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Conflict and cooperation in the societies of the clonal ant *Cerapachys biroi*

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Table of Contents

I. Introduction

Social insects (mainly in the insect groups Isoptera and Hymenoptera, plus a few other examples) have achieved an extremely high success in almost all of Earth's ecosystems. This dominance is probably due to the inter-individual cooperation within societies, which involves among others a division of reproductive labor and of ergonomic functions. Social complexity, together with colony-level coherence and efficiency, are reached via extremely sophisticated mechanisms of communication between colony members. This group-level coordination, and the presence of a (almost) unique, collectively shared reproductive interest within single societies, makes colonies comparable to single organisms. However, highly diverse social phenotypes have emerged over evolutionary time due to the action of different selective pressures. Studying the life history traits of social insects allows understanding how group-level cohesion is maintained, and which are the mechanisms behind its evolution. The Hymenoptera family Formicidae originated around 120 million years ago, and has become the most species-rich and ecologically diverse group of social insects (Ward 2010, Grimaldi & Engel 2005; Holldobler & Wilson 1990). Although scientists have already described around 13.000 species of ants, this number keeps growing, and the whole family is estimated to include around 25.000 species (Ward 2010). Ants are currently divided in 21 subfamilies (Rabeling et al. 2008, Ward 2007), many of which belong to a taxonomic group known as formicoid clade. This group includes the subfamilies Dolichoderinae, Formicinae, Myrmicinae, Myrmeciinae, Pseudomyrmecinae and a group of ants known as dorylomorphs (Bolton 1990; Brady 2003), which is in turn constituted by the army ants and some other closely related group (Ward 2010). *Cerapachys biroi*, the ant species I have used as model system for this thesis, belongs to the dorylomorph subfamily Cerapachyinae.

I.I The dorylomorph ants

Formerly known as the doryline taxonomic section of ants (Bolton 1990, 2003), the dorylomorph ants include six subfamilies: Aenictogitoninae, Cerapachyinae and Leptanilloidinae, plus the “true army ants” Aenictinae, Dorylinae and Ecitoninae (Figure 1). Aenictogitoninae include exclusively the genus *Aenictogiton* and is confined, as far as is currently known, to central Africa (Brown 1975). Until some years ago this subfamily was only known from male specimens. *Aenictogiton* queens are still unknown to scientists, and detailed information on the behavior of the subfamily is still missing. Leptanilloidinae have been collected very rarely and, with the exception of one record, are known exclusively from worker specimens (Longino 2003). According to Brandão et al. (1999), foraging trails and larval transport are reminiscent of army ants, but no other records exist of their behavior.

The three true army ants” subfamilies Aenictinae, Ecitoninae and Dorylinae, which have been more recently renamed “AenEcDo” (Kronauer 2009), have received the most attention from biologists because of their peculiar traits. The subfamily Cerapachyinae, to which *C. biroi* belongs, is for many aspects similar to AenEcDo army ants.

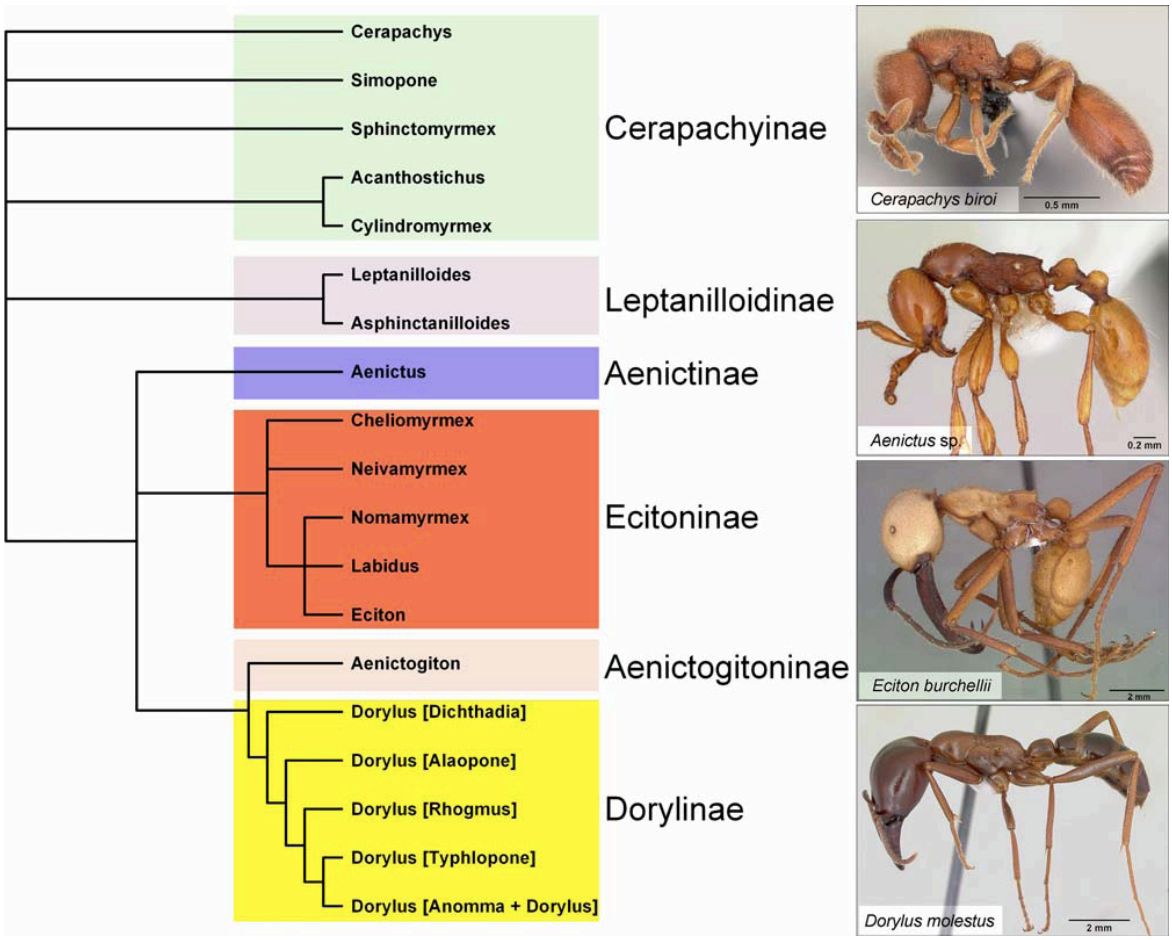

Figure 1. The current understanding of the internal phylogeny of the dorylomorph section of ants, based on molecular and morphological studies. Insert pictures are courtesy of AntWeb at www.antweb.org and April Nobile. From Kronauer (2009) with permission.

I.II The 'true army ants' and the army ant adaptive syndrome

AenEcDo army ants are found in tropical and subtropical areas of Africa, the Americas, Asia, and Indo-Australia, although a few species are found in more temperate regions (they are absent in regions with cold winters and from many remote islands; Kronauer 2009). The New World army ants are categorized in two tribes and five genera (Borgmeier 1955, Watkins 1976, Bolton et al. 2007, Ward 2007, Kronauer 2009). The tribe Cheliomyrmecini includes the single genus *Cheliomyrmex,* whereas the tribe Ecitonini contains four genera: *Neivamyrmex, Nomamyrmex, Labidus* and *Eciton*. The two Old World army ant subfamilies,

Dorylinae and Aenictinae, include just one genus each, respectively *Dorylus* and *Aenictus*. Dorylinae is primarily Afrotropical, with a few species occurring in the Oriental, Indo-Australian, and Palaeartic biogeographical regions. Most Aenictinae species occur in the Oriental and Indo-Australian regions, with some species living in the Afrotropical region (Wilson 1964; Bolton 1995).

All species within the AenEcDo army ants share behavioral and reproductive traits such as obligate collective foraging (group predation), nomadism, and highly modified permanently wingless queens (dichthadiigynes) that found new colonies accompanied by workers via obligatory dependent foundation (Wilson 1958; Schneirla 1971; Gotwald 1995; Kronauer 2009). The presence of these three traits has been referred to as the "Army ants adaptive syndrome" (Gotwald 1995; Brady 2003; Kronauer 2009). Group predation includes both group raiding and group retrieval of living prey (Gotwald 1995, Wilson 1958a). Nomadism implies that army ant colonies typically emigrate from one nest site to another, and it has possibly evolved because it allows colonies to adaptively change their hunting grounds periodically. AenEcDo army ant queens are unique in that they have worker-like shaped thoraxes (ergatoid queens) and abdomens with highly extendable intersegmental membranes. This, together with an extremely intense ovarian activity, allows the production of up to hundreds of thousands eggs per day in some species. Group raiding requires a large colony size, and consequently colony foundation via fission (i.e. a newly mated queen plus a large number of workers departing from the original colony) is an optimal way for new army ant colonies to maintain the minimal size for foraging efficiency.

Cerapachys biroi belongs to the dorylomorph subfamily Cerapachyinae. This subfamily is a paraphyletic group organized in three tribes: Acanthostichini (with the genus *Acanthostichus*), Cerapachyini (with the genera *Cerapachys*, *Simopone*, *Sphinctomyrmex, Tanipone* and *Vicinopone*), and Cylindromyrmecini (with the genus *Cylindromyrmex*). Many known cerapachyine species show behaviors that are somewhat similar to those of the true army ants (Wilson, 1958; Brown 1975; Hölldobler 1982; Buschinger et al. 1989; Fisher 1997; Ravary & Jaisson 2002, Kronauer 2009). *Cerapachys biroi* shares are somewhat similar to some AenEcDo army ants such as for example for the biphasic reproductive cycle typical of Ecitoninae.

Figure 2. A *Cerapachys biroi* worker tending a larva. Photo by Serafino Teseo.

I.III The study system *Cerapachys biroi*

Generalities and distribution. *Cerapachys biroi* (Forel 1907; Figure 2) is an ant belonging to the subfamily Cerapachyinae. It measures around 2-3 mm in length and lives underground in colonies of a few hundred individuals. *Cerapachys biroi* feeds exclusively on other ants' brood, even though under laboratory conditions it occasionally accepts soft-bodied larvae or pupae of other hymenopterans (such as bumblebees for example), and has been observed feeding on caterpillars in natural conditions (Wolcott 1948; Wetterer 2012). The native range of *C. biroi* is believed to extend from northern India and Nepal to southern China and Vietnam (Wetterer et al. 2012), but the species has reached a circumtropical distribution on islands around the world (Figure 3) probably around the beginning of the 20th century. This makes *C. biroi* the only known case of introduced species in the dorylomorph clade of the family Formicidae (Wetterer et al. 2012; Kronauer et al. 2012).

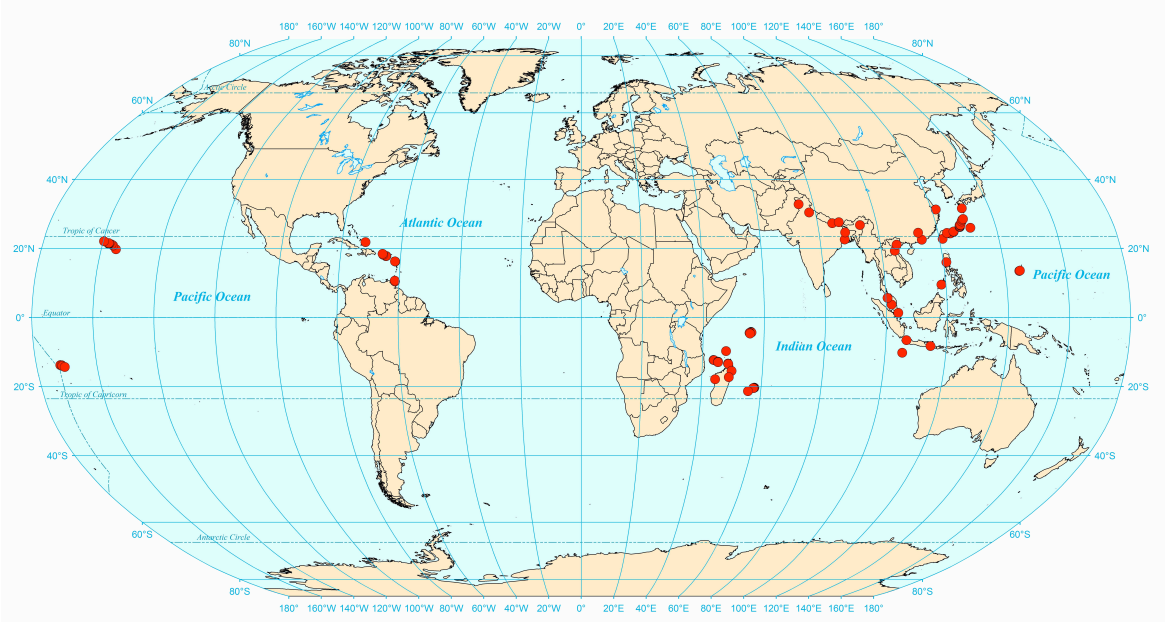

Figure 3. Worldwide distribution record of *Cerapachys biroi*. From Wetterer et al. (2012) with permission.

In the introduced range, colonies are typically found in disturbed and anthropized habitats such as parks and agricultural landscapes. According to a recent study

on the phylogeography of *C. biroi* (Kronauer et al. 2012), at least four distinct mitochondrial haplotypes are found in the introduced range (Figure 4), each of them associated with a certain number of characteristic, closely related nuclear microsatellite genotypes (multi-locus lineages or MLLs). This means that at least four independent genetic lineages have been introduced from the native range. According to Kronauer et al. (2012), these lineages appear to all be closely related when compared to samples from the putative native range (northern India, Nepal, Vietnam and China). On four introduced mitochondrial haplotypes, two are found on only a single island (haplotype C on Okinawa and haplotype D on American Samoa), while the other two (A and B) have achieved a wider distribution. Haplotype A is found on four different islands in Asia, and haplotype B is found in the Caribbean, the Indian Ocean and Asia.

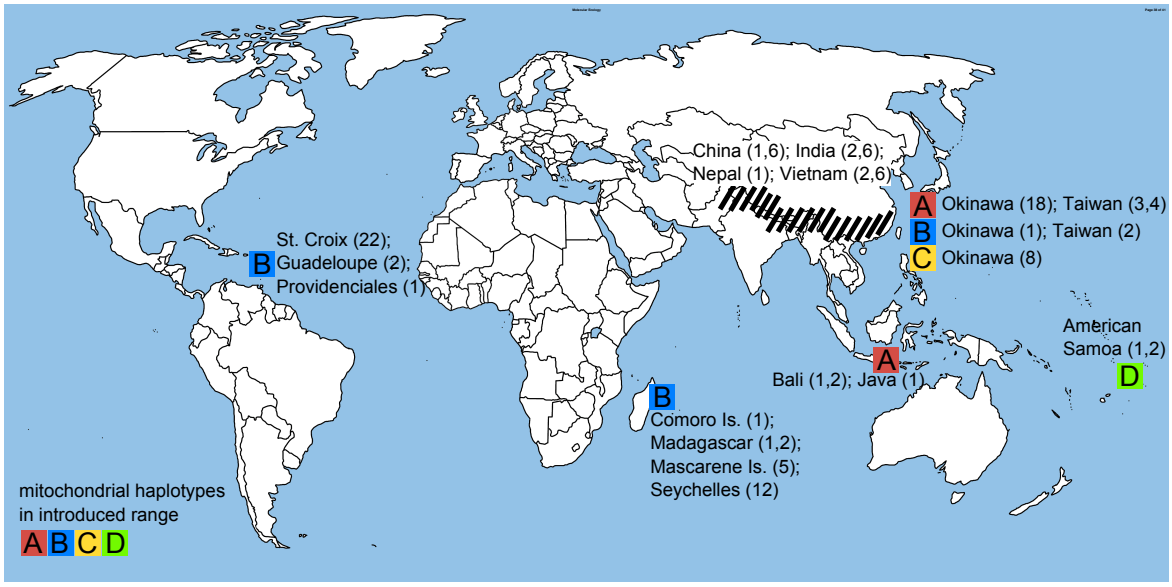

Figure 4. Global distribution of *Cerapachys biroi*. Samples of the introduced range are color-coded according to mitochondrial haplotype. The putative native range is hatched black. From Kronauer et al. (2012) with permission.

Given that winged queens are absent in *C. biroi* and that the species has a strictly subterranean lifestyle, the worldwide spread of the species is almost certainly human mediated, and might occur when colony fragments are accidentally transported in soil. The colonies used for the experiments described in the present manuscript come from the populations of Okinawa and Taiwan, and

belong to three different multi-locus lineages (Table 1).

Table 1. Names, clonal lineages, origin and collection dates of the colonies used in the experiments described in this thesis.

Colony	Clonal lineage	Origin	Field collection date
J1	MLL1 (A)	Java, Indonesia	2005
O4	MLL1 (A)	Okinawa, Japan	2006
O5	MLL1 (A)	Okinawa, Japan	2006
T4	MLL1 (A)	Taiwan	2001
T5	MLL1 (A)	Taiwan	2001
O6	MLL4 (B)	Okinawa, Japan	2006
T1C	MLL4 (B)	Taiwan	1997
T3	MLL4 (B)	Taiwan	2000
C10	MLL6 (C)	Okinawa, Japan	2008
C11	MLL6 (C)	Okinawa, Japan	2008
C3B	MLL6 (C)	Okinawa, Japan	2008
C9	MLL6 (C)	Okinawa, Japan	2008

Clonality. Unlike most social Hymenoptera, colonies of *C. biroi* lack sexual castes. Queens are absent, and males are produced very rarely (they are known exclusively from laboratory colonies). This sporadic production of males may indeed suggest the existence of some sexual population in the native range of the species. Evidence for a sexual recombination event has been found in one colony from Taiwan (T4, Table 1), and supports this hypothesis. Alternatively, given that no spermatheca has ever been observed in *C. biroi* (Tsuji & Yamauchi 1995), males might simply be a vestige of an ancestral sexual reproductive system. In *Cerapachys biroi* colonies all individuals reproduce at least for a period of their life, via obligatory thelytokous parthenogenesis. Intra-colonial variability is very low, with all individuals almost genetically identical (intra-colonial relatedness=0.99 in Kronauer et al. 2012). Thelytokous parthenogenesis in *C. biroi* is not determined by microorganisms such as *Wolbachia* (Wenseleers &

Billen 2000), which has been shown to produce parthenogenesis in many invertebrates (Stouthamer et al. 1999). As heterozigosity persists in the absence of sex and haploid males occur, automixis with central fusion is the most probable mechanism explaining thelytoky in this species (Kronauer et al 2012).

Biphasic reproductive cycle. The colonies of *C. biroi* develop according to a biphasic reproductive cycle where they alternate, in a stereotypical way, two different activity phases synchronized on the development of the brood (Ravary & Jaisson 2002, 2004; Ravary et al. 2006; Teseo et al. 2013, Figure 5). The foraging phase, which lasts on average 16 days, begins just after the synchronous emergence of the callow workers and the hatching of the eggs. During this phase, workers older than 4-5 months forage in search of prey, whereas the younger fertile workers stay inside the nest and take care of the developing larvae. The reproductive phase, which lasts approximately 18 days, starts at the beginning of larval pupation, which occurs in a synchronous way. During the reproductive phase, all colony members form dense aggregates, stop feeding and stay inside the nest until the onset of the next foraging phase. No extra-nidal activity is observed in the reproductive phase, and during this period individuals lay eggs and show low activity levels. Around four days after the onset of pupation, eggs are laid and amassed in clusters among metamorphosing pupae. After more or less 10 further days (14 days from the onset of pupation), pupae complete their development and emerge, and freshly hatched larvae consume their vestigial cocoon. Consistent oophagy by premature larvae on eggs occurs at this point. The average duration of a complete reproductive cycle (the sum of the durations of both activity phases) is approximately 34 days, and the development of a generation of brood, from egg to adult insects, extends on average around 45-60 days (Ravary & Jaisson 2002; Ravary 2003; Teseo personal observation). The reproductive phase has been previously referred to as statary phase (Ravary & Jaisson 2002, 2004; Ravary et al. 2006, 2007; Lecoutey et al. 2011), a term borrowed from the biphasic cycles of the army ant of the genus *Eciton* (Schneirla 1934). As it is not clear whether and how *C. biroi* migrate during the foraging phase, and at least in captivity no clear tendency to migrate is observed during the foraging phase, we prefer, and will use in this thesis, the

formulation “reproductive phase” to “statary phase”, the latter term making sense only for truly migrating ants.

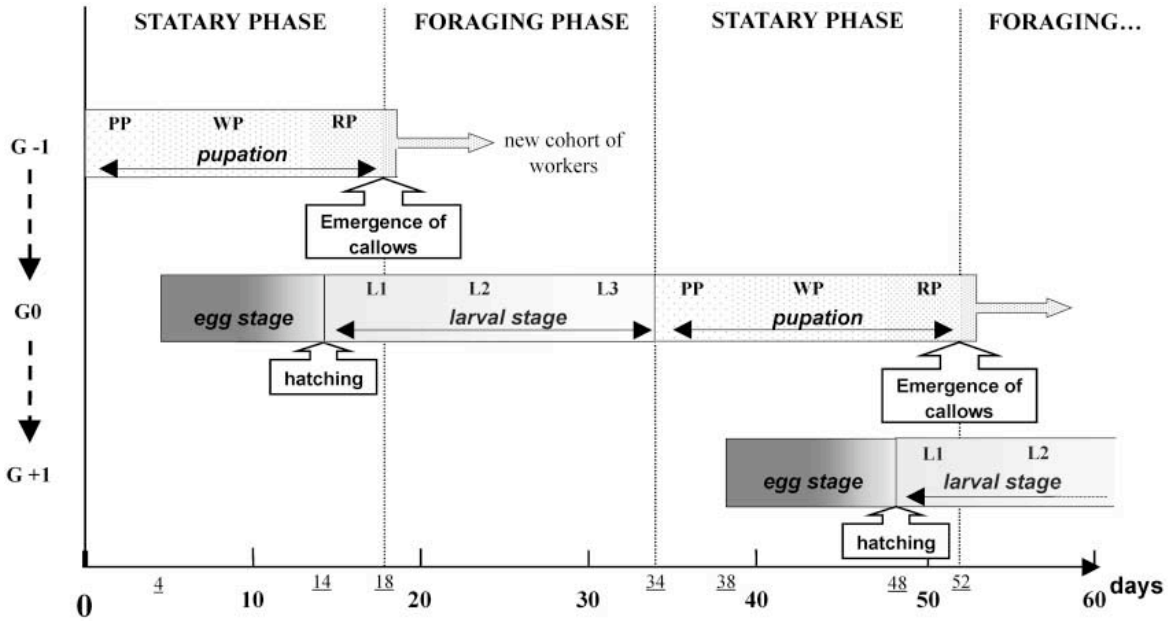

Figure 5. Reproductive cycle of *Cerapachys biroi* showing two alternating phases of activity synchronized with the brood stages. PP = prepupae, WP = white pupae, RP = reddish pupae, L1 = 1st larval instar, L2=2nd larval instar, L3 = 3rd larval instar, G = brood generation. From Ravary & Jaisson 2004, with permission.

