf) with glass needles made from pulled capillary tubes using a P-97 Pipette Puller. The exact settings were adjusted for every needle separately

in order to have an identical volume injected in the eggs for every injection session. Eggs stayed in the mesh, untouched, for two hours to recover from the injection. They were then removed carefully from the mesh and put into a petri dish containing a water reservoir for humidity where they developed for 10 days. Larvae were collected right after hatching (petri dishes monitored every four hours) and placed into recipient colonies (a group of workers without a queen) as they needed care and food to develop until pupation. We created one recipient colony for the control and one for the treatment for Exp. 1, whereas for Exp. 2 there were three recipient colonies for the control and three for the treatment in order to take into account the box effect of the recipient colonies on the developing larvae. A recipient colony contained 40 workers in total – 20 foragers taken in the foraging arena and 20 nurses taken from inside the nest. There was no crossfostering, so both the eggs and the workers raising the eggs came from the same colony. Individuals developing from the injected eggs were collected at the pupal stage, as worker and gyne are morphologically distinct at this developmental stage in *P. rugosus* and the caste was recorded.

For the analysis, we compared the proportion of gyne pupae between the control and treatment group. We used Rstudio (RStudio Team 2015) and the package *lme4* (Bates *et al.* 2015) to perform a linear mixed effects analysis, with caste as response variable, which is a binary categorical factor, and treatment type as explanatory variable. We added colony as a random effect for Exp. 1, and recipient colonies nested in colony for Exp. 2. We ran an additional analysis to test whether the treatment type might influence the survival (from egg to larvae and from larvae to pupae). We performed a linear mixed effects analysis with survival as response variable, treatment type as explanatory variable, and colonies as random effects.

Results

We did not find any difference in size between the treatment (injection of eggs with one of four different miRNAs) and control groups (injection with water) for either workers or gynes (see Chapter 1). However, we found a significantly higher proportion of gynes in the group treated with miRug-d1 compared to the control, in both experiments (Exp. 1: $\beta = -6.50$, SE = 3.10, $z = -2.10$, $p = 0.036$; Exp. 2: $\beta = -2.40$, SE = 1.13, $z = -2.13$, $p = 0.033$; Data of both experiments pooled together: $\beta = -3.77$, SE = 1.23, $z = -3.07$, $p = 0.002$). In Exp. 1 there was 48±16% of gynes in the treatment and 18±15% of gynes in the control (Figure 1A; average ± SE). In Exp. 2, there was 52±12% and 27±8% of gynes in the in the treatment and control groups, respectively (Figure 1B).

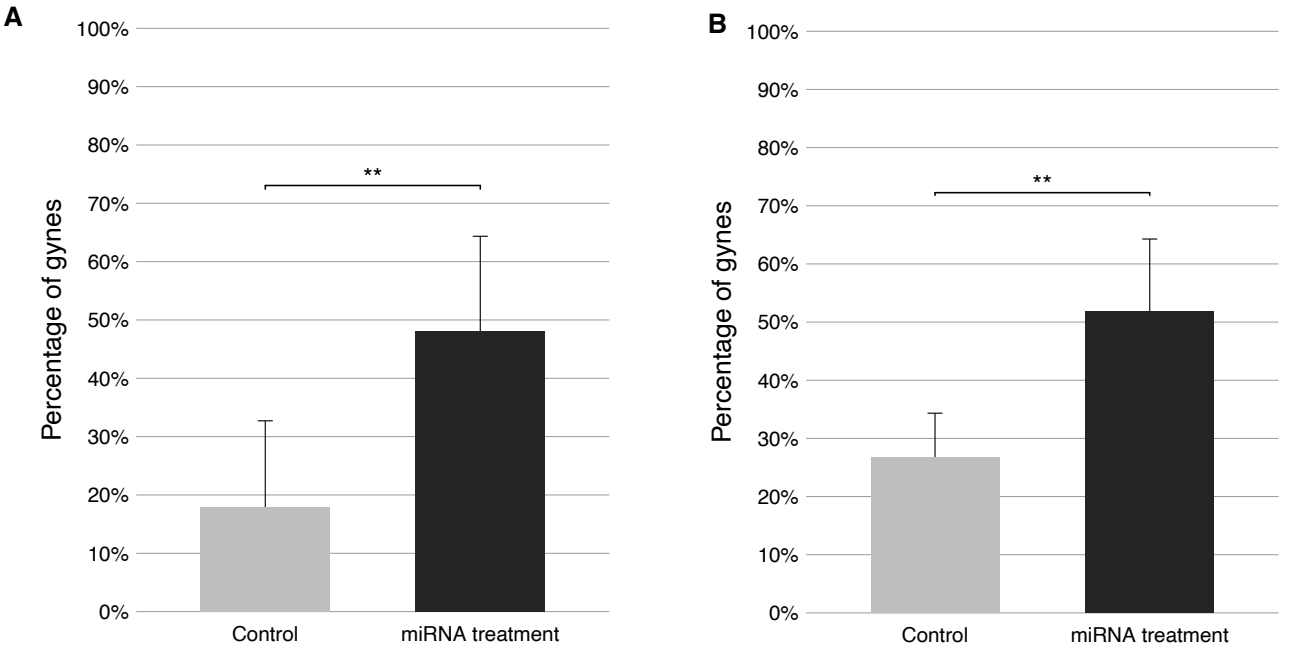


Figure 1. Average of the percentage of gynes treated with miRug-d1 or with the control, for Exp. 1 (**A**) and Exp. 2 (**B**).

Table 1. Number of worker and gyne pupae that developed from eggs injected with water (control treatment - **c**) or miRNA treatment - **t**). For Exp 1. (**A**) there was one recipient colony for the control and one for the treatment for each colony replicate, for Exp. 2 (**B**) there were three recipient colonies per colony replicate and treatment and the three numbers per cell show the number of pupae per recipient colony. Empty lines or cell means that no pupae developed in this recipient colony.

A

Sample size	1		81		117		130		173		303		316		12B		99A		Total	
	c	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**
worker	1	5	4	2	17				1		3			1	7	6			33	14
gyne						5	2	1	2	6	2			1			1		7	13

B

Sample size	1		81		117		130		173		193		303		316		12B		99A		Total	
	c	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**	**c**	**t**
worker	1 3 6	3	6 5 6	2 2 3	2 9 6	3	6		1 1	3	1		1 1 1	1 1 1	1	2 2 1	1 1 8	2 7	3 1	2 3	71	38
gyne	2 2	1 3		4 4 3	3 3	3 2 3	3 3	2 1		3 1	2 1	1 3 4	1				1 4	1 2	1		26	41

In both Exp. 1 and Exp. 2, there was no significant difference in survival between treatment and control groups for the transition from egg to larva (Exp. 1: $\beta = -1.72$, SE = 2.35, $t_{(20)} = -0.73$, $p = 0.48$; Exp. 2: $\beta = 4.27$, SE = 4.58, $t_{(20)} = 0.93$, $p = 0.37$) and larva to pupa (Exp. 1: $\beta = -3.80$, SE = 12.92, $t_{(20)} = -0.29$, $p = 0.78$; Exp. 2: $\beta = -1.94$, SE = 3.80, $t_{(20)} = -0.51$, $p = 0.62$; Table 2).

Table 2. Percentage of survival at two developmental stages: egg to larva and larva to pupa, for Exp 1 and Exp 2 (average ±SE).

Percentage of survival (average ±SE)		Exp. 1		Exp. 2	
		Egg to larva	Larva to pupa	Egg to larva	Larva to pupa
miRug-d1	Treatment	23.6±4.1%	29.9±10.3%	47.2 ±7.3%	19.8 ±5.0%
	Control	25.3±5.0%	33.7±8.8%	42.9 ±7.7%	21.8 ±4.5%

Discussion

This study provides the first evidence for the role of a miRNA in queen vs worker caste determination in the ant *P. rugosus*. This novel miRNA, miRNA-1, when down regulated in eggs, significantly increases the likelihood of gyne development. Although miRNA-1 has not previously been described, we suggest it is a good target for future research on ant caste determination. Furthermore, this is the first study to have successfully influenced caste fate by experimentally manipulating miRNA within eggs and we hope future research is able to utilize our methodology.

Paolucci *et al.* (unpublished data) found that miRNA-1 is expressed in eggs laid by established queens and under-expressed in eggs from founding queens. In our study, miRug-d1 was designed to inhibit miRNA-1, and we expected that its injection into eggs from established queens would result in the development of small workers. However, we instead found that the injection influenced the embryo's development by increasing the probability of developing into a gyne. Those two results could seem contradictory as no gynes develop at the founding stage even though miRNA-1 is under-expressed in eggs, but as discussed below, there are at least three non-mutually exclusive explanations for our findings.

First, trophic eggs had not been identified in this species when Paolucci *et al.* collected the eggs for miRNA expression investigation. Subsequently, their samples contained a mix of reproductive and trophic eggs of unknown ratio. Trophic eggs have been identified in the course of Exp. 1 (see Chapter 1), with a range between 30 - 50% of all eggs laid recorded as trophic (pers. obs). We assume that there was at least a similar percentage among the established colonies at the time of Paolucci *et al.*'s sampling. However, the percentage of trophic eggs laid by founding queens of this species is currently unknown. In *Solenopsis invicta* and *Atta sexdens*, the percentage of trophic eggs laid by founding queens was 46.8% ± 4.5% (average ± SE; Glancey *et al.* 1973) and 90% (average; Huber 1907), respectively, and for established queens it was 9.6% ± 6.6% (Glancey *et al.* 1973) and 7.7% (Della Lucia *et al.* 1990; Augustin *et al.* 2011), respectively. If *P. rugosus* follow the same pattern than *S. invicta* and *A. sexdens*, then it is a reasonable assumption that the percentage of trophic eggs laid by *P. rugosus* founding queens was potentially much higher at this early stage in comparison to the established stage. It is currently unknown what is actually in the trophic eggs, but if we assume that miRNA-1 is not present in them, this becomes of extreme relevance as then the titres per egg determined by Paolucci *et al.* are likely to have been diluted by the presence of trophic eggs in the sample. This would mean that the measured expression of miRNA-1 in a pooled eggs sample is inversely proportional to the proportion of trophic eggs in the sample.

Second, at the time of the experiment done by Paolucci *et al*, the established colonies were five years old but had not started producing gynes, although it is the approximate age where colonies start producing them (Gordon 1995). It is likely that the laboratory conditions and colony growth limitation due to the box size influenced the development of the colonies and thereby delayed the production of sexuals. Wild colonies reach 12'000 workers at five years old (Gordon 1995), while lab colonies count 800 workers maximum at the same age (pers. obs), and in several species, colonies need to reach a minimum size to start producing sexuals (Gordon 1995; Cole and Wiernasz 2000; Smith and Tschinkel 2006). In the absence of any recorded gyne

development, we can extrapolate that the level of miRNA-1 detected by Paolucci *et al.* came from worker-destined eggs. The established colonies used for Exp. 1 and 2 were 5 and 12 years old, respectively, and they had started producing gynes. Therefore, the inhibition of miRNA-1, through injections of miRug-d1, could have triggered the development of gynes. As a consequence, the fact that miRNA-1 was under-expressed in founding queen's eggs but that no gynes develop at this stage could be explained by the colony life cycle stage. Many factors, such as queen age, the food quality and quantity or the social environment may favor the development of workers and prevent the development of gynes at the founding stage (Schwander *et al.* 2008; Libbrecht *et al.* 2011; Taylor *et al.* 2019). At this critical stage, the queen needs to produce enough workers to develop the colony and the energy required for gyne production would be highly detrimental for the colony survival. The queen herself could influence the caste fate of her offspring by changing the content of her eggs (i.e. maternal effect; Schwander *et al.* 2008). The expression level of miRNA-1 could vary between life cycle stages, as it has been shown to vary between developmental stages. Feng *et al.* (2018) showed that miRNAs were highly expressed in the larval and adult stages and under-expressed in the pupal stage. The interaction between factors specific to the founding stage with the expression level of miRNA-1 could explain how the latter is under-expressed in founding queen's eggs without triggering gyne development, whereas its inhibition in established queens' eggs results in the development of gynes.

And finally, the difference in miRNA-1 expression level between eggs laid by founding and established queens detected by Paolucci *et al.* could be a false positive. This would explain why there is no gyne development at the founding stage if the level of miRNA-1 in the reproductive eggs is the same than at the established stage, when only workers are produced.

We find that miRNA-1, when down regulated in eggs, significantly increases the likelihood of gyne development. One alternative interpretation that is less compatible with our results is that the queen- and worker-destined eggs would have a differential survival probability when injected with miRNA-1. For instance, pre-determined eggs could have a differential mortality influenced by their caste fate, which could result in a higher number of gynes simply by merit of larger number of eclosing pupae. However differential survival alone cannot explain our results. We performed analyses to check for confounding differences in survival (from egg to larvae and larvae to pupae) and we did not find any difference between control and treatment groups. This allows us to exclude differential survival between castes as the main explanation for the high proportion of gynes observed in the treatment group.

A recent study from Guo *et al.* (2013) artificially manipulated the concentration of miRNAs in the royal jelly of the honeybee *A. melifera.* The queen-destined larvae fed with this royal jelly developed a worker-like morphology, and in addition, significant changes in mRNA expression were observed. Guo *et al.* (2013) and Zhu *et al.* (2017) found that in the honeybee, the worker jelly is enriched in miRNA complexity and abundance compared to the royal jelly, and they suggest that it is not the royal jelly that stimulates the differentiation of larvae into queens but rather the worker jelly which ensures the development of the larvae into workers. MiRNAs enriched in the worker jelly delay larval development and decrease body size, thereby preventing larval differentiation into queens and inducing development into worker bees (Zhu *et al.* 2017). Guo *et al.*

(2013) propose that miRNAs in the nurse bee secretions constitute an additional element in the regulatory control of caste determination in the honey bee.

One of the differences between the study of Guo *et al.* (2013) and ours is the fact that they have influenced caste-related traits at the larval stage, resulting in queen-destined pupae that developed worker-like traits. In our study, we managed to manipulate the caste fate of the individual earlier, at the egg stage, which is as early as possible in ant development without artificially removing eggs from the queen directly. As it is currently unknown when caste differentiation occurs in *P. rugosus*, we cannot know whether the miRNA inhibitor influenced gyne development right after injection or whether it stayed undegraded in the eggs and acted later in the embryo's development or even in the early larval stage. However, we obtained well developed queen pupae, not only worker pupae with a queen-like morphology, which suggests that miRNA-1 has a complete action in caste differentiation in *P. rugosus*. Investigations of miRNA expression at early developmental stages have thus far only been done in bee larvae where individual caste fate is known (Pereboom *et al.* 2005; Cameron *et al.* 2013), thanks to distinct worker versus queen cells (Shi *et al.* 2011).

Our study provides further compelling evidence that miRNAs are key factors involved in development. In insects, both maternal miRNAs and miRNAs produced by the embryo play important roles in developmental transitions (Asgari 2013; Ylla *et al.* 2017), sex determination (Fagegaltier *et al.* 2014) and phenotypic plasticity (Trionnaire *et al.* 2008; Legeai *et al.* 2010). For example, let-7 is one of the most conserved miRNA known, which is involved in several important developmental processes, such as the regulation of the developmental timing, the development of the nervous system, or the control of cell divisions in wing development (Asgari 2013). Seventeen miRNAs have been found involved in the development of the two sexual and asexual morphs in aphids (Trionnaire *et al.* 2008), and in the locust *Locusta migratoria,* the concentration of miRNAs was found to be nearly twice higher in the gregarious phase compared to the solitary phase (Wei et al. 2009). Within the eusocial Hymenoptera, several studies have identified differentially expressed miRNAs between queen and worker adult (Weaver *et al.* 2007; Ashby *et al.* 2016), queen- and worker-destined larvae (Guo *et al.* 2013; Shi *et al.* 2015; Collins *et al.* 2017; Zhu *et al.* 2017) or within the worker caste, between soldiers and workers (Matsunami *et al.* 2019). Liu *et al.* (2012) found that nine miRNAs are differentially expressed between bee nurses and foragers, and that some of the predicted target genes are associated with neural functions.

The importance of miRNAs cannot be understated for their role in development, however they are likely to be of even greater biological significance. In 2015, Søvik *et al.* found 5 genes associated with eusociality in Hymenoptera independent of phylogeny. They suggested that miRNAs might have contributed to the emergence of eusociality in insects. Comparisons of miRNA expression amongst castes have been conducted in eusocial Hymenoptera.

In conclusion, we offer evidence of a novel miRNA involved in caste determination in the ant *P. rugosus*. Experimentations with direct pharmacological manipulation supports the premise that suppression of miRNA expression at the egg stage can influence ultimate determination of gyne caste, irrespective of external factors. While there are no doubt additional factors at play (as the result is not black and white), this is strong evidence

of an actual important regulating aspect, as the gynes produced had distinctive gyne morphology and no worker-like characteristics (unlike in Guo *et al.* 2013).

Further investigations in miRNA expression in *P. rugosus* eggs and first instars larvae might give further resolution on the miRNAs involved in caste determination at those developmental stages. More precisely, determining the titres of miRNA-1 in the eggs as well as in queen- and worker-destined larvae would confirm whether miRNA-1 is naturally differentially expressed according to the caste fate of the individuals, and therefore corroborate our results. In addition, the accurate identification of specific miRNA cocktails combined with our injection technique and incubation process of manipulated eggs might further resolve the precise importance of early gene expression on caste.

Acknowledgments

We thank J. Buser, F. Pasquier Genoud and S. Vogel for ants care and assistance for the experiments. We are grateful to S. McGregor for his helpful comments on the chapter. This work was supported by an ERC grant and the Swiss NSF (LK).

References

Asgari, S. 2013. MicroRNA functions in insects. *Insect Biochem. Mol.* Biol. **43**:388–397.

Ashby, R., S. Forêt, I. Searle, and R. Maleszka. 2016. MicroRNAs in Honey Bee Caste Determination. *Sci. Rep.* **6**:18794.

Augustin, J. O., J. F. L. Santos, and S. L. Elliot. 2011. A behavioral repertoire of *Atta sexdens* (Hymenoptera, Formicidae) queens during the claustral founding and ergonomic stages. *Insectes Soc.* **58**:197–206.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw*. **67**:1–48.

Cameron, R. C., E. J. Duncan, and P. K. Dearden. 2013. Biased gene expression in early honeybee larval development. *BMC Genomics* **14**:903.

Cole, B. J., and D. C. Wiernasz. 2000. Colony size and reproduction in the western harvester ant, *Pogonomyrmex occidentalis. Insectes Soc*. **47**:249–255.

Collins, D. H., I. Mohorianu, M. Beckers, V. Moulton, T. Dalmay, and A. F. G. Bourke. 2017. MicroRNAs Associated with Caste Determination and Differentiation in a Primitively Eusocial Insect. *Sci. Rep.* **7**:1–9.

Della Lucia, T. M. C., E. F. Vilela, D. D. O. Moreira, J. M. S. Bento, and N. Dos Anjos. 1990. Egg-laying in Atta sexdens rubropilosa, under laboratory conditions. Pp. 173–179 in R. K. Vander Meer, K. Jaffé, and A. Cedeno, eds. Applied Myrmecology: A World Perspective. Westview Press, New York.

Fagegaltier, D., K. Annekatrin, A. Gordon, E. C. Lai, T. R. Gingeras, G. J. Hannon, and H. R. Shcherbata. 2014. A genome-wide survey of sexually dimorphic expression of *Drosophila* miRNAS identifies the steroid hormone-induced miRNA let-7 as a regulator of sexual identity. *Genetics* **198**:647–668.

Feng, X., J. Wu, S. Zhou, J. Wang, and W. Hu. 2018. Characterization and potential role of microRNA in the Chinese dominant malaria mosquito *Anopheles sinensis* (Diptera: Culicidae) throughout four different life stages. *Cell Biosci.* **8**:1–17.

Glancey, B. M., C. E. Stringer, and B. P. M. 1973. Trophic egg production in the imported fire ant, *Solenopsis invicta. Georg. Entomol. Soc.* **8**:217–220.

Gordon, D. M. 1995. The development of an ant colony's foraging range. Anim. Behav. **49**:649–659.

Guo, X., S. Su, G. Skogerboe, S. Dai, W. Li, Z. Li, F. Liu, R. Ni, Y. Guo, S. Chen, S. Zhang, and R. Chen. 2013. Recipe for a busy bee: MicroRNAs in honey bee caste determination. *PLoS One* **8**:1-10.

Huber, J. 1907. The founding of colonies of *Atta sexdens*.

Legeai, F., G. Rizk, T. Walsh, O. Edwards, K. Gordon, D. Lavenier, N. Leterme, A. Méreau, J. Nicolas, D. Tagu, and S. Jaubert-Possamai. 2010. Bioinformatic prediction, deep sequencing of microRNAs and expression analysis during phenotypic plasticity in the pea aphid, *Acyrthosiphon pisum*. *BMC Genomics* **11**:1-9.

Libbrecht, R., M. Corona, F. Wende, D. O. Azevedo, J. E. Serrão, and L. Keller. 2013. Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proc. Natl. Acad. Sci. U.S.A.* **110**:11050–11055.

Libbrecht, R., T. Schwander, and L. Keller. 2011. Genetic components to caste allocation in a multiple-queen ant species. *Evolution (N. Y).* **65**:2907–2915.

Liu, F., W. Peng, Z. Li, W. Li, L. Li, J. Pan, S. Zhang, Y. Miao, S. Chen, and S. Su. 2012. Next-generation small RNA sequencing for microRNAs profiling in Apis mellifera: Comparison between nurses and foragers. *Insect Mol. Biol.* **21**:297–303.

Matsunami, M., M. Nozawa, R. Suzuki, K. Toga, Y. Masuoka, K. Yamaguchi, K. Maekawa, S. Shigenobu, and T. Miura. 2019. Caste-specific microRNA expression in termites: insights into soldier differentiation. *Insect Mol. Biol.* **28**:86–98.

Pereboom, J. J. M., W. C. Jordan, S. Sumner, R. L. Hammond, and A. F. G. Bourke. 2005. Differential gene expression in queen-worker caste determination in bumble-bees. *Proc. R. Soc. B Biol. Sci.* **272**:1145–1152.

RStudio Team. 2015. RStudio: Integrated Development Environment for R. Boston, MA.

Schwander, T., J. Y. Humbert, C. S. Brent, S. H. Cahan, L. Chapuis, E. Renai, and L. Keller. 2008. Maternal Effect on Female Caste Determination in a Social Insect. *Curr. Biol.* **18**:265–269.

Shi, Y. Y., Z. Y. Huang, Z. J. Zeng, Z. L. Wang, X. B. Wu, and W. Y. Yan. 2011. Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, apidae). *PLoS One* **6**:2–7.

Shi, Y. Y., H. J. Zheng, Q. Z. Pan, Z. L. Wang, and Z. J. Zeng. 2015. Differentially expressed microRNAs between queen and worker larvae of the honey bee (*Apis mellifera*). *Apidologie* **46**:35–45.

Smith, C. R., and W. R. Tschinkel. 2006. The sociometry and sociogenesis of reproduction in the Florida harvester ant, *Pogonomyrmex badius*. *J. Insect Sci.* **6**:1–11.

Søvik, E., G. Bloch, and Y. Ben-Shahar. 2015. Function and evolution of microRNAs in eusocial Hymenoptera. *Front. Genet.* **6**:1–11.

Taylor, B. A., M. Reuter, and S. Sumner. 2019. Patterns of reproductive differentiation and reproductive plasticity in the major evolutionary transition to superorganismality. *Curr. Opin. Insect Sci.* **34**:40–47.

Trionnaire, G., J. Hardie, S. Jaubert-Possamai, J.-C. Simon, and D. Tagu. 2008. Shifting from clonal to sexual reproduction in aphids: physiological and developmental aspects. *Biol. Cell* **100**:441–451.

Weaver, D. B., J. M. Anzola, J. D. Evans, J. G. Reid, J. T. Reese, K. L. Childs, E. M. Zdobnov, M. P. Samanta, J. Miller, and C. G. Elsik. 2007. Computational and transcriptional evidence for microRNAs in the honey bee genome. *Genome Biol.* **8**:1-15

Wei, Y., S. Chen, P. Yang, Z. Ma, and L. Kang. 2009. Characterization and comparative profiling of the small RNA transcriptomes in two phases of locust. *Genome Biol.* 10:1–18.

Ylla, G., M. D. Piulachs, and X. Belles. 2017. Comparative analysis of miRNA expression during the development of insects of different metamorphosis modes and germ-band types. *BMC Genomics* **18**:1–13.

Zhu, K., M. Liu, Z. Fu, Z. Zhou, Y. Kong, H. Liang, Z. Lin, J. Luo, H. Zheng, P. Wan, J. Zhang, K. Zen, J. Chen, F. Hu, C. Y. Zhang, J. Ren, and X. Chen. 2017. Plant microRNAs in larval food regulate honeybee caste development. *PLoS Genet.* **13**:1–23.

Supplementary material

Table S1. Percentage of survival at two developmental stages: egg to larva and larva to pupa, for Exp 1 and Exp 2 (average ±SE).

Percentage of survival (average ±SE)		Exp. 1		Exp. 2	
		Egg to larva	Larva to pupa	Egg to larva	Larva to pupa
miRug-d1	Treatment	23.6±4.1%	29.9±10.3%	47.2 ±7.3%	19.8 ±5.0%
	Control	25.3±5.0%	33.7±8.8%	42.9 ±7.7%	21.8 ±4.5%
	Statistics	β = -1.72 SE = 2.35 $t_{(20)}$ = -0.73 p = 0.48	β = -3.80 SE = 12.92 $t_{(20)}$ = -0.29 p = 0.78	β = 4.27 SE = 4.58 $t_{(20)}$ = 0.93 p = 0.37	β = -1.94 SE = 3.80 $t_{(20)}$ = -0.51 p = 0.62
miRug-d2	Treatment	23.0±3.3%	29.1±7.0%	55.6±5.5% *	30.3 ±9.6%
	Control	24.5±3.4%	25.1±6.7%	41.9±7.5% *	29.4 ±8.8%
	Statistics	β = -1.50 SE = 2.33 $t_{(20)}$ = -0.65 p = 0.53	β = 4.00 SE = 7.91 $t_{(20)}$ = 0.51 p = 0.62	β = 13.97 SE = 4.41 $t_{(20)}$ = 3.17 p = 0.01	β = 0.90 SE = 11.58 $t_{(20)}$ = 0.08 p = 0.94
miRug-u3	Treatment	-	-	57.9 ±5.0%	32.5 ±5.8%
	Control	-	-	52.4 ±4.4%	35.4 ±8.0%
	Statistics	-	-	β = 5.55 SE = 3.42 $t_{(20)}$ = 1.62 p = 0.13	β = -2.85 SE = 5.49 $t_{(20)}$ = -0.52 p = 0.61

In Exp. 1 there is no significant difference in survival between treatment and control groups for the transition from egg to larva and larva to pupa. Exp. 2 there is significantly more larvae that hatch from the eggs injected with miRug-d2 than from the control, 55.6±5.5% for 41.9±7.5% respectively (average ± SE; β = 13.97, SE = 4.41, t(9) = 3.17, p = 0.011). There is no significant difference in survival for the other miRNAs in both transitions (egg to larva and larva to pupa).

CHAPTER 3

Effect of trophic eggs on larval caste fate in *Pogonomyrmex rugosus* ant

E. Genzoni, L. Keller and T. Schwander

Author contributions: EG, TS and LK designed the study. EG performed the experiment and analysed the data. EG wrote the manuscript, with input from all co-authors.

Abstract

Understanding how a single genome creates distinct phenotypes remains a fundamental challenge for biologists. Social insects provide a striking example of polyphenism, with queen and worker castes exhibiting morphological, behavioural, and reproductive differences. Although a number of factors are known to influence caste development in ants (e.g., food, worker: egg ratio, presence/absence of the queen or hormones), it is likely that several factors have not yet been identified. In this study, we report evidence supporting a role of trophic eggs in caste determination in the harvester ant *Pogonomyrmex rugosus*. Trophic eggs, unlike reproductive eggs, do not contain an embryo and are primarily regarded as an additional food source for the colony. We show that first instar larvae that had access to trophic eggs mostly developed into workers, whereas larvae without access to trophic eggs developed into gynes. This prompted us to investigate trophic egg content to identify which molecules could influence larval development in the direction of the worker caste. The results show that trophic eggs differ morphologically and quantitatively from reproductive eggs, with less protein, triglycerides, glycogen, sugar and small RNAs. Moreover, comparison of miRNAs fragment size distribution suggests differences in the composition of these miRNAs between the two egg types. These results raise the possibility that trophic eggs are an evolved maternal adaptation involved in caste determination in ants.

Keyword: Trophic eggs, *P. rugosus* ant, caste determination, triglycerides, glycogen, proteins, small RNA

Introduction

The textbook definition of trophic eggs are eggs that do not contain an embryo whose primary function has been described as a food source for developing offspring. The occurrence of this phenomenon is widespread, having been observed in several taxa, including insects, spiders, amphibians, marine invertebrates and sharks (Levin and Bridges 1995; Blake and Arnofsky 1999; Collin 2004; Kudo and Nakahira 2004; Perry and Roitberg 2006; Strathmann and Strathmann 2006; Gibson *et al.* 2012; López-Ortega and Williams 2018). In all cases, the widespread assumption is that these non-developing eggs are either (1) a mistake or by-product of failed reproduction, or (2) an evolved maternal adaptation to provide food resources for offspring and/or to reduce sibling cannibalism (Perry and Roitberg 2006). The only review on trophic eggs in eusocial insects was published by Crespi (1977) who put forwards two hypotheses providing an adaptive explanation of the evolution of trophic eggs. The first hypothesis assumes that trophic eggs serve as food for the colony members, and the second hypothesis is that trophic eggs provide an advantage for workers by keeping their ovaries active, which would be beneficial to rapidly switch to reproductive egg laying after the queen's death. None of the hypotheses explain the evolution of trophic egg laying by queens. Since Crespi's review, there has been no further theoretical debate on the evolutionary origins of trophic eggs in eusocial insects.

The morphological differences between reproductive and trophic eggs have been described in several ant species (*Acanthomyrmex careoscrobis*, *Anoplolepis gracilipes*, *Lasius niger*, *Messor semirufus, Myrmecia gulosa,* several *Myrmica* sp. and *Neoponera villosa*; Urbani 1991; CamargoMathias and Caetano 1995; Wardlaw and Elmes 1995; Dietemann *et al.* 2002; Helms Cahan *et al.* 2011; Lee *et al.* 2017; Yamada *et al.* 2018), however we found very little first-hand information about trophic eggs composition and production in ants across the literature. Only a few studies aimed at investigating trophic eggs directly, and the general assumption is that they are a food source for the colony and that they are laid by workers (*as per* Crespi 1977; Perry and Roitberg 2006). For the few species for which information is available, it is often incomplete and surprisingly unclear whether trophic eggs are laid by queens and/or workers. As a demonstration, we have collected all the data available on 60 species (to the best of our knowledge this is all the data available and comprises eight subfamilies; Table 1), and recorded whether or not the authors noted if the trophic eggs of that species were produced by queen(s) or workers. Trophic eggs are laid only by workers in three species, only by queens in three species, by both worker and queen in 10 species, and there are two species that do not produce trophic eggs at all. For the remaining 42 species, either workers or queens have been identified as producing trophic eggs, but it remains unclear whether the other caste lay or not trophic eggs.

It remains to be determined what factors influence trophic egg production, whether trophic eggs are laid all year-round or in specific periods (e.g. before and after hibernation) or how the timing of the colony life cycle (e.g. founding vs established stage) affects their production. Studies on several *Myrmica* species revealed that the production of trophic eggs by workers is influenced by the absence of the queen, the period of the year (before and after hibernation) and an increased exposure to light (Brian and Rigby 1978; Passera 1978; Choe 1988; Wardlaw and Elmes 1998), but not by worker size or age, the presence of brood or the size-class of the larvae. It remains to be clarified whether queens in *Myrmica* species are involved in trophic egg production. Additionally, there is almost no information regarding the content of trophic eggs, with the exception of one

study from Lorber and Passera (1981) which showed that trophic and reproductive eggs have the same composition in soluble proteins, but that the concentration is lower in trophic than in reproductive eggs.

Ultimately, the sweeping generalization that trophic eggs only provide a nutritional function is based on surprisingly little direct evidence and may be inaccurate. For example, recent studies on marine invertebrates revealed that trophic eggs contribute to the development of different morphs in the larvae of several spionid species (Gibson *et al.* 2012; Oyarzun and Brante 2015) and influence the duration of the larval stage (Simon 2015). In these species, trophic eggs are produced by the mother and are formed through an active developmental process, which lead Gibson *et al.* (2012) to conclude that trophic eggs are an evolved maternal adaptation. In ants, we were able to find a single study (Bartels 1988) in which the author described a potential influence of trophic eggs on larval development in the Argentine ants *Linepithema humile*. He observed a direct competition between queen and larvae to eat the trophic eggs laid by workers, with larvae failing to develop into queens without eating trophic eggs. The results from our own work on *P. rugosus* (see chapter 1, unpublished data) also suggest a role of trophic eggs on caste determination. A substantial research effort has been spent for years in eusocial insects to try to understand the factors influencing the caste fate of an egg. So far, several factors have been identified, such as environmental, nutritional, social, hormonal, pheromonal and genetic factors (Brian 1974; Bartels 1988; Anderson *et al.* 2008; Helms Cahan *et al.* 2011; Guo *et al.* 2013; Libbrecht *et al.* 2013), but despite this, the mechanism behind caste determination remains unclear. The aim of the present study is to investigate the potential role of trophic eggs in caste determination in *P. rugosus* ants. Firstly, we report on an experiment confirming a role of trophic eggs in caste determination wherein we discovered that when first instar larvae are raised in recipient colonies without trophic eggs, the percentage of gynes that developed was significantly higher than in recipient colonies where there were trophic eggs. Secondly, we investigate the structure and molecular composition of trophic eggs to test the hypothesis that the larval caste fate is directly influenced by some components found in trophic eggs. Finally, we discuss our results in the light of the theory of trophic eggs as an evolved maternal adaptation in *P. rugosus* and suggest that the notion of trophic eggs serving only as a nutritional supplement is inaccurate.

Table 1. List of ant species for which previous studies described the presence or absence of trophic eggs and whether they are laid by queens (q), workers (w), both (q & w) or neither (no trophic eggs). Question marks indicate that there is no information available concerning this caste or that further investigations are necessary to confirm or deny it. The absence of a caste means that it does not lay trophic eggs.

Subfamily	Species	T-eggs laid by:	References
Amblyoponinae	*Amblyopone silvestrii*	no trophic eggs	(Masuko 2003)
	Prionopelta kraepelini	w - q?	(Masuko 2003)
Dolichoderinae	*Dolichoderus quadripunctatus*	w - q?	(Fletcher and Ross 1985)
	Linepithema humile	w - q?	(Bartels 1988)
	Technomyrmex albipes	w & q	(Yamauchi *et al.* 1991)
Ectatomminae	*Gnamptogenys menadensis*	w	(Gobin *et al.* 1998, 1999)
	Ectatomma tuberculatum	w - q?	(Hora *et al.* 2007; Azevedo *et al.* 2011)
	Gnamptogenys costata	w - q?	(Gobin *et al.* 1998)
	Gnamptogenys dammemani	w - q?	(Gobin *et al.* 1998)
	Gnamptogenys moelleri	w - q?	(Gobin *et al.* 1998)
Formicinae	*Plagiolepis pygmaea*	w	(Passera 1980)
	Anoplolepis gracilipes	w - q?	(Lee *et al.* 2017)

	Cataglyphis floricola	w - q?	(Amor *et al.* 2017)
	Cataglyphis tartessica	w - q?	(Amor *et al.* 2017)
	Formica pergandei	w - q?	(Hung 1973)
	Oecophylla longinoda	w - q?	(Gobin *et al.* 1998)
	Lasius niger	w - q?	(Urbani 1991)
Myrmeciinae	*Myrmecia forceps*	w - q?	(Freeland 1958)
	Myrmecia gulosa	w - q?	(Dietemann *et al.* 2002)
Myrmicinae	*Pheidole pallidula*	q	(Lorber and Passera 1981)
	Pogonomyrmex rugosus	q	(Genzoni pers. obs.; Cahan *et al.* 2011)
	Solenopsis invicta	q	(Fletcher and Ross 1985)
	Pogonomyrmex barbatus	q - w?	(Volny *et al.* 2006)
	Pogonomyrmex J lineages	q - w?	(Helms Cahan *et al.* 2011)
	Acromyrmex sp.	w - q?	(Choe 1988; Dijkstra *et al.* 2005)
	Aphaenogaster cockerelli	w - q?	(Smith *et al.* 2008)
	Aphaenogaster rudis	w - q?	(Khila and Abouheif 2008, 2010)
	Aphaenogaster subterranea	w - q?	(Passera 1978; Choe 1988)
	Atta laevigata	w - q?	(Passera 1978)
	Leptothorax acervorum	w - q?	(Ito 2005)
	Leptothorax recedens	w - q?	(Choe 1988)
	Messor sp.	w - q?	(Choe 1988)
	Messor capitatus	w - q?	(Urbani 1991)
	Messor semirufus	w - q?	(Urbani 1991)
	Myrmica americana	w - q?	(Khila and Abouheif 2008)
	Novomessor cockerelli	w - q?	(Choe 1988)
	Pogonomyrmex badius	w - q?	(Passera 1978)
	Pogonomyrmex californicus	w - q?	(Smith et al. 2007)
	Temnothorax recedens (Nyl.)	w - q?	(Dejean and Passera 1974)
	Zacryptocerus varians (fr. smith)	w - q?	(Wilson 1976)
	Acanthomyrmex careoscrobis Moffett	w & q	(Yamada et al. 2018)
	Acanthomyrmex ferox	w & q	(Gobin and Ito 2000)
	Atta sp.	w & q	(Choe 1988; Dijkstra *et al.* 2005)
	Atta sexdens	w & q	(Della Lucia *et al.* 1990; Augustin *et al.* 2011)
	Crematogaster smithi Creighton	w & q	(Heinze *et al.* 1995)
	Myrmica rubra	w & q	(Wardlaw and Elmes 1995)
	Myrmica ruginodis	w & q	(Wardlaw and Elmes 1995)
	Myrmica schencki	w & q	(Wardlaw and Elmes 1995)
	Myrmica sulcinodis	w & q	(Wardlaw and Elmes 1995)
Nothomyrmeciinae	*Nothomyrmecia macrops*	w - q?	(Choe 1988)
Ponerinae	*Rhytidoponera mayri*	no trophic eggs	(Choe 1988)
	Hypoponera eduardi	q?	(Choe 1988)
	Odontomachus haematodes	q?	(Choe 1988)
	Rhytidoponera purpurea	q?	(Choe 1988)
	Pachycondyla apicalis	w	(Dietemann and Peeters 2000)
	Neoponera villosa	w - q?	(CamargoMathias and Caetano 1995)
	Pachycondyla krugeri	w - q?	(Gobin *et al.* 1998)
	Paraponera clavata	w - q?	(Peeters 2017)
	Diacamma rugosum	w?	(Choe 1988)
	Ophthalmopone berthoudi	w?	(Choe 1988)

Materials and methods

All established colonies used in this study were initiated by queens collected after a mating flight in 2008 (Bowie, Arizona, USA) or 2013 (Bowie and Florence, Arizona, USA). For all the sampling periods, the colonies were maintained at 28°C and 60% humidity, with a 12-h/12-h light:dark cycle and fed 24 hours before an egg collection session or once a week with grass seeds, flies and 20% honey water.

