Year : 2012

Division of labor in insect societies: Genetic components and physiological regulation

LIBBRECHT Romain

UNIL | Université de Lausanne
Faculté de biologie
et de médecine
Département d'Ecologie et d'Evolution

Division of labor in insect societies: Genetic components and physiological regulation

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

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

par

Romain LIBBRECHT

Diplômé en Biologie
Université de Rennes, France

Jury

Prof. Edward Farmer, Président
Prof. Laurent Keller, Directeur de thèse
Dr. Michel Chapuisat, Expert interne
Prof. Francis Ratnieks, Expert externe

Lausanne 2012

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	Monsieur	Prof. Edward E. **Farmer**
Directeur de thèse	Monsieur	Prof. Laurent **Keller**
Experts	Monsieur	Prof. Michel **Chapuisat**
	Monsieur	Prof. Francis **Ratnieks**

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

Monsieur Romain Libbrecht

Mater of Science de l' Université de Rennes 1

intitulée

Division of labor in insect societies:
Genetic components and physiological regulation

Lausanne, le 28 septembre 2012

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Edward E. Farmer

E. Farmer

Remerciements

Je tiens à remercier dans les lignes qui suivent toutes les personnes qui, de près ou de loin, ont partagé, facilité, animé ou égayé ces cinq années de thèse.

Merci à Laurent. Pour m'avoir accueilli dans son labo, pour la liberté qu'il m'a laissée, pour son efficacité, pour sa confiance et pour les séjours à Villars.

Merci à Michel, Ted Farmer et Francis Ratnieks. Pour avoir accepté d'être dans mon jury de thèse, pour leurs conseils et pour le temps qu'ils m'ont accordé.

Merci à Tanja Schwander, Miguel Corona, Dihego Azevedo, Jose Serrão et Franziska Wende. Pour le succès de nos collaborations scientifiques.

Merci à Jojo, Christophe, Mic, Tim, Colby, Eric, Tibo, Valou, Adria, Geoffrey, Mischa, Miguel, Yannick, Michel, Akiko, Nathalie, Danielle, Mingkwan et tous les membres du groupe fourmis, passé et présent. Pour l'ambiance dynamique, efficace, inventive, enjouée et pleine d'humour.

Merci à Christine, Céline, Hannes, Franck, France, Nadia, Elisa, Catherine, Vicky, Yvan, Dominique et Blaise. Sans eux pas de labo, de maison pour les fourmis, de contrat, de crédit, de lumière ou de colis.

Merci à Nicolas, Jacques, Franck, Caro A, Caro C, Guigui, Bernard et tous les assistants et étudiants de Roscoff. Pour l'eau, le vent, Chondrus, les cailloux, les anémones, Crispus, les crabes et les goélands.

Merci à Jojo pour son pessimisme éclairé, Christophe pour ses ronchonnements musicaux, Mic pour sa curiosité légendaire, Alex pour sa dextérité dactylographique, Auré pour ses larmes égarées et Tim pour son humour absurde.

Merci à Guigui, Colby, Eric, Thibault, Aline, Hélène, Valou, Fred, CaroA, CaroC, Loïc, Agnès, Cyril, Lucie, Alan, Rob, Biollay, Geoffrey, Sophie, Pierre B, Zoé, Christine L, Céline,

Christophe, Romain, Isaline, Vincent, Lucas, Nico, Gordon, Khadim, Céline, Farice, Walid, Olivier, Yannick, Greg, Godefroy, Pierre F, Charlène, Nadia, Eytan, Fernando, Christine G, Guillaume B. Pour avoir tâté de la boule ou du ballon, du shot ou de la pinte, de la raquette ou du jeton, et régulièrement mis 1.- dans la cagnotte.

Merci à Mark Fellowes et David Renaud. Pour avoir pavé mon chemin vers Lausanne.

Merci aux potes d'avant et de toujours, ceux avec qui j'ai grandi, et avec qui je vieillirai, qu'ils viennent du Texas, de Fée d'herbe ou de la Cité II.

Merci à mes parents et à mamie Lola. Pour leur amour, leur force, leur simplicité et leur humour.

Merci à Po, mon petit coeur à roulettes. Pour son amour, son intelligence et sa créativité. Pour me pousser à suivre mes passions, même si elles m'amènent à tripoter de la fourmi chez les suisses. Pour être là. Pour notre avenir.

Je dédie cette thèse à mamie Dora et mon pote Lockso, dont les beaux souvenirs me rappellent constamment que sourire à la vie est le plus beau cadeau qu'on puisse lui faire.

Summary

The shift from solitary to social organisms constitutes one of the major transitions in evolution. The highest level of sociality is found in social insects (ants, termites and some species of bees and wasps). Division of labor is central to the organization of insect societies and is thought to be at the root of their ecological success. There are two main levels of division of labor in social insect colonies. The first relates to reproduction and involves the coexistence of queen and worker castes: while reproduction is usually monopolized by one or several queens, functionally sterile workers perform all the tasks to maintain the colony, such as nest building, foraging or brood care. The second level of division of labor, relating to such non-reproductive duties, is characterized by the performance of different tasks or roles by different groups of workers. This PhD aims to better understand the mechanisms underlying division of labor in insect societies, by investigating how genes and physiology influence caste determination and worker behavior in ants.

In the first axis of this PhD, we studied the nature of genetic effects on division of labor. We used the Argentine ant *Linepithema humile* to conduct controlled crosses in the laboratory, which revealed the existence of non-additive genetic effects, such as parent-of-origin and genetic compatibility effects, on caste determination and worker behavior. In the second axis, we focused on the physiological regulation of division of labor. Using *Pogonomyrmex* seed-harvester ants, we performed experimental manipulation of hibernation, hormonal treatments, gene expression analyses and protein quantification to identify the physiological pathways regulating maternal effects on caste determination. Finally, comparing gene expression between nurses and foragers allowed us to reveal the association between vitellogenin and worker behavior in *Pogonomyrmex* ants.

This PhD provides important insights into the role of genes and physiology in the regulation of division of labor in social insect colonies, helping to better understand the organization, evolution and ecological success of insect societies.

Résumé

L'une des principales transitions évolutives est le passage de la vie solitaire à la vie sociale. La socialité atteint son paroxysme chez les insectes sociaux que sont les fourmis, les termites et certaines espèces d'abeilles et de guêpes. La division du travail est la clé de voûte de l'organisation de ces sociétés d'insectes et la raison principale de leur succès écologique. La division du travail s'effectue à deux niveaux dans les colonies d'insectes sociaux. Le premier niveau concerne la reproduction et implique la coexistence de deux castes : les reines et les ouvrières. Tandis que la reproduction est le plus souvent monopolisée par une ou plusieurs reines, les ouvrières stériles effectuent les tâches nécessaires au bon fonctionnement de la colonie, telles que la construction du nid, la recherche de nourriture ou le soin au couvain. Le second niveau de division du travail, qui concerne les tâches autres que la reproduction, implique la réalisation de différents travaux par différents groupes d'ouvrières. Le but de ce doctorat est de mieux comprendre les mécanismes sous-jacents de la division du travail dans les sociétés d'insectes en étudiant comment les gènes et la physiologie influencent la détermination de la caste et le comportement des ouvrières chez les fourmis.

Dans le premier axe de ce doctorat, nous avons étudié la nature des influences génétiques sur la division du travail. Nous avons utilisé la fourmi d'Argentine, *Linepithema humile*, pour effectuer des croisements contrôlés en laboratoire. Cette méthode nous a permis de révéler l'existence d'influences génétiques non additives, telles que des influences dépendantes de l'origine parentale ou des effets de compatibilité génétique, sur la détermination de la caste et le comportement des ouvrières. Dans le second axe, nous nous sommes intéressés à la régulation physiologique de la division du travail. Nous avons utilisé des fourmis moissonneuses du genre *Pogonomyrmex* pour effectuer des hibernations artificielles, des traitements hormonaux, des analyses d'expression de gènes et des mesures de vitellogénine, ce qui nous a permis d'identifier les mécanismes physiologiques régulant les effets maternels sur la détermination de la caste. Enfin, la comparaison d'expression de

gènes entre nourrices et fourrageuses suggère un rôle de la vitellogénine dans la régulation du comportement des ouvrières chez les fourmis moissonneuses.

En détaillant les influences des gènes et de la physiologie dans la régulation de la division du travail dans les colonies d'insectes sociaux, ce doctorat fournit d'importantes informations permettant de mieux comprendre l'organisation, l'évolution et le succès écologique des sociétés d'insectes.

“None preaches better than the ant,
and she says nothing”

Benjamin Franklin

“J’aime la force, et de la force que j’aime,
une fourmi peut en montrer autant qu’un éléphant”

Stendhal

GENERAL INTRODUCTION

Eusociality

One of the major transitions in evolution is the shift from solitary to social organisms, along with the transitions from prokaryotes to eukaryotes, asexual to sexual reproduction and unicellular to pluricellular organisms (Maynard Smith and Szathmary 1995; Szathmary and Maynard Smith 1995). Eusociality, the highest level of social organization, describes societies in which there are overlapping generations, cooperative care of the brood and coexistence of fertile and sterile individuals (Wilson 1971). Eusociality is widespread in the animal kingdom. It has mostly been described in insects (in Hymenoptera, Isoptera, Hemiptera, Thysanoptera and Coleoptera) (Holldobler and Wilson 1990; Ross and Matthews 1991; Stern and Foster 1996; Crespi et al. 1997) but also in crustaceans (in *Synalpheus* shrimps) (Duffy 1996; Duffy and Morrison 2000) and mammals (in two species of mole rat) (Burda et al. 2000). The recent discovery of eusocial fluke worms (Hechinger et al. 2011) suggests that eusociality may be even more common than generally realized (Newey and Keller 2010).

Eusociality has long appeared paradoxical to many theorists in the field of evolution. Darwin himself feared that the evolution of altruistic behaviors could be "fatal" to his theory (Darwin 1859). Although he did not resolve the paradox, Darwin understood the importance of family relationships that would later become the basis of kin selection theory. In 1964, Hamilton extended natural selection to relatives using a genetic framework (Hamilton 1964a, 1964b). This theory, later named kin selection theory (Maynard Smith 1964), rapidly became a keystone for our modern understanding of social evolution (Bourke and Franks 1995). Kin selection states that individuals can transmit copies of their genes not only by reproducing themselves (direct fitness), but also by helping relatives that share genes inherited from recent common ancestors (indirect fitness). This theory explains how the combination of high relatedness and particular ecological conditions can lead to the evolution of eusociality (Bourke and Franks 1995; Hughes et al. 2008; Boomsma 2009; Boomsma et al. 2011).

Eusociality is best exemplified by social insects, a group comprised mainly of ants, termites and some bee and wasp species, and which has achieved great ecological success. Ants, for instance, have colonized most terrestrial biomes and represent 15-20% of the terrestrial animal biomass (Schultz 2000). They occupy keystone positions in most terrestrial environments and have strong ecological impacts due to their varied roles as scavengers, predators, granivores and herbivores (Holldobler and Wilson 1990). Ants are also important in below ground processes through the alteration of the physical and chemical environment and through their effects on plants, microorganisms, and other soil organisms (Folgarait 1998). Understanding the ecological dominance of social insects requires investigation into the complex organization of their societies.

Division of labor in insect societies

Division of labor, the cornerstone of insect societies, is the phenomenon whereby different groups of individuals perform different tasks or roles. By enhancing colony performance, division of labor is thought to be one of the main factors responsible for the tremendous ecological success of social insects (Wilson 1971; Oster and Wilson 1979; Holldobler and Wilson 1990). There are two main levels of division of labor in insect societies: the first relates to reproduction and the second to all non-reproductive tasks necessary to take care of the colony.

Reproductive division of labor

While reproduction is usually monopolized by one or several queens, functionally sterile workers perform all the tasks involved in colony maintenance. Queens and workers usually show great differences in morphology, physiology and lifespan. For instance, queens outweigh minor workers by a factor of 130 in the leafcutter ant *Atta cephalotes* (Stradling 1978; Mintzer 1990) and produce up to 80 eggs per hour in the red imported fire ant *Solenopsis invicta* (Tschinkel 1988, 2006). In the black garden ant *Lasius niger*, queens can

reach the extreme age of 29 years, whereas workers live only 1–2 years (Kutter and Stumper 1969; Holldobler and Wilson 1990). These differences between queens and workers arise from a developmental switch which occurs during the larval stages (Wilson 1971; Holldobler and Wilson 1990). Understanding the mechanisms underlying the determination of the female caste in social insect colonies is crucial to understand the organization and ecological success of insect societies.

The proximate mechanisms regulating the production of queens and workers in social insects have received much attention. For several decades, it was assumed that social insect female brood were fully totipotent in their early stages, and that environmental factors alone determined whether an individual becomes a queen or a worker. However, several recent studies have revealed that genetic factors can, and often do, play an important role in female caste determination (Smith et al. 2008b; Schwander et al. 2010). These genetic influences range from plastic genotypes that are biased toward queen or worker development [e.g. *Pogonomyrmex rugosus* (Schwander and Keller 2008), *Acromyrmex echinatior* (Hughes and Boomsma 2008)] to a strictly genetic determination [e.g. *Pogonomyrmex* lineages (Helms Cahan et al. 2002; Julian et al. 2002; Volny and Gordon 2002, Helms Cahan and Keller 2003), *Solenopsis xyloni* (Helms Cahan and Vinson 2003), *Wasmannia auropunctata* (Fournier et al. 2005)]. Maternal factors such as temperature or queen age have also long been suspected to affect caste determination (Gösswald 1951; Bier 1954; Petersen-Braun 1977; Passera 1980; Vargo and Passera 1992). However, it is only recently that maternal effects on caste determination have been documented experimentally (Schwander et al. 2008). Cross-fostering of eggs between hibernated and non-hibernated *Pogonomyrmex* colonies revealed strong maternal effects on caste production, as only eggs produced by a hibernated queen could develop into queens, irrespective of the hibernation status of the rest of the colony (Schwander et al. 2008). Such maternal effects on the caste fate of the female offspring require that the hibernation triggers changes in the queen that regulate caste determination in the offspring.

Division of labor among workers

The second level of division of labor relates to the performance of tasks that are not related to reproduction, such as nest building, foraging or brood care. This division of labor among the worker force stems from differences between workers in their likelihood and ability to perform different tasks. A minority of ant species have morphologically differentiated worker subcastes, whose morphology is adapted to the performance of specific tasks (Wilson 1980; Detrain and Pasteels 1991; Robinson et al. 2009). In most species, however, workers are morphologically uniform in size and shape (Oster and Wilson 1979; Robinson 1992). In such species, within-colony task specialization results from workers differing in their responses to environmental signals indicating colony needs for specific tasks (Wilson 1971; Oster and Wilson 1979; Robinson and Page 1989a). Three main factors are known to influence worker thresholds for particular tasks. The first is age, as evidenced by workers frequently moving from one task to another as they become older (Wilson 1971; Seeley 1982). The second is individual experience, which has been shown to influence task preference in a few species (Theraulaz et al. 1998; Ravary et al. 2007). Finally, in almost all species studied, the genetic background of workers seems to affect their likelihood to undertake different tasks (Oldroyd and Fewell 2007).

Aim of the PhD

The aim of this PhD is to investigate the genetic components and physiological regulation of division of labor in insect societies. Specifically, this PhD was constructed around two axes. First, we used the Argentine ant *Linepithema humile* to study the nature of genetic influences on caste determination and worker behavior (Axis 1). Second, we focused on *Pogonomyrmex* ants to study how physiological changes in queens and workers affect caste production and behavior, respectively (Axis 2).

Axis 1: Genetic components to division of labor

Genetic effects on division of labor

Many studies have investigated the effects of genes on division of labor in insect societies, by comparing queen/worker ratio or worker task performance between subfamilies (matrilines or patrilines). This method revealed genetic effects on caste determination (Hughes and Boomsma 2008; Schwander and Keller 2008; Smith et al. 2008a; Frohschammer and Heinze 2009) and worker behavior (Calderone and Page 1988; Frumhoff and Baker 1988; Robinson and Page 1988, 1989b; Stuart and Page 1991; Snyder 1992; Snyder 1993; Estoup et al. 1994; Kaib et al. 1996; Blatrix et al. 2000; Kryger et al. 2000; Goodisman and Crozier 2003; Jones et al. 2004; Julian and Fewell 2004; Schwander et al. 2005; Waddington et al. 2010). Although authors usually discussed these effects as if they were simple additive genetic effects, the mere demonstration of maternal or paternal effects on division of labor in colonies with several matrilines or patrilines does not allow one to discriminate between additive and non-additive genetic effects such as parent-of-origin specific effects associated with imprinting, epistasis and genetic compatibility effects (Schwander and Keller 2008; Libbrecht et al. 2011). The only way to discriminate between additive and non-additive effects is to conduct controlled crosses to quantify paternal and maternal effects, as well as the interaction between parental effects.

The Argentine ant as a model system

The Argentine ant *Linepithema humile* is one of the most studied invasive species (Pysek et al. 2008). Native to South America, it has colonized many places throughout the world, particularly in Mediterranean climates, and occurs on six continents and many oceanic islands (Suarez et al. 2001; Wetterer et al. 2009; Vogel et al. 2010). This species is also highly polygynous (up to several hundred mated and functional queens coexist in a colony) (Newell 1909; Markin 1970) and has an unusual social organization in its introduced range,

whereby individuals mix freely within large supercolonies containing a high number of interconnected nests (Giraud et al. 2002; Holway et al. 2002; Ingram 2002). The combination of polygyny and unicoloniality results in a very low relatedness (indistinguishable from 0) between nestmates (Krieger and Keller 2000; Tsutsui and Case 2001; Giraud et al. 2002).

Another important feature of *L. humile* is that it is possible to obtain both males and queens, as well as induce mating, in the laboratory (Keller and Passera 1992). This provided us with the rare opportunity to conduct controlled crosses to test for the existence of non-additive genetic effects on caste allocation (**Chapter 1**) and worker behavior (**Chapter 2**).

Axis 2: Physiological regulation of division of labor

Physiological regulation of caste differentiation and worker behavior in bees

Among social insects, the honeybee *Apis mellifera* has been the primary focus of studies of the physiological regulation of caste differentiation and worker behavior. Several physiological pathways, known to interact with each other in solitary insect species, were found to be involved in the regulation of division of labor. Insect vitellogenin is a yolk protein expressed in the fat bodies, released to the hemolymph and internalized into competent oocytes where it is used as a source of amino acids for the developing embryo (Hagedorn and Kunkel 1979). The production of vitellogenin is affected by the juvenile hormone (Comas et al. 1999; Tatar et al. 2001), a major insect hormone with multiple effects on growth, reproduction, and longevity (Flatt et al. 2005). Several studies have established interactions between vitellogenin and juvenile hormone in the regulation of caste differentiation and worker behavior in honeybees (Barchuk et al. 2002; Amdam et al. 2003; Amdam et al. 2004; Amdam et al. 2007; Corona et al. 2007; Nelson et al. 2007; Ament et al. 2008; Nilsen et al. 2011; Wurm et al. 2011). Both vitellogenin and juvenile hormone are known to interact with the nutrient sensitive insulin signaling pathway, which regulates growth, reproduction and lifespan in insects (Tissenbaum and Ruvkun 1998; Stocker and Hafen 2000; Giannakou and

Partridge 2007). Key components of this pathway, such as the insulin-like peptides, have also been found to be involved in the regulation of caste differentiation and worker behavior (Corona et al. 2007; Ament et al. 2008; Daugherty et al. 2011; Nilsen et al. 2011). Despite important advances in the understanding of the physiological regulation of caste differentiation and worker behavior in *A. mellifera*, very little is known about the physiological regulation of queen production and worker behavior in ants.

The use of *Pogonomyrmex* ants as model systems

Harvester ants refer to species that collect and store seeds for later consumption. Half of the harvester ant species in the world belong to *Pogonomyrmex*, a genus of 60 species found in the deserts of North, Central and South America (Taber 1999) that have important effects on community structure and ecosystem functioning, as their presence affects plant species composition and diversity (MacMahon et al. 2000).

Pogonomyrmex ants have been primarily used as model systems to study caste determination. The first example of strictly genetic caste determination was described in *P. barbatus* (Helms Cahan et al. 2002; Julian et al. 2002; Volny and Gordon 2002; Helms Cahan and Keller 2003) while genetic compatibility effects between parental genomes were found to influence caste determination in *P. rugosus* (Schwander and Keller 2008). Interestingly, maternal effects on caste determination were also documented in this species, in which only eggs produced by a hibernated queen can develop into new queens, irrespective of the hibernation status of the rest of the colony (Schwander et al. 2008). Such maternal effects require that hibernation-triggered physiological modifications in queens regulate caste determination in the offspring. We used a combination of carefully-designed experimental manipulation of hibernation, hormonal treatments, gene expression analyses and vitellogenin quantification to identify the physiological mechanisms regulating maternal effects on caste determination in *P. rugosus* (**Chapter 3**). Finally, the genome of *P. barbatus* was recently sequenced (Smith et al. 2011b) and revealed the existence of two *vitellogenin*

genes in this species. We compared the expression of these genes between queens, nurses and foragers to investigate the role of vitellogenin in the physiological regulation of worker behavior in ants (**Chapter 4**).

CHAPTER 1

CHAPTER 1

Genetic components to caste allocation in a multiple-queen ant species

Romain Libbrecht, Tanja Schwander and Laurent Keller

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

Abstract

Reproductive division of labor and the coexistence of distinct castes are hallmarks of insect societies. In social insect species with multiple queens per colony, the fitness of nestmate queens directly depends on the process of caste allocation (i.e., the relative investment in queen, sterile worker and male production). The aim of this study is to investigate the genetic components to the process of caste allocation in a multiple-queen ant species. We conducted controlled crosses in the Argentine ant *Linepithema humile* and established single-queen colonies to identify maternal and paternal family effects on the relative production of new queens, workers and males. There were significant effects of parental genetic backgrounds on various aspects of caste allocation: the paternal lineage affected the proportion of queens and workers produced while the proportions of queens and males, and females and males were influenced by the interaction between parental lineages. In addition to revealing non-additive genetic effects on female caste determination in a multiple-queen ant species, this study reveals strong genetic compatibility effects between parental genomes on caste allocation components.

Introduction

One of the major transitions in evolution is the shift from solitary organisms to societies with reproductive division of labor (Maynard Smith and Szathmary 1995; Szathmary and Maynard Smith 1995). In eusocial Hymenoptera (ants, bees and wasps), reproductive division of labor is associated with morphological differences between the reproductive queens and the non-reproductive workers (Wilson 1971; Holldobler and Wilson 1990; Bourke and Franks 1995). These morphological differences, which can be extremely marked in some ant species, arise from a developmental switch during the larval stage (Wilson 1971; Holldobler and Wilson 1990).

For several decades, it was assumed that social insect female brood are fully totipotent, and that environmental factors alone determine whether an individual becomes a reproductive queen, or a functionally sterile worker. However, several recent studies have revealed that genetic factors can, and often do, play an important role in queen and worker caste determination [see (Smith et al. 2008b; Schwander et al. 2010) for review]. These genetic influences range from plastic genotypes that are biased toward queen or worker development [e.g. *Pogonomyrmex rugosus* (Schwander and Keller 2008), *Acromyrmex echinatior* (Hughes and Boomsma 2008)] to a strictly genetic determination [e.g. *Pogonomyrmex* lineages (Helms Cahan et al. 2002; Julian et al. 2002; Volny and Gordon 2002; Helms Cahan and Keller 2003), *Solenopsis xyloni* (Helms Cahan and Vinson 2003), *Wasmannia auropunctata* (Fournier et al. 2005)].

The occurrence of a genetic component to caste determination has important implications in species where colonies contain several queens, as this may influence each queen's relative reproductive success. Several studies have shown that queens within a colony may differ in their relative contribution to worker and queen production (Ross 1988; Bourke et al. 1997; Fournier et al. 2004). However, it is unknown whether these differences arise from competitive interactions among queens and other social effects or whether intrinsic genetic

differences among queens and/or their mates directly bias the developmental trajectories of their female brood.