Subcastes. In *C. biroi* all colony members ensure reproduction via thelytokous parthenogenesis. However, two groups of morphologically, behaviorally and reproductively distinct individuals are present within colonies (Figure 6). One of which are the low reproductive individuals or LRIs, formerly known as “workers” (Ravary & Jaisson 2002, 2004; Ravary et al. 2006, 2007; Lecoutey et al. 2011), and the other of which are the high reproductive individuals or HRIs, formerly known as “intercastes” (Ravary & Jaisson 2002, 2004; Ravary et al. 2006, 2007) and referred to as ergatoid queens in Lecoutey et al. (2011). HRIs are bigger and more fertile than LRIs, and specialize in intra-nidal tasks such as reproduction and brood care (Ravary & Jaisson 2004; Lecoutey et al. 2011; Teseo et al. 2013).

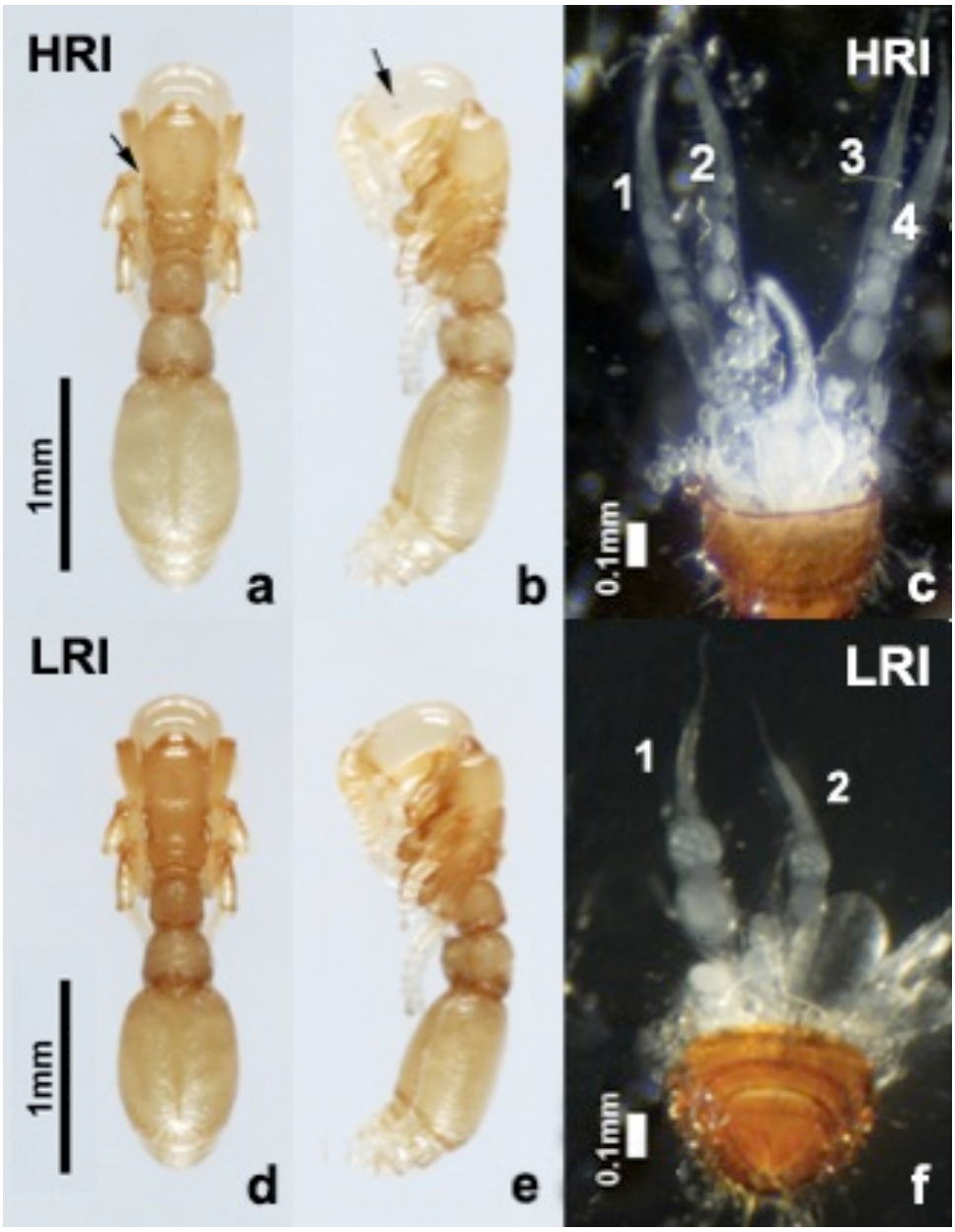

Figure 6. The two subcastes of *Cerapachys biroi*. Dorsal view (a) and lateral view (b) of a HRI pupa, and ovaries of a HRI (c). Dorsal view (d) and lateral view (e) of a LRI pupa, and ovaries of a LRI (f). HRIs are slightly larger and have more ovarioles than LRIs. In HRIs, thoracical sutures (arrow in a) are more developed, and they have visible vestigial eyes (arrow in b). Photos by Serafino Teseo.

In this thesis we will avoid the use of terms such as intercaste or ergatoid queen to indicate HRIs. Intercastes are defined as anomalous mosaics of winged queens and workers erratically produced by colonies through environmental or genetic perturbations (Molet et al. 2012), which is not the case in the HRIs of *C. biroi*. HRIs are in fact produced according to the overall fertility level of the

colonies (Lecoutey et al. 2011). Although they are able to lay many more eggs than LRIs, and exhibit morphological and behavioral features reminiscent of the shape and the behavior of "true" ant queens, HRIs cannot be defined as (ergatoid) queens because they lack spermatheca.

Representing around 95% of the population of a colony, LRIs have two or sometimes three ovarioles, and lay one or two eggs per cycle exclusively at the beginning of their life, between four and five months after their emergence, which roughly corresponds to two or three reproductive colony cycles (Ravary & Jaisson 2004). During this period, young LRIs stay in the nest to lay eggs and take care of the developing brood. Around three to four cycles later, their fertility declines and they become extra-nidal foragers. Because of this physiological and behavioral switch, two LRI groups exist from a behavioral perspective: the old sterile workers specialized in foraging, and the young fertile workers that dedicate to intra-nidal tasks such as brood care (Ravary & Jaisson 2004). This age-related change in behavior, referred to as centrifugal or age polyethism, is widespread in social Hymenoptera (Oster & Wilson 1978; Hölldobler & Wilson 1990; Robinson et al. 1994; Calderone & Page 1996), and is supposed to be adaptive at the colony level because it minimizes the cost of foraging by limiting it to the individuals with lower life expectancy and reproductive potential.

Representing around 5% of the total colony size in freshly collected colonies (Lecoutey 2009), HRIs have four to six ovarioles which, together with their higher longevity and long-lasting capacity to lay eggs, makes them more fertile than LRIs. These individuals are able to lay up to eight eggs during the same reproductive phase (Ravary & Jaisson 2004) and are never involved in the extra-nidal tasks. Besides reproductive anatomy and functionality, HRIs are also morphologically distinguishable from LRIs by the presence of more or less developed vestigial eyes, an ocellum on the posterior part of the head (almost only detectable through electronic microscopy), and more or less developed sutures between pro-, meso- and metathorax. Additionally, HRIs are overall significantly larger than LRIs, exhibiting a wider head and thorax, and a larger gaster (Ravary & Jaisson 2004).

Even though we use the terms HRI and LRI, individuals with ovaries varying in number from one to six are actually found in *C. biroi* colonies (Ravary & Jaisson 2004; Teseo et al. 2013; Teseo personal observation), and this variation might be

accompanied by a continuum in morphology and behavior. For example, it is probable that a continuum exists for the age of the switch from intra-nidal reproduction to extra-nidal foraging. Some HRIs might indeed become sterile foragers at some point in the end of their life, and some LRIs may start foraging directly after emergence without ever laying a single egg. Because of all these reasons, we consider all *C. biroi* females as workers, and HRIs and LRIs as worker subcastes.

Subcaste regulation. Societies of *C. biroi* alter subcaste ratios by significantly increasing HRI production when larvae are reared by non-fertile individuals. This can occur in two situations: either when senescent colonies are faced with food shortage, or when well-fed larvae are reared by callow workers (Lecoutey et al. 2011). The colony investment in reproduction is thus regulated via a feedback system that relies on the actual fertility level of the colony. The more fertile individuals (young LRIs and all HRIs) are present in a colony, the less HRIs are produced. This system ensures an equilibrium between colony reproductive and ergonomic functions. The proximate mechanism underlying this feedback regulation is however still unknown, and could be related either to differential nutrition of larvae, to a contact pheromone to which larvae get exposed when cared for by fertile individuals, or a combination of these two factors.

Predatory and alimentary habits. Cerapachyine ants are known to be specialized in ant brood predation and group foraging (Wilson 1958; Brown 1975; Hölldobler & Wilson 1990; Hölldobler 1982; Ravary & Jaisson 2002). They usually lead raids against colonies of Myrmicinae ants such as *Tetramorium* or *Pheidole*, during which workers kill or disperse (possibly using propaganda pheromones) adult individuals, and then seize the larvae and pupae. It is not clear whether colonies relocate frequently in order to find new prey ant nests, or whether they live more or less stably close to prey colonies that they parasitize. *Cerapachys biroi* colonies might establish within prey ant nests after raids, moving from a prey nest to the other and transporting larvae during their foraging phases. Observation of the behavior of wild colonies in the field is critical in order to shed light on this and many other aspects of *C. biroi* life history traits.

Raids usually start with some scouts returning to the colony after having found a prey nest (Hölldobler 1982). In captivity, when foragers find a prey item like a pupa or a larva of a prey species, they repeatedly bring their pygidium in contact with the substrate while going back to the nest, possibly deposing a chemical signal to communicate the location of prey to nestmates. This is followed by the formation of columns of foragers from the nest to the site in which prey items are located. Foragers often sting prey items before transporting them into the nest to feed the larvae. Stinging possibly involves the injection of a poison that blocks the development of prey and keeps them alive at the same time. This reduces the decaying process and maintains them viable for consumption during a longer time (Hölldobler 1982). Although this is probably a chemical process, nothing is known either about the poison of *C. biroi* or about the targeted metabolic pathways within prey ants. However, once prey has been transported into the nest, adults place the larvae directly on prey items, which allows them to feed in an autonomous way. As larvae are mobile and active, inter-larval cannibalism is frequent. Larval cannibalism from the adults also occurs very often.

I.V Conceptual framework and aims of the thesis

Colonies of most ant species, of most of social Hymenoptera and more generally most social groups of animals exhibit a certain degree of inter-individual genetic variation within each group. In insect societies inter-individual relatedness and thus colony-level genetic heterogeneity depend on the group-level social structure and the breeding system of species (e.g. how many worker patrilines are present, how many breeders etc.), and on the structure of populations. Genetic heterogeneity, together with the relatedness asymmetries due to the haplodiploid sex determination system characterizing Hymenoptera, creates conflicts of interest within colonies (Ratnieks et al. 2006).

The model system we used for the research described in this thesis, *C. biroi*, reproduces exclusively via thelytokous parthenogenesis and can thus be considered as clonal. A clone can be defined as a genetic copy of a previously existing biological entity. Such an entity can be interpreted broadly, across levels of biological organization, to include a particular stretch of DNA (a locus), an ensemble of physically linked loci, a genome of a somatic cell, the full genetic constitution of a multicellular organism (Avise 2008), up to a society that is genetically identical to a previously existing society. The clonal societies of *Cerapachys biroi* represent an extreme case in social Hymenoptera because they do not bear any matter of social conflict. In fact, because of clonality, intra-colonial conflicts of interests based on relatedness asymmetries and colony-level genetic heterogeneity should be absent from its societies. Being genetically identical to one another, *C. biroi* individuals interact in a maximally cooperative way, and colonies are conceptually analogous to 'true' multicellular organisms. According to a broad definition of an organism, based on conflict and cooperation between biological entities (Queller & Strassmann 2009), *C. biroi* societies can even be considered an organism *de facto*. Clonality makes *C. biroi* an incomparably valuable model system for the study of animal behavior, giving unique opportunities to investigate fundamental questions about social evolution.

Besides its interesting and unique biology, *Cerapachys biroi* is also an excellent model system from a practical perspective. Due to clonality, colonies are virtually immortal and can be kept in the laboratory over long periods of time, whereas

experimental colonies can be obtained by separating individuals from bigger stock colonies at any time. Collective and synchronized egg-laying and larval development determine the production of cohorts of individuals of the same age at relatively short intervals (34 days on average), which maximizes the possibilities of standardization of experimental protocols and allows obtaining experimental individuals at predictable time intervals.

The overall aim of the present work was to investigate conflict and cooperation at different levels of biological organization, using *C. biroi* societies as model system. We conducted two major projects. The first project has been dedicated to conflict and cooperation from an intra-colonial perspective, and has investigated a surprising phenomenon that according to kin selection theory (Hamilton 1964) is not expected to occur in clonal societies. In fact, aggressive inter-individual interactions regularly occur within the colonies of *C. biroi*, and we wanted to understand the causes of this phenomenon. This project was integrated with he investigation of the role of cuticular hydrocarbons (CHCs) in the colony-level reproductive dynamics and nestmate recognition.

The second main project of the present work was inspired by the experiments on conflict and cooperation between unrelated strains of social microorganisms obliged to associate in chimeric structures for dispersal (De Angelo et al. 1990; Strassmann et al. 2000; Foster et al. 2002; Fiegna et al. 2005; Buttery et al. 2009, 2010; Parkinson et al. 2011). The project was aimed at understanding how different genotypes of *C. biroi* interact within experimental polyclonal colonies. This meant investigating whether and how inter-clonal conflict arises when genetic heterogeneity is artificially introduced in homogeneous societies through mixing ants from different clonal lineages. The interesting results obtained with this project gave origin to a complementary series of experiments in which we investigated the effects of the environment of larval development on the adult behavior of individuals.

II. Enforcement of reproductive synchronization via policing in *C. biroi*

II.I Introduction

In biological systems, cooperation between selfish entities is the basis of the major evolutionary transitions to higher levels of complexity (Szathmáry & Maynard-Smith 1994). Lower-level entities cooperate within higher-level units, and natural selection acts simultaneously at different levels of organization. In cases where the reproductive interests of lower-level units are not perfectly aligned, conflicts over reproductive allocation arise that can destabilize the higher-level entity. This means that organisms, or organism-like social associations, harbor conflict-cooperation tradeoffs between the lower level entities that constitute them. From this perspective, much knowledge comes from social insects, where the inter-individual relatedness asymmetries due to the haplodiploid sex determination system shape the behavior of individuals (Ratnieks et al. 2006), or more precisely of the different parties within colonies (parties are for example the queen, the workers as single units or the workers as a whole, (Beekman et al. 2003; Beekman & Ratnieks 2003)). Individuals' reproductive choice depends so on 1) their individual interests, 2) the interests of the party to which they belong, 3) the colony-level interests and 4) the strategies of other colonies in the population (Hamilton & May 1977; Boomsma & Grafen 1991). These factors have a different weight depending on the features of the different societies, i.e. play different roles according to colony size, number of worker patrilines, workers' fertility etc., producing a plethora of different scenarios. For example, in monogynous monandrous societies such as the ones of the bumblebee *Bombus terrestris* (Alford 1975; Schmid-Hempel & Schmid-Hempel 2000; Lopez-Vaamonde et al. 2003, 2004; Alaux et al. 2004), a conflict exists over male production because workers are less related to the sons of the queen than to their sisters' sons. Because of this, it is advantageous for workers to breed nephews rather than brothers, and the queen kills worker-laid eggs because it is

advantageous for her to breed sons rather than grandsons. When the number of patrilines increases, and becomes higher than two, the situation is reversed, and workers are more related to brothers than to nephews. Because of this, workers prefer breeding brothers and kill worker-laid eggs, as occurs in honeybees (Ratnieks 1988). These examples clarify that, even though social insect colonies are often depicted as “superorganisms”, their members usually have different reproductive optima.

From this perspective, *Cerapachys biroi* is an exception. The species has lost sexual reproduction, and workers reproduce through obligate thelytokous parthenogenesis (Tsuji & Yamauchi 1995), living in clonal colonies in which a common single genotype is shared by all nestmates. Because of this, no conflict is expected on relatedness grounds. In other words, in *C. biroi*, the behavior of the single individuals depends exclusively on the colony-level interests. Despite this, aggressive behavior is regularly observed within colonies. During the foraging phase, some individuals are blocked, dragged out of the next and killed by nestmates in a process that can last hours or even days. Although manifestations of intra-colonial conflicts have been observed in other social insects exhibiting clonal reproduction, none of these species show the genetic homogeneity levels observed in *C. biroi*. For example, it had originally been proposed that in the asexual ponerine ant *Platythyrea punctata*, worker policing occurred in genetically homogeneous societies (Hartmann et al. 2003). However, it has since been found that natural colonies of this species are often chimeras of multiple coexisting clonal lineages, and levels of aggression have been shown to be higher in multiclonal than in monoclonal societies (Kellner et al. 2010; Kellner & Heinze 2011). In another asexual ant, the myrmicine *Pristomyrmex punctatus*, genetic heterogeneity is again observed within colonies. Policing in this species has not been reported, but genetically heterogeneous colonies show a lower tendency to assemble as compared to homogeneous ones (Nishide et al. 2007). Moreover, in the parthenogenetic cape honeybee *Apis mellifera capensis*, natural colonies are mosaics of different worker patrilines, only a few of which are able to lay thelytokous eggs (Moritz et al. 1996; Fuchs & Moritz 1999).

II.II Article. Enforcement of reproductive synchrony via policing in a clonal ant

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Summary

In insect societies, worker policing controls genetic conflicts between individuals and increases colony efficiency [1–6]. However, disentangling relatedness from colony-level effects is usually impossible [7–11]. We studied policing in the parthenogenetic ant *Cerapachys biroi*, where genetic conflicts are absent due to clonality [12, 13] and reproduction is synchronized through stereotyped colony cycles [14]. We show that larval cues regulate the cycles by suppressing ovarian activity and that individuals that fail to respond to these cues are policed and executed by their nestmates. These individuals are genetically identical to other colony members, confirming the absence of intracolonial genetic conflicts. At the same time, they bear distinct cuticular hydrocarbon profiles, which could serve as proximate recognition cues for policing. Policing in *C. biroi* keeps uncontrolled reproduction at bay and thereby maintains the colony-level phenotype. This study shows that policing can enforce adaptive colony-level phenotypes in societies with minimal or no potential genetic conflicts. In analogy to immunosurveillance on cancer cells in genetically homogeneous multicellular organisms [15–17], colony efficiency is improved via the control of individuals that do not respond properly to regulatory signals and compromise the functioning of the higher-level unit.

Results and Discussion

Worker policing in insect societies is often interpreted as a way to repress or reduce reproductive conflicts that arise between colony members because of intracolonial relatedness asymmetries [1–4]. Alternatively, it can serve as a regulatory mechanism to increase group-level efficiency [5, 6]. Although these two hypotheses are not mutually exclusive, their relative contribution has been much debated over the past decade, mostly because the two factors are hard to separate in any given species [7–11]. Parthenogenetic species with clonal societies provide important new insights, because conflicting individual interests can be excluded as an underlying selective factor. In the parthenogenetic ant *Cerapachys biroi* [12], nestmates are genetically identical or very nearly so (average within colony relatedness R = 0.99 [13]). Colonies consist only of workers, all of which reproduce during at least a period of their life. Dominance hierarchies, which can be the basis of aggressive behaviors in ants with totipotent workers [1, 9], are absent. Despite this, intracolonial aggressive behavior is

Figure 1. Worker Policing in *Cerapachys biroi*. The focal individual is spread-eagled by several workers, sometimes over the course of several days.

regularly observed in laboratory colonies, where single ants are dragged out of the nest, immobilized, spread-eagled by multiple aggressors, and often killed through biting and stinging over the course of several hours or even days (Figure 1; see also Movie S1 available online). We conducted a series of experiments aimed at understanding the causes of this behavior. Eleven colonies from three different clonal lineages (MLL1, MLL4, and MLL6 [18]; colony sizes were circa 500–5,000 individuals) were initially monitored for 13 months (see Supplemental Information section). The aggressed individuals and a subset of aggressing individuals were dissected to count the number of ovarioles. Of 201 aggressed individuals, 92.5% had four to six ovarioles (high-reproductive individuals, or HRIs, which constitute circa 5% of the individuals in normal colonies [19]), whereas 93.4% of 198 aggressing individuals had two ovarioles (low-reproductive individuals, or LRIs [19]). HRIs and LRIs were not randomly distributed among

aggressive and aggressed individuals (general linear mixed model [GLMM], colony as random factor, chi-square = 139.42, df = 1, $p < 0.0001$). Colonies of *C. biroi* undergo reproductive cycles similar to phasic army ants, such as *Eciton burchellii* and *Neivamyrmex nigrescens* [14, 20]. During the course of each cycle, a cohort of larvae develops synchronously during a 16 day foraging phase that starts with larval hatching and ends with pupation. The adults then lay a new batch of eggs at the beginning of an 18 day reproductive phase that ends with larval hatching and the emergence of a new cohort of adults. In the foraging phase, workers do not reproduce, and they conduct raids on the brood of other ant species to feed the developing larvae.

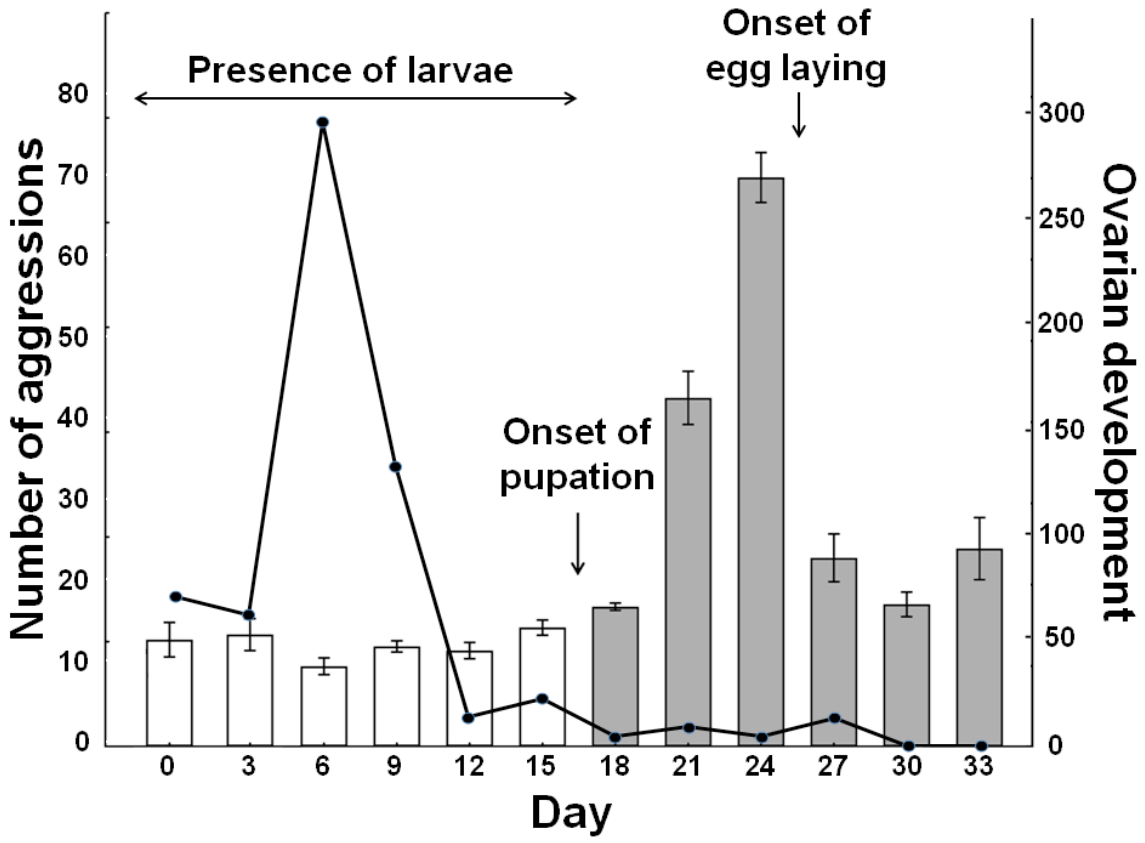

Figure 2. Course of Ovarian Development in HRIs and Worker Policing during the Colony Cycle. Ovarian development is measured as the square root of the picture's area of the biggest oocyte (mean 6 SEM). For each column, n = 30, except for day 21 (n = 35) and day 30 (n = 40). Gray and white histogram columns represent the reproductive and foraging phase, respectively. The curve describes the number of observed aggressions in the corresponding parts of the

colony cycle. Ovaries resume developing at the end of the reproductive phase, possibly because of the absence of larvae, and regress completely once the larvae have hatched.

