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Mechanisms for the Evolution of Superorganismality in Ants

Vikram Chandra

MECHANISMS FOR THE EVOLUTION OF SUPERORGANISMALITY IN ANTS

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Vikram Chandra
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Vikram Chandra, Ph.D.
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Ant colonies appear to behave as superorganisms; they exhibit very high levels of within-colony cooperation, and very low levels of within-colony conflict. The evolution of such superorganismality has occurred multiple times across the animal phylogeny, and indeed, origins of multicellularity represent the same evolutionary process. Understanding the origin and elaboration of superorganismality is a major focus of research in evolutionary biology. Although much is known about the ultimate factors that permit the evolution and persistence of superorganisms, we know relatively little about how they evolve. One limiting factor to the study of superorganismality is the difficulty of conducting manipulative experiments in social insect colonies. Recent work on establishing the clonal raider ant, *Ooceraea biroi*, as a tractable laboratory model, has helped alleviate this difficulty.

In this dissertation, I study the proximate evolution of superorganismality in ants. Using focussed mechanistic experiments in *O. biroi*, in combination with comparative work from other ant species, I study three major aspects of ant social behaviour that provide insight into the origin, maintenance, and elaboration of superorganismality.

First, I ask how ants evolved to live in colonies, and how they evolved a reproductive division of labour. A comparative transcriptomic screen across the ant phylogeny, combined with experimental manipulations in *O. biroi*, finds that reproductive ants have higher insulin levels than their non-reproductive nestmates, and that this likely regulates the reproductive division of labour. Using these data, as well as studies of the idiosyncrasies of *O. biroi*'s life history, I propose a mechanism for the evolution of the first colonies. It is possible that similar mechanisms underlie the

evolution of reproductive division of labour in other superorganisms, and of germ-soma separation in nascent multicellular individuals.

Second, I ask how ant workers assess colony hunger to regulate their foraging behaviour. I find that workers use larval signals, but not their own nutritional states, to decide how much to forage. In contrast, they use their nutritional states, but not larval signals, to decide how much to eat, suggesting that in at least some ant species, foraging and feeding have been decoupled. This evolution of colony-level foraging regulation has occurred convergently in hymenopteran superorganisms, and is analogous to the evolution of centralised regulation of foraging behaviour in multicellular animals.

Finally, I ask how an iconic collective foraging behaviour – the mass raids of army ants – evolved. I find that *O. biroi*, a relative of army ants, forages collectively in group raids, that these are ancestral to the mass raids of army ants, and that the transition from group to mass raiding correlates with expansion in colony size. I propose that the scaling effects of increasing colony size explain this transition. It is possible that similar principles underlie the evolution of disparate collective behaviours in other animal groups and among cells within developing animals.

Together, these studies illuminate the life history of *O. biroi*, and suggest mechanisms for the evolution of core aspects of cooperative behaviour in ant colonies. I draw comparisons to the evolution of superorganismality in other lineages, as well as to the evolution of multicellularity. I suggest that there may be additional similarities in the proximate evolutionary trajectories of superorganismality and multicellularity.

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CHAPTER 1: INTRODUCTION

1.1: The ant colony as an organism

Ants are one of the most successful lineages of animals (Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009), and their most conspicuous feature is that they live in colonies. These colonies - like those of termites, honeybees, and hornets - are fundamentally different from 'colonies' of guillemots, gulls, or marmosets. Ant colonies are not simply groups of animals living in proximity. Instead, the colonies behave like single organisms. The ants in a colony clearly share a home – a nest that they typically construct and maintain themselves. Their nests have skeletons with intricate anatomies (often built from muddy tunnels or hollow twigs, or sometimes the linked bodies of worker ants), with designated rubbish heaps and flood chambers, and a carefully controlled internal climate (Baudier and O'Donnell, 2016; Hölldobler and Wilson, 2009; Ireland and Garnier, 2018). Most ants in a typical colony are workers; the daughters of a queen who monopolises reproduction. To a first approximation, the queen is an ungainly, walking bag of ovaries. The workers do not normally reproduce, and instead perform all the other tasks. They tend the queen's brood (eggs, larvae, and pupae) in their nest almost indiscriminately, while simultaneously scrupulously and violently rejecting both con- and hetero-specific outsiders – in effect behaving as a colony-level immune system.

When the workers forage, they seek and retrieve food not just for themselves, but for the whole colony. Some ant species, like fire ants (*Solenopsis invicta*), have the colony-level equivalent of a circulatory system, with workers regurgitating liquid food into each other and into the larvae to distribute nutrition efficiently through the colony (Cassill and

Tschinkel, 1999). This cooperation is often evident in the act of foraging too. For instance, crazy ants (*Paratrechina longicornis*) work as teams that lift large food items, with a rotating band of workers that steers the food homewards (Feinerman *et al.*, 2018). More famously, army ants forage in groups of many thousands or millions of workers that collectively prey on colonies of other species of ants and wasps. Their feats of coordination have traditionally been described in a surfeit of military metaphors: these "Huns and Tartars of the insect world" forage in "raids" composed of columns of workers (including "soldiers") "marching" outwards from a "bivouac", performing "flanking movements" to surround and overwhelm their prey (Gotwald, 1995; Schneirla, 1971; Wheeler, 1910). Much of the coordination required for such collective behaviour is orchestrated by a complex system of chemical communication: workers lay and reinforce trail pheromones for their nestmates to find their way home, they warn and attract each other with alarm and recruitment pheromones, they adjust their foraging effort based on signals from the larvae, and so on.

In summary, in virtually every aspect of their behaviour, ant colonies appear to behave as single, coherent, unified wholes – as individuals[1]. Like organisms, ant colonies also behave as though their components are "contrived for the *same* purpose"[2] (Gardner, 2009). This idea is at least a century old. One of its clearest early[3] expressions came from

[1] Note that this does not mean there is no conflict within the individual.

[2] Emphasis in original

[3] Wheeler was not the first to note that ant colonies behaved as coherent entities. (Weismann, 1893) "[I]n the case of the ant colony, or rather state, the barren individuals or organs are metamorphosed only by selection of the germ-plasm from which the whole state proceeds. In respect of selection the whole state behaves as a single animal; the state is selected, not the single individuals; and the various forms behave exactly like the parts of one individual in the course of ordinary selection."

William Morton Wheeler (Wheeler, 1910; Wheeler, 1911), most explicitly in a lecture entitled "The ant-colony as an organism". As Wheeler recognised, and as the subsequent century of research has clarified, the similarities between the organisation of social insect colonies and solitary organisms are not simply superficial, nor simply a poetic metaphor. A strong argument can be made that they are outcomes of the *same* evolutionary process; that they are both true evolutionary individuals, albeit at different levels of organisation.

Ant colonies are not unique in the extent of their cooperation. Many corbiculate bee and vespid wasp species (especially honeybees and hornets), all termites, some snapping shrimp, an ambrosia beetle, naked mole rats, and siphonophores exhibit equivalent levels of collective coherence; these colonies all appear to be individuals. And similar – although less extreme - levels of cooperation can be seen in yet other social insects too, including in paper wasps, halictine bees, burying beetles, and so on (Davies *et al.*, 2013; Korb and Heinze, 2016; Wilson, 1971; Wilson, 2008).

1.2: The evolution of colony-level individuality is typically a fraternal major transition

The history of life on earth is marked by repeated transitions in the level of biological organisation at which the individual exists. In the most recent common ancestor of extant life forms, the individual was a single cell. At the origin of eukaryotes, one bacterium was engulfed by another – an endosymbiotic event that resulted in mitochondria. Within the eukaryotes, multicellularity has evolved often (Buss, 1987; Grosberg and Strathmann, 2007), and within multicellular animals, colony-level individuality has also evolved repeatedly. These 'major evolutionary transitions'

(Maynard Smith and Szathmáry, 1995) are responsible for the organisation of life as we know it. They occur repeatedly, sequentially, and recursively. David Queller (1997) distinguishes between two kinds of major evolutionary transitions. Individuals may arise from the collaboration of related or unrelated entities, and their organisation and behaviour can be predicted fairly well from the nature of their alliance.

Individuals that arise from the cooperation[4] of unlike parts – typically of multiple species – usually have a division of labour of their non-reproductive tasks. These instances of mutual benefit[5] occur from combining two entities that have independently evolved distinct specialisations. Because such individuals do not have a reproductive division of labour, Queller calls their alliance 'egalitarian'. The members of these egalitarian individuals are not typically interchangeable, and they are usually evenly represented in the offspring[6]. The endosymbiosis of mitochondria within early eukaryotes was clearly an egalitarian transition, as was the association of distinct genes to form chromosomes and genomes (Bourke, 2011). More esoteric egalitarian unions exist. For example, leafcutter ant workers farm a symbiotic fungus, which they then feed from (Hölldobler and Wilson, 2011; Schultz and Brady, 2008). They nourish the fungus with pulp from leaves that they harvest, but do not themselves eat. The fungus benefits from a protected environment inside the ant nest, and from being propagated by the ants – it is usually inherited vertically through generations of ant colonies, travelling in specialised morphological structures of new ant queens as they leave their natal nest to

[4] Cooperation is formally defined as a behaviour in which the recipient gains a direct fitness benefit (i.e., in lifetime number of offspring). The actor may or may not benefit, leading to two forms of cooperation: mutual benefit and altruism, defined in the footnotes below (Davies *et al.*, 2013; West *et al.*, 2007a).

[5] Mutual benefit is properly defined as an interaction between two entities that benefits the direct fitness of both (Davies *et al.*, 2013; West *et al.*, 2007a).

[6] Of course, some exceptions exist – for instance, the evolution of sex and sexual reproduction is egalitarian in that it is an instance of mutual benefit. However, there is usually no division of labour, and sexual conflict often centres around fair representation in the offspring.

found their own (Hölldobler and Wilson, 2011; Howe *et al.*, 2019; Weber, 1972). This is an alliance with a high level of interdependence; the ants subsist entirely on the fungi. Leafcutter ant colonies and their fungi also have conflicts that require resolution. For instance, the ants cannot eat and do not benefit from the reproductive mushrooms that the fungus develops, and so suppress the growth of mushrooms in favour of 'somatic' hyphae (Mueller, 2002; Poulsen and Boomsma, 2005). However, this conflict may not always be resolved (Shik *et al.*, 2016). In general, egalitarian individuals often evolve to control internal conflicts, since their members do not necessarily have aligned fitness interests (Bourke, 2011). Moreover, since these unions can in principle evolve from nearly any pair (or set) of interacting partners, and since their conflicts can only be resolved through coercion (which functions differently in different contexts), their evolution is often relatively idiosyncratic.