Another important factor affecting queen reproductive success in multiple-queen colonies is their relative contribution to male production. Social Hymenoptera have a haplodiploid sex determination system whereby diploid females develop from fertilized eggs while haploid males develop from unfertilized eggs (Crozier 1977). Both queens and workers have been shown to influence the proportion of new queens and males produced in their colonies. Queens may influence the sex ratio produced by altering the relative proportion of haploid and diploid eggs laid (Passera et al. 2001; Rosset and Chapuisat 2006) while workers may later affect sex ratio by selectively killing males or preferentially rearing females into queens rather than workers (Pamilo 1991; Aron et al. 1995; Passera et al. 1995; Keller et al. 1996; Sundstrom et al. 1996; Hammond et al. 2002). Accordingly, in multiple-queen colonies of ants such as *Linepithema humile*, *Pachycondyla sp.*, *Pheidole pallidula* and *Formica exsecta*, queens vary in their relative contribution to male and female (queens and workers) production (Fournier and Keller 2001; Heinze et al. 2001; Fournier et al. 2004; Kummerli and Keller 2007b). Similarly, a skew in the production of males or new queens has been reported in *L. humile* and *Leptothorax acervorum* (Bourke et al. 1997; Fournier and Keller 2001). However, it remains unknown whether these contribution differences among queens have genetic components and, if so, whether these components are additive or result from epistatic and pleiotropic effects.

The aim of this study is to investigate genetic effects on the process of caste allocation (the relative investment in queen, worker and male production) in an ant species with multiple queens per colony. For this purpose, we conducted controlled crosses in the Argentine ant *L. humile.* Colonies of this species contain numerous reproductive queens (Newell 1909; Markin 1970) and, in contrast to most other ants, it is possible to obtain both males and queens, as well as induce mating, in the laboratory (Keller and Passera 1992). After conducting controlled crosses, we established single-queen colonies to study the effects of

maternal and paternal genetic backgrounds, as well as the interaction between parental genomes on caste allocation.

Methods

Production of parental lineages

We collected *L. humile* colonies on 11 February 2008 in Port-Leucate (3°2'20"E, 42°51'22"N), southern France and set up 13 single-queen colonies with 2.5cm^3 (ca. 1000) workers. To ensure that colonies contained only brood from the mother queen, we removed all the brood present during the first two weeks. The queens were then allowed to lay eggs during eight weeks before being removed so as to stimulate the production of sexuals (new queens and males) (Keller and Passera 1992; Keller and Passera 1993). Colonies were then regularly checked to transfer all male and queen pupae to queenless and broodless recipient colonies, set up to receive the pupae of only a single sex and colony. This allowed us to obtain large numbers of unmated queens and males of the same lineage (i.e., produced by the same mother queen). These individuals were used to conduct the controlled crosses.

Controlled crosses

Of the 26 recipient colonies, six produced enough new queens and four produced enough males to conduct replicate crosses between these maternal and paternal lineages. Mating was obtained by placing one unmated queen with four to six males overnight in a 6.5-cm-diameter vial (Keller and Passera 1992). In *L. humile*, queens are inseminated by only one male even if they mate multiple times (Keller et al. 1992; Krieger and Keller 2000). These crosses allowed us to obtain between two and eight singly inseminated queens for 22 of the 24 possible maternal-by-paternal lineage combinations (Table 1). The 110 newly mated queens were then overwintered with ca. 1000 workers for three months in the dark at 10 ± 2°C, 60% humidity to trigger the production of sexual offspring (Vargo and Passera 1992).

MATERNAL LINEAGES	PATERNAL LINEAGES			
	Pat1	**Pat2**	**Pat9**	**Pat13**
Mat3	4	6	5	-
Mat5	4	3	8	7
Mat6	2	4	6	4
Mat7	-	6	2	5
Mat8	6	6	5	8
Mat12	2	6	5	6

Table 1 - The number of singly mated queens obtained per parental lineages combination. Each of these singly mated queens is a new queen from one of the maternal lineages (rows) inseminated by a male from one of the paternal lineages (columns).

After overwintering each mated queen was placed with a new set of ca. 600 workers (collected randomly in the same stock colony composed of a mix of several field colonies collected on 16 February 2009) and no brood, in 20x14x5cm transparent plastic boxes under a 12hr:12hr artificial light:dark cycle at 25°C, 60% humidity. Colonies were fed a mixture of mealworms, eggs, honey and vitamins three times a week. Queens were allowed to lay eggs during six weeks before being removed. Under field conditions, 90% of the queens are killed by the workers before the beginning of the reproductive season (Markin 1970; Keller et al. 1989). Thus, queen removal mimicked the conditions leading to the production of males and new queens in the field (Keller and Passera 1992; Keller and Passera 1993). Colonies were then monitored weekly to remove all pupae produced. As the pupal stage lasts more than seven days at 25°C in *L. humile* (R. Libbrecht, personal observation), this allowed us to count all queen, worker and male pupae produced, and estimate the worker/queen, male/queen, and male/female ratios.

Statistical analysis

Among the 110 colonies that overwintered successfully, 20 were removed from the analysis: five queens died during the experiment, four colonies did not produce any offspring, and 11 colonies did not produce any female offspring suggesting that the queens were not inseminated. To test for the effect of maternal and paternal lineages (taken as random variables) on colony-level offspring production, we conducted 2-way analyses of variance (ANOVAs) on models optimized to fit our data. The numbers of offspring, females and males were analyzed using a generalized linear model (GLM) with Poisson distributed errors. The worker/queen, male/queen and male/female proportions were analyzed using a GLM with binomial errors. The models were checked for overdispersion and corrected when needed using quasi-likelihood to specify an appropriate variance function. Correlation tests were carried out using Spearman rank correlation tests. All statistical analyses were performed with *R* (http://www.R-project.org).

Results

Every component of caste allocation varied considerably among the single-queen colonies. Both the proportion of the female offspring that developed into queens (female caste ratio) and the proportion of queens among the sexual offspring (sex ratio) ranged from 0 to 1 (female caste ratio: 0.091 ± 0.16 and sex ratio: 0.38 ± 0.36, mean ± sd) while the proportion of females among all the offspring produced ranged from 0.008 to 1 (0.78 ± 0.26, mean ± sd).

For each component of caste allocation, we found significant effects of either the paternal lineage or the interaction between parental lineages. The female caste ratio was significantly influenced by the paternal lineage ($F_{3,12}$ = 6.44, P = 0.007, Figure 1) while there was no significant effect of the maternal lineage ($F_{5,12}$ = 1.77, P = 0.19) and no significant interaction between maternal and paternal lineages ($F_{12,89}$ = 0.75, P = 0.69). The sex ratio and the

proportion of females among the offspring were not significantly influenced by the paternal (queen/male proportion: $F_{3,12}$ = 1.12, P = 0.38; female/male proportion: $F_{3,12}$ = 1.82, P = 0.2) nor the maternal lineage (queen/male proportion: $F_{5,12}$ = 1.18, P = 0.38; female/male proportion: $F_{5,12}$ = 0.70, P = 0.63). By contrast, there were significant interactions between maternal and paternal lineages on both of these proportions (queen/male proportion: $F_{12,88}$ = 2.06, P = 0.032, Figure 2; female/male proportion: $F_{12,89}$ = 3.29, P < 0.001, Figure 3). The effect sizes for the different components of caste allocation are summarized in Table 2.

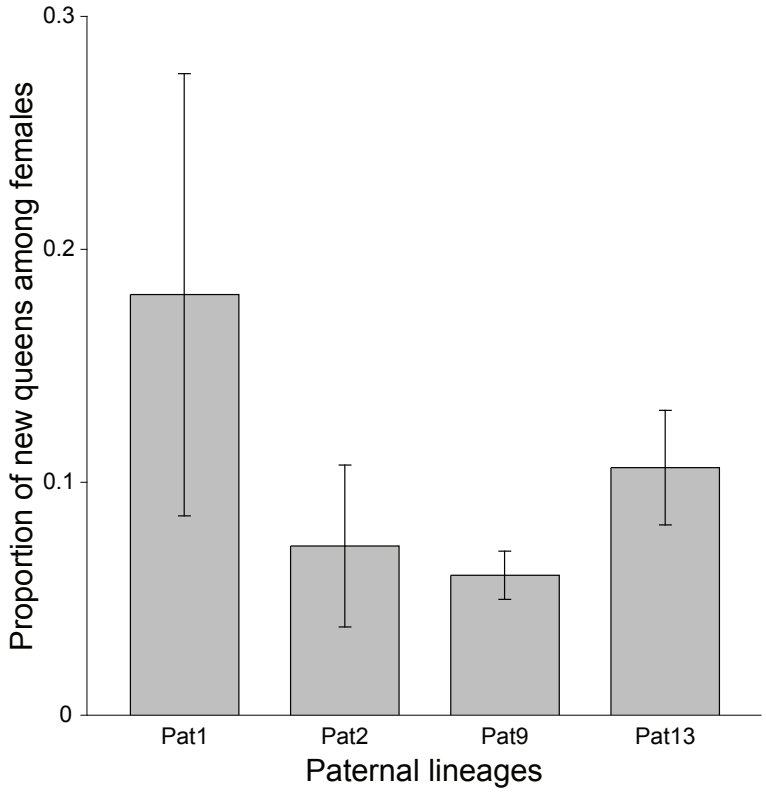

Figure 1 - The proportion of new queens among female offspring is significantly affected by the paternal lineage (mean ± se for each paternal lineage).

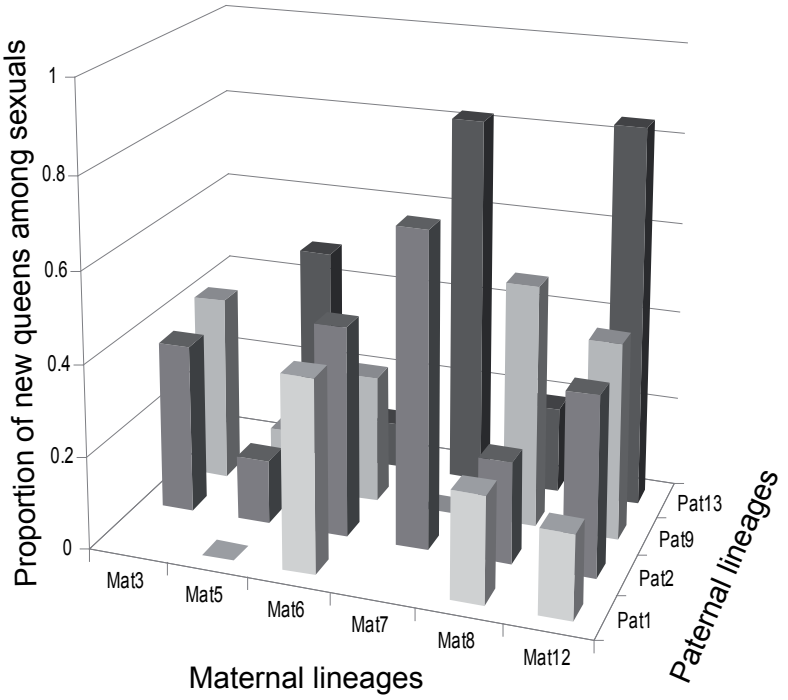

Figure 2 - The proportion of new queens among sexual offspring is significantly affected by the interaction between parental lineages (each bar depicts the mean for all queens per combination of parental lineages).

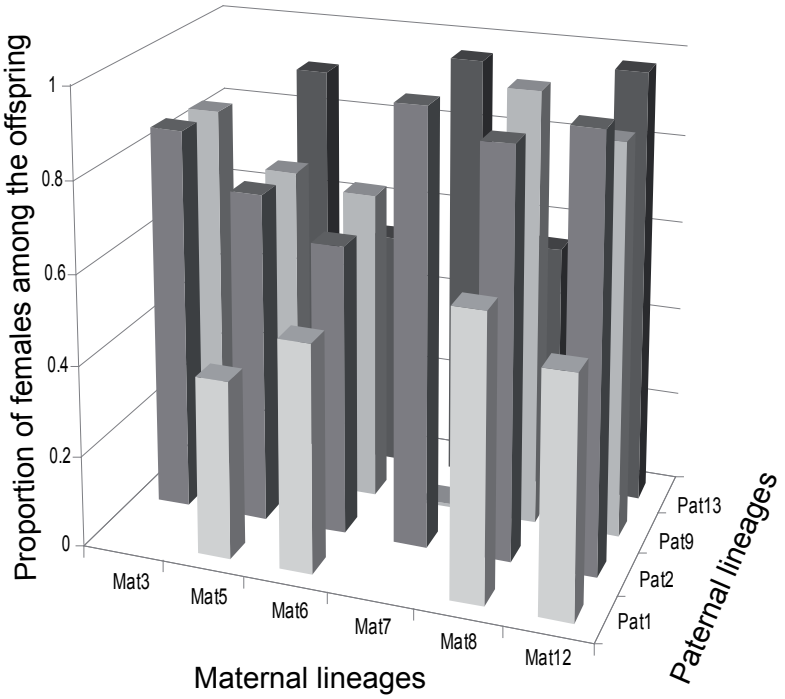

Figure 3 - The proportion of females among offspring is significantly affected by the interaction between parental lineages (each bar depicts the mean for all queens per combination of parental lineages).

Because there appeared to be extreme variation among colonies in the total number of offspring produced (range 23-234; 120.3 ± 45.6, mean ± sd), we also tested for parental lineage effects on numbers of different offspring produced. The total number of offspring was significantly affected by the maternal lineage ($F_{5,12}$ = 3.33, P = 0.04) but not by the paternal lineage ($F_{3,12}$ = 1.35, P = 0.30) nor by the interaction between maternal and paternal lineages ($F_{12,89}$ = 1.74, P = 0.076). By contrast, we found a different pattern when separately analyzing the numbers of males and females produced: neither the paternal (number of males: $F_{3,12}$ = 1.26, P = 0.33; number of females: $F_{3,12}$ = 2.21, P = 0.14) nor the maternal lineage (number of males: $F_{5,12}$ = 0.81, P = 0.56; number of females: $F_{5,12}$ = 1.52, P = 0.25) significantly affected these numbers, while there was a significant interaction between parental lineages (number of males: $F_{12,89}$ = 3.24, P < 0.001; number of females: $F_{12,89}$ = 2.45, P = 0.01). The effect sizes for the numbers of male and female offspring produced are summarized in Table 2.

	Maternal lineage	Paternal lineage	Interaction between maternal and paternal lineages
Relative proportion of new queens and workers	7.08%	**15.43% ****	9.58%
Relative proportion of new queens and males	11.12%	6.35%	**22.68% ***
Relative proportion of females and males	7.86%	12.24%	**26.9% *****
Number of offspring	**22.48% ***	5.46%	16.18%
Number of females	13.09%	11.46%	**20.68% ****
Number of males	9.68%	8.99%	**28.49% *****

Table 2 - Reduction of deviance obtained when the maternal lineage, the paternal lineage or the interaction between parental lineages is added to the model. These percentages thus represent the extent to which an explanatory variable improves the model's ability to account for the empirical data. The significance of maternal and paternal lineage effects and of their interaction is also notified (* = P<0.05; ** = P<0.01; *** = P<0.001).

Finally, to test for possible allocation trade-offs, we analyzed correlations between caste numbers produced. Significant negative correlations revealed trade-offs between queen and male (n = 90, rho = -0.24, P = 0.024, Figure 4), and female and male productions (n = 90, rho = -0.34, P = 0.001, Figure 5). There appeared to be no trade-off between queen and worker production as the number of queens and workers were positively correlated (n = 90, rho = 0.32 , P = 0.002, Figure 6).

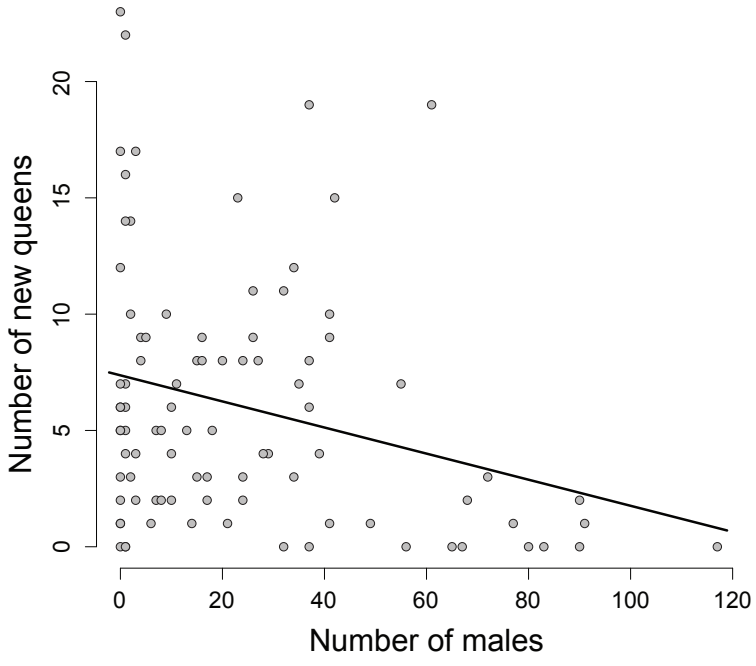

Figure 4 - Across colonies the number of new queens produced is negatively correlated with the number of males produced.

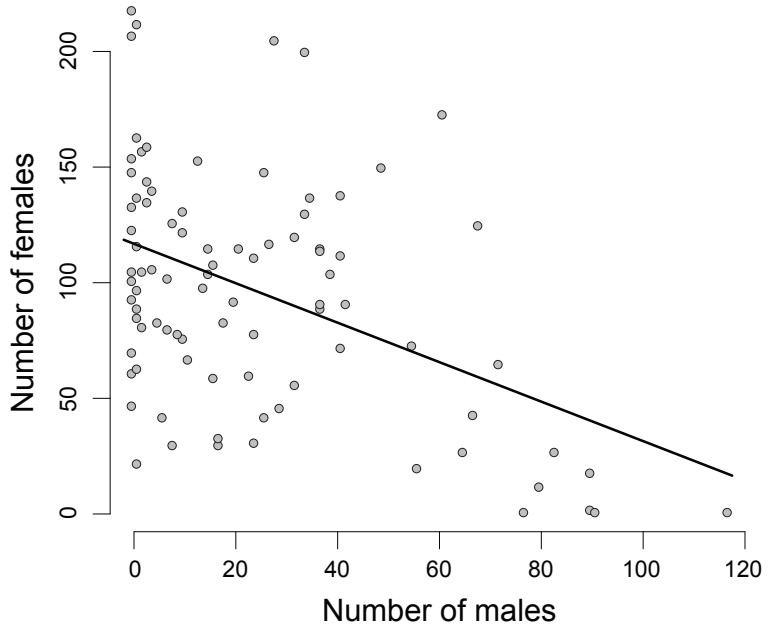

Figure 5 - Across colonies the number of females produced is negatively correlated with the number of males produced.

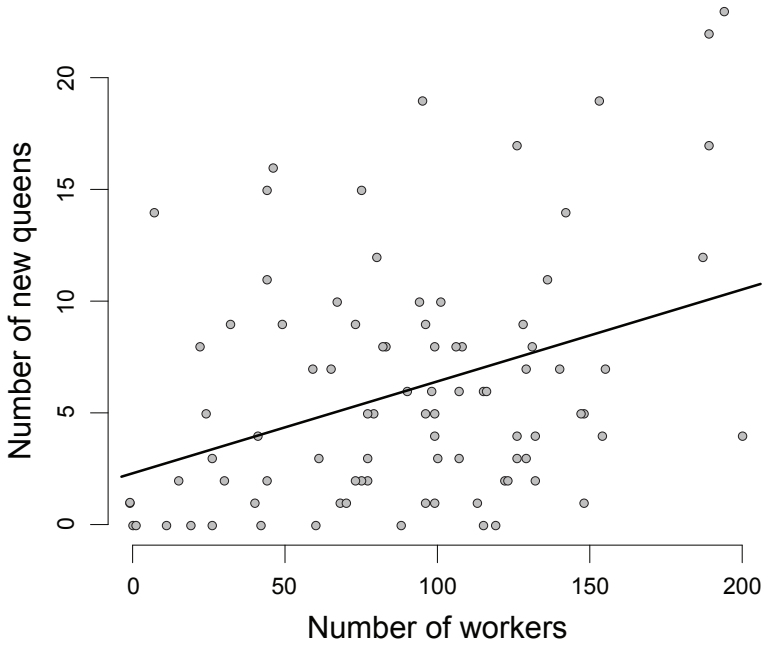

Figure 6 - Across colonies the number of new queens produced is positively correlated with the number of workers produced.

Discussion

This study demonstrates strong effects of the maternal and paternal lineages on offspring production and caste allocation (i.e., the proportion of queens, workers and males produced) in the Argentine ant *L. humile*. The maternal lineage had a significant effect on the number of offspring produced and the paternal lineage influenced the proportion of females developing into queens or workers (i.e., the process of caste determination). There were also significant interactions between parental lineages for the two other components of caste allocation, namely the proportion of offspring being queens or males and the relative production of males and females.

Several lines of evidence suggest that these parental lineage effects have genetic components. First, all the experiments were conducted under highly controlled laboratory conditions, with colonies containing similar numbers of workers, thus largely removing possible environmental effects. Second, the workers that reared the new queens and the males were unrelated to them and all came from the same stock colonies hence insuring a uniform social environment. Finally, after mating, queens of all lineages were placed in new colonies containing a new set of workers coming from the same stock colonies. Again, the number of workers was standardized in all colonies. As a result, mothers, fathers and grandmothers of the broods considered in the analyses were kept under similar environmental and social conditions. This design thus makes it highly likely that parental lineage effects on caste allocation and brood production stem from genetic variation among lineages, even if some environmental influences cannot completely be ruled out. Importantly, a genetic component on sex ratio and caste allocation may stem from both direct and indirect effects. Direct influences could originate from genetic differences in offspring survival and/or development whereas indirect effects could stem from workers altering brood care and/or rearing allocations in response to changes in brood composition (Linksvayer 2006). A combination of direct and indirect effects is also possible. For example, a direct genetic effect inducing a larger proportion of females to develop into queens may reduce the resources

available and lead workers to eliminate a greater proportion of males. In the following sections we discuss in more details the effect of parental lineages on each component of offspring production and caste allocation.

The first interesting finding of our study is that the paternal lineage affected the relative production of queens and workers while no significant effect of the maternal lineage was detected. This pattern reveals that the genetic effects on female caste determination in *L. humile* have a complex architecture, as classic additive effects would imply an influence of both parental lineages. This result is unlikely to stem from little statistical power for detecting maternal lineage effects. Because of haplodiploidy, the proportion of within-lineage additive genetic variation is smaller for the maternal than the paternal lineages (this is because full sisters share a larger portion of their genome than full brothers). As a consequence, additive genetic factors are more likely to generate significant effects of the maternal than the paternal lineage. The influence of only the paternal lineage on female caste fate is thus best consistent with parent of origin specific effects and/or other epigenetic factors. Thus, caste-biasing genes could be expressed in the female brood only if paternally inherited. Alternatively, heritable epigenetic changes that affect the likelihood for the female offspring to develop into queens could be triggered by male-dependent conditions.

One important issue discussed by previous studies that reported genetic effects on female caste determination is the maintenance of genetic variation for the trait, as alleles biasing caste development toward queens should quickly go to fixation (Crozier and Pamilo 1996). Four hypotheses have been proposed to account for the maintenance of genetic variation. First queen-biasing alleles are associated with costs such as decreased colony productivity (Bourke and Ratnieks 1999; Wenseleers and Ratnieks 2004). Second, queen-biasing alleles are deleterious when in the homozygous form (Keller and Ross 1998; Hayashi et al. 2007). Third, under sexual antagonism (Rice 1984), queen-biasing alleles, which are favored in females, decrease male fitness (Moritz et al. 2005). Finally, genetic influences on female caste can be maintained if the genetic architecture underlying caste biasing is complex

(Schwander et al. 2010). The finding of imprinting and/or epigenetic effects on caste determination is very interesting in this perspective because it reveals a new type of genetic influences, that are more complex than additive genetic effects. The traditional method used to test for genetic components to caste determination is to compare the relative representations of patrilines among new queens and workers in species with only one multiply-mated queen per colony (Hughes and Boomsma 2008; Schwander and Keller 2008; Smith et al. 2008a; Frohschammer and Heinze 2009). However, this experimental design does not allow inferring the genetic architecture underlying caste bias. We call for more studies on the influence of genetic architecture on the developmental fate of female brood to get a better understanding of the maintenance of genetic effects on caste determination in social insect species.