In the reproductive phase, the ants remain inside the nest chamber and lay eggs [14]. The stage of the colony cycle was noted for 167 of the observed aggressions to determine their chronological distribution. Of the aggressions, 85.45% occurred during the foraging phase, 4.84% during the reproductive phase, and 9.69% at the transition between the two phases (Figure 2).

Aggressions were then recorded twice a week during one cycle for ten colonies. Twenty-seven instances of aggression were observed in six of the colonies (4.5 6 2.9 SD per colony), 25 (92.6%) of which occurred during the foraging phase. The other two aggressions, although observed at the transition between foraging and reproductive phase, could have started during the foraging phase during the interval between two observations. Overall, the vast majority of aggressions were directed toward HRIs and occurred during the foraging phase.

Like other ants, *C. biroi* undergoes a process of melanization after emergence; i.e., workers darken as they age. To determine the age of aggressed individuals (n = 60, 10 from each of six colonies), we compared their cuticular melanization to individuals of known age (circa 2 weeks old, 1 month old, and 2 months old; n = 20 for each age group). Aggressed individuals were darker than 2-week-old individuals (linear mixed model [LMM], colony as random factor, F = 17, 58740; df = 4; $p < 0.0001$; least significant difference [LSD] post hoc test, $p < 0.001$) (Figure S1A), lighter than 2-month-old individuals (LSD post hoc test, $p < 0.01$), and not different from 1-month-old individuals (LSD post hoc test, $p = 0.157$).

Aggressed HRIs therefore received aggression during the foraging phase following their first reproductive phase, when their ovaries were activated for the first time. Because aggression was almost always directed toward HRIs during the foraging phase, we hypothesized that this behavior might have been linked to reproductive regulation in relation to the alternation of phases. We therefore determined the normal course of ovarian activity in HRIs throughout the colony cycle, in order to compare it to ovarian development in aggressed individuals. In normal HRIs, ovaries were activated only during the reproductive phase [LMM, colony as random factor, F(11, 358) = 64, 574; $p < 0.0001$] (Figure 2; details are

given in the Supplemental Information section). Significant differences were found between the ovarian development of aggressed HRIs, aggressing LRIs, nonaggressed HRIs collected during the reproductive phase, and nonaggressed HRIs collected during the foraging phase [LMM, colony as random factor, $F(3, 659) = 71, 289$; $p < 0.0001$] (Figure 3 and Table S1). The ovarian development of aggressed HRIs was not different from that of nonaggressed HRIs collected

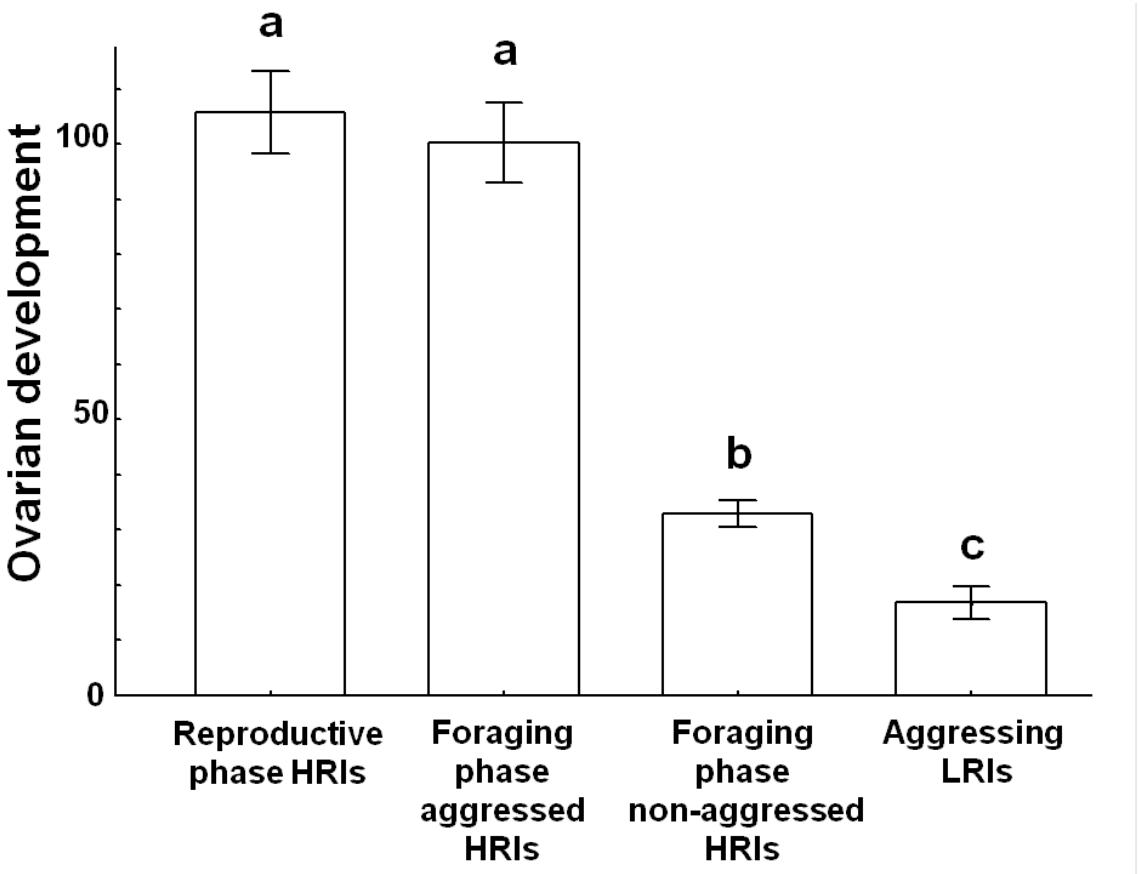

Figure 3. Ovarian Development in Different Groups of Individuals, Measured as in Figure 2 Letters indicate statistically significant differences (linear mixed model [LMM] with colony as random factor; least significant difference [LSD] post hoc test). The reproductive status of aggressed individuals was the same as that of reproductively active egg-layers during the reproductive phase. Aggressing LRIs are older foragers and show the lowest level of ovarian development. The reported statistics include only the six colonies for which all four groups were available (four colonies from MLL1 and two from MLL4) (Table S4). However, results do not change qualitatively when including all aggressed and aggressing individuals (Table S1). Additional information on the four groups is given in Figure

S1.

during the reproductive phase (LSD post hoc test, p = 0.4761), but it was higher than that of nonaggressed HRIs collected during the foraging phase (LSD post hoc test, p < 0.0001). Based on these results, we suspected that larvae inhibit ovarian development and thereby give rise to the colony cycles. We therefore monitored ovarian activity in experimental colonies with and without larvae. Individuals activated their ovaries in the absence of larvae, whereas larvae suppressed ovary development [LMM, colony as random factor, F(31, 1144) = 30, 863; p < 0.0001] (Figure 4; details in Supplemental Information section). This implies that aggressed individuals with active ovaries during the foraging phase did not respond to the larval inhibition of reproduction.

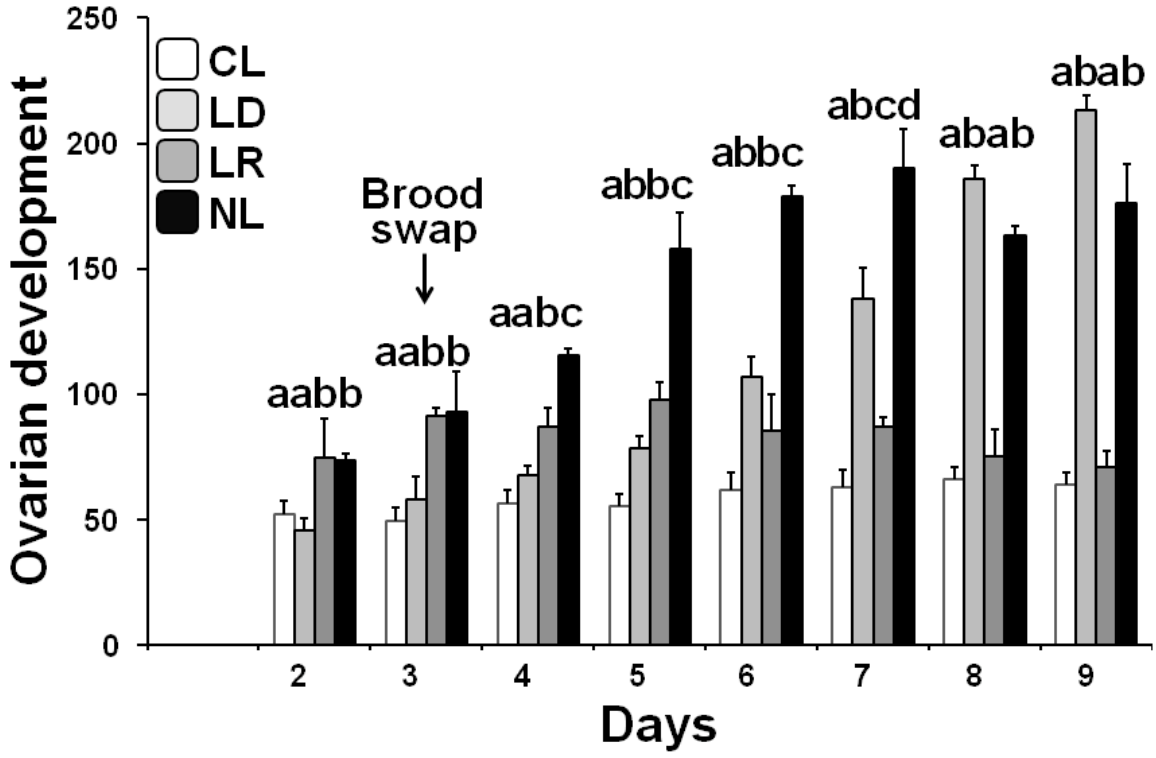

Figure 4. Larvae Inhibit Ovarian Development, Measured as in Figure 2 CL, control group with larvae; LD, larvae donor group; LR, larvae receiver group; NL, control group without larvae. Letters indicate statistically significant differences (LMM with colony as random factor, LSD post hoc test).

We then tested the hypothesis that aggressed HRIs act out of selfish genetic

interest, i.e., that they constitute genetically distinct parasitic lineages. Across eight colonies (Table S2), aggressed HRIs, aggressing LRIs, and nonaggressed HRIs were genetically identical over six to eight polymorphic microsatellite loci. Two additional MLL1 colonies contained two multilocus genotypes (MLGs) differing by only one allele, and there was no skew in the distribution of the two MLGs among the three groups in either colony (Fisher's exact tests $p = 1.0$ and $p = 0.81$). According to these results, aggressions were not related to genetic conflicts of interest or directed toward unrelated parasitic lineages.

Given that cuticular hydrocarbons (CHCs) signal reproductive and dominance status in ants [21], we hypothesized that the CHC profile was the proximate cue eliciting aggression; i.e., we expected aggressed individuals to exhibit a reproductive phase-like profile during the foraging phase. The profiles of aggressed HRIs, aggressing LRIs, nonaggressed HRIs collected during the reproductive phase, and nonaggressed HRIs collected during the foraging phase showed significant differences (discriminant analysis [DA] across 16 CHC peaks; Wilks' lambda test: 0.16829; F approximately (36, 2051) = 47, 170; $p < 0.0001$) (Figure S1B; compounds are listed in Table S3). Aggressed HRIs were different from the other three groups ($p < 0.0001$). No difference was found between reproductive phase nonaggressed HRIs and foraging phase nonaggressed HRIs [$F(1, 35225)$; df = 12, 694; $p = 0.1842$]. These results suggest that the unique CHC signature of aggressed HRIs, rather than specific fertility-related compounds, might serve as the proximate cue that elicits aggression. However, this requires additional confirmation. Moreover, compared to the other groups, aggressed HRIs had significantly lower amounts of all compounds (Table S3). Because CHC quantities usually increase with age in social Hymenoptera [22, 23], this is in accordance with our result based on melanization level that aggressed individuals were young.

The results of our study show that larvae of *C. biroi* restrict the colony's reproductive investment to coordinated cohorts of brood by regulating reproduction directly via oogenesis inhibition. By limiting egg-laying to a short time window after pupation, larvae act as pacemakers of the alternating phases.

Individuals that are not reproductively inhibited by the presence of larvae are costly because they threaten to disrupt the colony cycle: in the absence of policing, the alternation of phases would disappear. Eliminating those nonphasic

individuals is therefore adaptive even if, as our results show, they are not abundant in normal colonies. As is the case for ovarioles number [19], individual response thresholds to larval cues that inhibit oogenesis might vary along a continuum in *C. biroi*. Although the presence of larvae prevents most colony members from reproducing, some HRIs might have such a high response threshold that their ovaries remain active irrespective of the social environment. Less fertile LRIs have too low a threshold to be nonphasic, and this is probably why they hardly ever get aggressed. Given that aggressions occur regularly (we estimate that 0.09%–0.9% of all individuals are aggressed) and it seems improbable that allelic mutations occur at a similarly high rate, we suggest that the nonphasic phenotype is, at least in most cases, due to epigenetic differences. Although we cannot exclude the possibility that mutations could in some cases account for the occurrence of desynchronized HRIs, such mutant cheater lineages are expected to be unstable and therefore rare in clonal groups [24].

In insect societies, policing rarely results in the death of the focal individual (but see [25] and, in a different context, [26]). Contrarily, the death of policed individuals is the norm in *C. biroi* and serves to permanently eliminate dysfunctional individuals immediately after they have become reproductively active. According to our results, *C. biroi* can develop ovaries and lay eggs within 5–9 days in the absence of larvae. This means that, whereas ‘‘normal’’ egg-layers lay once per cycle, noninhibited egg-layers could lay more in the same time-lapse, increasing the reproductive output of a hypothetical nonphasic colony. These superproductive colonies should outcompete phasic colonies and spread in populations, but this is not what we observe. Selective pressures have likely favored the conservation of the reproductive cycle, and an effective policing system has evolved to enforce the alternation of phases (the adaptive value of the phasic cycle is discussed in the Supplemental Information section). Although we cannot exclude the possibility that worker policing has originated in a sexual ancestor of *C. biroi* as an adaptation to genetic conflicts (see, e.g., [27]) its main current function is clearly to increase colony efficiency.

Earlier studies on another parthenogenetic ant, *Platythyrea punctata*, suggested that policing occurs in clonal societies to establish dominance hierarchies and maximize the reproductive output of colonies [9]. However, it has since become clear that despite parthenogenetic reproduction, colonies of *P. punctata* are often

genetically heterogeneous due to colony fusions [10] and that high levels of policing are correlated with genetic heterogeneity [11]. Similarly, in the clonal ant *Pristomyrmex punctatus*, genetic heterogeneity within colonies negatively correlates with assembling behavior [28]. Even though we cannot exclude the possibility that chimeric colonies occur in some populations of *C. biroi*, genetic conflict would still seem an unlikely explanation for worker policing during the foraging phase. The reason is that desynchronized individuals that reproduce during the foraging phase are easily detected and removed from the colony. Instead, a social cheater lineage should show disproportionate reproduction during the reproductive phase. Due to clonality, individuals in *C. biroi* colonies act as genetically identical replicators. In this context, interindividual reproductive conflicts are largely absent, and cooperation is promoted as it enhances the fitness of the common unique genotype. The individuals disrupting this organismal-like harmony are adaptively eliminated. However, in other parthenogenetic social Hymenoptera, social parasitism by unrelated genetic lineages has been reported. In the ant *P. punctatus*, for example, parasitic lineages spread by horizontal transmission across host colonies of the same species [29, 30]; the Cape honeybee, *A.m. capensis*, parasitizes another honeybee subspecies [31]. Because of uncontrolled reproduction, selfishness, and transmissibility, these social parasites have been compared to specific types of transmissible cancer found in mammals such as the Tasmanian devil *Sarcophilus harrisii* [32–34]. The example of nonphasic HRIs in *C. biroi* allows us to develop the analogy to cancer much more generally.

Cancer is a disease where cellular proliferation is no longer under normal growth control, and the unrestrained division of cells interferes with the normal functioning of the organism [35]. The cell cycle is regulated through a series of transductional systems at the transitions between phases, and if this regulation is lost, cells may undergo uncontrolled proliferation [36]. There are several ways in which this phenomenon can occur, e.g., DNA mutations or epigenetic changes can constitutively activate oncogenes or inactivate tumor-suppressor genes, resulting, for example, in the deactivation or underexpression of membrane receptors of extracellular growth-suppressing factors [37–39]. Insensitivity to the larval inhibition of reproduction, which is in most cases probably mediated via epigenetic effects, produces an analogous phenotype in nonphasic HRIs. Remarkably, these

individuals exhibit specific chemical signatures and are detected and killed through the coordinate action of their colony-mates. This is analogous to immunosurveillance in multicellular organisms [15, 16], where cancer cells are detected and killed because they bear tumor-specific surface antigens [17]. These processes occur at different levels of organization (societies and multicellular organisms), involve selfish entities at the lower level (single ants or single cells), and are adaptive at the higher level. Policing in *C. biroi* is an example of how the regulation of individual reproduction is necessary in organismal associations to maintain group-level coherence, even in the absence of genetic conflicts. This selective pressure has produced analogous regulation systems at different levels of biological organization.

Experimental Procedures

Colonies

Twelve colonies of *C. biroi* were used in this study (details are given in Table S4). Colonies were housed in plastic boxes with a plaster of Paris floor containing a single nest chamber covered with red Plexiglas.

Cuticular Melanization Measurements

A picture of each individual was taken under standardized settings (see Supplemental Information section). Pictures were transformed to 32-bit grayscale, and melanization was measured as the average gray level value of a standard area in the center of the abdomen.

Reproductive Status

A picture of the ovaries was taken for each dissected individual. The status of ovarian development was assessed by measuring the picture surface area of the biggest egg, using the software ImageJ.

Larval Inhibition of Ovarian Development

Four experimental colonies were established from each of four stock colonies (16 in total), and each of those received a different treatment. On day 0, two experimental colonies were deprived of larvae (no larvae [NL] and larvae receiver

[LR] treatments); the other two received equal amounts of larvae (control larvae [CL] and larvae donor [LD] treatments). On day 3, larvae were removed from LD colonies and placed in LR colonies. Individuals from each experimental colony (five fertile LRIs and three to five HRIs, depending on their availability in the different colonies; total n = 1,335) were collected daily from day 2 to day 9 in order to follow ovarian development in the different treatments (details in the Supplemental Information section).

Genetic Analyses

Genotyping procedures and marker loci have been described in Kronauer et al. [18], and details are given in Table S3. The software GenClone 2.0 [40] was used to assign individuals to recurrent MLGs.

Chemical Analyses

An Agilent Technologies 7890A gas chromatography system connected to an Agilent Technologies 5975C mass spectrometer was used for chemical analyses.

Supplemental Information

Supplemental Information includes one figure, four tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.011.

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Supplemental Information

Supplemental figures

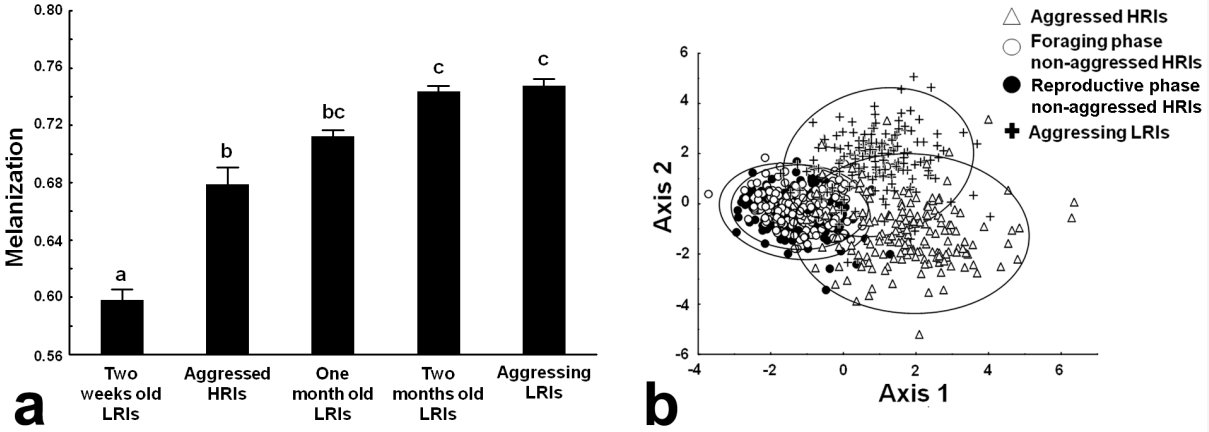

Figure S1. Melanization and chemical profiles of different categories of individuals, related to Figure 3 in the main text. **a)** Melanization levels of different groups of individuals. Columns and bars represent respectively mean values ± SEM. Letters indicate statistical significance. Significant differences were found between groups (ANOVA, $F(4, 176)=30,033$, $p<0.0001$). Aggressed individuals were significantly darker than two weeks old individuals (LSD post hoc test, $p<0.0001$) and significantly lighter than two months old individuals (LSD post hoc test, $p<0.0001$). No significant differences were found between aggressed individuals and one month old individuals (LSD post hoc test, $p=0.158$). **b)** Discriminant analysis of all 16 CHC peaks from foraging phase HRIs, reproductive phase HRIs, aggressed HRIs, and aggressing LRIs. Data from six colonies, four from MLL1 and two from MLL4. Axes 1 and 2 explain 64.8% and 34.6% of the variance, respectively. All groups are significantly different from one another (Lambda Wilk: 0,16829, F approx. (36,2051) = 47,170, $p<0.0001$) except for foraging phase non-aggressed HRIs and statary phase HRIs ($F(1,35225)$, dl 12,694, $p=0.1842$).