Individuals that arise from the collaboration of like parts are rather different. The parts are highly related (typically full siblings, clones, or offspring), and usually develop an extreme reproductive division of labour, with distinct reproductive and non-reproductive parts. For obvious reasons, Queller calls such individuals 'fraternal'. These transitions require altruism[7]; i.e., the non-reproductive members of the union must 'surrender' their reproductive potential, instead helping the other member(s) reproduce. These non-reproductive members are, of course, not directly represented in the individual's offspring. Most ant and termite colonies are clearly fraternal individuals, as are many other social insect colonies, as well as naked mole rats, siphonophores, etc. So are the vast majority of solitary animals, with their strict germ-soma separation. But other groups – including paper wasps (Hunt, 2007), halictine bees (Michener, 2007), salps

[7] Altruism is strictly defined as an interaction in which an actor pays a direct fitness cost, and a recipient gains a direct fitness benefit (Davies *et al.*, 2013; Hamilton, 1964a; Hamilton, 1964b; West *et al.*, 2007a).

(Huxley, 1852), choanoflagellates (Fairclough *et al.*, 2010), and dandelions (Janzen, 1977) - are less clear-cut. To decide systematically whether a group of organisms qualifies as a fraternal individual, one must first define individuality explicitly.

1.3: The nature and semantics of individuality

Unsurprisingly, the attempt to define individuality has been fraught with controversy, and a perusal of the sociobiology literature of the last several decades reveals a variety of often contradictory ideas (reviewed by Boomsma and Gawne (2018), and Strassmann and Queller (2010)). These disagreements have a long history. For instance, in a lecture preceding Darwin's Origin, Thomas Huxley listed a number of competing concepts of individuality, before suggesting that the individual is the sum of the forms produced by a single egg (Huxley, 1852). Since then, these disagreements – especially in the social insect literature - have continued to focus on the extent to which the individuality of colonies is simply metaphorical, the extent to which it is useful, the extent to which it may be unambiguously defined, and the extent to which such definitions are evolutionarily meaningful. Boomsma and Gawne (2018) extensively document the history of this debate in the social insects, where many terms have been constructed for transitions toward colony-level individuality (the most popular – aside from individuality (Buss, 1987; Janzen, 1977) - being eusociality (Batra, 1966; Wilson, 1971), organismality (Queller and Strassmann, 2009; Strassmann and Queller, 2010), and superorganismality (Wheeler, 1928)).

To briefly summarise this tangled web of terminology, individuality, eusociality, organismality, and superorganismality refer to roughly the same phenomenon – that of

groups that appear contrived for a common purpose. However, these terms vary in their sensitivity, their specificity, and – sometimes - the level of organisation at which they are applied. In essence, the individual is the answer to the question: 'for whose benefit is this adaptation?' More specifically, it is usually seen as the highest level of organisation at which a meaningful answer to this question exists. It is the primary phenotypic unit of natural selection (Bourke, 2011; Buss, 1987). Organismality is sometimes used as a synonym for individuality. And as Queller (1997) emphasised, we "designate something as an organism [i.e., an individual], not because it is *n* steps up on the ladder of life, but because it is a consolidated unit of design, the focal point where lines of adaptation converge. It is where history has conspired to make between-unit selection efficacious and within-unit selection impotent".

Eusociality is both more and less restrictive: it is typically defined as a life history that includes reproductive division of labour, cooperative brood care, and overlapping generations (Crespi and Yanega, 1995; Wilson, 1971). This includes many groups that are undeniably fraternal individuals, but it excludes fraternal individuals at the level of multicellularity, individuals that do not consist of arthropod or vertebrate groups, and individuals that evolved via an egalitarian transition. Moreover, strict application of the definition would include a number of groups that biologists do not usually call individuals of any kind.

Superorganismality is more nebulous, with multiple authors defining it in multiple, mutually incompatible ways. Following Wheeler, Boomsma and Gawne consider colonies of (most) ants and honeybees (and a few other social insects) to be superorganisms. For an entity to be a superorganism, they say, it must have an extreme reproductive division of labour, with permanently and morphologically differentiated

reproductive and sterile castes. Specifically, they say it is "defined by permanent membership of each colony member to a single morphologically distinct adult caste that is fixed for life during early individual development". Gardner and Grafen (2009) argue instead that sterile worker castes are neither necessary nor sufficient for superorganismality, and that a superorganism is "a group that wields adaptations in its own right". Hölldobler and Wilson, authors of a 2009 book titled 'The Superorganism' visualise the concept as "a colony with many of the attributes of an organism but one step up from organisms in the hierarchy of biological organisation. The basic elements of the superorganism are not cells and tissues but closely cooperating animals". They go on to provide two conflicting definitions of superorganismality. One view, that of Wilson, is that superorganismality is synonymous with eusociality. Another, that of Hölldobler, is that "the term *superorganism* may be applied only to colonies of an advanced state of eusociality, in which interindividual conflict for reproductive privilege is diminished and the worker caste is selected to maximise colony efficiency in intercolony competition"[8]. These disagreements are largely the result of differences in the desired specificity of the definition. Superorganismality, according to all these definitions, is some sort of colony-level individuality.

Rather than choosing a side, I see this debate instead as a consequence of the "tyranny of the discontinuous mind" (Dawkins, 2011). Obviously, sometimes, choosing a single level of individuality (or superorganismality) is difficult. For example, many paper wasp workers spend considerable fractions of their lives in their mother's nest, rearing their siblings instead of reproducing themselves. However, they are not

[8] Emphasis in original

obligately sterile, sometimes replacing their mother as the dominant reproductive, or leaving to found their own nests. Yet, these wasp colonies behave largely coherently (Gadagkar, 2016; Hunt, 2007; Sherman *et al.*, 1995). Sociobiologists frequently disagree about whether such groups qualify as eusocial, or as individuals (Boomsma and Gawne, 2018; Bourke, 2011; Korb and Heinze, 2016; Sherman *et al.*, 1995). Indeed, the two authors of the book 'The Superorganism' cannot agree between themselves which ant colonies deserve to be classified as superorganisms. These examples, with countless others like them, suggest that individuality, eusociality, and superorganismality are all continuous rather than binary. Groups can have variable degrees of individuality (or superorganismality, or eusociality). At least in principle, this degree could be quantified by the ratio of within- to between-group conflict (Queller, 1997). Another way to view this is that "eusocial societies are 'superorganismal' in some contexts but not others" (Bourke, 2011).

In this dissertation, for consistency with the literature, I will use the terms eusociality and individuality as they are conventionally defined, and I will use the term superorganismality somewhat in the sense of Hölldobler (i.e., as a high degree of colony-level individuality). I will avoid the term organismality.

1.4: The trajectories of fraternal transitions are constrained

A majority of fraternal individuals develop and evolve along strikingly similar trajectories, despite having originated independently, in distant neighbourhoods of the phylogeny. They virtually always begin with a strong genetic bottleneck, in both evolutionary and developmental time. Animals, plants, and other multicellular groups

are typically founded in development by a single-celled zygote, and they evolved from an ancestral single-celled protist. Similarly, ant and corbiculate bee colonies and other superorganisms are typically founded by a single, monogamous reproductive queen[9], and evolved from an ancestral solitary wasp (Boomsma, 2009; Boomsma and Gawne, 2018; Boomsma *et al.*, 2011; Davies *et al.*, 2013; Hughes *et al.*, 2008; Korb and Heinze, 2016; Linksvayer, 2009). This initial life history – of daughters failing to disperse, and instead remaining with (and helping) their mothers – is sometimes called subsociality (Davies *et al.*, 2013). The term is typically used in conjunction with colonial (or generally social) animals, but it could reasonably be applied to colonies of cells too.

Fledgling individuals are typically small in size, with few members. They generally develop strong reproductive division of labour (over evolutionary time) and grow in size (over evolutionary and developmental time). The former manifests as a dedicated germline cell lineage or queen that monopolises meiosis and individual reproduction. The latter permits the non-reproductive components of this individual to also specialise over evolutionary time, dividing their labour repeatedly to form new cell types or worker castes. The individual evolves elaborate communication systems for its specialist components to coordinate their behaviour. These communication systems – akin to colony-level nervous systems - are typically chemical in the social insects, with pheromones serving analogous roles to hormones and neurotransmitters in multicellular individuals (Friedman *et al.*, 2020)[10].

[9] Although exceptions exist, they are derived – they do not represent the ancestral superorganism, and their evolution from a bottlenecking ancestor requires additional forces (Boomsma, 2009; Boomsma *et al.*, 2011).

[10] Note that these trajectories can only be inferred in hindsight; I do not mean to imply that any evolution of cooperative behaviour must necessarily be followed by the evolution of eusociality or full individuality, and indeed, many groups appear to be stable at 'intermediate' levels of sociality.

These similarities in the evolutionary trajectories of fraternal individuals are not trivial; they need not – at least in principle - have always happened this way. Indeed, a few decades ago, many biologists believed that colony-level individuality may have evolved not via subsociality, but instead via an alternative route called parasociality or semisociality (Brockmann and Dawkins, 1979; Davies *et al.*, 2013; Korb and Heinze, 2016; West-Eberhard, 1975; West-Eberhard, 1987a; West-Eberhard, 1996). Here, multiple adults would aggregate after leaving their mothers. These adults may or may not have been related. Many extant social insects – such as allodapine bees and some paper wasps - are 'parasocial' in that they live in groups that were formed by the aggregation of adults. Moreover, some ant species - including some harvester ants, fire ants, and carpenter ants - also sometimes found colonies cooperatively, with multiple reproductive queens aggregating to jointly construct a nest (Bernasconi and Strassmann, 1999; Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009). However, there is no evidence that any fraternal evolutionary transition – either in the social insects or elsewhere on the tree of life – occurred via parasociality (Davies *et al.*, 2013; Hughes *et al.*, 2008; Johnson and Linksvayer, 2010; Korb and Heinze, 2016; Linksvayer, 2009).