Our study also revealed significant interactions between parental lineages on sex ratio (proportion of queens and males) while neither the maternal nor the paternal lineage affected this proportion. The lack of maternal and paternal lineage effects are difficult to interpret given that the source colonies used for setting up the parental lineages already showed a biased sex ratio (male bias for the paternal and queen bias for the maternal lineages). This may have altered the distribution of genetic variability for sex ratio between the maternal and paternal lineages. By contrast, several mechanisms could explain the interaction between parental lineages on sex ratio. Sex ratio could be influenced directly by interactions between parental lineages if compatibility between parental genomes affects the viability of female broods or influences the likelihood of egg fertilization. Alternatively, sex ratio may be influenced indirectly via changes in the proportion of females that develop into queens rather than workers. The latter explanation is unlikely in *L. humile* because, contrary to the proportion of males and females, the proportion of new queens and workers produced was not significantly affected by the interaction between parental lineages. Our analyses of brood numbers are best consistent with compatibility affecting fertilization probability, though additional effects on female brood viability or the worker propensity to preferentially raise

new queens and/or eliminate males remain possible. Indeed, the numbers of males and females produced were negatively correlated and both were significantly affected by interactions between the maternal and paternal lineages. This is the expected pattern if queens laid a fixed number of eggs independently of the ratio of haploid to diploid eggs among them, thereby generating a trade-off in the numbers of males and diploid brood produced. Queens may actively change sex allocation depending on qualities of the sperm transferred by their mate (Fjerdingstad and Boomsma 1997; Fjerdingstad 2004) or fertilization success may be passively influenced by compatibilities between parental genomes. Whatever the detailed mechanism, our results reveal that interactions between queens and males can affect the colony sex ratio. Previous studies showed that the queen influences the colony sex ratio in *S. invicta*, *F. selysi* and *Cardiocondyla kagutsuchi* (Passera et al. 2001; Rosset and Chapuisat 2006; Frohschammer and Heinze 2009). However, because these studies were not designed to detect potential effects of the interaction between the queens and their mates, it is impossible to infer whether the reported queen influences also stem from interaction effects or between-queen differences.

The finding that interactions between the queen and her mate may affect the relative production of new queens and males has important implications for sex ratio and conflict theory in social insects. Because of the haplodiploid mechanism of sex determination, there is a potential conflict between queens and their mates over the sex ratio produced (Haig 1998; Helantera and Ratnieks 2009), as males have all their genes in their daughters but none in the males produced. There is thus strong pressure on males to bias the sex ratio toward females (Haig 1998) while queens should favor balanced sex ratios because they are equally related to their daughters and sons. The finding that the interaction between queens and males can influence the sex ratio produced should be added to the traditional queen/worker framework when studying intra-colonial conflicts in social insects.

Our study also provides a new explanation for why queen in multi-queen societies often tend to specialize in the production of a single caste. For example, queens producing more males

produce fewer queens in *L. humile* (Fournier and Keller 2001) and *F. exsecta* (Kummerli and Keller 2007a; Kummerli and Keller 2007b). A trade-off between the contribution to worker and queen production has been reported in *Pheidole pallidula* (Fournier et al. 2004) and a trade-off between worker and male production in *Leptothorax acervorum* (Hammond et al. 2006). In these studies it was not possible to determine whether the queen specializations (Kummerli and Keller 2007a; Kummerli and Keller 2007b) resulted from competition and social interactions between queens or from intrinsic differences between the broods produced by queens. In our single-queen colonies, we found similar trade-offs for the relative investment into males and queens, as well as males and females, revealing that competition and social interactions between queens are not required to generate specializations. In addition, given that caste allocation in our colonies was also influenced by the interaction between parental lineages, queen specialization reported in the previous studies may at least partly stem from genetic differences. More generally, queen specialization is likely to be affected by complex genetic interactions between the queen and her mate. Such interaction effects between queens and males may help to explain the maintenance of queen specialization in social insect species with multiple queens per colony.

Finally, we also found that the total number of offspring produced in our experimental colonies was affected exclusively by the maternal lineage. This is not surprising given that both the number of eggs produced and the nutrients in the eggs depend on the mother queens. Since males only contribute their sperm to offspring production, paternal effects in this case would have to occur mainly via some type of chemical manipulation of the females, possibly in combination with effects on diploid brood viability. The effect of the maternal lineage on the number of offspring produced thus stems most likely from between-lineage variation in fecundity and/or egg viability. This variation may derive from genetic differences between queens from different lineages (Frohschammer and Heinze 2009), maternal effects (Schwander et al. 2008) and/or different environmental conditions experienced by different lineages during their development. As explained above, all colonies were maintained under

highly controlled conditions so that differences in environmental conditions should only have a minor contribution to differences between lineages compared to genetic or maternal effects.

In conclusion, the use of controlled crosses in the laboratory allowed us to demonstrate widespread effects of both parental genetic backgrounds on several components of caste allocation. Our study provides evidence that non-additive genetic effects account for between-queen and between-colony variations in the caste and sex ratios produced. Such diverse influences of non-additive genetic effects demonstrate overall complex architectures of the genetic components to caste allocation. More such studies are strongly needed to develop insights into the genetics of phenotypic plasticity and caste allocation in social insects.

Acknowledgments

We thank Colby Tanner and two anonymous reviewers for their helpful comments on the manuscript. This research was supported by several grants from the Swiss NSF and an ERC advanced grant.

CHAPTER 2

CHAPTER 2

Genetic compatibility affects division of labor in the Argentine ant *Linepithema humile*

Romain Libbrecht and Laurent Keller

Libbrecht, R. and Keller, L. 2012. Genetic compatibility affects division of labor in the Argentine ant *Linepithema humile*. **Evolution** In press

Abstract

Division of labor is central to the organization of insect societies. Within-colony comparisons between subfamilies of workers (patrilines or matrilines) revealed genetic effects on division of labor in many social insect species. Although this has been taken as evidence for additive genetic effects on division of labor, it has never been experimentally tested. To determine the relative roles of additive and non-additive genetic effects (e.g., genetic compatibility, epistasis and parent-of-origin imprinting effects) on worker behavior, we performed controlled crosses using the Argentine ant *Linepithema humile*. Three of the measured behaviors (the efficiency to collect pupae, the foraging propensity and the distance between non-brood-tenders and brood) were affected by the maternal genetic background and the two others (the efficiency to feed larvae and the distance between brood-tenders and brood) by the paternal genetic background. Moreover, there were significant interactions between the maternal and paternal genetic backgrounds for three of the five behaviors. These results are most consistent with parent-of-origin and genetic compatibility effects on division of labor. The finding of non-additive genetic effects is in strong contrast with the current view and has important consequences for our understanding of division of labor in insect societies.

Introduction

Division of labor is characterized by the performance of different tasks or roles by different groups of individuals. The most striking examples of division of labor in nonhumans occur in insect societies. By enhancing colony performance and homeostasis, division of labor is thought to be one of the main factors responsible for the tremendous ecological success of ants, termites, bees and wasps (Wilson 1971; Oster and Wilson 1979; Holldobler and Wilson 1990).

Task specialization within a colony results from workers differing in their responses to environmental signals indicating colony needs for specific tasks (Wilson 1971; Oster and Wilson 1979; Robinson and Page 1989a). Four main factors are known to influence worker thresholds for particular tasks. The first is worker size and morphology, both of which correlate with worker behavior in almost all species in which this has been studied [e.g., (Wilson 1980; Detrain and Pasteels 1991; Robinson et al. 2009)]. The second is age, as evidenced by workers frequently moving from one task to another as they become older (Wilson 1971; Seeley 1982). The third is individual experience, which has been shown to influence task preference in a few species (Theraulaz et al. 1998; Ravary et al. 2007). Finally, in almost all species studied, the genetic background of workers seems to affect their likelihood to undertake different tasks (Oldroyd and Fewell 2007). Besides some reports of artificial selection for honeybee worker behaviors (Rothenbuhler 1964; Pérez-Sato et al. 2009), evidence for genetic effects on task specialization comes primarily from the finding of differences between subfamilies (matrilines or patrilines) in the worker likelihood and efficiency to perform different tasks in species where colonies contain either several queens or one multiply-mated queen. Such genetic effects on task performance have been shown to influence a wide range of behaviors in ants (Stuart and Page 1991; Snyder 1992; Snyder 1993; Blatrix et al. 2000; Julian and Fewell 2004; Schwander et al. 2005; Waddington et al. 2010), bees (Calderone and Page 1988; Frumhoff and Baker 1988; Robinson and Page

1988, 1989b; Estoup et al. 1994; Kryger et al. 2000; Jones et al. 2004) and termites (Kaib et al. 1996; Goodisman and Crozier 2003).

The finding of between-matriline and between-patriline differences in task performance has lead to the realization that task specialization and division of labor are influenced by genetic effects. Authors usually discussed these effects as if they were simple additive genetic effects. However, the mere demonstration of maternal or paternal effects on division of labor in colonies with several matrilines or patrilines does not allow one to discriminate between additive and non-additive genetic effects such as parent-of-origin specific effects associated with imprinting, epistasis and genetic compatibility effects (Schwander and Keller 2008; Libbrecht et al. 2011). In both cases, one would expect an association between matriline and patriline affiliation and the performance of specific tasks. The only way to discriminate between additive and non-additive effects is to conduct controlled crosses to quantify paternal and maternal effects, as well as the interaction between parental effects.

So far such controlled crosses have only been used twice in social insects, the aim being to investigate the effects of parental genetic backgrounds on various aspects of caste allocation. In the seed-harvester ant *Pogonomyrmex rugosus* the process of caste determination was shown to be strongly influenced by interactions between the parental genomes, but neither by the paternal nor the maternal colony of origin (Schwander and Keller 2008). Similarly, in the Argentine ant *Linepithema humile*, controlled crosses revealed both significant parent-of-origin influences and interactions between parental genomes on various components of caste allocation (Libbrecht et al. 2011). Although there have been numerous studies investigating the roles of paternal and maternal effects on division of labor, surprisingly no controlled crosses have been conducted so far and it remains completely unknown whether genetic effects on division of labor stem from additive effects or more complex, non-additive effects such as parent-of-origin influences or interactions between the parental genomes.

The aim of this study was to conduct controlled crosses in the Argentine ant *Linepithema humile* to investigate the genetic components affecting worker behavior and patterns of division of labor. In contrast to most other ants it is possible to obtain both males and queens in the laboratory and to mate them under controlled conditions (Keller and Passera 1992; Libbrecht et al. 2011). By conducting controlled crosses, we investigated the effects of both the maternal and paternal genetic backgrounds, as well as their interaction, on five measures of worker behavior that were indicators of the likelihood of workers to perform several tasks and their efficiency to perform them.

Methods

Production of parental lineages

The first step of our experiment consisted in producing sufficient numbers of unmated queens and males to conduct the controlled crosses in the laboratory. To this end, we collected *Linepithema humile* colonies on February 11, 2008 in Port-Leucate (3°2'20"E, 42°51'22"N), southern France and established 13 single-queen colonies with each 2.5cm3 (ca. 1000) workers in the laboratory. Colonies were kept under a 12hr:12hr artificial light:dark cycle at 25°C, 60% humidity and were fed a mixture of mealworms, eggs, honey and vitamins three times a week. To ensure that colonies contained only brood from the mother queen, we removed all the brood present during the first two weeks. The queens were then allowed to lay eggs during eight weeks before being removed so as to stimulate the production of new queens and males (Keller and Passera 1992; Keller and Passera 1993). Colonies were then regularly checked to transfer all the male and queen pupae produced to queenless and broodless recipient colonies, set up to each receive the pupae of only a single sex and colony. This design allowed us to obtain large numbers of unmated queens and males of the same lineage (i.e., produced by the same mother queen). These individuals were used to conduct the controlled crosses.

Controlled crosses

Of the 26 (13 female and 13 male) recipient colonies, six produced enough new queens and four produced enough males to conduct replicate crosses between these maternal and paternal lineages. Mating was obtained by placing one unmated queen with four to six males overnight in a 6.5cm-diameter vial (Keller and Passera 1992). In *Linepithema humile*, queens are inseminated by a single male (Keller et al. 1992; Krieger and Keller 2000). The crosses allowed us to obtain between two and eight singly-inseminated queens for 22 of the 24 possible maternal-by-paternal lineage combinations. The 110 newly mated queens were then overwintered with ca. 1000 workers for three months in the dark at 10±2°C, 60% humidity to trigger the production of sexual offspring (Vargo and Passera 1992) which were used in another study (Libbrecht et al. 2011).

Single-cohort colonies

Worker behavior is known to be affected by age (Wilson 1971; Seeley 1982) and experience (Theraulaz et al. 1998). To control for these factors, all experiments were performed on single-cohort colonies (Giray and Robinson 1994) consisting of groups of workers of the same age with similar nursing and foraging experience. To produce such workers, each mated queen was placed with a new set of ca. 600 workers (collected randomly in a stock colony composed of a mix of several field colonies collected on February 16, 2009) and no brood, in 20x14x5cm transparent plastic boxes. To set up the single-cohort colonies, 50 worker pupae were collected from each colony and isolated with 5-7 marked workers which took care of them, ensuring the emergence of adult workers. The marked workers were removed from the single-cohort colonies as soon as the first workers emerged from the pupae. The workers used in the behavioral experiments were collected in the single-cohort colonies four weeks after the isolation of pupae. We quantified the efficiency of workers to feed larvae, their speed to collect pupae, their foraging propensity and their average distance to the brood.

Efficiency to feed larvae

The efficiency to feed larvae was assessed by measuring the mass gained by larvae when tended by workers for three days. For each single-cohort colony, 20 workers were collected and introduced in a 5cm-diameter plastic box containing 10 third-instar larvae randomly collected from a stock colony. The workers and larvae were kept for three days in complete darkness with water and food very close to the larvae, so that the workers did not have to forage. The larvae were weighed before and after the three days using a microbalance (Mettler Toledo MT5) to a precision of 1µg. The difference between the initial and final larval mass was used as a proxy for the efficiency of worker feeding behavior.

Efficiency to collect pupae

The efficiency to collect pupae was quantified as the time needed by 20 workers to collect 12 pupae. For each single-cohort colony, 20 workers were anesthetized with CO_2 and introduced in an arena (5x10.5cm) containing 12 pupae evenly distributed around the center of the arena. All these pupae were randomly collected from a stock colony. We videotaped the arena in the dark using an infrared camcorder (Sony HDR-XR200) and recorded the time needed to collect the twelfth pupae once the first worker woke up.

Foraging propensity

Once the workers had gathered the pupae in one or several piles, some workers tended to stay close to the brood (later referred to as brood-tenders) while others tended to move away from the brood and walk around the arena (later referred to as non-brood-tenders). Once the workers had gathered all the pupae we extracted, for each colony, screenshots for 20 time points in the dark (every 30s for 10 minutes) and 12 time points in the light (every 10s for 1 minute after the light was switched on and every 30s for the next 3 minutes). For each screenshot, we recorded the numbers of brood-tenders, defined as any worker with less than one ant-length to the nearest pupa, and non-brood-tenders, defined as all other workers with

more than one ant-length to the nearest pupa. The proportion of non-brood-tenders was used as a proxy for the foraging propensity.

Distance to the brood

The distance between workers and brood was measured for both brood-tending and non-brood–tending workers. We used the same screenshots (32 time points) as for the foraging propensity to record the distance between workers and brood in the dark and in the light. The software ImageJ (http://rsbweb.nih.gov/ij) was used to collect the spatial coordinates of each worker, as well as the center of each pile of pupae. These coordinates were then used to infer the distance between each worker and the center of the closest pile of pupae. Brood-tenders and non-brood-tenders were analyzed separately.

Statistical analysis

Among the 110 colonies that overwintered successfully, four colonies did not produce any offspring, 11 colonies did not produce any females (perhaps because the queens were not inseminated), 25 colonies did not produce enough brood to set up a single-cohort colony with 50 pupae and five colonies lost their queen. Therefore, a total of 65 single-cohort colonies could be used for all behavioral experiments, except the nursing of larvae and collection of pupae experiments for which 2 and 7 colonies, respectively, were discarded for technical reasons. To test for the effect of paternal and maternal lineages on worker behavior (as well as time when required), we conducted two-way analyses of variance (ANOVAs) on linear models optimized to fit our data. When needed, the data were transformed so that the residuals of the models followed a normal distribution. The proportion of non-brood-tenders and the distance between workers and brood were analyzed using a linear mixed-effect model with a Satterthwaite compilation of degrees of freedom. For each response variable, we used a single value (proportion or mean) per time point per colony and we specified the colony as a random factor to avoid pseudoreplication and take into account the non-independence of repeated measures. The time to collect pupae and the difference in larval

mass were analyzed using a linear model. Correlation tests were carried out using Spearman rank correlation tests.

Results

Our measures of worker behavior revealed that worker likelihood and efficiency to perform tasks varied greatly among the single-cohort colonies. The change in larval mass over three days varied from -0.44 to 0.69mg (0.17 ± 0.23, mean ± sd), the time to collect pupae from 227 to 1063s (515 ± 221), the proportion of non-brood-tenders from 0.1 to 0.8 (0.41 ± 0.12), the distance between workers and the center of the pile of pupae from 0.01 to 1.07cm (0.28 ± 0.14) for brood-tending and 0.21 to 8.6cm (2.19 ± 1.74) for non-brood tending individuals. There was a significant positive correlation between normal conditions (darkness) and stressful conditions (light) for the foraging propensity (rho = 0.64, $p < 0.0001$), and the distance between brood and either brood-tenders (rho = 0.83, $p < 0.0001$) or non-brood-tenders (rho = 0.59, $p < 0.0001$).

The maternal lineage had a significant effect on three of the five behavioral measures. These were the time needed to collect the 12 pupae (Figure 1, $F_{5,52} = 2.71$, $p = 0.03$), the proportion of non-brood-tenders (Figure 2, $F_{5,341} = 3.62$, $p = 0.003$) and the distance between non-brood-tenders and the brood (Figure 3, $F_{5,872} = 6.75$, $p < 0.0001$). The paternal lineage had no significant effect on any of these measures but significantly affected the two others, namely the change over time in the larval mass (Figure 4, $F_{3,44} = 3.30$, $p = 0.026$) and the distance between brood-tenders and the center of the closest pile of pupae (Figure 5, $F_{3,141} = 8.98$, $p < 0.0001$).

The interaction between parental lineages significantly affected three of the five measures of behavior. These were the change in larval mass over three days (Figure 4, $F_{10,44} = 2.22$, $p = 0.033$), the proportion of non-brood-tenders (Figure 2, $F_{10,341} = 2.3$, $p = 0.01$), and the distance between brood-tenders and the brood pile center (Figure 5, $F_{10,141} = 2.79$, $p = 0.0035$).

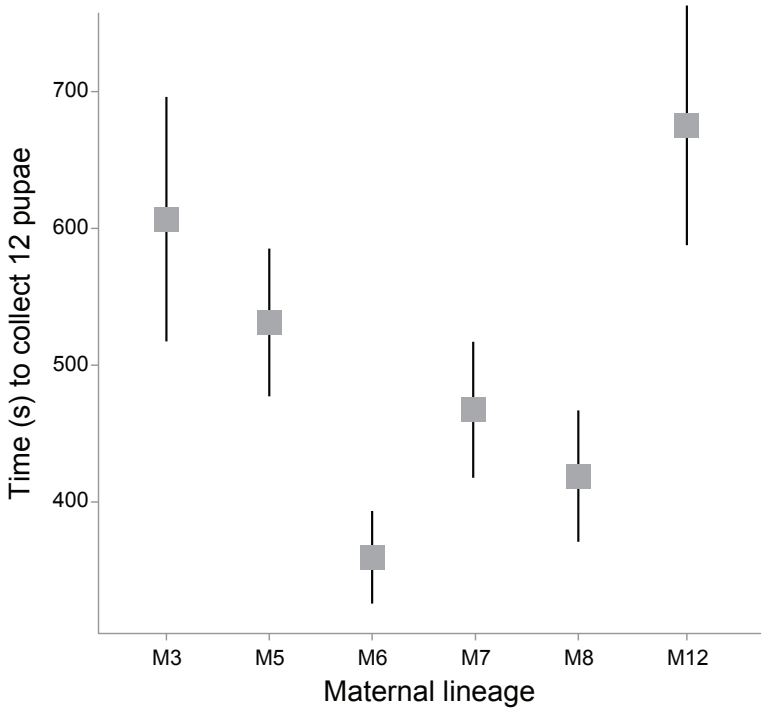

Figure 1 - The time to collect pupae (mean ± se) is significantly affected by the maternal lineage.

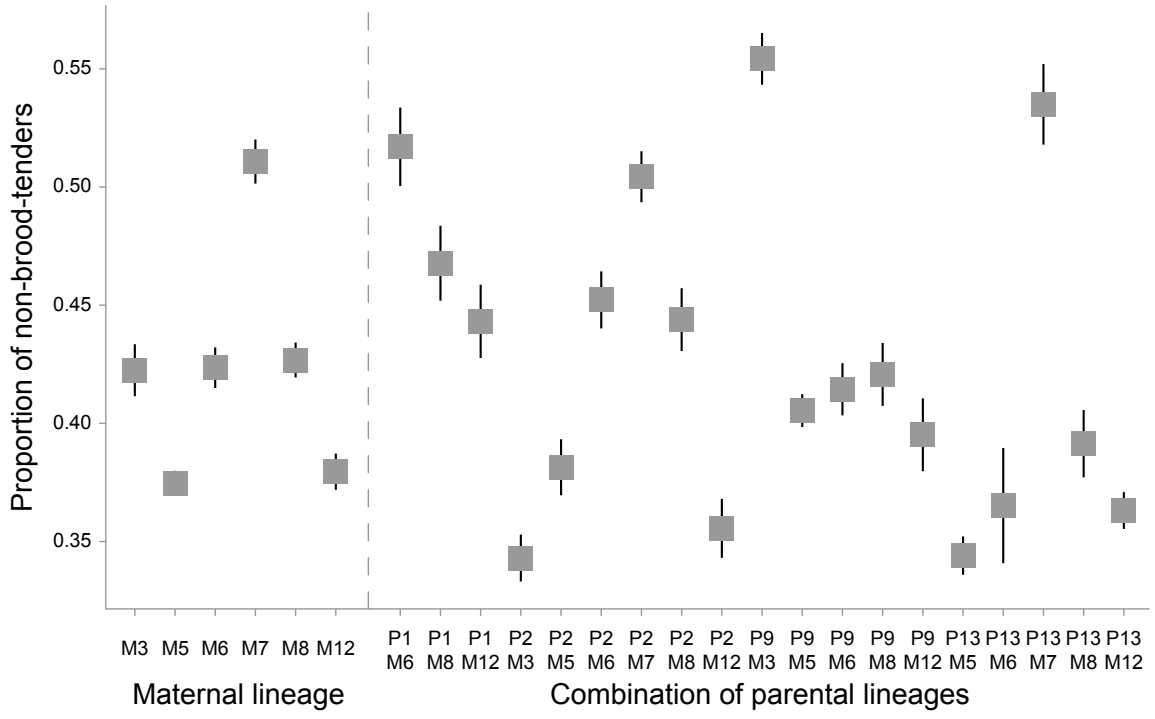

Figure 2 - The proportion of non-brood-tenders (mean ± se) is significantly affected by the maternal lineage and by the interaction between parental lineages.