Supplemental Tables

Table S1. LSD post hoc test p values of ovarian development in the different groups (LMM, F(3, 764)=86,030, p<0.0001). Related to Figure 3 in the main text. Results include aggressing LRIs and aggressed HRIs from all colonies where available.

	Aggressing LRIs	**Aggressed HRIs**	**Foraging phase HRIs**	**Reproductive phase HRIs**
Aggressing LRIs	-	<0.0001	0.012448	<0.0001
Aggressed HRIs		-	<0.0001	0.223379
Foraging phase HRIs			-	<0.0001
Reproductive phase HRIs				-

Table S2. Samples used for genetic analyses (number of individuals of the different categories for each colony). Related to Figure 3 in the main text. Colonies from MLL1 were genotyped for microsatellite loci ED32S, EGR4W, D71AW, D4XW2, ETJ3E and D8M16, colonies from MLL4 for ER4IH, D8CI3, EFAFC, D8ZOW, ETCR2, D4XW2, ETJ3E and D8M16, and colonies from MLL6 for ED32S, EGR4W, ETJ3E, EFAFC, D8ZOW, D8M16 and D9Y4L.

Colony	Aggressing LRIs	Aggressed HRIs	Non-aggressed HRIs	Clonal lineage
T4	13	14	10	MLL3
T5	10	11	11	MLL1
J1	10	13	13	MLL1
O5	11	11	14	MLL1
T1	10	10	12	MLL4
T3	11	10	12	MLL4
C3B	1	6	not available	MLL6
C9	3	9	not available	MLL6
C10	17	19	not available	MLL6
C11	4	11	not available	MLL6

Table S3. Mean absolute quantities of each CHC peak (areas of each peak divided by the area of the internal standard). Related to Figure 3 in the main text. The p-value (LMM with colony as random factor) refers to the comparison of aggressed HRIs vs. all other groups combined.

	Aggressing LRIs	Aggressed HRIs	Foraging phase HRIs	Reproductive phase HRIs	p
Pentacosane	0,00924	0,00278	0,00597	0,00508	p<0.0001
11- + 13-methylpentacosane	0,00375	0,00217	0,00497	0,00534	p<0.0001
2-methylpentacosane	0,09152	0,04003	0,07727	0,08222	p<0.0001
3-methylpentacosane	0,01395	0,00712	0,01071	0,01235	p<0.0001
Hexacosane	0,00923	0,00283	0,00728	0,00671	p<0.0001
10- + 12-methylhexacosane	0,0057	0,00314	0,00834	0,00949	p<0.0001
2- methylhexacosane	0,05241	0,01811	0,04582	0,05089	p<0.0001
Heptacosane	0,13448	0,03083	0,11054	0,09473	p<0.0001
11- + 13-methylheptacosane	0,12343	0,06506	0,17157	0,19636	p<0.0001
11,15-dimethylheptacosane	0,50854	0,23947	0,54875	0,61378	p<0.0001
4,15-dimethylheptacosane	0,03967	0,02149	0,04752	0,05517	p<0.0001
Octacosane	0,01512	0,00696	0,01459	0,01517	p<0.0001
10-methyloctacosane	0,00748	0,00391	0,01656	0,02041	p=0.0005
12-methyloctacosane	0,00657	0,00483	0,00975	0,01095	p=0.003
14- + 16-methyloctacosane	0,0099	0,00519	0,02151	0,02596	p=0.0004
Nonacosane	0,00726	0,00457	0,00918	0,00936	p<0.0001

Table S4. Colonies used in the study. Related to Figure 1 in the main text. Letters indicate the involvement of colonies in the different analyses and experiments: a. aggressing and aggressed individuals collected for analyses of ovarian development and CHCs; b. HRIs collected regularly during colony cycle; c. used for genetic analyses; d. used to test larval inhibition of ovarian development.

Colony	Clonal lineage	Origin	Field collection date	Experiments
J1	MLL1	Java, Indonesia	2005	a,b,c
O4	MLL1	Okinawa, Japan	2006	a
O5	MLL1	Okinawa, Japan	2006	a,b,c,d
T4	MLL3	Taiwan	2001	a,b,c
T5	MLL1	Taiwan	2001	a,b,c,d
O6	MLL4	Okinawa, Japan	2006	a,d
T1C	MLL4	Taiwan	1997	a,b,c
T3	MLL4	Taiwan	2000	a,b,c,d
C10	MLL6	Okinawa, Japan	2008	a,c
C11	MLL6	Okinawa, Japan	2008	a,c
C3B	MLL6	Okinawa, Japan	2008	a,c
C9	MLL6	Okinawa, Japan	2008	a,c

Supplemental Experimental Procedures

Collection of aggressive and aggressed individuals. Stock colonies were monitored at least twice a week during 13 months. Whenever an aggression was observed, both the aggressing and the aggressed individuals were collected with soft forceps and frozen.

Cuticular melanization measurements. Aggressed HRIs and aggressing LRIs were fixed on paper after chemical extractions, dissections and removal of the legs and the last part of the abdomen. Individuals of known age, isolated from their mother colonies in groups of 50 at emergence, received the same treatment but were not subject to the chemical extractions (which is unlikely to have affected cuticular melanization levels). A picture of each individual was taken with a Canon EOS 7D DSLR camera in standardized light condition and settings at 5:1 magnification. Pictures were transformed to 32-bit grayscale with the software ImageJ (http://rsbweb.nih.gov/ij/) and melanization was measured as the average gray level value of a standard area in the center of the abdomen. The melanization levels were calculated by using the formula m = 1 - g/r, where m is the melanization level, g is the average gray value of the standard area, and r is the reference white value (the average gray level of an A4 white paper sheet photographed under the same standardized conditions).

Reproductive status evaluation procedure. Each individual was dissected in water by removing the last three abdominal segments. A picture of the ovaries was taken with a Nikon D200 SLR camera mounted on a Zeiss STEMI 2000 C stereomicroscope for colonies J1, O4, O5, O6, T1, T3, T4 and T5. A JVC 3-CCD digital camera (model KY-F75U) mounted on a Leica MZ 12.5 Stereomicroscope with a 0.5x objective was used for colonies C3B, C9, C10 and C11. The total number of ovarioles was recorded and the status of ovarian development was assessed by measuring the picture surface area of the biggest egg in the software ImageJ. The ovarian development of individuals with no clearly visible or atrophic ovaries was scored as zero.

Reproductive status of normal HRIs during the colony cycle. To evaluate the normal course of ovarian status in HRIs, five non-aggressed HRIs were collected every third day throughout one colony cycle from six colonies (four colonies from MLL1 and two from MLL4. total of 375 HRIs). Three days after the beginning of pupation, ovaries were significantly more developed than in each of the sampling days during the foraging phase (LSD post hoc test, all $p<0.0001$). Eggs were ready to be laid after six days from the onset of pupation. After egg-laying, average ovarian activity was initially low but increased again towards the end of the reproductive phase and was significantly higher than on all the sampling days of the foraging phase (LSD post hoc test, all $p<0.02$) just before the larvae hatched. Once the larvae had hatched, ovarian development returned to base levels.

Larvae inhibit ovarian development. On day 0 of the experiment, four mother colonies were each split into four daughter colonies. Three categories of individuals (HRIs, older LRIs, and young LRIs in their first foraging phase) were equally distributed among daughter colonies. Five HRIs and five young LRIs (recognizable by their light color) were collected from each mother colony to assess the general ovarian status before treatment. HRIs and young LRIs were marked with a dot of enamel paint on the abdomen to make them readily recognizable. Each of the four daughter colonies (16 colonies total) received different treatments on the first day of the experiment (day 0): two of them were deprived of larvae (No Larvae (NL) and Larvae Receiver (LR) treatments), while the other two each received equal amounts of larvae (Control Larvae (CL) and Larvae Donor (LD) treatments). On day 3 of the experiment, larvae were removed from LD colonies and placed in LR colonies. Individuals from each experimental colony (five fertile LRIs and three to five HRIs, depending on their availability in the different colonies; total n=1335) were collected daily during nine days and dissected to evaluate their ovarian development. All experimental colonies were fed on days 0, 3, 6 and 9 with live pupae of the ant *Aphaenogaster senilis.* Fertile individuals in NL colonies already showed higher ovarian development than in CL and LD colonies on day 3 (LSD post hoc test, $p<0.0001$ and $p=0.0002$, respectively). The highest average levels of ovarian development (eggs ready to be laid) were observed on days 6 and 7, and the first eggs were observed in NL colonies between days 5 and 8. On the other hand, individuals in CL treatments

showed no significant changes in ovarian development during the experiment and never laid eggs. Individuals in LD colonies behaved like the NL ones (i.e. they reached maximal ovarian development and egg-laying), with a four-day delay due to removal of larvae on day 3, the fourth day of the experiment. On day 7 (corresponding to day 3 for CL and NL treatments), their ovarian development was higher than in CL colonies on day 3, and not significantly different from NL ones (LSD post hoc test, $p<0.0001$ and $p=0.3$, respectively). The first eggs were observed in LD nests between days 9 and 11. In LR colonies, ovarian development increased in the first three days as in NL colonies. On day 3, worker ovaries were significantly more developed than in CL and LD colonies (LSD post hoc test, $p=0.0006$ and $p=0.005$, respectively), and not significantly different from NL colonies (LSD post hoc test, $p= 0.764$). The highest ovarian development in LR colonies, on day 5, was significantly lower than in NL colonies on day 5 (LSD post hoc test, $p<0.0001$). After day 5, ovaries slowly regressed to base levels. On day 9, there were no significant differences between LR and CL colonies, and eggs were never laid. The transferred larvae thus prevented ovaries from developing completely in LR colonies.

Genetic analyses. We genotyped at least 10 aggressed HRIs, 10 aggressing LRIs, and 10 non-aggressed HRIs collected during foraging phases from six colonies (four from MLL1 and two from MLL4; Table S1). For another four colonies from MLL6, samples included only aggressed HRIs and aggressing LRIs. Six, eight, and seven polymorphic microsatellite loci were genotyped for a total of 141, 63, and 70 individuals from MLLs 1, 4, and 6, respectively (details are given in Table S1). The software GenClone 2.0 was used to assign individuals to recurrent multilocus genotypes (MLGs, i.e. individuals that shared the exact same genotype across all loci). Microsatellite markers used for genotyping of the different MLLs are given in Table S1. Chromatograms were analyzed with the software Peak Scanner 1.0 (www.appliedbiosystems.com).

Chemical analyses. Cuticular extracts were prepared by placing single ants in 200µl glass vials and washing them for 10 minutes in 20µl of a pentane solution with an internal size standard (C30). Individuals were then frozen in order to preserve them for subsequent dissection, DNA extraction, and analysis of

melanization. Two µl of each extract were manually injected in an Agilent Technologies 7890A Gas Chromatography System connected to an Agilent Technologies 5975C mass spectrometer. The GC column temperature was kept at 70°C for 1 minute, then increased in steps of 30°C/minute to 260°C, then in steps of 5°C/minute to 300°C, then in a step of 20°C/minute to 320°C, and then left at 320°C for 3 minutes. Gas chromatography/mass-spectrometry analyses were conducted on cuticular extracts of four groups of individuals: aggressed HRIs, aggressing LRIs, non-aggressed HRIs collected during the reproductive phase, and non-aggressed HRIs collected during the foraging phase. The areas of the 16 main CHC peaks were measured using the Agilent Technologies software MSD Chemstation (v. E 02.00.493) and either transformed in proportions and normalized for qualitative analyses following the method of Reyment and Aitchinson ($Z_{i,j} = \ln(Y_{i,j}/g(Y_j))$, where $Y_{i,j}$ is the area of peak i for the individual j, $g(Y_j)$ is the geometric mean of the areas of all peaks for individual j, and $Z_{i,j}$ is the transformed area of peak i for individual j [1, 2]), or divided by the area of the internal standard peak for quantitative analyses. Colonies of MLL6 were kept at Harvard University, while the chemical analyses were conducted at the University of Paris 13. Individuals from MLL6 therefore had to be treated with a modified protocol. Each individual was washed in pentane for 10 minutes; the extract was then evaporated and re-suspended in 20µl pentane prior to injection. Quantitative analyses were not performed on MLL6 individuals, and only aggressed HRIs and aggressing LRIs were analyzed. The two groups were significantly different in a discriminant analysis on the 16 CHC peaks (Lambda Wilk: 0,34334, F approx. (12,66)=10,519, $p<0.0001$). Aggressed HRIs and aggressing LRIs were correctly placed in a classification matrix in 88.37% and 91.66% of the cases, respectively.

Adaptive value of phasic colony cycles. Phasic colony cycles are known from a variety of army ant genera, such as *Eciton*, *Neivamyrmex*, *Cerapachys* and *Aenictus* [3]. However, their adaptive value has not been studied experimentally and is therefore not well understood. Based on what we know about the biology of phasic army ants, we present the following hypothesis. Coordinated brood development limits the presence of food-demanding larvae to the minimum possible time span (approximately the time a single larva needs to develop from hatching to pupation). Thus, intensive foraging activity is also restricted to the

foraging/nomadic phase. When food is patchy and restricted to fresh prey (e.g. ant brood in *C. biroi*), colonies are forced to emigrate frequently, and emigrations are probably costly, especially because the brood and queen become exposed. Short foraging phases might minimize the absolute number of emigrations and therefore minimize the associated costs. Moreover, as preying on other social insect colonies requires a great investment, light foraging activity would not be successful because it is not sufficient to overwhelm prey colonies. Mass foraging is the only efficient strategy when feeding mostly on other ant colonies. If a prey colony is overwhelmed, a big quantity of food becomes suddenly available and can sustain a large number of developing brood. Therefore it is probably adaptive to have synchronously developing larvae, which limits the period of time during which mass foraging has to be sustained. In the case of *C. biroi*, moreover, larvae are relatively mobile and display a strong tendency for cannibalism. Synchronized brood cycles might therefore also be a way to minimize cannibalism among otherwise overlapping brood stages (e.g. larvae together with immobile pupae and/or eggs). On the other hand, as larvae can be used as an emergency food source by other larvae, larval cannibalism might help colonies to produce cohorts of adults even when food sources are not abundant. In light of the Reproductive Ground Plan Hypothesis [4], the phasic colony cycle of army ants might be interpreted as a colony-level reversion to the phasic lifestyle of solitary insects, which similarly alternate between reproductive and foraging phases. Further work is needed to understand whether there is a link between the physiology and gene expression of phasic solitary insects and phasic insect societies throughout the colony cycle.

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II.III Article. Dysfunctional reproductive physiology, and not reproductive activation, triggers policing in experimental colonies of the clonal ant *Cerapachys biroi*

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Abstract

We previously reported the existence of a unique policing system in the clonal ant *Cerapachys biroi*, where individuals that fail to synchronize to the colony reproductive dynamics and reproduce without control are recognized and executed by their nestmates. These executions help maintain the alternation of reproductive and foraging phases, a colony-level adaptive phenotype. In our previous study, we hypothesized that the specific chemical signature of non-synchronized individuals rather than some fertility-related cues serve as the proximate factor triggering their execution. We here examined this hypothesis by testing whether reproductively active individuals introduced in colonies in foraging phase are the target of aggression. We show that introduced fertile individuals display clear behavioral differences from sterile individuals of the foraging colonies, but are never targeted with aggressive behavior. Foraging workers, which usually perform aggressions, are able to discriminate the introduced individuals' subcaste but not their reproductive status. Our results therefore demonstrate that ovarian activation is not enough to trigger policing in experimental colonies, supporting our previous hypothesis that aggressed individuals are not just unsynchronized, but possibly non-responsive to colony-level regulation cues and thus dysfunctional in their reproductive physiology.

Text

Colonies of the clonal ant *Cerapachys biroi* undergo stereotypic reproductive cycles made of two constantly alternating phases[1-3]. In the reproductive phase eggs are collectively laid while pupae of the previous generation complete their development, whereas in the foraging phase larvae feed on prey items provided by foraging workers, and there is no reproductive activity until the onset of pupation. In a previous study, we showed that colonies of *C. biroi* are subject to a strict regulation of reproduction, with larval cues inhibiting ovarian activation in fertile adults[4]. Nonetheless, some individuals seem to lack sensitivity to these cues and fail to synchronize to the colony-level reproductive cycle. Those individuals are executed during their first foraging phase as reproductively mature individuals, when they exhibit for the first time their unusual physiology (i.e. reproductive activation during a non-reproductive phase). Interestingly, almost all the executed individuals belong to a worker subcaste specialized in reproduction (highly reproductive individuals, or HRIs, which have four to six ovarioles, lay up to eight eggs per cycle and do not engage in foraging; in contrast, low reproductive individuals, or LRIs, which have 2 ovarioles, lay up to 2 eggs during few cycles and then become sterile foragers). Aggressed HRIs show significantly different cuticular hydrocarbons profiles compared to non-aggressed HRIs from both foraging and reproductive phases.[4] In our previous study, we hypothesized that the specific signatures of aggressed HRIs, rather than being fertility-related[5], are the proximate cues revealing their peculiar reproductive physiology, and trigger aggressive behavior. We concluded that this novel form of policing[6] is analogous to immunosurveillance on cancer cells in multicellular organisms[7,8], where cells that do not respond anymore to the organism-level growth inhibition signals are killed by the immune system. These cells bear cancer-specific surface antigens[9], which are the proximal cue triggering the action of immune cells. Accordingly, the profiles of *C. biroi*'s aggressed HRIs exhibit a significant chemical difference from the profiles of reproductively active non-aggressed HRIs, differing for both relative proportions and absolute quantities of cuticular hydrocarbons. This suggests that they might be different from normal reproducers. According to our hypothesis, individuals' response threshold to the larval inhibition of reproduction might be distributed along a continuum. While most individuals in a colony refrain from reproducing in the presence of larvae, at the extremes of the distribution of these

traits the individuals' thresholds to larval cues become extremely low or high (respectively left and right side of the curve in Figure 1), corresponding respectively to a permanently reproductively inactive or active phenotype. Aggressed HRIs are among those individuals with permanently activated ovaries, and thus not simply non-synchronized in reproduction, but probably extreme in their reproductive physiology and dysfunctional from a colony-level perspective.

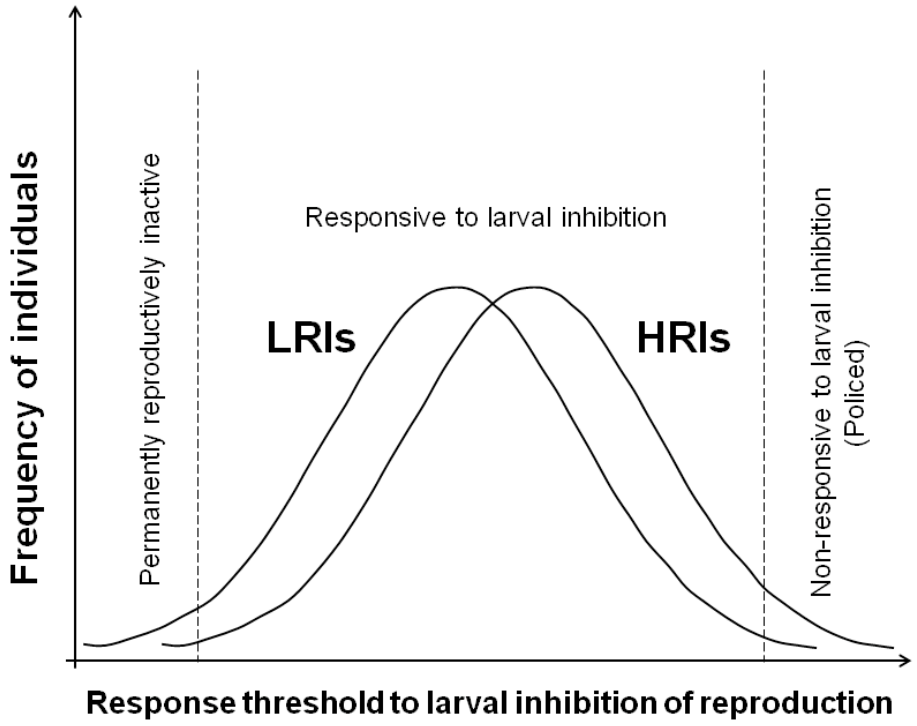

Figure 1. Distribution of responsiveness to the larval inhibition of reproduction for LRIs and HRIs in a *C. biroi* colony. We hypothesize that part of the HRIs do not respond to the larval inhibition of ovary activity, and get executed by nestmates in order to maintain the alternation of reproductive and foraging phases. Permanently sterile LRI might exist having a so low threshold to the larval inhibition cues that they never activate ovaries. These individuals are however difficult to identify because contrary to non-synchronized reproducers, they are not a threat for the colony and do not get aggressed by nestmates.

As it is possible to manipulate experimentally the reproductive status of individuals by exposing them to specific social contexts (i.e., if separated from larvae, fertile individuals produce fully developed eggs within roughly five days[4]), we tested whether individuals with artificially activated ovaries were targeted with aggressive behavior when introduced into foraging colonies. We conducted a first experiment in which we induced ovarian activation in some HRIs and LRIs, (details in the SI section) and observed the behaviors they performed and received during the days following their introduction in foraging recipient colonies made with splits of the same mother colony. Reproductively active individuals of both subcastes were less active than controls, spending more time in the nest chamber (LMM (linear mixed model), $F(1, 379)=35, 037$, $p<0.0001$, all detailed results are showed in the SI section). HRIs (both reproductively active and inactive) spent more time in the nest chamber than LRIs (LMM, $F(1, 379)=261, 69$, $p<0.0001$), which foraged more (LMM, $F(1, 379)= 309, 34$, $p<0.0001$). Interestingly, no differences were found between the behavior targeted at reproductively active or inactive individuals (LMM, all $p > 0.067$), including the only six observed episodes of biting (which indeed cannot be considered as true policing, in which individuals are normally immobilized and killed by nestmates) and the single observed immobilization (which was unexpectedly performed towards a LRI).

In a second experiment, we more precisely tested the reaction of foragers (which usually perform the aggressions) towards reproductively active and inactive HRIs and LRIs. We introduced experimentally treated ants in arenas positioned in the foraging areas of foraging colonies which contained two foraging workers, and observed interactions during two minutes per test. No aggressions were observed when foraging workers faced reproductively active individuals. Moreover, although foraging workers showed more interest in HRIs than LRIs (they antennated them significantly more (LMM, experimental colony and reproductive status used as random factors, $F(1,88)=11, 388$, $p=0.001$), showing that they are possibly able to discriminate them from LRIs), reproductive status of introduced individuals alone had no effect on the behavior of foraging workers (LMM, experimental colony and subcaste used as random factors, $F(1, 89)=0, 28308$). The interaction between reproductive status and subcaste showed differences exclusively between

subcastes (LMM, experimental colony as random factor F(3, 87)= 3, 9388; LSD post-hoc tests, all p= 0.002), confirming the previous results.