These constraints may in principle be explained partly by mechanistic, developmental features[11] of the ancestral organisms whose descendants underwent a fraternal evolutionary transition. However, it is unlikely that the same 'developmental' constraints could explain the broad similarities of these trajectories across multiple levels of biological organisation. Instead, these similarities are largely explained by ultimate[12]

[11] i.e., in this view, fraternal transitions would happen *this* way because there is no attainable alternative in the mutation space. Physical constraints limit the space of possible solutions.

[12] i.e., answers to questions of *why* a trait is the way it is, as opposed to possible alternatives (Gardner, 2013; Mayr, 1961; Tinbergen, 1963). The proximate evolutionary question asks *how* a trait has evolved. These questions are complementary, not conflicting, and a full understanding of the trait requires both.

factors. Specifically, the only evolutionarily stable form of altruism is one that satisfies Hamilton's rule; i.e., the altruistic actor must gain an indirect fitness benefit[13] that is greater than the (direct fitness) cost of her actions. The simplest way to maximise indirect fitness benefits is to only help close relatives, and the simplest way to help close relatives is not to leave them. The theory of inclusive fitness (Dawkins, 1976; Dawkins, 1982; Hamilton, 1963; Hamilton, 1964a; Hamilton, 1964b; Smith, 1964) formalises this logic, and it explains why subsociality so often precedes colony-level individuality while parasociality does not. These evolutionary considerations explain many aspects of the origin and maintenance of individuality. They explain why colonies are able to exist at all, and why similar conflicts and conflict-resolution mechanisms within colonies have evolved convergently across ants, bees, and wasps (Bourke, 2011; Davies *et al.*, 2013). The evolution of such altruism has fascinated sociobiologists for decades, and they have developed a vast body of work to explain it (Bourke, 2011; Bourke, 2014; Buss, 1987; Davies *et al.*, 2013; Field, 2005; Field and Brace, 2004; Foster, 2009; Foster, 2011; Gardner and Grafen, 2009; Grafen, 1984; Grafen, 2006; Grafen, 2007; Hamilton, 1963; Hamilton, 1964a; Hamilton, 1964b; Maynard Smith and Szathmáry, 1995; Queller, 1994; Smith, 1964; West *et al.*, 2007a; West *et al.*, 2007b). In summary, much is known about why fraternal evolutionary transitions happen in the way they do.

In this dissertation, I shall not discuss these ultimate explanations further, and shall instead focus on the proximate evolution of superorganismality, whose mechanisms are rather less well understood. In the next section, I shall discuss the proximate origins

[13] i.e., the direct benefit (usually measured as the lifetime number of offspring) to a recipient, scaled by the coefficient of relatedness between actor and recipient (Davies *et al.*, 2013; West *et al.*, 2007a).

and elaboration of this lifestyle in the most conspicuously superorganismal lineage of animals – the ants.

1.5: The origin of superorganismality in the ants

There are roughly 14,000 described species of ants, and they live on every continent save Antarctica. These species vary enormously in all aspects of their life history. Some ant species are largely vegetarian, while others – like many army ants and their relatives – are obligate predators of ants. Some are arboreal, others subterranean; some live in colonies of tens of millions of workers, while others live in colonies of no more than a dozen. Although I cannot hope to do justice to the breadth of diversity among ants, I will summarise the current (coarse) view of the life history of the most recent common ancestor of extant ants, and in the next section I will touch briefly on important subsequent transitions in life history.

The earliest ants lived – approximately 140 million years ago - in relatively small colonies consisting of a monogamous queen and a few hundred of her daughters (Burchill and Moreau, 2016; Hughes *et al.*, 2008; Moreau *et al.*, 2006; Ward, 2014). The queen was winged at eclosion, while the workers were not. The ants probably had an expanded olfactory repertoire (McKenzie *et al.*, 2016). The colonies were ground-nesting, and were predators of small arthropods on the forest floor (Lanan, 2014; Nelsen *et al.*, 2018). Workers probably foraged independently, without substantial coordination or communication. They tended the brood – i.e. the eggs, larvae, and pupae – actively. They probably had an age-based division of labour (Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009). Their nests may have been relatively simple, perhaps consisting of a single chamber that housed the queen, the workers, and all the brood, with one or a few

tunnels that served as entrances. The queens were not much larger than the workers, who were capable of reproducing (i.e., laying unfertilised, haploid eggs that could grow into males) but probably did not mate (Hölldobler and Wilson, 1990; Hughes *et al.*, 2008; Peeters and Ito, 2001).

Each of these colonies was founded by a single, 'semi-claustral' queen. When she had eclosed in her mother's nest, she would fly out, mate once, and then dig a burrow in the ground. She would then detach her wings, lay a batch of eggs, and rear the resulting larvae to adulthood single-handedly, leaving the nest regularly to forage for them (Hölldobler and Wilson, 2009; Peeters, 2010). Her first batch of brood were probably small and underfed workers, who then took over brood care while she focussed exclusively on reproduction. Over time, the colony would grow in size until it reached its equilibrium of a few hundred workers. The colony would then begin to rear new reproductive offspring – queens and males – who would fly out to repeat this cycle.

How did the first colonies evolve? The ancestry of the first eusocial ant colonies is somewhat obscured by the phylogenetic position of the ants. The closest living relatives of the ants are the members of the Apoidea (Branstetter *et al.*, 2017; Peters *et al.*, 2017; Sann *et al.*, 2018). These include all bees, as well as a number of lineages of 'sphecoid' wasps. The common ancestor of the Apoidea was likely a parasitoid wasp similar to many of its 'sphecoid' members. The closest relatives to the common ancestor of ants and Apoidea are also parasitoid wasps, similar to cockroach wasps and other 'sphecoids'. Given the phylogeny, this is very likely to also have been the solitary ancestral state for the ants. This ancestral parasitoid could likely dig a burrow, and then hunt and paralyse a small arthropod (such as a cockroach or cricket). She would then transport it into the burrow, lay an egg on or in it, seal the burrow and leave. The egg would hatch into a larva, and

would feed on the arthropod until it had grown to pupation. It would eventually leave the burrow as an adult to repeat this life cycle (Carey, 2001; Hunt, 2007; O'Neill, 2001).

Clearly, multiple changes must have occurred for this life history to turn into that of the first ant colonies. First, the wasps must have evolved increased brood care. This may have happened by switching the order of events in the ancestral parasitoid life history: i.e., instead of finding a host, leading it to a burrow, and laying an egg in it, the wasps may have evolved to first construct a burrow, then lay an egg, and finally provision the eggs with food (arthropods they have incapacitated or killed), before leaving to repeat this cycle. This life history, together with that of the parasitoids, is called 'mass provisioning', and is widespread in the Hymenoptera (Field, 2005; Hunt, 2007; Michener, 2007). Next, the wasps must have evolved active, continuous brood care. This may have happened partly by another switch in the order of events in their life history: they would now provision the offspring with food only after it had hatched into a larva. The wasps must also have evolved to perform this provisioning actively, for the duration of larval growth. Exactly how and why they first evolved this ability remains unknown, but this lifestyle (known either as 'progressive provisioning' or as 'subsociality'[14]) has evolved a number of times in the Hymenoptera (Hines *et al.*, 2007; Hunt, 2007; Kelstrup *et al.*, 2018; West-Eberhard, 1978; West-Eberhard, 1987a; West-Eberhard, 1987b; West-Eberhard, 2005; Wilson, 2008).

Progressive provisioners typically undergo reproductive cycles. The wasp digs a burrow or builds a nest, and lays an egg inside. Then, when the egg hatches into a larva, the wasp ceases reproduction, and instead cares for the larva, leaving the nest every day

[14] Note that this is a subtly different usage of the term than in the context of the abstract nature of fraternal evolutionary transitions (Section 1.4).

to hunt insects for it. In the digger wasp *Ammophila campestris*, adult wasps often build and maintain up to four nests (each with a single larva) simultaneously. Every morning, the wasp inspects each larva, assesses how much food it needs, and then provisions each nest accordingly (Tinbergen, 1984). This shows that in at least some progressive provisioners, there is communication between adult and larva; a trait that must have evolved after the evolution of progressive provisioning. Once the larvae have grown to pupation, the adult often leaves to build a new burrow and repeat this cycle all over again. Sociobiologists consider this 'subsocial' life cycle to precede eusociality – and superorganismality – in the ants (as well as in paper wasps, and some other social insects). This was first suggested by Wheeler (1910), and was later explicitly enunciated by West-Eberhard (1987a).

How does eusociality evolve from progressive provisioning? The most important change that must have occurred is that most daughters of the wasp that founds the nest must have remained with her, must not have mated or reproduced, and must have spent their lives helping her raise her other offspring (i.e., their siblings). In other words, the reproductive cycle of subsocial progressive provisioning wasps must have been somewhat 'broken', with the founding wasp (or ant) 'fixed' in the reproductive phase of the ancestral cycle (i.e., as an obligate egg-layer) and most of her daughters 'fixed' in the brood care phase (i.e., as obligate non-reproductive 'workers'). The ancestral reproductive cycle was not necessarily lost entirely; as mentioned above, the queens of extant eusocial ant colonies appear to 'recapitulate' some version of this cycle when they first found their nests. However, the mechanisms by which such an ancestral reproductive cycle evolved into eusociality remain largely unknown, and until recently, have been difficult to study.

1.6: The maintenance and elaboration of superorganismality within the ants

After ants first evolved to live in colonies, they evolved further adaptations that appeared to increase their individuality. The strongest evidence for this is the repeated evolution of obligate sterility in ant workers. Although the earliest ant workers – like those of many extant species - were capable of reproduction, some ant lineages have evolved adaptations to prevent this (Boomsma and Gawne, 2018). Most strikingly, fire ant workers do not develop ovaries, which makes reproduction impossible. In other lineages, ant workers have gained ovarian modifications that prevent viable eggs from being laid (Khila and Abouheif, 2010). Such adaptations minimise the potential for reproductive conflict; they help maintain individuality, and sometimes, such as when they widen the reproductive asymmetry within a colony, they are arguably an increase in the individuality – and superorganismality – of ant colonies.