Trophic eggs influence on the larval caste fate

To determine whether trophic eggs influence the process of caste determination, we conducted an experiment where small queenless colonies (hereafter called recipient colonies) raised larvae until pupal stage. In half of the colonies, larvae were provided trophic eggs and in the other half, they did not receive trophic eggs. Once the pupal stage was reached, we compared the proportion of gynes and worker pupae that developed in recipient colonies. We conducted the experiment in October and November, 2020 using twenty-two *P. rugosus* colonies. Each day queens were isolated for 16 hours and eggs were collected twice every eight hours. The queens were then put back in their colony for eight hours until the next isolation session. After three days under this isolation schedule, the queens were given an uninterrupted 24-hour break. After collection, the eggs were sorted by type, trophic or reproductive, based on their color, shape and chorion resistance (see result section & Figure 1 for details). Reproductive eggs were kept in a petri dish containing a water reservoir and developed for 10 days until larvae hatched and trophic eggs were discarded. We created two recipient colonies per colony, one was used for the treatment group (with trophic eggs) and the other one for the control (no trophic eggs). Each recipient colony contained 10 foragers taken from the foraging arena and 10 nurses taken from inside the nest. Right after hatching, each larva from the treatment group was introduced in the corresponding recipient colony with three 0-4-hours old trophic eggs (queens were isolated again every day once larvae started hatching to provide freshly laid trophic eggs), whereas larvae from the control group were put in the recipient colony without trophic eggs. The same number of larvae (15 larvae on average) were placed in the two recipient colonies (control and treatment). There was no cross-fostering between colonies, so both the larvae and the workers raising the larvae came from the same colony. Individuals were collected at the pupal stage, as worker and gyne are morphologically distinct at this developmental stage in *P. rugosus* and the caste was recorded.

To compare the proportion of gyne pupae between the group with and without trophic eggs, we used Rstudio (RStudio Team 2015) and the package *lme4* (Bates *et al.* 2015) to perform a binomial linear mixed effects analysis, with caste as response variable (binary categorical factor) and presence/absence of trophic eggs as an explanatory variable. Colony was included as a random effect. We considered that an alternative explanation of a differential caste ratio between control and treatment (other than an effect of trophic eggs on the larval caste fate) could be a differential mortality with larvae eating trophic eggs having a higher survival rate than larvae without trophic eggs. To test for this, we performed a linear mixed effects analysis with mortality as response variable, presence/absence of trophic eggs as explanatory variable, and colonies as random effects. As we found a significantly higher survival rate in larvae eating trophic eggs than larvae without trophic eggs, we tested whether the survival might be correlated to the percentage of gynes by performing a linear

mixed effects analysis with the percentage of gyne as response variable, number of dead larvae as an explanatory variable and colonies as random factor.

Content of trophic and reproductive eggs

The egg collections were carried out in March-October 2021 in twelve colonies. Queens were isolated for 10 hours (7am to 5pm CET) and kept in the dark in a petri-dish with three workers and a water supply. Eggs were collected every hour using a fine brush, sorted and pooled by type, trophic or reproductive, according to the different protocol requirements: 6 eggs were pooled for RNA extraction and 20 eggs pooled for fat-sugar-protein extraction. Right after, the samples were flash-frozen in liquid nitrogen, so all eggs were a maximum of one hour old. Egg samples were kept at -80°C to preserve their content until the extractions were performed. After the 10 hours isolation, queens and workers were put back into their colony until the next isolation session. There were two replicates per egg type and colony for both RNA and fat-sugar-protein extractions.

Total and small RNA

RNA (>200 nt) and small RNA isolations were performed using the miRNeasy Mini Kit (Qiagen, cat. no. 217004) and RNeasy® MinElute® Cleanup Kit (Qiagen, cat. no. 74204), respectively. In brief, eggs were homogenized in QIAzol Lysis Reagent (Qiagen) with beads using a Precellys Evolution tissue homogenizer coupled with a Cryolys Evolution (Bertin Technologies SAS). Chloroform was added and after centrifugation, the upper aqueous phase containing the RNA was carefully removed and transferred in a new reaction tube. Ethanol was added to the sample and after vortexing, it was pipetted into a RNeasy Mini spin column. After centrifugation, the flow-through containing the small RNAs was transferred into a RNeasy MinElute spin column and after several washes, small RNAs were eluted in RNase-free water. The RNeasy Mini spin column was washed as well, and the RNA (>200 nt) were eluted in RNase-free water. RNA (>200 nt) and small RNA concentrations were measured with a QuantiFluor® RNA System (Promega). RNA (>200 nt) integrity was examined with an Agilent Fragment Analyzer (at the Lausanne Genomic Technologies Facility) using a High Sensitivity Assay and small RNA were examined using the small RNA kit (at the Gene Expression Core Facility at EPFL). All RNA samples were stored at 4°C for future potential analyses.

For the analyses, we compared the miRNA and RNA (>200nt) concentrations between the reproductive and trophic egg groups. We took the average between the two replicates per colony and used Rstudio (RStudio Team 2015) to perform a paired t-test. To detect potential differences in small RNA identity between trophic and reproductive eggs, we used the electropherogram of the samples and did a Mantel test on the fragment distribution, between 10 to 40 nucleotides for the miRNAs (Figure 6C) and 50 to 90 for the RNA transfer (tRNA). As the RNA messenger (mRNA) fragments are too large to be detected by the Fragment Analyzer, we used the range containing the mRNA degraded fragments (from 200 to 2000 nucleotides; Figure 7A) to indirectly evaluate the mRNA fragment distributions. Differences in mRNA fragment distributions between trophic and reproductive eggs would be an indication of differential mRNAs between the two egg types. We also compared the mRNA degradation between the two egg types by calculating the area under the curve for the fragments between 200 and 2000 nucleotides. We did a linear mixed model analysis with the residues to take the RNA concentration into account.

Triglycerides, glycogen, glucose, and proteins quantification

Triglycerides, glycogen and glucose were quantified as described by Tennessen *et al.* (2014), and protein levels were measured using a Bradford assay (Bradford 1976). The 20 one-hour old eggs per sample were homogenized with beads in 200µl of PBS buffer in a Precellys Evolution tissue homogenizer coupled with a Cryolys Evolution (Bertin Technologies SAS). The homogenate was separated in aliquots to perform the three Bradford, Glycogen/Glucose and Triglycerides assays. All isolated homogenates that could not be immediately processed were stored at -80°C to avoid degradation.

For the Bradford assay, 10*µ*l of the homogenate (in duplicates) were put in a clear-bottom 96-well plate with 300*µ*l of Coomassie Plus Reagent (Thermo Scientific: 23200) and incubated for 10 minutes at room temperature. Protein standard (Sigma: P5369) was used as standard (ranging from 0-0.5mg/ml) and protein absorbance was read at 595nm on a Hidex Sense Microplate Reader.

To get an estimation of the lipid content in trophic and reproductive eggs, we extracted and quantified triglycerides, as they are the basic unit of lipids. For the triglycerides assay, 90*µ*l of homogenate were heat treated at 70°C for 10 minutes, then 40*µ*l were mixed with 40*µ*l of Triglyceride Reagent (Sigma: T2449) for digestion and 40*µ*l were mixed with PBS buffer for free glycerol measurement. After a 30 minutes incubation at 37°C, 30*µ*l of each sample and standards were transferred to clear-bottom 96-well plate (in duplicate). We added 100*µ*l of Free Glycerol Reagent (Sigma: F6428) to each sample, mixed well by pipetting and incubated five minutes at 37°C. Glycerol standard solution (Sigma: G7793) was used as standard (ranging from 0-1.0 mg/ml TAG) and absorbance was read at 540nm on a Hidex Sense Microplate Reader. The triglycerides concentration in each sample was determined by subtracting the absorbance of free glycerol in the corresponding sample.

Glucose is another major source of energy, which is stored in the form of glycogen. To estimate the glucose and glycogen content in trophic and reproductive eggs, we used a glycogen assay, as described in Tennessen *et al.* (2014). The assay is based on the conversion of glycogen to glucose by the amyloglucosidase enzyme and on its measurement by the glucose assay reagent. The 90 *µ*l aliquot was heat treated at 70°C for 10 min and then diluted 1:2 with PBS. The standard curves for glucose (Sigma, G6918) and glycogen (Sigma: G0885) were made by diluting stocks to 160 *µ*g/ml, making 1:1 serial dilution for 160, 80, 40, 20 and 10 *µ*g/ml. 40 *µ*l of each sample was pipetted in duplicates of a clear microplate, and 30 *µ*l of each glucose or glycogen standard was pipetted in duplicates. Amyloglucosidase enzyme (Sigma, A1602) was diluted 3 *µ*l into 2000 *µ*l of PBS, and 40 *µ*l diluted enzyme was pipetted to the glycogen standards and to one well of the sample (for total glucose determination), 40 *µ*l PBS was pipetted to the glucose standards and to the other sample well (for free glucose determination). The plate was incubated at 37°C for 60 minutes. 30*µ*l of each standard and samples (in duplicates) were transferred to a UV 96-well plate and 100 *µ*l Glucose Assay Reagent (G3293) was pipetted to each well. The plate was incubated at room temperature for 15 min and the absorbance was read at 340 nm on a Hidex Sense Microplate Reader. The free glucose concentration was calculated based on the glucose standard curve. The glycogen concentration was quantified by subtracting the free glucose absorbance from the total glycogen + glucose absorbance.

Concentrations of each compound (protein, triglycerides, glycogen, and glucose) between the reproductive eggs and trophic eggs were compared with Rstudio (RStudio Team 2015) and the package *lme4* (Bates *et al.* 2015) to perform a linear mixed effects analysis, with the concentration as response variable and egg type as explanatory variable. We added colony and extraction batch as random effects.

Worker and queen egg laying behaviour

To determine whether *P. rugosus* workers lay trophic and/or reproductive eggs, we isolated ten workers from each of 12 queenless colonies and divided them randomly into two groups of five, each placed in a petri-dish. The workers remained in these petri dishes for 12 hours every other day for two weeks. We collected the eggs at the end of each isolation and sorted them by type, trophic or reproductive. All eggs were put into a petri dish containing a water reservoir for humidity where they developed for 10 days to confirm their type. The number of larvae hatching from reproductive eggs was recorded.

To test whether queens lay reproductive and trophic eggs non-randomly (Figure 3), we performed a Wald–Wolfowitz runs test for each queen's egg laying sequence using Rstudio (package *snpar* v.1.0; RStudio Team 2015). This is a non-parametric test which calculates the likelihood that a binomial data sequence is random.

Results

Characteristics of trophic and reproductive eggs in *P. rugosus*

P. rugosus queens lay both reproductive eggs (Figure 1A-C), that develop into either worker or gyne, and trophic eggs that do not contain a developing embryo (Figure 1D-F). It remains unclear whether they lay unfertilized eggs that develop into males, as males very rarely develop in queenright colonies in the lab (pers. obs). However, Smith *et al.* (2007) found that in *P. badius*, the majority of the males (>97%) produced in queenright colonies develop from haploid eggs laid by the queen. We found that *P. rugosus* workers start laying eggs approximately three weeks after queen's removal. None of the eggs laid by the workers were trophic, they were all reproductive eggs; approximately 90% of them successfully hatched and only approximately 5% succeeded to develop until the pupal stage; 100% of the pupae were males. We therefore conclude that workers do not lay trophic eggs but can lay reproductive haploid eggs in queenless colonies that, despite being unfertilized, develop into males.

Trophic and reproductive eggs are morphologically different in *P. rugosus* and can be distinguished under a microscope as soon as they are laid (Figure 1A,D). Trophic eggs are about a third larger than reproductive eggs (94.3±4.3nL vs. 63.3±1.6nL, respectively), slightly oval in shape, white and semi-transparent, with a smooth surface containing granular looking content and a fragile chorion (Figure 1D-F). Reproductive eggs have a distinct oval shape, white with a bright surface, a homogenous content, and a solid chorion (Figure 1A-C).

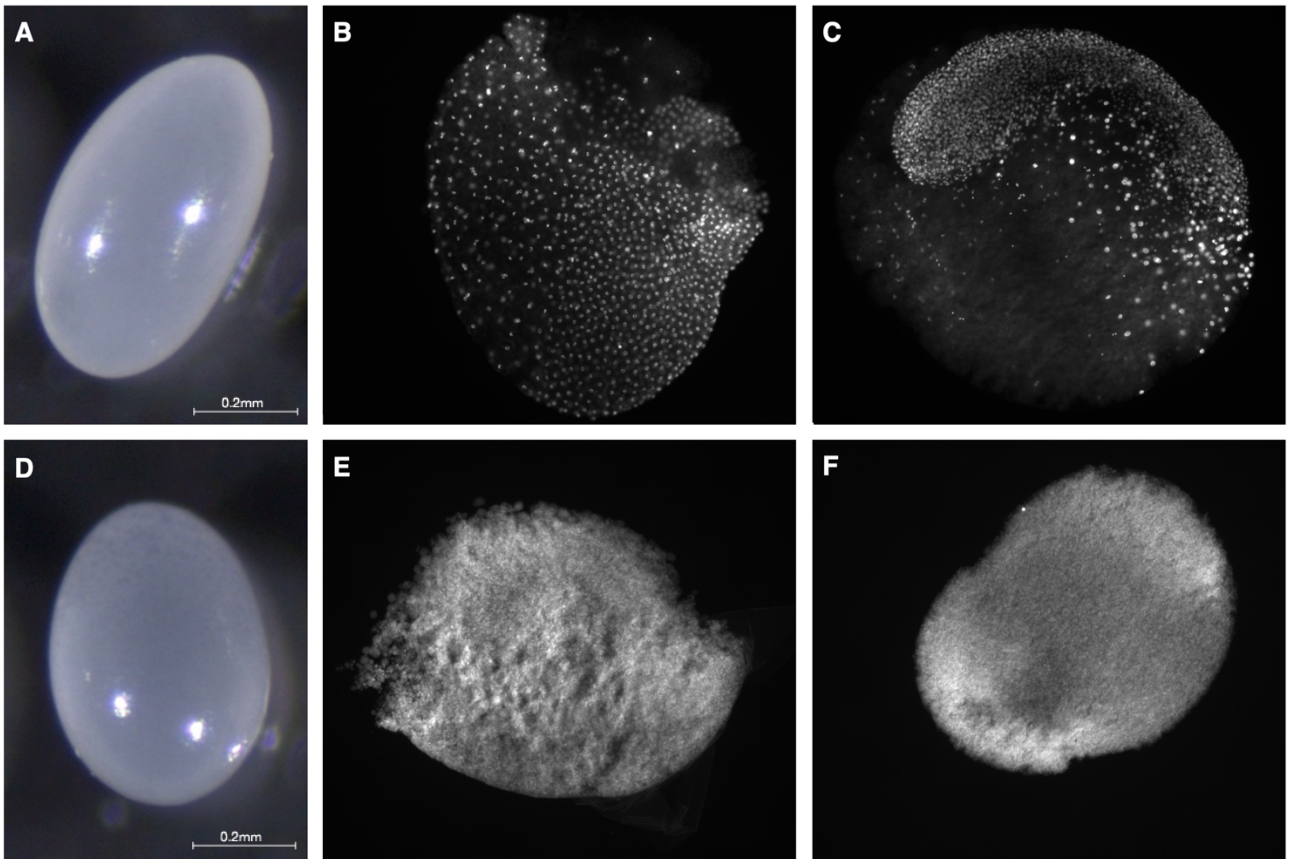


Figure 1. Morphology and development of eggs laid by *P. rugosus* queens. Reproductive eggs (**A-C**) with the embryo's development at approximately 25 hours (**B**) and 65 hours (**C**). The white dots are nuclei dyed with Dapi. In trophic eggs (**D-F**), we do not observe any embryonic development at 25 hours (**E**), nor at 65 hours (**F**). The bright white dot on (**F**) could be the maternal nucleus of the oocyte, as we found that trophic eggs contain DNA (see Figure 5C), but approximately half the amount found in reproductive eggs right after egg laying, which suggests that trophic eggs are not fertilized.

Trophic eggs represent on average 56.6±1.3% of the total number of eggs laid by queens (mean± SE; n=43 colonies). There is a significantly higher percentage of trophic eggs before hibernation (61.6±1.4%) than after (50.3±2.0%; β= 11.20, SE=2.21, t(86)=5.04, $p=9x10^{-6}$), which is caused by an increase in the number of reproductive eggs laid after hibernation compared to before, whereas the number of trophic eggs remain stable throughout the year (Figure 2).

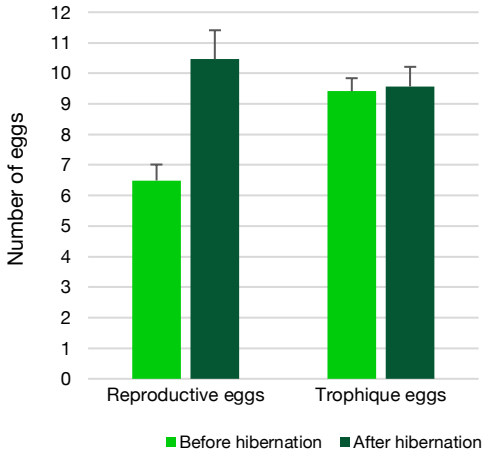


Figure 2. Average number of reproductive and trophic eggs laid by the queens in 24 hours, before and after hibernation.

Queens lay eggs by batches consisting solely of either reproductive or trophic eggs (Figure 3), non-randomly, as supported by the significant p-values from the Wald-Wolfowitz runs tests (p-values in Table 2). Each queen's egg laying sequence was tested independently and in each case, the sequence was determined to be non-random. Within each batch, we recorded an average of 6.1±0.5 eggs (mean±SE) regardless of the egg type. The fact that eggs are laid by batch of same type and neither randomly, or one by one (one reproductive, one trophic and so on), support the fact that the production of the two egg types is different and therefore that trophic eggs are not failed reproductive eggs. This assortative laying behavior might suggest that production of both egg types is under the control of the queen.

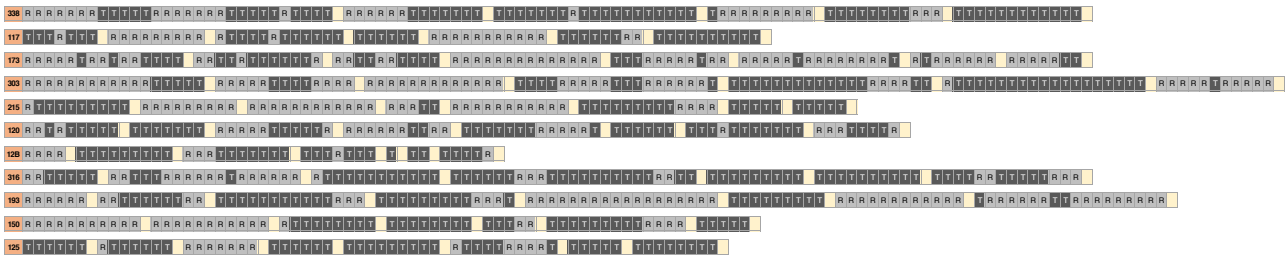


Figure 3. Egg laying sequences from eleven *P. rugosus* queens. Every row shows the eggs laid by one queen (queen ID in the orange cell), with **R** for reproductive and **T** for trophic eggs. Each egg laying session lasted 10 hours. Each yellow square separates two egg laying sessions and represents an interval of minimum 16 hours to several days.

Table 2. Results of the Wald-Wolfowitz runs tests on the queen's egg sequence. The significant p-values indicate that queens do not lay reproductive and trophic eggs in a random sequence.

Queen ID	P-value for egg sequence	P-value of random sequence	Number of eggs per sequence
338	$8.2e^{-12}$	0.419	94
117	$8.7e^{-08}$	0.567	63
173	$3.4e^{-04}$	0.755	92
303	$1.0e^{-13}$	0.765	110
215	$2.0e^{-11}$	0.292	70
120	$2.8e^{-06}$	0.518	75
12B	$3.7e^{-04}$	0.298	38
316	$6.9e^{-10}$	0.737	93
193	$8.6e^{-13}$	0.655	101
150	$2.9e^{-11}$	0.630	62
125	$3.0e^{-06}$	0.404	58

Trophic eggs as a new factor influencing caste determination

There was a significantly higher percentage of larvae that developed into gynes in the control (without trophic eggs; 83±10%; mean ± SE) than in the treatment group (with trophic eggs; 27±9%; $\beta = 2.95$, SE = 0.64, $z = 4.25$, $p = 2x10^{-5}$; Figure 4). To determine whether this could be due to differential mortality between larvae eating or not trophic eggs, rather than an effect of trophic eggs on the larval caste fate, we performed a linear mixed effects analysis with mortality as response variable and presence/absence of trophic eggs as explanatory variable (colony added as random factor). This analysis revealed a significantly higher survival of the larvae that received trophic eggs (30.2±6.7% of survival; mean ±SE) compared to the larvae that did not receive trophic eggs (16.9±3.8% of survival) for the transition from larva to pupa ($\beta = 0.85$, SE = 0.32, $z = 2.66$, $p = 0.008$). However, there was no correlation between the percentage of gynes and number of deaths among larvae ($\beta = 0.88$, SE = 0.91, $z = 0.97$, $p = 0.34$). This allows us to exclude differential survival between castes as an explanation for the high proportion of gynes observed in the control group.

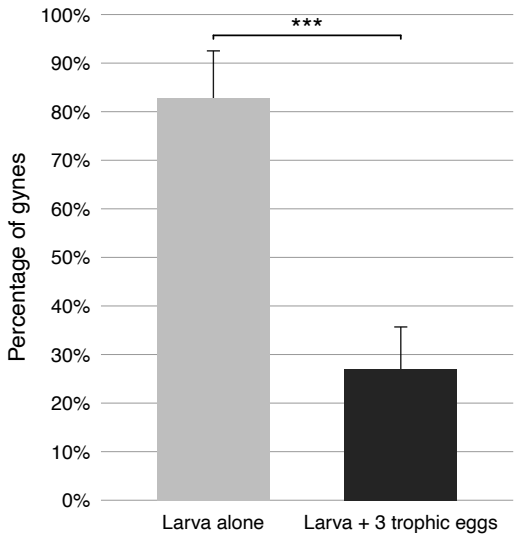


Figure 4. Percentage of gynes which developed from larva transferred in the recipient colony without (grey) or with (black) trophic eggs.

Table 3. Number of worker and gyne pupae that developed from larva without trophic eggs (**W**) or with three trophic eggs (**3T**). Empty cells mean that no pupae developed in this recipient colony.

Sample size	50		61		66		69		75		81		117		120		125		130		150		173	
	W	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**
worker		5		12	1	2	1			4		1		1	1	2		1	1		1	2		
gyne	2	3	3		1		1	1	2	1	1				1						2	1	2	

Sample size	193		215		235		286		303		316		338		12B		59A		99A		Total	
	W	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**	**W**	**3T**
worker						1		4	1			1		2		5		3			6	46
gyne	3	3		2			2			1	2	1	1	1	4		2	1		2	29	17

Quantitative and qualitative evaluation of RNA in eggs

There was a significantly higher amount of small RNA in reproductive eggs (44.3±1.4ng, mean ± SE) than in trophic eggs (22.3±1.1ng; $t_{(23)} = 15.9$, $p = 6.5*10^{-14}$; Figure 5A). The same is observed in RNA (>200 nt) with 7.6±2.1 ng per reproductive eggs and 3.6±0.3ng per trophic egg ($t_{(23)} = 7.2$, $p = 2.7*10^{-7}$; Figure 5B).

To determine whether trophic eggs were fertilized or not (i.e. in which case we would expect half the DNA than that of reproductive eggs), we quantified DNA in both reproductive and trophic eggs and used the amount of DNA in reproductive eggs as a reference for the quantity of DNA in fertilized eggs. There was significantly less DNA in trophic eggs (8.8±1.9ng/μl; $t_{(4.7)} = 2.7$, $p = 0.045$; Figure 5C) compared to reproductive eggs (15.9±1.9ng/μl), corresponding approximately to half that of reproductive eggs. This suggests that trophic eggs are not fertilized.

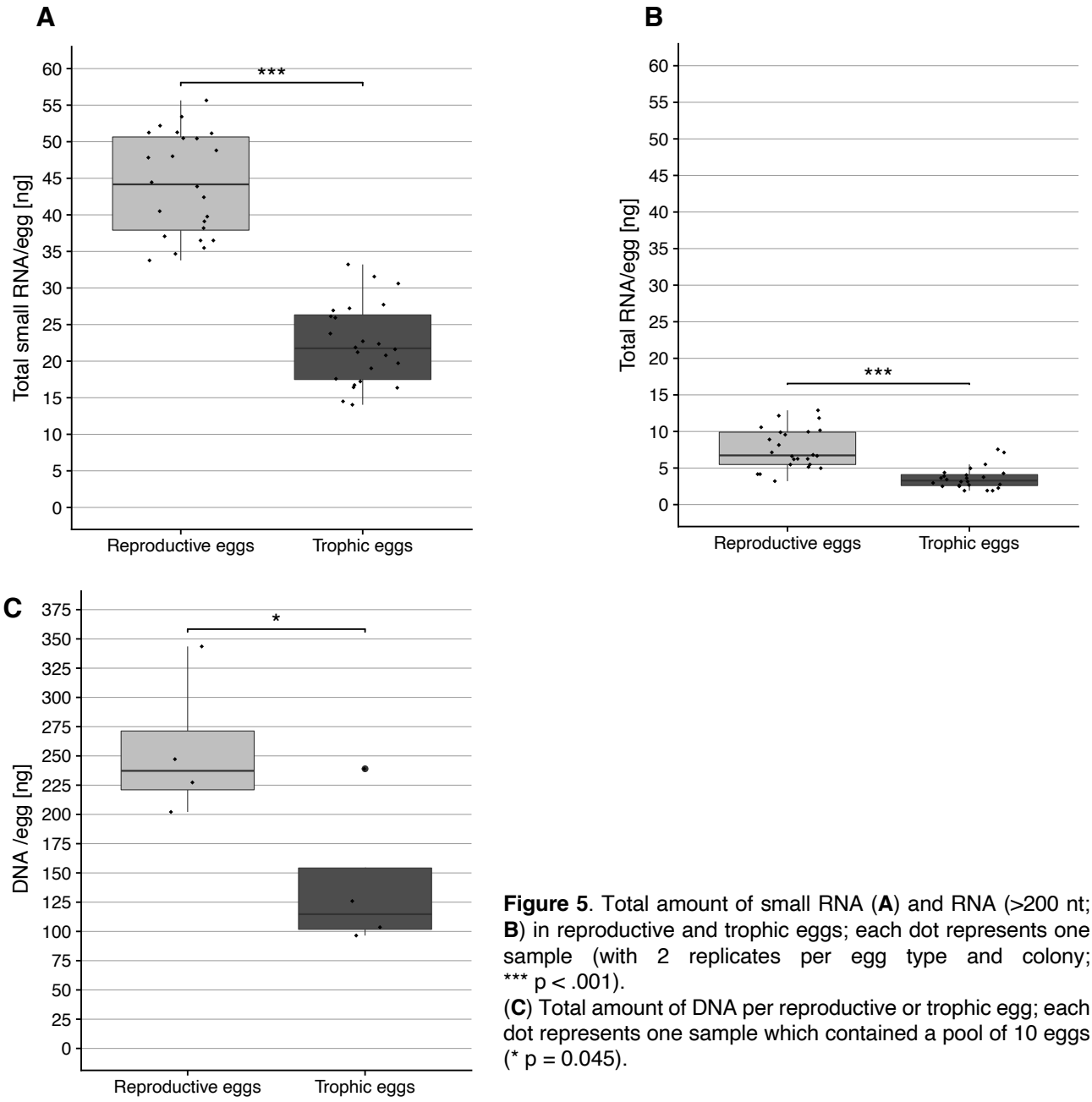


Figure 5. Total amount of small RNA (**A**) and RNA (>200 nt; **B**) in reproductive and trophic eggs; each dot represents one sample (with 2 replicates per egg type and colony; *** $p < .001$).
(**C**) Total amount of DNA per reproductive or trophic egg; each dot represents one sample which contained a pool of 10 eggs (* $p = 0.045$).

There was a significant difference in the miRNA fragment size distribution between reproductive and trophic eggs ($r_M = 0.26$, $p<.0001$). A principal component analysis (PCA) revealed that 56.5% of the total variation in the fragment size was explained by the first principal component (PC1; Figure 6A) which separates trophic and reproductive eggs into two clusters; 24.3% of the remaining variation was explained by the second principal component (PC2). There was no difference in the tRNA fragment size distribution between the two egg types ($r_M = -0.01$, $p=0.49$), as shown on the PCA (Figure 6B) where there is no separation of the samples by egg types.

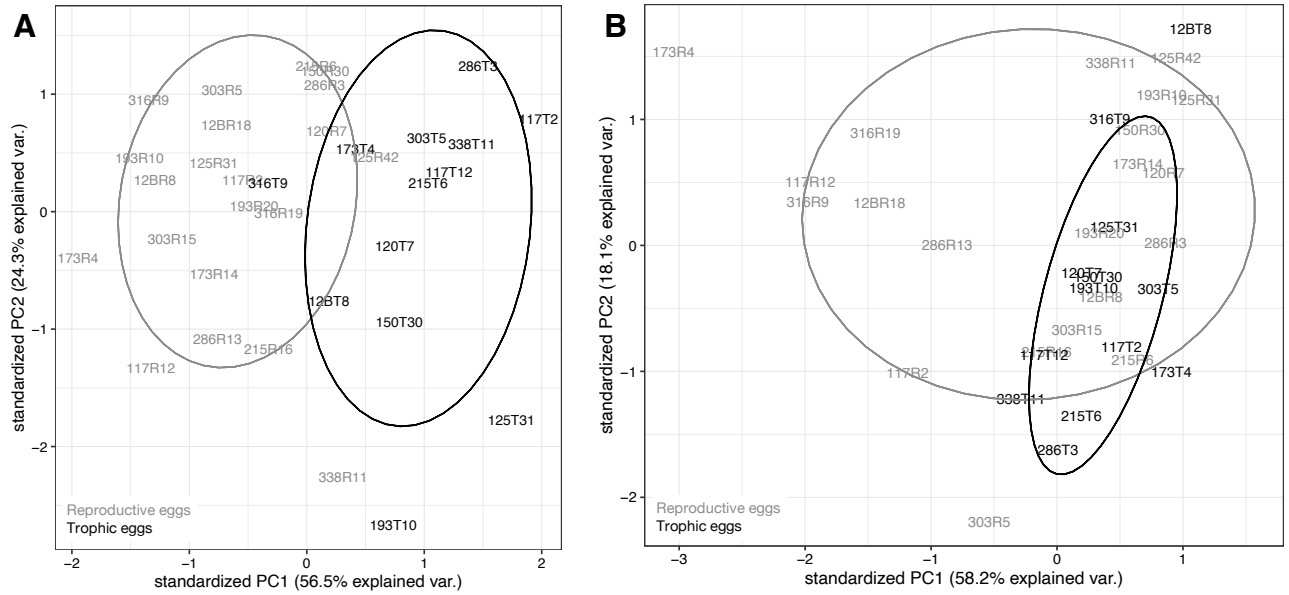


Figure 6. First two principal components (PC1 and PC2) explaining size distribution variation for (**A**) miRNA and (**B**) tRNA across egg samples, with reproductive eggs in grey and trophic eggs in black. There is a separation of the samples by egg types for miRNAs, whereas there is no separation for tRNA, as both trophic and reproductive egg samples overlap. Ellipses enclose each of the egg type groups.
(**C**) Profile of the small RNA electropherogram, with the peaks showing the size distribution of RNA fragments shorter than 200 nucleotides. The yellow squares show the ranges, for miRNA (from 10 to 40 nucleotides) and tRNA (from 50 to 90 nucleotides), used to compare the fragment size distributions between egg types.

Although not statistically significant, there was a trend with a higher RNA (>200nt) degradation in reproductive than trophic eggs (β = -28447, SE = 16616, t = -1.71, p = 0.09; Figure 7B).

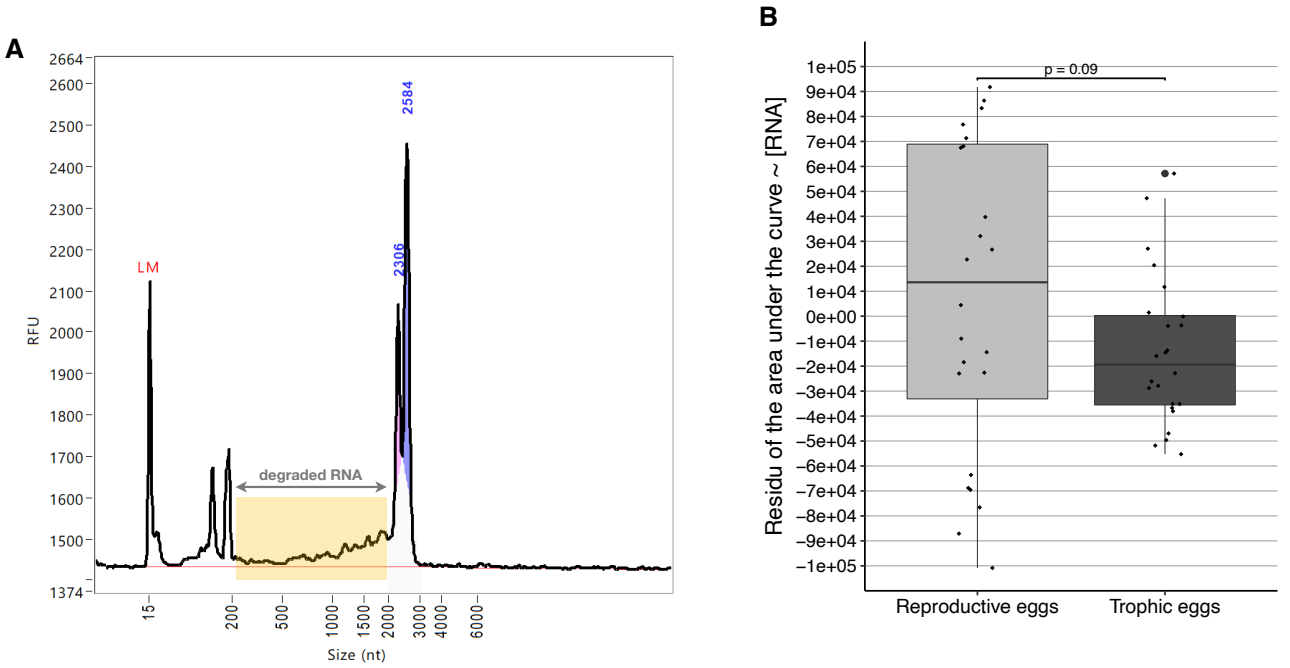


Figure 7. (**A**) Profile of the RNA electropherogram, with the peaks showing the size distribution of RNA and degraded RNA fragments. The yellow square shows the range (from 200 to 2000 nucleotides) used to evaluate RNA degradation between egg types. (**B**) Residue of the size distribution of the degraded RNA (>200nt) fragments in reproductive and trophic eggs.

Triglycerides, glycogen, glucose, and proteins concentrations

The concentrations were consistently significantly higher in reproductive eggs than in trophic eggs for the four quantified compounds: protein (β = -3.93, SE = 0.30, t = -13.11, p <.0001), triglycerides (β = -3.70, SE = 0.32, t = -11.66, p <.0001), glycogen (β = -1.24, SE = 0.10, t = -11.98, p <.0001), and glucose (β = -1.61, SE = 0.09, t = -18.60, p <.0001; Figure 8).

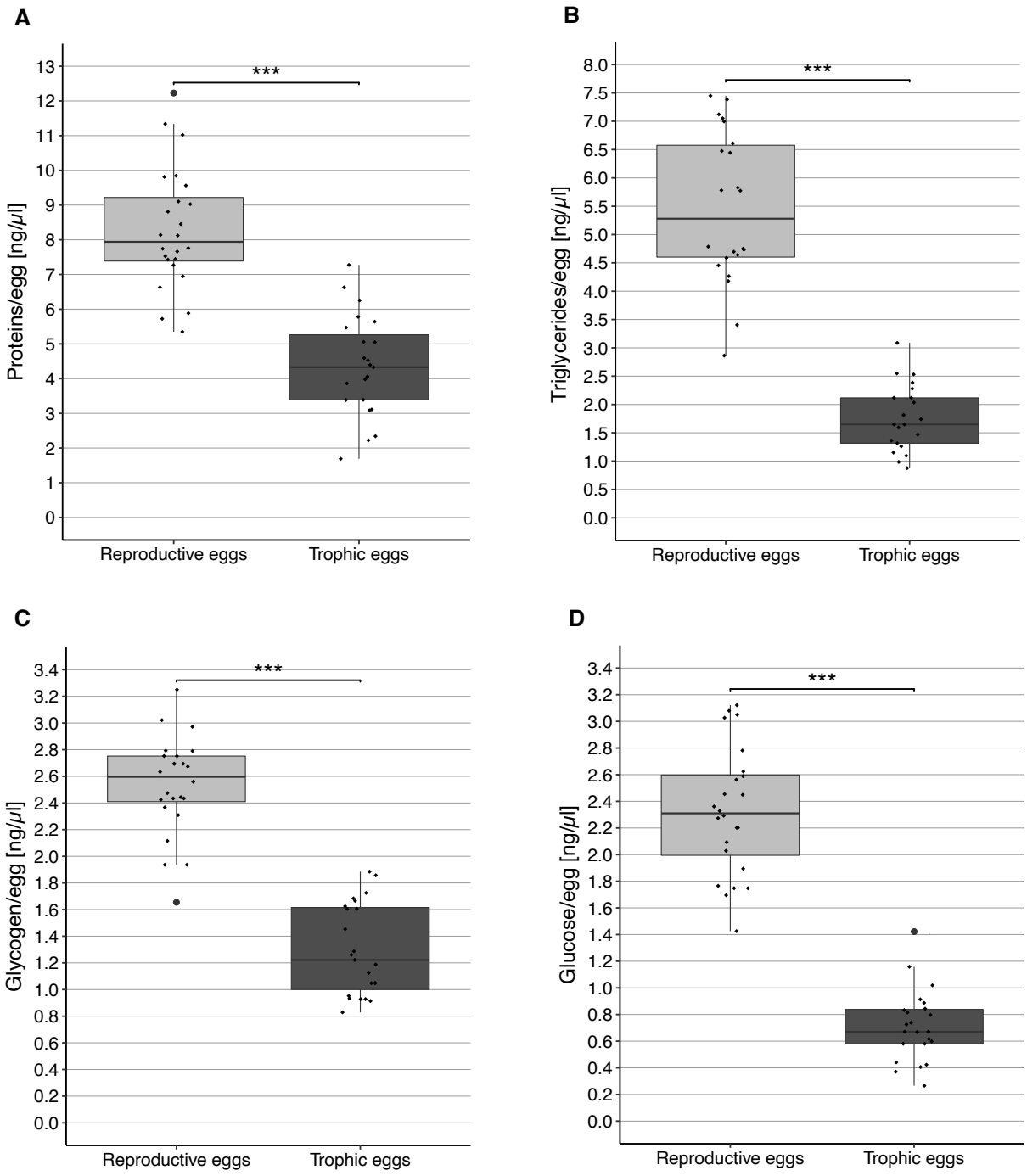


Figure 8. Concentration of protein (**A**), triglycerides (**B**), glycogen (**C**) and glucose (**D**) per reproductive and trophic egg. On the figure, each dot represents one sample (with 2 replicates per egg type and colony).