The results of our two experiments show that reproductive activation does not produce specific reactions in foraging individuals. Indeed, as we showed in our previous study, fertile *C. biroi* are able to regress rapidly their ovarian status by re-absorbing developing eggs in the presence of larvae. Reproductive de-synchronization is thus not enough to trigger aggressive behavior, and this supports our hypothesis that "naturally" aggressed individuals are indeed dysfunctional in their reproductive physiology and probably exhibit maladaptive extreme response thresholds to social colony-level cues. However, policing in *C. biroi* has been reported to occur in colonies of 500-5000 individuals, and for this reason we cannot exclude that the small size of our experimental colonies (150 individuals) might have influenced the behavioral tests. Further work on the relation between policing and colony-level life history traits in *C. biroi* is needed to elucidate this issue.

Material and methods

Experiment 1. A stock colony (T1, clonal line MLL4[10], around 5090 individuals, 9.3% HRIs) was split in two parts at the beginning of a foraging phase (larvae at first developmental stage (L1)). 1000 individuals were deprived of larvae and put in a separate nest; another 1000 individuals were put in a separate nest with larvae. The goal of the procedure was to induce ovary activation in the larvae-less colony fragment, and to keep ovaries inactive in the other fragment. 20 colonies were made with the remaining 3000 individuals to be used as recipient colonies for the experimentally treated ants. The experimental procedure started 5 days after the fragmentation of the mother colony, which is the time needed by fertile ants to produce new eggs. The experiment consisted in introducing five reproductively inactive and active HRIs or LRIs (respectively from the 1000 individuals fragments with and without larvae) in recipient colonies and observing their behavior during the following two days.

Experiment 2. The same procedure of de-synchronization was applied to colony T1 (20 months after experiment 1), in order to focus on the individual interactions of foraging ants with reproductively active or inactive HRIs and LRIs.

Experimentally treated individuals (n=25 for each of four groups) were introduced in arenas placed in the foraging areas of recipient colonies, which contained two different foraging workers for each test. Interactions were observed during two minutes per test.

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Supplementary Information

Material and methods

Experiment 1. A stock colony (T1, clonal line MLL4, around 5090 individuals, 9.3% HRIs) was split in several parts at the beginning of a foraging phase (larvae at first stage (L1)). 1000 individuals were deprived of larvae and put in a separate nest; another 1000 individuals were put in a separate nest with larvae. This procedure was adopted to have, after ca. one week, the two colony fragments at two opposite moments of the colony cycle, i.e. one fragment with fully activated ovaries and the other with inactive ovaries and young larvae. The goal of the procedure was to introduce the treated individuals in foraging colonies, and to see e.g. whether reproductively active individuals were aggressed by foraging workers. 20 colonies (150 individuals each with the original HRI/LRI ratio, plus larvae) were made with the remaining 3000 individuals, to be used as recipient colonies for the experimentally treated ants. We paint-marked 10 LRIs or 10 HRIs (the subcastes can be easily distinguished because of morphological differences, i.e. HRIs have visible sutures on the thorax and vestigial eyes, whereas LRIs do not) in recipient colonies to be able to remove them rapidly before the introduction of experimentally manipulated individuals. This avoided recipient colonies to perceive the introduced individuals as supernumerary reproducers. In the two 1000 individuals fragments, we marked individually 100 LRIs and 100 HRIs in order to observe their behavior during the following days. The experiment consisted in introducing in recipient colonies individuals belonging to 4 different categories: reproductively active HRIs and LRIs (fertile, from the 1000 individuals fragment without larvae), and reproductively inactive HRIs and LRIs (non-fertile, from the 1000 individuals fragment that kept larvae). Ten of 20 recipient colonies received 5 reproductively active and 5 reproductively inactive HRIs, while the other 10 colonies received 5 reproductively active and 5 reproductively inactive LRIs. Individuals were released in the mini-colonies nest chamber and observed during 10 minutes. From the day after introduction, recipient colonies were scanned every thirty minutes for 12 hours per day during two days, and the behavior of each introduced individual was recorded. The reproductive activation of individuals in larvae-less fragment was checked by dissecting randomly collected individuals in the colony fragments with and without larvae prior to the experiment. Besides

checking the localization of individuals within experimental nests, we looked at several different behaviors. Antennation and licking the body of another ant were considered as an expression of interest, as well as "boxing" (stimulation of the body of another ant by drumming with anterior legs, possibly performed to stimulate inactive individuals, comparable to the vibrational signal in honey bees;[11] biting and stinging were considered as aggressive behaviors, so manifestations of hostility, as well as blocking an individual.

Experiment 2. The same procedure of de-synchronization was applied to colony T1, after 20 months of pause from experiment 1, in order to focus on the individual interactions of foraging ants with reproductively activated or inactive HRIs and LRIs. Reproductively activated and inactive individuals (n=25 for each of four groups) were introduced in arenas placed in the foraging areas of recipient colonies, which contained two different foraging workers for each test. Introduction tests were filmed during two minutes, and videos were analyzed by recording the behaviors received by focal individuals. The reproductive activation of individuals was checked by dissecting randomly collected individuals in the colony fragments with and without larvae prior to the experiment.

Results

Experiment 1. For each of the tests we performed, we considered four different factors, i.e. observation day, subcaste, experimental colony and reproductive status. According to the different question we asked, we considered one factor (or the interaction of two factors) as fixed, and the others as random.

Localization. Overall, individuals spent time in different zones of the nest according to the subcaste, with LRIs more often in the foraging area than HRIs (LMM (linear mixed model), $F(1, 379)=35, 037$, $p<0.0001$) and HRIs more often on or in the proximity of brood (LMM, $F(1,379)= 261, 69$ and $125,70$ respectively, both $p<0.0001$). LRIs and HRIs did not differ in their presence in the nest chamber far from the brood (LMM, $F(1,379)=0, 19009$, $p=0.66$). The desynchronization had a significant effect on the localization of individuals in the nest, with reproductively inactive individuals found more often than reproductively active ones in the foraging area and far from the brood inside the nest (LMM, $F(1,379)= 6, 6299$ and $30, 641$

respectively, p= 0.01 and p<0.0001 respectively) and reproductively active individuals found more often on or in proximity of the brood (LMM, F(1,379)= 35, 037 and 45, 771 respectively, both p<0.0001).

Reproductively active HRIs were the group spending more time on the brood pile and in its proximity, followed by reproductively inactive HRIs, reproductively active LRIs and reproductively inactive LRIs; all groups showed significant differences (LMM, F(3,378)= 102, 69, LSD post hoc tests, all p<0.012). Reproductively inactive HRIs were more often found far from the brood within the nest than the other three groups (LMM, F(3,378), p<0.001, LSD post hoc tests, all p<0.03). For the presence in the foraging area, there was no difference between reproductively inactive and reproductively active HRIs (LMM, F(3,378)=124,46, LSD post hoc test, p=0.57), but both were found significantly less often in the foraging area compared to reproductively inactive and reproductively active LRIs (LMM, F(3,378)=124,46, LSD post hoc test, all p<0.0001). Reproductively inactive LRIs were found in the foraging area significantly more often than reproductively active LRIs (LMM, F(3,378)=124,46, LSD post hoc test, all p<0.0001).

Performed behaviors. Overall, individuals explored more in the first than in the second day (LMM, F(1,379)=14, 605, p<0.001). LRIs foraged more, performed less brood care, more exploration, less antennation and less boxing than HRIs (LMM, respectively: F(1, 379)=309,34, p<0.0001; F(1, 379)=132, 25, p<0.0001; F(1, 379)=13,476, p<0.001; F(1, 379)=4, 7231, p=0.03; F(1, 379)=206, 02, p<0.0001), HRIs performed overall significantly more brood care than LRIs (LMM, F(1,379)=132,25, p< 0.0001) whereas no differences were found between reproductively inactive and reproductively active HRIs (LMM, F(3,378)=46,254, p=0.22). Reproductively active LRIs performed significantly more brood care than reproductively inactive LRIs (LMM, F(3,378)=46,254, p=0.029). Reproductively inactive HRIs performed more exploration and antennation than reproductively active HRIs and reproductively inactive and reproductively active LRIs (LMM, respectively: F(3,378)=7, 6086; F(3,378)=3, 7854; all p<0.01); reproductively active HRIs explored more than reproductively inactive LRIs (LMM, F(3,378)=7,6086, p=0.03), whereas no differences were found between reproductively active HRIs and both reproductively inactive and reproductively active LRIs for antennation (LMM, F(3, 378)=3, 7854, all p > 0.7). Reproductively

active HRIs showed the highest levels of boxing compared to the other groups (LMM, $F(3, 378)=77, 412$, $p<0.0001$, LSD post hoc test, all $p<0.0001$). Reproductively inactive and active LRIs did not differ for boxing levels (LMM, $F(3, 378)=77, 412$, $p<0.0001$, LSD post hoc test, $p=0.9$). Licking, self grooming and biting did not differ between the 4 groups of individuals.

Received behaviors. Among all the behaviors received, only antennation showed a significant decrease in the second day of observation (LMM, $F(1, 379)=23, 348$, $p<0.0001$). HRIs received overall more boxing (LMM, $F(1,379)=15, 047$, $p=0.0001$), and more biting than LRIs (LMM, $F(1,379)=6, 1838$). While all the other received behaviors did not change according to the caste.

For what concerns the interaction between subcaste and reproductive status, we found significant differences only in boxing (LMM, $F(3, 378)= 5, 8408$, $p<0.001$): reproductively active HRI received significantly more boxing than reproductively inactive LRI (LSD post hoc test, $p=0.014$), while reproductively active HRIs received more boxing than reproductively active and inactive LRIs (LSD post hoc tests, respectively $p<0.01$ and $p<0.001$).

III. Epistasis between adults and larvae induces a social cheater phenotype in ants

III.I Introduction

Biological entities face an everlasting trade-off between cooperation and selfishness when obliged to coexist within higher-level units (Hamilton 1964, Szathmáry & Maynard-Smith 1994). When biological systems are genetically homogeneous cooperation between lower-level entities is a stable strategy, whereas a compromise between selfishness and cooperation is adaptive in presence of genetic heterogeneity. Studies on conflict and cooperation within societies often focus on humans, primates, other cooperative vertebrates or social insects, but many interesting insights come increasingly from microorganisms (Crespi 2001; Foster et al. 2007; West et al. 2007). While it was commonly assumed that most microbes lived asocially as individual cells, this idea has completely been changed in the last ten-twenty years thanks to the work of many microbiologists. Microbes have in fact being found to be involved in a variety of social cooperative behaviors. Microorganisms such as the amoeba *Dictyostelium discoideum* or the myxobacterium *Myxococcus xantus*, which produce cooperative multicellular dispersing structures, have become important model systems to understand the basis of conflict and cooperation, and thus the fundamental principles of social evolution (Fiegna et al. 2005; Strassmann & Queller 2011). In these species, multicellular fruiting bodies made of a stalk and a head full of spores are constituted by free-living individuals that aggregate when the medium becomes poor of nutrients or prey (Raper 1935). Several cells constitute each of these structures, and while the stalk is sterile, the spores are fertile. This means that some individuals forego reproduction and 'help' other individuals by increasing the height at which the spore are located, increasing thus the probability of being dispersed. According to kin selection theory (Hamilton 1964), altruistic behaviors are stable when cooperators are genetically related, meaning that the behavior of a stalk-contributing amoeba (or myxobacterium) is selected only if it enhances the reproduction of a genetically

related spore-contributing individual. In other words, while genetically identical individuals should cooperate in the production of a fruiting body in an organismal-like way, there should be competition between non-related amoebas over the production of spores in genetically heterogeneous fruiting bodies.

The completely clonal system of *C. biroi*, together with the existence of two subcastes differing for reproductive behavior and physiology is strikingly analogous to a social microorganism. In fact, in a given colony of *C. biroi*, LRIs can be compared to stalk cells in a social microorganism because besides being less reproductive, they start behaving exclusively as helpers when they switch from intranidal egg-layers to sterile foragers. On the other hand, HRIs can be compared to spores because they benefit of the cooperative behavior of LRIs and achieve the maximum levels of reproduction. The conflict between different clonal lineages of *C. biroi* coexisting in the same experimental colony can be studied by observing whether and how the production of HRI varies in presence of genetic heterogeneity.

III.II Article (submitted): Epistasis between adults and larvae induces a social cheater genotype in ants

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Abstract. Understanding the effects of genetic heterogeneity on the balance between selfishness and cooperation is a central topic in social evolution, and studies on social microorganisms have revealed context-dependent clone-specific reproductive strategies in experimental chimeras. Here we show for the first time that different genotypes in animal societies also adopt context-dependent strategies in response to experimentally introduced genetic heterogeneity. Comparing two clonal lineages (A and B) of the parthenogenetic ant *Cerapachys biroi*, we found that lineage A was more fertile than lineage B in pure colonies, but that lineage B outcompeted lineage A in chimeric colonies. The reason for this was that lineage B produced a higher proportion of individuals that specialized in reproduction rather than cooperation. This proportion increased dramatically when, in a brood cross-fostering experiment, lineage B larvae were reared by lineage A workers. Epistatic interactions between worker and larval genotypes, which maintain a stable subcaste ratio in natural monoclonal colonies, therefore underlie an inducible social cheater phenotype in the presence of genetic heterogeneity. In nature, the success of each strategy, i.e. facultative parasitism on partner clones vs. fast colony growth in isolation, will depend on the population structure, with lineages B and A performing better in populations with and without genotype mixing, respectively. As a model system, *C. biroi* uniquely combines experimental accessibility with the behavioral richness of animal societies, opening up new avenues in the study of social evolution.

Introduction

According to inclusive fitness theory, cooperation in biological systems is more easily achieved when cooperators are genetically related, and conflicts arise in genetically heterogeneous systems (1). Facing increased genetic heterogeneity, i.e. when unrelated strains coexist, social microorganisms respond with inter-clonal reproductive competition (2), antagonism (3), decreased group-level efficiency (4, 5), and facultative social cheating (6-9). Predicting the effects of genetic heterogeneity in animal societies is often difficult because interactions are influenced by factors other than relatedness, e.g. the advantages of living within a group (10) or receiving help from group-mates (11). Moreover, studies of animal societies are usually limited by a lack of control over the genetic composition of groups.

In the societies of the ant *Cerapachys biroi* all individuals are genetically identical and reproduce via obligatory thelytokous parthenogenesis (12-14), while dominance hierarchies are absent (15, 16). Similar to social microorganisms, the species is ideally suited to study the effects of genetic heterogeneity by experimentally creating chimeric (polyclonal) colonies. Natural colonies of *C. biroi* consist of two worker subcastes that differ in morphology, behavior and reproductive physiology (16, 17) (Figure 1).

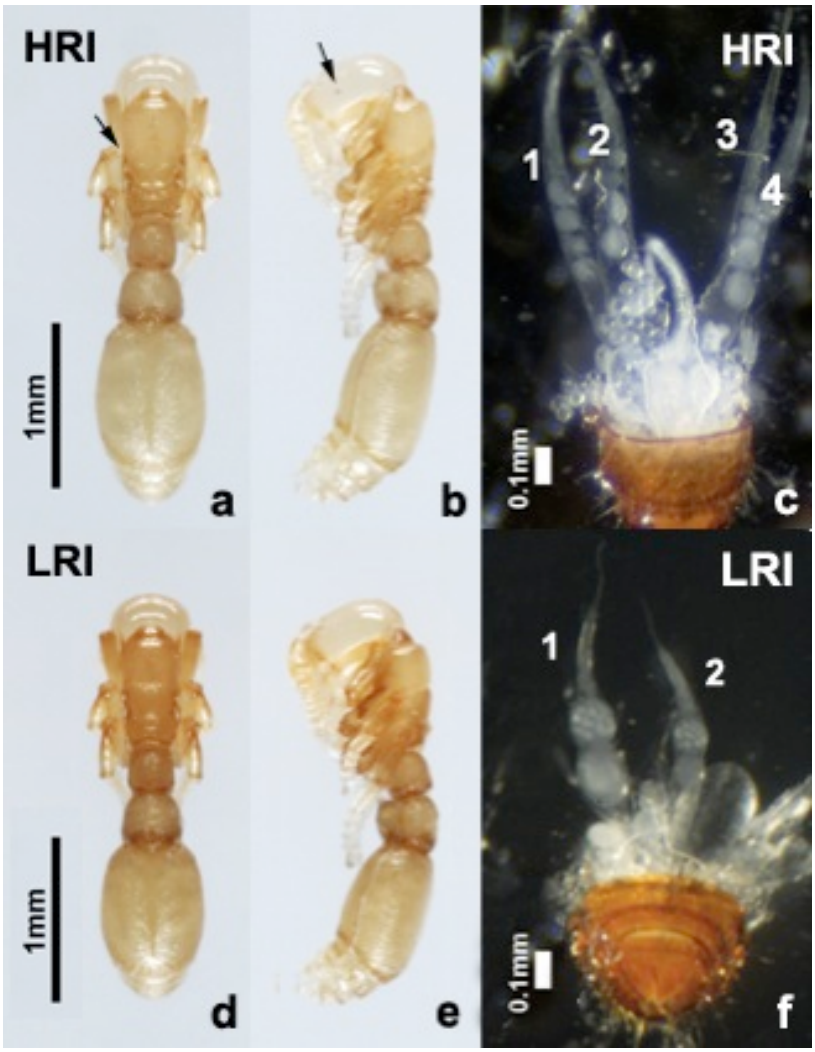

Figure 1. The two subcastes of *C. biroi*. Dorsal view (**a**) and lateral view (**b**) of a HRI pupa, and ovaries of a HRI (**c**). Dorsal view (**d**) and lateral view (**e**) of a LRI pupa, and ovaries of a LRI (**f**). HRIs are slightly larger and have more ovarioles than LRIs. In HRIs, thoracical sutures (arrow in **a**) are more developed, and they have visible vestigial eyes (arrow in **b**).

High reproductive individuals (HRIs) do not forage and lay up to eight eggs per reproductive cycle (in *C. biroi*, larval cues coordinate collective reproduction in 34-day cycles (16, 17)), while low reproductive individuals (LRIs) lay at most two eggs per cycle and become sterile foragers at four to five months of age (16,17). Subcaste differentiation is regulated via a feedback system where the production of HRIs is inversely proportional to group fertility, i.e., the higher the proportion of egg-layers in a colony, the fewer HRIs are produced (18). This leads to an

equilibrium between reproductive and ergonomic colony function. As conflicts of interest exist between unrelated individuals, clonal lineages are expected to compete for group resources when mixed in experimental chimeric colonies. One potential way to compete is social cheating (or facultative parasitism), which means 1) adjusting the reproductive strategy in a context-dependent manner and 2) getting more than the fair share, i.e., reproductively outcompeting the partner clone. This could be achieved by investing less in cooperative tasks like foraging and increasing the reproductive output instead (6-9), and should come at a cost to colony-level efficiency, potentially leading to a "tragedy of the commons"(19). Indeed, "tragedies of the commons" as a result of genetic heterogeneity have been observed in social microorganisms, where organismal-like aggregations of individuals belonging to unrelated strains were less efficient than genetically homogeneous aggregations (4, 5).

In this study, we investigated social conflict and cooperation within an insect society. Our aim was to bring the issues that are usually explored in unicellular microorganisms to a higher level of biological organization. We conducted experiments using two different clonal lineages of *C. biroi* in order to understand how the behaviour of clones in isolation relates to competitive scenarios, i.e. 1) whether clones alter their HRI investment in chimeric vs. monoclonal colonies and 2) whether a clone consistently outcompetes another clone in chimeras.

Results and discussion

We set up chimeric and monoclonal colonies with LRIs from two clonal lineages (lineages A and B; see material and methods for details) and bred them for six generations (in *C. biroi*, a new cohort of adults emerges synchronously every 34 days (15)). Each generation of workers was paint marked upon emergence to *a posteriori* monitor changes in clone ratios and HRI allocation during colony growth. The individuals that emerged at generations one, two, and six in chimeric colonies were microsatellite genotyped to assign them to clonal lineages (253, 379 and 770 individuals for generations one, two, and six, respectively; 1422 individuals in total). After six generations, monoclonal A colonies were larger than monoclonal B and chimeric colonies (Kruskal-Wallis test, Chi sq. 8.2324, Df= 2, $p=0.016$, Bonferroni correction, $\alpha=0.016$, both $p<0.001$, Figure 2a), showing that A colonies are more fertile than B colonies. B and chimeric colonies did not differ significantly in size (Bonferroni correction, $\alpha=0.016$, $p=0.9$). Based on these results, we expected A to outcompete B in chimeras. During the first generation, clone A indeed produced more offspring than clone B, but no differences were found at generation two (generalized linear mixed model, GLMM, respectively, Chi sq.=6.3792, Df=1, $p=0.01$ and Chi sq.=0.6812, Df=1, $p=0.4$, Figure 2b). By the sixth generation, however, significantly more B than A individuals were produced (GLMM, Chi sq.=84.609, Df=1, $p<0.0001$, Figure 2b). Moreover, while in clone A the HRI proportion did not change between monoclonal and chimeric colonies (GLMM, Chi sq. 61.891, Df=3, $p<0.0001$, Bonferroni correction, $\alpha=0.008$, $p=0.04$), the HRI proportion produced by clone B was significantly higher in chimeras (Bonferroni correction, $\alpha=0.008$, $p=0.002$). As a result, almost all HRIs in chimeras (98%) were produced by clone B, which explains why B outcompeted A.

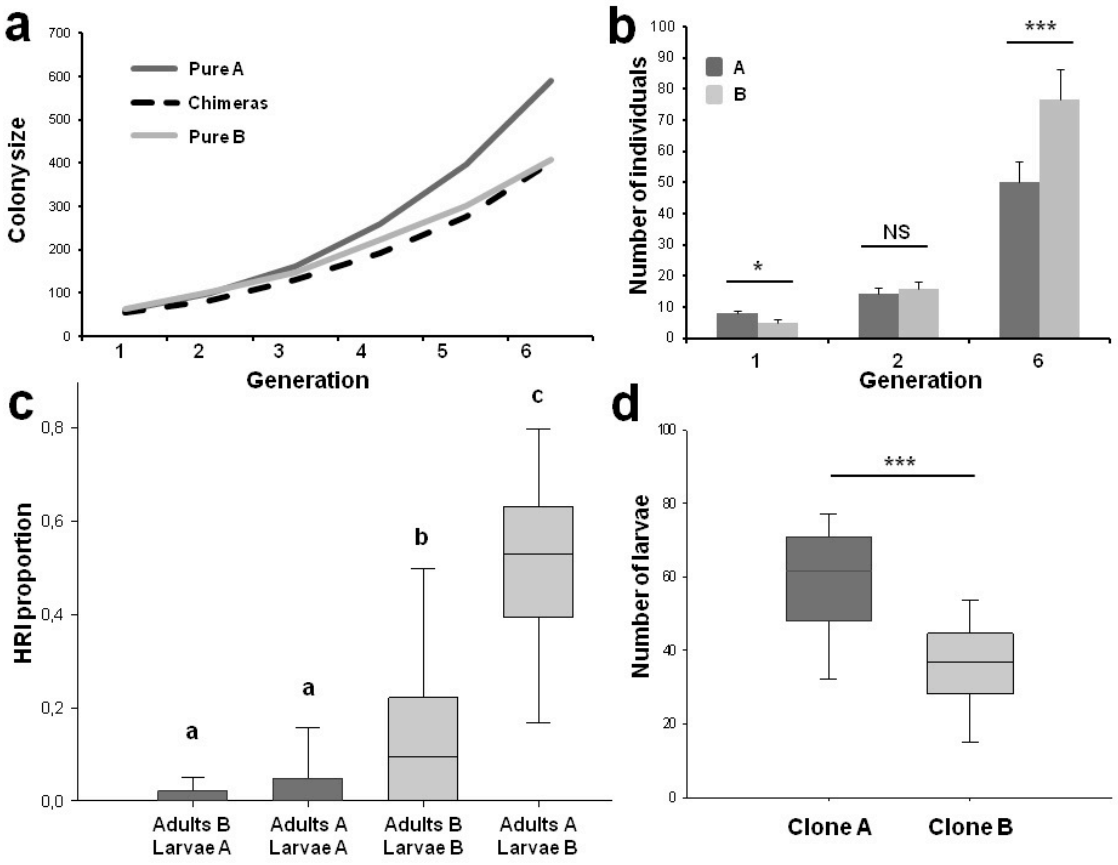

Figure 2. Results of the cross-fostering and clone-mixing experiments. **a**, average colony size in monoclonal and chimeric colonies over six generations. **b**, number of individuals for each clone at generation one, two and six in chimeric colonies (mean ± SEM). **c**, HRI proportions across the four adult-larvae combinations in the cross-fostering experiment (letters indicate statistically significant differences). **d**, number of larvae produced by each clone in isolation before cross-fostering.