Traits that are not directly related to reproduction are harder to associate with degrees of superorganismality. But at least superficially, the extent of coordination within ant colonies appears to have increased over evolutionary time. One example of this is the multiple independent expansions in colony size in the subfamilies Ponerinae, Dorylinae, Formicinae, and Myrmicinae (Burchill and Moreau, 2016). Concordant with this expansion in colony size comes an increase in the extent of their division of labour. Many ant species have independently gained additional worker castes, and larger colonies have more specialised workers (Dornhaus *et al.*, 2012; Ferguson-Gow *et al.*, 2014; Gautrais *et al.*, 2002; Jeanson *et al.*, 2007; Trible and Kronauer, 2017; Ulrich *et al.*, 2018). The clade of non-doryline "formicoid" ants evolved to be substantially vegetarian rather than (or in

addition to) predatory, and a major part of their diet is nectar. Concordant with this, they have evolved to rely heavily on trophallaxis to distribute such liquid food through the colony – the equivalent of a colony-level circulatory system (Greenwald *et al.*, 2018; Hölldobler and Wilson, 2009; Leboeuf *et al.*, 2016). Many other such examples can be adduced from the abundant literature of ant natural history (and see also Section 1.1). Indeed, entire books have been written about the complex innovations that specific lineages of ants have evolved (Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009; Hölldobler and Wilson, 2011; Rettenmeyer, 1963; Schneirla, 1971). In summary, these innovations help reinforce and maintain eusociality as a life history. Moreover, they represent elaborations of the eusocial ancestral state into something more unambiguously superorganismal.

However, although the field boasts an exceptional record of both expansive and rigorous natural history, as well as a long record of theoretical work explaining why natural selection favours specific forms of cooperation (Hölldobler and Wilson, 1990; Oster and Wilson, 1978), we do not yet know much about how these colony-level behaviours are regulated, or how they evolved.

1.7: The difficulties of studying ant colonies

Ant colonies – like other social insect colonies, with the partial exception of honeybees - are typically very difficult to maintain in laboratory conditions for substantial periods of time. New queens and males usually cannot be induced to undertake mating flights indoors, which prohibits colonies from reproducing. Ant colonies often have very long generation times – they typically grow for several years

before beginning to make new reproductive offspring, which seriously restricts the feasibility of experiments and observations of sociogenesis (i.e. colony growth and development). The workers within a colony vary in a number of ways: in age, genotype, physiology, and experience, and each of these factors affects their behaviour (Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009; Ulrich *et al.*, 2020). These factors are hard to control in most ant colonies. Studying reproductive division of labour in most ant species is difficult for the slightly different reason that reproductive caste is usually irreversibly determined during development (Kronauer and Libbrecht, 2018). Moreover, pharmacological and genetic manipulations are challenging to do rigorously, especially with high numbers of replicates. Other difficulties, including in observing the inside of most ant colonies, in tracking individual ants, and in inducing ant colonies to perform their most impressive feats of cooperation under cameras in the lab, also contribute to hindering the experimental study of social behaviour and its evolution.

1.8: A solution: the experimentally tractable clonal raider ant

For the last several years, the lab of my advisor, Daniel Kronauer, has worked to establish one ant species as a lab model. This is the clonal raider ant, *Ooceraea biroi* (subfamily Dorylinae). Unlike most other ants, O. *biroi* colonies can be kept and propagated in the lab indefinitely. The species has secondarily lost queens, and instead only has workers. The workers are blind. They are also totipotent; each worker can do essentially all tasks, including reproduction. The workers are all female, and they reproduce clonally by thelytokous parthenogenesis (Ravary and Jaisson, 2002; Ravary and Jaisson, 2004; Ravary *et al.*, 2006; Tsuji and Yamauchi, 1995).

Despite this unusual biology, *O. biroi* is eusocial. Workers display cooperative brood care, colonies contain overlapping generations of adults, and reproductive asymmetry exists within colonies (Teseo *et al.*, 2013). Indeed, because *O. biroi* colonies are clonal, have no predicted conflict within them, have extensive within-colony cooperation, and are almost completely unable to gain fitness in solitude, they are also unambiguously individuals. Whether they qualify as superorganisms or not would, in this case, appear to depend entirely on one's favoured definition, but it is clear that the species meets the fundamental requirements to be at least partially superorganismal.

O. biroi colonies also undergo stereotyped reproductive cycles. The workers all lay eggs in synchrony. The eggs then develop in synchrony, hatching into larvae within a day of each other. The larvae then suppress adult reproduction and induce brood care. The workers forage, nurse, and do other non-reproductive tasks until the larvae have grown to pupation, at which point this suppression is released and the workers activate their ovaries to lay another batch of eggs. Meanwhile, the pupae develop and eclose as adults in synchrony too (Oxley *et al.*, 2014; Ravary and Jaisson, 2002; Ravary *et al.*, 2006). This cycle is fascinating mechanistically (one wishes to ask how larvae control so much of adult behaviour) and evolutionarily (it resembles the reproductive cycle of subsocial wasps, and perhaps also that of the subsocial ancestor of the ants). Moreover, it is also experimentally convenient; it allows us to control the genotypes, ages, and rearing environments of workers with unique precision. The colonies are also highly modular, and can be assembled experimentally from nearly arbitrary groups of *O. biroi* workers.

The lab has developed efficient techniques for the establishment of experimental colonies, for the delivery of pharmacological and other compounds to ants at various life stages, and for the high-throughput automated behavioural tracking of individually

tagged workers over entire colony cycles (Gal *et al.*, 2020; Ulrich *et al.*, 2018). The lab has also generated high quality genomes and attendant genomic resources for *O. biroi* (Libbrecht *et al.*, 2016; Libbrecht *et al.*, 2018; McKenzie and Kronauer, 2018; McKenzie *et al.*, 2014; McKenzie *et al.*, 2016; Oxley *et al.*, 2014). Moreover, recent advances in our understanding of the phylogeny of ants have substantially clarified both the somewhat recent (Borowiec, 2019) and more ancient (Borowiec *et al.*, 2019) history of *O. biroi*, allowing us to make comparisons across a large range of evolutionary timescales.

1.9: Dissertation outline

In this dissertation, I will make use of all these technological and methodological advances to study the mechanisms and evolution of superorganismality in the ants. Throughout, I use focussed experimental work in *O. biroi*, with some comparative analyses – of varying degrees of formality - of its relatives to study mechanisms for and the evolution of traits that illustrate the origin, maintenance, and elaboration of superorganismality in the ants.

In Chapter 2, I use experimental investigations of *O. biroi*'s unusual life history, with a comparative transcriptomic screen across the ant phylogeny, to ask how an ancestral subsocial reproductive cycle could evolve into the fixed reproductive division of labour that characterises eusocial – and superorganismal - ant colonies. I propose a mechanism for the origin of the first colonies.

Once eusocial (or somewhat superorganismal) colonies have evolved, they must evolve adaptations to behave as a unified whole; to coordinate the behaviour of their members, and to maintain colony-level homeostasis. In Chapter 3, I study an important

aspect of colony-level cooperation: the social regulation of foraging. Using experimental manipulations of *O. biroi* workers' nutritional states and social environment, I ask how workers maintain the colony's nutritional homeostasis. Specifically, I ask to what extent their nutritional states – as opposed to social signals – regulate their foraging and their feeding behaviour.

Ant colonies perform some of the most iconic collective behaviours known to biologists. Many of these behaviours are only possible because they function as integrated individuals, with negligible within-colony conflict. In Chapter 4, using automated tracking, I quantitatively describe the foraging behaviour of *O. biroi* for the first time. Drawing from the wealth of natural history observations of its relatives, I find that it represents the ancestral state to a superficially rather different (and rather dramatic) form of foraging behaviour: the mass raids of army ants. I propose a mechanism for the evolution of army ant raids.

In Chapter 5, I discuss the implications of these findings for our understanding of the life history of *O. biroi*, and more generally, for the evolution of superorganismality – and individuality - in the ants, across the social insects, and across multiple levels of biological organisation.

CHAPTER 2: SOCIAL REGULATION OF INSULIN SIGNALLING AND THE EVOLUTION OF EUSOCIALITY IN ANTS

The fundamental feature that separates superorganisms from other groups is their extreme reproductive division of labour. This must have evolved first; before they developed the sophisticated communication systems or worker castes or adaptations for complex collective behaviour that make them conspicuous today. Understanding the origin of their reproductive division of labour would thus explain how they first evolved to live in colonies. In this chapter, I shall refer to the social state as 'eusociality' rather than superorganismality, because this more properly includes early ant colonies.

As discussed in the previous chapter, eusociality in ants and in many other Hymenoptera likely evolved from a subsocial state in which a female wasp would lay an egg and then care for the resulting larva until pupation (Hunt, 2007; Wheeler, 1910; Wilson, 1971). Such brood care may have been induced by larval signals, and observations of extant subsocial wasps are consistent with this scenario (Field, 2005; Hunt, 2007; Wheeler, 1910). This temporal reproductive and behavioural plasticity was then modified into a fixed reproductive asymmetry between queens and workers in eusocial colonies (West-Eberhard, 1987a; Wheeler, 1910). This raises three important mechanistic questions: first, how are subsocial reproductive cycles regulated? Second, how is the eusocial reproductive division of labour regulated, i.e. what allows queens to lay eggs but prevents workers from doing so? And third, what is the evolutionary trajectory that gave rise to fixed eusocial division of labour from subsocial cycles? Here we suggest that, in ants, evolutionary innovations in insulin signalling may have played a crucial role in each case.

2.1: Seeking candidate regulators of the reproductive division of labour

Eusociality evolved once in a common ancestor of ants and, with the exception of a few derived social parasites, all extant ants are eusocial (Ward, 2014) (Fig. 2.1). To identify conserved potential regulators of division of labour between reproduction and brood care in ants, we conducted an unbiased screen for differentially expressed genes between whole brains or heads of reproductives and non-reproductives across seven ant species, including four previously published datasets (Fig. 2.1; Appendix A) (Gospocic *et al.*, 2017; Li *et al.*, 2014; Libbrecht *et al.*, 2016; Patalano *et al.*, 2015). We sampled a range of reproductive strategies, from species with morphologically distinct queens and workers to queenless species (that independently lost queens). Among all 5,581 identified single-copy orthologs shared among these seven species, we found only one such gene: insulin-like peptide 2 (*ilp2*). *ilp2* was always significantly upregulated in reproductives (Fig. 2.1). Thus, the differential expression of *ilp2* is likely conserved across ants. Consequently, the most recent common ancestor of ants likely had *ilp2* expression that was high in reproductives and low in non-reproductives.