Discussion

Trophic eggs, an evolved maternal adaptation in *P. rugosus* ants

Our study provides several lines of evidence supporting the hypothesis that trophic eggs in *P. rugosus* are an evolved maternal adaptation, and not just a by-product of egg production. First, trophic and reproductive eggs are morphologically distinct, similar to what has been described in other ant species (Wilson 1976; Wardlaw and Elmes 1995; Dietemann *et al.* 2002; Lee *et al.* 2017) and they can be distinguished as soon as they are laid (Figure 1; Gobin *et al.* 1998; Perry and Roitberg 2006). Dissections of the ponerine ants *Gnamptogenys menadensis* and *Pachycondyla apicalis* showed that trophic and reproductive eggs are already distinguishable in the ovaries (Gobin *et al.* 1998; Dietemann and Peeters 2000), with trophic and reproductive oocytes even found to be developing in the same ovariole in *Aphaenogaster rudis* workers (from queenless colonies; Khila and Abouheif 2010). This suggests that trophic and reproductive eggs could be the product of two fundamentally different developmental pathways. We found approximately half the amount of DNA in trophic eggs compared to reproductive eggs, which suggests that trophic eggs are not fertilized (*see also* Urbani 1991; Elgar and Crespi 1992). However, as unfertilized eggs usually develop into males across Hymenopteran species, the fact that trophic eggs are unfertilized but do not develop into males further support that they follow a different developmental pathway than reproductive eggs. One promising candidate for trophic egg development might be the localization of the maternal determinant (*Vasa*) during oocyte development. Khila and Abouheif (2008, 2010) reported that in *Aphaenogaster* ants, oocytes follow a common developmental pathway up until *Vasa* localization after which development diverges between reproductive and trophic oocytes, the latter being characterized by the absence of *Vasa* localization.

Second, trophic and reproductive eggs show differences in their nutritional content, with trophic eggs having fewer proteins, glycogen, glucose and triglycerides than reproductive eggs. This is consistent with Crespi's (1977) suggestion on the evolution of cannibalism and trophic eggs asserting that trophic eggs must be less costly to produce than reproductive ones. Weeks *et al.* (2004) reported that larvae are fed with proteins in higher proportions than with lipids or carbohydrates and suggested that early protein provision assists development. This is supported by our finding that protein is the most abundant constituent of *P. rugosus* trophic eggs, consistent with what was found in *Pheidole pallidula* (Lorber and Passera 1981). Our results found that trophic eggs contain a quantifiably lower amount of lipids, proteins and carbohydrates, which means that the investment into trophic eggs is likely less than that of reproductive eggs. This observation corroborates the hypothesis of Crespi (1977) that trophic eggs are less costly to produce than reproductive eggs. Our results also showed that protein is the largest constituent part of trophic eggs, and if it is the case that trophic eggs are a maternal adaptation, this might be advantageous for early larval development (as per the observation of Weeks *et al.* 2004).

Third, we found a higher percentage of trophic eggs (57%) than reproductive eggs, which is higher than what was has been reported in other species (30% of trophic eggs in *Myrmica rubra,* Wardlaw and Elmes 1995; 49% of trophic eggs in *Acanthomyrmex ferox*, Gobin and Ito 2000). This high percentage of trophic eggs is inconsistent with the by-product hypothesis as it predicts a low number of failed reproductive eggs such that

there is no measurable adaptive benefit to the offspring. It is important to note that first instar larvae cannot process solid food (Freeland 1958), therefore workers cannot feed them with seed bread or insects, as in later instar. Trophic eggs therefore represent an important nutritional intake for freshly hatched larvae (Crespi 1977), which may significantly enhance larval survival and contribute to their faster development into workers, compared to the longer developmental time that we observe in gynes without trophic eggs (on average in *P. rugosus*, 57 days for gynes development from egg to adult emergence vs 41 days for workers, pers. obs). Taken together, these results provide clear evidence for two fundamentally different egg types and support the hypothesis that trophic eggs are an evolved maternal adaptation in *P. rugosus*.

Influence of trophic eggs on larval caste-fate

The first part of our study showed that larvae fed with trophic eggs are significantly more likely to develop into workers than larvae without access to trophic eggs. This suggests that the function of ant trophic eggs may go further than the provision of early larval nutrition and is also involved in caste determination. Maternal effect on caste determination has been shown in several species, either by queen behaviour on developing larvae, or egg content, effect of hibernation on queen's physiology (Schwander *et al.* 2008; Libbrecht *et al.* 2013), and we now provide evidence for the first time that the provision of trophic eggs influences caste fate in *P. rugosus*. Using trophic eggs, which are the first food source for freshly hatched larvae (Freeland 1958; Brian and Rigby 1978), the queen could transfer compounds to the larva and influence its development along the worker pathway. Our results are consistent with findings in other species (including Argentine ant and spionid worms; Bartels 1988; Gibson *et al.* 2012; Oyarzun and Brante 2015), suggesting that the use of trophic eggs as a mechanism for phenotype differentiation may be substantially more widespread than what has been previously hypothesized in the literature on trophic eggs (*see* introduction). Ant species that do not have trophic eggs might use other mechanisms (e.g. trophallaxis) to transfer or exchange nutrients and other molecules between colony members (Crespi 1977; Dietemann *et al.* 2002), such as caste-influencing compounds, chemicals or hormones (LeBoeuf *et al.* 2016).

Our results predict that under natural condition, should a colony experience a decrease in trophic eggs, there would be an increase in gyne production. There are two situations where we observe the development of gynes in *P. rugosus* colonies, (1) after hibernation (such as in *P. badius*, Smith and Tschinkel 2006) and (2) when the queen dies or is removed from the colony (also observed in *Myrmecia gulosa*, Dietemann *et al.* 2002). We found that the trophic:reproductive egg ratio is lower after hibernation compared to before, which means that there are less trophic eggs available per larva after hibernation. As trophic eggs are produced only by the queen and not by the workers in *P. rugosus*, when the egg laying rate of the queen decreases (winter period) or stops (queen's death), there are less or no trophic eggs available for the larvae developing from the last batch of eggs laid by the queen, which coincides with an increase of gyne development, further supporting trophic eggs playing a role in the process of caste determination.

It has been shown in several ant species that workers lay only trophic eggs in queenright colonies but mostly (if not only) reproductive eggs (that develop into males) in queenless colonies (pers. obs. in *P. rugosus*; Dejean and Passera 1974; Passera 1980; Wardlaw and Elmes 1998; Dietemann and Peeters 2000; Gobin and Ito

2000; Dietemann *et al.* 2002; Smith *et al.* 2007, 2008; Khila and Abouheif 2008; Amor *et al.* 2017). This suggests that even in ant species where trophic eggs are laid by workers, the number of trophic eggs decrease in the absence of the queen as workers focus their efforts on producing their own reproductive male offspring. As the number of trophic eggs decline within the colony, this coincides with an increase in the proportion of larvae that developed into gynes. Although the above correlation between the decline in trophic egg number and the increase in gyne percentage is empirically untested, it merits investigation to determine if this further supports a role of trophic eggs in caste-determination, as demonstrated here.

RNA content in trophic and reproductive egg

The overall RNA concentration (small RNA and longer RNA together) was higher in reproductive eggs than in trophic eggs, although both egg types shared the same proportion of small RNA to longer RNA in a ratio of 4:1. Small RNAs (i.e. miRNAs, tRNAs, 5S and 5.8S) comprised the dominant fraction with 85% of the total RNAs (comparatively similar proportion in trophic and reproductive eggs), whereas the minor fraction represented longer RNA (i.e. mRNAs, 18S and 28S rRNAs). The same pattern was observed in ovaries of roach *Rutilus rutilus* (Kroupova *et al.* 2011), in oocytes of the frog *Xenopus laevis* (Picard *et al.* 1980) and in the oocytes of nine teleost fishes (Mazabraud *et al.* 1975) with higher concentration of small RNAs than longer RNAs. Our results are consistent with what has been shown in eggs from other species.

The difference in miRNA fragments size distribution between the two egg types suggests differences in the identity of miRNAs. miRNAs have already been shown to influence larval caste determination in the honeybee (Guo *et al.* 2013) with previous studies finding differences in miRNA complexity between the royal jelly and worker jelly, the latter being enriched in miRNA (Guo *et al.* 2013; Zhu *et al.* 2017). These studies suggest that it is not the royal jelly that stimulates larval differentiation into queen, but rather the worker jelly which stimulates the development of larva into worker. We can make a parallel between the honeybee worker jelly and the ant trophic eggs. Both jelly and trophic eggs are eaten by larvae, both at the first larval instar and both might transfer molecular compounds to the larvae through the medium of a food packet. When honeybee larvae are fed with worker jelly, they develop into workers, which is identical to what we found in *P. rugosus* with larvae developing into workers when they are fed with trophic eggs. Our findings are analogous to those of Guo *et al.* (2013) and we suggest that trophic eggs in *P. rugosus* are analogous to honey bee jelly in the honey bee, confirming the hypothesis of Bartels (1988) for a role of trophic eggs in caste determination. We suggest that small RNAs are good candidates to trigger larval development into one caste or the other. miRNA sequencing would allow us to confirm the suspected difference in miRNAs composition between trophic and reproductive eggs as well as to identify miRNAs that could influence the larval caste fate. For this, the miRNAs with the highest concentration in trophic eggs should be selected, their inhibitor synthesized, and then injected into trophic eggs to artificially manipulate the miRNAs composition (as per Guo *et al.* 2013). By feeding those manipulated trophic eggs to first instar larvae, we could observe whether they influence larval development into gynes, by comparison with unmanipulated trophic eggs as a control where larvae would develop into workers. We predict either gyne pupae or worker pupae with measurable gyne-like phenotype.

Our results show a trend of higher RNA degradation in reproductive eggs than in trophic eggs. If the analysis of additional samples reveal that there is indeed a difference in RNA degradation between reproductive and trophic eggs, several explanations can be put forward. Previous studies on *Drosophila* found that the degradation of the maternal RNA starts upon egg activation at the oviposition time (Vastenhouw *et al.* 2019). The *Drosophila* egg is blocked in the first meiotic division until the oviposition, which activates the eggs to complete the meiosis and initiates its development (Went 1982). Observations of egg laying in bumblebees and honeybees (Reinhardt 1960; Went 1982) suggest that in Hymenoptera Aculeata, the egg is activated as well by the oviposition, and more precisely by its deformation or compression caused by its passage in the uterus and/or vagina (cf. figure page 33 in Wheeler 1910). In our study, eggs were collected and flash frozen maximum one hour after being laid. If maternal RNA starts to degrade upon egg activation as supposed, it could explain the RNA degradation observed in reproductive eggs. As we found low RNA degradation in trophic eggs and as no embryo develops in them, we can assume that trophic eggs are not activated. There are two potential explanations for this, (1) as trophic eggs do not possess a solid chorion, like that of reproductive eggs (Went 1982; Klowden 2013), their passage through the uterus/vagina does not compress them or move their content around and therefore does not activate them (Went 1982; Klowden 2013) and/or (2) trophic eggs contain a protein or 3' 5' RNA modifications which protects the RNA against degradation (Wang *et al.* 2010; Towler *et al.* 2015; Gagliardi and Dziembowski 2018). If there is such a mechanism in trophic eggs protecting miRNAs from degradation, it would make miRNAs even more likely to be able to mediate gene expression if they are ingested by a larva through trophic eggs. Further investigations on proteins or RNA modifications in trophic egg would help understanding the mechanisms protecting and stabilizing miRNA from degradation.

Conclusion

Our study provides evidence dismissing the by-product hypothesis and raising the possibility that trophic eggs are a maternal adaptation in *P. rugosus* ants. We propose that trophic eggs function not just as a food source, as we showed that they also directly influence larval caste fate by triggering the worker developmental pathway. Finally, we propose a potential molecular mechanism similar to that described in the honeybee (Guo *et al.* 2013), whereby miRNAs might act on and influence larval development. Ongoing quantification of juvenile hormone and ecdysteroids will provide a better understanding of which molecules influence larval development into workers. Further investigation is still necessary to fully understand the role and influence of trophic eggs on caste determination in *P. rugosus* and in other ant species.

Acknowledgments

We thank C. Berney for technical assistance to develop wet lab protocols. We are grateful to S. McGregor for his helpful comments on the manuscript. This work was supported by an ERC grant and the Swiss NSF (LK).

References

Amor, F., P. Ortega, R. Boulay, and X. Cerdá. 2017. Frequent colony orphaning triggers the production of replacement queens via worker thelytoky in a desert-dwelling ant. *Insectes Soc.* **64**:373–378.

Anderson, K. E., T. a Linksvayer, and C. R. Smith. 2008. The causes and consequences of genetic caste determination in ants (Hymenoptera : Formicidae). *Ecology* **11**:119–132.

Augustin, J. O., J. F. Santos, and S. L. Elliot. 2011. A behavioral repertoire of *Atta sexdens* (Hymenoptera, Formicidae) queens during the claustral founding and ergonomic stages. *Insectes Soc.* **58**:197–206.

Azevedo, D. O., J. C. Zanuncio, J. H. C. Delabie, and J. E. Serrão. 2011. Temporal variation of vitellogenin synthesis in *Ectatomma tuberculatum* (Formicidae: Ectatomminae) workers. *J. Insect Physiol.* **57**:972–977.

Bartels, P. J. 1988. Reproductive caste inhibition by Argentine ant queens: New mechanisms of queen control. *Insectes Soc.* **35**:70–81.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**:1–48.

Blake, J. A., and P. L. Arnofsky. 1999. Reproduction and larval development of the spioniform Polychaeta with application to systematics and phylogeny. *Hydrobiologia*. **402**:57-106

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**:248–254.

Brian, M. V. 1974. Caste differentiation in *Myrmica rubra*: The role of hormones. *J. Insect Physiol.* **20**:1351–1365.

Brian, M. V, and C. Rigby. 1978. The trophic eggs of *Myrmica rubra L. Insect Mol. Biol.* **25**:89–110.

CamargoMathias, M., and F. Caetano. 1995. Trophic eggs in workers of *Neoponera villosa* ants (Hymenoptera:Ponerinae). *J. Adv. Zool.* **16**:62–66.

Choe, J. C. 1988. Worker reproduction and social evolution in ants (Hymenoptera: Formicidae). Advances in myrmecology. 163-187

Collin, R. 2004. Phylogenetic effects, the loss of complex characters, and the evolution of development in calyptraeid gastropods. *Evolution.* **58**:1488–1502.

Crespi, B. J. 1977. Cannibalism and trophic eggs in subsocial and eusocial insects. Pp. 176–213 in M. A. Elgar and B. J. Crespi, eds. Cannibalism: Ecology and Evolution Among Diverse Taxa.

Dejean, A., and L. Passera. 1974. Ponte des ouvrières et inhibition royale chez la Fourmi *Temnothorax recedens* (Nyl.) (Formicidae, Myrmicinae). *Insectes Soc.* **21**:343–355.

Della Lucia, T. M. C., E. F. Vilela, D. D. O. Moreira, J. M. S. Bento, and N. Dos Anjos. 1990. Egg-laying in Atta sexdens rubropilosa, under laboratory conditions. Pp. 173–179 in R. K. Vander Meer, K. Jaffé, and A. Cedeno, eds. Applied Myrmecology: A World Perspective. Westview Press, New York.

Dietemann, V., B. Hölldobler, and C. Peeters. 2002. Caste specialization and differentiation in reproductive potential in the phylogenetically primitive ant *Myrmecia gulosa. Insectes Soc.* **49**:289–298.

Dietemann, V., and C. Peeters. 2000. Queen influence on the shift from trophic to reproductive eggs laid by workers of the ponerine ant *Pachycondyla apicalis*. *Insectes Soc.* **47**:223–228.

Dijkstra, M. B., D. R. Nash, and J. J. Boomsma. 2005. Self-restraint and sterility in workers of *Acromyrmex* and *Atta* leafcutter ants. *Insectes Soc.* **52**:67–76.

Elgar, M. A., and B. E. Crespi. 1992. Cannibalism: Ecology and Evolution among diverse taxa. Oxford.

Fletcher, D. J. C., and K. G. Ross. 1985. Regulation of reproduction in Eusocial hymenoptera. *Annu. Rev. Entomol.* **30**:319–343.

Freeland, J. 1958. Biological and social patterns in the Australian bulldog ants of the genus *Myrmecia*. *Aust. J. Zool.* **6**:1–18.

Gagliardi, D., and A. Dziembowski. 2018. 5' and 3' modifications controlling RNA degradation: from safeguards to executioners. *Phil. Trans. R. Soc. B.* **373**:1–5.

Gibson, G., C. Hart, C. Coulter, and H. Xu. 2012. Nurse eggs form through an active process of apoptosis in the spionid *Polydora cornuta* (Annelida). *Integr. Comp. Biol.* **52**:151–160.

Gobin, B., J. Billen, and C. Peeters. 1999. Policing behaviour towards virgin egg layers in a polygynous ponerine ant. *Anim. Behav.* **58**:1117–1122.

Gobin, B., and F. Ito. 2000. Queens and major workers of *Acanthomyrmex ferox* redistribute nutrients with trophic eggs. *Naturwissenschaften* **87**:323–326.

Gobin, B., C. Peeters, and J. Billen. 1998. Production of trophic eggs by virgin workers in the ponerine ant *Gnamptogenys menadensis*. *Physiol. Entomol.* **23**:329–336.

Guo, X., S. Su, G. Skogerboe, S. Dai, W. Li, Z. Li, F. Liu, R. Ni, Y. Guo, S. Chen, S. Zhang, and R. Chen. 2013. Recipe for a busy bee: MicroRNAs in honey bee caste determination. *PLoS One* **8**:1–10.

Heinze, J., S. Cover, and B. Hölldobler. 1995. Neither worker, nor queen: an ant caste specialized in the production of unfertilized eggs. *Psyche A J. Entomol.* **102**:173–185.

Helms Cahan, S., C. J. Graves, and C. S. Brent. 2011. Intergenerational effect of juvenile hormone on offspring in *Pogonomyrmex harvester* ants. *J. Comp. Physiol. B.* **181**:991–999.

Hora, R. R., C. Poteaux, C. Doums, D. Fresneau, and R. Fénéron. 2007. Egg cannibalism in a facultative polygynous ant: Conflict for reproduction or strategy to survive? *Ethology.* **113**:909-916.

Hung, A. C. F. 1973. Reproductive biology in dulotic ants: preliminary report (Hymenoptera: Formicidae). *Entomol. News.* **84**:253–259.

Ito, F. 2005. Mechanisms regulating functional monogyny in a Japanese population of *Leptothorax acervorum* (Hymenoptera, Formicidae): Dominance hierarchy and preferential egg cannibalism. *Belgian J. Zool.* **135**:3–8.

Khila, A., and E. Abouheif. 2010. Evaluating the role of reproductive constraints in ant social evolution. *Philos. Trans. R. Soc. B.* **365**:617–630.

Khila, A., and E. Abouheif. 2008. Reproductive constraint is a developmental mechanism that maintains social harmony in advanced ant societies. *Proc. Natl. Acad. Sci. U.S.A.* **105**:17884–17889.

Klowden, M. J. 2013. Developmental Systems. Pp. 149–196 in M. J. Klowden, ed. Physiological Systems in Insects (Third Edition). Academic Press.

Kroupova, H., A. Trubiroha, S. Wuertz, and W. Kloas. 2011. Stage-dependent differences in RNA composition and content affect the outcome of expression profiling in roach (*Rutilus rutilus*) ovary. *Comp. Biochem. Physiol. Part A* **159**:141–149.

Kudo, S., and T. Nakahira. 2004. Effects of Trophic-Eggs on Offspring Performance and Rivalry in a Sub-Social Bug. *Oikos*. **107**:28–35.

LeBoeuf, A. C., P. Waridel, C. S. Brent, A. N. Gonçalves, L. Menin, D. Ortiz, O. Riba-Grognuz, A. Koto, Z. G. Soares, E. Privman, E. A. Miska, R. Benton, and L. Keller. 2016. Oral transfer of chemical cues, growth proteins and hormones in social insects. *Elife*. **5:e20375**:1-27

Lee, C. C., H. Nakao, S. P. Tseng, H. W. Hsu, G. L. Lin, J. W. Tay, J. Billen, F. Ito, C. Y. Lee, C. C. Lin, and C. C. S. Yang. 2017. Worker reproduction of the invasive yellow crazy ant *Anoplolepis gracilipes*. *Front. Zool.* **14**:1–12.

Levin, L. A., and T. S. Bridges. 1995. Pattern and diversity in reproduction and development. Pp. 1–48 in L. McEdward, ed. Ecology of marine invertebrate larvae. CRC Press.

Libbrecht, R., M. Corona, F. Wende, D. O. Azevedo, J. E. Serrão, and L. Keller. 2013. Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proc. Natl. Acad. Sci. U.S.A.* **110**:11050–11055.

López-Ortega, M., and T. Williams. 2018. Natural enemy defense, provisioning and oviposition site selection as maternal strategies to enhance offspring survival in a sub-social bug. *PLoS One* **13**:1–18.

Lorber, B., and L. Passera. 1981. Etude comparative des protéines solubles des oeufs de la fourmi *Pheidole pallidula* Nyl. Bull Intérieur SF-UIEIS 97–99.

Masuko, K. 2003. Larval oophagy in the ant *Amblyopone silvestrii* (Hymenoptera, Formicidae). *Insectes Soc*. **50**:317–322.

Mazabraud, A., M. Wegnez, and H. Denis. 1975. Biochemical research on oogenesis. RNA accumulation in the oocytes of teleosts. *Dev. Biol*. **44**:326–332.

Oyarzun, F. X., and A. Brante. 2015. A new case of poecilogony from South America and the implications of nurse eggs, capsule structure, and maternal brooding behavior on the development of different larval types. *Biol. Bull.* **228**:85–97.

Passera, L. 1980. La fonction inhibitrice des reines de la fourmi *Plagiolepis pygmaea* Latr.: Rôle des phéromones. *Insectes Soc*. **27**:212–225.

Passera, L. 1978. Une nouvelle catégorie d'oeufs alimentaires: les oeufs alimentaires émis par les reines vierges de *Pheidole pallidula* (Nyl.) (Formicifae, Myrmicinae). *Insectes Soc*. 25:117–126.

Peeters, C. 2017. Independent colony foundation in *Paraponera clavata* (hymenoptera: Formicidae): First workers lay trophic eggs to feed queen's larvae. *Sociobiology* **64**:417–422.

Perry, J. C., and B. D. Roitberg. 2006. Trophic egg laying: Hypotheses and tests. *Oikos* **112**:706–714.

Picard, B., M. le Maire, M. Wegnez, and H. Denis. 1980. Biochemical Research on Oogenesis: Composition of the 42-S Storage Particles of *Xenopus laevis* Oocytes. *European Journal of Biochemistry* **109**:359-368

Reinhardt, E. 1960. Kernverhaltnisse, Eisystem und Entwicklungsweise von Drohnen- und Arbeiterin- neneiern der Honigbiene (*Apis mellifera*). *Zool. Jahrbücher, Abteilung für Anat. und Ontog. derTiere* **78**:167–234.

RStudio Team. 2015. RStudio: Integrated Development Environment for R. Boston, MA.

Schwander, T., J. Y. Humbert, C. S. Brent, S. Helms Cahan, L. Chapuis, E. Renai, and L. Keller. 2008. Maternal Effect on Female Caste Determination in a Social Insect. Curr. Biol. **18**:265–269.

Simon, C. A. 2015. Observations on the composition and larval developmental modes of polydorid pests of farmed oysters (*Crassostrea gigas*) and abalone (*Haliotis midae*) in South Africa. *Invertebr. Reprod. Dev.* **59**:124–130.

Smith, A. A., B. Hölldobler, and J. Liebig. 2008. Hydrocarbon signals explain the pattern of worker and egg policing in the ant *Aphaenogaster cockerelli. J. Chem. Ecol.* **34**:1275–1282.

Smith, C. R., C. Schoenick, K. E. Anderson, J. Gadau, and A. V Suarez. 2007. Potential and realized reproduction by different worker castes in queen-less and queen-right colonies of *Pogonomyrmex badius. Insect. Soc.* **54**:260-267

Smith, C. R., and W. R. Tschinkel. 2006. The sociometry and sociogenesis of reproduction in the Florida harvester ant, *Pogonomyrmex badius. J. Insect Sci.* **6**:1–11.

Strathmann, M. F., and R. R. Strathmann. 2006. A vermetid gastropod with complex intracapsular cannibalism of nurse eggs and sibling larvae and a high potential for invasion. *Pacific Sci.* **60**:97–108.

Tennessen, J. aso. M., W. E. Barry, J. Cox, and C. S. Thummel. 2014. Methods for studying metabolism in *Drosophila*. *Methods* **68**:105–115.

Towler, B. P., C. I. Jones, and S. F. Newbury. 2015. Mechanisms of regulation of mature miRNAs. *Biochem. Soc. Trans.* **43**:1208–1214.

Urbani, C. B. 1991. Indiscriminate oophagy by ant larvae: an explanation for brood serial organization? *Insectes Soc.* **38**:229–239.

Vastenhouw, N. L., W. X. Cao, and H. D. Lipshitz. 2019. The maternal-to-zygotic transition revisited. *Development*. **146**:1–20.

Volny, V. P., M. J. Greene, D. M. Gordon, V. P. Volny, M. J. Greene, and D. M. Gordon. 2006. Brood Production and Lineage Discrimination in the Red Harvester Ant (*Pogonomyrmex barbatus*). *Ecology*. **87**:2194–2200.

Wang, K., S. Zhang, J. Weber, D. Baxter, and D. J. Galas. 2010. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res*. **38**:7248–7259.

Wardlaw, J. C., and G. W. Elmes. 1995. Trophic eggs laid by fertile *Myrmica* queens (Hymenoptera: Formicidae). *Insectes Soc*. **42**:303–308.

Wardlaw, J. C., and G. W. Elmes. 1998. Variability in oviposition by workers of six species of *Myrmica* (hymenoptera, formicidae). *Insectes Soc.* **45**:369–384.

Weeks, R. D., L. T. Wilson, S. B. Vinson, and W. D. James. 2004. Flow of carbohydrates, lipids, and protein among colonies of polygyne red imported fire ants, *Solenopsis invicta* (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am.* **97**:105–110.

Went, D. F. 1982. Egg Activation and Parthenogenetic Reproduction in Insects. *Biol. Rev.* **57**:319–344.

Wheeler, W. M. 1910. Ants: Their Structure, Development, and Behavior. The Columbia University Press, New York.

Wilson, E. O. 1976. A social ethogram of the neotropical arboreal ant *Zacryptocerus varians* (Fr. Smith). *Anim. Behav.* **24**:354–363.

Yamada, A., F. Ito, R. Hashim, and K. Eguchi. 2018. Queen polymorphism in Acanthomyrmex careoscrobis Moffett, 1986 in Peninsular Malaysia (Hymenoptera: Formicidae: Myrmicinae), with descriptions of hitherto unknown female castes and males. *Asian Myrmecology.* **10**:1-19.

Yamauchi, K., T. Furukawa, K. Kinomura, H. Takamine, and K. Tsuji. 1991. Secondary Polygyny by Inbred Wingless Sexuals in the Dolichoderine Ant *Technomyrmex albipes*. *Behav. Ecol. Sociobiol*. **29**:313–319.

Zhu, K., M. Liu, Z. Fu, Z. Zhou, Y. Kong, H. Liang, Z. Lin, J. Luo, H. Zheng, P. Wan, J. Zhang, K. Zen, J. Chen, F. Hu, C. Y. Zhang, J. Ren, and X. Chen. 2017. Plant microRNAs in larval food regulate honeybee caste development. *PLoS Genet.* **13**:1–23.

CHAPTER 4

Caste biased gene expression during early development in *Pogonomyrmex* J lineages

E. Genzoni, C. Morandin, S. McKenzie, L. Keller and T. Schwander

Author contributions: EG, TS and LK designed the study. EG collected the data. SMcK did the custom gene annotation and read mapping. EG and CM analysed the data. EG wrote the manuscript, with input from all co-authors.

Abstract

Phenotypic plasticity, the capacity of a single genome to produce different phenotypes in response to environmental cues, has been a topic of interest for the past decade. However, there is limited understanding of the molecular mechanisms underlying this phenomenon. Eusocial insects provide a good model to study phenotypic plasticity thanks to the presence of reproductive queens and non-reproductive workers that differ greatly in morphology, physiology, behavior and life history. This study aims at investigating the molecular mechanisms underlying gyne and worker caste differentiation in early developmental stages in ants. To address this, we used *Pogonomyrmex* J lineages ants as their genetic caste-determination system allows us to determine the caste-fate at any developmental stage via genotyping approaches. We used mRNA-seq to identify caste-biased genes and profile gene expression dynamics at five early developmental stages ranging between 24-hour old eggs and second instar larvae. We found differences in gene expression between gyne and worker-destined embryos as early as 24 hours after egg laying, and two developmental stages showed a burst of caste-biased genes, in six day old eggs and in the first instar larvae, which suggests that in *P. rugosus* J lineages, caste differentiation starts early in embryonic development. There were more worker-biased than gyne-biased genes in all five developmental stages, which supports the idea that the queen phenotype is the default developmental pathway in ants and worker developmental programs are actively switched on. More studies on gene expression in worker and gyne-destined ants at the egg stage are necessary to broaden our understanding of caste differentiation mechanisms.

Keywords: Genetic caste determination, caste-biased gene expression, early developmental stages, ants, *Pogonomyrmex* J lineages.

Introduction

The key characteristic of eusocial insects is the reproductive division of labor between female castes, with the queen specializing in reproduction and the workers specialized in all the non-reproductive tasks. It has been documented in different eusocial species that whether a female egg develops into a worker or into a gyne (virgin queen) is influenced by environmental, nutritional, social, hormonal, pheromonal, epigenetic and/or genetic factors (Haydak 1970; Wheeler 1986; Hölldobler and Wilson 1990; Richards and Packer 1994; Schwander *et al.* 2008; Smith *et al.* 2008; Bignell *et al.* 2010; Miura and Scharf 2011; Guo *et al.* 2013; Walker 2017; Taylor *et al.* 2019). However, in ants it remains unknown whether one of these factors alone is sufficient to trigger caste differentiation. In the honeybee *Apis mellifera*, the quantity and quality of food is known to be responsible for caste differentiation (Asencot and Lensky 1985; Leimar *et al.* 2012; Slater 2017; Slater *et al.* 2020). In ants, many factors have been found to influence caste development with variations between species: food, worker:egg ratio, queen hibernation, queen absence, pheromones, age or condition (Libbrecht *et al.* 2011). The genotype of the nurses taking care of the developing larvae can influence caste fate as well (Linksvayer 2006; Linksvayer *et al.* 2011; Teseo *et al.* 2014; Villalta *et al.* 2016). All factors are described as influencing the probability of gyne production, however it remains unknown whether one or several of these factors are necessary or sufficient for gyne development. Finally, in some species of the genus *Pogonomyrmex* (Helms Cahan *et al.* 2002; Julian *et al.* 2002; Schwander *et al.* 2010; Sirviö *et al.* 2011), *Solenopsis* (Hung and Vinson 1977; Helms Cahan and Vinson 2003) and *Messor* (Norman et al. 2016), the genotype of the individual is the main determinant of the caste, with queens and workers having different genomic compositions. However, despite the genotype determining the caste fate, additional factors are required for successful queen development (Schwander *et al.* 2008).

In contrast to the many identified environmental factors affecting queen vs worker production, little is known about the molecular mechanisms regulating queen vs worker development. Whilst there are some genomic studies that have identified genes that are differentially expressed between castes at the adult stage of different social insects (Sumner *et al.* 2006; Gräff *et al.* 2007; Grozinger *et al.* 2007; Hoffman and Goodisman 2007; Toth *et al.* 2007; Hunt *et al.* 2010; Colgan *et al.* 2011; Ometto *et al.* 2011), adult individuals are already morphologically and physiologically differentiated and therefore do not provide information on caste determination. Investigations at early developmental stages have thus far only been done in bees where individual caste fates are known (Pereboom *et al.* 2005; Cameron *et al.* 2013) thanks to distinct worker vs queen cells (Shi *et al.* 2011). Cameron *et al.* (2013) found early differences in gene expression in honeybees between worker- and gyne-destined larvae six hours after hatching. During this early phase, larval gene expression changed in response to royal jelly (newly hatched larvae were grafted into queen rearing cells), biasing development towards queens, while retaining the ability to reverse to the worker developmental pathway. Royal jelly contains histone deacetylase inhibitors (Bonasio *et al.* 2012; Sumner *et al.* 2018) and noncoding microRNAs that have been proposed as regulators of caste differentiation (Guo *et al.* 2013; Ashby *et al.* 2016). After 84 hours, a peak of juvenile hormone (JH) induces a unique set of genes in queen larvae which marks the irreversible bifurcation in the developmental pathway (Cameron *et al.* 2013) and later in development, *A. mellifera* larvae express a distinct set of worker- or queen-related genes (Evans & Wheeler,

2000). Four differentially expressed genes in *A. mellifera* are also differentially expressed in the bumblebee *Bumbus terrestris* (Pereboom *et al.* 2005), however there is little further similarities between the two species, as the expression pattern varies between species; in the bumblebee, most of the genes that are upregulated in early-instar queen-destined larvae are also upregulated in worker-destined larvae but later on in their development (Pereboom *et al.* 2005).

In summary, there is very little information on molecular mechanisms of caste differentiation in social insects, with the exception of the honeybee, where the caste fate can be assigned early in development, before any morphological differences, depending on whether the larva is in a worker or queen cell. Recent data from bumblebees suggest that the molecular mechanisms underlying caste differentiation in different bee species feature little convergence (Collins *et al.* 2021) and does not allow us to draw further conclusions about whether or not caste differentiation is a conserved process across the social insects. Here, we will use an ant species with genetic caste determination, which allows to determine the individual's caste fate early in the development, to investigate molecular mechanisms underlying caste differentiation in ants and to provide an additional comparison to the existing literature on bees.

In the ant genus *Pogonomyrmex,* historical hybridization between *P. rugosus* and *P. barbatus* with environmental caste determination (ECD) gave rise to several co-dependent lineages characterized by genetic caste determination (GCD; Helms Cahan *et al.* 2002; Julian *et al.* 2002; Volny and Gordon 2002; Helms Cahan and Keller 2003). These lineages are characterized by an unusual reproductive system, called social hybridogenesis (Figure 1; Helms Cahan *et al.* 2002; Julian *et al.* 2002), in which the lineages are completely dependent on one another to produce the workers. In this system, the virgin queen needs to mate with at least two males during the mating flight, one from her own lineage and one from the other lineage. Within lineage fertilization gives rise to gyne-destined eggs, whereas eggs fertilized by sperm from male of the other lineage develop into workers. Males are haploid and develop from unfertilized eggs laid by queens from both lineages. This genetic difference between pure lineage gynes and inter-lineage workers allows us to accurately determine the caste of the individual through genotyping at any developmental stage, especially before visible morphological differences between gynes and workers.

In both *P. rugosus and P. barbatus* (ECD), and *Pogonomyrmex* lineages (GCD), the development of gynes is triggered by specific conditions. Only eggs laid by queens of at least 3 years old and having hibernated are able to go through the development to become adult gynes (Schwander *et al.* 2008). Libbrecht *et al.* (2013) showed that the hibernation of the queen increases the gene expression of the insulin/insulin-like growth factor signaling and JH, which increase the production of vitellogenin, and the high proportion of vitellogenin in the egg influences its development into gyne. Although queens from GCD colonies lay gyne-destined eggs all year round, only the eggs laid after hibernation can develop successfully into gynes until the adult stage. The rest of the year, gyne-destined eggs stop developing at late egg-early larval stage (Helms Cahan *et al.* 2004; Clark *et al.* 2006; Volny *et al.* 2006), but how and why this happens remains unknown.

In this project, we took advantage of being able to determine the individual's caste at any developmental stage to compare gene expression during early development, where we expect the caste determination to occur, as

worker- and gyne-destined larvae from third instar exhibit morphological differences (Smith and Tschinkel 2006). This study had three main aims. The first one was to profile gene expression differences between queen- and worker-destined individuals of *Pogonomyrmex* J lineages at early developmental stages and determine when gene expression starts differing between castes. The second aim was to characterize the gene expression dynamics throughout the embryonic and early larval-instar stages and the third aim was to identify promising candidate genes and GO-terms that may be involved in caste differentiation.

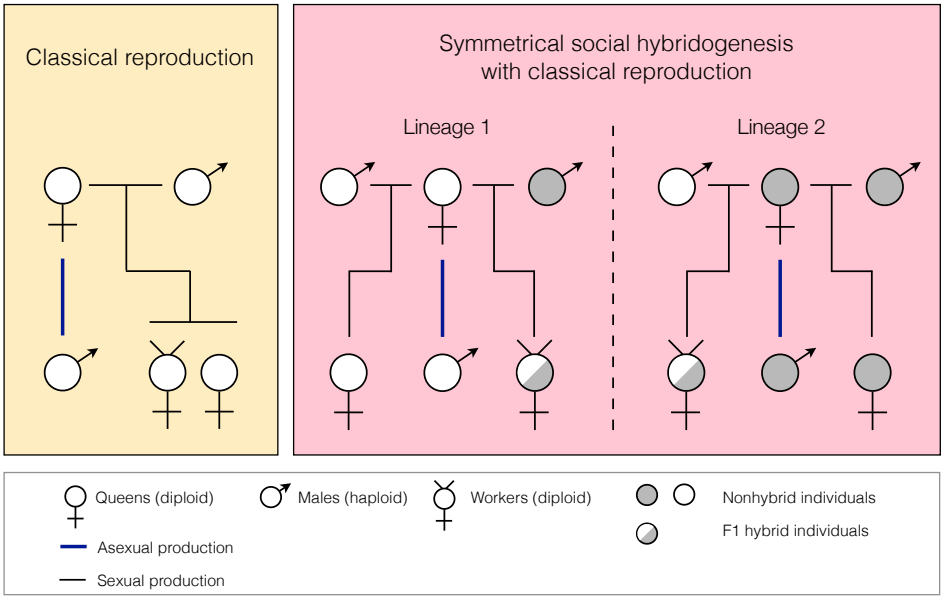


Figure 1. Classical reproduction mode in *P. rugosus* and *P. barbatus* with environmental caste determination and social hybridogenesis experienced by the *Pogonomyrmex* genetic lineages (figure adapted from Darras *et al.* 2019).