As lineage B changed its HRI production in a context-dependent way, we suspected that this could be due to social influences on larval differentiation, i.e. that adults influence larval fate via epistasis, a phenomenon in which genetic expression depends on the expression of other genes (located in the same or a different genome) which act as regulators or modifiers (20, 21). We therefore assessed the propensity of larvae to develop into HRIs when raised by workers of their own or the other clonal lineage. When raised by clone-mates, B larvae showed a higher tendency to develop into HRIs than A larvae (on average 16.3±16.5(s.d.)% vs. 3.4±5.6%, GLMM, Chi sq.=77.72, Df=3, $p<0.0001$,

Bonferroni correction, α=0.008, p=0.001, Figure 2c). While A larvae did not significantly change their HRI differentiation rate when raised by B workers (Bonferroni correction, α=0.008, p=0.1, Figure 2c), B larvae showed a dramatically increased propensity to develop into HRIs when raised by A workers (on average 51.7±21%, Bonferroni correction, α=0.008, all p<0.0001, Figure 2c). Possibly due to cannibalism between larvae, larval mortality was high (37.5±14.6(s.d.)%) but did not differ across the four workers-larvae combinations (GLMM, Chi sq.=1.0351, Df=3, p=0.79). Prior to cross-fostering, larvae produced by each experimental colony were counted in order to evaluate the fertility of each clonal lineage. Lineage A produced significantly more larvae than B (GLMM, Chi sq.=53.413, Df=1, p<0.0001, Figure 2d), confirming the higher fertility observed in the previous experiment.

The elevated proportion of B HRIs raised by A adults (Figure 2c) suggests that the same epistatic interactions between larval and worker genomes underlie the elevated proportion of B HRIs raised in chimeric colonies. Larvae of different clones have possibly different response thresholds to a “fertility signal” that inhibits HRI development (18), and adults of different clones inhibit HRI differentiation at different intensities (Figure 3). Lineage B larvae have a low HRI differentiation threshold, but B adults exhibit strong inhibition that prevents them from developing into HRIs. On the other hand, A larvae have a high threshold to develop into HRIs, and A adults display a lower level of inhibition. According to this interpretation, B larvae were only weakly inhibited when raised by A adults, and their HRI differentiation rate became extremely high. The HRI differentiation rate of clone A was already low in control colonies (3.4±5.6(s.d.)%) and decreased even further when cross-fostered in B colonies (Figure 2a). However, this decrease was not statistically significant, possibly due to the small medium effect size of clone A (Cohen’s d (22, 23) = 0.43±0.6(95%CI) vs. Cohen’s d = 1.9±0.7 for clone B, indicating a large effect size). The results obtained with chimeric colonies are in line with this interpretation, suggesting that the mix of A and B adults produced an intermediate level of larval inhibition, resulting in an increased proportion of B larvae and a decreased proportion of A larvae developing into HRIs.

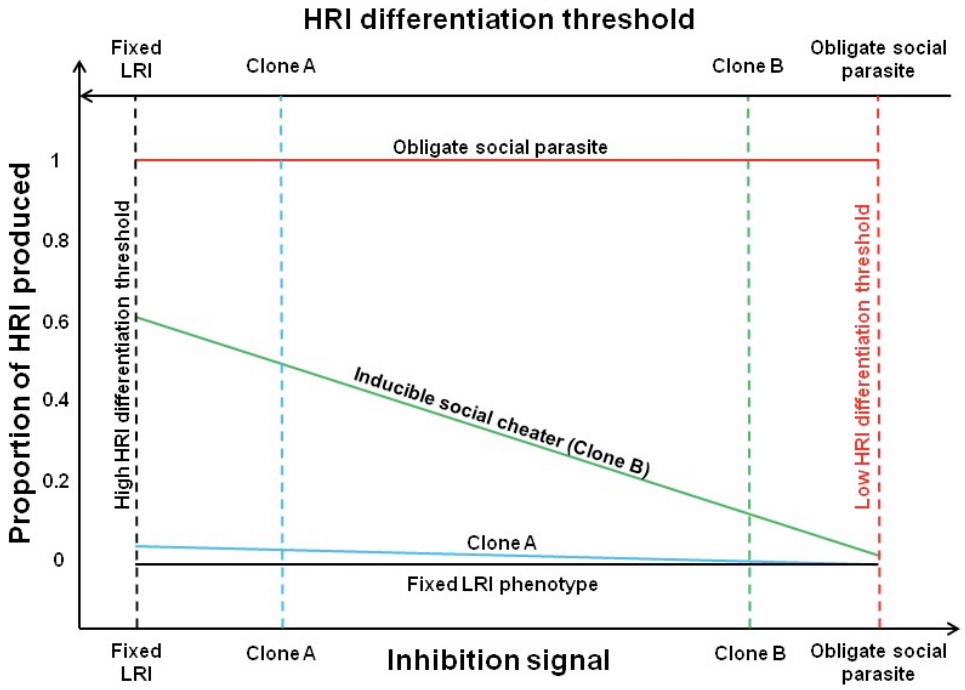

Figure 3. Threshold model for the differentiation of *C. biroi* larvae into HRIs. We hypothesize that the reproductive strategies of different *C. biroi* clones are distributed along a continuum which includes the strategies of A and B. At the extremes of the continuum, phenotypic plasticity is lost, and only one subcaste is produced (LRI or HRI).

In social insects, castes evolve, disappear and re-emerge over evolutionary time via a process known as genetic accommodation, which occurs when novel phenotypes are induced and incorporated in populations through selection on genes controlling their frequency and form of expression (24, 25). In *C. biroi*, the proportion of HRIs differs across different clones, possibly depending on two genetically based individual-level traits, the HRI differentiation threshold of larvae and the level of larval fate inhibition by adults. Selection on the quantitative variability of these two traits generates differently calibrated epistatic adult-larva interactions, which get then positively selected and genetically stabilized in wild populations, possibly by undergoing genetic accommodation (26-28). This

ultimately gives rise to the different reproductive strategies of the clonal lineages, including A and B (Figure 3). As LRIs become sterile at four to five months of age, while HRIs stay fertile for over a year, the strategy of B (slower growth in isolation but high investment in HRIs) would be better suited to preserve colony-level fertility over long periods of non-reproduction. Cerapachyinae ants are specialized in preying on ant brood (15, 29-32), and colonies of *C. biroi* would be forced to undergo diapause-like reproductive interruptions e.g. during winters, when prey ant colonies might not contain brood to prey on. A high proportion of HRIs providing the possibility to lay eggs after those periods should be adaptive in fluctuating environments. The strategy of clonal lineage A (fast growth with almost no HRIs), on the other hand, should be advantageous in constant conditions, e.g. with no seasonality and stable food sources.

From a population-level perspective, the differential strategies of *C. biroi* clones could be adaptive in a further context. Clones with an A-like strategy would grow faster and outcompete clones with a B-like strategy in populations where genotypes never mix in chimeric colonies. On the other hand, there is an evolutionary trade-off between the reproductive optimum in isolation vs. chimeras in populations where genotypes mix. The optimal strategy, i.e. the optimal "calibration" of the epistatic interaction between larval threshold and adult inhibition, depends on how frequently mixing occurs, as well as on the larval thresholds and levels of inhibition displayed by other clones in the population. In chimeras, clonal lineage B increases its reproductive investment and decreases its allocation to cooperative tasks such as foraging, thereby outcompeting A. This plasticity is characteristic for a facultative social parasite. As other *C. biroi* clones exist (13), the clone-specific reproductive strategies might be distributed along a continuum (Figure 3). At one extreme of this continuum, the HRI differentiation threshold might be extremely high, the LRI phenotype fixed, and the subcaste polyphenism lost. Moving towards the other extreme, the HRI differentiation threshold decreases and the HRI proportion increases, until the HRI phenotype is fixed and the polyphenism is again lost. Because HRIs do not forage under normal conditions, social parasitism would then become a possible lifestyle. This condition might indeed exist in *C. biroi*, and is found in another clonal ant, *Pristomyrmex punctatus,* where parasitic clonal lineages spread horizontally between host colonies (33). Interestingly, neither social parasites in *P. punctatus*

nor HRIs in *C. biroi* forage, and both have more ovarioles and lay more eggs than average workers (16, 17, 33).

The behaviour of *C. biroi* in chimeras is strikingly similar to the behaviour of the social amoeba *Dictyostelium discoideum*. When starved, individual cells aggregate to form a fruiting body consisting of a sterile stalk and a capsule with fertile spores. While stalk cells serve to lift the capsule off the ground, the spores disperse to reach a food-rich medium (34). Similar to the different HRI allocation ratios in *C. biroi*, different strains of *D. discoideum* have fixed spore allocation levels in isolation that undergo a partner-dependent shift in chimeras (6-8). Also in this case, differentially calibrated strain-specific systems interact via inter-genomic epistasis, resulting in altered spore-to-stalk allocation ratios in chimeric partners. For example, if strain one increases the stalk cell allocation of strain two, while its own spore cell allocation increases due to the weaker stalk cell induction by strain two, this eventually results in strain one prevailing over strain two. A similar scenario is found in the myxobacterium *Myxococcus xanthus*, where some strains produce more spores in chimeras than in pure culture (3). These evolutionary convergences show that the same selective pressures produce similar forms of social organization across different levels of biological complexity, from microorganismal aggregations to the societies of eusocial insects.

More than a decade ago, scientists began to view social microorganisms as ideal model systems to study social evolution, mainly because they did not exhibit the experimental limitations associated with insect societies (2). Here we show that the ant *Cerapachys biroi* has great potential to become a new model system for the study of social evolution and behavior, combining unparalleled experimental accessibility with the behavioral complexity of an animal society.

Material and methods

Clone mixing experiment

Experimental colonies were established with callow LRIs (clonal lineage A: colonies O3 and T5 (respectively from Okinawa, Japan and Taiwan); clonal lineage B: colonies O6 and T1 (also respectively from Okinawa and Taiwan); A and B are two multi-locus lineages (MLLs) referred to respectively as MLL1 and MLL4 in Kronauer et al. 2012 (13)). We used callow individuals in order to 1) standardize fertility (there could be age-related changes in egg-production) and 2) avoid potential aggression due to different chemical signatures (callow individuals are chemically insignificant and acquire their cuticular hydrocarbon profile during early adult life). We established 20 experimental colonies by mixing individuals from A and B stock colonies (20 individuals each, total colony size N=40; 12 O3xO6 and eight T1xT5). Thirteen colonies were used as controls (eight BxB (T1xO6) and five AxA (O3xT5)). Colonies were fed *ad libitum* with live pupae of the ant *Aphaenogaster senilis* during six colony cycles (roughly seven months). Each new cohort of adult individuals was marked at emergence with a dot of enamel paint on the abdomen. Colonies were killed by freezing when the sixth generation of workers emerged. For DNA extractions we used the protocol described in Kronauer et al. 2013 (14). Each genotyped individual was assigned to one or the other clonal lineage on the basis of two characteristic microsatellite loci (we used different combinations of loci ED32S, ETJ3E, D4XW2, D8M16, ER4IH, D8ZOW, ETCR2, and EFOHK (13)). We used a Generalized Linear Mixed Model (GLMM) to compare the proportion of HRIs produced by each clonal lineage in chimeric and monoclonal colonies over the six generations combined. The model included the treatment (experimental or control) and the clonal lineage (A or B) as a single four-level fixed factor, while the original stock colony and the experimental colony were used as random factors. The overall proportion of individuals produced by each clone in chimeric colonies at generations one, two and six was analyzed with GLMMs including the clonal lineage as fixed factor and the combination of stock colonies (T1xT5, O3xO6) as random factor. All HRIs produced by chimeric colonies from generations one to six were genotyped (n=533). While all the LRIs of generations one could be genotyped due to the small size of cohorts (on average 14±11(s.d.) individuals per colony), we genotyped a subsample of LRIs of generations two and six due

to the large size of cohorts (30.3±14.9(s.d.) and 127.3±49.1(s.d.) individuals per colony for generation two and six, respectively). We obtained an estimation of the number of LRIs produced by each clone by multiplying the total number of LRIs produced in that generation by the proportion of LRIs produced by each clone among the genotyped LRIs. All statistical analyses were performed with the software R (35).

Cross-fostering experiment

Experimental colonies were established with 50 callow LRIs (one to two weeks old) to exclude effects of age-related changes in fertility. As HRI production depends on the colony-level proportion of fertile individuals, ref lecoutey the exclusive use of LRIs guaranteed no fertility-related biases in the HRI proportion of the focal cohorts of larvae. We used individuals from the same four stock colonies used in the cross-fostering experiment, (A: colonies O3 and T5; B: colonies O6 and T1), covering all possible pairwise adult-larvae combinations (at least five repetitions each, 86 experimental colonies in total). Experimental pairs of colonies were established simultaneously, so that egg-laying and larval hatching occurred in synchrony and cross-fostering could be performed with larvae at the same developmental stage (early-hatched L1). Colonies were initially fed identical amounts (0.020610±0.00057(s.d.) g) of live pupae of *Aphaenogaster senilis*, and were not fed again until after cross-fostering. Afterwards, colonies were fed *ad libitum* with live *A. senilis* pupae until larvae pupated. As larvae inhibit ovary development in *C. biroi* (17), no eggs were laid during the development of the focal cohort (the individuals cross-fostered as larvae), excluding the possibility of clone-mixed cohorts of larvae. Just before callow emergence, the HRI/LRI ratio of the focal cohort was calculated by classifying individuals according to the presence of thoracic sutures and vestigial eyes. We used a generalized linear mixed model (GLMM) to compare the proportion of HRIs developing from larvae raised by their own or the other clonal lineage. The four combinations between adults and larvae were used as a single four-level fixed factor, while the colony of origin and the adopting colony were used as random factors. To evaluate differences in larval mortality we used a GLMM with clone combination (AxA, AxB, BxB, BxA) as fixed factor and the original stock colony of adults and larvae as random factors. The model we used

to detect quantitative clone-related differences in brood production included the clonal lineage as fixed factor and the original stock colony as random factor. All statistical analyses were performed with the software R (35).

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IV. The variability of cuticular hydrocarbons in *Cerapachys biroi*

IV.I Introduction

Cerapachys biroi has a peculiar colony-level social structure in which all individuals are genetically identical and reproduce by thelytokous parthenogenesis (Tsuji & Yamauchi 1995; Kronauer 2012). Colonies regulate their subcaste ratio (Lecoutey et al. 2011; Teseo et al in preparation) and their reproductive synchrony (Teseo et al. 2013) via decentralized collective mechanisms, but the proximate factors involved in these regulations are in fact unknown. Cuticular hydrocarbons (CHC) bear information about caste, age and reproductive status of individuals within colonies of social Hymenoptera (Denis et al. 2006, Ayasse et al. 1999; Tentschert et al. 2002; Cuvillier-hot et al. 2001; Ichinose & Lenoir 2009; Liebig et al. 2000; Monnin 2006; Le Conte & Hefetz 2008; Smith et al. 2008), often serving as inter-individual signals that optimize colony-level reproductive and ergonomic functions. Part of the work we conducted for this thesis was aimed 1) at investigating of the role of cuticular hydrocarbons in the C. biroi peculiar colony-level dynamics, and 2) at having an idea of how chemical signatures vary across colonies and clonal lineage.

IV.II article (in preparation): Cuticular hydrocarbons variability in the clonal ant *Cerapachys biroi*

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Abstract

Although cuticular hydrocarbons (CHCs) have received much attention from biologists because of their important role in insect communication, few studies have addressed the chemical ecology of clonal species of eusocial insects. In this study we investigated whether and how CHCs relate to the ecology and the colony-level reproductive dynamics of the parthenogenetic ant Cerapachys biroi. We collected individuals of different age and subcastes from several colonies belonging to four clonal lineages, and analyzed their cuticular chemical signature. CHCs varied according to colonies and clonal lineages in two independent data sets, and correlations were found between genetic and chemical distances between colonies. This supports the results of previous research showing that C. biroi workers discriminate between nestmates and non-nestmates, especially when they belong to different clonal lineages. In C. biroi, the production of individuals of a morphological subcaste specialized in reproduction is inversely proportional to colony-level fertility. As chemical signatures usually correlate with fertility and reproductive activity in social Hymenoptera, we asked whether CHCs could function as fertility-signaling primer pheromones determining larval subcaste fate in C. biroi. Interestingly, and contrary to findings for several other ant species, fertility and reproductive activity showed no correlation with chemical signatures, suggesting the absence of fertility related CHCs. This implies that other cues are responsible for subcaste differentiation in this species.

Introduction

In social Hymenoptera, the hydrocarbons present on the surface of the cuticle (cuticular hydrocarbons or CHCs) play an essential role in social communication (Howard & Blomquist 2005), while their primary function is to limit desiccation and infiltration of microorganisms (Gibbs & Crockett 1998; Gibbs 2002; Martin et al. 2009). Much research on social evolution and behavior, where insect societies are extensively used as model systems, has focused on the evolution and the functional role of CHC variability. A significant amount of work has been conducted to understand how chemical signatures can, at the same time, enable colony-level coherence and bear information about caste, age and reproductive status of individuals within colonies (Denis et al. 2006, Ayasse et al. 1999; Tentschert et al. 2002; Cuvillier-hot et al. 2001; Ichinose & Lenoir 2009; Liebig et al. 2000; Monnin 2006; Le Conte & Hefetz 2008; Smith et al. 2008). From this perspective, clonal hymenopteran species have received little attention, despite their potential importance in the understanding of these issues. Genetic homogeneity within colonies allows experimental control over the genotype of individuals , and clonal species can therefore be used to tease apart the effects of genetics versus environment or caste (either behavioral or morphological) on chemical signatures. In the cerapachyine ant Cerapachys biroi, females lack a spermatheca (i.e. they cannot be inseminated) and reproduce via thelytokous parthenogenesis (Tsuji & Yamauchi 1995). Male production occurs exceedingly rarely in laboratory colonies (Kronauer et al. 2012). Even though several clonal lineages have been found in C. biroi (Kronauer et al. 2012), as far as we know colonies are always monoclonal, i.e. all the individuals in a colony belong to the same clonal lineage (intra-colonial relatedness=0.99 (Kronauer et al. 2013)). A recent study has shown that individuals are able to discriminate between nestmates and non-nestmates, especially when they are of different clonal origin (Kronauer et al. 2013). As social recognition in ants is based on the divergence of CHC profiles, we explored the inter-colonial variability of chemical signatures in C. biroi and the relationship between genetic and chemical distances between colonies from the same or different clonal lineages.

The colonies of C. biroi include two worker subcastes that differ in morphology, behavior and fertility levels, referred to as high and low reproductive individuals, or HRIs and LRIs (Teseo et al. 2013) (also called intercastes and workers by

Ravary & Jaisson (2004) and ergatoid queens and workers by Lecoutey et al. (2011)). LRIs have two ovarioles and lay eggs exclusively during their first four-five months of life, in which they also provide care to the developing brood. After this stage, they become nonreproductive foragers (Ravary & Jaisson 2004). HRIs, which constitute approximately 5% of the individuals in a colony (Ravary & Jaisson 2004), show low activity levels and produce up to eight eggs per cycle (they have four to six ovarioles in total). These individuals probably remain fertile for a much longer time compared with LRIs. From a functional perspective, colonies therefore comprise two groups: old LRIs that behave as reproductively inactive foragers, as well as young LRIs and HRIs of all ages that act as nurses and are fertile. In C. biroi, the production of HRIs is regulated via a feedback system based on the actual fertility level of the colony (Lecoutey et al 2011). The more fertile a colony is (the greater its proportion of HRIs and young LRIs), the less HRIs it produces, and vice versa. In social Hymenoptera, CHC signatures are often correlated with fertility, and are used by reproducers to signal their presence and reproductive status (reviewed by Monnin 2006). As a previous study on C. biroi excluded the possibility that the HRI regulation system is based on volatile chemical signals (Lecoutey et al 2011), we hypothesized that non-volatile, cuticular chemical cues could signal fertility and/or reproductive status. We further hypothesized that these cues act as primer pheromones that inhibit the HRI developmental pathway. Cerapachys biroi colonies undergo stereotypic reproductive cycles in which they alternate between a reproductive and a foraging phase (Ravary & Jaisson 2002, 2004; Ravary et al. 2006). In the reproductive phase, all individuals aggregate while a new batch of eggs is laid by fertile individuals, whereas in the foraging phase, young individuals and HRIs perform brood care while older LRIs forage for prey. We analyzed the CHC profiles of HRIs (the most fertile individuals) throughout the reproductive cycle, in order to assess whether the phasic ovarian activity of egg-layers (Teseo et al. 2013) correlated with changes in cuticular profiles. A reproductive activity-related change in chemical signatures could possibly play a role in the feedback regulation of HRI production. We thus compared intranidal (more fertile) workers and foragers (less fertile workers) in the foraging phase, when the two types of individuals can be easily distinguished. This allowed us to assess whether

chemical differences between both groups could be involved in this fertility-related cue.

Materials and methods

Colonies. The *C. biroi* colonies used in this study (origin, clonal lineage and collection date are given in Table 1) were kept in the laboratory at constant conditions of 27 °C, ~70% humidity and 12h L:12h D photoperiod. Nests were made of ~30cmx30cmx10 cm plastic boxes with Fluon®-coated edges, filled for one fourth with plaster of Paris. A single chamber was dug in the center of the nest and covered with a red glass sheet. Colonies were fed twice a week with live pupae of the ant *Aphaenogaster senilis* during foraging phases.

Table 1. Colonies used in this study.