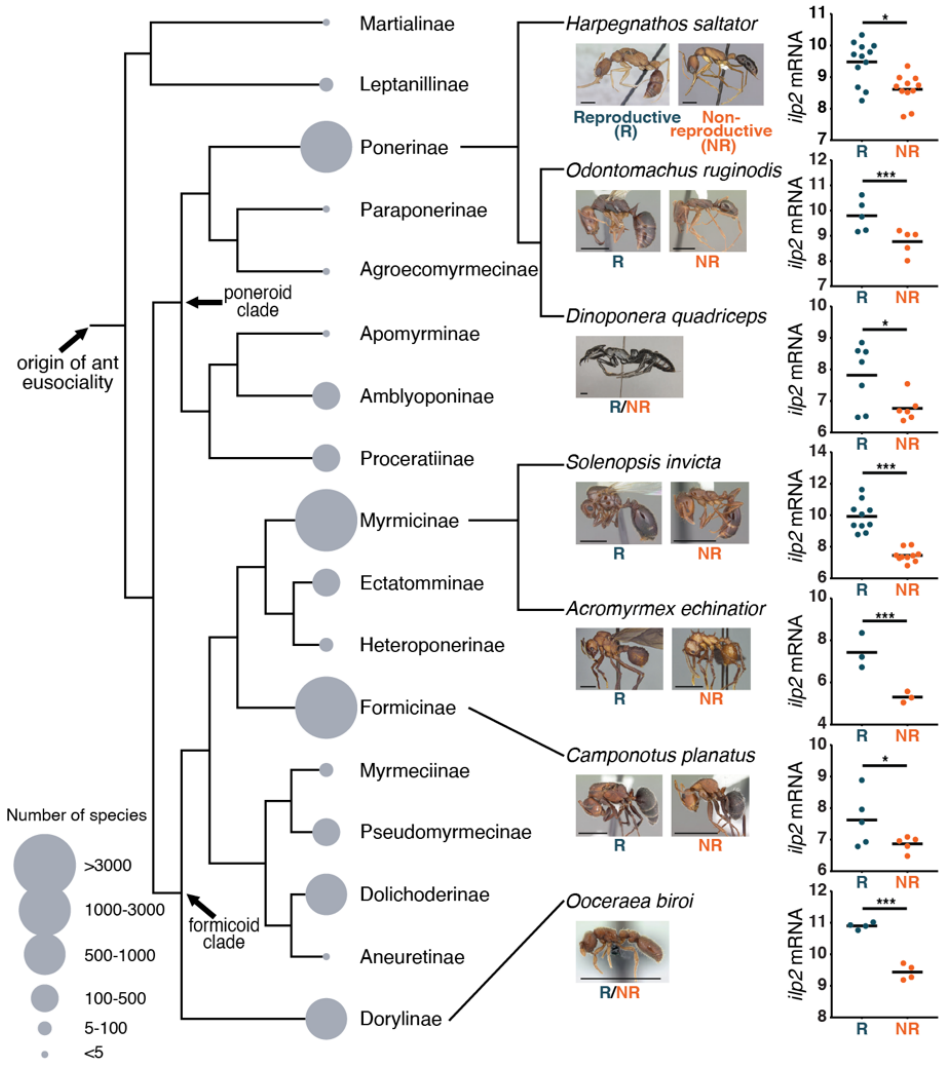


Figure 2.1: Brain gene expression in seven ant species identifies one conserved differentially expressed gene. The figure shows the summary cladogram of the seven ant species used in this study in the context of the entire ant phylogeny with all subfamilies labelled. Five of the focal species have queens, while two (*D. quadriceps* and *O. biroi*) are queenless. Although *H. saltator* is not queenless, the data compared reproductive and non-reproductive workers (Appendix A). The dot plots show variance-stabilised transformed read counts for *ilp2*. Blue and orange dots indicate reproductive and non-reproductive ants, respectively. Horizontal bars indicate means, and asterisks indicate statistically significant differences between groups (Wald test: * $p<0.05$; *** $p<0.001$). All images except for *A. echinatior* are from A. Nobile, S. Hartman, and E. Prado (www.antweb.org). Scale bars represent 2mm. The phylogeny is based on (Borowiec *et al.*, 2017). Species numbers are from (Ward, 2014).

Although our approach is conservative and probably misses genes, it has the advantage of eliminating false positives. When we relaxed the statistical stringency for classifying genes as differentially expressed, the screen still returned *ilp2* as the single candidate gene. Relaxing other inclusion criteria divulged additional genes that might be expected to vary with reproductive state. For example, a total of 24 genes were consistently differentially expressed in subsets of five of the seven studied species.

This list includes insulin-like peptide 1 (*ilp1*), as well as other genes implicated in insulin signalling (Fig. 2.2). Non-single-copy orthologs were excluded from the screen. One example is vitellogenin (*vg*), a gene that has undergone repeated duplications in ants (Corona *et al.*, 2013). The vitellogenin protein is a lipid carrier that provisions developing oocytes with yolk and constitutes a reliable indicator of female reproductive activity (Corona *et al.*, 2013; Oxley *et al.*, 2014). Studies of bees and other insects have shown that vitellogenin interacts with insulin signalling (Badisco *et al.*, 2013; Corona *et al.*, 2007; Nilsen *et al.*, 2011). *vg* indeed showed consistently higher expression in reproductives in our screen, even though this difference was not statistically significant in two of the ponerines (Fig. 2.2). These findings further bolster the conclusion that insulin signalling played a major role in the evolution of reproductive division of labour in ants.

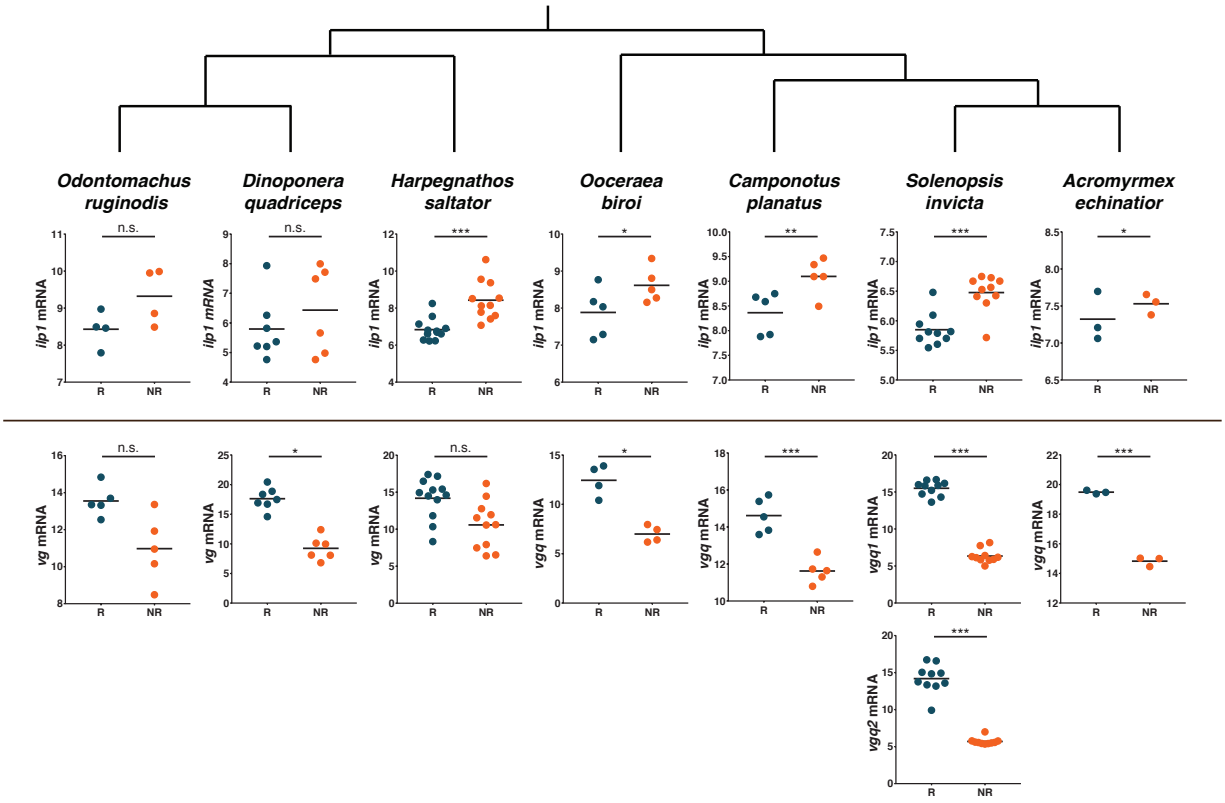


Figure 2.2: Variance-stabilised transformed read counts for *ilp1* and vitellogenin (*vg*) in the seven ant species in the comparative screen. *ilp1* always tends to have higher expression in the non-reproductives, although it is not significantly differentially expressed in two ponerine species: *O. ruginodis* and *D. quadriceps*. *vg* is duplicated in the formicoids, and here we only show its pro-ortholog, queen vitellogenin (*vgq*), as it is the formicoid *vg* paralog that is associated with reproduction (Oxley *et al.*, 2014). This duplication was likely followed by a loss in the formicines and, as a result, *C. planatus* only appears to have one *vg*. *vgq* is duplicated in *S. invicta*. While the average expression of *vgq* is higher in reproductives of all seven species, this difference is not statistically significant in two ponerine species: *H. saltator* and *O. ruginodis*. Horizontal bars indicate means and asterisks indicate statistically significant differences between groups (Wald test with 5% FDR correction: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s.: not significant).

Insulin regulates reproduction and food-seeking behaviour across a wide range of organisms, making it a prime candidate for the regulation of subsocial cycles and eusocial division of labour (Toth and Robinson, 2007). Most studied hymenopterans have two ILPs: ILP1 and ILP2. While ILP1 resembles insulin-like growth factor, ILP2 is similar to canonical insulin (Mizoguchi and Okamoto, 2013; Okamoto *et al.*, 2009; Southey *et al.*, 2008; Veenstra, 2000). In other holometabolous insects, these ILPs regulate larval growth, adult metabolism, and reproduction (Brown *et al.*, 2008; Toth and Robinson, 2007; Wang *et al.*, 2013). Moreover, caste determination in most ant species relies on nutritional asymmetries during development: queen-destined larvae eat more than worker-destined larvae, which likely explains how queens acquire higher ILP2 levels (Trible and Kronauer, 2017). A study of *Diacamma sp.* found that the asymmetry in reproductive potential between ants was correlated with insulin receptor expression in the ovaries (Okada *et al.*, 2010). This suggests a possible secondary mode of reproductive control downstream of ILPs that may augment or replace the initial reproductive asymmetry reflected by differential *ilp2* expression in the brain. ILPs have not been studied functionally in eusocial insects in the context of reproductive division of labour between adults. However, insulin signalling has been implicated in other contexts, such as caste development and non-reproductive division of labour (Ament *et al.*, 2008; de Azevedo and Hartfelder, 2008; Wang *et al.*, 2013; Wheeler *et al.*, 2006).