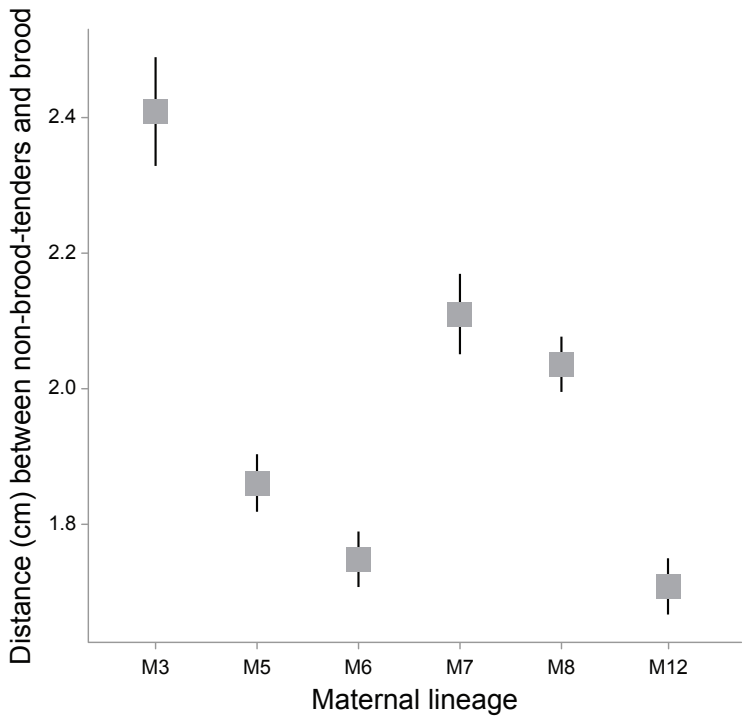

Figure 3 - The distance between non-brood-tenders and the brood (mean ± se) is significantly affected by the maternal lineage.

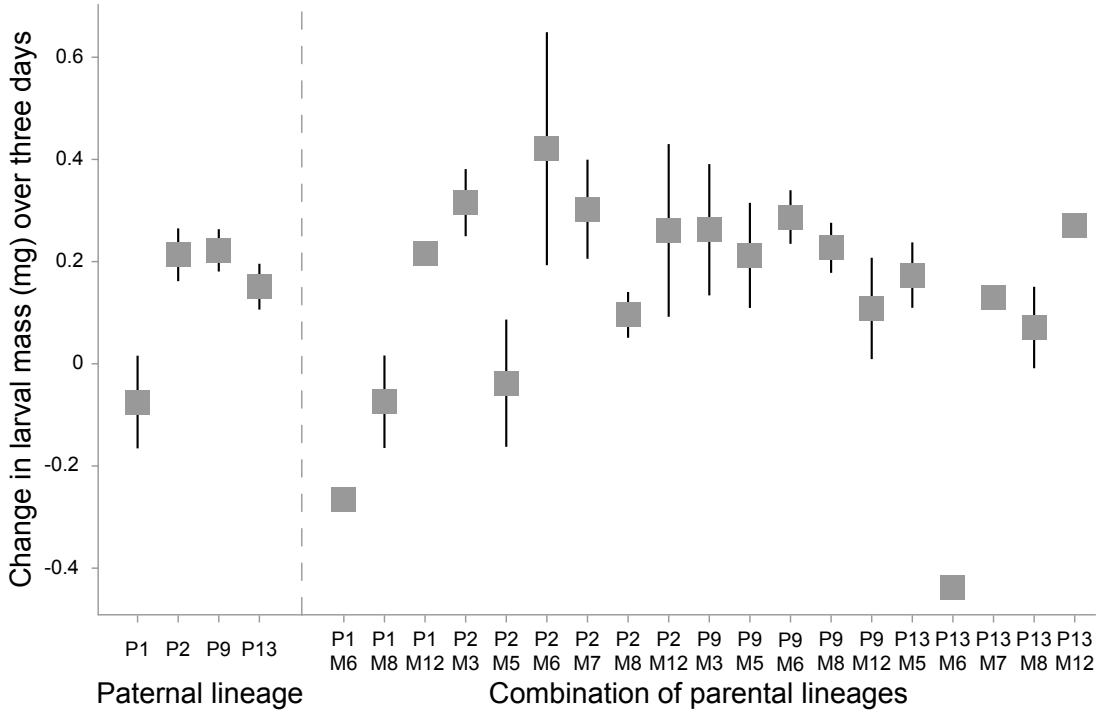

Figure 4 - The change in larval mass over three days (mean ± se) is significantly affected by the paternal lineage and by the interaction between parental lineages.

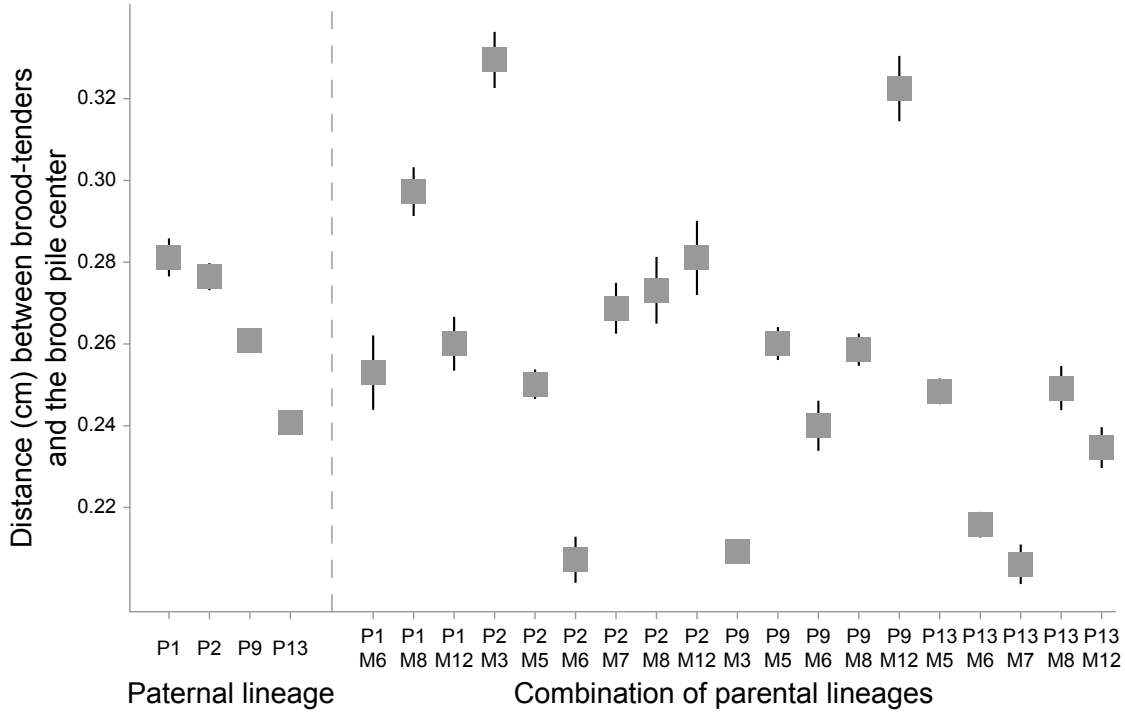

Figure 5 - The distance between brood-tenders and the brood pile center (mean ± se) is significantly affected by the paternal lineage and by the interaction between parental lineages.

Discussion

This study demonstrates strong effects of the maternal and paternal lineages on worker behavior in the Argentine ant *Linepithema humile*. While the maternal lineage affected the efficiency to collect pupae, the foraging propensity and the distance between non-brood-tenders and the brood, the paternal lineage influenced the efficiency to feed larvae and the distance between brood-tenders and the brood pile center. Furthermore, there was a significant interaction between maternal and paternal lineages in the efficiency to feed larvae, the foraging propensity and the distance between brood-tenders and the brood pile center.

Three lines of evidence indicate that the effects of the parental lineages are mostly or only due to genetic influences. First, the experiments were performed on workers that developed in colonies that experienced the same environment (colony size, food, temperature, humidity, light cycle). Second, the use of single-cohort colonies allowed us to ensure that the workers were of the same age and had similar nursing and foraging experience at the time of the

experiments. Finally, while we cannot completely rule out the existence of maternal effects during oviposition or larval development, the paternal effects must have been of genetic nature only, as males died after mating and never encountered their progenies.

The finding that three of the five behavioral measures were affected by the maternal lineage and the two others by the paternal lineage is surprising. If there were additive genetic effects on worker behavior, one would expect a given behavior to be similarly affected by the maternal and paternal lineages (Schwander and Keller 2008; Libbrecht et al. 2011). The finding of all tested behaviors being affected by either the maternal or the paternal lineage is best explained by parent-of-origin specific effects through epigenetic changes. This interpretation is consistent with recent findings indicating that social insects are capable of epigenetic modifications. First DNA methylation was found to occur widely among ants, bees and wasps (Kronforst et al. 2008). Second the genome sequences of several bee and ant species revealed the conservation of a DNA methylation toolkit across social Hymenoptera (Wang et al. 2006; Bonasio et al. 2010; Nygaard et al. 2011; Smith et al. 2011a; Smith et al. 2011b; Suen et al. 2011; Wurm et al. 2011; Gadau et al. 2012). Parent-of-origin specific effects could occur if genes that affect behavior were imprinted and differed in expression depending on whether they were maternally or paternally inherited, as found in mammals (Isles et al. 2006; Garfield et al. 2011).

Importantly, this study also revealed that three of the five measured behaviors (the efficiency to feed larvae, the foraging propensity and the distance between brood-tenders and the brood pile center) were significantly affected by the interaction between parental lineages. This indicates widespread genetic compatibility effects on worker behavior. These findings are important, in particular because the worker intrinsic propensity and efficiency to perform a given task are the basis of division of labor in social insects (Wilson 1971; Oster and Wilson 1979).

The finding of non-additive genetic effects, such as parent-of-origin and genetic compatibility effects on behavior have important implications for our understanding of the role of genetic diversity in social insects. Genetic effects on division of labor have been documented in many species of social insects, primarily through the finding of within-colony behavioral differences between matrilines or patrilines (Oldroyd and Fewell 2007). As these genetic effects were typically assumed to be additive, it was argued that an increase in genetic diversity among the worker force would increase behavioral diversity and colony performance (Crozier and Page 1985). In line with this view a greater genetic diversity has been shown to facilitate division of labor (Page and Robinson 1991; Mattila and Seeley 2007; Oldroyd and Fewell 2007) and enhance colony performance (Oldroyd et al. 1992; Fuchs and Schade 1994; Page et al. 1995; Costa and Ross 2003; Jones et al. 2004; Wiernasz et al. 2004; Mattila and Seeley 2007; Oldroyd and Fewell 2007) in some social insects. However, in some other species, studies failed to find a link between genetic diversity and colony performance (Sundstrom and Ratnieks 1998; Fjerdingstad et al. 2003; Rosset et al. 2005; Fournier et al. 2008). The discrepancy between these studies could stem from between-species differences in the genetic architecture underlying division of labor. Of interest would be to investigate whether additive and non-additive genetic effects on division of labor produce different links between genetic diversity and colony performance, and to what extent such differences impact colony performance.

In conclusion, the use of controlled crosses allowed us to investigate for the first time the genetic architecture of division of labor in a social insect species. Maternal and paternal effects were found to each affect several behaviors but surprisingly, no behavior was simultaneously affected by both the maternal and paternal lineages. Such a pattern is not expected under simple additive genetic effects and rather suggests parent-of-origin specific effects on behavior. Our study also revealed genetic compatibility effects between parental genomes on three of the five behaviors recorded. These findings, together with the recent empirical (Schwander and Keller 2008; Libbrecht et al. 2011) and theoretical (Dobata and

Tsuji 2012) documentation of similar effects on caste and sex allocation, indicate that non-additive genetic effects play an important and unrecognized role in the organization of social insect colonies. Such effects are likely to have important implications in our understanding of social organization, the resolution of kin conflicts, the maintenance of multiple mating and other important issues in social insect biology.

Acknowledgments

We thank Bill Hughes, Eric Lucas, Tanja Schwander and two anonymous reviewers for their helpful comments on the manuscript. This research was supported by several grants from the Swiss NSF and an ERC advanced grant.

CHAPTER 3

CHAPTER 3

Interplay between insulin signaling, juvenile hormone and vitellogenin regulates maternal effects on polyphenism in ants

Romain Libbrecht, Miguel Corona, Dihego Azevedo, Jose Serrão and Laurent Keller

Abstract

Polyphenism is the phenomenon where alternative phenotypes are produced by a single genotype in response to environmental cues. An extreme case is found in social insects, where reproductive queens and sterile workers that greatly differ in morphology and behavior can arise from a single genotype. The first experimental evidence for maternal effects on caste determination, the differential larval development toward the queen or worker caste, was recently documented in *Pogonomyrmex* seed-harvester ants, in which only colonies with a hibernated queen produce new queens. However, the proximate mechanisms behind these intergenerational effects have remained elusive. We used a combination of artificial hibernation, hormonal treatments, gene expression analyses and vitellogenin quantification to investigate how the combined effect of environmental cues and hormonal signaling affects the process of caste determination in *Pogonomyrmex* ants. The results show that the interplay between insulin signaling, juvenile hormone and vitellogenin regulates maternal effects on the production of alternative phenotypes and set vitellogenin as a likely key player in the intergenerational transmission of information. This study reveals how environmental cues experienced by one generation can translate into phenotypic variation in the next generation.

Introduction

Polyphenism, whereby alternative phenotypes are produced by a single genotype (Michener 1961; Mayr 1963; Stearns 1989; West-Eberhard 1989), allows adequate responses to environmental cues such as temperature, nutrition and population density in many species (Simpson et al. 2011). The most striking example of polyphenism is found in insect societies (Wheeler 1986), where the reproductive division of labor implies the coexistence of fertile queens and sterile workers that greatly differ in morphology and behavior (Wilson 1971; Holldobler and Wilson 1990). Even though recent studies revealed genetic influences on caste determination in social insects [reviewed in (Schwander et al. 2010)], female caste fate is primarily influenced by environmental factors in most species studied (Winter and Buschinger 1986; Keller et al. 1997; Moritz et al. 2005; Hartfelder et al. 2006; Hayashi et al. 2007; Hughes and Boomsma 2008; Schwander and Keller 2008; Smith et al. 2008a; Frohschammer and Heinze 2009; Koyama et al. 2009; Rabeling et al. 2009; Libbrecht et al. 2011).

Many studies have focused on the proximate mechanisms regulating the development of alternative phenotypes in response to environmental changes [reviewed in (Simpson et al. 2011)]. In social insects, the honeybee *Apis mellifera* has been the primary focus of studies of caste differentiation. In this species, worker-triggered differences in larval diet affect the insulin/insulin-like growth factor signaling (IIS) pathway (Wheeler et al. 2006; Patel et al. 2007; de Azevedo and Hartfelder 2008; Mutti et al. 2011), which is known to regulate the release of neuropeptides (e.g. allatostatin, allatotropin) that affect the production of juvenile hormone (JH) by the corpus allatum (Tu et al. 2005; Mutti et al. 2011). Changes in JH modulate the expression of several genes (Dubrovsky et al. 2000; Li et al. 2007; Minakuchi et al. 2008), including genes regulating vitellogenesis (Comas et al. 1999; Tatar et al. 2001), resulting in differences in vitellogenin (Vg) levels between honeybee queen- and worker-destined brood (Barchuk et al. 2002). The same pathways may also play a role in the regulation of caste differentiation in ant larvae, as caste-specific expressions of genes

involved in the IIS pathway were documented in *Solenopsis invicta* (Lu and Pietrantonio 2011) and *Diacamma* sp. (Okada et al. 2010). Interestingly, caste-specific differences in IIS, JH and Vg were also documented in adult ants and bees (Amdam et al. 2004; Amdam et al. 2007; Corona et al. 2007; Nelson et al. 2007; Ament et al. 2008; Nilsen et al. 2011; Wurm et al. 2011), suggesting further roles of these pathways in the regulation of social life (Amdam et al. 2003; Amdam et al. 2004).

Maternal effects on polyphenism, through which the environment experienced by the mother is translated into phenotypic variation in the offspring, have long been documented in solitary insects but their proximate mechanisms remain poorly understood (Hunter-Jones 1958; Sutherland 1969; Saiful Islam et al. 1994; Miller et al. 2008). In ants, several studies suggested that maternal factors such as temperature or queen age may affect caste determination (Gösswald 1951; Bier 1954; Petersen-Braun 1977; Passera 1980; Vargo and Passera 1992). However, it is only recently that the first example of maternal effects on female caste polyphenism was documented experimentally (Schwander et al. 2008). Cross-fostering of eggs between hibernated and non-hibernated *Pogonomyrmex* colonies revealed strong maternal effects on caste production, as only eggs produced by an hibernated queen had a chance to develop into queens, irrespective of the hibernation status of the rest of the colony (Schwander et al. 2008). Such maternal effects on the caste fate of the female offspring require that the hibernation triggers changes in the queen that regulate polyphenism in the offspring. JH may be involved in this process, as *Pogonomyrmex* queens treated with JH were found to produce bigger workers (Cahan et al. 2011).

We propose that the interplay between IIS, JH and Vg regulates maternal effects on caste polyphenism by translating the environmental conditions experienced by the queen into the production of alternative phenotypes in the offspring. Under this hypothesis, IIS would translate environmental cues into changes in JH and JH would affect the production of Vg. The Vg content in queens would then influence the Vg content in eggs, thus affecting the caste fate of the offspring. This hypothesis makes four predictions. First, a pharmacological

increase of JH in queens should mimic the effect of hibernation and stimulate the production of queens. Second, hibernation should affect IIS and the production of JH in queens. Third, both hibernation and a JH increase should stimulate the production of Vg in queens. Finally, Vg content should differ between queen- and worker-destined eggs. We tested these predictions by performing artificial hibernation, hormonal treatments, gene expression analyses and vitellogenin quantification in *Pogonomyrmex rugosus*, an ant species where temperature-triggered changes in the queen had previously been shown to affect the relative production of queens and workers. Each of the four predictions was confirmed by our experiments thus revealing that the interplay between IIS, JH and Vg regulates maternal effects on caste polyphenism in *P. rugosus*.

Methods

Ant collection

Pogonomyrmex rugosus founding queens were collected during nuptial flights on July 15th, 2008 in Bowie, Arizona, USA (N32°18'54''//W109°29'03''). After worker eclosion, the colonies were kept in laboratory conditions (30°C, 60% humidity and 12h/12h light:dark cycle) in plastic boxes containing a nest, a foraging area and water tubes, and were fed once a week with grass seeds and a mixture of eggs, honey and crushed mealworms. The experiments were performed on 92 2.5 years old colonies that had never been exposed to cold and never produced queens. The colonies were divided in four groups: acetone (n=26), non-acetone (n=15), hibernation (n=25) and methoprene (n=26).

Experimental manipulations

The first phase was set up to test the effect of an exposure to cold. Colonies from the hibernation group were kept for 2.5 months in a dark climate chamber at 13°C ± 1°C and 60% humidity. The transition to and out of hibernation was done over a period of two weeks by progressively decreasing or increasing temperature in a 8h/16h light:dark cycle. All the

other colonies (acetone, non-acetone and methoprene groups) were kept in the usual laboratory conditions (30°C, 60% humidity and 12h/12h light:dark cycle). The first phase terminated at week 0, when the second phase started. The second phase was set up to test the effect of JH treatment. To do so we used methoprene (Sigma-Aldrich), a synthetic analog of JH. The colonies from the methoprene group were fed four mealworms crushed with 0.1mg of methoprene in 0.1 ml of acetone each week for eight weeks (from week 0 to week 7). Colonies from the hibernation and acetone groups received four mealworms crushed in acetone, while colonies from the non-acetone group received four crushed mealworms without acetone. The proportion of queens produced did not differ significantly between the acetone and non-acetone groups (acetone: n = 26; non-acetone: n = 15; Mann-Whitney U-test: U = 204, P = 0.15). Thus, all further mentions of a control group will refer to the acetone group.

Sample collection

Samples were collected in each colony to assess the proportion of queens among the female offspring produced, the number, size and Vg content of eggs produced, the expression of candidate genes and the JH and ecdysteroid titers in queens. All the pupae produced were collected from week 3 until no brood remained and observations of size and morphology allowed the assignation of each pupae to the queen or worker caste. The proportion of queens among the offspring produced was then calculated for each colony (except one which did not produce enough offspring; control: n= 26, hibernation: n= 25, methoprene: n= 25). At week 4, the queen of each colony was isolated for 24 hours in a 2ml plastic tube closed with wire mesh and placed in the colony. Thus, the queen could still communicate with workers, reducing the stress of isolation. This method allowed us to collect and count the number of eggs produced by each queen in 24 hours (control: n= 26, hibernation: n= 25, methoprene: n= 25). At week 5, a batch of eggs was collected in each colony (between 5 and 52 eggs per colony; 26.1 ± 8.9, mean ± sd) and weighed using a microbalance (Mettler Toledo MT5) to a precision of 1 µg (control: n= 26, hibernation: n= 25, methoprene: n= 25).

The eggs were then stored at -80°C for further measurement of vitellogenin content, successfully performed on eggs produced by 40 colonies (control: n= 15, hibernation: n= 11, methoprene: n= 14). At week 7, the queen was collected in each colony, flash-frozen in liquid nitrogen and stored at -80°C for later RNA extraction (control: n= 13, hibernation: n= 13, methoprene: n= 13).

Gene expression analysis

Whole body queen samples were used to measure the expression of genes involved in the IIS pathway (two insulin-like peptide genes: *ILP1* and *ILP2*), JH production (one gene coding for JH epoxidase: *JHepox*) and vitellogenesis (two vitellogenin genes: *Vg1* and *Vg2*). RNA extractions were performed using a modified protocol including the use of Trizol (Invitrogen) for the initial homogenization and the RNeasy plus micro extraction kit (Qiagen). For each individual queen, cDNAs were synthesized using 500 ng of total RNA, random hexamers and Applied Biosystems reagents. Levels of mRNA were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) using ABI Prism 7900 sequence detector and SYBR green. All qPCR assays were performed in triplicates and subject to the heat-dissociation protocol following the final cycle of the qPCR in order to check for amplification specificity. qRT-PCR values of each gene were normalized by using an internal control gene (*RP49*). Paralog-specific primers (sequences available in appendix 1) were designed using sequence alignment (Thompson et al. 1997) and primer analysis (Rychlik 2007) programs. Primer sequences overlapped coding regions split by introns, allowing the specific amplification of cDNA levels over potential genomic DNA contaminations. Transcript quantification calculations were performed by using the ΔΔCT method (Livak and Schmittgen 2001).

Vitellogenin content in eggs

The eggs were macerated in 100 µL of extraction buffer (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 2.5 mM EDTA, 0.5% Tritom X-100, 5% glycerol), followed by centrifugation at 8000 g for 10 min. The supernatant was collected and the amount of total proteins was measured by the

Bradford method (Bradford 1976), using a BSA standard curve. The amount of Vg was calculated by dot-blotting using *Ectatomma tuberculatum* (Formicidae: Ectatomminae) anti-Vg antibodies (Azevedo et al. 2011). Different dilutions of *E. tuberculatum* Vg (ranging from 0,003 to 45 µg of proteins) and 10 µg of total protein of each *P. rugosus* egg extract were applied (2 µL/dot) on a nitrocellulose membrane. The membrane was incubated with 5% nonfat dry milk in PBST (0.1M PBS pH 8.0 plus 0.1% Tween-20) for 1 hour, followed by 1h30 incubation with rabbit anti-vg antibody diluted 1:500 in PBST plus 2.5% nonfat dry milk, washing 3 times in PBST and incubating for 1h30 with anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) diluted 1:5000 in PBST + 2.5% nonfat dry milk, washing 3 times with PBST and revelation with DAB/H_2O_2 solution. The membrane was dried and scanned, and the optical density of the dots analyzed with the software ImajeJ (http://rsbweb.nih.gov/ij/) after background correction and calculation of the median grey value. The *E. tuberculatum* Vg dilutions were used to build a standard curve plotting Vg quantity against the optical density of the dot (median grey value). The calculation of Vg amount per total protein in each egg extract was based on this curve.

Statistical analyses

To test for the effect of the treatments on the proportion of queens among the offspring, gene expression and egg number and weight, we conducted analyses of variance (ANOVAs) on models optimized to fit our data. The proportion of queens was fit using a generalized linear model with quasi-binomial errors. The gene expression data were fit using a general linear model with normal errors. The Vg measures could not be normalized and were analyzed using Kruskal-Wallis and Mann-Whitney non-parametric tests. All statistical analyses were performed with R (http://www.R-project.org).