Materials and methods

In this study, we are using colonies from the J lineages system (Volny and Gordon 2002; Helms Cahan and Keller 2003); three J2 colonies and one J1 colony. The colonies developed in the lab from single multiply-mated queens collected after mating flights in Scottdale (Arizona, USA) in 2012 and 2015. As queens need to be exposed to cold to produce gynes (Schwander *et al.* 2008), we started the sampling seven days after the colonies came out of eight weeks of hibernation (at 16°C, 60% humidity, 12-h/12-h light:dark cycle; sampling from February 2019). For all the sampling period, the colonies were maintained at 28°C with 60% humidity, with a 12-h/12-h light:dark cycle.

Sample collection – queen isolation

Queens were isolated for egg laying for 12 hours per day (7am to 7pm) and kept in the dark in a petri-dish with three workers and a water supply. Eggs were collected every four hours using a soft brush and transferred to a humid petri-dish. Queens and workers were put back into their colony for 12 hours during the night until the next isolation session. The eggs laid within the same four hours (collection batch) were attributed to one of the five following age groups: 24 hours old, 6 days old, 9 days old (when the larvae started moving inside the eggs), 1st larval instar (24 hours after larvae hatching), 2nd larval instar (72 hours after larvae hatching). We focused on eggs and early larval instars as worker- and queen-destined larvae can be distinguished visually by the last instar (Smith and Tschinkel 2006), so caste determination likely occurs before the 3rd instar larvae. In the meantime, eggs were kept per collection batch in a humid petri dish until flash freezing in liquid nitrogen. For the samples flash frozen at the larval stage, freshly hatched larvae were collected and put in recipient colonies, each containing 20 workers (picked randomly from the nest and foraging arena), to be fed and cared for until flash freezing. Recipient colonies were fed every two days with sugar water and seeds. After flash-freezing, samples were kept at -80°C until DNA and RNA extraction.

Egg and larval caste determination

DNA and RNA were extracted from whole individuals (eggs or larvae) using TRIzol (Life Technologies). The protocol was adapted for small tissue quantities (see SI Text 1 for details). Eggs and larvae were classified as gyne (same-lineage) or worker (inter-lineage) based on their genotype after DNA amplification at eight microsatellite loci [Pb5, Pb8, Pb9, Pb10 (Volny and Gordon 2002), Po3, Po8 (Wiernasz *et al.* 2004), Pr1 (Gadau *et al.* 2003) and L18 (Foitzik *et al.* 1997; see Table S5 for primer sequences and Table S6 for PCR conditions). Amplifications were performed on a VERITI thermal cycler (model 9902, Applied Biosystem) and PCR products were run on an ABI 3100 sequencer. Fluorescent signals were visualized using GeneMapper v.4.1 (Applied Biosystems, Foster City, CA, USA). Dead eggs have degraded DNA and cannot be successfully amplified (Schwander *et al.* 2006). Eggs with allele peak heights over 400 relative fluorescence units (RFUs) were considered alive. The genotype of all individuals and their inferred caste are available in Table S7.

Sample choice for libraries and sequencing

We found different paternal genotypes among workers belonging to the same colony (Table 3), which corroborates previous studies showing that most *Pogonomyrmex* queens mate with several males (Hölldobler 1976; Volny and Gordon 2002; Gadau *et al.* 2003; Wiernasz *et al.* 2004). To reduce the variability between samples within caste and colony for the differential gene expression analyses, we selected individuals from a single patriline (within caste). The genotyping revealed that the J1 queen probably did not mate with a J1 male, as there was not a single gyne-destined egg or larvae among the samples. We therefore excluded the J1 colony for the first gene expression analyses. After this first selection, the samples with the highest RNA concentration (quantified using RiboGreen Reagent) and the lowest RNA degradation (done by Fragment Analyzer) were chosen for the libraries. We sequenced worker and gyne-destined samples from the three J2 colonies (30 libraries in total, one sample per age, caste and colony, representing three biological replicates for each developmental stage; Table 1). The sequencing libraries were constructed by the Lausanne Genomic Technologies Facility using a NEBnext Ultra II preparation kit (Illumina; poly(A) selected RNA-seq libraries). Paired-end sequencing of 150-bp was performed with a Hiseq 4000 sequencer (Illumina). The libraries were sequenced on three lanes, one lane containing 10 samples (5 per caste) from the same colony. Post sequencing quality checks revealed that the RNAseq libraries had been contaminated by human exome libraries at the step of PCR amplification. After removing the contaminant human reads, the samples still contained more than 30 million reads. To avoid potential biases stemming from this contamination, we sequenced a second set of samples from the three J2 colonies (again 30 libraries in total, one sample per age, caste and colony, representing three biological replicates for each developmental stage; Table 1) and from the single J1 colony (15 libraries in total, three samples per age for worker caste). For the second sequencing, the 45 libraries were multiplexed and run together on five lanes.

Table 1. Description of samples included in this study. There were two runs of sequencing. In the 1st run, only gyne and worker samples from J2 lineage were sequenced. Three J2 colonies (A, B and C) were used for the sampling, so there are three samples per developmental stage and caste (one sample per colony). In total, 30 samples were sequenced in the 1st run, 10 samples per lane per colony (3 lanes in total). For the 2nd run, we sequenced again 3 samples per developmental stage and caste (one sample per J2 colony) as well as 3 worker samples per developmental stage for J1, all coming from the same unique J1 colony (D). In total, 45 samples were sequenced in the 2nd run, all samples were multiplexed and sequenced on 5 lanes. See Table S3 for patriline information.
+ At 6d, one interlineage sample from the J1-lineage colony (D) was suspected to be an interlineage gyne and was therefore removed from the analyses (analyses were also re-run without this exclusion and are available in the supplementary material). ▲ The two worker samples from colony A at 6d were removed as well as they appeared as extreme outliers on the PCA plot. ■ One gyne sample from L2 for was also removed following quality checks as the result of severe degradation.

		J2 lineage		J1 lineage		
	Developmental stage	**Gyne**	**Worker**	**Gyne**	**Worker**	**Total**
1st sequencing	24h	3	3	-	0	6
	6d	3	2▲	-	0	6
	9d	3	3	-	0	6
	L1	3	3	-	0	6
	L2	2■	3	-	0	5
					Total	**28**
2nd sequencing	24h	3	3	-	3	9
	6d	3	2▲	-	2+	9
	9d	3	3	-	3	9
	L1	3	3	-	3	9
	L2	3	3	-	3	9
					Total	**43**

Updating reference genome annotation

Raw read quality was assessed with FastQC tools (www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and FastQ Screen (Wingett and Andrews 2018). FastQC data quality analysis revealed minimal adapter content in libraries (< 1%), and as STAR read aligner is highly robust to adapters, no further adapter trimming/filtering was deemed necessary. Genes that are not annotated in the available reference genome of *Pogonomyrmex barbatus* would not be included in our gene expression comparisons, and we therefore updated the available annotations with our new transcriptome information. Reads were mapped to the latest NCBI RefSeq *Pogonomyrmex barbatus* genome (v101, GCF_000187915.1) using the STAR RNA-seq read aligner (Dobin *et al.* 2013) guided by splice junctions from the corresponding RefSeq annotation. A custom gene annotation was then created by building separate reference guided transcript assemblies for each condition (genotype x age) using Stringtie2 (v2.1.4; Kovaka *et al.* 2019), again using the RefSeq v101 annotation to guide assembly (-G option) and using stranded settings (--rf option). These were then combined with the Stringtie2 "merge" function, again using the RefSeq v101 annotation to guide merging. Reads mapping to transcripts in this annotation were then tallied using the featureCounts program from the Subread suite (Liao *et al.* 2014). Approximately 80-95% of mapped reads were successfully assigned to features in this annotation, vs. ~60-80% when using the RefSeq annotation. As such, our custom annotation was used in downstream analysis.

Differential expression analysis

The resulting expected counts were used in the differential gene expression analysis with the R Bioconductor package, EdgeR (Robinson *et al.* 2009). Reads generated by the first and second sequencing runs were used as replicates. Similarity between our replicates was assessed by the biological coefficient of variation implemented in EdgeR. Reads generated from the 71 samples (28 from 1st sequencing runs and 43 from the second 2nd; for details see Table 1) were used as replicates, and comparisons were made across castes within each developmental stage. For all comparisons, we first filtered out transcripts with very low read counts by removing loci lower than one per kilobase of exons per million fragments mapped in at least half of the sequenced libraries, as recommended by EdgeR. TMM normalization was applied to account for compositional differences between libraries. We implemented generalized linear models (GLM) to identify differentially expressed genes between castes including colony (three colony J2 and one colony J1) and batch (first or second sequencing run) as a covariate in the model to control for variation introduced by differences between nests and sequencing runs. Expression differences were considered significant after correction for multiple testing using a false discovery rate of FDR < 0.05. We used the R packages UpSetR v.1.4.0 (Conway *et al.* 2017) and ggplot2 v.3.3.5 (Wickham 2016) to visualize the number of caste-biased genes (or GO-terms) that were unique to a developmental stage or shared between them. We used the SuperExactTest v.1.0.7 (Wang *et al.* 2015) to test whether the overlap of caste-biased genes between the five developmental stages was greater than what is expected by chance. A Principal Component Analysis (PCA) plot was drawn to measure the similarity between samples using EdgeR package. We visualized the caste-biased gene expression across developmental stages using logFC with pheatmap v.1.0.12 (Kolde 2019). A two-tailed Fisher's exact test, as implemented in the exact2x2 R package v.1.6.6 (Fay 2010), was used to compare the number of genes biased towards a caste or a developmental stage relative to the total number of genes.

Functional analysis of differentially expressed genes

The NCBI nr database was downloaded from the NCBI BLAST FTP site on June 7th, 2021. Transdecoder (http://transdecoder.github.io) was used to find ORFs and translated all transcripts from the reference-guided Stringtie annotation. These protein sequences were searched against the nr database using blastp with all default settings. The eggNOG-mapper tool (http://eggnog-mapper.embl.de) was used to infer functional annotation of the *de novo* transcriptome gene set using structural similarity. The GOstat package for R v.1.7.4 (Falcon and Gentleman 2007) was used to conduct GO term enrichment analysis on differentially expressed genes, using all genes having GO terms as the universe. A two-tailed Fisher's exact test, as implemented in the exact2x2 R package v.1.6.6 (Fay, 2010), was used to compare the number of enriched GO-terms between castes and developmental stages relative to the overall number of genes.

Enriched GO terms ($p < 0.05$) were then semantically clustered using rrvgo R package v.1.6.0 (Sayols 2020), to avoid redundancy of GO terms and to help interpretation. For each developmental stage and Biological Process GO term, similarity matrices were created using the "Rel" (Relevance) as the method and "org.Dm.eg.db" (*Drosophila melanogaster*) as a reference database. Similarity matrices were reduced using a threshold of 0.7 and using –log(*p* value) as scores, meaning a lower *p* value equals a higher score.

Results

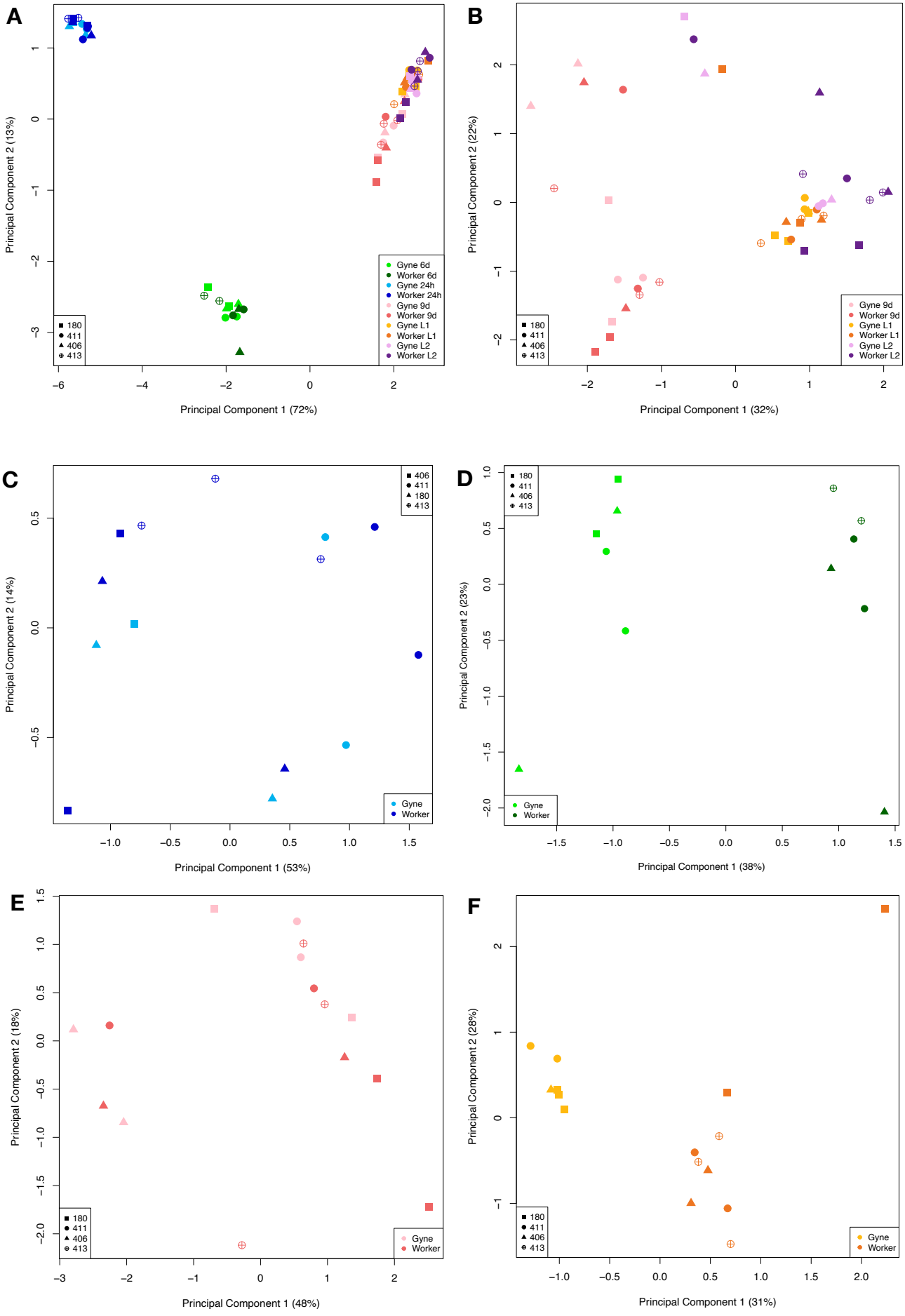
We investigated overall gene expression profiles from whole bodies of worker-destined and gyne-destined eggs at 24 hours (24h), six days (6d), nine days (9d) after egg laying and worker-destined and gyne-destined larvae in first larval instar (L1; 24h after larvae hatching) and second larval instar (L2; 72 hours after larvae hatching). Sequencing using the Illumina HiSeq4000 platform yielded 2275.9 million 150-bp paired-end sequence reads (BioProject ID PRJNA802583, www.ncbi.nlm.nih.gov/bioproject/802583).

Gene expression patterns

Visual inspection of the PCA plots show three main results (Figure 2). First, when all developmental stages are plotted together, the samples cluster by developmental stage and not by genotype/caste (pure J2J2 gyne versus inter-lineage J1J2 worker). This shows that the developmental stage brings more variation than genotype/caste. One 6d sample, which was genetically a worker (inter-lineage J1J2), clustered with the pure lineage gynes (J2J2; Figure S1A). This sample came from the single J1 colony that did not produce any brood with gyne genotypes. We suspected that this sample would have developed into an inter-lineage gyne. Previous studies showed that, although very rare, inter-lineage individuals can occasionally develop into gynes, especially when the queen did not mate with a male of her own lineage and there is therefore no competition with pure-lineage brood (Helms Cahan *et al.* 2002; Schwander *et al.* 2007). We therefore proceeded to run all analyses twice, first with the exclusion of this sample, and then a second time with this sample considered as an inter-lineage gyne. For clarity, only the results without this sample will be shown here, results with this individual considered as a gyne are available in the supplementary material.

The second observation from the PCA plots is that developmental stage differences are smaller between the three oldest samples, 9d-L1-L2, which cluster together with an overlap between L1 and L2 samples (Figure 2B), whereas samples from 24h and 6d cluster in two clearly distinct groups. This is probably linked to the differences in time between the developmental stages (120 hours between 24h and 6d, 72 hours between 6d and 9d, 48 hours between 9d-L1 and L1-L2) as well as perhaps more major developmental transitions in embryos than young larvae.

Third, when all developmental stages are plotted separately, a clear cluster by caste is observed at 6d and L1 (Figure 2D,F). No effect of caste is detected in the samples of 24h, 9d and L2 (Figure 2C,E,G, respectively). However, when we plotted PC1 and PC3 for 9d and L2, there was a clear separation by caste (Figure S1B-C), which suggests that other factors explain more variation than the caste at 9d and L2. At 24h, no principal component showed a separation of the samples by caste although we were still able to detect significant gene expression differences between castes at 24h (see below).


A
Principal Component 2 (13%)
Principal Component 1 (72%)
180
411
406
413
Gyne 6d
Worker 6d
Gyne 24h
Worker 24h
Gyne 9d
Worker 9d
Gyne L1
Worker L1
Gyne L2
Worker L2
B
Principal Component 2 (22%)
Principal Component 1 (32%)
C
Principal Component 2 (14%)
Principal Component 1 (53%)
Gyne
Worker
D
Principal Component 2 (23%)
Principal Component 1 (38%)
E
Principal Component 2 (18%)
Principal Component 1 (48%)
F
Principal Component 2 (28%)
Principal Component 1 (31%)

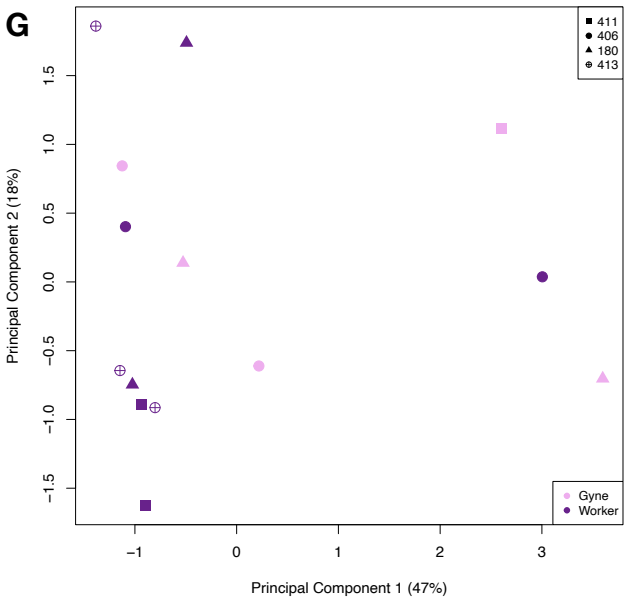


Figure 2. First two principal components (PC1 and PC2) explaining transcriptomic variation across samples from all developmental stages (**A**) or only the three oldest ones showing an overlap (**B**). Each developmental stage is plotted individually from **C-G**. Colors show the developmental stage (24h in blue, 6d in green, 9d in pink, L1 in orange and L2 in purple) and the caste (light color for gynes and dark color for workers). Filled symbols refer to the three J2-lineage colonies and open symbol refer to the single J1-lineage colony.

Number of caste-biased genes

We found differences in gene expression between queen- and worker-destined eggs as early as 24 hours after egg laying, as well as in all the following developmental stages (i.e. 6d, 9d, L1 and L2; data on additional file Table S1). The number of caste-biased genes varied between developmental stages, with an abrupt significant increase from 24h to 6d (with 42 respectively 416 caste-biased genes $p < .001$, Fisher's exact test, Figure 3), and a significantly lower number of caste-biased genes at 9d compared to all the other developmental stages (all pairwise comparisons $p < .001$, Fisher's exact test). The number of worker-biased genes were higher in every developmental stage compared to gynes-biased genes (worker vs gyne, 343 vs 73 at 6d, 212 vs 31 at 9d, 360 vs 41 at L1 and 284 vs 108 at L2; Figure 3, $p < .001$, Fisher's exact test). The 42 caste-biased genes at 24h were all biased towards workers, there were no gyne-biased genes at 24h.

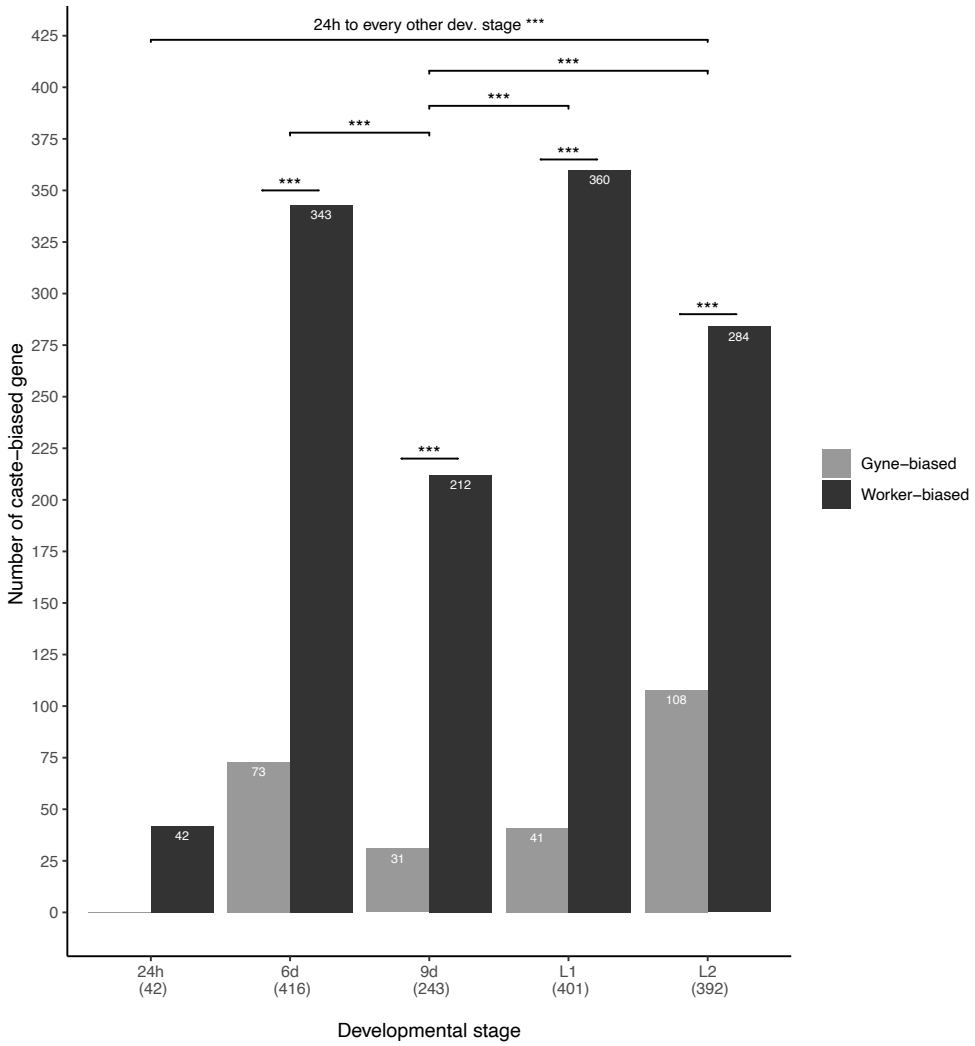


Figure 3. The number of caste-biased genes varied significantly between castes at all developmental stages and the developmental stages 24h and 9d are significantly different to all the other developmental stages (Fisher's exact test, *** $p < .001$). The x-axis indicates the five developmental stages, with the total number of caste-biased genes per developmental stage in brackets. The y-axis indicates the number of DEG found up-regulated in gynes (light grey) or workers (dark grey). Numbers in the bars give the number of up-regulated genes per caste.

Caste-biased gene dynamics between developmental stages

More genes were over-expressed in workers than in gynes, and the few gyne-biased genes tend to be more specific to one developmental stage, compared to the worker-biased genes. Only 9.8% of gyne-biased genes were found overexpressed in two or more developmental stages, compared to 34.5% for worker-biased genes ($p < .001$, Fisher's exact test; Figure 4). Nine genes (out of 987 caste-biased genes) were found consistently worker-biased in all five developmental stages, and 71 genes were worker-biased in four developmental stages from 6d to L2 (Figure 4B). A single gene (PogoMSTRG.12666, no functional annotation) was consistently gyne-biased in the four developmental stages from 6d to L2. Thirty-three genes were caste-biased in at least two developmental stages, but the direction of expression changed between caste: 13 genes were first worker-biased and then switched to gyne-biased, 17 genes were first gyne-biased and then switched to worker-biased

and three genes changed direction three times over three or four developmental stages. No caste-biased gene switched more than three times between developmental stages.

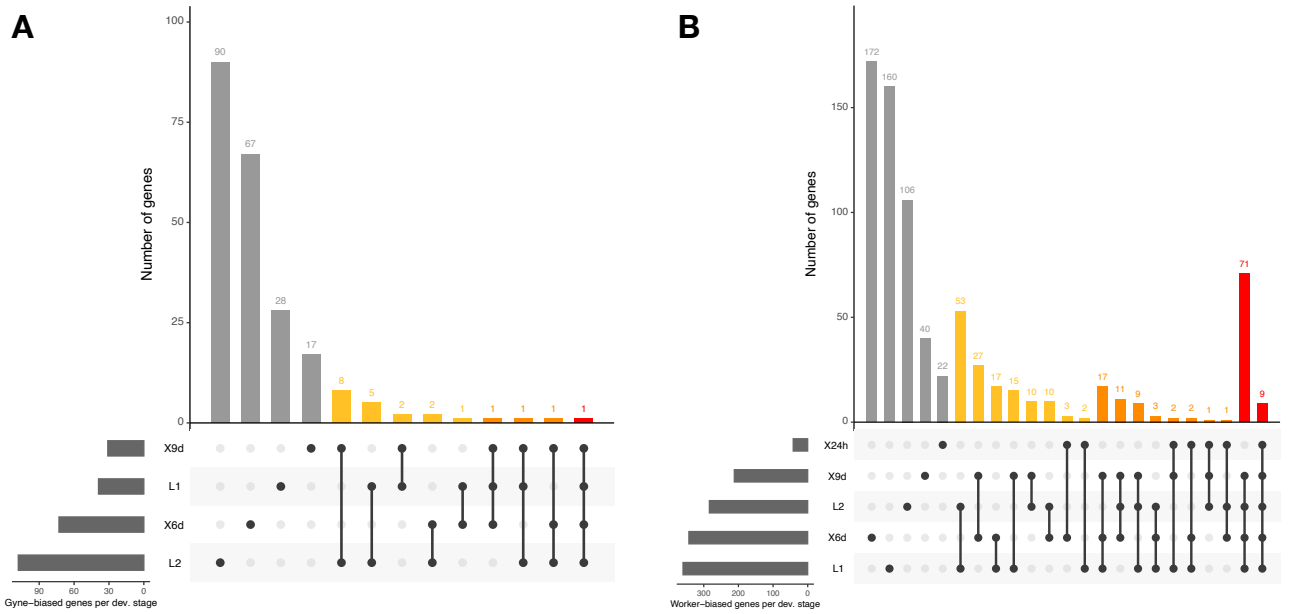


Figure 4. Number of caste-biased genes shared between developmental stages. The vertical bars of the UpSetR plots show the number of (**A**) gyne-biased and (**B**) worker-biased genes expressed in a single developmental stage (in grey) or shared between two (yellow), three (orange) and four or five (red) developmental stages. The dot plot below the graph indicates which developmental stages share the caste-biased genes shown in the vertical bar. In cases where genes are shared between two or more developmental stages, dots are linked with a vertical line. Horizontal lines adjacent to the dot plot show the total number of caste-biased gene per developmental stage. The number of genes shared between all five stages (all worker-biased) was greater than expected by chance (observed overlap = 9, expected overlap = $3x10^{-5}$, Exact test of multi set intersections: p = $2x10^{-47}$). All pairwise overlaps also contained more genes than expected by chance (detailed results in Table S8).

We looked for functional annotations of the 42 worker-biased genes at 24h in the NCBI nr. database. More than half of them (23/42) had functional annotations (Figure 5), however we did not find related information in previous studies. Among the genes consistently worker-biased through all five or four (from 6d to L2) developmental stages, approximately half of them were annotated (36/71 and 5/9, respectively; Figure 5 and Figure S5). The overall proportion of genes with a functional annotation did not differ between worker-biased and gyne-biased genes (51±4% and 54±2%, respectively).

Figure 5. Caste-biased genes shared across development. The heatmap shows the 42 caste-biased genes (all worker-biased) at 24h stage with their annotations and their expression in the five developmental stages. Genes in red are worker-biased, blue are gyne-biased. Saturation of the colors increases with the $\log_2$FC. Genes without a label have no annotation, annotation in green is for genes that were caste-biased in all five stages (all worker-biased).

Fourteen key genes known to play a role in social insect caste differentiation (in *A. mellifera*, *B. terrestris* and several ant species; Sieber *et al.* 2021) were found caste-biased in one or more developmental stages (see Table 2). Eight genes for cytochrome P450 (CYP) were found caste-biased at 6d, 9d, L1 and/or L2. CYP 4C1 stayed up-regulated in workers from 6d to L2 and four other CYP genes were expressed in workers in at least one or all three developmental stages between 6d and L2. Two genes for CYP 4g15 were up-regulated in gynes, at 9d and L2. Four fatty-acid synthase-like genes were up-regulated in workers in several developmental stages from 6d to L2, the myosin light chain kinase gene was worker-biased at 24h and 6d and the heat shock protein gene (HSP70) was up-regulated in workers at L1.

Table 2. List of caste-biased genes previously described as involved in caste-differentiation or sex-differentiation, per developmental stage.

Gene		Up-regulated in: Caste	Dev. stages	Possible function(s) from previous studies	References
Cytochrome P450	CYP 4c1	Worker	6d, 9d, L1, L2	Overexpressed in worker compared to gyne in 3rd instar larvae	(Evans and Wheeler 2001)
	CYP 6k1	Worker	6d, L1, L2	Expressed throughout development in *Blattella germanica* Associated with sex differentiation in *Bombus terrestris* larvae	(Wen and Scott 2001) (Harrison *et al.* 2015)
	CYP 9e2-like CYP 304a1	Worker	L1	NA Potentially involved in the metabolism of insect hormones	Flybase database
	CYP 18a1 CYP 6a14	Worker	L2	Essential for proper insect development and metamorphosis (up-regulated by ecdysteroids in *D. melanogaster*) NA	(Sztal *et al.* 2012; Wan *et al.* 2013)
	CYP 4g15	Gyne	9d, L2	Biosynthesis of endogenous compounds, pheromone metabolism, diapause in different insects	(Wan *et al.* 2013)
	CYP 301a1, mitochondrial	Gyne	9d	Mitochondrial role in the detoxification of compounds Potentially involved in ecdysteroid metabolism, 20-hydroxyecdysone signaling or regulation during adult cuticle formation	(Wan *et al.* 2013) (Sztal *et al.* 2012)
Myosin light chain kinase, smooth muscle-like		Worker	24h, 6d	Overexpressed in adult workers (compared to queens) in 16 ant species	(Morandin *et al.* 2016)
Fatty acid synthase-like		Worker	6d, 9d, L1, L2	(4 genes in total, 2 per developmental stage)	
Hsp70 family protein		Worker	L1	Over expressed in young bipotential *A. mellifera* larvae	(Evans and Wheeler 2001)
Juvenile hormone acid O-methyltransferase-like		Gyne	L2	Involved in JH biosynthesis, overexpressed in *A. mellifera* queen-destined individuals in second and fifth instar larvae, and in pupae	(Li *et al.* 2013)
Sex-determining region Y protein-like		Worker	6d	Sex-biased in mammals and presumed role in gonadogenesis or gonadal differentiation non-mammals, including in insects	(Herpin and Schartl 2015)
Nose resistant to fluoxetine protein 6-like		Worker	6d, L1	Associated with sex differentiation in *Bombus terrestris* larvae	(Harrison *et al.* 2015)

Caste-biased gene enrichment analysis

The overall number of GO-terms related to caste-biased genes increases with the developmental stages ($p < .001$, Fisher's exact test, Figure 7), except between 6d and 9d where there is no significant increase. The number of GO-terms related to gyne-biased genes is low in all developmental stages except at L2 where we observe a substantial increase. There are significantly more GO-terms related to the worker-biased genes than

to the gynes in all developmental stages (*** p < .001), except at L2 where there appear to be more GO-terms related to gyne-biased genes, however the difference is not significant.

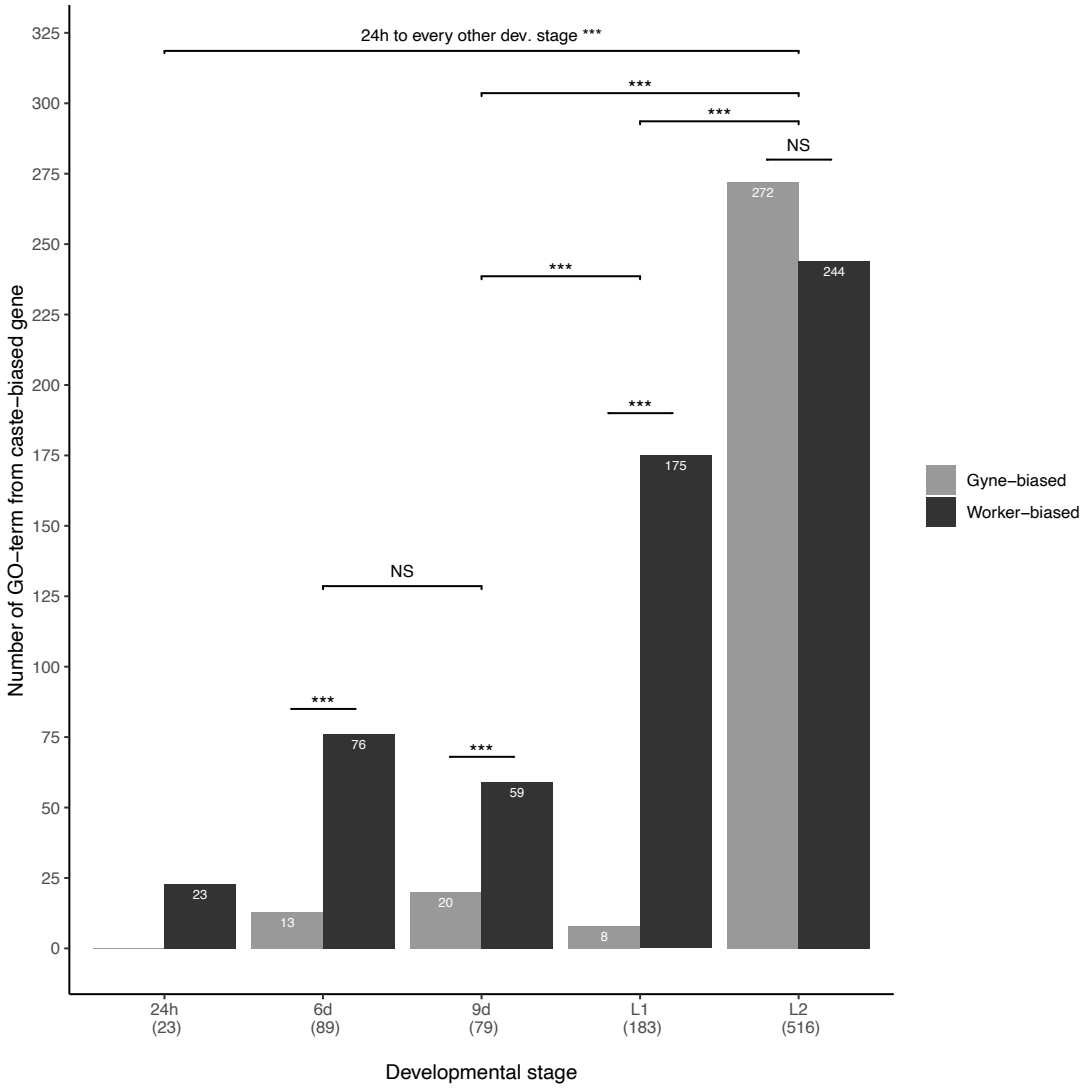


Figure 7. The number of enriched GO-terms related to caste-biased genes increases with the progression through the developmental stages (*** p < .001), except between 6d and 9d. There are significantly more GO-terms related to the worker-biased genes than to the gynes in all developmental stages (*** p < .001), except at L2 where there are more GO-terms related to gyne-biased genes, however the difference is not significant. The x-axis indicates the five developmental stages, with the total number of GO-terms related to caste-biased genes per developmental stage in brackets. The y-axis indicates the number of enriched GO-terms related to caste-biased genes up-regulated in gynes (light grey) or workers (dark grey). Numbers in the bars give the number of GO-terms per caste.

In workers, 7 enriched GO terms were found shared between all five developmental stages, and 104 were shared between two to four developmental stages (Figure 8B). The number of enriched GO terms specific to one developmental stage increased with developmental stage (11 enriched GO terms found at 24h, 26 at 6d, 23 at 9d, 80 at L1 and 149 at L2; Figure 8B) in workers. In gynes, the overall number of enriched GO-terms was quite low, except at L2 where we found 264 GO terms specific to L2 gynes (Figure 8A), and only nine enriched GO terms were found shared between developmental stages.

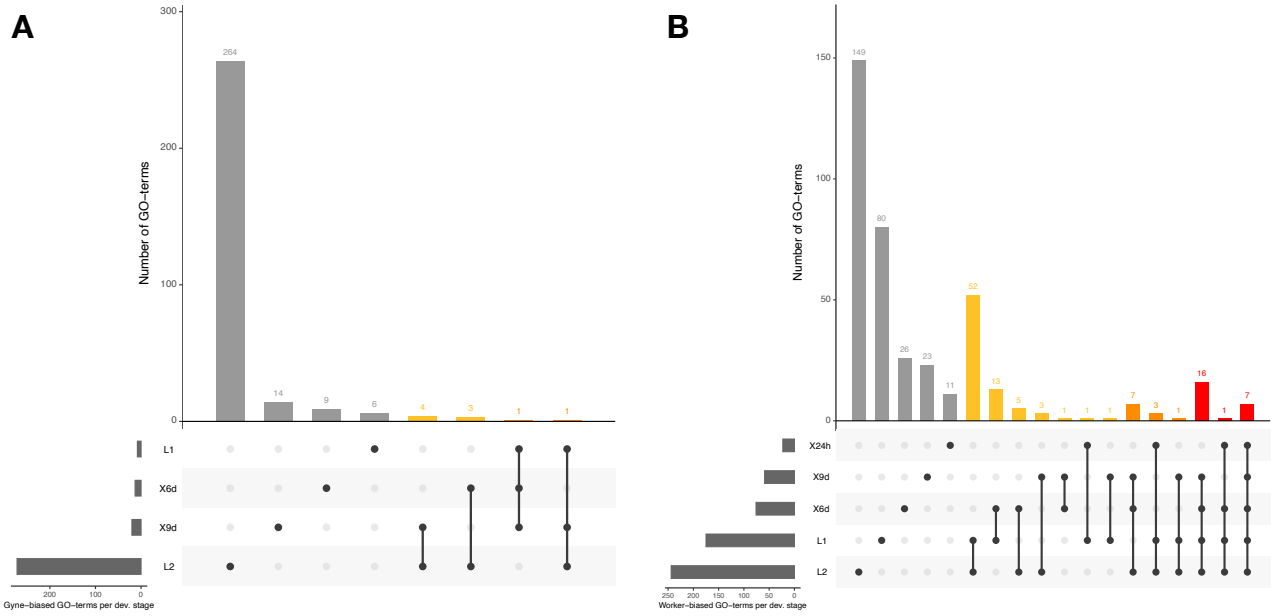


Figure 8. Number of GO-terms shared between developmental stages. The UpSetR plots show the number of (**A**) gyne-biased and (**B**) worker-biased GO-terms found in a single developmental stage (in grey) or shared between two (yellow), three (orange) and four or five (red) developmental stages. The dot plot below the graph indicates which developmental stages share the GO-terms shown in the vertical bar. In cases where GO-terms are shared between two or more developmental stage, dots are linked with a vertical line. Horizontal lines adjacent to the dot plot show the total number of caste-biased GO-terms per developmental stage.