2.2: The regulation of ILP2 production in *O. biroi*, a model ant species

We used the clonal raider ant *Ooceraea biroi* to study ant ILP2. As discussed in the previous chapter, *O. biroi* has secondarily lost queens, resulting in a species in which workers reproduce synchronously and asexually (Oxley *et al.*, 2014; Teseo *et al.*, 2013). Colonies alternate between reproductive and brood care phases. This colony cycle is regulated by the periodical presence of larvae, which suppress reproduction and induce brood care behaviour in adults, and is reminiscent of the subsocial cycle presumed to precede eusociality in ants.

We found that antibody-staining of ILP2 exclusively localised to the brain, primarily in a single medial cluster of ca. 15 cells in the pars intercerebralis (Fig. 2.3, A to C). These insulin-producing cells coincide in location with those of other insects (Géminard *et al.*, 2009; Riehle *et al.*, 2006). We quantified ILP2 in the insulin-producing cells, and found that its levels are higher in the brood care than in the reproductive phase (Fig. 2.3D). Peptide levels are thus anti-correlated with transcription. This pattern is known from *D. melanogaster*, where the rate of ILP secretion correlates with the rate of *ilp* transcription (Géminard *et al.*, 2009). This suggests that the mechanisms of *ilp* expression and ILP secretion are conserved in holometabolous insects.

Because larvae regulate the *O. biroi* colony cycle, we asked whether larval communication altered *ilp2* expression in adults. When larvae are removed from colonies in the brood care phase, *ilp2* expression levels in adult brains increase dramatically within 12 hours (Fig. 2.3E) (Libbrecht *et al.*, 2017). This increase occurs under identical nutritional conditions. Conversely, when ants in the reproductive phase are given larvae, their *ilp2* levels decrease (Fig. 2.3E). *vgq*, the vitellogenin gene upregulated in ant queens, responds similarly, albeit slower, to these changes (Fig. 2.4A), raising the possibility that ILP2

regulates reproduction at least partly by acting on *vgq*. Although this experiment is highly suggestive, the addition of larvae was always correlated with the removal of pupae, and changes in expression occurring after the 24h time point were confounded by nutritional differences (because colonies were fed if they had larvae). We therefore repeated this experiment without pupae and under nutritionally-controlled conditions. We removed larvae from colonies in the brood care phase, waited until the ants in these colonies activated their ovaries, and then compared brain gene expression between these and control colonies. Again, the removal of larvae increased *ilp2* (Fig. 2.3F) and *vgq* (Fig. 2.4B) expression. This suggests that social signals can mediate insulin signalling independently of internal nutritional state, and that this is a key regulatory mechanism underlying the *O. biroi* colony cycle. Given the conserved association of caste and *ilp2* expression in all ants, social regulation of *ilp2* may also underlie the life cycle of the subsocial ancestor.

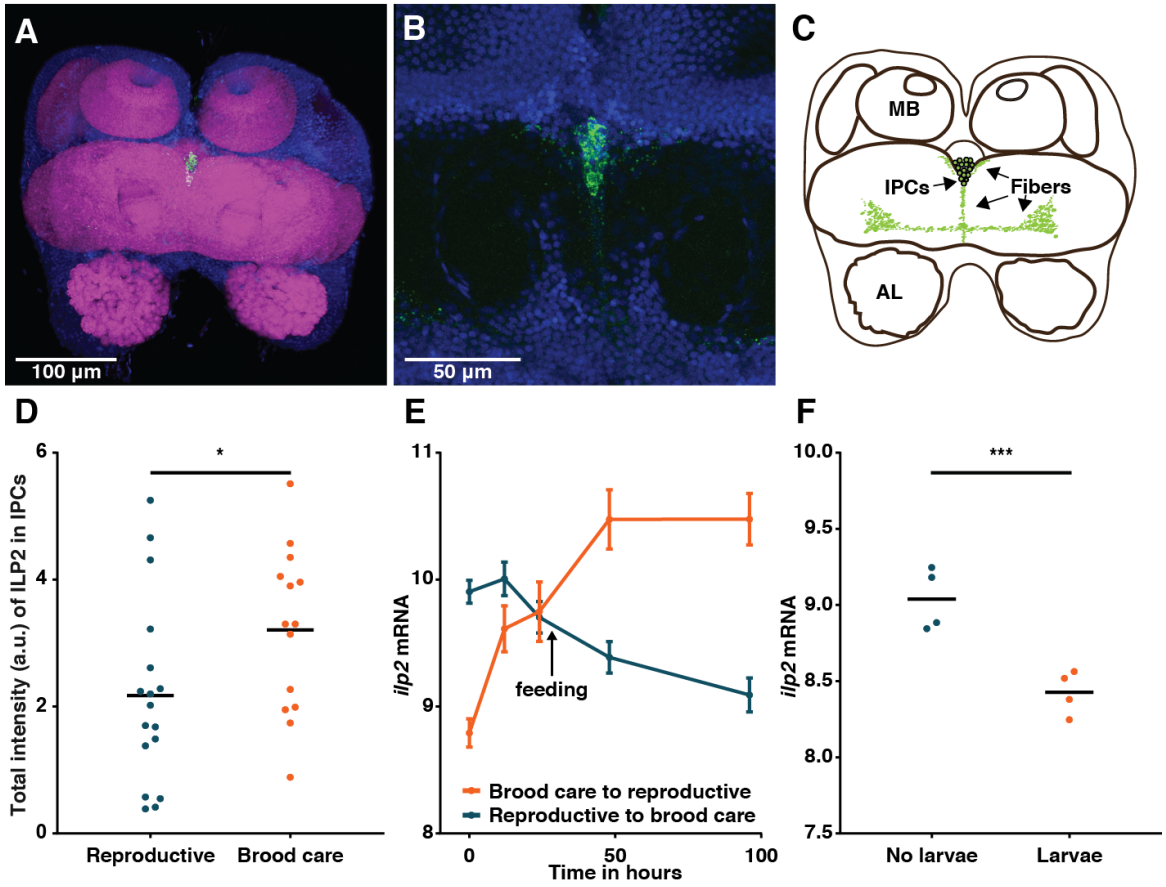


Figure 2.3: Larvae regulate *ilp2* in adults. (A-C) Immunohistochemistry with anti-ILP2 antibody on an *O. biroi* brain localizes ILP2 peptide to a single cluster of insulin-producing cells (IPCs) in the pars intercerebralis (body-axis dorsal view). Green: anti-ILP2; blue: DAPI; magenta: phalloidin. MB: mushroom body; AL: antennal lobe. (D) Total intensity of ILP2 in the insulin-producing cells is higher in the brood care phase than in the reproductive phase (n≥14, t-test; p=0.046). (E) RNA-Seq time course shows that the addition of larvae downregulates *ilp2*, whereas the removal of larvae upregulates *ilp2* (n≥4, time:transition interaction, Likelihood Ratio Test with 5% FDR correction; $p<10^{-15}$). The black arrow indicates when ants with larvae were fed, i.e. changes in expression beyond that time point are confounded by differences in nutrition. Error bars depict SEM. Data from (Libbrecht *et al.*, 2017). (F) RNA-Seq on ant brains shows that under nutritionally controlled conditions, *ilp2* is upregulated eight days after larvae are removed from *O. biroi* workers in the brood care phase (n=4, Wald test with 5% FDR correction; $p<10^{-6}$). Data are variance-stabilised transformed read counts. Horizontal bars indicate means.

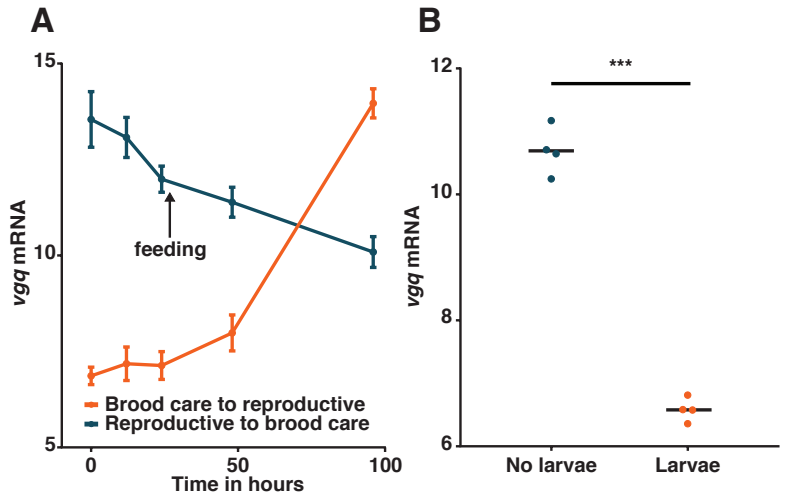


Figure 2.4: *vgq* expression is suppressed by larval signals. (A) Brain RNA-Seq time course shows that the addition of larvae downregulates *vgq*, while the removal of larvae upregulates *vgq* (time:transition interaction, Likelihood Ratio Test; $p<10^{-15}$). (B) Brain RNA-Seq shows that *vgq* is upregulated eight days after larvae are removed from *O. biroi* workers in the brood care phase under nutritionally controlled conditions (n=4, Wald test with 5% FDR correction; $p<10^{-13}$). The data are variance-stabilized and transformed read counts. Horizontal bars in (B) indicate means.

2.3: ILP2 is a core regulator of *O. biroi* reproduction

In *D. melanogaster* insulin signalling is necessary and sufficient to regulate the terminal differentiation of germline stem cells into oocytes. Moreover, it promotes yolk uptake in developing oocytes and is crucial for ovary activation (LaFever and Drummond-Barbosa, 2005). It is therefore plausible that the differential expression of *ilp2* in ants has a causal role in regulating ovary activation and reproductive division of labour. We further hypothesized that if the regulation of *ilp2* were freed, at least partially, from larval control, this would yield ants whose physiology is less susceptible to reproductive suppression. Such a mechanism would allow the evolution of distinct

reproductive and non-reproductive castes from an ancestral subsocial cycle. To test this hypothesis, we injected synthetic *O. biroi* ILP2 mature peptide into workers in colonies with larvae. As a control, we injected the inactive B chain of this peptide, which has no activity on its own (Brown *et al.*, 2008) (Fig. 2.7A). Injecting ILP2 mature peptide caused strong ovary activation despite the presence of larvae (Fig. 2.5, Fig. 2.6A).