Results

To investigate the mechanisms of caste allocation, we compared the production of queens between control, hibernated and methoprene-treated *P. rugosus* colonies. There was a great variation among colonies in the proportion of queens among the offspring produced, ranging from 0 to 0.47 (0.05 ± 0.11, mean ± sd). There was a significant effect of the treatments on the proportion of queens produced ($F_{2,73}$ = 40.51, P < 0.001; Figure 1). Hibernation significantly increased the proportion of queens among the female offspring (t = 2.06, P = 0.04). The methoprene treatment had a similar – albeit stronger – effect, as the queen/worker ratio among the female offspring was significantly higher in colonies fed methoprene-treated food compared to control colonies (t = 5.39, P < 0.001). When only pupae that did not receive any treatment during larval development but were produced by treated queens (thus those collected after week 11) were considered, there was also a significant difference between control and methoprene-treated colonies in the proportion of queens produced (t = -5.56, P < 0.001).

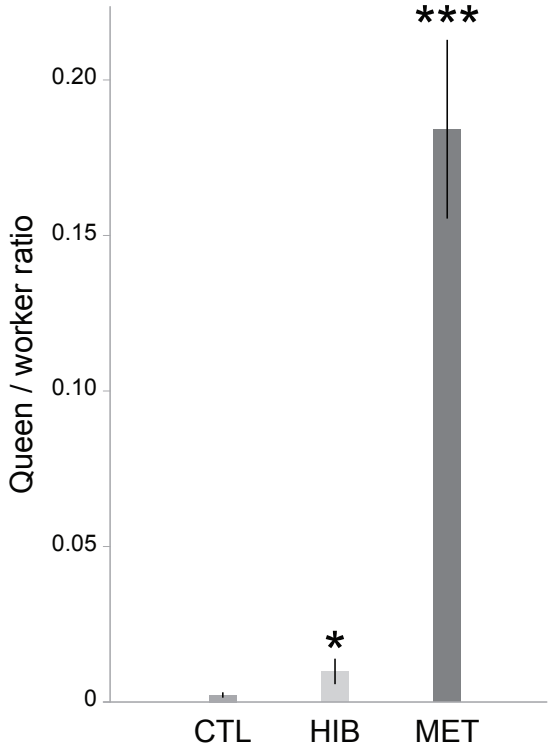

Figure 1 – The proportion of queens among the offspring produced was increased in hibernation (HIB) and methoprene (MET) treatments compared to control (CTL). *: P < 0.05; ***: P < 0.001.

The treatments also significantly affected the expression of all the genes tested (*ILP1*: $F_{2,36}$ = 5.30, P = 0.01); *ILP2*: $F_{2,36}$ = 19.47, P < 0.001; *JHepox*: $F_{2,36}$ = 4.12, P = 0.02; *Vg1*: $F_{2,36}$ = 11.15, P < 0.001; *Vg2*: $F_{2,36}$ = 7.93, P = 0.001). Compared to the control group, both hibernation and methoprene treatments upregulated the expression of *ILP1* (hibernation: t = 1.92, P = 0.06; methoprene: t = 3.24, P = 0.003; Figure 2), *ILP2* (hibernation: t = 4.02, P < 0.001; methoprene: t = 6.14, P < 0.001; Figure 2), *JHepox* (hibernation: t = -2.28, P = 0.03; methoprene: t = -2.65, P = 0.01; Figure 3), *Vg1* (hibernation: t = 2.20, P = 0.03; methoprene: t = 4.72, P < 0.001; Figure 4) and *Vg2* (hibernation: t = 2.15, P = 0.04; methoprene: t = 3.98, P < 0.001; Figure 4).

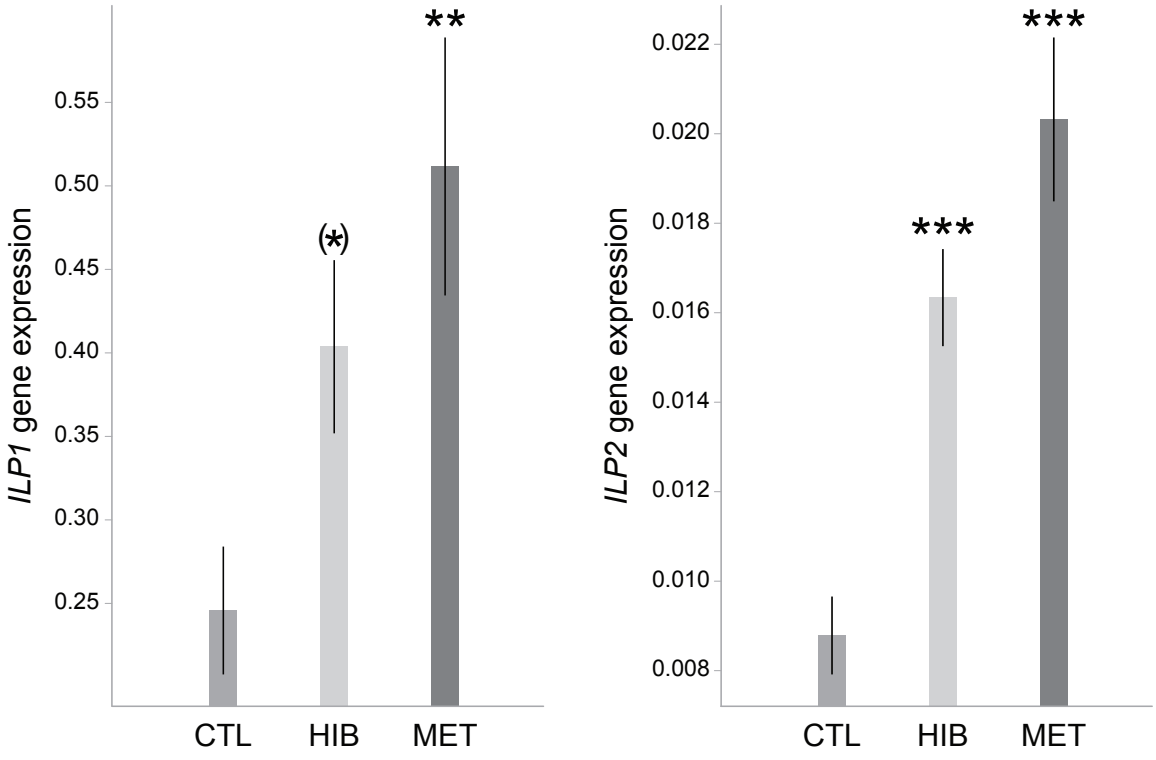

Figure 2 – *ILP1* and *ILP2* were upregulated in hibernation and methoprene treatments. The y axis indicates the relative gene expression in queens, corresponding to the *ILP1* and *ILP2* mRNA levels relative to the *RP49* (control) mRNA level. (*): P = 0.06; **: P < 0.01; ***: P < 0.001.

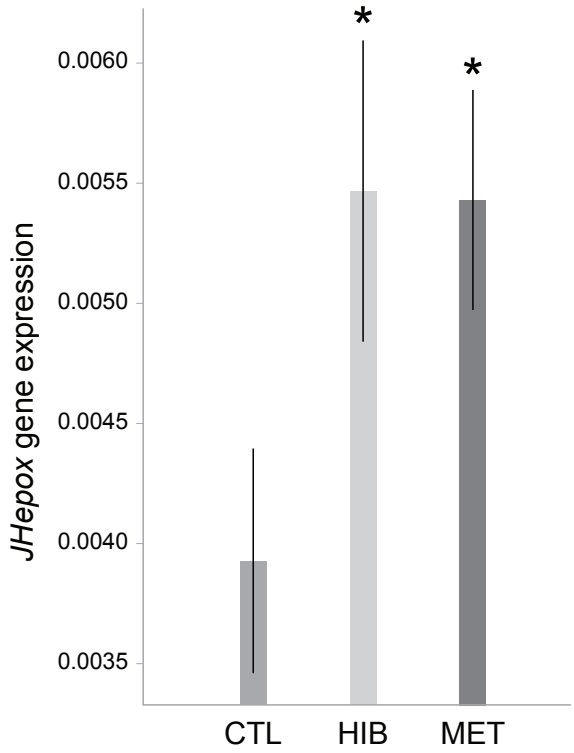

Figure 3 – *JHepox* was upregulated in hibernation and methoprene treatments. The y axis indicates the relative gene expression in queens, corresponding to the *JHepox* mRNA level relative to the *RP49* (control) mRNA level. *: P < 0.05.

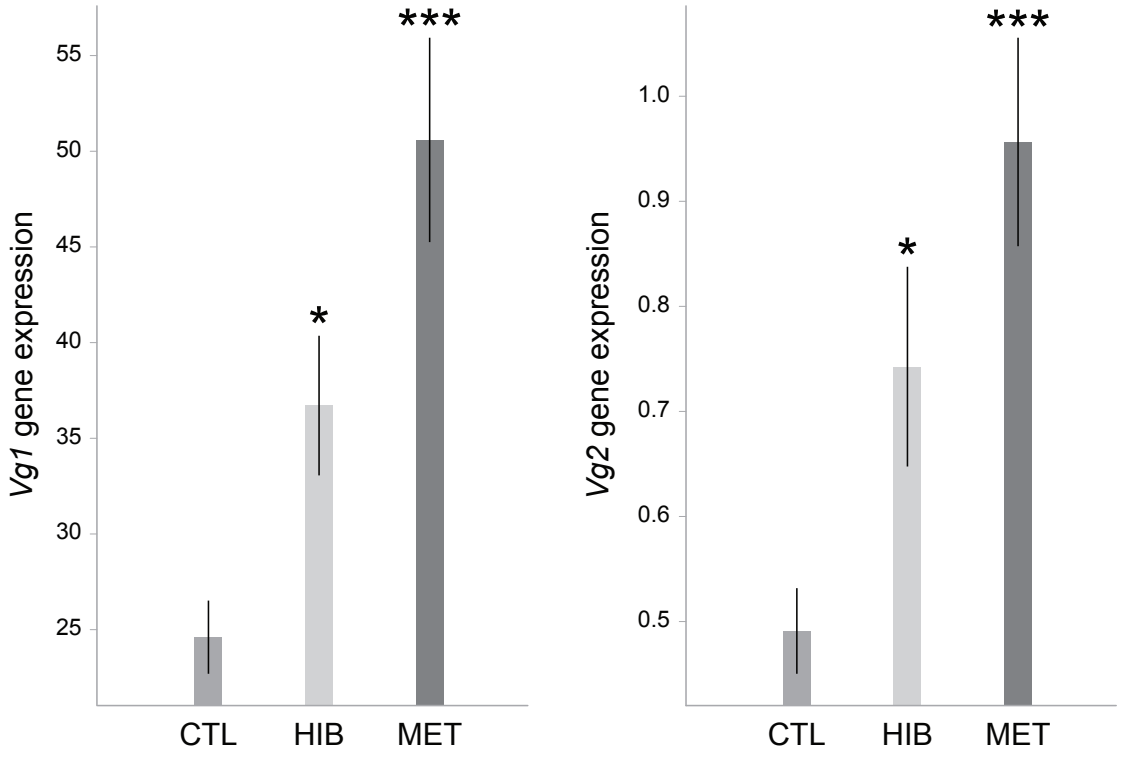

Figure 4 – *Vg1* and *Vg2* were upregulated in hibernation and methoprene treatments. The y axis indicates the relative gene expression in queens, corresponding to the *Vg1* and *Vg2* mRNA levels relative to the *RP49* (control) mRNA level. *: P < 0.05; ***: P < 0.001.

There was no significant difference between treatments in the number ($F_{2,72}$ = 1.35, P = 0.27) and weight ($F_{2,72}$ = 1.09, P = 0.34) of eggs produced. However, the treatments significantly affected the proportion of Vg among total proteins (Kruskal-Wallis χ^2 = 6.63, P = 0.04, Figure 5). The proportion of Vg among the protein content of eggs produced by both hibernated (*U* = 42, P = 0.038) and methoprene-treated (*U* = 53.5, P = 0.026) queens was significantly higher than in eggs produced by control queens. By contrast, this proportion did not differ significantly between eggs produced by hibernated and methoprene-treated queens (*U* = 79, P = 0.93).

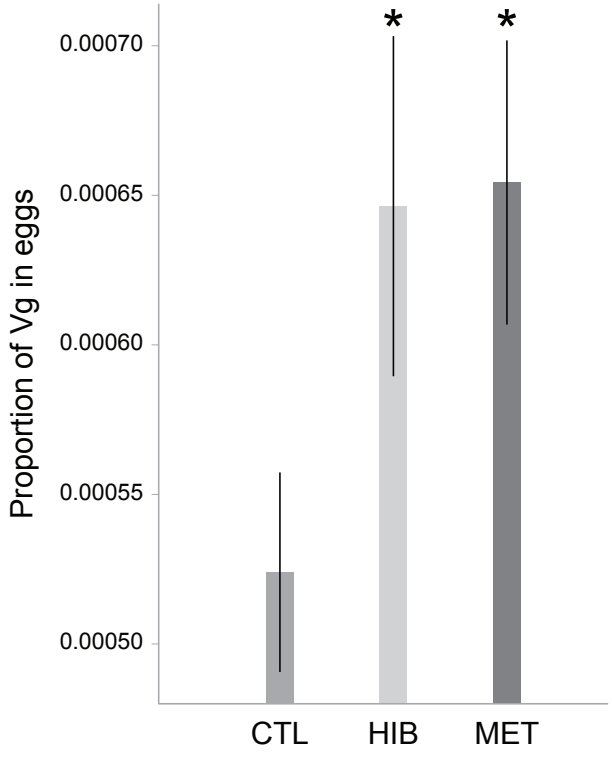

Figure 5 – The proportion of Vg among total proteins was increased in eggs produced in hibernation and methoprene treatments. *: P < 0.05.

Discussion

Each of the four predictions developed under the hypothesis that the interplay between IIS, JH and Vg regulates maternal effects on caste polyphenism in *P. rugosus* was confirmed by this study. In line with the first prediction that an artificial increase of JH in queens should stimulate the production of queens, the feeding of *P. rugosus* colonies with a juvenile hormone analog (methoprene) mimicked the effect of hibernation, with both hibernated and methoprene-treated colonies showing an increased production of queens. These results reveal a role of JH in the regulation of caste polyphenism in *P. rugosus*. In this species, maternal effects were previously found to stimulate the production of queens in response to hibernation, as only colonies headed by an hibernated queen produced queens, whether or not the workers had been exposed to cold (Schwander et al. 2008). The exposure to cold therefore triggers changes in queens that make them more likely to lay queen-destined eggs. In this study, the methoprene treatment also targeted the queen, as evidenced by an increase in the proportion of queens among the offspring developing from eggs laid by methoprene-treated queen. Similar results were found in *Pheidole pallidula*, where direct topical application of JH on the queen stimulated the production of queens (Passera and Suzzoni 1979). Overall, the observed effects of hibernation and methoprene treatments show that hibernation-triggered JH changes in queens are involved the production of queens in *P. rugosus*.

The second prediction was that hibernation should affect IIS and JH in queens. In line with this prediction, our results revealed that genes involved in IIS (*ILP1* and *ILP2*) were upregulated in *P. rugosus* hibernated queens. This suggests that hibernation can translate into changes in the IIS pathway. Low temperature or the associated photoperiod changes could directly affect IIS, as reported in the regulation of insect diapause (Sim and Denlinger 2008). Alternatively, the effect of exposure to cold could have been mediated by a change in the queen nutritional status due to decreased activity and metabolism (Mellanby 1939) or lower food intake during hibernation. Such effects of nutrition on IIS have been reported in

Drosophila (Britton et al. 2002; Ikeya et al. 2002; Puig and Tjian 2006). Changes in IIS usually result in the release of neuropeptides (e.g., allatostatin, allatotropin) that influence the production of JH by the corpus allatum (Tu et al. 2005). Accordingly, the exposure to cold also upregulated the expression of *JHepox*, coding for JH epoxidase, an enzyme involved in the production of JH. These results show that in *P. rugosus* queens, IIS regulates JH in response to environmental changes such as those experienced during hibernation.

The third prediction was that both hibernation and an artificial increase in JH should stimulate the production of Vg. In our experiments, both hibernation and methoprene treatments stimulated the production of queens and upregulated the expression of Vg genes (*Vg1* and *Vg2*) in queens. The effect of hibernation on vitellogenesis is likely to have been triggered by the increase in JH production due to temperature-triggered changes in IIS. This is supported by the finding that the methoprene treatment also upregulated *Vg* expression These results show that JH-regulated vitellogenesis in adult *P. rugosus* queens is involved in the regulation of caste polyphenism.

Finally, the fourth prediction was that the Vg content in eggs should correlate positively with their likelihood of developing into queens. This prediction was also supported by our data. While neither the number nor the weight of eggs produced differed between control, hibernated and methoprene-treated queens, the proportion of Vg in the protein content was significantly higher in eggs produced by both hibernated and methoprene-treated queens than control queens. It is likely that the increased production of Vg in hibernated and methoprene-treated queens translated into in a higher Vg content in the eggs, increasing their likelihood of developing into queens. How the Vg content in eggs alters the caste fate remains to be investigated but, as Vg is thought to act as a nutritive source for the embryo (Hagedorn and Kunkel 1979), more Vg in the egg could result in more energy during early development, facilitating the path toward queen development. The finding of a higher proportion of Vg in eggs produced by queen-producing hibernated and methoprene-treated queens is consistent with our fourth prediction, and shows that the quantity of Vg injected in

the eggs is involved in the early regulation of caste allocation and plays a role in the intergenerational transmission of information required for maternal effects on polyphenism to happen.

Overall, this study describes the mechanisms that allow the environmental cues experienced by one generation to be translated into phenotypic variation in the next generation. IIS in queens translates environmental cues into changes in JH and JH regulates the production of Vg. The Vg content in queens affects the quantity of Vg injected into the eggs produced, influencing their development toward the queen or worker caste. In addition to the insights provided on the regulation of maternal effects on caste determination in social insects, this study may also provide new routes to study the mechanisms regulating intergenerational effects on insect polyphenism. Maternal effects have been found to be involved in the regulation of well-described examples of polyphenism, such as the density-dependent phase change in locusts (Hunter-Jones 1958) or the seasonally triggered production of winged individuals in aphids (Sutherland 1969). This study raises the possibility that the interplay between IIS, JH and Vg is also involved in the maternal regulation of such polyphenisms.

Acknowledgments

We thank Gro Amdam and Eric Lucas for their helpful comments on the manuscript. This research was supported by several grants from the Swiss NSF and an ERC advanced grant.

CHAPTER 4

CHAPTER 4

Behavioral and caste specific expression of vitellogenin genes in the ant *Pogonomyrmex barbatus*

Romain Libbrecht*, Miguel Corona* and Laurent Keller

*these authors contributed equally to the work

Abstract

Division of labor, the cornerstone of insect societies, implies the coexistence of individuals differing in reproduction and behavior. The reproductive ground plan hypothesis asserts that physiological pathways regulating reproduction in solitary insects have been co-opted to regulate worker behavior in insect societies. Although well-supported by studies in the honeybee *Apis mellifera*, in which the vitellogenin (*Vg*) gene regulates the onset of foraging, this hypothesis have remained poorly tested in other social insects. Ant species which genome harbors multiple *Vg* genes, such as *Pogonomyrmex barbatus* and *Solenopsis invicta*, provide great opportunities to investigate the role of *Vg* genes. In this study, we compared the expression of the two *Vg* genes present in the genome of *P. barbatus* between queens, nurses and foragers. The expression of *Vg1* differed among queens, nurses and foragers while that of *Vg2* only differed between nurses and foragers. Such patterns of expression reveal that *Vg1* is associated with reproduction and behavior, and *Vg2* with behavior only. This is consistent with *Vg* genes regulating worker behavior in ants and suggests that the co-option of reproductive pathways, thus not restricted to bees, plays a major role in social evolution. Furthermore, gene expression and phylogenetic analyses suggest that the *Vg* genes in ants, which duplication occurred after the divergence from bees, have underwent neo or sub-functionalization to acquire caste and behavioral specific functions.

Introduction

Division of labor is the cornerstone of insect societies and implies the coexistence of individuals that differ in morphology, reproduction and behavior in social insect colonies (Wilson 1971; Holldobler and Wilson 1990). There are usually two main levels of division of labor. The first relates to reproduction, which is monopolized by one or several queens while sterile workers perform all the tasks to take care of the colony. The second level of division of labor occurs among workers, with groups of individuals being specialized in different tasks.

Work in the honeybee *Apis mellifera* has lead to the development of the reproductive ground plan hypothesis, which asserts that the physiological pathways regulating reproduction also regulate worker behavior in insect societies (Amdam et al. 2003; Amdam et al. 2004). This hypothesis is based on the early findings that the reproductive status influences the behavior in solitary wasps: females with developed ovaries (before oviposition) stay in the nest to build cells while females with undeveloped ovaries (after oviposition) leave the nest to forage for food (West-Eberhard 1987). The mechanisms regulating reproduction (and hence behavior) in solitary insect species would have been co-opted to regulate the behavior of functionally sterile workers in social species (Amdam et al. 2004). Several studies support this hypothesis in *Apis mellifera*. The comparison of workers with different foraging strategies (high or low pollen-hoarding strains) revealed differences in the number of ovarioles (Amdam et al. 2006) and the vitellogenin (Vg) content and gene expression (Amdam et al. 2004; Ihle et al. 2010 but see Oldroyd and Beekman 2008). In many social insect species, including *A. mellifera*, age is one of the factors influencing worker behavior, as evidenced by workers frequently moving from nursing to foraging tasks as they become older (Wilson 1971; Seeley 1982). Together with the juvenile hormone (JH), Vg also regulates the temporal switch between nurse and forager activities in *A. mellifera* workers (Bloch et al. 2002; Amdam and Omholt 2003; Guidugli et al. 2005; Oldroyd and Beekman 2008). A causal link between Vg and behavior has also been documented, as workers with repressed *Vg* gene expression

foraged earlier (Nelson et al. 2007). This last study showed that a single reproductive gene could regulate the behavior of functionally sterile workers in *A. mellifera*.

To understand whether the co-option of reproductive pathways plays a major role in social evolution would require to investigate the link between reproductive physiology and behavior in other social insects (Amdam et al. 2004; Amdam and Page 2010), preferentially those, such as ants, having evolved sociality independently from bees (Brady et al. 2006; Moreau et al. 2006). A recent study reported JH differences between nurses and foragers in the ant *Pogonomyrmex californicus* (Dolezal et al. 2012). These results are consistent with the co-option of reproductive pathways to regulate behavior, as JH is known to interact with Vg in insects (Robinson and Vargo 1997; Comas et al. 1999; Tatar et al. 2001), including in *Pogonomyrmex* ants (see chapter 3). However, the link between JH and Vg differs between species (Amdam and Omholt 2003) and developmental stages (Hartfelder and Engels 1998; Bloch et al. 2002), and no study so far has investigated the link between Vg and worker behavior in ants. Interestingly, the seed-harvester ant *Pogonomyrmex barbatus* and the fire ant *Solenopsis invicta* have more than one *Vg* genes in their genome (Wurm et al. 2011). For instance, the genome of *S. invicta* harbors four *Vg* genes, among which two are preferentially expressed in queens (*Vg2* & *Vg3*) and two in workers (*Vg1* & *Vg4*), suggesting a role of *Vg* genes in the regulation of reproduction (Wurm et al. 2011). The question of whether *Vg* genes also play a role in the regulation of worker behavior in ants remains to be investigated.

In this study, we analyzed the patterns of *Vg* genes (*Vg1* and *Vg2*) expression in queens, nurses and foragers in *Pogonomyrmex barbatus*. This analysis allowed us to test whether Vg genes expression was associated with reproduction and behavior, and to compare the expression patterns of *Vg1* and *Vg2* genes. Finally, the construction of a phylogenetic tree of the known *Vg* gene sequences in Hymenoptera provided information on the origin and evolution of multiple *Vg* genes in ants.