Colony	Clonal lineage	Origin	Field collection date	Used in Set
J1	A (MLL1)	Java, Indonesia	2005	1, 2
O4	A (MLL1)	Okinawa, Japan	2006	1
O5	A (MLL1)	Okinawa, Japan	2006	1,2
T4	D (MLL3)	Taiwan	2001	1,2
T5	A (MLL1)	Taiwan	2001	1, 2
O6	B (MLL4)	Okinawa, Japan	2006	1
T1C	B (MLL4)	Taiwan	1997	1, 2
T3	B (MLL4)	Taiwan	2000	1, 2
C10	C (MLL6)	Okinawa, Japan	2008	1
C9	C (MLL6)	Okinawa, Japan	2008	1

Genetic analyses

Prior to chemical analyses, one individual of each of ten colonies (Table 1) was genotyped at 30 microsatellite loci and sequenced for two mitochondrial gene fragments (658 bp of cytochrome oxidase I and 575 bp of cytochrome oxidase II)

to determine the clonal origin of each colony (data reported in Kronauer et al. (2012)). Ten individuals were then randomly collected from each colony and genotyped at 17 polymorphic microsatellite loci (CKPWC, D6CNC, B3KAG, EFOHK, D3N3P, ESOCS, E27C5, B8PND, DK371, ESI77, ETWBP, EH2OX, ESA52, E324Z, ED6BM, EPCI6, D8EP1) as in Kronauer et al. (2012), in order to estimate the inter-individual relatedness within our colonies and to assure that the initially selected worker was representative for the genetic makeup of the colony. The software GenClone 2.0 (Arnaud-Haond and Belkhir 2007) was used to assign individuals to multilocus genotypes (MLGs) based on these 17 microsatellite loci. MLGs were used to produce matrices containing pair-wise Euclidean genetic distances between individuals of the same clonal lineages. Based on these matrices, we calculated the average pairwise relatedness within colonies as r = (A - D) / A, where A is the maximum possible allele distance given the markers, and D is the average observed allele distance between individuals in a given colony (Kronauer et al 2013). Given that C. biroi reproduces asexually and that the studied populations show very low clonal diversity (Kronauer et al. 2012), we did not perform standard calculations of pairwise regression relatedness (e.g. Queller and Goodnight 1989).

Chemical analyses

Identification of the chemical profile of *C. biroi*. Initially, a set of one hundred individuals was used for the identification of the CHC peaks of C. biroi. Fifty individuals were randomly collected in the foraging area and fifty individuals in the nest chamber of one colony (T5, clonal lineage A, Table 1). Ants were frozen and pooled, and their CHCs were extracted in 2 ml pentane during one hour. The extract was analysed with a VGM250Q GC/MS equipped with a DB-5 column (30 m x 0,32 mm x 0,25 µm, J & W scientific column, Agilent Technologies, Palo Alto, CA, USA). Helium was used as carrier gas, with a 28,57 cm/s flow. The column temperature was held at 150 °C during two minutes, then was increased to 300 °C at 5 °C/min and finally held at 300 °C for 30 min. The injection port was maintained at 200 °C. The MS detector was a Fisons MD 800 (Foremost Equipment, Rochester, NY, USA) set at 70eV. This experimental step was conducted as a first exploration of the chemical signature of C. biroi. We used

a slow method to maximize the definition of the part of the spectrum including CHC peaks, thus optimizing their identification.

Origin and preparation of samples. We used two further independent sets of individuals to explore different aspects of the chemical signature of C. biroi. Set 1 included foragers (which show low fertility levels on average) and intranidal workers (higher fertility on average) collected at the beginning of the foraging phase from different colonies belonging to different clonal lineages. Set 2 included HRIs collected throughout the complete colony reproductive cycle from different colonies from different clonal lineages. Analyzing individuals from set 1 allowed exploring the CHC variability within and between colonies and clonal lineages, thereby highlighting CHC-related differences on the basis of the behavioral subcastes of individuals and their clonal origin. Set 2 individuals were used to investigate whether and how chemical signatures correlate with the changes in reproductive activity related to the biphasic colony cycle of C. biroi.

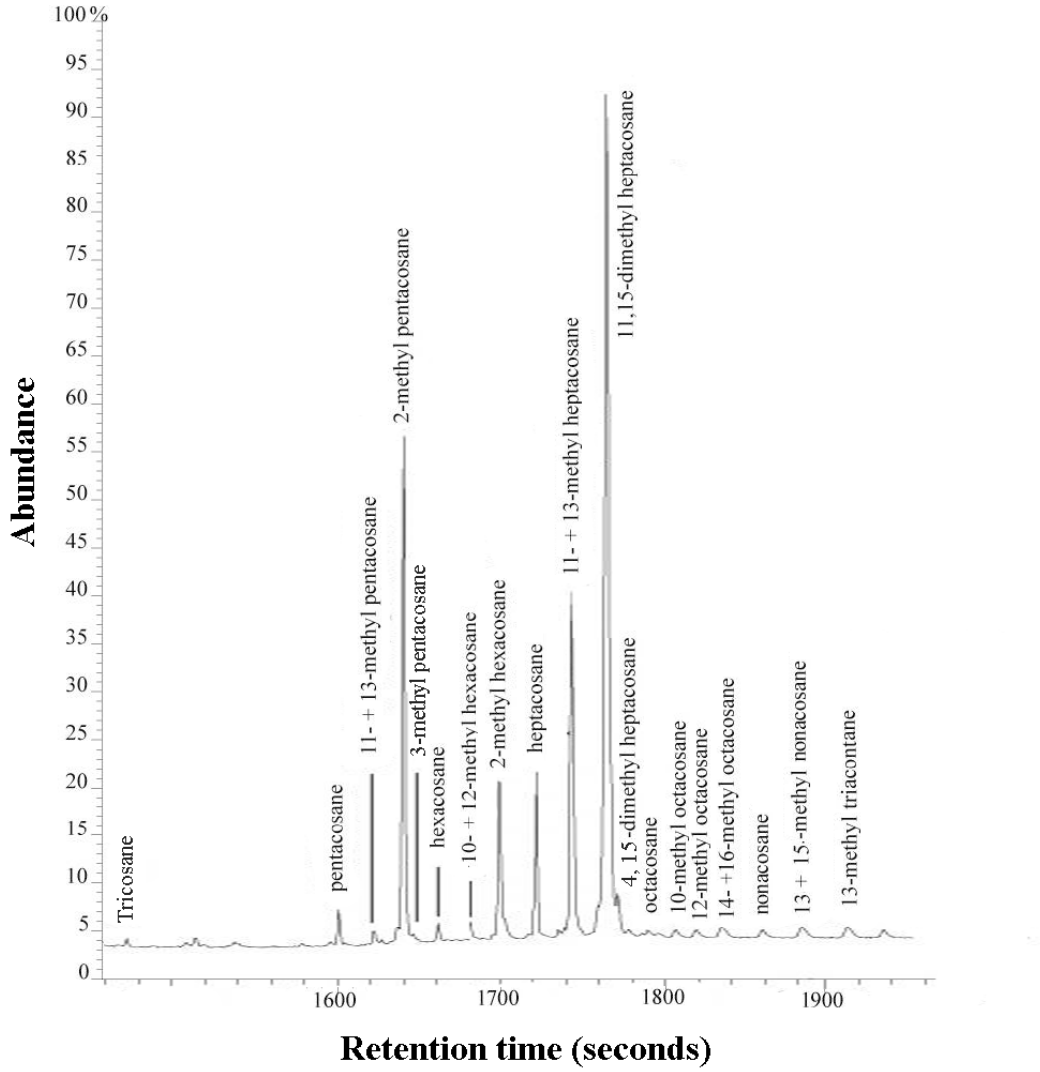

Figure 1. Cuticular hydrocarbon peaks in a representative profile of *C. biroi*. The tricosane, the 13 + 15-methyl nonacosane and the 13-mehtyl triacontane peaks appeared persistently only in set 1, while the 3-methyl pentacosane peak appeared persistently only in set 2.

Set 1.

For each colony, 80 callow LRIs, 40 foraging LRIs and 40 HRIs were collected five days after the beginning of the foraging phase without previous feeding. Since ants do not forage and eat during the statary phase, which lasts around 18 days, the collected individuals had not eaten during at least the previous 23 days, thus excluding any diet-dependent influences on the chemical profiles. All collected individuals were killed by freezing, except for 40 callow LRIs per colony that were placed in separate nests with larvae during one reproductive colony cycle (around 34 days). These individuals were then collected after the beginning of the following foraging phase for chemical analysis, and were considered as young fertile intranidal LRIs. Thus, four morphologic/behavioral subcastes were analyzed for each colony: 1) five to seven days old callow LRIs with undeveloped ovaries, 2) intranidal fertile LRIs (nurses), 1 to 1.5 months old, 3) foragers (sterile LRIs older than 4 months), and 4) HRIs, fertile and at least one month old. For each of ten colonies and each of these four categories, five groups of eight individuals were pooled and extracted during one hour in 200 µl of pentane containing 10µl/l C14 and 15µl/l C24 as internal standards. Chemical analyses (200 samples in total) were carried out using a Varian GC 3900 gas chromatographer equipped with a VARIAN type Factor four VF-5ms column (30 m x 0,32 mm x 0,25 µm). The GC injection port was set to 220 °C and the transfer line to 300 °C. The column temperature was held at 60 °C during two minutes, then was increased to 300 °C at 10 °C/min and finally held at 300 °C for 10 min. Helium was used as carrier gas at 1 ml/min.

Set 2.

To evaluate possible variation during the two phases of the colony cycle, five HRIs were collected every third day throughout one colony cycle from six colonies (three colonies from A, two from B and one for D, total of 375 HRIs). We chose HRIs to be sure that the analyzed individuals were fertile, thus able to lay eggs and showing, if present, fertility-related components in their cuticular profiles. Cuticular washes were prepared by immersing single ants for 10 minutes in 20µl of a pentane solution with an internal standard (C30). We used a lower quantity of solvent because ants were extracted individually and not in groups of eight, and in this way we maximized the CHC concentration in the samples. Two µl of each

extract were manually injected in an Agilent Technologies 7890A Gas Chromatography System connected to an Agilent Technologies 5975C mass spectrometer. The GC column temperature was kept at 70°C for 1 minute, then increased in steps of 30°C/minute to 260°C, then in steps of 5°C/minute to 300°C, then in a step of 20°C/minute to 320°C, and then left at 320°C for 3 minutes. This method was aimed at minimizing the time of each run by focusing exclusively on the part of the spectrum including the previously identified CHC peaks.

Statistical analyses

Eighteen peaks corresponding to CHCs appeared in all samples of Set 1, while only sixteen peaks appeared persistently in Set 2. We thus used eighteen peaks for the statistical analyses of set 1 and sixteen peaks for the statistical analyses of Set 2 (details in Figure 1). The relative concentrations of the compounds used for the discriminant analyses were transformed in proportions and then imported in the software PRIMER (Clarke & Gorley 2006) with the PERMANOVA^{+} add-on package. Data were square root normalized and transformed in matrices of Euclidean inter-individual distances prior to statistical analyses. We used PERMANOVA tests in order to include random factors in our statistical design. This allowed taking the inter-colony and inter-subcaste variability into account, while pooling individuals for statistical tests.

Set 1.

CHC profiles were analyzed using a first PERMANOVA design including three factors: Subcaste (four levels (HRIs and callow, young and old LRIs), fixed), Clone (four levels (A, B, C, D), random) and Colony (ten levels (Table 1), random, nested within Clone). This test was aimed at investigating the subcaste-related variability in chemical signatures within colonies. A second PERMANOVA design included the same three factors but with a different hierarchy: Clone (fixed), Colony (random, nested within Clone) and Subcaste (random). This was aimed at testing whether differences could be found in relation to the clonal origin of the ten colonies we included in the study. For both PERMANOVA designs, p values were obtained using 999 permutations of residuals.

Set 2.

CHC profiles were analyzed with two different PERMANOVA designs using the same three factors with different hierarchies. The first PERMANOVA included the factors Phase (two levels (reproductive or foraging phase), fixed), Clone (random) and Colony (six levels (Table 1), random, nested within Clone), and was designed to investigate the influence of reproductive state of fertile individuals (HRIs) on chemical signatures. The second PERMANOVA design included the factors Clone (fixed), Colony (random, nested within Clone) and Phase (random). This was aimed at understanding whether and what type of inter-clonal differences could be found in the chemical signatures of the analyzed individuals. Also in these cases, for both statistical designs, p values were obtained using 999 permutations of residuals.

Chemical and genetic distances. In order to assess any potential associations between CHC profiles and genetic relatedness, we performed Mantel correlation tests (Mantel 1967) based on 9999 random permutations using the software GenoDive (Meirmans & Tienderen 2004). We correlated matrices containing chemical Euclidean pairwise distances between colony centroids (obtained in the software PRIMER (Clarke & Gorley 2006) from the square root transformed areas of CHC peaks) and genetic distances between colonies. Euclidean genetic distances were obtained with the software GenoDive based on 30 nuclear microsatellite loci analyzed for ten individuals (one for each colony).

Results

Genetic analyses. The 10 individuals (one per colony) sequenced for two mitochondrial DNA fragments and 30 nuclear microsatellite loci belonged to four previously described asexual lineages from Okinawa and Taiwan (MLL1, MLL4, MLL6 and MLL3 in Kronauer et al. 2012, referred to as A, B, C and D, respectively in this study (see Table 1)). Four individuals had A genotypes, three had B genotypes, two had C genotypes, and one had the D genotype. Based on the 17 polymorphic microsatellite loci analyzed for ten individuals per colony, we detected a single clonal lineage in each colony. On average, we detected 1.75 multilocus genotypes (MLGs; usually differing by a single allele across all 17 loci) per colony for the eight colonies belonging to clonal lineages A, B and D. As predicted, average pairwise relatedness within colonies was extremely high (r=0.985 on average). Similar results have been reported previously for our two colonies from clonal lineage C (Kronauer et al. 2013). This confirms that colonies in our study were genetically homogeneous, and shows that the genotype of a single individual per colony reliably represents the genetic makeup of that colony.

Chemical analyses

Set 1.

We found significant differences between subcastes (PERMANOVA, Pseudo F=2, 1788, df=3, p=0.043), and a subsequent pairwise PERMANOVA revealed that significant differences were present exclusively between callow LRIs and HRIs and between callow LRIs and young LRIs (p=0.036 and p=0.023, respectively); the difference between callow LRIs and old LRIs was marginally non-significant (p=0.063). These results suggest that callow LRIs bear distinct chemical signatures from all the other groups (with the possible exception of old LRIs), while the remaining three groups are identical in their CHC profiles. Significant differences were found between colonies of different clonal lineages (PERMANOVA, Pseudo F=3, 4406, df=3, p=0.008, Figure 2). While clones A, B and D were not significantly different from one another (pairwise PERMANOVA, all p>0.18), clone C was significantly different from clones A, B and D (pairwise PERMANOVA, all p<0.019). A positive correlation was found between chemical and genetic distances between colonies (Mantel test, r=0.58, p=0.0005).

Within clonal lineages, significant differences were found between colonies of clone A (PERMANOVA, Pseudo F=2, 9686, df=3, p=0.019) and clone B (PERMANOVA, Pseudo F=6, 0295, df=2, p=0.013), whereas colonies of clonal lineage C were not significantly distinct and clone D was represented by only one colony.

Set 2.

No significant differences were found between the chemical signatures of fertile individuals in different phases of the colony cycle, i.e. there was no influence of reproductive status (activity or inactivity) on chemical signatures (PERMANOVA, Pseudo F=1, 3203, df=1, p=0.34). Moreover, no significant differences were found between cuticular signatures among any of the groups of HRIs collected every third day during a complete colony cycle (PERMANOVA, random factors: colony nested in clones; fixed factor: group of HRIs collected the same day; Pseudo F=0, 8904, df=11, p=0.6; pairwise PERMANOVA, all p>0.089). Contrary to Set 1, clones A and B showed significant differences in their chemical signature (PERMANOVA, Pseudo F=13, 119, df=1, p=0.015). However, the correlation between genetic and chemical distances was marginally non-significant (Mantel test, 9999 permutations, r=0.7, p=0.064).

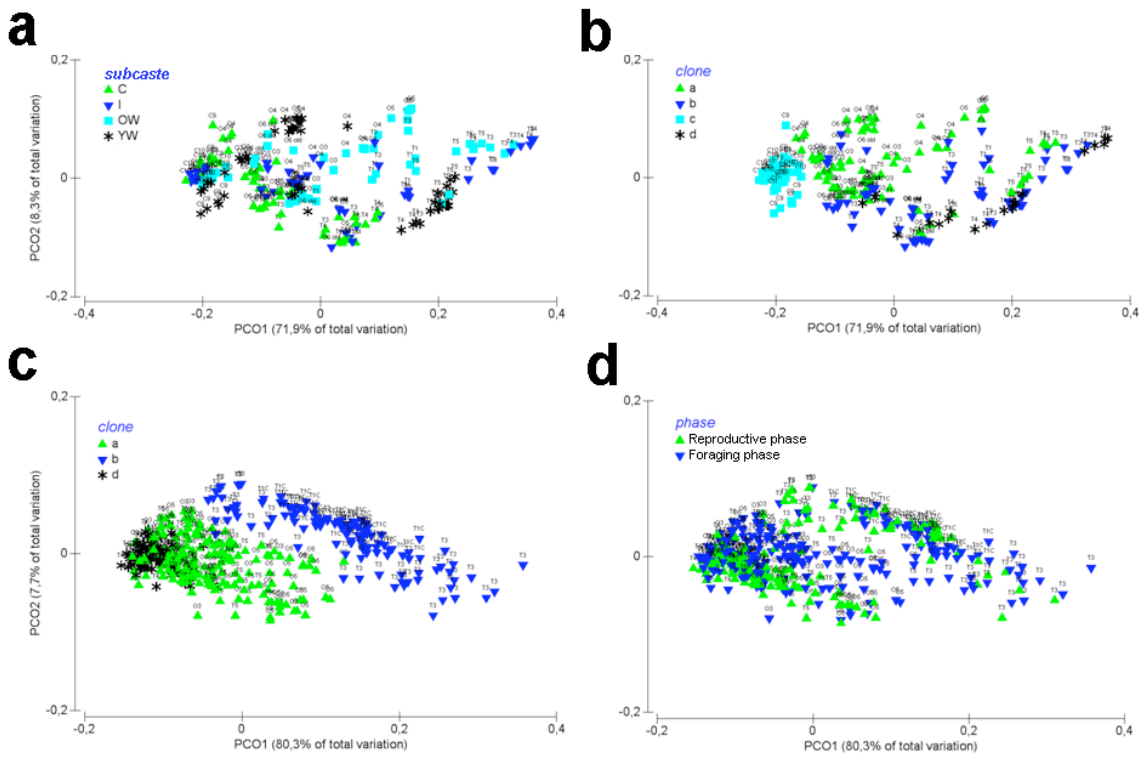

Figure 2. Principal coordinates analyses on CHC data sets 1 and 2. **a** and **b**, samples of set 1 labeled by subcaste and by clone, respectively. **c** and **d**, samples of set 2 labeled by clone and phase of colony cycle, respectively.

Set 2.

No significant differences were found between the chemical signatures of fertile individuals in different phases of the colony cycle, i.e. there was no influence of reproductive status (activity or inactivity) on chemical signatures (PERMANOVA, Pseudo F=1, 3203, df=1, p=0.34). Contrary to Set 1, clones A and B showed here significant differences in their chemical signature (PERMANOVA, Pseudo F=13, 119, df=1, p=0.015). However, Set 1 included more colonies than Set 2, with a wider diversity of profiles. This higher diversity might have at the same time 1) increased the diversity between colonies of the same clone, and 2) reduced the differences between clones.

A Mantel correlation test between the allelic and chemical inter-colony distances confirmed this result (9999 permutations, r=0.7, p=0.064). The correlation obtained was indeed not significant, but this was probably due to the low number of colonies we could include in the analysis (n=6).

Discussion

Within-colony CHC variability

Our chemical analyses revealed a high degree of homogeneity in cuticular signatures within *C. biroi* colonies, with only callow LRIs being different from all the other groups we considered. However, this finding is probably due to the fact that the cuticular profile of recently eclosed individuals is not completely matured, and as a result they are chemically different from older nestmates (Breed et al. 2004; Ichinose & Lenoir 2009; Teseo et al. 2013).

In many species of social Hymenoptera, CHC profiles signal fertility and/or reproductive activity (see Monnin 2006), which allows non-reproductive individuals to perceive the presence of reproductives and refrain from egg-laying (Slessor et al. 2005; Le Conte & Hefetz 2008; Holman et al. 2010) ensuring a balance between reproductive and ergonomic colony function. In some ant species where all nestmates are able to mate and produce female offspring, fertility-related cuticular hydrocarbons serve to maintain colony-level reproductive dominance hierarchies (Monnin et al. 1998; Monnin & Peeters 1999; Peeters et al. 1999; Heinze et al. 2002; Cuvillier-Hot et al. 2004; Monnin 2006). In the ponerine ant *Platythyrea punctata*, where individuals are able to produce female brood asexually, fertility and dominance signaling via cuticular signatures maintains a single reproductive individual per colony (Hartmann et al. 2003, 2005). *Cerapachys biroi* is completely different in its colony-level reproductive dynamics and social structure, in that all individuals reproduce (Ravary and Jaisson 2002), and there are no reproductive dominance hierarchies. Cuticular signatures are thus not expected to bear fertility signals related to dominance or inducing nestmates to refrain from laying eggs. It seems thus improbable that the

colony-level regulation of reproduction relies on cuticular signals related to individual fertility or reproductive activity. However, HRI production in *C. biroi* varies depending on the average colony-level fertility, implying the existence of some regulation acting on larval fate (Lecoutey et al. 2011). The lack of correlation between fertility levels and cuticular signatures suggests that the regulation of HRI development is either non-chemical or does not depend on signals derived from CHCs. One possibility is that a non-CHC, non-volatile chemical signal that is present on the cuticular surface or secreted from other glandular sources is involved. Moreover, the quality of the food that may be admixed with some glandular products of adults can direct larval development toward different pathways, which underlies caste determination in many social Hymenoptera (Hölldobler & Wilson 1990; Wheeler 1986, 1991). Primer pheromones transmitted from adults to larvae via direct contact during parental care might play a complementary role in caste differentiation. Indeed, workers regularly perform a peculiar behavior during brood care that consists in licking the developing larvae ventrally under the head capsule (Lecoutey, personal observations). Further studies on the mandibular secretions of nurses are therefore needed to investigate whether they play a role in larval differentiation in *C. biroi*.

Subcaste differentiation might also exclusively rely on the quantity of food available to larvae. Quantitative differences in food intake during pre-imaginal stages have major effects on development in insects, and give rise not only to differences in adult size, but also differential expression of adult polyphenisms (Emlen 1994; Hunt & Simmons 1997; Mockzek & Emlen 2000). Adult *C. biroi* could quantitatively limit larval feeding in several ways, e.g. by actively keeping

larvae far from prey items within the nest, or simply by competing with larvae for food. HRI production is inversely proportional to the proportion of fertile individuals in a colony, and fertile individuals might need a higher quantity of food in order to produce eggs. Thus, the more fertile individuals are present in a colony, the less food might be available for larvae, which possibly limits HRI production. Other types of influences on larval fate, such as mechanical stress on developing larvae due to biting from adults (Brian 1973; Penick & Liebig 2012), could also be involved. Observations on the behavior of adult individuals towards larvae during the foraging phase will clarify the proximate factors determining subcaste differentiation in *C. biroi*.