Differences in the gene enrichment profiles are observed between castes at the five developmental stages, and associated processes often have plausible links to caste determination and overlap with findings from previous studies (see Table 3 for details). At 24h, caste-biased genes (all worker-biased) were enriched for GO-terms related to diverse metabolic and biosynthetic processes, with no clear association to caste differentiation (Figure S8A, data on additional file Table S9).

At 6d, workers showed gene enrichment in diverse metabolic, biosynthetic and catabolic processes, in transport, in histone/protein modification and in regulation of mating type switching (Figure S8B), which may play a role in caste determination. Gynes showed gene enrichment in guanosine monophosphate and purine nucleoside biosynthetic processes, as well as in N-terminal protein amino acid methylation and modification.

At 9d, gynes showed gene enrichment in response to chemical stimulus, in fatty acid/lipid metabolic processes and in response to oxidative stress (Figure S8C). Genes worker-biased at 9d were enriched for GO-terms related to metabolic and biosynthetic processes, transmembrane transport, nitrate assimilation and production of small RNA, with no clear association to caste determination.

At L1, worker-biased genes were enriched for GO-terms related to regulation of circadian rhythm, quinolinate metabolic process, positive regulation of hippo signaling, olfactory behaviour and like at 6d, regulation of mating type switching (Figure S8D). Gynes were enriched in DNA metabolic process and innate immune response.

At L2, caste-biased genes stayed enriched for GO-terms related to immune response like at L1 and to cytokine signaling pathways like at 9d. Worker biased genes were also enriched in circadian rhythm and regulation of hippo signaling like at L1. Several GO-terms related to gyne development were found at L2. Gyne-biased genes were enriched in viral processes, wing morphogenesis, positive regulation of TOR signaling and mitotic spindle organization (Figure S8E).

Table 3. List of GO-terms that are potentially good candidate to be involved in caste determination, per caste and developmental stages.

Dev. stage	Caste	Semantic similarity terms	Related GO-terms
24h	Worker	• Metabolic and biosynthetic processes	*proteolysis, nucleic acid metabolic process, fructose metabolic process, DNA biosynthetic process*
6d	Worker	• Metabolic, biosynthetic and catabolic processes	*lipid catabolic processes, proteolysis, nitrogen cycle metabolic process*
		• Transport	*fatty acid transport, oligopeptide transport*
		• Histone/protein modification	*positive regulation of histone acetylation, regulation of histone modification*
		• Regulation of mating type switching	*mating type switching, mating type determination*
	Gyne	• Guanosine monophosphate (GMP) and purine nucleoside biosynthetic processes • N-terminal protein amino acid methylation and modification	
9d	Worker	• Metabolic and biosynthetic processes • Transmembrane transport • Nitrate assimilation • Production of small RNA	
	Gyne	• Response to chemical stimulus	*chemokine-mediated signaling pathway, response to cytokine*
		• Fatty acid/lipid metabolic processes	*fatty acid biosynthetic process, cellular lipid metabolic process*
		• Response to oxidative stress	
L1	Worker	• Regulation of circadian rhythm • Quinolinate metabolic process • Positive regulation of hippo signaling • Olfactory behaviour • Regulation of mating type switching	*circadian behaviour, circadian sleep/wake cycle process*
	Gyne	• DNA metabolic process • Innate immune response	
L2	Worker	• Immune response pathway • Cytokine signaling pathway • Circadian rhythm • Regulation of hippo	
	Gyne	• Immune response pathway	*imaginal disc-derived wing morphogenesis, wing disc development*
		• Cytokine signaling pathway	
		• Mitotic spindle organization	*mitotic spindle pole body organization, spindle elongation, mitotic spindle assembly*
		• Positive regulation of TOR signaling	
		• Viral processes	*negative regulation of viral process, modulation by virus of host gene expression, viral RNA genome replication*
		• Wing morphogenesis	

Developmental stage-biased gene enrichment analysis

Six GO-terms were consistently enriched in workers across all developmental stages: metabolic process, DNA biosynthetic process, DNA metabolic process, DNA integration, proteolysis and nucleic acid phosphodiester bond hydrolysis. In gynes, DNA metabolic process was found in 6d, 9d and L1, and proteolysis at L2. No enriched GO-term was found in gyne across all developmental stages.

Discussion

Our main goal was to profile gene expression differences in queen- and worker-destined individuals of *Pogonomyrmex* J lineages at early developmental stages when caste determination is expected to occur. When plotting gene expression of all samples together, we observe that they cluster by developmental stage rather than by caste (Figure 2). This pattern has been observed in several social insect species comparing gene expression in larvae and pupae (Ometto *et al.* 2011; Harrison *et al.* 2015; Collins *et al.* 2021), however when a single developmental stage is analyzed, samples separate by caste (Morandin *et al.* 2015; De Souza *et al.* 2018; Figure 2).

Gene expression patterns differ between queen- and worker-destined eggs as early as 24 hours old after the egg was laid. There is an abrupt increase in the number of caste-biased genes between 24 hour old eggs and 6 day old eggs, but further studies with more frequent sampling rates would be required to discern whether caste-biased genes are added gradually throughout the five day period of time that separates our sampling schedule or whether this significant increase is indicative of an abrupt 'spike' in developmental expression. There is a drop in the number of caste-biased genes at 9 days old compared to 6d, and a second peak in the first larval instar (L1), which raises the same question, but the number of caste-biased genes as well as the clear clustering by caste at 6d and L1 does suggest that the caste differentiation may follow a 'bursty' pattern with periods of greater caste-specific development, perhaps starting at 6 days old and then again at L1. Our inference is further supported by the observation that the developmental stage 9d corresponds to the pre-hatching stage, where the larva prepares to hatch from the egg (samples were collected less than 24 hours before hatching). In the ant *Monomorium pharaonis*, this corresponds to a period where no major developmental changes happen (stage 16; Pontieri *et al.* 2020). The decrease in caste-biased genes observed at 9d (Figure 2) likely reflects this critical step in *Pogonomyrmex* J lineages and, samples not clustering by caste, support the fact that other factors influence gene expression more than the caste at this developmental stage. We also observed a lower RNA concentration at 9d than at 6d (Figure S1), which supports the idea that the larva at 9d is preparing for molting and is not going through major developmental changes. In *P. rugosus*, the first larval molt occurs between 24 and 48 hours after the larva had hatched from the egg (pers. obs. and unpublished data from Dr Eduardo Fox), therefore at L2 (72 hours after hatching) the first larval molt had occurred already. It did not appear that larvae collected at L2 were in a critical developmental stage that could explain by which factor(s) the caste-biased gene expression were hidden.

One major concern in the conception of this project was the fact that the worker and gyne gene expression is influenced by a different genetic background and that it would be challenging to attribute differential expression to either caste (i.e. worker vs gyne) or genotype (i.e. inter-lineage J1J2 workers vs pure lineage J1J1 gynes). The presence of a single inter-lineage sample that clustered clearly within the pure lineage gynes at 6d eases this concern. If we are correct in our assumption that this sample is an inter-lineage gyne, it means that the effect of the genotype (pure lineage versus inter-lineage) does not conceal any caste effects. Moreover, a study on the whitefish *Coregonus clupeaformis* found that very few transcripts differed in mean expression level between normal and inter-lineage hybrid fishes at the embryonic stage (which lasts about 6 to 8 weeks),

in contrast to 16-week-old juvenile fishes (Renaut *et al.* 2009). We can therefore assume that the differentially expressed genes observed in this study were more linked to caste than to genotype.

A second aim of our study was to characterize the gene expression dynamics throughout the egg stage and first two larval stages. Although only 42 genes were caste-biased at 24h, they were all worker-biased and nine of them remained consistently worker-biased throughout all developmental stages. In all five developmental stages, there were more worker-biased genes than gyne-biased genes. To our knowledge, this study is the first to show an early bias towards worker-transcribed genes from eggs at 24h old until the second larval instar. A shift from worker- to gyne-bias in gene expression during development was found in several eusocial insect species (with variation in developmental stage between species), where little gyne-biased expression was detected until the late-larval, pupal or even emerging adult stages (Ometto *et al.* 2011; Morandin *et al.* 2015; Collins *et al.* 2021). Data presented by Severson *et al.* (1989) also suggested an early bias toward worker-transcribed genes followed by a general bias toward queen-specific gene expression in pre-pupae and pupae, periods during which much of the reproductive differentiation between queens and workers take place in *A. mellifera*. Our data further support the idea that queen is the developmental pathway by default and the worker pathway is actively switched on (Severson *et al.* 1989; Thompson *et al.* 2006; Guo *et al.* 2013; Wojciechowski *et al.* 2018).

The third aim was to find genes that could be involved in caste differentiation. None of the worker-biased genes at 24h have been described as involved in caste differentiation in previous studies on eusocial insect species. However, in the following four developmental stages measured, several caste-biased genes we detected have previously been described as involved in caste differentiation in several social insects (Table 2) and could potentially represent critical genes involved in the caste differentiation of *Pogonomyrmex* J lineages.

Several cytochrome P450 (CYP) genes have previously been linked to caste determination. CYP genes were found caste-biased in the several *Apis* species in all larval instars (Corona *et al.* 1999; Evans and Wheeler 1999; Pereboom *et al.* 2005; Cameron *et al.* 2013; Kang *et al.* 2021), in late-instar larvae in the bumblebee *Bombus terrestris* (Pereboom *et al.* 2005), in freshly emerged females in the wasp *Polistes canadensis* (Sumner *et al.* 2006), in the red imported fire ant, *Solenopsis invicta* (Liu and Zhang 2004) as well as in termites (Cornette *et al.* 2006; Tarver *et al.* 2012). We found several CYP genes biased towards worker in all developmental stages except at 24h (see Table 2). *CYP4C1* was proposed to participate in fatty acid oxidation (which provides an energy supply) in the cockroach (Lu *et al.* 1999) and CAP6k1, was shown to be essential for proper development and metamorphosis in other insects (Collins *et al.* 2021). The single CYP that we found up-regulated in gynes at 9d and L2 (*CYP4g15*) was found up-regulated in newly emerged workers in *Melipona* bees (Judice *et al.* 2004). This indicates that modulation of these CYP genes could be associated with energy requirements and the morphological changes in development associated with the castes in *Pogonomyrmex* J lineages.

The two enzyme families, glutathione transferase and cytochrome P450, play a central role in the detoxification of both endogenous and xenobiotic compounds, including oxygen free radicals (Diao *et al.* 2018). Glutathione transferase participates as well in the oxidative stress response and is involved in intracellular transport and

biosynthesis of hormones (Allen and Sohal 1986; Enayati *et al.* 2005). Two GO-terms related to glutathione transport have been found enriched in workers at 6d and stayed enriched in all following developmental stages until L2. Cameron *et al.* (2013) and Kang *et al.* (2021) found that *A. mellifera* and *A. cerana* workers, respectively, have higher expression of glutathione-s-transferase genes in larval and adult stages, which support our results.

Several caste-biased genes and GO-terms that we found in this study overlap with findings in previous research on social insects, however their role in caste determination remains unclear. These include the myosin light chain kinase, worker-biased at 24h and 6d, which was found to be consistently worker-biased in 16 ant species (at the adult stage; Morandin *et al.* 2016). Although its role remains unclear, myosin light chain kinase is likely a housekeeping gene part of a multicomponent signaling pathway (Morandin *et al.* 2016; Isobe *et al.* 2020). A fatty acid synthase-like gene was consistently up-regulated in workers from 6d to L2. Sumner *et al.* (2006) found this gene up-regulated in newly emerged workers in *P. canadensis*, supporting the likelihood that this gene might be involved in a pathway specific to worker development. The heat shock protein gene (HSP70) was worker-biased at L1, which is consistent with previous studies which found heat shock protein genes differentially expressed between castes or developmental stages in larvae and adults of *A. mellifera* (Evans and Wheeler 2001; Xu *et al.* 2010). Two genes (e.g. S*ex-determining region Y* and *nose-resistant to fluoxetine*, upregulated in 6d and 6d&L1 workers, respectively) have been linked to caste differentiation in *B. terrestris* (upregulated in queen-destined larvae) and to sex differentiation in other insects (Table 2). Moreover, several GO-terms related to mitotic spindle organization (enriched in L2 gynes) and to mating-type switch (enriched in workers at 6d and L1) have also been linked to sex differentiation in insects and yeast, respectively, which is consistent with reports linking the processes of caste- and sex differentiation in other eusocial species (Klein *et al.* 2016; Kapheim 2019; Warner *et al.* 2019).

Finally, we looked for caste-biased gene enrichment in all five developmental stages that could be related to caste differentiation. Several pathways have already been described as regulating the queen and worker caste determination, such as insulin growth signaling, target of rapamycin (TOR), Fat/Hippo signaling pathway, juvenile hormone/ecdysteroids, and vitellogenin (Barchuk *et al.* 2007; Wheeler *et al.* 2014; Ashby *et al.* 2016; Corona *et al.* 2016; Yin *et al.* 2018). However, only two of those pathways were differentially expressed between castes in this study: TOR and Fat/Hippo signaling pathways. TOR and Fat/Hippo signaling pathways are involved in the regulation of organ size through their respective functions in cell size or cell number regulation (Tumaneng *et al.* 2012). We found three enriched GO-terms related to *positive regulation of TOR signaling* that were up-regulated in gynes at L2. TOR is a highly conserved serine/threonine kinase that controls cell growth and metabolism in response to nutrients, growth factors, cellular energy, and stress. Wheeler *et al.* (2014) found that TOR is more highly expressed in *A. mellifera* queen larvae at 40 hours post-hatching. Previous studies showed that TOR gene knockdown blocks queen fate and results in individuals with worker morphology (Patel *et al.* 2007; Wheeler *et al.* 2014; Wang *et al.* 2021) and therefore suggested that this pathway is involved in caste determination in the honeybee *A. mellifera*. Our result support those previous findings and suggest that TOR might be involved in caste determination in the ant *Pogonomyrmex* J lineages as well. *Pogonomyrmex* queens are morphologically much larger than workers, therefore size and metabolism are likely an important facet of development.

Hippo signaling is essential for the earliest cell fate decisions in mammalian embryos (Barry and Camargo 2013) and in *Drosophila*, mutations in this pathway result in organized overgrowth of imaginal discs resulting in normal, but enlarged organs (Gotoh *et al.* 2015). In this study, we found enriched GO-terms related to the *positive regulation of hippo signaling* in workers at both 6d and L1 developmental stages. The fact that 6d and L1 developmental stages show an enrichment for genes involved in the Hippo signaling pathway and have the highest number of caste-biased genes plus show a clear separation by caste on the PCA plot, supports the fact that the Hippo pathway has a role in caste differentiation, as proposed by Gotoh *et al.* (2015). The development of morphological differences between gyne and worker castes requires a complex fine-tuning that may be regulated by the hippo signaling pathway, through the control of organ growth and cell proliferation.

We did not find caste-biased genes involving vitellogenin, although vitellogenin was described as involved in caste differentiation in several studies. Higher vitellogenin content was found in eggs laid by overwintered queens compared to non-overwintered queens (Libbrecht *et al.* 2013). Vitellogenin is incorporated in the egg during its production by the queen and used by the developing embryo as a nutritive source (Wu *et al.* 2021) but is not produced by the embryo, which explains why we did not find gene expression related to vitellogenin in any of the five developmental stages. The higher levels of vitellogenin in post hibernation eggs and the likely expensive cost of gyne development may be one reason why vitellogenin titres are different, but are not a prime reason for caste differentiation, rather they are a limiting factor. Indeed, if a gyne does not have enough vitellogenin, she cannot complete her more energetically costly development. A single gene related to juvenile hormone (*juvenile hormone acid O-methyltransferase-like*) was overexpressed in gynes at L2. Li *et al.* (2013) found this gene overexpressed in *A. mellifera* queen-destined larvae in second and fifth instar, which supports a role of juvenile hormone acid methyltransferase in caste differentiation.

At developmental stage 6d, we found several GO-terms enriched in histone acetylation in workers and genes enriched in *N-terminal protein amino acid methylation* in gynes. Modifications of histones has been proposed as a modifier of gene expression in social insects (Sieber *et al.* 2021). Wojciechowski *et al.* (2018) demonstrated that chromatin modifications have a key role in the establishment and maintenance of caste-specific transcriptional programs in *A. mellifera* larvae at 96 hours. Higher levels of methylation in *A. mellifera* induce the development of the worker phenotype, while having comparatively less methylation induces a queen phenotype (Sieber *et al.* 2021). In adult ants, a higher degree of methylation was observed in queens than in workers (Smith *et al.* 2012; Morandin *et al.* 2019). However, data showing methylation degree in egg and first instars larvae in ants are missing. Post-translational modifications of histones (i.e. histone acetylation) was shown to influence the generation of distinct ant worker castes (i.e. minor vs major workers; Bonasio 2014; Simola *et al.* 2016). Another study suggested that histone acetylation is involved in the molecular regulation of adult worker reproduction in the ant *Temnothorax rugatulus* (Choppin *et al.* 2021). We also found GO-terms enriched in the *regulation of circadian cycle* in 6d workers. Libbrecht *et al.* (2020) suggested a role for histone acetylation in controlling and regulating circadian rhythms in adult ants. It is therefore possible that the GO-terms for histone acetylation and circadian cycle work together in early developmental stage in workers, by modifying gene expression.

Last but not least, among the numerous GO-terms found in L2 gynes, five of them showed enrichment for genes related to imaginal disc wing morphogenesis. Recent studies showed that genes related to imaginal disc wing are expressed worker-destined larvae not for wing development, but in the soldier and minor worker subcaste differentiation (Abouheif and Wray 2002; Koch *et al.* 2021). In our study only L2 gynes expressed those genes, which suggests that gyne-destined larvae in second instar (L2) are already in a well differentiated developmental pathway with development of caste specific organs.

Even if the knowledge on gene expression between social insect castes is growing, it is difficult to make meaningful connections between caste-biased genes, their related GO-terms, and caste determination. As mentioned above, most of the caste-biased genes differ between species and developmental stages, therefore more studies on gene expression in social insects are necessary, as well as gene knockdowns to confirm gene function, to improve our understanding of caste determination.

Conclusion

To our knowledge, this study is the first to describe the dynamics of caste-biased gene expression in early developmental stages in ants, before any visible morphological difference between castes. Overall, our results show that several genes are caste-biased at a very early developmental stage in *Pogonomyrmex* J lineages, with two hotspots of caste differentiation at 6 day old eggs and first instar larvae. Our data support what has been previously suggested, that the worker developmental pathway is actively switched on from a default queen developmental program. We were able to identify a number of genes that might be involved in caste determination and differentiation among those already described as differentially expressed in later developmental stages (when castes are morphologically differentiated) in other eusocial insect species. More studies on gene expression in worker and queen-destined ants at the egg stage are necessary to broaden our understanding of caste differentiation and development. It would also be particularly interesting to look for caste-biased genes in an ant species with an environmental caste determination to compare caste differentiation between species with an environmental or genetic caste determination system.

Acknowledgments

We thank Dr. E. Cash for kindly giving us the colonies used in this study, J. Buser and F. Pasquier Genoud for ants' care, C. Berney for technical assistance to develop wet lab protocols, M. Labédan, C. Stoffel, F. Dolivo and C. La Mendola for helping with RNA and DNA extractions and J. Djordjevic for helping with some transcriptomic analyses. This study was performed using the infrastructure of the Lausanne Genomic Technologies Facility for libraries and sequencing. We are grateful to S. McGregor for his helpful comments on the manuscript. This work was supported by an ERC grant and the Swiss NSF (LK).

References

Abouheif, E., and G. A. Wray. 2002. Evolution of the gene network underlying wing polyphenism in ants. *Science*. **297**:249–252.

Allen, R. G., and R. S. Sohal. 1986. Role of Glutathione in the Aging and Development of Insects. *Insect Aging*. 168–181.

Asencot, M., and Y. Lensky. 1985. The phagostimulatory effect of sugars on the induction of "queenliness" in female honeybee (Apis mellifera L.) larvae. *Comp. Biochem. Physiol*. **81A**:203–208.

Ashby, R., S. Forêt, I. Searle, and R. Maleszka. 2016. MicroRNAs in Honey Bee Caste Determination. *Sci. Rep.* **6**:1–15. Nature Publishing Group.

Barchuk, A. R., A. S. Cristino, R. Kucharski, L. F. Costa, Z. L. Simões, and R. Maleszka. 2007. Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. *BMC Dev. Biol*. **7**:1–19.

Barry, E. R., and F. D. Camargo. 2013. The Hippo superhighway: Signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. *Curr. Opin. Cell Biol.* **25**:247–253. Elsevier Ltd.

Bonasio, R. 2014. The role of chromatin and epigenetics in the polyphenisms of ant castes. *Brief. Funct. Genomics.* **13**:235–245.

Bonasio, R., Q. Li, J. Lian, N. S. Mutti, L. Jin, H. Zhao, P. Zhang, P. Wen, H. Xiang, Y. Ding, Z. Jin, S. S. Shen, Z. Wang, W. Wang, J. Wang, S. L. Berger, J. Liebig, G. Zhang, and D. Reinberg. 2012. Genome-wide and Caste-Specific DNA Methylomes of the Ants *Camponotus floridanus* and Harpegnathos saltator. *Curr. Biol.* **22**:1755–1764.

Cameron, R. C., E. J. Duncan, and P. K. Dearden. 2013. Biased gene expression in early honeybee larval development. *BMC Genomics* **14**:1–12.

Choppin, M., B. Feldmeyer, and S. Foitzik. 2021. Histone acetylation regulates the expression of genes involved in worker reproduction in the ant *Temnothorax rugatulus*. *BMC Genomics* **22**:1–13.

Clark, R. M., K. E. Anderson, J. Gadau, and J. H. Fewell. 2006. Behavioral regulation of genetic caste determination in a *Pogonomyrmex* population with dependent lineages. *Ecology*. **87**:2201–2206.

Colgan, T. J., J. C. Carolan, S. J. Bridgett, S. Sumner, M. L. Blaxter, and M. J. F. Brown. 2011. Polyphenism in social insects: Insights from a transcriptome-wide analysis of gene expression in the life stages of the key pollinator, *Bombus terrestris*. *BMC Genomics.* **12**:1–20.

Collins, D. H., A. Wirén, M. Labédan, M. Smith, D. C. Prince, I. Mohorianu, T. Dalmay, and A. F. G. Bourke. 2021. Gene expression during larval caste determination and differentiation in intermediately eusocial bumblebees, and a comparative analysis with advanced eusocial honeybees. *Mol. Ecol.* **30**:718–735.

Conway, J. R., A. Lex, and N. Gehlenborg. 2017. UpSetR: An R package for the visualization of intersecting sets and their properties. *Bioinformatics.* **33**:2938–2940.

Cornette, R., S. Koshikawa, M. Hojo, T. Matsumoto, and T. Miura. 2006. Caste-specific cytochrome P450 in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae). *Insect Mol. Biol.* **15**:235–244.

Corona, M., E. Estrada, and M. Zurita. 1999. Differential expression of mitochondrial genes between queens and workers during caste determination in the honeybee *Apis mellifera. J. Exp. Biol.* **202**:929–938.

Corona, M., R. Libbrecht, and D. E. Wheeler. 2016. Molecular mechanisms of phenotypic plasticity in social insects. *Curr. Opin. Insect Sci.* **13**:55–60.

Darras, H., A. Kuhn, and S. Aron. 2019. Evolution of hybridogenetic lineages in *Cataglyphis ants. Mol. Ecol.* **28**:3073–3088.

De Souza, D. A., O. Kaftanoglu, D. De Jong, R. E. Page, G. V Amdam, and Y. Wang. 2018. Differences in the morphology, physiology and gene expression of honey bee queens and workers reared in vitro versus in situ. *Biol. Open.* **7**:1-8

Diao, Q., L. Sun, H. Zheng, Z. Zeng, S. Wang, S. Xu, H. Zheng, Y. Chen, Y. Shi, Y. Wang, F. Meng, Q. Sang, L. Cao, F. Liu, Y. Zhu, W. Li, Z. Li, C. Dai, M. Yang, S. Chen, R. Chen, S. Zhang, J. D. Evans, Q. Huang, J. Liu, F. Hu, S. Su, and J. Wu. 2018. Genomic and transcriptomic analysis of the Asian honeybee *Apis cerana* provides novel insights into honeybee biology. *Sci. Rep.* **8**:1–14.

Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R. Gingeras. 2013. STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics***. 29**:15–21.

Enayati, A. A., H. Ranson, and J. Hemingway. 2005. Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol.* **14**:3–8.

Evans, J. D., and D. E. Wheeler. 1999. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera. Proc. Natl. Acad. Sci. USA.* **96**:5575–5580.

Evans, J. D., and D. E. Wheeler. 2001. Expression profiles during honeybee caste determination. *Genome Biol*. **2**:1–6.

Falcon, S., and R. Gentleman. 2007. Using GOstats to test gene lists for GO term association. *Bioinformatics.* **23**:257–258.

Fay, M. P. 2010. Confidence intervals that match Fisher's exact or Blaker's exact tests. *Biostatistics.* **11**:373–374.

Foitzik, S., M. Haberl, J. Gadau, and J. Heinze. 1997. Mating frequency of *Leptothorax nylanderi* ant queens determined by microsatellite analysis. *Insectes Soc.* **44**:219–227.

Gadau, J., C. P. Strehl, J. Oettler, and B. Hölldobler. 2003. Determinants of intracolonial relatedness in *Pogonomyrmex rugosus* (Hymenoptera; Formicidae): Mating frequency and brood raids. *Mol. Ecol.* **12**:1931–1938.

Gotoh, H., J. A. Hust, T. Miura, T. Niimi, D. J. Emlen, and L. C. Lavine. 2015. The Fat/Hippo signaling pathway links within-disc morphogen patterning to whole-animal signals during phenotypically plastic growth in insects. *Dev. Dyn.* **244**:1039–1045.

Gräff, J., S. Jemielity, J. D. Parker, K. M. Parker, and L. Keller. 2007. Differential gene expression between adult queens and workers in the ant *Lasius niger. Mol. Ecol.* **16**:675–683.

Grozinger, C. M., Y. Fan, S. E. Hoover, and M. L. Winston. 2007. Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). *Mol. Ecol.* **16**:4837–4848.

Guo, X., S. Su, G. Skogerboe, S. Dai, W. Li, Z. Li, F. Liu, R. Ni, Y. Guo, S. Chen, S. Zhang, and R. Chen. 2013. Recipe for a busy bee: MicroRNAs in honey bee caste determination. *PLoS One.* **8**:1-10

Harrison, M. C., R. L. Hammond, and E. B. Mallon. 2015. Reproductive workers show queenlike gene expression in an intermediately eusocial insect, the buff-tailed bumble bee *Bombus terrestris. Mol. Ecol.* **24**:3043–3063.

Haydak, M. 1970. Honey Bee Nutrition. *Annu. Rev. Entomol.* **15**:143–156.

Helms Cahan, S., G. E. Julian, S. W. Rissing, T. Schwander, J. D. Parker, and L. Keller. 2004. Loss of phenotypic plasticity generates genotype-caste association in harvester ants. *Curr. Biol.* **14**:2277–2282.

Helms Cahan, S., and L. Keller. 2003. Complex hybrid origin of genetic caste determination in harvester ants. *Nature.* **424**:306–309.

Helms Cahan, S., J. D. Parker, S. W. Rissing, R. A. Johnson, T. S. Polony, M. D. Weiser, and D. R. Smith. 2002. Extreme genetic differences between queens and workers in hybridizing *Pogonomyrmex harvester* ants. *Proc. R. Soc. B Biol. Sci.* **269**:1871–1877.

Helms Cahan, S., and S. B. Vinson. 2003. Reproductive Division of Labor between Hybrid and Nonhybrid Offspring in a Fire Ant Hybrid Zone. *Evolution (N. Y).* **57**:1562–1570.

Herpin, A., and M. Schartl. 2015. Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpators. *EMBO Rep.* **16**:1260–1274.

Hoffman, E. A., and M. A. Goodisman. 2007. Gene expression and the evolution of phenotypic diversity in social wasps. *BMC Biol.* **5**:1-9

Hölldobler, B. 1976. The behavioral Ecology of Mating in Harvester Ants (Hymenoptera: Formicidae: *Pogonomyrmex*). *Behav. Ecol. Sociobiol.* **1**:405–423.

Hölldobler, B., and E. O. Wilson. 1990. The Ants. Harvard University. Harvard University Press, Harvard USA.

Hung, A. C. F., and S. B. Vinson. 1977. Interspecific Hybridization and Caste Specificity of Protein in Fire Ant. *Science*. **196**:1458–1460.

Hunt, J. H., F. Wolschin, M. T. Henshaw, T. C. Newman, A. L. Toth, and G. V Amdam. 2010. Differential gene expression and protein abundance evince ontogenetic bias toward castes in a primitively eusocial wasp. *PLoS One*. **5**:1-10

Isobe, K., V. Raghuram, L. Krishnan, C. L. Chou, C. R. Yang, and M. A. Knepper. 2020. Crispr-cas9/phosphoproteomics identifies multiple noncanonical targets of myosin light chain kinase. *Am. J. Physiol. Ren. Physiol.* **318**:F600–F616.

Judice, C., K. Hartfelder, and G. A. Pereira. 2004. Caste-specific gene expression in the stingless bee *Melipona quadrifasciata* - Are there common patterns in highly eusocial bees? *Insectes Soc.* **51**:352–358.

Julian, G. E., J. H. Fewell, J. Gadau, R. A. Johnson, and D. Larrabee. 2002. Genetic determination of the queen caste in an ant hybrid zone. *Proc. Natl. Acad. Sci. USA.* **99**:8157–8160.

Kang, I., W. Kim, J. Y. Lim, Y. Lee, and C. Shin. 2021. Organ-specific transcriptome analysis reveals differential gene expression in different castes under natural conditions in *Apis cerana. Sci. Rep.* **11**:1–12.

Kapheim, K. M. 2019. Synthesis of Tinbergen's four questions and the future of sociogenomics. Behav. Ecol. Sociobiol. **73**:1–15.

Klein, A., E. Schultner, H. Lowak, L. Schrader, J. Heinze, L. Holman, and J. Oettler. 2016. Evolution of Social Insect Polyphenism Facilitated by the Sex Differentiation Cascade. *PLoS Genet.* **12**:1–16.

Koch, S., R. Tahara, A. Vasquez-Correa, and E. Abouheif. 2021. Nano-CT imaging of larvae in the ant Pheidole hyatti reveals coordinated growth of a rudimentary organ necessary for soldier development. *J Exp Zool B Mol Dev Evol.* **336**:540–553.

Kolde, R. 2019. pheatmap: Pretty Heatmaps.

Kovaka, S., A. V Zimin, G. M. Pertea, R. Razaghi, S. L. Salzberg, and M. Pertea. 2019. Transcriptome assembly from long-read RNA-seq alignments with StringTie2. *Genome Biol.* **20**:1–13. Genome Biology.

Leimar, O., K. Hartfelder, M. D. Laubichler, and R. E. Page. 2012. Development and evolution of caste dimorphism in honeybees - a modeling approach. *Ecol. Evol.* **2**:3098–3109.

Li, W., Z. Y. Huang, F. Liu, Z. Li, L. Yan, S. Zhang, S. Chen, B. Zhong, and S. Su. 2013. Molecular Cloning and Characterization of Juvenile Hormone Acid Methyltransferase in the Honey Bee, *Apis mellifera*, and Its Differential Expression during Caste Differentiation. *PLoS One.* **8**:1–12.

Liao, Y., G. K. Smyth, and W. Shi. 2014. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. **30**:923–930.

Libbrecht, R., M. Corona, F. Wende, D. O. Azevedo, J. E. Serrão, and L. Keller. 2013. Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proc. Natl. Acad. Sci. USA.* **110**:11050–11055.

Libbrecht, R., D. Nadrau, and S. Foitzik. 2020. A Role of Histone Acetylation in the Regulation of Circadian Rhythm in Ants. *iScience*. **23**:100846.

Libbrecht, R., T. Schwander, and L. Keller. 2011. Genetic components to caste allocation in a multiple-queen ant species. *Evolution (N. Y)*. **65**:2907–2915.

Linksvayer, T. A. 2006. Direct, maternal, and sibsocial genetic effects on individual and colony traits in an ant. Evolution (N. Y). **60**:2552.

Linksvayer, T. A., O. Kaftanoglu, E. Akyol, S. Blatch, G. V Amdam, and R. E. Page. 2011. Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen-worker dimorphism. *J. Evol. Biol.* **24**:1939–1948.

Liu, N., and L. Zhang. 2004. CYP4AB1, CYP4AB2, and Gp-9 gene overexpression associated with workers of the red imported fire ant, *Solenopsis invicta* Buren. *Gene*. **327**:81–87.

Lu, K. H., J. Y. Bradfield, and L. L. Keeley. 1999. Juvenile hormone inhibition of gene expression for cytochrome P4504C1 in adult females of the cockroach, *Blaberus discoidalis*. *Insect Biochem. Mol. Biol.* 29:667–673.

Miura, T., and M. E. Scharf. 2011. Molecular Basis Underlying Caste Differentiation in Termites. Pp. 211–253 in D. E. Bignell, Y. Roisin, and N. Lo, eds. Biology of Termites: A Modern Synthesis.

Morandin, C., V. P. Brendel, L. Sundström, H. Helanterä, and A. S. Mikheyev. 2019. Changes in gene DNA methylation and expression networks accompany caste specialization and age-related physiological changes in a social insect. *Mol. Ecol.* **28**:1975–1993.

Morandin, C., K. Dhaygude, J. Paviala, K. Trontti, C. Wheat, and H. Helanterä. 2015. Caste-biases in gene expression are specific to developmental stage in the ant *Formica exsecta*. *J. Evol. Biol.* **28**:1705–1718.

Morandin, C., M. M. Tin, S. Abril, C. Gómez, L. Pontieri, M. Schiøtt, L. Sundström, K. Tsuji, J. S. Pedersen, H. Helanterä, and A. S. Mikheyev. 2016. Comparative transcriptomics reveals the conserved building blocks involved in parallel evolution of diverse phenotypic traits in ants. *Genome Biol.* **17**:1–19.

Norman, V., H. Darras, C. Tranter, S. Aron, and W. O. Hughes. 2016. Cryptic lineages hybridize for worker production in the harvester ant *Messor barbarus*. *Biol. Lett.* **12**:1-5

Ometto, L., D. Shoemaker, K. G. Ross, and L. Keller. 2011. Evolution of gene expression in fire ants: The effects of developmental stage, caste, and species. *Mol. Biol. Evol*. **28**:1381–1392.

Patel, A., M. K. Fondrk, O. Kaftanoglu, C. Emore, G. Hunt, K. Frederick, and G. V Amdam. 2007. The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. *PLoS One.* **2**:1–7.

Pereboom, J. J., W. C. Jordan, S. Sumner, R. L. Hammond, and A. F. Bourke. 2005. Differential gene expression in queen-worker caste determination in bumble-bees. *Proc. R. Soc. B Biol. Sci.* **272**:1145–1152.

Pontieri, L., A. Rajakumar, A. M. Rafiqi, R. S. Larsen, E. Abouheif, and G. Zhang. 2020. From egg to adult: a developmental table of the ant Monomorium pharaonis. bioRxiv 2020.**12**.22.423970.

Renaut, S., A. W. Nolte, and L. Bernatchez. 2009. Gene expression divergence and hybrid misexpression between lake whitefish species pairs (*Coregonus* spp. Salmonidae). *Mol. Biol. Evol.* **26**:925–936.

Rheindt, F. E., J. Gadau, C. P. Strehl, and B. Hölldobler. 2004. Extremely high mating frequency in the Florida harvester ant (*Pogonomyrmex badius*). *Behav. Ecol. Sociobiol*. **56**:472–481.

Richards, M. H., and L. Packer. 1994. Trophic aspects of caste determination in *Halictus ligatus*, a primitively eusocial sweat bee. *Behav. Ecol. Sociobiol*. **34**:385–391.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2009. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* **26**:139–140.

Sayols, S. 2020. rrvgo: a Bioconductor package to reduce and visualize Gene Ontology terms.

Schwander, T., S. Helms Cahan, and L. Keller. 2007. Characterization and distribution of *Pogonomyrmex* harvester ant lineages with genetic caste determination. *Mol. Ecol.* **16**:367–387.

Schwander, T., S. Helms Cahan, and L. Keller. 2006. Genetic caste determination in *Pogonomyrmex* harvester ants imposes costs during colony founding. *J. Evol. Biol*. **19**:402–409.

Schwander, T., J. Y. Humbert, C. S. Brent, S. Helms Cahan, L. Chapuis, E. Renai, and L. Keller. 2008. Maternal Effect on Female Caste Determination in a Social Insect. *Curr. Biol*. **18**:265–269.

Schwander, T., N. Lo, M. Beekman, B. P. Oldroyd, and L. Keller. 2010. Nature versus nurture in social insect caste differentiation. *Trends Ecol*. Evol. **25**:275–282.

Severson, D. W., J. L. Williamson, and J. M. Aiken. 1989. Caste-specific transcription in the female honey bee. *Insect Biochem*. **19**:215–220.

Shi, Y. Y., Z. Y. Huang, Z. J. Zeng, Z. L. Wang, X. B. Wu, and W. Y. Yan. 2011. Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, apidae). *PLoS One*. **6**:2–7.

Sieber, K. R., T. Dorman, N. Newell, and H. Yan. 2021. (Epi)genetic mechanisms underlying the evolutionary success of eusocial insects. *Insects.* **12**:1–20.