Methods

Sample collection

Pogonomyrmex barbatus founding queens were collected during mating flights on July 15th, 2008 in Bowie, Arizona, USA (N32°18'54''//W109°29'03''). After worker eclosion, the colonies were kept in laboratory conditions (30°C, 60% humidity and 12h/12h light:dark cycle) in 15*13*5cm plastic boxes with water tubes, and were fed once a week with grass seeds and a mixture of eggs, honey and smashed mealworms. Samples were collected in seven *Pogonomyrmex barbatus* colonies on December 16th, 2010. The queen (n = 1 per colony) was collected from the seven colonies while nurses (n = 12 per colony) and foragers (n = 12 per colony) could only be collected from five colonies because we failed to assign enough ants to the nurse or forager behavioral group in two colonies. Nurses were defined as ants taking care of brood in the nest tube. To collect foragers, each colony was connected with a cardboard-made bridge to a foraging area composed of a plastic box containing grass seeds. Foragers were defined as ants handling a food item in the foraging area. Ant samples were flash-frozen in liquid nitrogen and kept at -80°C for further RNA extraction. Although task performance in workers is age related, with nurses tending to be younger than foragers (Holldobler and Wilson 1990), the association between physiology and behavior was recently found to be independent of age in *Pogonomyrmex californicus* (Dolezal et al. 2012).

Gene expression analysis

Whole body worker samples were used to measure the expression of *Vg1* and *Vg2* genes. RNA extractions were performed using a modified protocol including the use of Trizol (Invitrogen) for the initial homogenization step, RNeasy extraction kit and DNAse I (Qiagen) treatment to remove genomic DNA traces. For each individual worker, cDNAs were synthesized using 500 ng of total RNA, random hexamers and Applied Biosystems reagents. Levels of mRNA were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) using ABI Prism 7900 sequence detector and SYBR green. All qPCR assays were

performed in duplicates and subject to the heat-dissociation protocol following the final cycle of the qPCR in order to check for amplification specificity. qRT-PCR values of each gene were normalized by using an internal control gene (*RP49*). Paralog-specific primers (sequences available in appendix 1) were designed using sequence alignment (Thompson et al. 1997) and primer analysis (Rychlik 2007) programs. Primer sequences overlapped coding regions split by introns, allowing the specific amplification of cDNA levels over eventual genomic DNA contaminations. Transcript quantification calculations were performed by using the ΔΔCT method (Livak and Schmittgen 2001).

Phylogenetic tree

The phylogenetic tree of Vg genes was constructed as follows. Initial protein alignments were performed using ClustalW2 (Larkin et al. 2007) and then edited using Jalview (Waterhouse et al. 2009). Edited sequences were realigned using ClustalX 2.0.12. The parsimony tree was established using PAUP 4.0 b10 (Swofford 2003) and was rooted using the most divergent sequence in each group as the outgroup. Bootstrap support for internal branches was evaluated from 10,000 full-heuristic searches, and groups with a frequency greater than 50% were retained in the consensus tree.

Statistical Analysis

All data were analyzed using R (http://www.r-project.org/) and the R packages lme4 (Bates 2005) and languageR (Baayen 2008). The effect of caste on gene expression relative values was analyzed using linear mixed effects models. To avoid pseudoreplication, the colony was included as a random effect. We checked for normality and homogeneity by visual inspections of plots of residuals against fitted values. To assess the validity of the mixed effects analyses, we performed likelihood ratio tests to test that the models with fixed effects differed significantly from the null models with only the random effects. Throughout the paper, we present MCMC-estimated p-values that are considered significant at the $\alpha=0.05$ level. All significant results remained significant after Bonferroni correction.

Results

We analyzed the expression patterns of *Vg1* and *Vg2* using the whole body of queens, nurses and foragers. The expression of both genes was associated with both the caste (queen or worker) and the behavior (nurse or forager) (Figure 1).

On average, the *Vg1* gene was 4.1 times more expressed in queens than in nurses (pMCMC < 0.0001) and 779 times more than in foragers (pMCMC < 0.0001). Therefore the expression of *Vg1* was 190 times higher in nurses compared to foragers (pMCMC < 0.0001).

The expression of *Vg2* did not differ significantly between queens and nurses (pMCMC = 0.84). However *Vg2* was on average 4.8 times more expressed in foragers than in queens (pMCMC = 0.0004) and 6.5 times more expressed in foragers than in nurses (pMCMC < 0.0001).

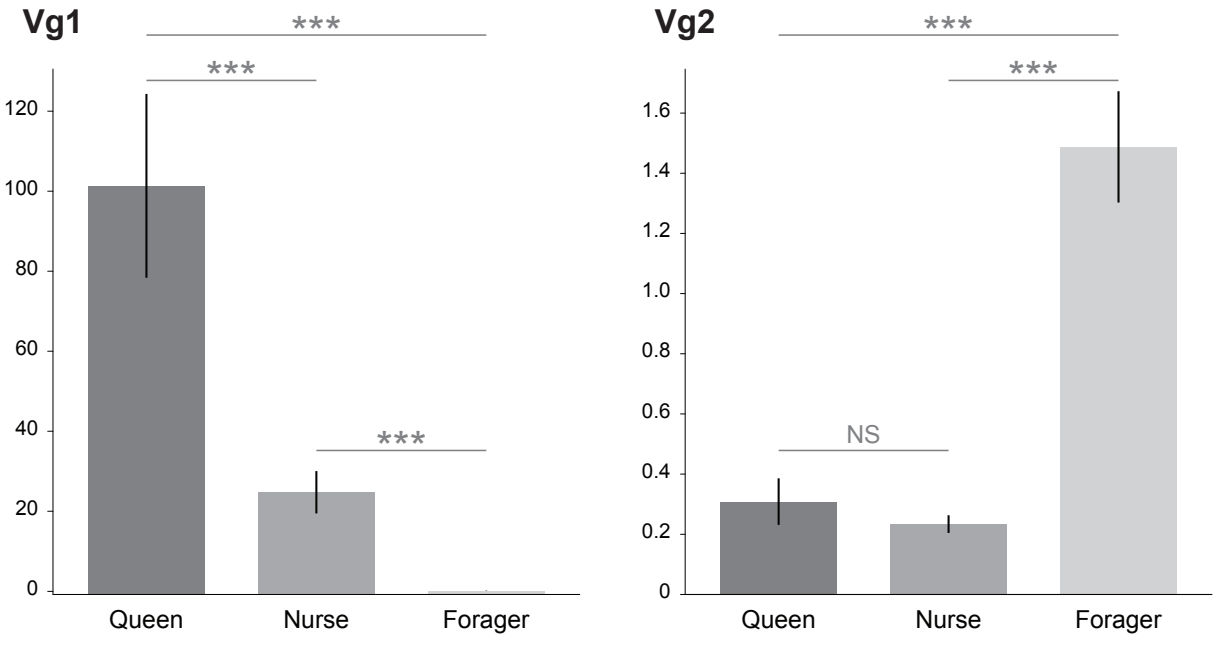

Figure 1 – The y-axes indicate the relative gene expression, corresponding to the *Vg1* or *Vg2* mRNA levels relative to the *RP49* (control) mRNA level. *Vg1* was significantly more expressed in queens than in nurses or foragers, as well as more expressed in nurses than in foragers. *Vg2* was significantly more expressed in foragers than in queens or nurses and its expression did not differ significantly between queens and nurses. NS: pMCMC > 0.05; ***: pMCMC < 0.0001.

In both queens and nurses, *Vg1* was significantly more expressed than *Vg2* (327 times more in queens, pMCMC = 0.0016; 107 times more in nurses, pMCMC < 0.0001). On the contrary, *Vg2* was 11.5 times more expressed than *Vg1* in foragers (pMCMC < 0.0001).

A phylogenetic analysis (Figure 2) revealed that the ancestral *Vg* gene underwent a first duplication after ants split from bees. This duplication resulted in two daughter *Vg* genes: one is the ancestor of the *P. barbatus Vg1* gene (Pogonomyrmex_Vg1 in the figure) and two *S. invicta Vg* genes (Solenopsis_Vg2 and Solenopsis_Vg3), the other is the ancestor of the *P. barbatus Vg2* gene (Pogonomyrmex_Vg2) and the two other *S. invicta Vg* genes (Solenopsis_Vg1 and Solenopsis_Vg4). The phylogenetic analysis also revealed that the four *Vg* genes in *S. invicta* resulted from two independent rounds of *Vg* duplication after the divergence from *Pogonomyrmex*.

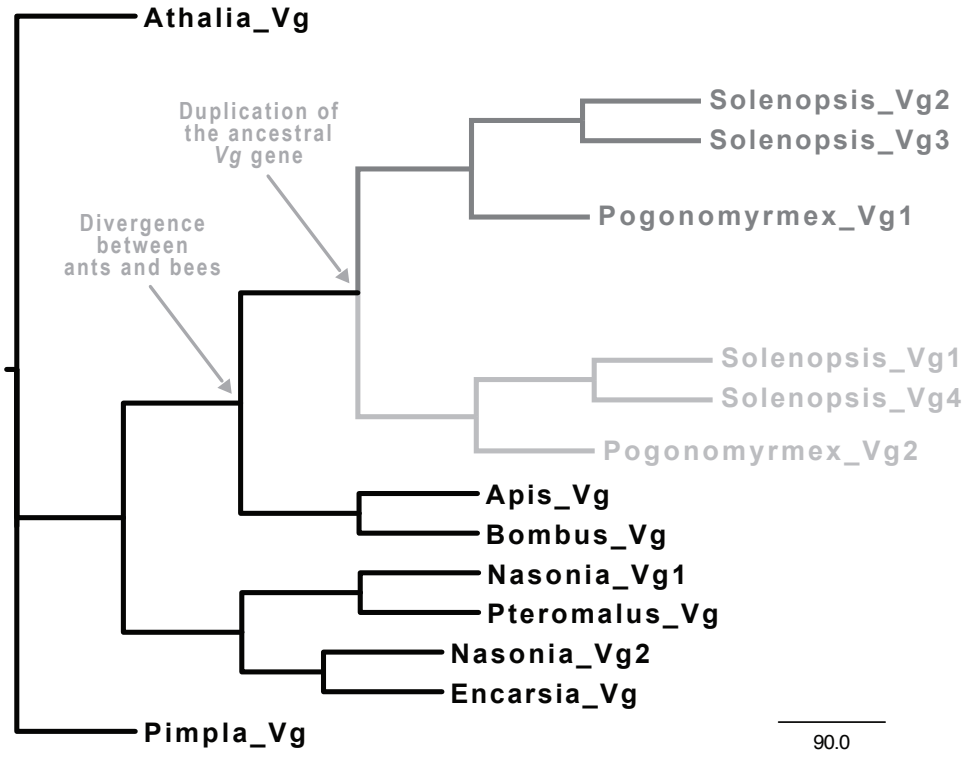

Figure 2 – Parsimony tree of known hymenopteran vitellogenin protein sequences suggests that the first round of vitellogenin duplication occurred after the split between ants and other hymenopterans including bees and wasps.

Discussion

The results of this study show that Vg is associated with both reproduction and behavior in the ant *Pogonomyrmex barbatus*. There are two *Vg* genes in the genome of *P. barbatus* and both (*Vg1* and *Vg2*) were expressed in queens, nurses and foragers. *Vg1* was more highly expressed in queens than in either nurses or foragers. This pattern is similar to that of the *Vg* gene in *A. mellifera* (Corona et al. 2007) and is consistent with Vg playing a predominant role in the regulation of reproduction. *Vg2* was also expressed in the queen but the expression was very low compared to *Vg1*. This, together with the finding that *Vg2* expression does not differ between fertile (queens) and sterile (nurses) individuals, suggests that *Vg2* is not involved in the regulation of reproduction.

The finding that both *Vg1* and *Vg2* genes expression differ between nurses and foragers suggests a role of *Vg* genes in the regulation of worker behavior in *P. barbatus*. The expression of *Vg1* was higher in nurses while the pattern was reversed for *Vg2*, which expression was higher in foragers. The results also highlight opposite differential expression of *Vg* genes in nurses and foragers, as *Vg1* was more expressed than *Vg2* in nurses while *Vg2* was more expressed than *Vg1* in foragers. The pattern of *Vg1* expression (higher in nurses, lower in foragers) is similar to that of the *Vg* gene in *A. mellifera* workers, where decreased expression triggers the onset of foraging (Fluri et al. 1982; Nelson et al. 2007).

Contrary to the expression of *Vg1*, which was associated with both reproduction and behavior, the level of expression of *Vg2* gene was associated with behavior but not reproduction: its expression differed between foraging and non-foraging (queens and nurses) individuals but not between reproducing (queens) and non-reproducing (nurses) individuals. Interestingly, the genome of *S. invicta* also harbors more than one *Vg* genes and in this species, two of the four *Vg* genes are preferentially expressed in workers (Wurm et al. 2011). This suggests that *P. barbatus* may not be the only ant species where some *Vg* genes are implicated in worker behavior but not in reproduction.

The phylogenetic tree of known *Vg* genes in Hymenoptera provides some interesting information on the existence of multiple *Vg* genes in ants. First it shows that the first duplication of the ancestral *Vg* gene occurred after ants diverged from bees, explaining why ant species have two or more *Vg* genes while bees have only one. Two independent rounds of duplications occurred in the *Solenopsis* lineage after it diverged from the *Pogonomyrmex* lineage, explaining why there are four *Vg* genes in the *S. invicta* genome and only two in the *P. barbatus* genome. Second, it is interesting to note that the *Vg* genes preferentially expressed in queens in both *P. barbatus* (Pogonomyrmex_Vg1) and *S. invicta* (Solenopsis_Vg2 and Solenopsis_Vg3) (Wurm et al. 2011) cluster together on one side of the tree while the *Vg* genes preferentially expressed in foragers in both species (Pogonomyrmex_Vg2, Solenopsis_Vg1 and Solenopsis_Vg4) cluster together on the other side of the tree. This suggests that the first duplication of the ancestral *Vg* gene in ants produced two paralogs: one is the ancestor of *Vg* genes involved in reproduction and potentially behavior (e.g. *Vg1* in *P. barbatus*) and the other is the ancestor of *Vg* genes that have lost their role in reproduction and correlate with worker behavior (e.g. *Vg2* in *P. barbatus*).

In conclusion, the results of this study are consistent with Vg having been co-opted to regulate worker behavior in the ant *P. barbatus*, as found to happen in the honeybee *A. mellifera*. Given that ants and bees evolved sociality independently, this suggests that the co-option of reproductive pathways to regulate the behavior of sterile individuals may be a major director of social evolution. Interestingly, the ancestral *Vg* gene was duplicated in ants after the divergence from bees (Wurm et al. 2011), resulting in the presence of several *Vg* genes in *P. barbatus*, *S. invicta* and probably other ant species. The finding that *Vg2* has lost its reproductive function and correlates with worker behavior in *P. barbatus* suggests that, after the initial duplication in ants, the *Vg* genes underwent neo- or subfunctionalization to acquire caste and behavioral specific functions. More such studies are needed in other ant species to

extend the understanding of the roles of *Vg* genes in the regulation of social life in ant societies.

Acknowledgments

This research was supported by several grants from the Swiss NSF and an ERC advanced grant.

GENERAL DISCUSSION

Summary of the main findings

The cornerstone of insect societies is division of labor, whereby different groups of individuals specialize in the performance of specific tasks or roles (Wilson 1971; Oster and Wilson 1979; Holldobler and Wilson 1990). The aim of this PhD was to investigate the genetic components and physiological regulation of division of labor in social insects.

In **chapter 1**, we investigated the genetic components of the process of caste allocation in the Argentine ant *Linepithema humile*. The use of controlled crosses in the laboratory allowed us to reveal the existence of non-additive genetic effects on female caste determination, as well as an effect of the interaction between queens and males on sex allocation. In **chapter 2**, we combined the same methods with behavioral analyses to investigate the nature of genetic effects on worker behavior in *L. humile*. This study revealed parent-of-origin and genetic compatibility effects on division of labor among the worker force. In **chapter 3**, we used a combination of artificial hibernation, hormonal treatments, gene expression analysis and vitellogenin quantification to investigate the process of caste determination in *Pogonomyrmex rugosus*. This study revealed crucial roles of insulin signaling, juvenile hormone and vitellogenin in the regulation of maternal effects on caste determination and set vitellogenin as a likely key player in the intergenerational transmission of information. In **chapter 4**, we compared the expression of *vitellogenin* genes between queens, nurses and foragers in *P. barbatus* and performed phylogenetic analyses to investigate the role of vitellogenin in the regulation of worker behavior in ants. The results of this study are consistent with vitellogenin having been co-opted to regulate worker behavior in *P. barbatus* and suggest that the *vitellogenin* genes underwent neo- or subfunctionalization to acquire caste and behavioral specific functions after the duplication of the ancestral *vitellogenin* gene in ants.

Axis 1: Genetic components to division of labor

The main message of the first axis of this PhD, presented in chapters 1 and 2, is the importance of non-additive genetic influences, such as genetic compatibility effects, on division of labor in ants. This was supported by several original findings, such as the first report of non-additive genetic components to caste allocation in a multiple-queen ant species, the first report of effects of the interaction between queens and males on the sex ratio produced and the first report of genetic compatibility effects between parental genomes on worker behavior in social insects.

The results of chapter 1 revealed an effect of the interaction between queens and males on the sex ratio produced. One of the hypotheses proposed to explain this result is that queens may actively change sex allocation depending on qualities of the sperm transferred by their mate. Under this hypothesis, male lineages are expected to differ in sperm quality. We developed a preliminary experiment to compare sperm quantity and viability between several lineages of males in *L. humile*. The methods and results of this experiment are detailed in appendix 2. We combined techniques of male genital tract dissection and fluorescence microscopy to measure the total number of sperm and their viability. The results revealed that the male lineages differed in sperm number but not in sperm viability. This experiment was performed as a follow-up to chapter 1 and used different lineages of males, thus making impossible the direct association between the number of sperm per male and the sex ratio produced. Nevertheless, the result of this experiment is interesting, as it does not rule out the hypothesis that queens may actively change sex allocation depending on the quantity of sperm receive during mating.

AXIS 2 - Physiological regulation of division of labor

The main messages of the second axis of this PhD (chapters 3 and 4) are first, the importance of hormones (e.g. insulin, juvenile hormone) in the sensing of environmental

changes and second, the co-option of vitellogenin, originally involved in reproduction, to regulate crucial aspects of social life in ants. Chapters 3 and 4 presented original findings, such as the first description of physiological mechanisms regulating maternal effects on polyphenism in insects and the first report of the role of vitellogenin in the regulation of worker behavior in ants.

Conclusion

Division of labor in insect societies involves the coexistence of fertile queens and sterile workers, as well as task specialization among the worker force. Because it enhances colony performance and productivity, division of labor is thought to be at the root of the ecological success of social insects (Wilson 1971; Oster and Wilson 1979; Holldobler and Wilson 1990). The findings of this PhD contribute to a better understanding of division of labor, as they reveal the nature of genetic and physiological processes involved in the determination of the caste and the regulation of worker behavior. Several results of this PhD also suggest that the co-option of existing pathways to regulate social life was not restricted to bees (Amdam et al. 2004) but also occurred in ants, providing important information on the evolution of sociality.

Perspectives

In the first axis of this PhD, we revealed the existence of non-additive genetic effects on division of labor in the Argentine ant *Linepithema humile*. The use of controlled crosses is a powerful, yet seldom used, tool to detect such effects and future studies should apply this method to other social insect species. Additionally, it would be interesting to determine whether epigenetic mechanisms are involved in the regulation of parent-of-origin effects on caste determination and worker behavior. To do so will require to investigate whether paternally and maternally inherited alleles show differential expression, in particular for genes involved in the process of division of labor. Finally, more efforts should be made to study the effect of males on sex allocation. Chapter 1 showed an effect of the interaction between

queens and males on the sex ratio produced and a preliminary experiment revealed that male lineages differ in sperm quantity in *L. humile*. Future studies should investigate whether such differences between males lineages are associated with differences in sex allocation, whether different lineages of queens show different strategies of sex allocation in response to different sperm quantity and to what extent the effect of the interaction between queens and males on sex allocation has to be taken in account in studies of intracolonial conflicts over sex ratio in insect societies.

In the second axis of this PhD, we highlighted the importance of the *vitellogenin* genes in the regulation of social life. Seven ant genomes are currently available (Bonasio et al. 2010; Nygaard et al. 2011; Smith et al. 2011a; Smith et al. 2011b; Suen et al. 2011; Wurm et al. 2011; Gadau et al. 2012) and more should be sequenced in the near future. Preliminary investigations suggest that many ant species have several *vitellogenin* genes in their genome. Future studies should investigate the role of these *vitellogenin* genes by comparing their expression between queens and workers, and between different behavioral castes among the worker force. The use of RNAi should also be considered, as silencing of *vitellogenin* genes would be a powerful tool to understand their functions. Such studies, combined with phylogenetic analyses, will provide crucial information on the duplication of the ancestral *vitellogenin* gene in ants, the neo- or subfunctionalization of the daughter *vitellogenin* genes and their implication in the regulation of social life.

Finally, in this PhD, we investigated the genetic components and physiological regulation of division of labor by tackling the effects of genes and physiology independently. Of interest would be to study how genes and physiology interact to regulate division of labor in insect societies. In that perspective, future studies should investigate how the expression of genes involved in candidate physiological pathways (e.g. insulin signaling, juvenile hormone or vitellogenin) differs between different genetic lineages or different combination of parental lineages, and whether or not this is associated with the regulation of caste allocation in queens, caste determination in larvae and behavior in workers. Together with the results of

this PhD, such investigations will provide a better understanding of division of labor in insect societies.