CHC variability among clones

Our study shows that the colony-level chemical signatures of *C. biroi* vary according to the clonal lineage, with chemical distances between colonies growing with genetic distances. The fact that we observed only a marginally non-significant correlation between genetic and chemical distances for colonies of Set 2 was probably due to the low number of colonies included in that analysis (n=6). Colonies belonging to the same clonal lineage also show some variability in their cuticular signature, even though the chemical distances among those are lower than distances among colonies from different clones. Overall, our findings support the results of a previous study on the invasive *C. biroi* population in Okinawa, where individuals were able to discriminate between nestmates and non-nestmates, especially when non-nestmates belonged to unrelated asexual lineages (Kronauer et al. 2013). According to our results, chemical signatures might be the proximate cues indicating genetic dissimilarity between interacting

individuals, which in turn might prevent fusions between unrelated colonies. This might be a possible reason explaining why natural *C. biroi* colonies have been found to all be monoclonal (Kronauer et al 2013). However, given that the putative native range of *C. biroi* remains still largely unexplored, it cannot be excluded that in natural populations different clones mix in chimeric colonies.

Invasive populations of ants are likely to originate via the introduction of few individuals, i.e. population bottlenecks which most of the time produce a strong founder effect (Tsutsui & Suarez 2003). As a result, invasive populations are overall genetically less diverse than native populations. This can result in individuals from different colonies displaying similar cuticular hydrocarbons profiles, potentially leading to a loss of aggression even between non-nestmates (Tsutsui & Suarez 2003). This loss of aggression is thought to promote the formation of supercolonies (Giraud et al. 2002). For example, many invasive species forming supercolonies exhibit negligible levels of between-colony aggression, even between individuals taken from nests separated by several kilometers (Giraud et al 2002; Drescher et al. 2010; Blight et al. 2012). *Cerapachys biroi* is invasive (Wetterer et al. 2012; Kronauer et al. 2012, 2013), but to our knowledge it does not form supercolonies. This might in part be due to the maintenance of non-nestmate discrimination between colonies from different clonal lineages (Kronauer et al. 2013), but as non-nestmate discrimination between colonies from the same clonal lineage is low, it would not explain the absence of monoclonal supercolonies. One further possible explanation is that strict myrmechophagy, which is characteristic of *C. biroi*, might keep the abundance of the species to low levels. Even though ant brood is ubiquitous, which might have contributed in *C. biroi*'s worldwide spread (Wetterer et al. 2012),

its availability is possibly too low to determine the growth rate needed for the formation of *C. biroi* supercolonies.

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V. Conclusion

The work we conducted on the model system *C. biroi* presented in this thesis has produced scientific advances at different levels. For example, new aspects of the biology of the species itself have been discovered, and some of those might be useful to understand the biology and the evolution of traits such as the phasic colony-level reproductive cycle, which is present in closely related ant species and has convergently appeared in other more distant ones. On a different level, this thesis might help completing the picture we have on the multi-level adaptivity of the social control on single individuals' contribution in group-level dynamics; policing in its broader sense is indeed not limited to insect societies, but is a widespread intrinsic feature of all social groups, including human communities. Our research also considered basic issues on conflict and cooperation on a more general level. Studying the outcome of the interactions between different genotypes coexisting in the same social group is in fact an issue that can be rarely explored outside the world of social microorganisms. The model system *C. biroi* allowed trespassing this limit and gave us the opportunity to understand, for example, how different reproductive strategies can emerge and be adaptive in different social contexts within groups of socially interacting metazoans.

V.I HRI and LRI: discrete variability or continuum?

Classically, colonies of *C. biroi* have been considered as constituted by two discrete worker subcastes, HRIs and LRIs (Ravary & Jaisson 2004; Lecoutey et al. 2011; Teseo et al. 2013). These two subcastes, as described in the introduction section of this thesis, differ in morphology, reproductive potential and behavior. However, previous work based on ovarian dissections of *C. biroi* has already evidenced the existence of several classes of individuals based on the number of ovarioles (from two to six; Ravary & Jaisson 2004), suggesting that the reproductive potential of individuals might vary also within, and not only between, worker subcastes. Moreover, during the work we conducted on *C. biroi*, we observed 1) that differences exist within the same subcaste in external morphology, i.e. the characters defining the subcaste can vary between

individuals, and 2) that it is sometimes difficult to attribute an individual to one or the other subcaste based exclusively on morphology. Also, I occasionally collected old sterile individuals in the act of foraging, which showed four or more ovarioles when dissected. Thus it is possible that also HRIs, at some point, become sterile and undergo the same physiological/behavioral switch occurring in LRIs at around four-five months of age. A continuum might indeed exist between LRIs and HRIs that is not exclusively limited to reproductive physiology and morphology, but also involves behavioral traits such as the switch to foraging that accompanies the physiological shift towards sterility. While there could be individuals that never lay eggs and start foraging immediately after emergence (freshly emerged callow LRI are occasionally seen foraging outside the nest chamber), there could be other individuals that never become sterile (I have personally observed HRIs reaching up to two years of age and still behaving as intra-nidal egg-layers and brood-carers). As the variability between those two extremes might be large and continuous, with individuals switching to foraging at any age, further work is needed to test and quantify this continuum. This will enable us to understand what the factors are that actually underlie the behavioral, morphological and reproductive traits of individuals within colonies of *C. biroi*, and what the relation between morphological features, reproductive potential and behavioral profile might be.

V.II Ovarian cycle of *C. biroi*

Cerapachys biroi colonies follow stereotypical reproductive cycles alternating foraging and reproductive phases (Ravary & Jaisson 2002, 2004; Ravary et al 2006, 2007; Lecoutey et al. 2011; Teseo et al. 2013). Our work on reproductive activity, which has been conducted through ovarian dissections of individuals in different phases of the reproductive cycle (or under various experimental treatments), has shown that the timing of the colony-level reproductive cycle depends on the presence/absence of larvae. In other words, larvae are the pacemakers of the alternation of phases, inhibiting ovarian development in fertile individuals (Teseo et al. 2013). Whenever larvae are experimentally removed from a colony, fertile adult ants almost immediately start activating their ovaries,

and lay eggs within around five days. Knowing how ovaries change along the phasic reproductive cycle and which are the factors determining the reproductive activation and de-activation could be helpful for further research. For example, it will allow maximal control of experiments on the phasic lifestyle. An idea of the advantages of using *C. biroi* as a model system can be given by citing the pioneering studies of Hagan on army ants (1954a, b, c), which showed phasic reproductive activity in queens of the genus *Eciton* (subfamily Ecitoninae). For his studies, Hagan needed several queens from different colonies and even different species, which he analysed exclusively *a posteriori* via dissection. Each queen had to be collected together with the whole colony, which means an enormous logistical effort. There was little control on the exact timing of colony collection, and many colonies had to be collected in order to have a representative sample of the stages of the reproductive cycle. As in the colonies of *C. biroi* many individuals reproduce regularly, the dynamics of the phasic reproductive cycle can be investigated in an inexpensive, non-invasive and controlled way. The increased knowledge on the ovarian cycle of *C. biroi* will aid in further advances in the study of the phasic reproductive cycle that is found in several ant groups (Gotwald & Brown 1966; Buschinger et al. 1989; Maschvitz et al. 1989; Brandão et al. 1999, 2008; Donoso et al. 2006; Kronauer 2009; Schmidt 2013). For example, comparative testing of the larval inhibition of ovarian activity in all the other ant groups where phasic reproduction has been observed could help in understanding how the phasic colony-level reproductive cycle has been selected for during evolutionary time.

V.III Adaptive value of ‘phasicity’ in ants

In the article “Enforcement of reproductive synchrony via policing in a clonal ant” we point at the adaptive value of the phasic reproductive cycle as the ultimate factor promoting policing in societies of *C. biroi*. According to the results of our study, ‘phasicity’ is adaptive for *C. biroi* because it probably allows optimal exploitation of a patchy food source such as ant brood. Coordinated brood development minimizes the time lapse in which food-demanding larvae are present in the colony, and thus also the costly foraging activity that is maintained

in order to feed them. Moreover, as prey ant brood is patchy and short-lived, predating colonies have to emigrate frequently to retrieve fresh prey, and thus short foraging phases possibly minimize the cost of those emigrations (Kronauer et al. 2009). In addition, the light foraging activity of single individuals is not sufficient to overwhelm prey colonies, whereas collective raiding is probably the only efficient strategy when feeding on ant brood. If a prey colony is overwhelmed, a large quantity of food suddenly becomes available and can sustain a large number of developing larvae. These hypotheses on the adaptive characteristics of the phasic colony cycle open a series of questions regarding the evolution of phasicity, first of all because other groups of phasic ants (as e.g. the well-studied genus *Eciton*) do not share with *C. biroi* the alimentary specialization on ant brood (Schneirla 1971; Holldobler & Wilson 1990). With regards to this difference, we can hypothesize that myrmecophagy has had a role in the evolution of phasicity, in that the transition from non-phasicity to phasicity has co-evolved with the alimentary specialization on ant brood. From the success of myrmecophagy, colonies have possibly been able to reach a large size (such as occurs in *Eciton*, for example), which might have allowed them to switch back to generalist predation, while phasicity could have been maintained because of its success. In fact, phasic reproduction coupled with collective coordinated raiding is probably the optimal strategy to harvest as much prey as possible while minimizing energetic costs.

Whether and where *C. biroi* is in this hypothetic evolutionary route is an impossible question to answer, at least for the moment; moreover, it is unknown whether phasicity is an ancestral trait of Dorylomorph ants or if it emerged convergently in Cerapachyine and the other phasic ant groups. However, as the habits of Cerapachyinae ants are still largely unknown (and the same is true for several ant groups exhibiting synchronized brood development; Gotwald & Brown 1966; Buschinger et al. 1989; Maschvitz et al. 1989; Brandão et al. 1999, 2008; Donoso et al. 2006; Kronauer 2009; Schmidt 2013), a comparative study on reproductive behavior, ecology and genomics of the subfamily might be useful to explore the evolution of phasicity. In general, an interesting scenario would be offered by a ‘facultatively phasic’ ant species showing phasic and non-phasic populations, which could be compared in order to disentangle the origin and selection pressures that could have favored phasic reproduction. Whether this

species exists is not yet known; however, we have observed that one of the clones of *C. biroi* from the invasive range (multilocus lineage 6) shows less regular cycles compared to the other known clones. Because of this, we cannot exclude the existence of populations of *C. biroi* where reproductive cycles are not well defined or do not occur at all. A comparison of the ecology of *C. biroi* populations (which at least in their huge putative native range could offer high diversity) might help shed light on the evolution of phasicity.

Finally, more advances in the knowledge of phasic activity in ants might arise from studies stemming from a theory known as "Reproductive Ground Plan Hypothesis" or RGPH (Amdam 2004), which we mention in our study on policing in *C. biroi*. According to this theory, division of labor in insect societies is possibly linked to a caste-specific differential expression of genes that were activated either during foraging or reproductive phases in the solitary ancestors of social hymenoptera. The oscillation between foraging and reproductive phases that we observe in *C. biroi* could arise from the alternate expression of the genes of solitary ancestors. In light of this hypothesis, it would be interesting and fruitful to examine the differential expression patterns of 'phasic genes' not only between the two subcastes of *C. biroi* and between the two phases of the reproductive cycle, but also in relation to the physiological switch from fertility to sterility occurring in *C. biroi* LRIs.

V.IV Policing as colony-level adaptive trait in ants

Policing has evolved in insect societies in order to repress intra-colonial conflict and optimize colony-level adaptive traits (Monnin & Ratnieks 2001; Otsuki & Tsuji 2009; Beekman & Ratnieks 2003; Ratnieks et al. 2006; Ratnieks 1988; Pirk et al. 2003; Hammond & Keller 2004; Wenseleers & Ratnieks 2006; Hartmann et al. 2003). The novelty of our study about policing in *C. biroi* relies on the fact that it is the only known ant model system that allows disentangling of the conflict between parties within colonies and the colony-level adaptive behaviors of individuals. This is to our knowledge impossible to perform with other ant models where policing occurs (Kellner et al 2010; Kellner & Heinze 2011). In our study we hypothesized that individuals respond at different levels to the larval inhibition of ovarian

activation, and that some individuals have such a high threshold to this inhibition that they cannot refrain from being reproductively active all the time. On the other hand, while these individuals are detected and killed by colony-mates because of their negative impact on colony-level dynamics, the individuals with an extremely low threshold to larval inhibition are difficult to individuate because they are not aggressed. With regards to this potential individual variability of reproductive output, a point that needs to be investigated is whether and how developmental processes influence reproductive physiology, shaping phasic and non-phasic reproductive phenotypes in *C. biroi*. Alternatively, the non-phasic phenotypes could be due to individual-specific patterns of expression of the 'phasic genes' that according to the RGPH might underlie the alternation of phases in C. biroi. Investigating the genome methylation pattern of aggressed and non-aggressed individuals might be an interesting route for further research.

Further work is also needed in order to understand whether a true correlation exists between individuals' fertility level and responsiveness to the larval inhibition of reproduction. From this perspective, in fact, non-phasicity could be a secondary aspect of extremely high fertility, i.e. some individuals are so fertile that they cannot refrain from laying eggs all the time (which would explain also why almost only HRIs are aggressed). However, if fertility is defined as the number of eggs laid in a given period of time, the most fertile individuals should be those with six ovarioles (the maximum number of ovarioles found in *C. biroi* HRIs), and this is not what is observed. There is no evident tendency for aggressed individuals to exhibit six ovarioles, which contradicts the hypothesis that fertility and responsiveness to larval inhibition are tightly linked. Non-phasicity might indeed be related to physiological 'errors' occurring during development that might affect exclusively HRIs.

The work we have conducted on the chemical ecology of *C. biroi* has revealed that there is no correlation between reproductive activity and cuticular hydrocarbon signatures, despite aggressed individuals showing a completely distinct chemical profile. Our study focused on the policing aspect of aggressions, defining non-phasic individuals as dysfunctional from a colony-level perspective, who are killed after being detected by policing colony mates. However, from a different perspective, the specific chemical signature of the aggressed individuals can be interpreted as an altruistic 'kill me signal' evolved as an adaptive response

to colony-level malfunctioning. This signal is interestingly both selfish and altruistic at the same time, because the individuals that exhibit it are genetically identical to the other members of the colony. Therefore, by essentially committing suicide, these individuals actually increase their own inclusive fitness.

V.V Inter-genomic epistasis as regulation mechanism of a colony-level phenotype

In our study "Epistasis between adults and larvae induces a cheater phenotype in ants", we have investigated for the first time the dynamics of a conflict between clones within an animal society. Conflict and cooperation have traditionally been explored in social microorganisms (Strassmann et al. 2000; Foster et al. 2002, 2007; Fiegna et al. 2005; West et al. 2007; Strassmann & Queller 2011), and occasionally in other organisms such as colonial Tunicates (Sabbadin & Zaniolo 1979; Stoner et al. 1999; Ben-Shlomo et al. 2008) and clonal social insects. For example, one study on social clonal aphids (Abbott et al. 2001) shows that individuals migrating in galls containing colonies belonging to an unrelated clone behave as cheaters when challenged with a threat. In fact, when faced with a potential predator (a larva of a dipteran), individuals that had migrated into a gall containing an unrelated colony did not cooperate with the local group to defend the colony, behaving thus as selfish social parasites. While the study by Abbott et al. (2001) focused on the existence of this type of conflict in societies of clones, we investigated the specific mechanistic aspects of cheating in *C. biroi*. The most important point emerging from our study is that epistatic interaction between the larval and the worker genome during development underlies caste determination in social insects, which is a novel insight of great general relevance for the understanding of fundamental concepts in social evolution. By perturbing these epistatic interactions, we revealed the dynamics of the conflict occurring when clones are mixed in the same colonies, and reproduced a possible scenario underlying the divergence and subsequent evolution of a social parasite from its host. Analogous inter-genomic epistatic interactions underlie simple reproductive strategies in social microorganisms (Buttery et al. 2009, 2010; Parkinson et al. 2011), and this shows that again (as for policing in *C. biroi*) the same selective

pressures can produce similar outcomes at different levels of biological organization.

From an ecological perspective, moreover, our study suggests the existence of different reproductive strategies underlying the behavior of different clones. In the two clonal lineages, we observed a differential allocation of the reproductive/ergonomic investment, and this could be due to a trade-off between environmental pressures (such as the climate and the food availability, for example) and the general population-level strategies. To what extent clones of *C. biroi* mix within natural populations is not yet clear, and mixed colonies might be transient and thus difficult to detect. Overall, a deeper knowledge of the population structure and the general biology of the species within its native range is needed in order to continue investigating inter-clonal conflict and cooperation. The reproductive strategies of the different clones of *C. biroi* might not be limited to differential investments in reproduction and work. Our study “Behavioral and physiological ontogeny in the clonal ant *Cerapachys biroi*: combined effect of genes and social environment on larval development”, allowed exploration of a further aspect of *C. biroi* epistatic interactions between adults and larvae. There we showed that inter-clonal cross-breeding determines not only a bias in caste differentiation within colonies, but can even produce long lasting effects on the behavior of individuals, possibly influencing the ontogeny of behavior in a colony-level adaptive way.

Overall, our work on inter-clonal conflict and epistatic effects on caste differentiation and behavior lacks a deeper investigation of how these processes work at the molecular level. Interesting new results might come from the exploration of larval and adult gene expression in further brood cross-fostering experiments.

VI. Future directions

The rapid advances in technology of molecular tools and the growing amount of evolutionary and behavioral research on social insects open up new scenarios in the study of social evolution. Due to its unique features such as clonality and collective reproduction, and thanks to the easy laboratory breeding and to the growing amount of knowledge on its biology, *C. biroi* has the potential to become a well-established model system in studies of animal behavior and evolutionary biology.

Combining next-generation genetic and genomic techniques with behavioral experiments could be a first interesting approach to continue research on *C. biroi*. In particular, it would be important to investigate two main aspects of the biology of the species in which our research has been recently focusing: 1) the developmental plasticity and behavioral flexibility at the individual level, and 2) the division of labor and reproductive tasks among colony members. A multilevel approach would give the important advantage of investigating the same issue at different levels of biological organization simultaneously (for example, studying the genetic expression patterns, behavior, physiology and chemical signaling at the individual and group levels). This should involve behavioral experiments on larval and adult individuals experimentally exposed to varying conditions during their development. In particular, after the encouraging results obtained and presented in this thesis, it would be interesting to test at the molecular level the influence of the social environment on the caste differentiation and behavioral ontogenetic processes, using my, now well-established, brood cross-fostering protocol.

As previous experiments on *C. biroi* have shown that individual experience plays a fundamental role in the division of labor within colonies (Lecoutey et al. 2007) another promising route would be to investigate whether and how gene expression varies in the behavioral development of adult individuals, and the possible influence of the social environment on these individual-level processes.

Cerapachys biroi is originally from Asia, and has been introduced to tropical and subtropical islands around the world since the beginning of the last century (Wetterer et al 2012). The genetic structure of populations in the introduced range

has been widely explored and is quite clearly understood (Kronauer et al. 2012); contrarily, populations in the huge native range are still almost untouched, and genetic variability might be much larger than in the invasive range (Kronauer et al. 2012). With regards to this, it is important to note that for *C. biroi*, contrary to other invasive species such as the pharaoh ant *Monomorium pharaonis* (Wetterer 2010), we do have an idea of where the native range of the species is located, which might help in the general comprehension of the dynamics of biological invasions.

Overall, a next fundamental step for the comprehension of *C. biroi* biology will be collecting more clonal lineages from the native range. The experiments conducted up to the present time are limited to three clonal lineages, whereas the more variable the range of genotypes we stock in laboratory, the wider the range of experiments that can be planned.

VIII. Collaborations and supervision activity

Collaboration: David Baracchi, Iacopo Petrocelli, Ginevra Cusseau, Lucia Pizzocaro, **Serafino Teseo** and Stefano Turillazzi (2013) Facial markings in the hover wasps: quality signals and familiar recognition cues in two species of Stenogastrinae. Animal Behaviour 85(1), 203-212

Supervision: "Master 2 Recherche d'Ethologie" of Steven Birot De La Pommeraye. Influence de l'adoption croisée sur le comportement de deux lignées clonales de la fourmi parthénogénétique *Cerapachys biroi*

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Résumé: Les conflits et la coopération sont des caractéristiques intrinsèques des associations d'organismes, y compris chez les insectes sociaux. L'objectif de cette thèse est d'étudier les conflits et la coopération dans les groupes sociaux en utilisant des colonies de la fourmi clonale sans reines *Cerapachys biroi* comme modèle. Chez cette espèce, les mâles sont absents et tous les individus se reproduisent au moins pendant une période de leur vie par parthénogénèse thélytoque. Mon travail comprend une série d'expériences visant à comprendre les causes des agressions intra-coloniales observées chez *C. biroi.* Ces agressions sont en effet inattendues du fait de l'homogénéité génétique des sociétés. Les résultats ont montré que le conflit apparent entre les individus est en fait un moyen pour la colonie d'optimiser sa reproduction en éliminant les individus qui ne sont pas sensibles aux signaux coloniaux qui régulent l'activité reproductrice. Ce phénomène est similaire à l'immunosurveillance des cellules cancéreuses chez les organismes multicellulaires. Une deuxième partie de mon travail a été conduite à un niveau supérieur d'organisation biologique. J'ai exploré la dynamique des conflits et de la coopération entre des lignées clonales non apparentées de *C. biroi* obligées à coexister dans des colonies polyclonales expérimentales. Les résultats montrent que certains clones peuvent prendre avantage de la présence de lignées non apparentées en se comportant comme des parasites sociaux. Ce comportement montre des similarités frappantes avec celui des microorganismes sociaux. D'une manière générale, ce travail montre que des pressions de sélection similaires peuvent entrainer des adaptations similaires entre des unités biologiques qui coexistent à différents niveaux d'organisation.

Conflict and cooperation in the societies of the clonal ant *Cerapachys biroi*

Abstract. Conflict and cooperation are intrinsic traits of organismal associations, including insect societies. The aim of this thesis was to investigate conflict and cooperation in social groups by using the colonies of the clonal queenless ant *Cerapachys biroi* as a model system. In colonies of this species, males are absent and all individuals reproduce at least for a period of their life via thelytokous parthenogenesis. My work aimed at understanding the causes of the intra-colonial aggressions that are regularly observed in *C. biroi* colonies, which are not expected to occur in genetically homogeneous societies. The results revealed that the apparent inter-individual conflict is in fact a way for the colony to optimize its reproductive output by eliminating those individuals that are insensitive to the colony-level cues regulating individuals' reproductive activity. This phenomenon is analogous to the immunosurveillance on cancer cells in multicellular organisms. Another part of my work was conducted on a higher level of biological organization. I explored the dynamics of conflict and cooperation between unrelated clonal lineages of *C. biroi* forced to coexist in experimental polyclonal colonies. The results showed that clones may take advantage of the presence of unrelated lineages by behaving as social parasites. This behaviour shows striking similarities with social microorganisms. Overall, my work shows that similar selective pressures can produce similar adaptations in coexisting biological entities at different levels of organization.

Discipline: Ethologie

Mots clés: *Cerapachys biroi*, insectes sociaux, différenciation, caste, division du travail, seuil de réponse, comportement, parthénogenèse thélytoque, hydrocarbures cuticulaires, cancer social, polyphenisme, fourmis, conflit et coopération

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