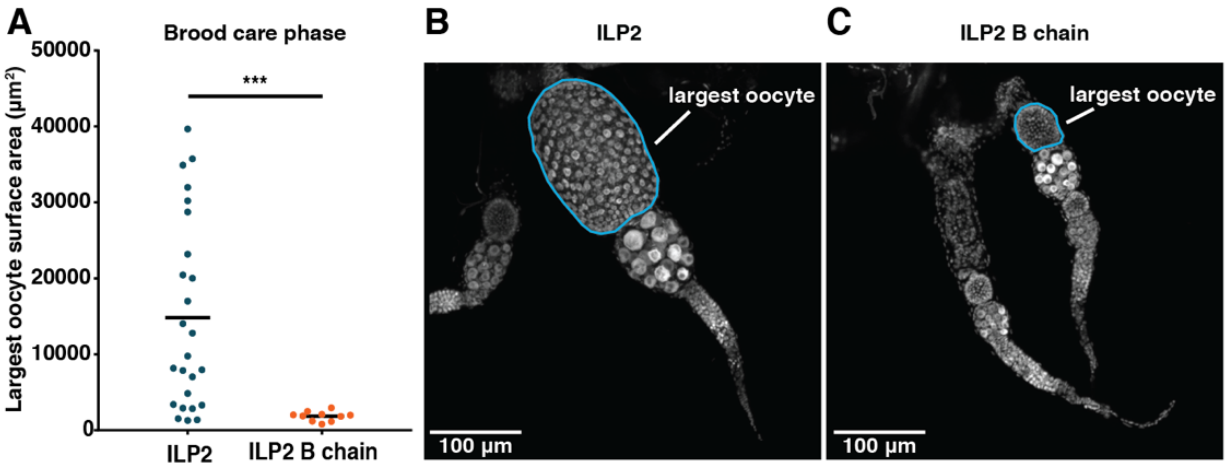


Figure 2.5: ILP2 supplementation overrides larval suppression of adult reproduction. (**A**) Workers injected with 100 μM ILP2 in the brood care phase activate their ovaries relative to controls injected with 100 μM ILP2 B chain despite being in contact with larvae ($n \geq 10$, Welch's t-test with Bonferroni correction; $p=0.0005$). (**B and C**) Confocal images of ovaries from ants injected with either 100 μM ILP2 (**B**) or 100 μM ILP2 B chain (**C**). Shown are the pairs of ovaries closest to the mean value from each treatment; the largest oocyte in each pair is circled in blue.

Higher doses of ILP2 caused increased ovary activation, as well as the simultaneous development of more eggs (Fig. 2.6, B and C), suggesting that quantitative differences in ILP2 levels vary the ants' positions along a spectrum of reproductive potential. To ensure that ILP2 does not have inhibitory effects during the opposite phase

of the colony cycle, we injected ants in the reproductive phase with ILP2, and found no detectable effect on ovary state (Fig. 2.7, B and C). This is consistent with data in mosquitoes showing that insulin-like peptides are only gonadotropic within a specific range of concentrations (Brown *et al.*, 2008); ants in the reproductive phase already have permissive levels of ILP2, and additional peptide appears to be unable to accelerate egg development.

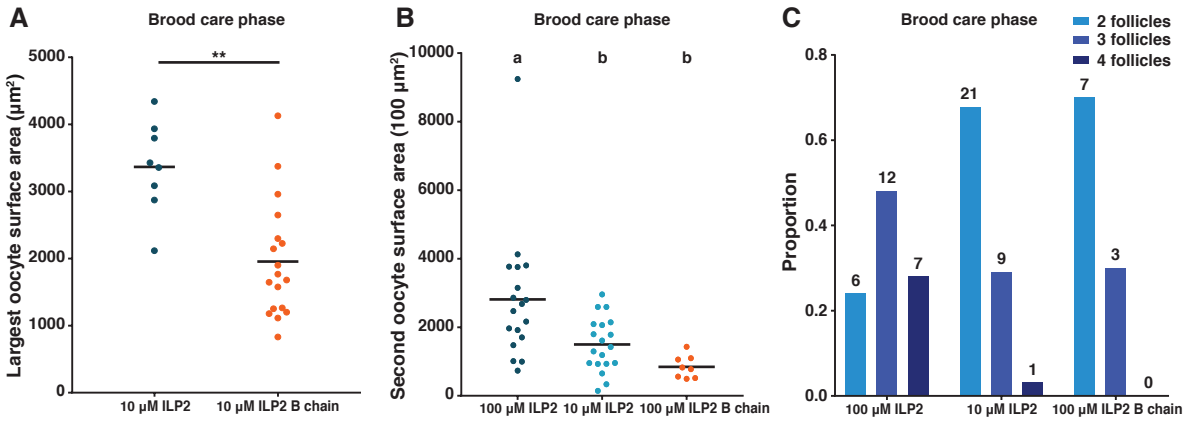


Figure 2.6: Ovaries activate in proportion to the concentration of injected ILP2. (**A**) 10µM ILP2 causes ants in the brood care phase to activate their ovaries relative to control workers injected with 10µM B chain, despite being in the presence of larvae (≥8, Welch's t-test with Bonferroni correction; p=0.002). (**B**) 100µM ILP2 injections also cause the smaller of two ovarioles to have more developed oocytes than injections with 10µM ILP2 or B chain control (n≥8, letters above the columns indicate significant differences at p<0.05 after a Kruskal-Wallis test with Dunn's correction on post-hoc pairwise comparisons). Horizontal bars indicate means on all dot plots. (**C**) The amount of ILP2 injected alters the number of eggs an ant can develop simultaneously (chi-squared test; p=0.0032). Y-axis shows proportion of ants with 2, 3, or 4 follicles in each treatment. Numbers above each bar indicate sample size.

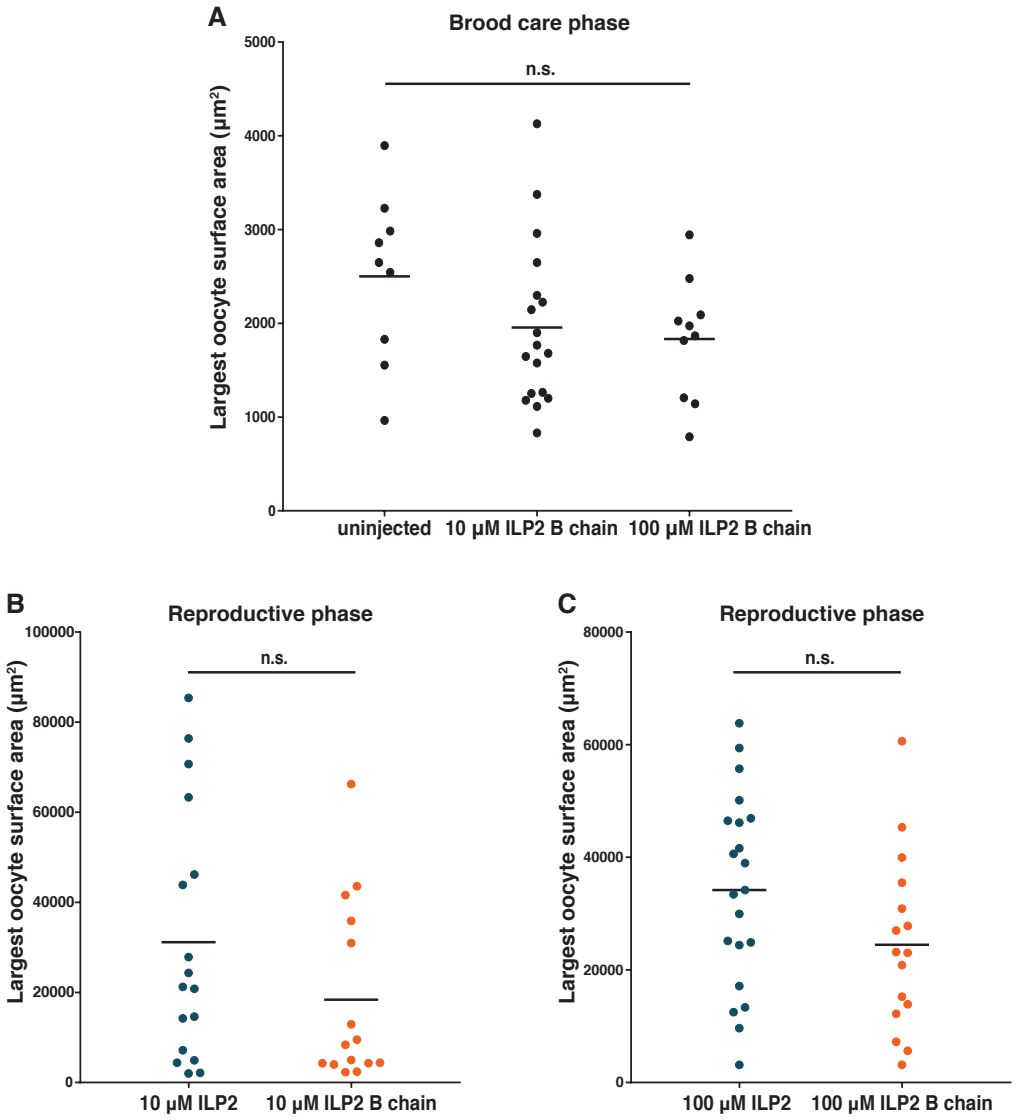


Figure 2.7: ILP2 injection controls. (**A**) Ants in the brood care phase injected with ILP2 B chain have oocytes that are indistinguishable from uninjected ants (n≥9, pairwise t-tests followed by Bonferroni correction: p>0.05), showing that the B chain is an appropriate negative control. Injecting ILP2 at 10 µM (n≥15, Welch's t-test; p=0.14) (**B**) and 100 µM (n≥16, Welch's t-test; p=0.08) (**C**) does not significantly further activate ovaries in the reproductive phase, likely because these ants already have levels of ILP2 permissive to ovary activation and their ovaries are already active.