REFERENCES

- Amdam, G. V., A. Csondes, M. K. Fondrk, and R. E. Page, Jr. 2006. Complex social behaviour derived from maternal reproductive traits. Nature 439: 76-78
- Amdam, G. V., K. A. Nilsen, K. Norberg, M. K. Fondrk, and K. Hartfelder. 2007. Variation in endocrine signaling underlies variation in social life history. Am. nat. 170: 37-46
- Amdam, G. V., K. Norberg, M. K. Fondrk, and R. E. Page, Jr. 2004. Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. Proc. Nat. Acad. Sci. USA 101: 11350-11355
- Amdam, G. V., K. Norberg, A. Hagen, and S. W. Omholt. 2003. Social exploitation of vitellogenin. Proc. Nat. Acad. Sci. USA 100: 1799-1802
- Amdam, G. V. and S. W. Omholt. 2003. The hive bee to forager transition in honeybee colonies: The double repressor hypothesis. J. Theor. Biol. 223: 451-464
- Amdam, G. V. and R. E. Page. 2010. The developmental genetics and physiology of honeybee societies. Anim. Behav. 79: 973-980
- Ament, S. A., M. Corona, H. S. Pollock, and G. E. Robinson. 2008. Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. Proc. Nat. Acad. Sci. USA 105: 4226-4231
- Aron, S., E. L. Vargo, and L. Passera. 1995. Primary and secondary sex-ratios in monogyne colonies of the fire ant. Anim. Behav. 49: 749-757
- Azevedo, D. O., J. C. Zanuncio, J. H. C. Delabie, and J. E. Serrao. 2011. Temporal variation of vitellogenin synthesis in *Ectatomma tuberculatum* (Formicidae: Ectatomminae) workers. J. Insect Physiol. 57: 972-977
- Baayen, R. H. 2008. Analyzing linguistic data: a practical introduction to statistics using R. Cambridge University Press, Cambridge
- Barchuk, A. R., M. M. G. Bitondi, and Z. L. P. Simões. 2002. Effects of juvenile hormone and ecdysone on the timing of vitellogenin appearance in hemolymph of queen and worker pupae of *Apis mellifera*. J. Insect Sci. 2:1-8
- Bates, D. 2005. Fitting linear mixed models in R. R News 5: 27-30
- Bier, K. 1954. Ueber den Saisondimorphismus des Oogenese von Formica rufa rufo-pratensis minor Gössw. und dessen Bedeutung für die Kastendetermination. Biol. Zentralbl. 73: 170–190
- Blatrix, R., J. L. Durand, and P. Jaisson. 2000. Task Allocation Depends on Matriline in the Ponerine Ant Gnamptogenys striatula Mayr. J. Insect Behav. 13: 553-562
- Bloch, G., D. E. Wheeler, and G. E. Robinson. 2002. Endocrine influences on the organization of insect societies. Horm. brain and behav. 3:195-235
- Bonasio, R., G. Zhang, C. Ye, N. S. Mutti, X. Fang, N. Qin, G. Donahue, P. Yang, Q. Li, C. Li, P. Zhang, Z. Huang, S. L. Berger, D. Reinberg, J. Wang, and J. Liebig. 2010. Genomic comparison of the ants *Camponotus floridanus* and *Harpegnathos saltator*. Science 329: 1068-1071
- Boomsma, J. J. 2009. Lifetime monogamy and the evolution of eusociality. Phil. Trans. R. Soc. B Biol. Sci. 364: 3191-3207
- Boomsma, J. J., M. Beekman, C. K. Cornwallis, A. S. Griffin, L. Holman, W. O. H. Hughes, L. Keller, B. P. Oldroyd, and F. L. W. Ratnieks. 2011. Only full-sibling families evolved eusociality. Nature 471: E4-E5
- Bourke, A. F. G. and N. R. Franks. 1995. Social evolution in ants. Princeton University Press, Princeton

- Bourke, A. F. G., H. A. A. Green, and M. W. Bruford. 1997. Parentage, reproductive skew and queen turnover in a multiple-queen ant analysed with microsatellites. Proc. R. Soc. Lond. B Biol. Sci. 264: 277-283
- Bourke, A. F. G. and F. L. W. Ratnieks. 1999. Kin conflict over caste determination in social Hymenoptera. Behav. Ecol. Sociobiol. 46: 287-297
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254
- Brady, S. G., T. R. Schultz, B. L. Fisher, and P. S. Ward. 2006. Evaluating alternative hypotheses for the early evolution and diversification of ants. Proc. Nat. Acad. Sci. USA 103: 18172-18177
- Britton, J. S., W. K. Lockwood, L. Li, S. M. Cohen, and B. A. Edgar. 2002. *Drosophila*'s insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev. Cell 2:239-249
- Burda, H., R. L. Honeycutt, S. Begall, O. Locker-Grotjen, and A. Scharff. 2000. Are naked and common mole-rats eusocial and if so, why? Behav. Ecol. Sociobiol. 47: 293-303
- Cahan, S. H., 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
- Calderone, N. W. and R. E. Page. 1988. Genotypic variability in age polyethism and task specialization in the honey bee, *Apis mellifera* (Hymenoptera: Apidae). Behav. Ecol. Sociobiol. 22: 17-25
- Comas, D., M. Piulachs, and X. Bellos. 1999. Fast induction of vitellogenin gene expression by juvenile hormone III in the cockroach *Blattella germanica* (L.)(Dictyoptera, Blattellidae). Insect Biochem. Mol. Biol. 29: 821-827
- Corona, M., R. A. Velarde, S. Remolina, A. Moran-Lauter, Y. Wang, K. A. Hughes, and G. E. Robinson. 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc. Nat. Acad. Sci. USA 104: 7128-7133
- Costa, J. T. and K. G. Ross. 2003. Fitness effects of group merging in a social insect. Proc. R. Soc. Lond. B Biol. Sci. 270: 1697-1702
- Crespi, B. J., a. Carmean, D.A., and T. W. Chapman. 1997. Ecology and evolution of galling thrips and their allies. Ann. Rev. Entomol. 42: 51-71
- Crozier, R. 1977. Evolutionary genetics of the Hymenoptera. Ann. Rev. Entomol. 22: 263-288
- Crozier, R. and R. Page. 1985. On being the right size: male contributions and multiple mating in social Hymenoptera. Behav. Ecol. Sociobiol. 18: 105-115
- Crozier, R. H. and P. Pamilo. 1996. Evolution of social insect colonies, sex allocation and kin-selection. Oxford University Press, Oxford
- Darwin, C. 1859. On the origin of species
- Daugherty, T., A. Toth, and G. Robinson. 2011. Nutrition and division of labor: Effects on foraging and brain gene expression in the paper wasp *Polistes metricus*. Mol. Ecol. 20: 5337-5347
- de Azevedo, S. and K. Hartfelder. 2008. The insulin signaling pathway in honey bee (*Apis mellifera*) caste development-differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. J. Insect Physiol. 54: 1064-1071
- Detrain, C. and J. Pasteels. 1991. Caste differences in behavioral thresholds as a basis for polyethism during food recruitment in the ant, *Pheidole pallidula* (Nyl.)(Hymenoptera: Myrmicinae). J. Insect Behav. 4: 157-176
- Dobata, S. and K. Tsuji. 2012. Intragenomic conflict over queen determination favours genomic imprinting in eusocial Hymenoptera. Proc. R. Soc. Lond. B Biol. Sci.

- Dolezal, A. G., C. S. Brent, B. Holldobler, and G. V. Amdam. 2012. Worker division of labor and endocrine physiology are associated in the harvester ant, *Pogonomyrmex californicus*. J. Exp. Biol. 215: 454-460
- Dubrovsky, E. B., V. A. Dubrovskaya, A. L. Bilderback, and E. M. Berger. 2000. The isolation of two juvenile hormone-inducible genes in *Drosophila melanogaster*. Dev. Biol. 224: 486-495
- Duffy, J. E. 1996. Eusociality in a coral-reef shrimp. Nature 381: 512-514
- Duffy, J. E. and C. L. Morrison. 2000. Multiple origins of eusociality among sponge-dwelling shrimps. Evolution 54: 503-516
- Estoup, A., M. Solignac, and J. M. Cornuet. 1994. Precise assessment of the number of patrilines and of genetic relatedness in honeybee colonies. Proc. R. Soc. Lond. B Biol. Sci. 258: 1-7
- Fjerdingstad, E. and J. Boomsma. 1997. Variation in size and sperm content of sexuals in the leafcutter ant *Atta colombica*. Insectes Sociaux 44: 209-218
- Fjerdingstad, E. J. 2004. Multiple paternity and colony homeostasis in Lasius niger ants. Behav. Ecol. Sociobiol. 56: 50-58
- Fjerdingstad, E. J., P. J. Gertsch, and L. Keller. 2003. The relationship between multiple mating by queens, within-colony genetic variability and fitness in the ant *Lasius niger*. J. Evol. Biol. 16: 844-853
- Flatt, T., M. P. Tu, and M. Tatar. 2005. Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. BioEssays 27: 999-1010
- Fluri, P., M. Luscher, H. Wille, and L. Gerig. 1982. Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. J. Insect Physiol. 28: 61-68
- Folgarait, P. J. 1998. Ant biodiversity and its relationship to ecosystem functioning: a review. Biodiversity Cons. 7: 1221-1244
- Fournier, D., S. Aron, and L. Keller. 2004. Significant reproductive skew in the facultatively polygynous ant *Pheidole pallidula*. Mol. Ecol. 13: 203-210
- Fournier, D., G. Battaille, I. Timmermans, and S. Aron. 2008. Genetic diversity, worker size polymorphism and division of labour in the polyandrous ant *Cataglyphis cursor*. Anim.Behav. 75: 151-158
- Fournier, D., A. Estoup, J. Orivel, J. Foucaud, H. Jourdan, J. Le Breton, and L. Keller. 2005. Clonal reproduction by males and females in the little fire ant. Nature 435: 1230-1234
- Fournier, D. and L. Keller. 2001. Partitioning of reproduction among queens in the Argentine ant, *Linepithema humile*. Anim. Behav. 62: 1039-1045
- Frohschammer, S. and J. Heinze. 2009. A heritable component in sex ratio and caste determination in a *Cardiocondyla* ant. Frontiers Zool. 6:27
- Frumhoff, P. C. and J. Baker. 1988. A genetic component to division of labour within honey bee colonies. Nature 333: 358-361
- Fuchs, S. and V. Schade. 1994. Lower performance in honeybee colonies of uniform paternity. Apidologie 25: 155-168
- Gadau, J., M. Helmkampf, S. Nygaard, J. Roux, D. F. Simola, C. R. Smith, G. Suen, Y. Wurm, and C. D. Smith. 2012. The genomic impact of 100 million years of social evolution in seven ant species. Trends Genet. 28: 14-21
- Garfield, A. S., M. Cowley, F. M. Smith, K. Moorwood, J. E. Stewart-Cox, K. Gilroy, S. Baker, J. Xia, J. W. Dalley, and L. D. Hurst. 2011. Distinct physiological and behavioural functions for parental alleles of imprinted Grb10. Nature 469: 534-538

- Giannakou, M. E. and L. Partridge. 2007. Role of insulin-like signalling in *Drosophila* lifespan. Trends Biochem. Sci. 32: 180-188
- Giraud, T., J. S. Pedersen, and L. Keller. 2002. Evolution of supercolonies: The Argentine ants of southern Europe. Proc. Nat. Acad. Sci. USA 99: 6075-6079
- Giray, T. and G. E. Robinson. 1994. Effects of intracolony variability in behavioral development on plasticity of division of labor in honey bee colonies. Behav. Ecol. Sociobiol. 35: 13-20
- Goodisman, M. A. D. and R. H. Crozier. 2003. Association between caste and genotype in the termite *Mastotermes darwiniensis* Froggatt (Isoptera : Mastotermitidae). Aust. J. Entomol. 42: 1-5
- Gösswald, K. 1951. Ueber den Lebenslauf von Kolonien der Roten Waldameise. Zool. Jb. System. Oekol. u. Geogr. 80: 27–63
- Guidugli, K. R., A. M. Nascimento, G. V. Amdam, A. R. Barchuk, S. Omholt, Z. L. P. Simoes, and K. Hartfelder. 2005. Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. FEBS Letters 579: 4961-4965
- Hagedorn, H. and J. Kunkel. 1979. Vitellogenin and vitellin in insects. Ann. Rev. Entomol. 24: 475-505
- Haig, D. 1998. Mother's boy or daddy's girl? Sex determination in Hymenoptera. Trends Ecol. Evol. 13: 380-381
- Hamilton, W. D. 1964a. The genetical evolution of social behaviour. I. J. Theor. Biol. 7: 1-16
- Hamilton, W. D. 1964b. The genetical evolution of social behaviour. II. J. Theor. Biol. 7: 17-52
- Hammond, R. L., M. W. Bruford, and A. F. G. Bourke. 2002. Ant workers selfishly bias sex ratios by manipulating female development. Proc. R. Soc. Lond. B Biol. Sci. 269: 173-178
- Hammond, R. L., M. W. Bruford, and A. F. G. Bourke. 2006. A test of reproductive skew models in a field population of a multiple-queen ant. Behav. Ecol. Sociobiol. 61: 265-275
- Hartfelder, K. and W. Engels. 1998. Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. Curr. Top. Dev. Biol. 40: 45-77
- Hartfelder, K., G. R. Makert, C. C. Judice, G. A. G. Pereira, W. C. Santana, R. Dallacqua, and M. M. G. Bitondi. 2006. Physiological and genetic mechanisms underlying caste development, reproduction and division of labor in stingless bees. Apidologie 37: 144-163
- Hayashi, Y., N. Lo, H. Miyata, and O. Kitade. 2007. Sex-linked genetic influence on caste determination in a termite. Science 318: 985-987
- Hechinger, R. F., A. C. Wood, and A. M. Kuris. 2011. Social organization in a flatworm: trematode parasites form soldier and reproductive castes. Proc. R. Soc. B Biol. Sci. 278: 656-665
- Heinze, J., B. Trunzer, B. Holldobler, and J. H. C. Delabie. 2001. Reproductive skew and queen relatedness in an ant with primary polygyny. Insectes Sociaux 48: 149-153
- Helantera, H. and F. Ratnieks. 2009. Sex allocation conflict in insect societies: who wins? Biol. Lett. 5: 700-704
- 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. Lond. B Biol. Sci. 269: 1871-1877
- Helms Cahan, S. H. and L. Keller. 2003. Complex hybrid origin of genetic caste determination in harvester ants. Nature 424: 306-309
- Helms Cahan, S. H. and S. B. Vinson. 2003. Reproductive division of labor between hybrid and nonhybrid offspring in a fire ant hybrid zone. Evolution 57: 1562-1570
- Holldobler, B. and E. Wilson. 1990. The ants. Belknap Press

- Holway, D. A., L. Lach, A. V. Suarez, N. D. Tsutsui, and T. J. Case. 2002. The causes and consequences of ant invasions. Ann. Rev. Ecol. Syst. 33: 181-233
- Hughes, W. O. H. and J. Boomsma. 2008. Genetic royal cheats in leaf-cutting ant societies. Proc. Nat. Acad. Sci. USA 105: 5150-5153
- Hughes, W. O. H., B. P. Oldroyd, M. Beekman, and F. L. W. Ratnieks. 2008. Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320: 1213-1216
- Hunter-Jones, P. 1958. Laboratory studies on the inheritance of phase characters in locusts. Anti-Locust Bull
- Ihle, K. E., R. E. Page, K. Frederick, M. K. Fondrk, and G. V. Amdam. 2010. Genotype effect on regulation of behaviour by vitellogenin supports reproductive origin of honeybee foraging bias. Anim. Behav. 79: 1001-1006
- Ikeya, T., M. Galic, P. Belawat, K. Nairz, and E. Hafen. 2002. Nutrient-dependent expression of Insulin-like Peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. Curr. Biol. 12: 1293-1300
- Ingram, K. K. 2002. Flexibility in nest density and social structure in invasive populations of the Argentine ant, *Linepithema humile*. Oecologia 133: 492-500
- Isles, A. R., W. Davies, and L. S. Wilkinson. 2006. Genomic imprinting and the social brain. Phil. Trans. R. Soc. B Biol. Sci. 361: 2229-2237
- Jones, J. C., M. R. Myerscough, S. Graham, and B. P. Oldroyd. 2004. Honey bee nest thermoregulation: diversity promotes stability. Science 305: 402-404
- Julian, G. E. and J. H. Fewell. 2004. Genetic variation and task specialization in the desert leaf-cutter ant, *Acromyrmex versicolor*. Anim. Behav. 68: 1-8
- 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. Nat. Acad. Sci. USA 99: 8157-8160
- Kaib, M., C. Husseneder, C. Epplen, J. Epplen, and R. Brandl. 1996. Kin-biased foraging in a termite. Proc. R. Soc. Lond. B Biol. Sci. 263: 1527-1532
- Keller, L., S. Aron, and L. Passera. 1996. Internest sex-ratio variation and male brood survival in the ant *Pheidole pallidula*. Behav. Ecol. 7: 292
- Keller, L. and L. Passera. 1992. Mating system, optimal number of matings, and sperm transfer in the Argentine ant *Iridomyrmex humilis*. Behav. Ecol. Sociobiol. 31: 359-366
- Keller, L. and L. Passera. 1993. Incest avoidance, fluctuating asymmetry, and the consequences of inbreeding in *Iridomyrmex humilis*, an ant with multiple queen colonies. Behav. Ecol. Sociobiol. 33: 191-199
- Keller, L., L. Passera, and D. Cantoni. 1992. Repeated mating by queens results in a single insemination in the Argentine ant *Iridomyrmex humilis*. Biol. Evol. Soc. Insects 35-39
- Keller, L., L. Passera, and J. P. Suzzoni. 1989. Queen Execution in the Argentine ant, *Iridomyrmex humilis*. Physiol. Entomol. 14: 157-163
- Keller, L. and K. Ross. 1998. Selfish genes: a green beard in the red fire ant. Nature 394: 573-575
- Keller, L., L. Sundstrom, and M. Chapuisat. 1997. Male reproductive success: paternity contribution to queens and workers in Formica ants. Behav. Ecol. Sociobiol. 41: 11-15
- Koyama, S., T. Takagi, S. Martin, T. Yoshida, and J. Takahashi. 2009. Absence of reproductive conflict during queen rearing in *Apis cerana*. Insectes Sociaux 56: 171-175
- Krieger, M. J. B. and L. Keller. 2000. Mating frequency and genetic structure of the Argentine ant *Linepithema humile*. Mol. Ecol. 9: 119-126

- Kronforst, M., D. Gilley, J. Strassmann, and D. Queller. 2008. DNA methylation is widespread across social Hymenoptera. Curr. Biol. 18: 287-288
- Kryger, P., U. Kryger, and R. F. A. Moritz. 2000. Genotypical variability for the tasks of water collecting and scenting in a honey bee colony. Ethology 106: 769-779
- Kummerli, R. and L. Keller. 2007a. Extreme reproductive specialization within ant colonies: some queens produce males whereas others produce workers. Anim. Behav. 74: 1535-1543
- Kummerli, R. and L. Keller. 2007b. Reproductive specialization in multiple-queen colonies of the ant *Formica exsecta*. Behav. Ecol. 18: 375-383
- Kutter, H. and R. Stumper. 1969. Sixth international congress of the International Union for the Study of Social Insects. Bern, Switzerland
- Larkin, M., G. Blackshields, N. Brown, R. Chenna, P. McGettigan, H. McWilliam, F. Valentin, I. Wallace, A. Wilm, and R. Lopez. 2007. Clustal W and Clustal X version 2.0. Bioinfo. 23: 2947-2948
- Li, Y., Z. Zhang, G. E. Robinson, and S. R. Palli. 2007. Identification and characterization of a juvenile hormone response element and its binding proteins. J. Biol. Chem. 282: 37605-37617
- Libbrecht, R., T. Schwander, and L. Keller. 2011. Genetic components to caste allocation in a multiple-queen ant species. Evolution 65: 2907-2915
- Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the -ΔΔCT method. Methods 25: 402-408
- Lu, H. L. and P. V. Pietrantonio. 2011. Insect insulin receptors: insights from sequence and caste expression analyses of two cloned hymenopteran insulin receptor cDNAs from the fire ant. Insect Mol. Biol. 20: 637-649
- MacMahon, J. A., J. F. Mull, and T. O. Crist. 2000. Harvester ants (*Pogonomyrmex* spp.): their community and ecosystem influences. Ann. Rev. Ecol. Syst. 31: 265-291
- Markin, G. 1970. The seasonal life cycle of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae), in southern California. Ann. Entomol. Soc. USA 63: 1238-1242
- Mattila, H. R. and T. D. Seeley. 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science 317: 362-364
- Maynard Smith, J. and E. Szathmary. 1995. The major transitions in evolution. Oxford University Press
- Maynard Smith, J. 1964. Group selection and kin selection. Nature 201: 1145-1147
- Mayr, E. 1963. Animal species and evolution. Belknap Press.
- Mellanby, K. 1939. Low temperature and insect activity. Proc. R. Soc. Lond. B Biol. Sci. 127: 473-487
- Michener, C. D. 1961. Social polymorphism in Hymenoptera. Symp. R. Entomol. Soc. Lond. 1: 43-56
- Miller, G. A., M. S. Islam, T. D. Claridge, T. Dodgson, and S. J. Simpson. 2008. Swarm formation in the desert locust Schistocerca gregaria: isolation and NMR analysis of the primary maternal gregarizing agent. J. Exp. Biol. 211: 370-376
- Minakuchi, C., X. Zhou, and L. M. Riddiford. 2008. Kruppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of *Drosophila melanogaster*. Mech. Dev. 125: 91-105
- Mintzer, A. 1990. Foundress female weight and cooperative foundation in *Atta* leaf-cutting ants. Applied Myrmecology - a World Perspective: 180-183
- Moreau, C. S., C. D. Bell, R. Vila, S. B. Archibald, and N. E. Pierce. 2006. Phylogeny of the ants: diversification in the age of angiosperms. Science 312: 101-104

- Moritz, R., H. Lattorff, P. Neumann, F. Kraus, S. Radloff, and H. Hepburn. 2005. Rare royal families in honeybees, *Apis mellifera*. Naturwissenschaften 92: 488-491
- Mutti, N. S., A. G. Dolezal, F. Wolschin, J. S. Mutti, K. S. Gill, and G. V. Amdam. 2011. IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. J. Exp. Biol. 214: 3977-3984
- Nelson, C. M., K. E. Ihle, M. K. Fondrk, R. E. Page, and G. V. Amdam. 2007. The gene vitellogenin has multiple coordinating effects on social organization. PLoS Biol. 5:e62
- Newell, W. 1909. The life history of the Argentine ant. J. Econom. Entomol. 2: 174-192
- Newey, P. and L. Keller. 2010. Social evolution: war of the worms. Curr. Biol. 20: R985-R987.
- Nilsen, K. A., K. E. Ihle, K. Frederick, M. K. Fondrk, B. Smedal, K. Hartfelder, and G. V. Amdam. 2011. Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. J. Exp. Biol. 214: 1488-1497
- Nygaard, S., G. Zhang, M. Schiott, C. Li, Y. Wurm, H. Hu, J. Zhou, L. Ji, F. Qiu, M. Rasmussen, H. Pan, F. Hauser, A. Krogh, C. J. Grimmelikhuijzen, J. Wang, and J. J. Boomsma. 2011. The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. Genome Res 21: 1339-1348
- Okada, Y., S. Miyazaki, H. Miyakawa, A. Ishikawa, K. Tsuji, and T. Miura. 2010. Ovarian development and insulin-signaling pathways during reproductive differentiation in the queenless ponerine ant *Diacamma* sp. J. Insect Physiol. 56: 288-295
- Oldroyd, B. P. and M. Beekman. 2008. Effects of selection for honey bee worker reproduction on foraging traits. PLoS Biol. 6:e56
- Oldroyd, B. P. and J. H. Fewell. 2007. Genetic diversity promotes homeostasis in insect colonies. Trends Ecol. Evol. 22: 408-413
- Oldroyd, B. P., T. E. Rinderer, J. R. Harbo, and S. M. Buco. 1992. Effects of intracolonial genetic diversity on honey bee (Hymenoptera: Apidae) colony performance. Ann. Entomol. Soc. America 85: 335-343
- Oster, G. and E. Wilson. 1979. Caste and ecology in the social insects. Princeton University Press
- Page, R. E., Jr., G. E. Robinson, M. K. Fondrk, and M. E. Nasr. 1995. Effects of worker genotypic diversity on honey bee colony development and behavior (*Apis mellifera L.*). Behav. Ecol. Sociobiol. 36: 387-396
- Page, R. E. and G. E. Robinson. 1991. The genetics of division of labour in honey bee colonies. Adv. Insect Physiol. 23: 117-169
- Pamilo, P. 1991. Evolution of colony characteristics in social insects. I. Sex allocation. Am. Nat. 137: 83-107
- Passera, L. 1980. The laying of biased eggs by the ant *Pheidole pallidula* (Nyl.) (Hymenoptera, Formicidae). Insectes Sociaux 27: 79-95
- Passera, L., S. Aron, and D. Bach. 1995. Elimination of sexual brood in the Argentine ant *Linepithema humile* - Queen effect and brood recognition. Entomol. Exp. et App. 75: 203-212
- Passera, L., S. Aron, E. Vargo, and L. Keller. 2001. Queen control of sex ratio in fire ants. Science 293: 1308-1310
- Passera, L. and L. Keller. 1992. The period of sexual maturation and the age at mating in *Iridomyrmex humilis*, an ant with intranidal mating. J. Zool. 228: 141-153
- Passera, L. and J. P. Suzzoni. 1979. The role of the queen of *Pheidole pallidula* (Nyl.) (Hymenoptera, Formicidae) in the brood sexualization after JH treatment. Insectes Sociaux 26: 343-353