Simola, D. F., R. J. Graham, C. M. Brady, B. L. Enzmann, C. Desplan, A. Ray, L. J. Zwiebel, R. Bonasio, D. Reinberg, J. Liebig, and S. L. Berger. 2016. Epigenetic (re)programming of caste-specific behavior in the ant *Camponotus floridanus*. *Science*. **351**: aac6633

Sirviö, A., P. Pamilo, R. A. Johnson, R. E. Page, and J. Gadau. 2011. Origin and evolution of the dependent lineages in the genetic caste determination system of *Pogonomyrmex* ants*. Evolution (N. Y).* **65**:869–884.

Slater, G. P. 2017. Eat to reproduce: the role of diet quantity on honey bee (*Apis mellifera*) caste determination. Thesis. 1-128

Slater, G. P., G. D. Yocum, and J. H. Bowsher. 2020. Diet quantity influences caste determination in honeybees (*Apis mellifera*): Caste determination in honey bees. *Proc. R. Soc. B Biol. Sci*. **287**:1-10

Smith, C. R., K. E. Anderson, C. V Tillberg, J. Gadau, and A. V Suarez. 2008. Caste determination in a polymorphic social insect: Nutritional, social, and genetic factors. *Am. Nat*. **172**:497–507.

Smith, C. R., N. S. Mutti, W. C. Jasper, A. Naidu, C. D. Smith, and J. Gadau. 2012. Patterns of DNA methylation in development, division of labor and hybridization in an ant with genetic caste determination. *PLoS One***.** **7**:1–9.

Smith, C. R., and W. R. Tschinkel. 2006. The sociometry and sociogenesis of reproduction in the Florida harvester ant, *Pogonomyrmex badius*. *J. Insect Sci.* **6**:1–11.

Sumner, S., E. Bell, and D. Taylor. 2018. A molecular concept of caste in insect societies. *Curr. Opin. Insect Sci.* **25**:42–50.

Sumner, S., J. J. Pereboom, and W. C. Jordan. 2006. Differential gene expression and phenotypic plasticity in behavioural castes of the primitively eusocial wasp, *Polistes canadensis*. *Proc. R. Soc. B Biol. Sci.* **273**:19–26.

Sztal, T., H. Chung, S. Berger, P. D. Currie, P. Batterham, and P. J. Daborn. 2012. A cytochrome p450 conserved in insects is involved in cuticle formation. *PLoS One.* **7**:1–9.

Tarver, M. R., M. R. Coy, and M. E. Scharf. 2012. Cyp15F1: A novel cytochrome p450 gene linked to juvenile hormone-dependent caste differentiation in the termite *Reticulitermes flavipes*. *Arch. Insect Biochem. Physiol.* **80**:92–108.

Teseo, S., N. Châline, P. Jaisson, and D. J. Kronauer. 2014. Epistasis between adults and larvae underlies caste fate and fitness in a clonal ant. *Nat. Commun*. **5**:1-8

Thompson, G. J., R. Kucharski, R. Maleszka, and B. P. Oldroyd. 2006. Towards a molecular definition of worker sterility: Differential gene expression and reproductive plasticity in honey bees. *Insect Mol. Biol*. **15**:637–644.

Toth, A. L., K. Varala, T. C. Newman, F. E. Miguez, S. K. Hutchison, D. A. Willoughby, J. F. Simons, M. Egholm, J. H. Hunt, G. M. E, and G. E. Robinson. 2007. Wasp Gene Expression Supports an Evolutionary Link Between Maternal Behavior and Eusociality. *Science*. **318**:441–444.

Tumaneng, K., R. C. Russell, and K. L. Guan. 2012. Organ size control by Hippo and TOR pathways. *Curr. Biol*. **22**:R368–R379.

Vastenhouw, N. L., W. X. Cao, and H. D. Lipshitz. 2019. The maternal-to-zygotic transition revisited. *Development.* **146**:1–20.

Villalta, I., O. Blight, E. Angulo, X. Cerdá, and R. Boulay. 2016. Early developmental processes limit socially mediated phenotypic plasticity in an ant. *Behav. Ecol. Sociobiol*. **70**:285–291.

Volny, V. P., and D. M. Gordon. 2002. Genetic basis for queen-worker dimorphism in a social insect. *Proc. Natl. Acad. Sci. USA*. **99**:6108–6111.

Volny, V. P., M. J. Greene, D. M. Gordon, V. P. Volny, M. J. Greene, and D. M. Gordon. 2006. Brood Production and Lineage Discrimination in the Red Harvester Ant (*Pogonomyrmex barbatus*). *Ecology*. **87**:2194–2200.

Walker, K. E. 2017. DNA Methylation as a Mechanism for Caste System Determination in *Solenopsis invicta*: Do the Genes Determine the Queen ? University of South Florida St. Petersburg. Thesis

Wan, P. J., X. Q. Shi, Y. Kong, L. T. Zhou, W. C. Guo, T. Ahmat, and G. Q. Li. 2013. Identification of cytochrome P450 monooxygenase genes and their expression profiles in cyhalothrin-treated Colorado potato beetle, *Leptinotarsa decemlineata*. *Pestic. Biochem. Physiol*. **107**:360–368.

Wang, M., Y. Xiao, Y. Li, X. Wang, S. Qi, Y. Wang, L. Zhao, K. Wang, W. Peng, G. Z. Luo, X. Xue, G. Jia, and L. Wu. 2021. RNA m6A Modification Functions in Larval Development and Caste Differentiation in Honeybee (*Apis mellifera*). *Cell Rep*. **34**:108580.

Wang, M., Y. Zhao, and B. Zhang. 2015. Efficient Test and Visualization of Multi-Set Intersections. *Science.* Rep. **5**:1–12.

Warner, M. R., L. Qiu, M. J. Holmes, A. S. Mikheyev, and T. A. Linksvayer. 2019. Convergent eusocial evolution is based on a shared reproductive groundplan plus lineage-specific plastic genes. *Nat. Commun.* **10**:1–11.

Wen, Z., and J. G. Scott. 2001. Cloning of two novel p450 cDNAs from German cockroaches, *Blattella germanica* (L.): CYP6K1 and CYP6J1. *Insect Molecular Biology*. **10**:131-137

Wheeler, D. E. 1986. Developmental and Physiological Determinants of Caste in Social Hymenoptera: Evolutionary Implications. Am. Nat. **128**:13–34.

Wheeler, D. E., N. A. Buck, and J. D. Evans. 2014. Expression of insulin/insulin-like signalling and TOR pathway genes in honey bee caste determination. *Insect Mol. Biol*. **23**:113–121.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wiernasz, D. C., C. L. Perroni, and B. J. Cole. 2004. Polyandry and fitness in the western harvester ant, *Pogonomyrmex occidentalis*. *Mol. Ecol.* **13**:1601–1606.

Wingett, S. W., and S. Andrews. 2018. Fastq screen: A tool for multi-genome mapping and quality control [version 2; referees: 4 approved]. F1000Research **7**:1–13.

Wojciechowski, M., R. Lowe, J. Maleszka, D. Conn, R. Maleszka, and P. J. Hurd. 2018. Phenotypically distinct female castes in honey bees are defined by alternative chromatin states during larval development. *Genome Res*. **28**:1532–1542.

Wu, Z., L. Yang, Q. He, and S. Zhou. 2021. Regulatory Mechanisms of Vitellogenesis in Insects. *Front. Cell Dev. Biol.* **8**:1–11.

Xu, P. J., J. H. Xiao, Q. Y. Xia, B. Murphy, and D. W. Huang. 2010. *Apis mellifera* has two isoforms of cytoplasmic HSP90. *Insect Mol. Biol.* **19**:593–597.

Yin, L., K. Wang, L. Niu, H. Zhang, Y. Chen, T. Ji, and G. Chen. 2018. Uncovering the Changing Gene Expression Profile of Honeybee (*Apis mellifera*) Worker Larvae Transplanted to Queen Cells. *Front. Genet.* **9**:1–11.

Supplementary Information

Additional Tables S1 and S9 available for download on the following dropbox link:
www.dropbox.com/transfer/AAAAABmxhk47dcDkPCND18T8quYhbR54RsfGqKb9pdUy8qWxYmWgMt4

Confirmation of the caste

We managed to infer intra and inter-lineage ancestry for 715 individuals out of 895 (Table). For the other 180 individuals, either the DNA extraction and/or microsatellites PCR amplification failed, or the genotype did not allow the attribution to the worker or gyne caste.

Table S2. Numbers of DNA extractions done on the J1-lineage colony and on the three J2-lineage colonies with successful caste attribution (worker or gyne), inconclusive genotype or failed DNA extraction. Inconclusive genotype contains individuals for which different microsatellite makers indicated different caste fates.

Lineage	Colony	Age	Worker	Gyne	Inconclusive genotype	Failed DNA extraction	Total samples extracted	Total
J2	1	24h	22	13	2	5	42	234
		6d	5	12	7	24	48	
		~9d	12	9	8	18	47	
		L1	17	17	6	10	50	
		L2	24	17	3	3	47	
	2	24h	41	12	1	0	54	246
		6d	22	31	0	0	53	
		~9d	31	25	0	6	62	
		L1	22	22	1	0	45	
		L2	20	12	0	0	32	
	3	24h	53	9	0	0	62	225
		6d	38	14	1	0	53	
		~9d	34	5	2	3	44	
		L1	30	12	0	0	42	
		L2	15	9	0	0	24	
J1	4	24h	25	0	3	7	35	190
		6d	17	0	5	14	36	
		~9d	11	0	4	19	34	
		L1	28	0	5	7	40	
		L2	29	0	9	7	45	

Table S3. Numbers of patrilines per colony and caste. **P1-P3**: worker patrilines, **P4-P7**: gyne patrilines. There is no relationship in patriline numbers between colonies (i.e. P1 in colony 1 is not the same than P1 in colony 2). **Unassigned** samples represent workers whose genotype could not be assigned to a patriline because of incomplete/unique genotypes.

Lineage	Colony	Worker patrilines				Gyne patrilines				Total number of patrilines per caste (worker - gyne)
		P1	P2	P3	Unassigned	P4	P5	P6	P7	
J2	1	52	27		1	25	19	13	11	2 - 4
	2	130	4	2		54	48			3 - 2
	3	80	76	5	9	14	14	13	8	3 - 4
J1	4	55	44	10	1					2 - 0

RNA concentration and quality

The RNA average concentration (elution done in 50*µ*l for every sample) per caste and age shows a surprisingly low RNA concentration in 9 day old eggs compared to 6 days old and 11 days old (first larval stage; Figure). We collected 9 days old samples once we could see the larva moving in the egg (i.e. close to hatching time which happens at ~10 days old). This low RNA concentration in 9 day old eggs does not seem to be explained by RNA degradation (Table S4), suggesting that transcription rates may be reduced, perhaps because of a transient pause in development until hatching.

Another unexpected result is the very high RNA degradation observed in 6 day old eggs, which is consistent in both worker and gyne samples (Table S4). It is probably too late in the development to be the maternal-to-zygote transition as it happens much earlier (2 to 32 hours after fertilization) in the classical model species (*Drosophila*, *C. elegans*, Zebrafish, mouse, human; (Vastenhouw *et al.* 2019). However, it is possible that another important developmental transition occurs at 6 days. Further investigations are required to confirm this observation.

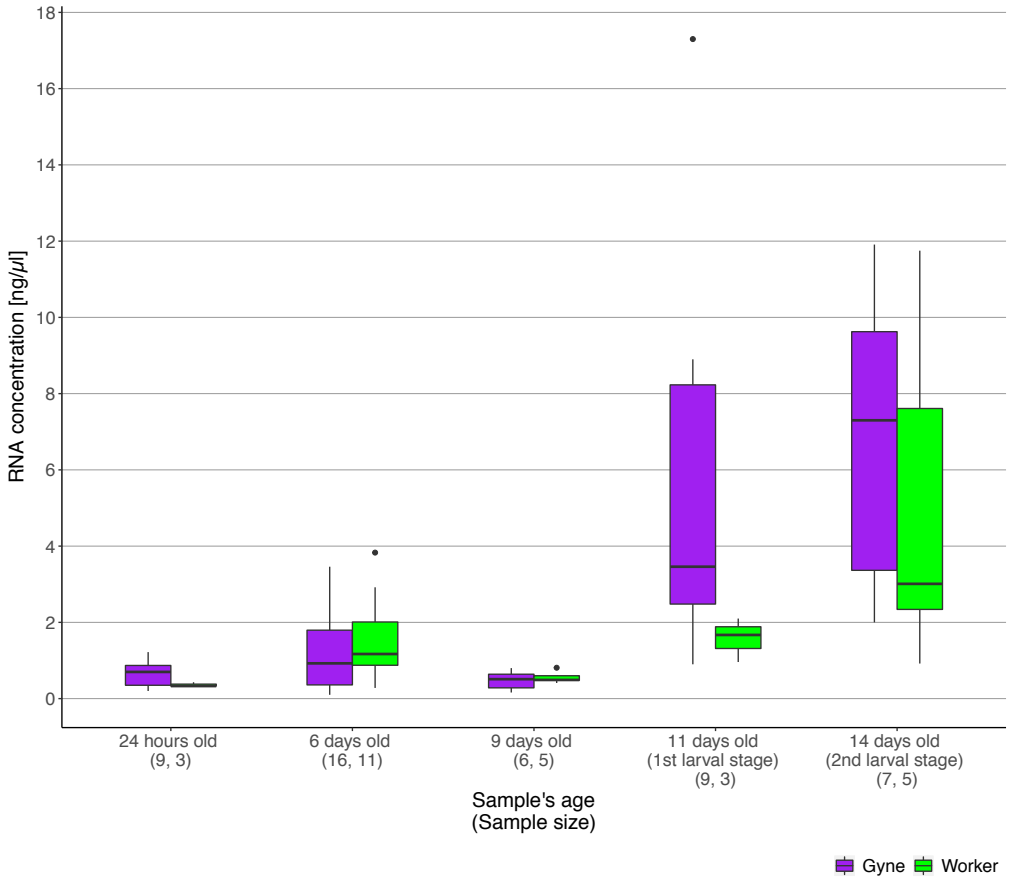


Figure S1. RNA average concentration [ng/*µ*l; RNA elution done in 50*µ*l] per egg for the five developmental stages, for gyne and worker caste (measures done by Ribogreen). Sample size (gyne, worker) is given in brackets under each age.

Table S4. RNA quality (evaluation by eyes based on Fragment Analyzer profiles). Percentage of samples with good quality/slightly degraded RNA and borderline/degraded RNA, per caste and egg age. The sample size is indicated in brackets.

Genotype	Egg age	Ok or slightly degraded		Borderline or degraded	
gyne	24 hours old	100%	(8)	0%	(0)
	6 days old	10%	(1)	90%	(9)
	9 days old	100%	(4)	0%	(0)
	1st larval stage (11 day old)	78%	(7)	22%	(2)
	2nd larval stage (14 days old)	75%	(6)	25%	(2)
worker	24 hours old	100%	(3)	0%	(0)
	6 days old	11%	(1)	89%	(8)
	9 days old	100%	(3)	0%	(0)
	1st larval stage (11 days old)	100%	(3)	0%	(0)
	2nd larval stage (14 days old)	60%	(3)	40%	(2)

SI Text 1

Protocol for double RNA/DNA extraction using TRIzol® with specific steps for small RNA amount extraction (24 hour old egg, one egg/sample) for *Pogonomyrmex* ant

Before starting

- Cool down the centrifuge at 4°C (fast temp)
- Make fresh 75% ethanol and put it at -20°C freezer to add it cold in the samples
- Incubate milliQ water at 60°C (to facilitate pellet dissolution)
- Defrost the Glycogen blue

Homogenization

Work **under the hood** from here

- Make a mix of TRIzol and ß-mercaptoethanol (number of samples + 10%)
  Per sample = 1ml of TRIzol + 10μl of ß-mercaptoethanol/ml of TRIzol (protect the RNA from degradation)
- Add 1010μl of the mix per tube containing the sample
- Homogenize tissue sample in TRIzol 2x 30 seconds at 6.500 using a machine with dry ice
- Label 2 new tubes per samples (1.5ml with lid)
- Transfer the content into new tube ✍ important step to keep the liquid without the beads (for DNA extraction later)

Separation phase

- Incubate 5 minutes at room temperature (RT)
- Add 200μl of chloroform per ml of TRIzol used (to denature protein in action with TRIzol)
- Shake vigorously for 15 seconds, the 2 phases have to be well mixed
- Incubate 3 minutes at RT
- Centrifuge 15 minutes at 12 000g at 4°C (to separates the mixture into 3 phases: the upper transparent phase contain the RNA. With egg and small larva there is no visible interphase. The lower pink phase is used for DNA extraction)
- Transfer the upper aqueous phase containing the RNA (~500μl) into a new 1.5ml labelled tube
- Add 500μl of 2-propanol (half of the TRIzol quantity used in step 1; to desalt and concentrate RNA) + 1µl Glycogen blue (RNAse-free, Invitrogen, 15 mg/ml; to increase the visibility of the pellet) and vortex ✍ sample can be stored at -80°C, for max 2 weeks
- If you want to extract the DNA as well, put the RNA + 2-propanol samples in the freezer until the end of RNA extraction. If no DNA extraction, jump directly to RNA extraction.

DNA isolation for 24 hour old eggs (low DNA amount)

Work **on ice** under the hood

- After having taken the aqueous phase with RNA, spin down the tubes which contain the interphase/organic phase with TRIzol at 12,000g for 5 min. at 4°C
- Carefully remove any remaining aqueous phase which would contaminate the DNA sample with RNA
  ✍ Removal of the remaining aqueous phase before DNA precipitation is a critical step for the quality of the isolated DNA.
- Add 700μl of re-extraction buffer (4M guanidine thiocyanate (powder), 50mM sodium citrate and 1M Tris, pH 8.0-8.5)
- Mix intensively by inverting for at least 3 min (or put on a shaker for 10 min), **/!\ DO NOT VORTEX** (shearing)
- Centrifuge at 12000g for 15-30 min at 4°C
- Transfer the upper phase to the 1.5 ml eppendorf tube previously labelled
- Add 1µl Glycogen blue (RNAse-free, Invitrogen, 15 mg/ml; carrier) + 360μl of isopropanol, mix by inversion and incubate for 5 min. at RT

- Centrifuge at 12,000g for 30 min. at 4°C (to pellet DNA)
- Remove the supernatant
- Wash the pellet with 1ml of 75% ETOH
- Centrifuge at 12,000g for 5 min. at 4°C
- Remove ethanol and dry the DNA pellet for 3 min by air-drying
- Dissolve pellet in 16 µl of TE buffer (=10 mM Tris / 0.1 mM EDTA) by pipetting up and down all around the bottom of the tube for ~2 min. to be sure the DNA pellet is well dissolved.

DNA isolation for eggs from 3 days old until small larvae

- Add 300 µl of 100% ethanol and 1µl of Glycogen blue
- Mix by inversion **/!\ DO NOT VORTEX**
- Incubate 3 minutes at RT
- Centrifuge 5 minutes at 2000g (=4300rpm) at 4°C (to pellet DNA)
- Remove the phenol/ethanol supernatant
- Add 1 ml of 0.1M trisodium citrate 10% ethanol pH 8.5 solution (washing step)
- Incubate at RT, with occasional mixing, for AT LEAST 30 minutes ✍ DO NOT reduce the time samples remain in the washing solution. 30 minutes is the absolute minimum time for efficient removal of phenol from the DNA (maximum 2 hours).
- Centrifuge for 5 minutes at 2000g at 4°C
- Remove the supernatant
- Repeat the washing step: add 1 ml of 0.1M trisodium citrate 10% ethanol solution, incubate at RT AT LEAST 30 minutes, centrifuge for 5 minutes at 2000g at 4°C and remove the supernatant.
- Add 1.5ml of 75% ethanol in the tube with the pellet
- Incubate for 15 minutes at RT
- Centrifuge at 2000g for 5 minutes at 4°C
- Remove the ethanol by flipping the tube, then quick spin and remove the last drop with the pipette.
- Dry the DNA pellet for 3 minutes by letting the tubes opened ✍ DO NOT over-dry the pellet, otherwise it cannot be solubilized anymore
- Make a mix NaOH-HEPES (sample number+10%):
  Per sample: 15µl of NaOH 8mM + 1.35µl of HEPES 0.1M to adjust pH at 8.4 = 16.35µl/sample
- Dissolve the pellet in 16.35µl of mix NaOH 8mM-HEPES 0.1M with repeated slow pipetting up and down

RNA isolation

Work **on ice** under the hood

- Centrifuge 10 minutes at 12 000g at 4°C.
- Remove the supernatant (the RNA precipitate will form a blue pellet (Glycogen) on the side-bottom of the tube)
- Add 1ml of 75% cold ethanol (to remove residual protein)
- Vortex
- Centrifuge 5 minutes at 12000g at 4°C.
- Remove the ethanol by flipping the tube and remove the last drop carefully with a pipet. Dry the RNA pellet for 5 minutes by air-drying.
  ✍ DO NOT over-dry the pellet, otherwise it cannot be solubilized anymore.
- Add 20-40µl of RNase free milliQ water at 60°C and pipette up and down for 2 min. to ensure complete dissolution.

Table S5. The caste of *P. rugosus* eggs and larvae was determined by microsatellite genotyping using the indicated primer sequences. (Primer names and sequences, fluorescent dye added to the 5' end of the forward primer, locus amplified in the same multiplex, repeat motif type, number of observed alleles (N_A), corresponding size range of the amplified fragment and GenBank accession numbers are given for each locus.)

Locus	Primer (5'-3')	Dye	Multiplex	Motif	N_A	Size range (bp)	GenBank
Pb5	F: CGCCGATGTCGCTATACC R: CTCAGAAGACGCAGGAACG	FAM	1	$(CTTT)_4TT(CT)_{16}$	4	220-240	AF481937
Pb8	F: GAGATGGGCAAGGAACAGGAC R: GGAAGAATCTGCGGAGTGC	FAM	1	$(CT)_4TT(CT)_{17}$	6	260-293	AF481940
Pb10	F: GCTGCTCTCGTAACTAAGTCG R: CCGTACTTTACCGTGCTGG	NED	1	$(CAA)_{10}$	3	266-290	AF481942
L18	F: TGA ATT TGG ATG GCG GTA GAC R: ACC TAA TGC ACG CTT TAG AAT	NED	1	$(GA)_{29}G\ (GA)_2$	8	140-210	na
Pb9	F: GTCGTGAAATAATAATCAGTACG R: GAACACAATAGAAATCCAGC	FAM	2	$(CA)_{21}AA(CG)_9$	7	315-360	AF481941
Po3	F: ATTGCTTTCCCACTTGACC R: TGAAGAGCGATAGGCAGAGT	NED	2	$(TC)_{24}$	9	150-180	AY301007
Po8	F: ACCACCAACCTCATTACGA R: GCTCAGCATACTGTTCTCCA	FAM	2	$(ATC)_6(CAG)_{16}$	6	200-270	AY301009
Pr1	F: AATCTCAGCAGGCGAGAAAG R: GAGAGCGTAGACGGAAATGC	NED	2	na	2	380-400	AF521193

Table S6. PCR conditions for the amplification at the 8 loci (see Table S5) to determine the genotype of *P. rugosus* eggs and larvae.

Cycling conditions	Temp	Time	Cycles
Denaturation initial	95°C	15 min	
Denaturation	94°C	30 sec	30
Annealing	58°C (multiplex 1) 52°C (multiplex 2)	90 sec	
Extension	72°C	1 min	
Final extension	60°C	30min	
	12°C	xxx	

Table S7. Genotypes of the eggs and larvae at 8 microsatellite loci. Gynes genotypes are pure J1J1 lineage, whereas workers genotypes are inter-lineage J1J2. Paternal alleles are given in italics.

age	sampleID	Colony	Batch	Father	Pb9		Po3		Po8		Pb10		Pb5		Pb8		L18		Pr1	
Gyne																				
	318sII	1	1st	M6	315	*333*	177	*181*	232	*241*	270	*270*			289	*289*	154	*156*	398	*398*
	551sII	1	2nd	M3	315	*315*	169	*177*	232	*241*	270	*270*	222	*222*	289	*287*	154	*154*	398	*398*
24h	916Q	2	1st	M3	315	*315*	177	*177*	238	*238*	270	*270*			287	*291*	154	*161*	398	*398*
	582Q	3	1st	M7	315	*315*	165	*177*	238	*238*	270	*270*	224	*224*	287	*279*	154	*154*	398	*398*
	164Q	3	2nd	M5	315	*315*	165	*165*	238	*238*	270	*270*			279	*287*	156	*161*	398	*398*
	322sII	1	1st	M6	315	*333*	177	*181*	232	*241*	270	*270*	224	*222*	289	*289*	163	*156*	398	*398*
	039sII	1	2nd	M4	315	*315*	169	*181*	232	*238*	270	*270*	224	*222*	289	*279*	163	*156*	398	*398*
6d	251Q	2	1st	M3	315	*315*	177	*177*	238	*238*	270	*270*	224	*222*	287	*291*	154	*161*	398	*398*
	329Q	2	2nd	M3	315	*315*	165	*177*	238	*238*	270	*270*	224	*222*	287	*291*	154	*161*	398	*398*
	098Q	3	1st	M7	315	*315*	165	*177*	238	*238*	270	*270*	222	*224*	279	*279*	154	*154*	398	*398*
	182Q	3	2nd	M5	315	*315*	165	*165*	238	*238*	270	*270*	222	*222*	279	*287*	156	*161*	398	*398*
	665sII	1	1st	M6	333	*333*							222	*222*	289	*289*			398	*398*
	095sII	1	2nd	M4	315	*315*	177	*181*			270	*270*	222	*222*	289	*279*	154	*156*	398	*398*
9d	694Q	2	1st	M3	315	*315*	177	*177*	238	*238*	270	*270*			291	*291*	154	*161*	398	*398*
	445Q	2	2nd	M3	315	*315*	165	*177*	238	*238*	270	*270*	224	*222*	291	*291*	156	*161*	398	*398*
	937Q	3	1st	M7	315	*315*	165	*177*	238	*238*	270	*270*			287	*279*	154	*154*	398	*398*
	1616Q	3	2nd	M6	315	*315*	165	*181*	238	*238*	270	*270*	222	*224*	287	*283*	156	*154*	398	*398*
	683sII	1	1st	M6	333	*333*							224	*222*	289	*289*			398	*398*
	286sII	1	2nd	M4	333	*315*							222	*222*	289	*279*			398	*398*
L1	887Q	2	1st	M3	315	*315*	165	*177*	238	*238*	270	*270*	224	*222*	287	*291*	154	*161*	398	*398*
	606Q	2	2nd	M3	315	*315*	165	*177*	238	*238*	270	*270*			287	*291*	156	*161*	398	*398*
	557Q	3	1st	M7	315	*315*	165	*177*	238	*238*	270	*270*	224	*224*	279	*279*	154	*154*	398	*398*
	388Q	3	2nd	M5	315	*315*	165	*165*	238	*238*	270	*270*	224	*224*	279	*287*	156	*161*	398	*398*
	698sII	1	1st	M6	333	*333*							224	*222*	289	*289*			398	*398*
	890sII	1	2nd	M4	315	*315*	177	*181*	241	*238*	270	*270*	222	*222*	289	*279*	163	*156*	398	*398*
L2	962Q	2	1st	M3	315	*315*	177	*177*	238	*238*	270	*270*	224	*222*	291	*291*	154	*161*	398	*398*
	899Q	2	2nd	M3	315	*315*	177	*177*	238	*238*	270	*270*	224	*222*	291	*291*	154	*161*	398	*398*
	815Q	3	2nd	M5	315	*315*	165	*165*	238	*238*	270	*270*	222	*224*	279	*287*	156	*161*	398	*398*
Worker																				
	459sII	1	1st	M1	315	*355*	177	*171*	232	*244*	270	*270*	224	*230*	289	*267*	154	*184*	398	*386*
	564sII	1	2nd	M2	333	*341*	177	*173*	232	*244*	270	*270*			289	*267*	154	*154*	398	*386*
	481Q	2	1st	M1	315	*341*	177	*173*	238	*244*	270	*274*	224	*230*	287	*267*	154	*182*	398	*386*
	022Q	2	2nd	M1	315	*341*	165	*173*	238	*244*	270	*274*	224	*230*	287	*267*	154	*182*	398	*386*
24h	915Q	2	2nd	M1	315	*341*	165	*173*	238	*244*	270	*274*	224	*230*	291	*267*	154	*182*	398	*386*
	740Q	3	1st	M2	315	*355*	165	*171*	238	*244*	270	*270*	222	*230*	279	*267*	156	*182*	398	*386*
	029Q	3	2nd	M1	315	*341*	165	*171*	238	*244*	270	*270*	222	*230*	279	*267*	154	*184*	398	*386*
	023sII	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	161	*161*	386	*398*
	233sII	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	161	*161*	386	*398*
	319sII	4	2nd	M1	355	*335*	173	*169*	244	*238*	270	*274*			267	*279*	153	*153*	386	*398*
	274sII	1	1st	M1	315	*355*	169	*171*	232	*244*	270	*270*	222	*230*	289	*267*	163	*184*	398	*386*
	669Q	2	1st	M1	315	*341*	177	*173*	238	*244*	270	*274*	224	*230*	291	*267*	154	*182*	398	*386*
	642Q	2	2nd	M1	315	*341*	165	*173*	238	*244*	270	*274*	224	*230*	287	*267*	154	*182*	398	*386*
6d	053Q	3	1st	M2	315	*355*	165	*171*	238	*244*	270	*270*	222	*230*	279	*267*	156	*182*	398	*386*
	176Q	3	2nd	M1	315	*341*	165	*171*	238	*244*	270	*270*	222	*230*	279	*267*	156	*184*	398	*386*
	066sII	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	154	*154*	386	*398*
	258sII	4	2nd	M3	355	*335*							230	*222*	267	*279*			386	*398*
	910sII	4	2nd	M1	355	*335*	173	*169*	244	*238*	270	*274*	230	*226*	267	*279*	153	*153*	386	*398*
	194sII	1	1st	M1	315	*355*					270	*270*	222	*230*	289	*267*	163	*184*		
	960sII	1	2nd	M2	315	*341*	169	*173*	241	*244*									398	*386*
	627Q	2	1st	M1	315	*341*	165	*173*	238	*244*	270	*274*			291	*267*	154	*182*	398	*386*
9d	321Q	2	2nd	M1	315	*341*	177	*173*	238	*244*	270	*274*	224	*230*	291	*267*	154	*182*	398	*386*
	840Q	3	1st	M2	315	*355*	165	*171*	238	*244*	270	*270*			279	*267*	156	*182*	398	*386*
	1065Q	3	2nd	M1	315	*341*	165	*171*	238	*244*	270	*270*	222	*230*	287	*267*	156	*184*	398	*386*
	323sII	4	2nd	M1	355	*335*							230	*226*	267	*279*			386	*398*

	799sll	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	161	*161*	386	*398*
	822sll	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	161	*161*	386	*398*
	284sll	1	1st	M1	333	*355*							224	*230*	289	*267*			398	*386*
	247sll	1	2nd	M2	333	*341*							222	*230*	289	*267*			398	*386*
	716Q	2	1st	M1	315	*341*	177	*173*	238	*244*	270	*274*	224	*230*	287	*267*	154	*182*	398	*386*
	1023Q	2	2nd	M1	315	*341*	165	*173*	238	*244*	270	*274*	224	*230*	291	*267*	154	*182*	398	*386*
L1	709Q	3	1st	M2	315	*355*	165	*171*	238	*244*	270	*270*			279	*267*	156	*182*	398	*386*
	703Q	3	2nd	M1	315	*341*	165	*171*	238	*244*	270	*270*			287	*267*	156	*184*	398	*386*
	296sll	4	2nd	M3	355	*335*							230	*222*	267	*279*			386	*398*
	329sll	4	2nd	M1	355	*335*							230	*226*	267	*279*			386	*398*
	708sll	4	2nd	M3	355	*335*							230	*222*	267	*279*			386	*398*
	584sll	1	1st	M1	315	*355*	177	*171*	241	*244*	270	*270*	222	*230*	289	*267*	154	*184*	398	*386*
	196sll	1	2nd	M2	333	*341*							224	*230*	289	*267*			398	*386*
	771Q	2	1st	M1	315	*341*	165	*173*	238	*244*	270	*274*	224	*230*	291	*267*	154	*182*	398	*386*
	770Q	2	2nd	M1	315	*341*	165	*173*	238	*244*	270	*274*	224	*230*	287	*267*	154	*182*	398	*386*
L2	392Q	3	1st	M2	315	*355*	165	*171*	238	*244*	270	*270*	224	*230*	279	*267*	156	*182*	398	*386*
	812Q	3	2nd	M1	315	*341*	165	*171*	238	*244*	270	*270*	222	*230*	287	*267*	154	*184*	398	*386*
	560sll	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	162	*162*	386	*398*
	676sll	4	2nd	M1	355	*335*							230	*226*	267	*279*			386	*398*
	986sll	4	2nd	M3	355	*335*	173	*169*	244	*238*	270	*274*	230	*222*	267	*279*	161	*161*	386	*398*
Interlineage gyne sample																				
6d	661sll	1	2nd	M1	315	*333*					270	*270*	224	*230*	289	*267*			398	*386*

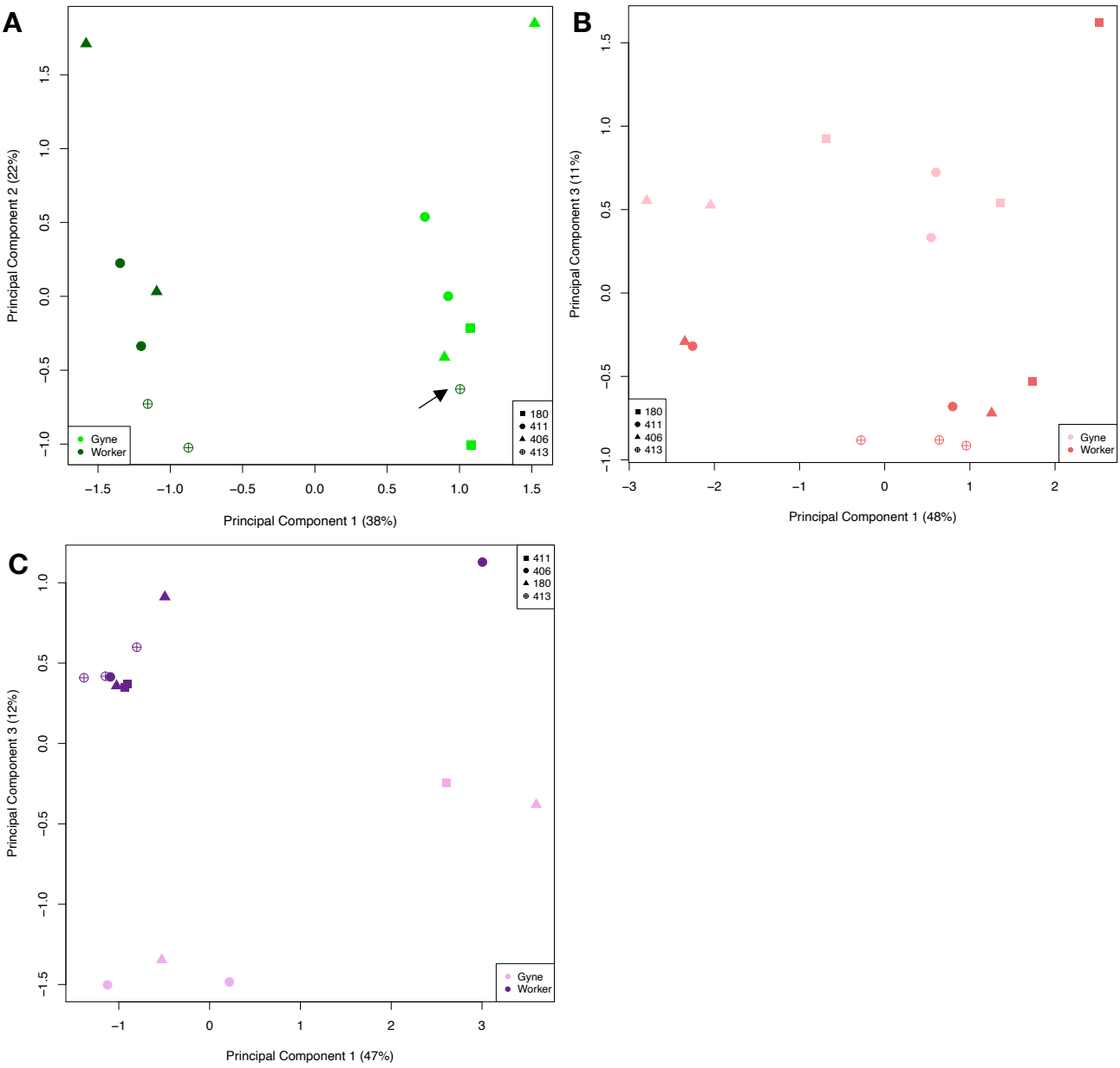


Figure S2. Principal component plots. (**A**) First two principal components (PC1 and PC2) explaining transcriptomic variation across samples for the developmental stage 6d with the inter-lineage gyne sample from J1 colony (arrow). First and third principal components (PC1 and PC3) for developmental stages 9d (**B**) and L2 (**C**) showing a clear separation by caste, which was not the case with PC1 and PC2. Colors show the caste (light color for gynes and dark color for workers). Filled symbols refer to the three J2-lineage colonies and open symbol refer to the single J1-lineage colony.

Table S8. Exact test of multi set intersections of gyne- and worker-biased genes between developmental stages.