- 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: e509
- Pérez-Sato, J. A., N. Châline, S. J. Martin, W. Hughes, and F. L. W. Ratnieks. 2009. Multi-level selection for hygienic behaviour in honeybees. Heredity 102: 609-615
- Petersen-Braun, M. 1977. Untersuchungen zur sozialen Organisation der PharaoameiseMonomorium pharaonis L.(Hymenoptera, Formicidae) II. Die Kastendeterminierung. Insectes Sociaux 24: 303-318
- Puig, O. and R. Tjian. 2006. Nutrient availability and growth: regulation of insulin signaling by dFOXO/FOXO1. Cell cycle (Georgetown, Tex.) 5: 503
- Pysek, P., D. M. Richardson, J. Pergl, V. Jarok, Z. Sixtov, and E. Weber. 2008. Geographical and taxonomic biases in invasion ecology. Trends Ecol. Evol. 23: 237-244
- Rabeling, C., J. Lino-Neto, S. C. Cappellari, I. A. Dos-Santos, U. G. Mueller, and M. Bacci. 2009. Thelytokous parthenogenesis in the fungus-gardening ant *Mycocepurus smithii* (Hymenoptera: Formicidae). PLoS ONE 4: e6781
- Ravary, F., E. Lecoutey, G. Kaminski, N. Chaline, and P. Jaisson. 2007. Individual experience alone can generate lasting division of labor in ants. Curr. Biol. 17: 1308-1312
- Rice, W. R. 1984. Sex chromosomes and the evolution of sexual dimorphism. Evolution 38: 735-742
- Robinson, E., O. Feinerman, and N. Franks. 2009. Flexible task allocation and the organization of work in ants. Proc. R. Soc. Lond. B Biol. Sci. 276: 4373-4380
- Robinson, G. E. 1992. Regulation of division of labor in insect societies. Ann. Rev. Entomol. 37: 637-665
- Robinson, G. E. and R. Page. 1989a. Genetic basis for division of labor in an insect society. The genetics of social evolution 61-80
- Robinson, G. E. and R. E. Page, Jr. 1988. Genetic determination of guarding and undertaking in honey-bee colonies. Nature 333: 356-358
- Robinson, G. E. and R. E. Page, Jr. 1989b. Genetic determination of nectar foraging, pollen foraging, and nest-site scouting in honey bee colonies. Behav. Ecol. Sociobiol. 24: 317-323
- Robinson, G. E. and E. L. Vargo. 1997. Juvenile hormone in adult eusocial hymenoptera: Gonadotropin and behavioral pacemaker. Arch. Insect Biochem. Physiol. 35: 559-583
- Ross, K. 1988. Differential reproduction in multiple-queen colonies of the fire ant *Solenopsis invicta* (Hymenoptera: Formicidae). Behav. Ecol. Sociobiol. 23: 341-355
- Ross, K. G. and R. W. Matthews. 1991. The social biology of wasps. Cornell University Press
- Rosset, H. and M. Chapuisat. 2006. Sex allocation conflict in ants: when the queen rules. Curr. Biol. 16: 328-331
- Rosset, H., L. Keller, and M. Chapuisat. 2005. Experimental manipulation of colony genetic diversity had no effect on short-term task efficiency in the Argentine ant *Linepithema humile*. Behav. Ecol. Sociobiol. 58: 87-98
- Rothenbuhler, W. C. 1964. Behaviour genetics of nest cleaning in honey bees. I. Responses of four inbred lines to disease-killed brood. Anim. Behav. 12: 578-583
- Rychlik, W. 2007. OLIGO 7 primer analysis software. Methods mol. biol. 402:35
- Saiful Islam, M., P. Roessingh, S. J. Simpson, and A. R. McCaffery. 1994. Parental effects on the behaviour and colouration of nymphs of the desert locust *Schistocerca gregaria*. J. Insect Physiol. 40: 173-181

- Schultz, T. R. 2000. In search of ant ancestors. Proc. Nat. Acad. Sci. USA 97:14028.
- 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
- Schwander, T. and L. Keller. 2008. Genetic compatibility affects queen and worker caste determination. Science 322: 552
- Schwander, T., N. Lo, M. Beekman, B. Oldroyd, and L. Keller. 2010. Nature versus nurture in social insect caste differentiation. Trends in Ecol. Evol. 25: 275-282
- Schwander, T., H. Rosset, and M. Chapuisat. 2005. Division of labour and worker size polymorphism in ant colonies: the impact of social and genetic factors. Behav. Ecol. Sociobiol. 59: 215-221
- Seeley, T. D. 1982. Adaptive significance of the age polyethism schedule in honeybee colonies. Behav. Ecol. Sociobiol. 11: 287-293
- Sim, C. and D. L. Denlinger. 2008. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc. Nat. Acad. Sci. USA 105: 6777-6781
- Simpson, S. J., G. A. Sword, and N. Lo. 2011. Polyphenism in insects. Curr. Biol. 21: 738-749
- Smith, C., K. Anderson, C. Tillberg, J. Gadau, and A. Suarez. 2008a. Caste determination in a polymorphic social insect: nutritional, social, and genetic factors. Am. Nat. 172: 497-507
- Smith, C., A. Toth, A. Suarez, and G. Robinson. 2008b. Genetic and genomic analyses of the division of labour in insect societies. Nature Rev. Genetics 9: 735-748
- Smith, C. D., A. Zimin, C. Holt, E. Abouheif, R. Benton, E. Cash, V. Croset, C. R. Currie, E. Elhaik, C. G. Elsik, M. J. Fave, V. Fernandes, J. Gadau, J. D. Gibson, D. Graur, K. J. Grubbs, D. E. Hagen, M. Helmkampf, J. A. Holley, H. Hu, A. S. Viniegra, B. R. Johnson, R. M. Johnson, A. Khila, J. W. Kim, J. Laird, K. A. Mathis, J. A. Moeller, M. C. Munoz-Torres, M. C. Murphy, R. Nakamura, S. Nigam, R. P. Overson, J. E. Placek, R. Rajakumar, J. T. Reese, H. M. Robertson, C. R. Smith, A. V. Suarez, G. Suen, E. L. Suhr, S. Tao, C. W. Torres, E. van Wilgenburg, L. Viljakainen, K. K. Walden, A. L. Wild, M. Yandell, J. A. Yorke, and N. D. Tsutsui. 2011a. Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). Proc. Nat. Acad. Sci. USA 108: 5673-5678
- Smith, C. R., C. D. Smith, H. M. Robertson, M. Helmkampf, A. Zimin, M. Yandell, C. Holt, H. Hu, E. Abouheif, R. Benton, E. Cash, V. Croset, C. R. Currie, E. Elhaik, C. G. Elsik, M. J. Fave, V. Fernandes, J. D. Gibson, D. Graur, W. Gronenberg, K. J. Grubbs, D. E. Hagen, A. S. Viniegra, B. R. Johnson, R. M. Johnson, A. Khila, J. W. Kim, K. A. Mathis, M. C. Munoz-Torres, M. C. Murphy, J. A. Mustard, R. Nakamura, O. Niehuis, S. Nigam, R. P. Overson, J. E. Placek, R. Rajakumar, J. T. Reese, G. Suen, S. Tao, C. W. Torres, N. D. Tsutsui, L. Viljakainen, F. Wolschin, and J. Gadau. 2011b. Draft genome of the red harvester ant *Pogonomyrmex barbatus*. Proc. Nat. Acad. Sci. USA 108: 5667-5672
- Snyder, L. E. 1992. The genetics of social behavior in a polygynous ant. Naturwissenschaften 79: 525-527
- Snyder, L. E. 1993. Non-random behavioural interactions among genetic subgroups in a polygynous ant. Anim. Behav. 46: 431-439
- Stearns, S. C. 1989. The evolutionary significance of phenotypic plasticity. Bioscience 39: 436-445
- Stern, D. L. and W. A. Foster. 1996. The evolution of soldiers in aphids. Biol. Rev. 71: 27-79
- Stocker, H. and E. Hafen. 2000. Genetic control of cell size. Curr. opinion in genetics & development 10: 529
- Stradling, D. 1978. The influence of size on foraging in the ant, *Atta cephalotes*, and the effect of some plant defence mechanisms. J. Anim. Ecol. 173-188

- Stuart, R. J. and R. E. Page. 1991. Genetic component to division of labor among workers of a leptothoracine ant. Naturwissenschaften 78 :375-377
- Suarez, A. V., D. A. Holway, and T. J. Case. 2001. Patterns of spread in biological invasions dominated by long-distance jump dispersal: Insights from Argentine ants. Proc. Nat. Acad. Sci. USA 98: 1095-1100
- Suen, G., C. Teiling, L. Li, C. Holt, E. Abouheif, E. Bornberg-Bauer, P. Bouffard, E. J. Caldera, E. Cash, A. Cavanaugh, O. Denas, E. Elhaik, M. J. Fave, J. Gadau, J. D. Gibson, D. Graur, K. J. Grubbs, D. E. Hagen, T. T. Harkins, M. Helmkampf, H. Hu, B. R. Johnson, J. Kim, S. E. Marsh, J. A. Moeller, M. C. Munoz-Torres, M. C. Murphy, M. C. Naughton, S. Nigam, R. Overson, R. Rajakumar, J. T. Reese, J. J. Scott, C. R. Smith, S. Tao, N. D. Tsutsui, L. Viljakainen, L. Wissler, M. D. Yandell, F. Zimmer, J. Taylor, S. C. Slater, S. W. Clifton, W. C. Warren, C. G. Elsik, C. D. Smith, G. M. Weinstock, N. M. Gerardo, and C. R. Currie. 2011. The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. PLoS Genet. 7:e1002007.
- Sundstrom, L., M. Chapuisat, and L. Keller. 1996. Conditional manipulation of sex ratios by ant workers: a test of kin selection theory. Science 274: 993
- Sundstrom, L. and F. L. W. Ratnieks. 1998. Sex ratio conflicts, mating frequency, and queen fitness in the ant *Formica truncorum*. Behav. Ecol. 9: 116-121
- Sutherland, O. 1969. The role of crowding in the production of winged forms by two strains of the pea aphid, *Acyrthosiphon pisum*. J. Insect Physiol. 15: 1385-1410
- Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony. Version 4.
- Szathmary, E. and J. Maynard Smith. 1995. The major evolutionary transitions. Nature 374: 227-232
- Taber, S. W. 1999. The world of the harvester ants. Texas A & M University Press.
- Tatar, M., A. Kopelman, D. Epstein, M. P. Tu, C. M. Yin, and R. Garofalo. 2001. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science's STKE 292: 107
- Theraulaz, G., E. Bonabeau, and J. Deneubourg. 1998. Threshold reinforcement and the regulation of division of labour in social insects. Proc. R. Soc. Lond. B Biol. Sci. 265: 327-332
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic acids research 25: 4876-4882
- Tissenbaum, H. A. and G. Ruvkun. 1998. An insulin-like signaling pathway affects both longevity and reproduction in *Caenorhabditis elegans*. Genetics 148: 703-718
- Tschinkel, W. R. 1988. Social control of egg laying rate in queens of the fire ant, *Solenopsis invicta*. Physiol. Entomol.13: 327-350
- Tschinkel, W. R. 2006. The fire ants. Belknap Press
- Tsutsui, N. D. and T. J. Case. 2001. Population genetics and colony structure of the argentine ant (*Linepithema humile*) in its native and introduced ranges. Evolution 55: 976-985
- Tu, M. P., C. M. Yin, and M. Tatar. 2005. Mutations in insulin signaling pathway alter juvenile hormone synthesis in *Drosophila melanogaster*. General and Comparative Endocrinology 142: 347-356
- Vargo, E. L. and L. Passera. 1992. Gyne development in the Argentine ant *Iridomyrmex humilis* - Role of overwintering and queen control. Physiol. Entomol. 17: 193-201
- Vogel, V., J. S. Pedersen, T. Giraud, M. J. B. Krieger, and L. Keller. 2010. The worldwide expansion of the Argentine ant. Diversity and Distributions 16: 170-186

- Volny, V. P. and D. M. Gordon. 2002. Genetic basis for queen-worker dimorphism in a social insect. Proc. Nat. Acad. Sci. USA 99: 6108-6111
- Waddington, S. J., L. A. Santorelli, F. R. Ryan, and W. O. H. Hughes. 2010. Genetic polyethism in leaf-cutting ants. Behav. Ecol. 21: 1165-1169
- Wang, Y., M. Jorda, P. L. Jones, R. Maleszka, X. Ling, H. M. Robertson, C. A. Mizzen, M. A. Peinado, and G. E. Robinson. 2006. Functional CpG methylation system in a social insect. Science 314: 645-647
- Waterhouse, A. M., J. B. Procter, D. M. A. Martin, M. Clamp, and G. J. Barton. 2009. Jalview Version 2 multiple sequence alignment editor and analysis workbench. Bioinformatics 25: 1189-1191
- Wenseleers, T. and F. Ratnieks. 2004. Tragedy of the commons in *Melipona* bees. Proc. R. Soc. B Biol. Sci. 271: S310
- West-Eberhard, M. J. 1987. Flexible strategy and social evolution. Animal societies: theories and facts 35-51
- West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. Ann. Rev. Ecol. System. 20: 249-278
- Wetterer, J. K., A. L. Wild, A. V. Suarez, N. Roura-Pascual, and X. Espadaler. 2009. Worldwide spread of the Argentine ant, *Linepithema humile* (Hymenoptera: Formicidae). Myrmecological News 12: 187-194
- Wheeler, D. 1986. Developmental and physiological determinants of caste in social Hymenoptera: Evolutionary implications. Am Nat 128: 13
- Wheeler, D. E., N. Buck, and J. D. Evans. 2006. Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera*. Insect Mol. Biol. 15: 597-602
- 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
- Wilson, E. O. 1971. The insect societies. Cambridge, MA: Harvard University Press.
- Wilson, E. O. 1980. Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: Atta). Behav. Ecol. Sociobiol. 7: 143-156
- Winter, U. and A. Buschinger. 1986. Genetically mediated queen polymorphism and caste determination in the slave-making ant, *Harpagoxenus sublaevis* (Hymenoptera: Formicidae). Entomologia generalis 11: 125-137
- Wurm, Y., J. Wang, O. Riba-Grognuz, M. Corona, S. Nygaard, B. G. Hunt, K. K. Ingram, L. Falquet, M. Nipitwattanaphon, D. Gotzek, M. B. Dijkstra, J. Oettler, F. Comtesse, C. J. Shih, W. J. Wu, C. C. Yang, J. Thomas, E. Beaudoing, S. Pradervand, V. Flegel, E. D. Cook, R. Fabbretti, H. Stockinger, L. Long, W. G. Farmerie, J. Oakey, J. J. Boomsma, P. Pamilo, S. V. Yi, J. Heinze, M. A. Goodisman, L. Farinelli, K. Harshman, N. Hulo, L. Cerutti, I. Xenarios, D. Shoemaker, and L. Keller. 2011. The genome of the fire ant *Solenopsis invicta*. Proc. Nat. Acad. Sci. USA 108: 5679-5684

APPENDICES

Appendix 1:

Primer sequences for all genes tested in chapters 3 and 4

	Forward	Reverse
RP49	CGATAGATATGACAAACTCAAACGCAAC	GTATTGGCCCTTGAAACGTCTGCG
ILP1	GGATAACACGGGAGTCTATCGC	AAGGGGCTTGCAATATCGTTC
ILP2	CGATTACCCATTCGCCTACGAG	GCGACTCCCTTCGATAACGTCT
JHepox	ATACTTCAAGCCGAGTTGGAC	AAAGCTTCCGTCATTGGCAAG
Vg1	ACAGGACGATGTTGTTTCGGAATTA	TCGTCACGGATGATTGAATGGTATAT
Vg2	TCTAATGATGGAGTTCTTTCGAGATCA	ACGGAAGACTGAATAGTGAAGCGTT

Primer sequences used for qRT-PCR (5'-3' order)

Appendix 2:

Do male lineages differ in sperm quality in *Linepithema humile*?

Romain Libbrecht, Elodie Gaide and Laurent Keller

This experiment was conducted as a follow-up to chapter 1, which revealed an effect of the interaction between queens and males on the sex ratio produced in *L. humile* colonies. One of the hypotheses proposed to explain this result is that queens may actively change sex allocation depending on qualities of the sperm transferred by their mate. Under this hypothesis, male lineages are expected to differ in sperm quality. This experiment aimed to compare sperm quantity and viability between several lineages of males in *L. humile*.

Methods

Production of male lineages

We collected *L. humile* colonies on 7 September 2010 in Port-Leucate (3°2'20"E, 42°51'22"N), southern France and set up 16 single-queen colonies with 2.5cm^3 (ca. 1000) workers. To ensure that colonies contained only brood from the mother queen, we removed all the brood present during the first two weeks. The queens were then allowed to lay eggs during three weeks before being removed so as to stimulate the production of sexuals (new queens and males) (Keller and Passera 1992; Keller and Passera 1993). Colonies were then regularly checked to transfer all male pupae to queenless and broodless recipient colonies. This allowed us to obtain large numbers of unmated males of the same lineage (i.e., produced by the same mother queen). Sixty males belonging to five different lineages

(lineages PS01, PS06, PS07, PS09, PS10; 12 males per lineage) were used to measure sperm quality and viability.

Dissection protocol

Males were dissected in a drop of a buffer solution (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7) on a petri dish kept on ice under a Leica stereomicroscope (magnification 8X). The internal reproductive system of males is attached to the genitalia and can easily be extracted from the abdomen by withdrawing the genitalia with minute forceps (Keller and Passera 1992). Male testes degenerate rapidly after emergence (Passera and Keller 1992), and the sperm are stored in the seminal vesicles, which were extracted and crushed in 15 µl of buffer. This solution was then vortexed for 2 minutes. Live and dead sperm were then differentially stained using the Live/Dead™ sperm viability kit (L-7011, Molecular Probes), which consists of a membrane-permeant nucleic acid stain for live sperm (SYBR-14) and a dead cell stain (propidium iodide). For each measurement, 5 µl SYBR-14 working solution (SYBR-14 stock diluted 250X in buffer solution) was added to the 15 µl of sperm solution and incubated for 10 min at 36°C. Afterwards, 10 µl of propidium iodide working solution (propidium iodide 2.4mM diluted 66X in buffer solution) was added to each sample and incubated for 10 minutes at 36°C, resulting in 30 µl of sperm solutino per male.

Measure of sperm quantity

5 µl of sperm solution was transferred in a Helber hematocytometer with field volumes of $1.25e^{-06}$ mm^3. The number of sperm present in 6 fields of the hematocytometer was counted in neutral light under a microscope (Leica DM5500, DFC 480 camera, magnification 400X). Sperm quantity was then calculated by multiplying the mean number of sperm per field by 24000 (=0.03 ml / $1.25E^{-06}$ mm^3, i.e. the ratio of the volume of the solution in which the seminal vesicles were crushed to that of a field).

Measure of sperm viability

10 µl of sperm solution was transferred on a SuperFrost microscope slide. The number of live and dead sperm was then counted in 16 randomly selected fields using a fluorescence microscope (Leica DM5500, DFC 480 camera, 480 nm GFP and 516 nm rhodamine filters, magnification 400X). Sperm viability was calculated for each sample as the percentage of live sperm in the total number of sperm counted.

Statistical analyses

To test for the effect of male lineages on sperm quantity and viability, we conducted 1-way analyses of variance (ANOVAs) on models optimized to fit our data. The proportion of live sperm (square-root arcsine transformed) and the number of sperm were analyzed using general linear models. We checked for normality and homogeneity by visual inspections of plots of residuals against fitted values.. The correlation between sperm viability and sperm quantity was tested using Spearman rank correlation test. All statistical analyses were performed with *R* (http://www.R-project.org).

Results

The quantity of sperm per male ranged from 140000 to 600000 (354564 ± 95797) and was significantly affected by the male lineage ($F_{4,55}$ = 3.08, P = 0.02; Figure 1).

The proportion of live sperm ranged from 0.74 to 0.99 (0.92 ± 0.05, mean ± sd) and did not differ significantly between male lineages ($F_{4,55}$ = 0.57, P = 0.68; Figure 2).

Finally, there was no significant correlation between sperm quantity and viability (n = 60, rho = -0.06, P = 0.62).

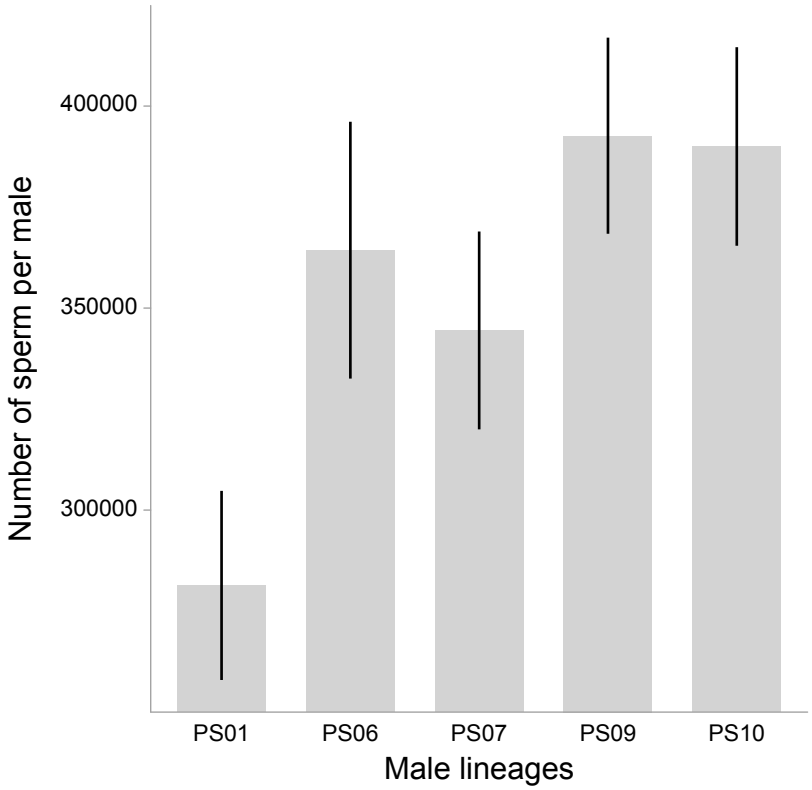

Figure 1 – Sperm quantity (mean ± se) was significantly affected by the paternal lineage.

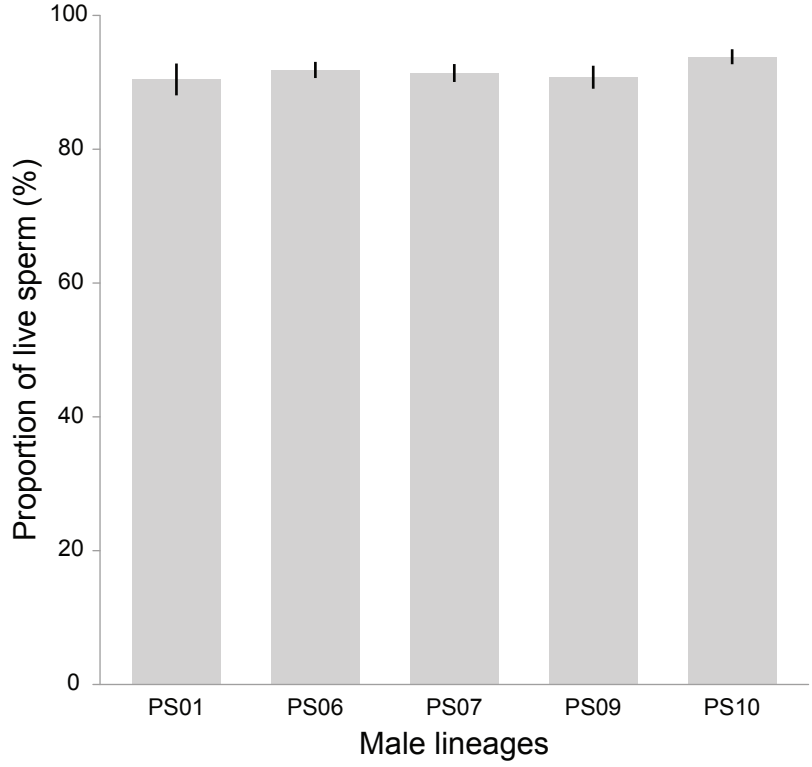

Figure 2 – Sperm viability (mean ± se) did not significantly differ between paternal lineages.

Conclusion

Chapter 1 revealed an effect of the interaction between queens and males on the sex ratio produced in *L. humile* colonies. One of the hypotheses proposed to explain this result is that queens may actively change sex allocation depending on qualities of the sperm transferred by their mate. This hypothesis would require differences in sperm quality between male lineages. Consistent to this prediction, this preliminary experiment revealed differences in sperm quantity (but not in sperm viability) among 5 male lineages in *L. humile*. However, this experiment did not allow us to test whether such differences were directly associated with differences in the sex ratio produced. Studies combining controlled crosses and techniques of male dissection and sperm measures will be needed to answer this question.

Thèse de doctorat déposée le 2 Août 2012

Thèse de doctorat soutenue devant le jury le 6 Septembre 2012

Thèse de doctorat soutenue publiquement le 28 Septembre 2012

Lausanne, 2012