Intersections	Degree	Observed Overlap	Expected Overlap	FE	P-value
Workers					
L2	1	284	NA	NA	NA
L1	1	211	NA	NA	NA
L1 & L2	2	68	4,98	13,67	7.31e-60
9d	1	212	NA	NA	NA
9d & L2	2	111	5,00	22,21	3.39e-130
9d & L1	2	55	3,71	14,81	5.07e-50
9d & L1 & L2	3	37	0,09	422,55	6.03e-87
6d	1	343	NA	NA	NA
6d & L2	2	105	8,09	12,98	3.61e-92
6d & L1	2	60	6,01	9,99	7.81e-44
6d & L1 & L2	3	35	0,14	247,05	1.81e-73
6d & 9d	2	135	6,04	22,36	6.96e-164
6d & 9d & L2	3	91	0,14	639,30	2.51e-240
6d & 9d & L1	3	44	0,11	416,06	1.79e-103
6d & 9d & L1 & L2	4	34	2.49e-03	13635,42	1.04e-131
24h	1	42	NA	NA	NA
24h & L2	2	11	0,99	11,11	2.31e-09
24h & L1	2	9	0,74	12,23	3.54e-08
24h & L1 & L2	3	5	0,02	288,23	9.31e-12
24h & 9d	2	12	0,74	16,23	4.50e-12
24h & 9d & L2	3	10	0,02	573,73	1.53e-25
24h & 9d & L1	3	6	0,01	463,34	3.88e-15
24h & 9d & L1 & L2	4	5	3.05e-04	16375,87	1.52e-20
24h & 6d	2	15	1,20	12,54	2.38e-13
24h & 6d & L2	3	10	0,03	354,61	2.02e-23
24h & 6d & L1	3	7	0,02	334,11	1.74e-16
24h & 6d & L1 & L2	4	5	4.94e-04	10121,53	1.71e-19
24h & 6d & 9d	3	9	0,02	427,54	6.69e-22
24h & 6d & 9d & L2	4	9	4.96e-04	18132,82	1.35e-36
24h & 6d & 9d & L1	4	5	3.69e-04	13559,03	3.93e-20
24h & 6d & 9d & L1 & L2	5	5	8.70e-06	575065,3	2.77e-28
Gynes					
L2	1	108	NA	NA	NA
L1	1	41	NA	NA	NA
L1 & L2	2	7	0.37	44305,00	6.70e-08
9d	1	31	NA	NA	NA
9d & L2	2	11	0.28	39.57	1.30e-15
9d & L1	2	5	0.111	47.38	5.65e-08
9d & L1 & L2	3	2	9.46e-04	2113.85	4.19e-07
6d	1	73	NA	NA	NA
6d & L2	2	4	0.65	44506,00	4.14e-03
6d & L1	2	5	0.25	44550,00	4.50e-06
6d & L1 & L2	3	1	0.002	448.83	2.23e-03
6d & 9d	2	3	0.19	15.97	8.50e-04
6d & 9d & L2	3	2	0.002	1187.23	1.34e-06
6d & 9d & L1	3	2	6.40e-04	3127.34	1.90e-07
6d & 9d & L1 & L2	4	1	5.73e-06	174392.70	5.73e-06

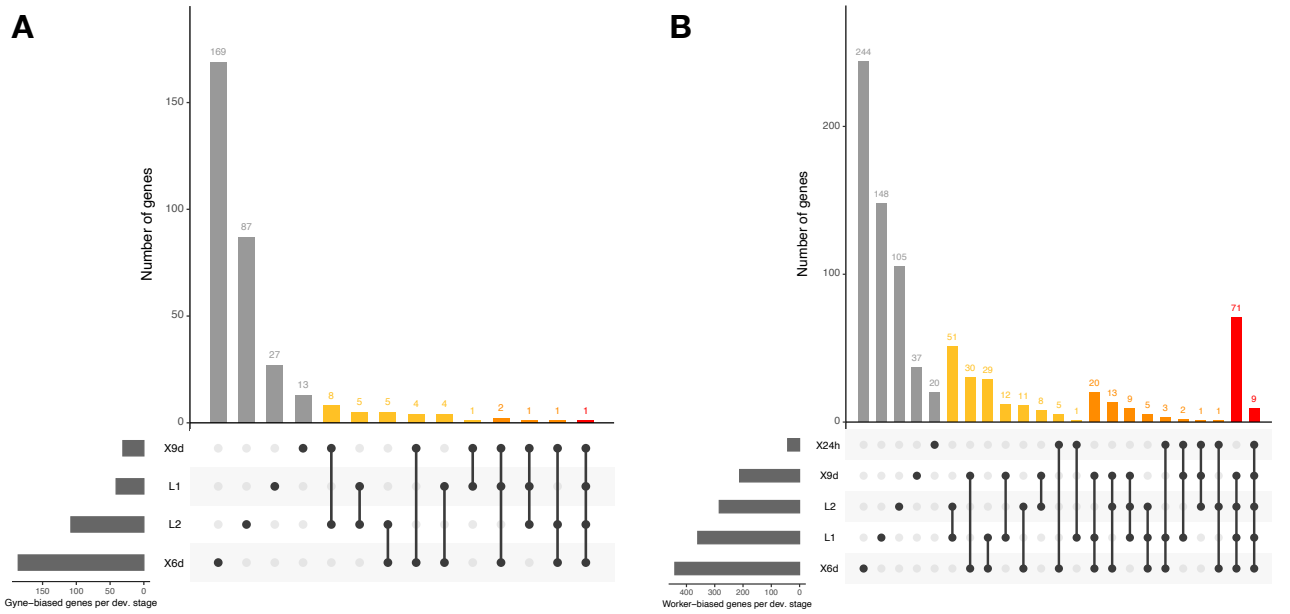


Figure S3. Number of caste-biased genes shared between developmental stages, with the inter-lineage gyne sample from J1 colony. The UpSetR plots show the number of (**A**) gyne-biased and (**B**) worker-biased genes expressed in a single developmental stage (in grey) or shared between two (yellow), three (orange) and four or five (red) developmental stages.

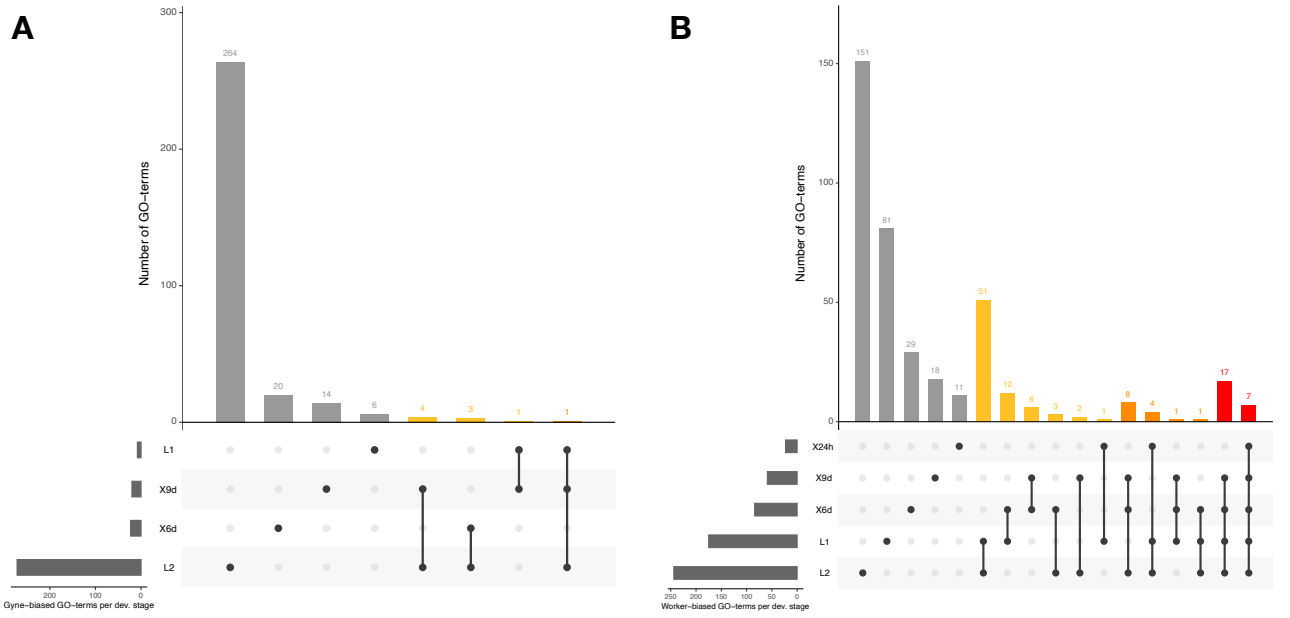


Figure S4. Number of GO-terms shared between developmental stages, with the inter-lineage gyne sample from J1 colony. The UpSetR plots show the number of (A) gyne-biased and (B) worker-biased GO-terms found in a single developmental stage (in grey) or shared between two (yellow), three (orange) and four or five (red) developmental stages.

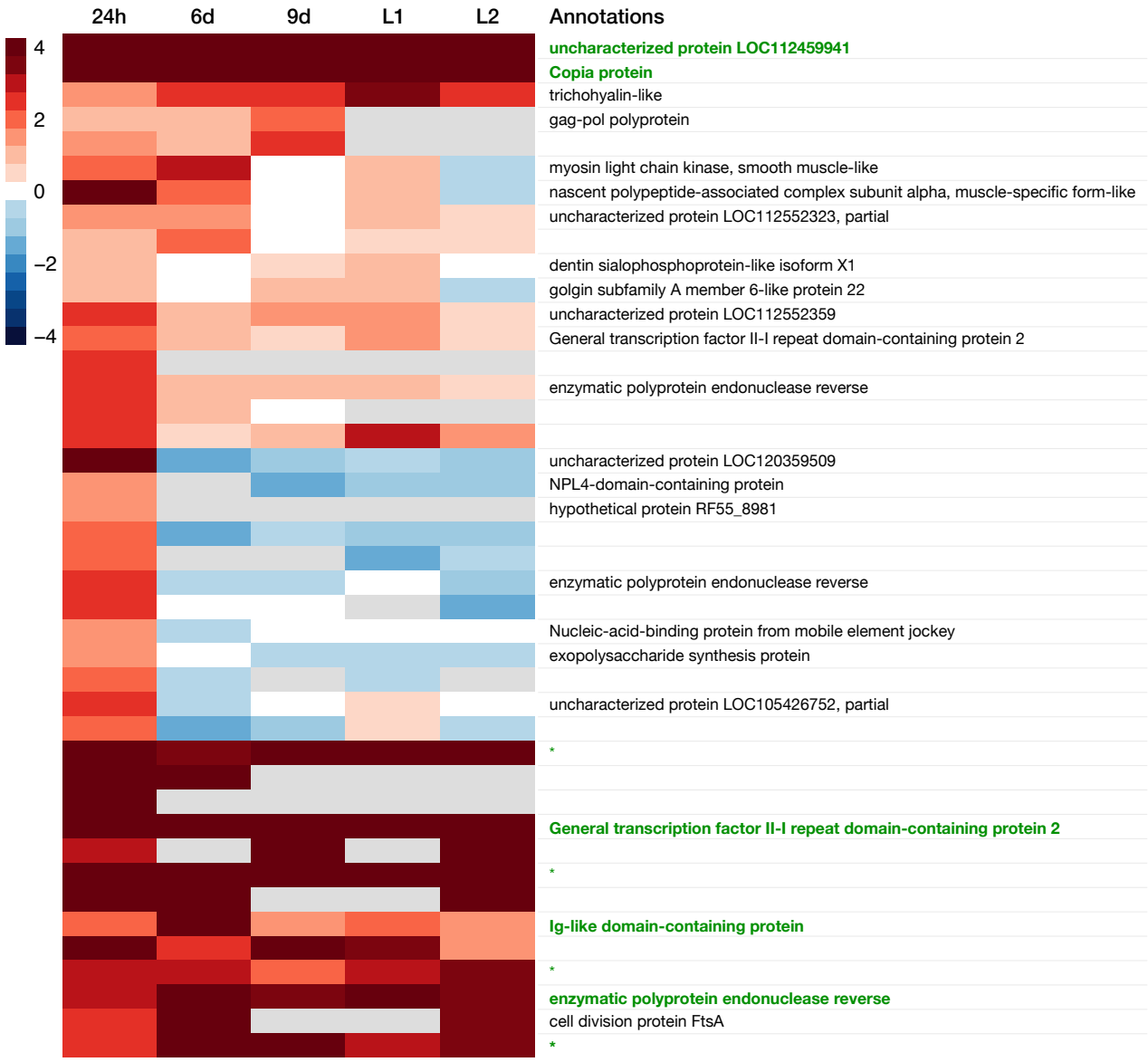


Figure S5. Caste-biased genes shared across development, with the inter-lineage gyne sample from J1 colony. The heatmap shows the 42 caste-biased genes (all worker-biased) at 24h stage with their annotations and their expression in the five developmental stages. Genes in red are worker-biased, blue are gyne-biased. Saturation of the colors increases with the log2FC. Genes without a label have no annotation, annotation in green is for genes that were caste-biased in all three stages (all worker-biased). The tree shows the relation between genes according to Euclidian distance.

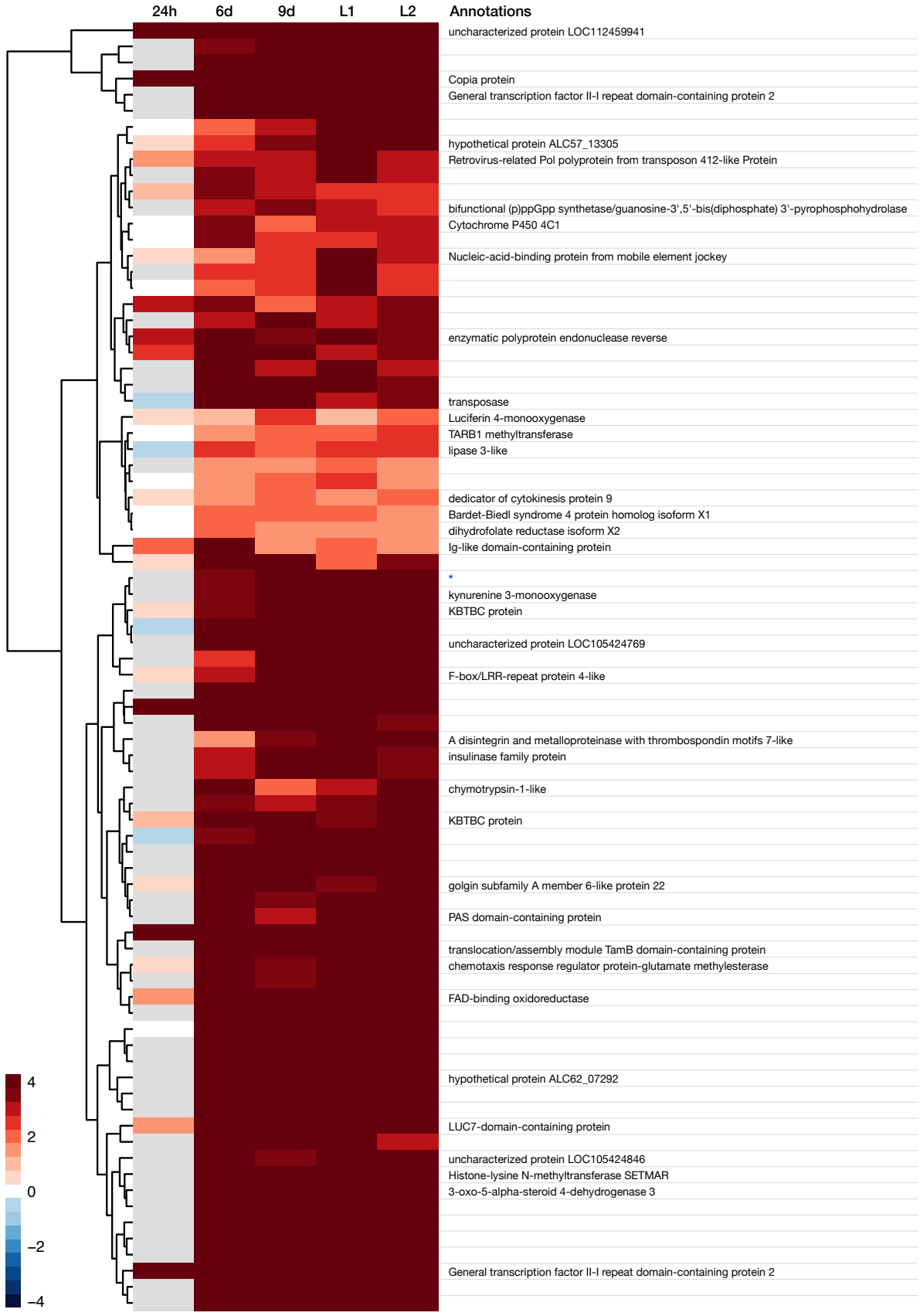


Figure S6. Caste-biased genes shared across development from 24h or 6d developmental stages, without the inter-lineage gyne sample from J1 colony. The heatmap shows the 80 genes caste-biased (all worker-biased) in all five or four developmental stages (from 24h or 6d, respectively). Genes in red are worker-biased, blue are gyne-biased. Saturation of the colors increases with the log2FC. Genes without a label have no annotation, annotation in blue is

for gene that was not found in the analysis with the inter-lineage gyne sample. The tree shows the relation between genes according to Euclidian distance.

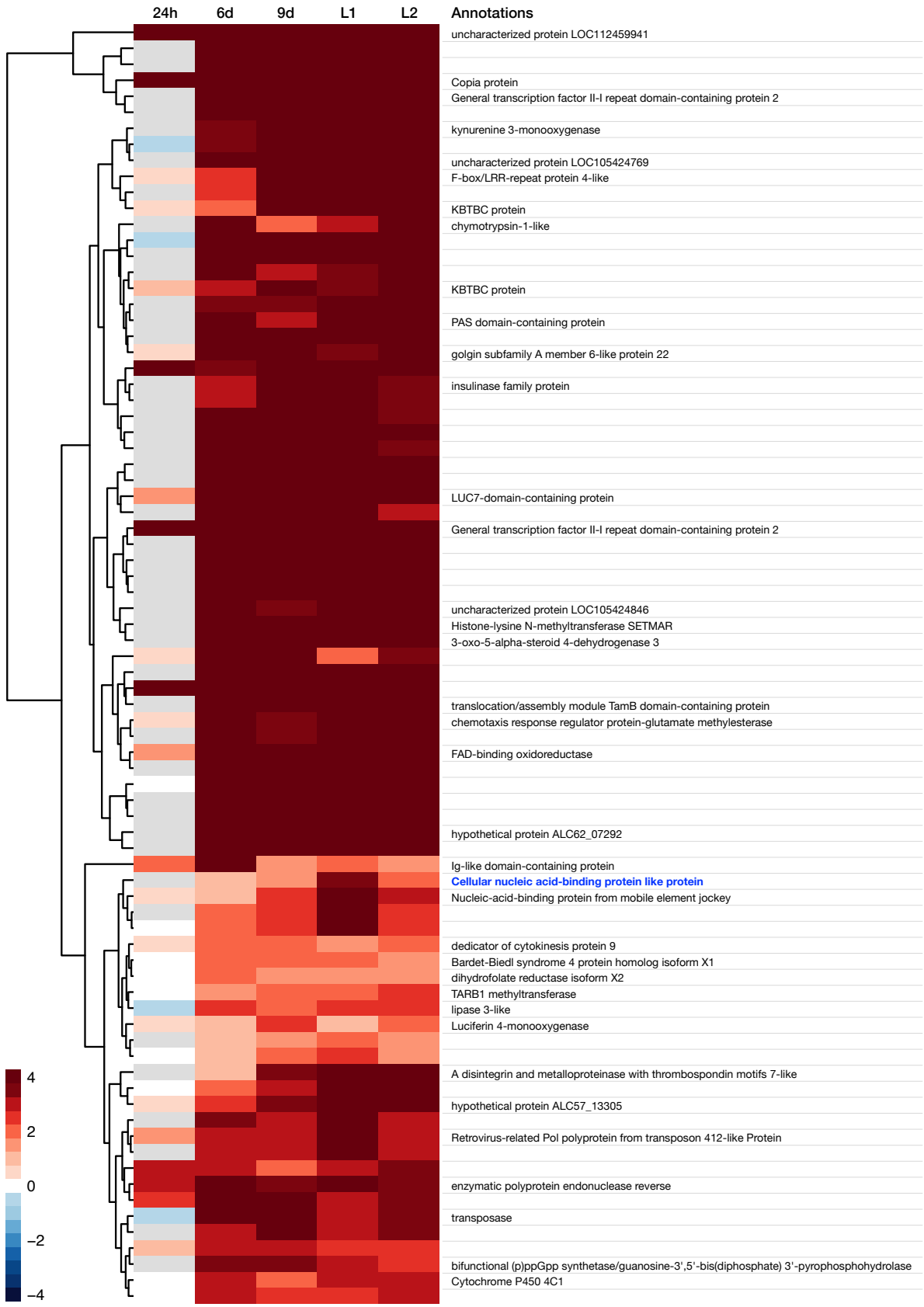
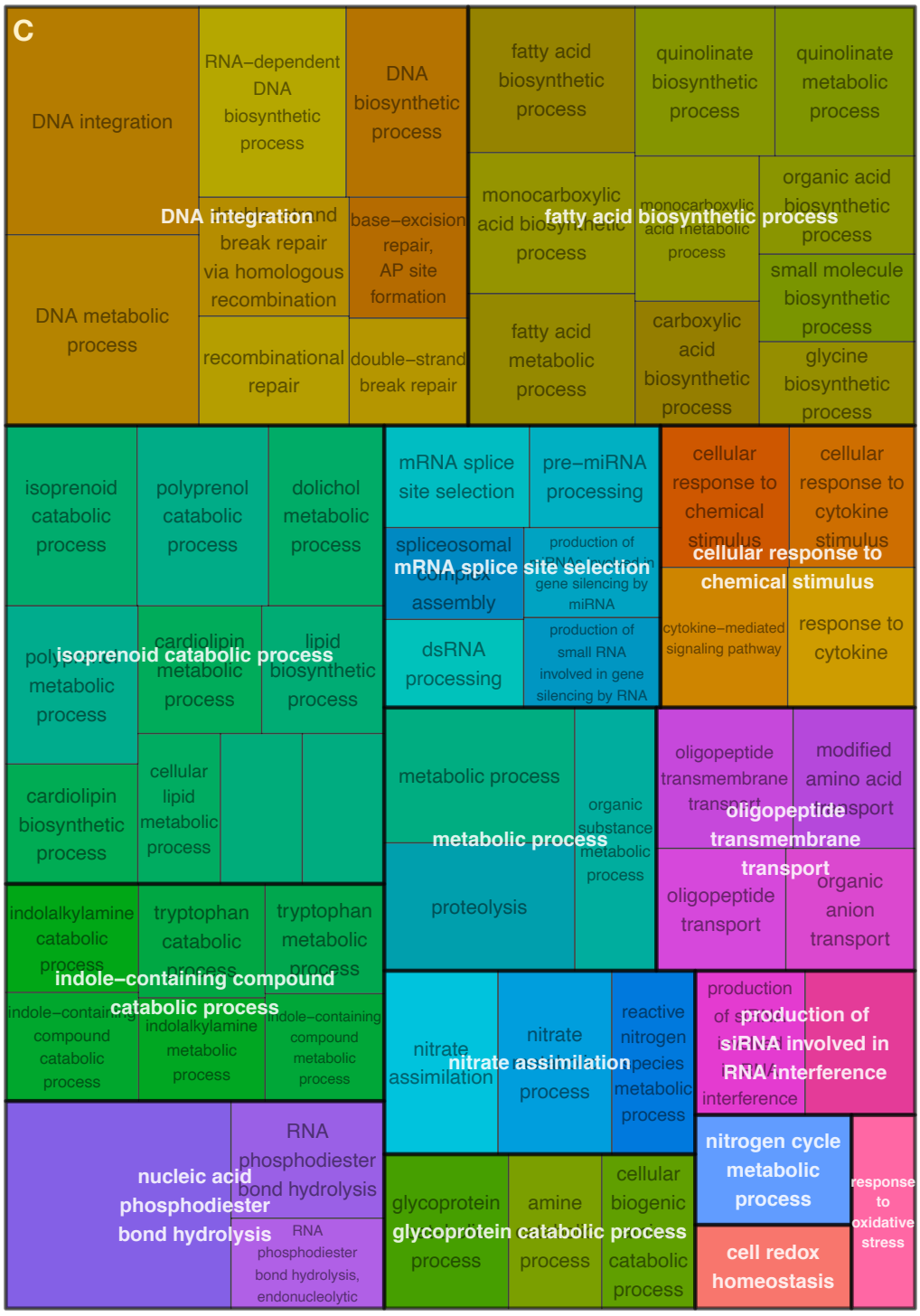


Figure S7. Caste-biased genes shared across development from 24h or 6d developmental stages, with the inter-lineage gyne sample from J1 colony. The heatmap shows the 80 genes caste-biased (all worker-biased) in all five or four developmental stages (from 24h or 6d, respectively). Genes in red are worker-biased, blue are gyne-biased. Saturation of the colors increases with the $\log_2$FC. Genes without a label have no annotation, annotation in blue is

for gene that was not found in the analysis without the inter-lineage gyne sample. The tree shows the relation between genes according to Euclidian distance.

Figure S8. TreeMap view (obtained with "rrvgo" R package) of GO Biological process enriched terms generated from caste-biased genes at 24h (**A**), 6d (**B**), 9d (**C**), L1 (**D**) and L2 (**E**). Similar colors denote semantic similarity and the size of the rectangles reflects the p-value of enrichment. Not all the terms are reported due to space constraints (see Table S1 on additional file for details).


B
RNA–dependent DNA biosynthetic process
DNA integration
RNA–dependent DNA biosynthetic process
DNA biosynthetic process
DNA metabolic process
double–strand break repair
cobalamin transport
carbohydrate transmembrane transport
icosanoid secretion
icosanoid transport
fatty acid transport
oligopeptide transmembrane transport
arachidonate transport
cobalamin transport
long–chain fatty acid transport
proton transmembrane transport
modified amino acid transport
arachidonic acid secretion
oligopeptide transport
monocarboxylic acid transport
isoprenoid catabolic process
dolichol metabolic process
cellular lipid catabolic process
sphingomyelin catabolic process
sphingomyelin metabolic process
cardiolipin biosynthetic process
polyprenol catabolic process
polyprenol catabolic process
polyprenol metabolic process
cardiolipin metabolic process
sphingolipid metabolic process
lipid catabolic process
GMP biosynthetic process
D–gluconate metabolic process
quinolinate metabolic process
GMP metabolic process
GMP biosynthetic process
aldonic acid metabolic process
quinolinate biosynthetic process
guanosine–containing compound biosynthetic process
purine nucleoside biosynthetic process
purine ribonucleoside biosynthetic process
mating type switching
regulation of mating type switching
mating type switching
reproduction of a single–celled organism
reproductive process in single–celled organism
proteolysis
proteolysis
metabolic process
nitrate assimilation
nitrate metabolic process
nitrate assimilation
reactive nitrogen species metabolic process
N–terminal protein amino acid methylation
positive regulation of peptidyl–lysine acetylation
N–terminal protein amino acid modification
regulation of histone acetylation
N–terminal protein amino acid methylation
positive regulation of histone acetylation
positive regulation of protein acetylation
regulation of peptidyl–lysine acetylation
positive regulation of histone modification
nucleic acid phosphodiester bond hydrolysis
nucleic acid metabolic process
cellular biogenic amine metabolic process
cellular biogenic amine metabolic process
cellular amine metabolic process
mating type determination
regulation of cell fate commitment
cell fate commitment
N–glycan fucosylation
protein in Golgi
nitrogen cycle metabolic process
acid secretion


C
DNA integration
RNA–dependent DNA biosynthetic process
DNA biosynthetic process
DNA integration
DNA metabolic process
double–strand break repair via homologous recombination
base–excision repair, AP site formation
recombinational repair
double–strand break repair
fatty acid biosynthetic process
quinolinate biosynthetic process
quinolinate metabolic process
monocarboxylic acid biosynthetic process
monocarboxylic acid metabolic process
organic acid biosynthetic process
small molecule biosynthetic process
fatty acid metabolic process
carboxylic acid biosynthetic process
glycine biosynthetic process
isoprenoid catabolic process
polyprenol catabolic process
dolichol metabolic process
polyprenol metabolic process
cardiolipin metabolic process
lipid biosynthetic process
cardiolipin biosynthetic process
cellular lipid metabolic process
mRNA splice site selection
pre–miRNA processing
spliceosomal complex assembly
production of siRNA involved in gene silencing by miRNA
dsRNA processing
production of small RNA involved in gene silencing by RNA
cellular response to chemical stimulus
cellular response to cytokine stimulus
cytokine–mediated signaling pathway
response to cytokine
metabolic process
organic substance metabolic process
proteolysis
oligopeptide transmembrane transport
modified amino acid transport
oligopeptide transport
organic anion transport
indolalkylamine catabolic process
tryptophan catabolic process
tryptophan metabolic process
indole–containing compound catabolic process
indolalkylamine metabolic process
indole–containing compound metabolic process
nitrate assimilation
nitrate assimilation
nitrate process
reactive nitrogen species metabolic process
production of siRNA involved in RNA interference
RNA interference
nitrogen cycle metabolic process
response to oxidative stress
cell redox homeostasis
nucleic acid phosphodiester bond hydrolysis
RNA phosphodiester bond hydrolysis
RNA phosphodiester bond hydrolysis, endonucleolytic
glycoprotein catabolic process
glycoprotein process
amine process
cellular biogenic catabolic process


D
DNA integration
DNA metabolic process
DNA integration
RNA–dependent DNA biosynthetic process
DNA biosynthetic process
DNA recombination
somatic cell DNA recombination
proteolysis
metabolic process
proteolysis
organic substance metabolic process
macromolecule metabolic process
nitrogen compound metabolic process
primary metabolic process
organic cyclic compound metabolic process
organonitrogen compound metabolic process
steroid biosynthetic process
lipid metabolic process
steroid metabolic process
isoprenoid catabolic process
polyprenol metabolic process
cellular lipid biosynthetic process
steroid biosynthetic process
polyprenol catabolic process
farnesyl diphosphate biosynthetic process
farnesyl diphosphate metabolic process
dolichol metabolic process
farnesyl diphosphate biosynthetic process, mevalonate pathway
isoprenoid biosynthetic process via mevalonate
organic anion transport
oligopeptide transmembrane transport
organic acid transport
modified amino acid transport
arachidonic acid secretion
icosanoid transport
arachidonate transport
icosanoid secretion
oligopeptide transport
histidine metabolic process
aldonic acid metabolic process
histidine metabolic process
quinolinate metabolic process
quinolinate biosynthetic process
alpha–amino acid metabolic process
small molecule catabolic process
positive regulation of proteasomal ubiquitin–dependent protein catabolic process
small molecule catabolic process
dicarboxylic acid catabolic process
organic acid catabolic process
10–formyltetrahydrofolate catabolic process
10–formyltetrahydrofolate metabolic process
pteridine–containing compound catabolic process
pteridine–containing compound catabolic process
folic acid–containing compound metabolic process
anesthesia–resistant memory
olfactory learning
thermosensory behavior
thermosensory behavior
olfactory behavior
nucleic acid phosphodiester bond hydrolysis
heterocycle metabolic process
nucleic acid metabolic process
cellular aromatic compound metabolic process
regulation of bicoid mRNA localization
regulation of intracellular mRNA localization
regulation of oskar mRNA localization
pole plasm
RNA localization
bicoid mRNA localization
cellular response to ethanol
cellular response to xenobiotic stimulus
xenobiotic catabolic process
xenobiotic catabolic process
xenobiotic metabolic process
positive regulation of hippo signaling
negative regulation of smoothened signaling
positive regulation of smoothened signaling pathway
regulation of hippo signaling
negative regulation of DNA–templated transcription, initiation
pole plasm oskar mRNA localization
pole plasm
pole plasm mRNA localization
cytoplasm organization
pole plasm RNA localization
mating type switching
regulation of mating type switching
regulation of reproductive process
anterior/posterior pattern specification, imaginal disc morphogenesis
transposition, DNA–mediated
transposition, DNA–mediated transposition
circadian behavior
circadian sleep/wake cycle, sleep
regulation of circadian sleep/wake cycle
regulation of circadian sleep/wake cycle
regulation of circadian rhythm
circadian sleep/wake cycle
rhythmic behavior
negative regulation of nucleocytoplasmic transport
mRNA export from nucleus
negative regulation of nucleocytoplasmic transport
ribonucleoprotein complex export from nucleus
negative regulation of intracellular transport
sprouting of injured axon
neuron projection regeneration
sprouting of injured axon
axon regeneration
regeneration
regulation of actin cytoskeleton reorganization
oocyte microtubule cytoskeleton polarization
innate immune response
positive regulation of type I interferon production
DNA unwinding involved in DNA replication
benzene–containing compound metabolic process
myeloid leukocyte activation


E
segment polarity determination
periodic partitioning
blastoderm segmentation
embryonic pattern specification
embryo development
anterior region determination
zygotic determination of anterior/posterior axis, embryo
segmentation
pattern specification process
embryonic axis specification
tripartite regional subdivision
axis specification
anatomical structure development
developmental process
multicellular organism development
animal organ morphogenesis
wing disc development
instar larval or pupal development
open tracheal system development
respiratory system development
imaginal disc–derived wing morphogenesis
DNA integration
DNA metabolic process
DNA replication, Okazaki fragment processing
elongation involved in DNA replication
DNA biosynthetic process
lagging strand elongation
DNA strand elongation
torso signaling pathway
intracellular receptor signaling pathway
positive regulation of receptor signaling pathway via STAT
regulation of receptor signaling pathway via JAK–STAT
regulation of receptor signaling pathway via STAT
regulation of smoothened signaling pathway
negative regulation of signal transduction
regulation of smoothened signaling pathway
torso signaling pathway via STAT
receptor signaling pathway via JAK–STAT
negative regulation of smoothened signaling pathway
negative regulation of signaling
aminoglycan metabolic process
carbohydrate derivative catabolic process
aminoglycan catabolic process
peptidoglycan metabolic process
chitin metabolic process
glycosaminoglycan metabolic process
amino sugar catabolic process
carbohydrate derivative metabolic process
glycoprotein catabolic process
lipid metabolic process
isoprenoid catabolic process
polyprenol catabolic process
lipid catabolic process
dolichol metabolic process
steroid metabolic process
polyprenol metabolic process
cardiolipin metabolic process
mitotic spindle elongation
actin cytoskeleton reorganization
spindle elongation
mitotic spindle assembly
mitotic spindle organization
microtubule depolymerization
microtubule cytoskeleton organization involved in mitosis
spindle assembly
metabolic process
organic substance metabolic process
primary metabolic process
nitrogen compound metabolic process
organic cyclic compound metabolic process
positive regulation of hippo signaling
positive regulation of TORC1 signaling
regulation of TORC1 signaling
protein kinase A signaling
positive regulation of TOR signaling
circadian behavior
circadian sleep/wake cycle process
circadian sleep/wake cycle
circadian sleep/wake cycle, sleep
circadian rhythm
regulation of circadian sleep/wake cycle
regulation of circadian rhythm
compound eye pigmentation
regulation of compound eye pigmentation
regulation of developmental pigmentation
pigmentation
cell elongation involved in imaginal disc–derived wing morphogenesis
unidimensional cell growth
cell growth
cell growth involved in morphogenesis
quinolinate biosynthetic process
GMP metabolic process
dTDP biosynthetic process
dTDP metabolic process
quinolinate metabolic process
dTTP biosynthetic process
pyrimidine nucleoside diphosphate metabolic process
NAD biosynthetic process
dTTP metabolic process
quinolinate biosynthetic process
nucleoside diphosphate metabolic process
monophosphate biosynthetic process
nicotinamide nucleotide biosynthetic process
nucleoside diphosphate biosynthetic process
GMP biosynthetic process
pyrimidine nucleoside diphosphate biosynthetic process
small molecule biosynthetic process
organic acid biosynthetic process
negative regulation of cysteine–type endopeptidase activity
negative regulation of cysteine–type endopeptidase activity
negative regulation of cellular metabolic process
regulation of pole plasm oskar mRNA localization
regulation of intracellular mRNA localization
regulation of pole plasm oskar mRNA localization
bicoid mRNA localization
negative regulation of multi–organism process
tetrahydrofolate biosynthetic process
pteridine–containing compound biosynthetic process
regulation of syncytium formation by plasma membrane fusion
leg disc pattern formation
anterior/posterior pattern specification
imaginal disc
leg disc development
tissue development
viral genome replication
modulation by host of viral process
antifungal humoral response
defense response to fungus
peptidoglycan recognition protein signaling pathway
viral life cycle
negative regulation of viral genome replication
regulation of peptidoglycan recognition protein signaling pathway
regulation of viral process
biological process involved in interaction with symbiont
viral process
regulation of viral life cycle
modulation of viral process by host of symbiont process
viral gene expression
biological process involved in symbiotic interaction
viral transcription
response to fungus
negative regulation of viral process
virus maturation
antimicrobial humoral immune response mediated by antimicrobial peptide
organ or tissue specific immune response
negative regulation of immune system process
negative regulation of response to biotic stimulus
negative regulation of response to biotic stimulus
regulation of response to biotic stimulus
negative regulation of response to external stimulus
regulation of response to external stimulus
negative regulation of defense response to bacterium
negative regulation of defense response
imaginal disc–derived wing margin morphogenesis
pole plasm oskar mRNA localization
cytoplasm organization
cell wall macromolecule metabolic process
anatomical structure maturation
sprouting of injured axon
neuron projection regeneration
regulation of ecdysteroid metabolic process
mitochondrial protein catabolic process
oligopeptide transmembrane transport
activation of GTPase activity
long–term memory
olfactory learning
cognition
long–term memory
memory
organonitrogen compound catabolic process
cellular amide catabolic process
organic substance catabolic process
response to oxygen–containing compound
response to alcohol
negative regulation of response to cytokine stimulus
response to lipid
chemosensory behavior
olfactory behavior
cellular response to ethanol
response to chemical
response to molecule of origin
response to insecticide
regulation of response to cytokine stimulus
cellular response to cytokine stimulus
cytokine–mediated signaling pathway
response to cytokine
nucleic acid phosphodiester bond hydrolysis
nucleic acid phosphodiester bond hydrolysis
regulation of transcription by RNA polymerase II
transcription by RNA polymerase II
positive regulation of transcription, DNA–templated
meiotic sister chromatid segregation
meiosis II
meiotic chromosome segregation
proteolysis
proteolysis
organonitrogen compound metabolic process
ecdysis, chitin–based cuticle
molting cycle
chitin–based cuticle
regulation of cell development
nitrate assimilation
reactive nitrogen species metabolic process
cellular biogenic amine metabolic process
regulation of mitotic sister chromatid segregation
thermosensory behavior
regulation of behavior
negative regulation of cytokine production
cellular component macromolecule biosynthetic process
brain development
nitrogen cycle metabolic process
cell–cell fusion
cellular response to molecule of bacterial origin
drug metabolic process

- GENERAL DISCUSSION -

GENERAL DISCUSSION

Understanding the molecular basis of phenotypic plasticity represents a fundamental challenge in evolutionary biology. In this thesis, my main objective was to contribute to answering the unresolved question “What is the molecular basis of caste determination in ants?” and more specifically “Which genes are involved in queen and worker caste differentiation in ants during the early developmental stage?”. To investigate these questions, I worked with *Pogonomyrmex rugosus* ECD (environmental caste determination) in which I identify new factors potentially influencing caste fate, and with *Pogonomyrmex* J lineages GCD (genetic caste determination) where I looked for caste-biased gene expression patterns in early developmental stages.

In this general discussion, I outline the main findings of my thesis and place them in the context of the current collective knowledge of caste determination in ants. I explain how these results contribute to a broader understanding of caste determination in ants and I provide details for future studies that could be conducted on *Pogonomyrmex* to continue to broaden our knowledge and understanding of the proximate and ultimate mechanisms of caste determination.

Main conclusions

Maternal effect on worker size

The aim of this first chapter was to confirm the role of previously identified miRNAs in worker size. For this, synthetic miRNAs were designed to recreate the internal environment of eggs laid by founding queens, by altering the molecular composition of eggs laid by established queens. Using microinjection, I manipulated miRNA levels in reproductive eggs and predicted I would obtain workers with a smaller size than workers from the control, as synthetic miRNAs should have influenced the embryo’s development towards the nanitic phenotype. However, the experiment did not generate any quantifiable differences in worker size for any of the four targeted miRNAs and I was unable to confirm whether or not these miRNAs had an effect on worker size.

There are four potential explanations to explain this negative result. First, at the time of Paolucci *et al.*’s experiment, where they found the differences in miRNA level between eggs laid by founding and established queens, they were not aware of the presence of trophic eggs among eggs laid by *P. rugosus* queens. Therefore, the miRNAs suspected to be involved in worker size might have been falsely identified due to the presence of trophic eggs in the samples. For example, if the percentage of trophic eggs laid differ between founding and established queens, this could have affected the miRNA concentrations measured (*See* chapter 1 for details). This is likely to be the case, as *P. rugosus* is a species that does not perform trophallaxis (pers. obs, Smith *et al.* 2007) and uses a fully claustral colony-founding strategy. As such, new queens must rely on stored fat and muscle reserves to feed the first developing brood (Hölldobler and Wilson 1990; Hahn *et al.* 2004), likely through the synthesis of nutrient rich trophic eggs (as supported by our study in chapter 3). If this assumption is correct, we would predict an even higher proportion of trophic eggs at the colony founding stage

(compared to the established stage in which we found an average of 57% of trophic eggs) to be able to feed developing brood, as no other food sources are available. It has also been demonstrated, in other ant species, that queens lay trophic eggs during the founding stage (Cassill 2002; Volny *et al.* 2006), which further validates this explanation. If correct, such a high percentage of trophic eggs in the sample (from Paolucci *et al.*) would have diluted the miRNAs present in reproductive eggs. Of course, this explanation would only be valid if reproductive and trophic eggs do not contain the same type and amount of miRNA, which is what is suggested by our results of Chapter 3 (regarding small RNA concentration in trophic and reproductive egg). In order to shed light on this hypothesis, it is necessary to determine whether *P. rugosus* queens lay trophic eggs at the founding stage and if yes, in which proportion. If the proportion of trophic eggs varies between founding and established queens, then the experiment of Paolucci *et al.* should be redone with samples containing only reproductive eggs. The results should then be compared to the first experiment of Paolucci *et al.* to determine whether previously identified miRNAs used for the experiment in chapter 1 remain the same or not. If the two results (from Paolucci *et al*, and from the additional sampling without trophic eggs) are similar, it means that the fact that I was not able to detect a difference in worker size does not stem from miRNA misidentification. Otherwise, the entire manipulation experiment should be repeated with new synthetic miRNAs.

Second, one synthetic miRNA alone, and the combination of the four of them, may not have been enough to sufficiently influence worker size to be able to detect a difference, or their concentration was not appropriate to mimic or inhibit endogenous miRNAs, and therefore did not affect the phenotype as expected. An inappropriate concentration explanation is rather unlikely as we found an influence of one synthetic miRNA (miRug-d1) on gyne development, which support the fact that the concentration used were appropriate to generate a response. However it remains possible that, as miRNAs are not all present in the egg in the same concentration (Paolucci *et al.*), we could have injected the other miRNAs at inadequate concentrations. For the injection of the four miRNAs together, we cannot exclude an interaction between them that could have prevented miRug-d1 to produce the same response than when it was injected alone.

Thirdly, the combinations of several maternal factors, in addition to the miRNAs, might be necessary to create the nanitic phenotype, which could explain why we did not influence worker size by only injecting synthetic miRNA(s). For instance, in GCD *Pogonomyrmex* J lineages, the level of juvenile hormone in queens influenced the adult body mass of worker offspring as well as the concentration of ecdysteroids in eggs which was found to be negatively correlated to worker body size (Helms Cahan *et al.* 2011). The authors suggested that queens could bias worker size by varying ecdysteroid deposition in their eggs. Therefore the modification of miRNA level alone, without the influence of other maternal factors, might not have been enough to influence worker size.

Finally, assuming that the finding of Paolucci *et al.* is not biased by trophic egg percentage, the identified miRNAs may simply not be involved in worker size. Although the main observable difference between workers developing in founding or established colonies is their size (with the former being much smaller), it is extremely likely that other differences exist during embryonic development between the two colony life cycle stages. As in our study we only measured head width and tibia length, it is possible that the targeted miRNAs would have other morphological or physiological effects, that we did not detect. For example, in termites in addition to size,

Matsuura and Kobayashi (2010) found that the number, distribution and symmetry of setae of nanitic soldiers differed between nanitic soldiers and mature soldiers in two termite species. It is therefore possible that the observed differences in miRNA generate other morphological, physiological and/or behavioural differences between nanitics and workers.

MiRNA manipulation triggers gyne development

Although in Chapter 1, I was not able to show that the targeted miRNAs are involved in worker size, the microinjection technique I have developed remains valid and is an extremely useful method for future projects requiring manipulation of egg content. Using this technique, eggs can be injected with liquids without damaging or killing the developing embryo. Indeed, in chapter 2, I showed that the microinjection of inhibitor miRug-d1 influenced the development of embryos towards the gyne developmental pathway, which is evidence in itself for the viability of this method. This result, coupled with recent studies showing that miRNAs are involved in physiology and development in insects, and in caste determination in social insects (Guo *et al.* 2013; Lucas *et al.* 2015; Duan *et al.* 2021; Vieira *et al.* 2021; Zafar *et al.* 2021) makes miRNAs interesting and promising molecules to further investigate. The combination of the microinjection technique and generation of synthetic miRNAs to manipulate egg content can contribute to a better understanding of miRNAs function and implications in caste determination, and will broaden our understanding of developmental plasticity. For instance, as our results on trophic egg content (chapter 3) found that they contain miRNAs, we could use the microinjection technique to manipulate miRNA levels in trophic eggs and, by feeding the latter to first instar larvae, try to influence their caste fate (see next section).

Effect of trophic eggs on larval caste fate

It was during the projects of Chapter 1 and 2 that I discovered trophic eggs in *P. rugosus*. After the experimental injections, I was keeping the eggs in petri dishes, separated from the colonies, until larval hatching. I observed embryonic development every day and observed that some eggs failed to develop and dried out after a few days. Using morphological descriptions from other species in the literature, I was able to confirm that these eggs were trophic eggs.

While investigating queen and worker egg laying behaviour in *P. rugosus*, we found that workers start laying eggs approximately three weeks after the removal of the queen from the colony, similarly to what was observed in *P. badius* where workers start laying eggs 28 days after queen's removal or queen death (Smith *et al.* 2007). Smith and Tschinkel (2006) recorded the first and, to date, only *Pogonomyrmex* species, *P. badius*, in which workers lay reproductive eggs that develop into males; in this thesis we report a second species, *P. rugosus*, where workers exhibit the same behaviour. In addition, we found that *P. rugosus* workers do not lay trophic eggs, which disproves the hypothesis of Smith *et al.* (2007) that all *Pogonomyrmex* workers are expected to have some degree of ovary development for trophic egg laying. Smith's hypothesis was a reiteration of Crespi

(1977), stating that trophic eggs could provide an advantage for workers by keeping their ovaries active, which would be beneficial to rapidly switch to reproductive egg laying after the queen's death. We can therefore rule this out in *P. rugosus*, as workers do not lay trophic eggs but are able to lay reproductive eggs in queenless colonies.

After having characterized trophic eggs in *P. rugosus*, I performed the experiment described in Chapter 3 which found that trophic eggs likely have another role in ants other than simply providing a food source for larvae. Our result suggests that trophic eggs influence larval development in the direction of the worker caste, as in the absence of trophic eggs, larvae mostly develop into gynes. In the entirety of the *Pogonomyrmex* genus, trophic eggs have only previously been recorded in *P. barbatus* (without mention of which caste lays them, V.P. Volny, personal observation) and in GCD *Pogonomyrmex* J and H lineages where trophic eggs are also laid only by queens (E. Genzoni, personal observation; Helms Cahan *et al.* 2011). As stated in chapter 3, there is an overall lack of knowledge about trophic eggs in ants, and therefore characterizing trophic eggs in several *Pogonomyrmex* species would be necessary to determine whether the use of trophic eggs to influence the larval caste fate is an evolutionary novelty in *P. rugosus* or if this trait is shared across *Pogonomyrmex* genus. Additional studies are necessary to determine whether trophic eggs provide a greater role (other than a food source) in the eusocial Hymenoptera in general. A promising avenue for further research would be a comparative approach (see Sommer 2020); collecting data on trophic eggs across a range of species and using phylogenetic models to determine whether there are any shared evolutionary patterns between species that utilize trophic eggs.

The result of chapter 3 highlights trophic eggs as a promising (and potentially overlooked) mechanism involved in caste determination. Previous studies have already identified ecdysteroids and juvenile hormone in ant eggs and suggested that they are involved in caste determination (Schwander *et al.* 2008; Helms Cahan *et al.* 2011; Libbrecht *et al.* 2013) and so we have taken the approach to search for these molecules in trophic eggs. Ongoing quantification of juvenile hormone and ecdysteroids might subsequently reveal further candidate molecules within trophic eggs that could influence the larval caste fate.

Caste differentiation in *Pogonomyrmex* J lineages

To date, most of the studies concerning caste determination in eusocial insects, including ants, consider only larvae, pupae or the adult stage, as in these developmental stages, the caste fate of the individuals can be attributed based on the morphology. The earliest developmental stage (i.e. egg stage) has never been studied until now.

The study presented in this thesis is the first one to show caste-biased gene expression pattern during the early developmental stage (from 24 hours old embryo), before visible morphological differences exist between gynes and workers. This was made possible with the acquisition of an ant with a genetic caste determination, *Pogonomyrmex* J lineages. The genetic difference between pure lineage gynes and inter-lineage workers allowed me to accurately determine the caste of each individual via genotyping at any developmental stage.

The results showed that 24 hours after the egg is laid, there are already genes that are differentially expressed between gyne- and worker-destined embryos. At six days, we observed a peak of caste-biased genes, suggesting that important caste-specific developments occur at this stage. Additional sampling between 24 hours and 6 days would provide higher resolution longitudinal data on how caste-biased gene expression changes over time. This would provide a means to confirm whether or not the peak at 6 days old reflects an important developmental stage in caste differentiation, which would be a working hypothesis if no prior temporal stages of development express higher peaks of caste-biased gene expression.

Some unanswered questions and perspectives

This thesis has improved our understanding of several aspects of caste determination in *Pogonomyrmex* ants. However, there are still a number of gaps in our knowledge of *Pogonomyrmex* biology and evolution, that could, if they were filled, allow us to have a better understanding of the factors involved in and triggering caste determination, as well as the genetic basis of caste determination.

Trophic eggs, a new potential factor contributing to caste determination

- I initiated the quantification (data not shown in this thesis) of juvenile hormone and ecdysteroids in trophic and reproductive eggs, aiming at identifying which molecules could influence the larval caste fate towards worker developmental pathway. While preliminary results seemed to indicate the presence of juvenile hormone in both egg types, and most probably at higher concentration in reproductive eggs, a precise quantification in both egg types with replicates is necessary to confirm this trend and put this in light with the most recent studies showing the role and importance of juvenile hormone and ecdysteroid in development, and more precisely in caste determination in social insects (Libbrecht *et al.* 2013). Tests and improvements of ecdysteroid extraction protocol and quantification by LC-MS are ongoing. The samples for those investigations have already been collected and will be processed as soon as the protocols are approved.

- Investigation of trophic egg content could go further looking at other molecules or compounds already described as having a role in caste differentiation, such as vitellogenin and insulin (Libbrecht *et al.* 2013; Wheeler *et al.* 2014). Metabolomics is one of the newest '-omics' technologies, which has rapidly expanded over the last decade, providing an integral new approach to the study of biological systems, including insects as reviewed in Snart *et al.* (2015). Metabolomic studies would provide a wider picture of the egg content by detecting the full metabolome in trophic and reproductive eggs, and such metabolomic studies have already been performed successfully, for example in the honeybee brain (Pratavieira *et al.* 2020) or in the Asian corn borer (Su *et al.* 2021).

- We determined miRNA concentration, quality and size distribution in trophic and reproductive eggs. We found differences in miRNA concentration which suggests differences in miRNA identity. miRNA sequencing would allow us to confirm our results as well as to go a step further towards identifying miRNA candidates that could influence larval caste fate. Similar to the method described in the first two chapters, we would use the miRNA candidates as a template to design synthetic miRNAs. Then, using the microinjection technique that we have developed, we would inject these synthetic miRNAs into trophic eggs to change the miRNA levels in order to manipulate the caste fate of the developing larvae. We predict that larvae eating manipulated trophic eggs would develop into gynes, whereas larvae eating unmanipulated trophic eggs would develop into workers.

Investigation of caste-biased genes expression in early developmental stages in *Pogonomyrmex rugosus* with environmental caste determination

- Investigating the molecular basis of caste differentiation in a species with environmental caste determination would broaden our knowledge and understanding of caste determination. We could use the results from *Pogonomyrmex* J lineages (GCD) to investigate caste-biased genes and expression patterns in *P. rugosus* (ECD) that might be linked to caste determination. We could look for clusters at 6d and L1 in *P. rugosus*, as those two dev. stages showed clear clusters by caste in J lineages. As we are unable to determine the caste of ECD individuals prior to analysis, a cluster analysis at or around dev. stages 6d and L1 (as those two developmental stages showed clear clusters by caste in J lineages) might permit separation of the samples into two groups representative of worker and gyne. Collecting samples at two different time points would also help distinguishing samples by caste; after hibernation to get samples containing gynes and workers, and before hibernation to have only worker samples (Figure 1). Despite the fact that gyne development is known to occur only in the post-hibernation period in *P. rugosus* (Schwander *et al.* 2008), we cannot exclude the possibility that we would collect some gyne samples at an early developmental stage in the sampling before hibernation. However, we would expect only a small proportion, which should cluster with the gyne samples collected after hibernation. It should therefore be possible to determine the sample caste. We could then attribute the caste to the unknown samples based on the clusters and carry out a differential gene expression analysis. If the caste-biased genes are the same in *Pogonomyrmex* J lineages and *P. rugosus* ECD, it would give stronger support to genes identified in this thesis as involved in caste differentiation.

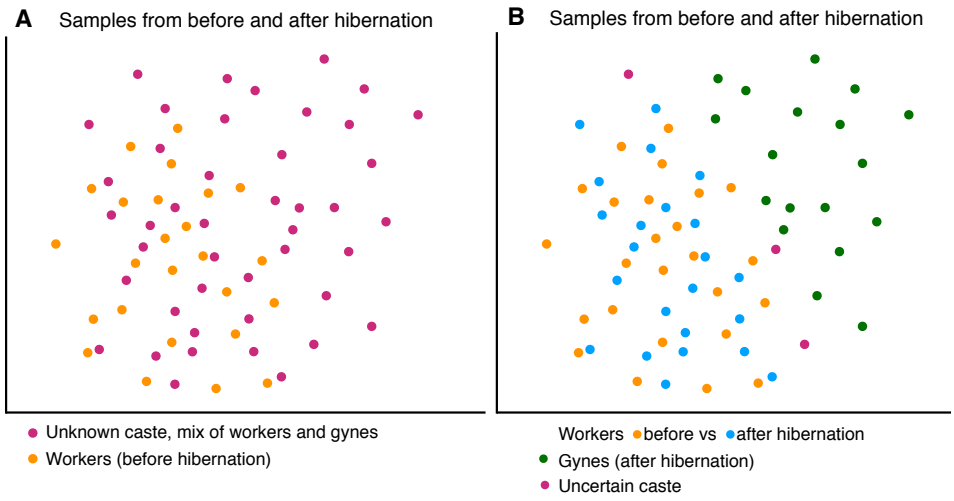


Figure 1. Hypothetical cluster analysis of *P. rugosus* ECD samples, with (A) samples collected before and after hibernation, with known and unknown caste, respectively and (B) caste attribution to the unknown samples based on the clustering with worker samples.

References

Agrawal, A. A, D. D. Ackerly, F. Adler, A. E. Arnold, C. Cáceres, D. F. Doak, E. Post, P. J. Hudson, J. Maron, K. A. Mooney, M. Power, D. Schemske, J. Stachowicz, S. Strauss, M. G. Turner, and E. Werner. 2007. Filling key gaps in population and community ecology. *Front. Ecol. Environ.* **5**:145–152.

Ananthakrishnan, T., and D. Whitman. 2005. Insect Phenotypic Plasticity: Diversity of Responses. Science Publishers, Enfield (New Hampshire).

Asencot, M., and Y. Lensky. 1985. The phagostimulatory effect of sugars on the induction of “queenliness” in female honeybee (*Apis mellifera L.)* larvae. *Comp. Biochem. Physiol.* **81A**:203–208.

Asgari, S. 2013. MicroRNA functions in insects. *Insect Biochem. Mol. Biol.* **43**:388–397. Elsevier Ltd.

Ashby, R., S. Forêt, I. Searle, and R. Maleszka. 2016. MicroRNAs in Honey Bee Caste Determination. *Sci. Rep.* **6**:1–15. Nature Publishing Group.

Barchuk, A. R., A. S. Cristino, R. Kucharski, L. F. Costa, Z. L. . Simões, and R. Maleszka. 2007. Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera. BMC Dev. Biol.* **7**:1–19.

Bolnick, D. I., and B. M. Fitzpatrick. 2007. Sympatric Speciation: Models and Empirical Evidence. *Annu. Rev. Ecol. Evol. Syst.* **38**:459–487.

Boomsma, J. J., and R. Gawne. 2018. Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation. *Biol. Rev.* **93**:28–54.

Bradshaw, A. D. 1965. Evolutionary Significance of Phenotypic Plasticity in Plants. *Adv. Genet.* **13**:115–155.

Brown, M. J. ., and S. Bonhoeffer. 2003. On the evolution of claustral colony founding in ants. *Evol. Ecol. Res.* **5**:305–313.

Bush, G. . 1975. Sympatric Speciation in Phytophagous Parasitic Insects. Pp. 187–206 in P. W. Price, ed. Evolutionary Strategies of Parasitic Insects and Mites. Springer, Boston, MA.

Bush, G. L. 1969. Sympatric host race formation and speciation in frugivorous flies of the genus. *Evolution (N. Y).* **23**:237–251.

Cameron, R. C., E. J. Duncan, and P. K. Dearden. 2013. Biased gene expression in early honeybee larval development. *BMC Genomics* **14**:1–12.

Casas, L., F. Saborido-Rey, T. Ryu, C. Michell, T. Ravasi, and X. Irigoien. 2016. Sex Change in Clownfish: Molecular Insights from Transcriptome Analysis. *Sci. Rep.* **6**:1–19.

Cassill, D. 2002. Brood care strategies by newly mated monogyne *Solenopsis invicta* (Hymenoptera: Formicidae) queens during colony founding. *Ann. Entomol. Soc.* Am. **95**:208–212.

Colgan, T. J., J. C. Carolan, S. J. Bridgett, S. Sumner, M. L. Blaxter, and M. J. F. Brown. 2011. Polyphenism in social insects: Insights from a transcriptome-wide analysis of gene expression in the life stages of the key pollinator, *Bombus terrestris. BMC Genomics* **12**:1–20.

Corona, M., R. Libbrecht, and D. E. Wheeler. 2016. Molecular mechanisms of phenotypic plasticity in social insects. *Curr. Opin. Insect Sci.* **13**:55–60.

Crespi, B. J. 1977. Cannibalism and trophic eggs in subsocial and eusocial insects. Pp. 176–213 in M. A. Elgar and B. J. Crespi, eds. Cannibalism: Ecology and Evolution Among Diverse Taxa.

Crespi, B. J., and D. Yanega. 1995. The definition of eusociality. *Behav. Ecol.* **6**:109–115.

Dambroski, H. R., and J. L. Feder. 2007. Host plant and latitude-related diapause variation in *Rhagoletis pomonella*: A test for multifaceted life history adaptation on different stages of diapause development. *J. Evol. Biol*. **20**:2101–2112.

Dambroski, H. R., C. Linn, S. H. Berlocher, A. Andrew, H. R. Dambroski, C. Linn, S. H. Berlocher, W. Roelofs, and J. L. Feder. 2005. The Genetic Basis for Fruit Odor Discrimination in *Rhagoletis* Flies and Its Significance for Sympatric Host Shifts. *Evolution (N. Y).* **59**:1953–1964.

Déjardin, J., and G. Cavalli. 2004. Transgenesis in *Drosophila*. *Protocol*.

Duan, T.-F., L. Li, Y. Tan, Y.-Y. Li, and B.-P. Pang. 2021. Identification and functional analysis of microRNAs in the regulation of summer diapause in *Galeruca daurica*. *Comp. Biochem. Physiol. - Part D Genomics Proteomics* **37**:1–10. Elsevier Inc.

Engel, K., T. Schreder, and R. Tollrian. 2014. Morphological defences of invasive *Daphnia lumholtzi* protect against vertebrate and invertebrate predators. *J. Plankton Res.* **36**:1140–1145.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. *Proc. Natl. Acad. Sci. U.S.A.* **91**:7990–7994.

Feder, J. L., J. B. Roethele, B. Wlazlo, and S. H. Berlocher. 1997. Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly. Proc. Natl. Acad. Sci. U. S. A. **94**:11417–11421.

Filchak, K. E., J. B. Roethele, and J. L. Feder. 2000. Natural selection and sympatric divergence in the apple maggot Rhagoletis pomonella. *Nature* **407**:739–742.

Gompel, N., and E. A. Schröder. 2015. *Drosophila* germline transformation. *Protocol*.

Gordon, D. G., A. Zelaya, I. Arganda-Carreras, S. Arganda, and J. F. Traniello. 2019. Division of labor and brain evolution in insect societies: Neurobiology of extreme specialization in the turtle ant *Cephalotes varians. PLoS One* **14**:1–16.

Gräff, J., S. Jemielity, J. D. Parker, K. M. Parker, and L. Keller. 2007. Differential gene expression between adult queens and workers in the ant *Lasius niger*. *Mol. Ecol.* **16**:675–683.

Grozinger, C. M., Y. Fan, S. E. . Hoover, and M. L. Winston. 2007. Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). *Mol. Ecol.* **16**:4837–4848.

Guo, X., S. Su, G. Skogerboe, S. Dai, W. Li, Z. Li, F. Liu, R. Ni, Y. Guo, S. Chen, S. Zhang, and R. Chen. 2013. Recipe for a busy bee: MicroRNAs in honey bee caste determination. *PLoS One* **8**:1–10.

Hahn, D. A., R. A. Johnson, N. A. Buck, and D. E. Wheeler. 2004. Storage protein content as a functional marker for colony-founding strategies: A comparative study within the harvester ant genus *Pogonomyrmex*. *Physiol. Biochem. Zool*. **77**:100–108.

Helms Cahan, S., C. J. Graves, and C. S. Brent. 2011. Intergenerational effect of juvenile hormone on offspring in *Pogonomyrmex harvester* ants. *J. Comp. Physiol.* B **181**:991–999.

Helms Cahan, S., G. E. Julian, T. Schwander, and L. Keller. 2006. Reproductive isolation between *Pogonomyrmex rugosus* and two lineages with genetic caste determination. *Ecology* **87**:2160–2170.

Helms Cahan, S., and L. Keller. 2003. Complex hybrid origin of genetic caste determination in harvester ants. *Nature* **424**:306–309.

Helms Cahan, S., J. D. Parker, S. W. Rissing, R. A. Johnson, T. S. Polony, M. D. Weiser, and D. R. Smith. 2002. Extreme genetic differences between queens and workers in hybridizing *Pogonomyrmex* harvester ants. *Proc. R. Soc. B Biol. Sci.* **269**:1871–1877.

Helms Cahan, S., and S. B. Vinson. 2003. Reproductive Division of Labor between Hybrid and Nonhybrid Offspring in a Fire Ant Hybrid Zone. *Evolution (N. Y).* **57**:1562–1570.

Hoffman, E. A., and M. A. . Goodisman. 2007. Gene expression and the evolution of phenotypic diversity in social wasps. *BMC Biol.* **5**:1–9.

Hölldobler, B. 1976. The behavioral Ecology of Mating in Harvester Ants (Hymenoptera: Formicidae: *Pogonomyrmex*). *Behav. Ecol. Sociobiol.* **1**:405–423.

Hölldobler, B., and E. O. Wilson. 1990. The Ants. Harvard Un. Harvard University Press, Harvard USA.

Hung, A. C. F., and S. B. Vinson. 1977. Interspecific Hybridization and Caste Specificity of Protein in Fire Ant. *Science* **196**:1458–1460.

Hunt, J. H., F. Wolschin, M. T. Henshaw, T. C. Newman, A. L. Toth, and G. V Amdam. 2010. Differential gene expression and protein abundance evince ontogenetic bias toward castes in a primitively eusocial wasp. *PLoS One* **5**:1–10.

Jandt, J. M., E. A. Tibbetts, and A. L. Toth. 2014. Polistes paper wasps: A model genus for the study of social dominance hierarchies. *Insectes Soc.* **61**:11–27.

Julian, G. E., J. H. Fewell, J. Gadau, R. A. Johnson, and D. Larrabee. 2002. Genetic determination of the queen caste in an ant hybrid zone. *Proc. Natl. Acad. Sci. U.S.A.* **99**:8157–8160.

Laland, K., T. Uller, M. Feldman, K. Sterelny, G. B. Müller, A. Moczek, E. Jablonka, J. Odling-Smee, G. A. Wray, H. E. Hoekstra, D. J. Futuyma, R. E. Lenski, T. F. . Mackay, D. Schluter, and J. E. Strassmann. 2014. Does evolutionary theory need a rethink? *Nature* **514**:161–164.

Leimar, O., K. Hartfelder, M. D. Laubichler, and R. E. Page. 2012. Development and evolution of caste dimorphism in honeybees - a modeling approach. *Ecol. Evol.* **2**:3098–3109.

Libbrecht, R., M. Corona, F. Wende, D. O. Azevedo, J. E. Serrão, and L. Keller. 2013. Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proc. Natl. Acad. Sci. U.S.A.* **110**:11050–11055.

Linksvayer, T. A. 2006. Direct, maternal, and sibsocial genetic effects on individual and colony traits in an ant. *Evolution (N. Y).* **60**:2552.

Linksvayer, T. A., O. Kaftanoglu, E. Akyol, S. Blatch, G. V Amdam, and R. E. Page. 2011. Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen-worker dimorphism. *J. Evol. Biol.* **24**:1939–1948.

Lucas, K. J., B. Zhao, S. Liu, and A. S. Raikhel. 2015. Regulation of physiological processes by microRNAs in insects. *Curr. Opin. insect Sci.* **11**:1–7.

MacKay, W. P. 1981. A Comparison of the Nest Phenologies of Three Species of *Pogonomyrmex* Harvester Ants (Hymenoptera: Formicidae). *Psyche* **88**:25–74.

Manfredini, F., M. Arbetman, and A. L. Toth. 2019. A Potential Role for Phenotypic Plasticity in Invasions and Declines of Social Insects. *Front. Ecol. Evol.* **7**:1–17.

Matsuura, K., and N. Kobayashi. 2010. Termite queens adjust egg size according to colony development. *Behav. Ecol.* **21**:1018–1023.

McLeod, L. 2007. Further investigations of the effect of low temperature on the phenotype of the adults of *Precis octavia* (Cramer) (Lepidoptera: Nymphalidae). *Metamorphosis* **18**:48–55.

Miura, T. 2005. Developmental regulation of caste-specific characters in social-insect polyphenism. *Evol. Dev.* **7**:122–129.

Moczek, A. P. 2010. Phenotypic plasticity and diversity in insects. *Philos. Trans. R. Soc. B Biol. Sci.* **365**:593–603.

Moczek, A. P., S. Sultan, S. Foster, C. Ledón-Rettig, I. Dworkin, H. F. Nijhout, E. Abouheif, and D. W. Pfennig. 2011. The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B Biol. Sci.* **278**:2705–2713.

Mollinari, C., and C. González. 1998. Microinjection of Drosophila Eggs. Pp. 587–603 in A. Cid-Arregui *et al.* (eds.), ed. Microinjection and Transgenesis.

Norman, V., H. Darras, C. Tranter, S. Aron, and W. O. . Hughes. 2016. Cryptic lineages hybridize for worker production in the harvester ant *Messor barbarus*. *Biol. Lett.* **12**:1–5.

Ometto, L., D. Shoemaker, K. G. Ross, and L. Keller. 2011. Evolution of gene expression in fire ants: The effects of developmental stage, caste, and species. *Mol. Biol. Evol.* **28**:1381–1392.

Oster, G. F., and E. O. Wilson. 1978. Caste and Ecology in the Social Insects. Princeton University Press, Princeton USA.

Patel, A., M. K. Fondrk, O. Kaftanoglu, C. Emore, G. Hunt, K. Frederick, and G. V Amdam. 2007. The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. *PLoS One* **2**:1–7.

Pereboom, J. J. ., W. C. Jordan, S. Sumner, R. L. Hammond, and A. F. . Bourke. 2005. Differential gene expression in queen-worker caste determination in bumble-bees. *Proc. R. Soc. B Biol. Sci.* **272**:1145–1152.

Pigliucci, M. 2001. Phenotipic plasticity: beyond nature and nurture. The Johns Hopkins University Press, Baltimore and London.

Pontieri, L., A. Rajakumar, A. M. Rafiqi, R. S. Larsen, E. Abouheif, and G. Zhang. 2020. From egg to adult: a developmental table of the ant Monomorium pharaonis. bioRxiv 2020.12.22.423970.

Porter, S. D., and W. R. Tschinkel. 1986. Adaptive Value of Nanitic Workers in Newly Founded Red Imported Fire Ant Colonies (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am.* **79**:723–726.

Pratavieira, M., A. Ribeiro Da Silva Menegasso, T. Roat, O. Malaspina, and M. S. Palma. 2020. In Situ Metabolomics of the Honeybee Brain: The Metabolism of l-Arginine through the Polyamine Pathway in the Proboscis Extension Response (PER). *J. Proteome Res.* **19**:832–844.

Prokopy, R. J., S. R. Diehl, and S. S. Cooley. 1988. Behavioral evidence for host races in *Rhagoletis pomonella* flies. *Oecologia* **76**:138–147.

Puthiyakunnon, S., Y. Yao, Y. Li, J. Gu, H. Peng, and X. Chen. 2013. Functional characterization of three MicroRNAs of the Asian Tiger Mosquito, *Aedes albopictus*. *Parasites and Vectors* **6**:1–10.

Revely, L., S. Sumner, and P. Eggleton. 2021. The Plasticity and Developmental Potential of Termites. *Front. Ecol. Evol.* **9**:1–13.

Scharf, M. E., C. E. Buckspan, T. L. Grzymala, and X. Zhou. 2007. Regulation of polyphenic caste differentiation in the termite *Reticulitermes flavipes* by interaction of intrinsic and extrinsic factors. *J. Exp. Biol.* **210**:4390–4398.

Schlichting, C. D., and M. Pigliucci. 1998. Phenotypic evolution: a reaction norm perspective. Sinauer associates incorporated.

Schwander, T., J. Y. Humbert, C. S. Brent, S. Helms Cahan, L. Chapuis, E. Renai, and L. Keller. 2008. Maternal Effect on Female Caste Determination in a Social Insect. *Curr. Biol.* **18**:265–269.

Schwander, T., L. Keller, and S. Helms Cahan. 2007. Two alternate mechanisms contribute to the persistence of interdependent lineages in *Pogonomyrmex* harvester ants. *Mol. Ecol.* **16**:3533–3543.

Schwander, T., N. Lo, M. Beekman, B. P. Oldroyd, and L. Keller. 2010. Nature versus nurture in social insect caste differentiation. *Trends Ecol. Evol.* **25**:275–282. Elsevier Ltd.

Shaw, K. A., M. L. Scotti, and S. A. Foster. 2007. Ancestral plasticity and the evolutionary diversification of courtship behaviour in threespine sticklebacks. *Anim. Behav.* **73**:415–422.

Shi, Y. Y., Z. Y. Huang, Z. J. Zeng, Z. L. Wang, X. B. Wu, and W. Y. Yan. 2011. Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, apidae). *PLoS One* **6**:2–7.

Shi, Y. Y., H. J. Zheng, Q. Z. Pan, Z. L. Wang, and Z. J. Zeng. 2015. Differentially expressed microRNAs between queen and worker larvae of the honey bee (*Apis mellifera*). *Apidologie* **46**:35–45.

Sieber, K. R., M. Saar, C. Opachaloemphan, M. Gallitto, H. Yang, and H. Yan. 2021. Embryo injections for crispr-mediated mutagenesis in the ant *Harpegnathos saltator*. *J. Vis. Exp.* **168**:1–14.

Sirviö, A., P. Pamilo, R. A. Johnson, R. E. Page, and J. Gadau. 2011. Origin and evolution of the dependent lineages in the genetic caste determination system of *Pogonomyrmex* ants. *Evolution (N. Y).* **65**:869–884.

Slater, G. P. 2017. Eat to reproduce: the role of diet quantity on honey bee (*Apis mellifera*) caste determination. *Thesis*.

Slater, G. P., G. D. Yocum, and J. H. Bowsher. 2020. Diet quantity influences caste determination in honeybees (*Apis mellifera*): Caste determination in honey bees. *Proc. R. Soc. B Biol. Sci.* **287**:1–10.

Smith, C. R., C. Schoenick, K. E. Anderson, J. Gadau, and A. V Suarez. 2007. Potential and realized reproduction by different worker castes in queen-less and queen-right colonies of *Pogonomyrmex badius*. *Insect. Soc*. **54**:260-267.

Snart, C. J. ., I. C. . Hardy, and D. A. Barrett. 2015. Entometabolomics: Applications of modern analytical techniques to insect studies. *Entomol. Exp. Appl*. **155**:1–17.

Sommer, R. J. 2020. Phenotypic plasticity: From theory and genetics to current and future challenges. *Genetics* **215**:1–13.

Su, L., C. Yang, J. Meng, L. Zhou, and C. Zhang. 2021. Comparative transcriptome and metabolome analysis of *Ostrinia furnacalis* female adults under UV-A exposure. *Sci. Rep*. **11**:1–14.

Sumner, S., J. J. . Pereboom, and W. C. Jordan. 2006. Differential gene expression and phenotypic plasticity in behavioural castes of the primitively eusocial wasp, *Polistes canadensis. Proc. R. Soc. B Biol. Sci.* **273**:19–26.

Teseo, S., N. Châline, P. Jaisson, and D. J. . Kronauer. 2014. Epistasis between adults and larvae underlies caste fate and fitness in a clonal ant. *Nat. Commun*. **5**:1–8.

Thompson, J. D. 1991. Phenotypic plasticity as a component of evolutionary change. *Trends Ecol. Evol.* **6**:246–249.

Toth, A. L., K. Varala, T. C. Newman, F. E. Miguez, S. K. Hutchison, D. A. Willoughby, J. F. Simons, M. Egholm, J. H. Hunt, G. M. E, and G. E. Robinson. 2007. Wasp Gene Expression Supports an Evolutionary Link Between Maternal Behavior and Eusociality. *Science* **318**:441–444.

Traniello, J. 2010. Pheidole ants: Sociobiology of a highly diverse genus. Pp. 699–706 in M. D. Breed and J. Moore, eds. Encyclopedia of Animal Behavior.

Vieira, J., F. C. de Paula Freitas, A. Santos Cristino, D. Guariz Pinheiro, L. R. Aguiar, M. A. Framartino Bezerra Laure, L. M. Rosatto Moda, Z. L. Paulino Simões, and A. R. Barchuk. 2021. Molecular underpinnings of the early brain developmental response to differential feeding in the honey bee *Apis mellifera. Biochim. Biophys. Acta - Gene Regul. Mech*. **1864**:1–11.

Villalta, I., O. Blight, E. Angulo, X. Cerdá, and R. Boulay. 2016. Early developmental processes limit socially mediated phenotypic plasticity in an ant. *Behav. Ecol. Sociobiol*. **70**:285–291.

Volny, V. P., and D. M. Gordon. 2002. Genetic basis for queen-worker dimorphism in a social insect. *Proc. Natl. Acad. Sci. U.S.A.* **99**:6108–6111.

Volny, V. P., M. J. Greene, D. M. Gordon, V. P. Volny, M. J. Greene, and D. M. Gordon. 2006. Brood Production and Lineage Discrimination in the Red Harvester Ant (*Pogonomyrmex barbatus*). *Ecology* **87**:2194–2200.

Wei, H., X. J. He, C. H. Liao, X. B. Wu, W. J. Jiang, B. Zhang, L. Bin Zhou, L. Z. Zhang, A. B. Barron, and Z. J. Zeng. 2019. A Maternal Effect on Queen Production in Honeybees. *Curr. Biol.* **29**:2208-2213

West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford University Press.

West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst*. **20**:249–278.

Wheeler, D. E. 1994. Nourishment in ants: patterns in individuals and societies. Pp. 245–278 in J. H. Hunt and C. A. Nalepa, eds. Nourishment and Evolution in Insect Societies. Westview Press, Boulder.

Wheeler, D. E., N. A. Buck, and J. D. Evans. 2014. Expression of insulin/insulin-like signalling and TOR pathway genes in honey bee caste determination. *Insect Mol. Biol.* **23**:113–121.

Whitford, W. G., P. Johnson, and J. Ramirez. 1976. Comparative ecology of the harvester ants *Pogonomyrmex barbatus* (F. Smith) and *Pogonomyrmex rugosus* (Emery). *Insectes Soc.* **23**:117–132.

Whitman, D. W., and A. A. Agrawal. 2009. What is Phenotypic Plasticity and Why is it Important? Phenotypic Plast. insects Mech. consequences 1–63.

Wilson, E. O. 1971. The insect societies. Harvard University Press, Princeton.

Wilson, R. S., and C. E. Franklin. 2002. Testing the beneficial acclimation hypothesis. *Trends* **17**:66–70.

Woltereck, R. 1909. Weitere experimentelle Untersuchungen über Artänderung, speziell über das Wesen quantitativer Artunterschiede bei Daphniden. *Verhandlungen der Dtsch. Zool. Gesellschaft Leipzig* **19**:110–173.

Woolgar, L., S. Trocini, and N. Mitchell. 2013. Key parameters describing temperature-dependent sex determination in the southernmost population of loggerhead sea turtles. *J. Exp. Mar. Bio. Ecol.* **449**:77–84.

Yan, H., C. Opachaloemphan, G. Mancini, H. Yang, M. Gallitto, J. Mlejnek, A. Leibholz, K. Haight, M. Ghaninia, L. Huo, M. Perry, J. Slone, X. Zhou, M. Traficante, C. A. Penick, K. Dolezal, K. Gokhale, K. Stevens, I. Fetter-Pruneda, R. Bonasio, L. J. Zwiebel, S. L. Berger, J. Liebig, D. Reinberg, and C. Desplan. 2017. An Engineered orco Mutation Produces Aberrant Social Behavior and Defective Neural Development in Ants. *Cell* **170**:736-747.e9.

Zafar, J., Y. Zhang, J. Huang, S. Freed, R. F. Shoukat, X. Xu, and F. Jin. 2021. Spatio-temporal profiling of *Metarhizium anisopliae*— responsive microRNAs involved in modulation of *Plutella xylostella* immunity and development. *J. Fungi* **7**:1–16.

Zhu, K., M. Liu, Z. Fu, Z. Zhou, Y. Kong, H. Liang, Z. Lin, J. Luo, H. Zheng, P. Wan, J. Zhang, K. Zen, J. Chen, F. Hu, C. Y. Zhang, J. Ren, and X. Chen. 2017. Plant microRNAs in larval food regulate honeybee caste development. *PLoS Genet.* **13**:1–23.