Finally, we hypothesized that, as developmental nutritional asymmetries determine caste in most ants, this might be a general and natural mechanism that produces asymmetries in baseline adult ILP2 levels and consequently in reproductive potential. While most *O. biroi* workers have two ovarioles, some ('intercastes') have four or more (Ravary and Jaisson, 2004; Teseo *et al.*, 2013) (Fig. 2.8, A and B). These differences can be determined by the amount of food a larva receives. Intercastes have longer and more active ovaries than regular workers in the brood care phase, suggesting that they are less sensitive to larval signals that suppress ovarian activity (Fig. 2.8C, Fig. 2.9A). This is consistent with previous work showing that some intercastes fail to regress their ovaries during the brood care phase (Teseo *et al.*, 2013).

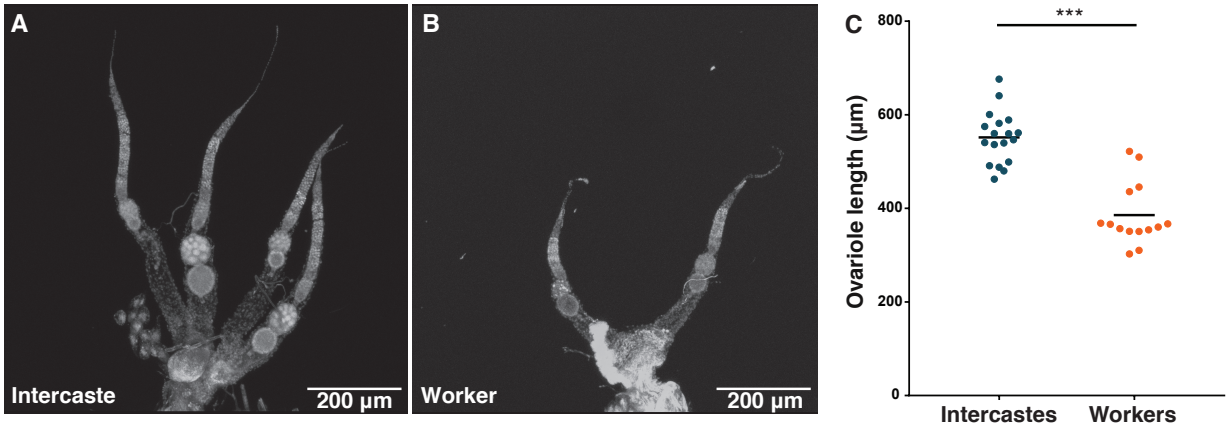


Figure 2.8: Intercastes have larger ovaries than regular workers. (**A**) Intercastes tend to have four ovarioles, while (**B**) regular workers tend to have two. In the brood care phase, intercaste ovarioles are also more developed (Fig. 4A). (**C**) Quantification of ovariole length between young phase-matched intercastes and regular workers in the brood care phase shows that intercastes have longer ovarioles ($n \geq 14$, Welch's t-test; $p < 0.0001$).

We found that the insulin-producing cells of intercastes contained more ILP2 than those of regular workers (Fig. 2.9, B and C). As we have shown above, ILP2 levels in the IPCs are negatively correlated with *ilp2* expression, ovary state and, by extension, circulating ILP2 levels in workers between the different phases of the cycle, likely due to higher rates of peptide release during the reproductive phase (Fig. 2.3F). We would expect to see the same pattern when comparing intercastes between phases. The phase-matched comparisons between different types of individuals, on the other hand, show that intercastes consistently have higher ILP2 levels in their IPCs and, given their more active ovaries and decreased sensitivity to larval signals (Ravary and Jaisson, 2004), it is likely that they also have consistently higher circulating ILP2 levels than workers. Overall, these data suggest that baseline ILP2 levels correlate with minor variation in baseline reproductive physiology in ants, as is seen in *O. biroi*. This limited polymorphism is comparable to the range of baseline reproductive physiologies present in solitary relatives of ants (Ohl and Linde, 2003). Moreover, this association between nutrition during development, adult baseline insulin levels, and adult reproductive potential is ancient and conserved at least across the holometabolous insects (Green and Extavour, 2014; Trible and Kronauer, 2017). This ancestral association is a sufficient substrate for natural selection to drive phenotypic divergence towards permanent reproductive and non-reproductive castes in ants.

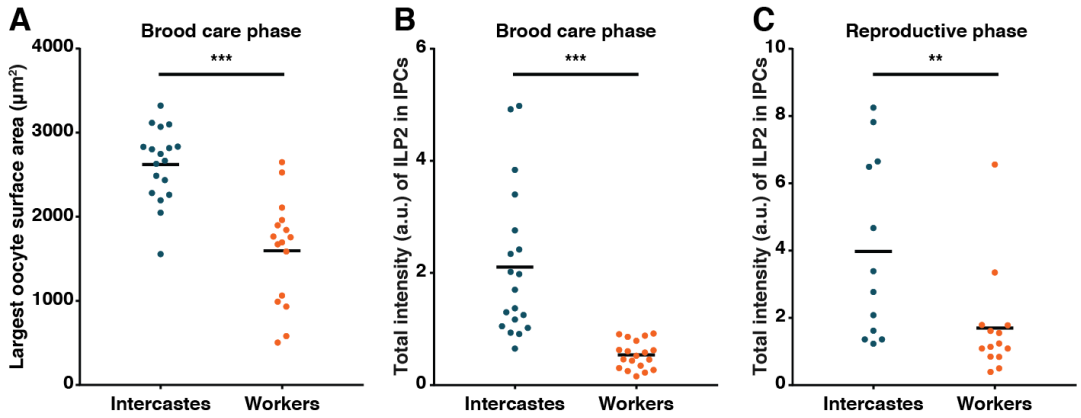


Figure 2.9: Intercastes respond less to larvae and have more ILP2 than regular workers. (**A**) Intercastes have more active ovaries than age-matched regular workers in the brood care phase, despite both being in contact with larvae (n≥16, Welch's t-test; p<0.0001). (**B**) In the brood care phase (n=19, Mann-Whitney U test; p<0.0001) and (**C**) in the reproductive phase (n≥12, Mann-Whitney U test; p=0.0043), intercastes have more ILP2 in their insulin-producing cells than age-matched regular workers. Horizontal bars indicate means on all dot plots.

2.4: A model for the evolution of the first ant colonies

How the ancestral subsocial cycle was regulated remains unknown. However, assuming that similar mechanisms underlie the *O. biroi* colony cycle, these findings suggest a plausible scenario for the evolution of ant sociality. First, during the transition from solitary to subsocial, some signalling systems (perhaps including insulin signalling) in adults must have become responsive to larval signals. This allowed behavioural and physiological responses in adults to be appropriately modified for the nutritional requirements of the larvae. During the transition from subsocial to eusocial, increased developmental variation may have caused some adults to emerge from the pupa with low nutritional stores and low ILP2 levels. These sub-fertile individuals would have been more sensitive to larval signals that suppress reproduction and would consequently have

foregone nest-founding and ovary activation and instead assumed brood care roles. Other adults, meanwhile, would have emerged with high nutritional stores and high ILP2 levels. These adults would have had reduced sensitivity to larval signals and would have been more likely to reproduce despite the presence of larvae. This reproductive asymmetry could then have been enhanced or modified by natural selection to ultimately produce the obligately reproductive queens and sterile workers of advanced eusocial species (Fig. 2.10). This scenario constitutes an explicit – albeit somewhat modified - molecular version of Mary Jane West-Eberhard's model for the evolution of hymenopteran eusociality (West-Eberhard, 1987a).

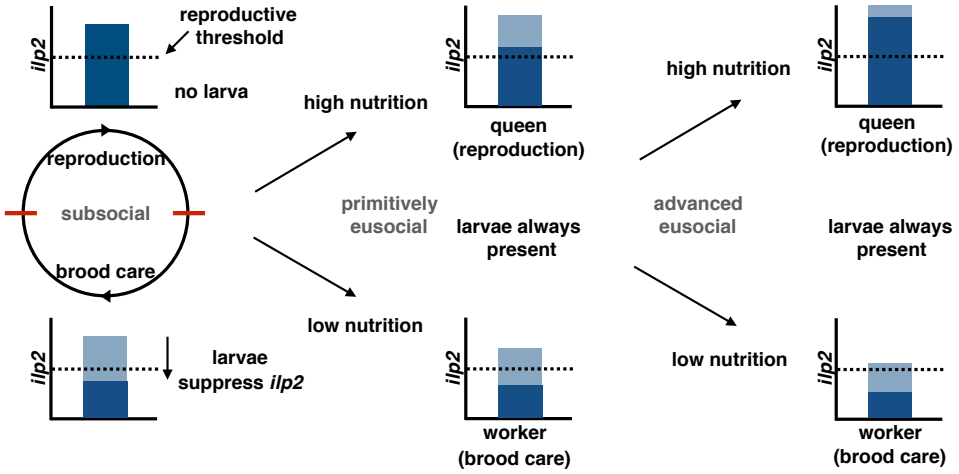


Figure 2.10: Schematic illustrating the putative origin and elaboration of eusociality from a subsocial cycle in ants. The dark bar in each bar graph illustrates realized *ilp2* expression, whereas the light bar shows maximum potential *ilp2* expression. *ilp2* expression in adults is reduced in the presence of larvae, thereby explaining the ancestral subsocial cycle. In the primitively eusocial state, queens have slightly higher maximum potential *ilp2* levels than workers do, and larval signals (or similar inhibitors of reproduction, such as aggression or queen pheromones) amplify this developmentally determined reproductive asymmetry. This allows queens to reproduce permanently instead of cyclically, and workers are permanently inhibited from reproducing. Over evolutionary time, this reproductive DOL may be further elaborated, as seen in the advanced eusocial condition.

This model makes two major assumptions. We assume that larvae suppress adult *ilp2* in the ancestral subsocial ant. The ovaries of workers of a few ant species are so far known to be sensitive to larval signals (Schultner *et al.*, 2017), and to the best of our knowledge, no ants are known to be insensitive. We hope that comparative work in the future will fully test this assumption. Note however that this assumption is not critical to the core of our model, for two reasons. First, larval suppression of adult insulin could be replaced by other social interactions, such as aggression (which may suppress worker insulin (Gospocic *et al.*, 2017)). Second, even without any social suppression of adult insulin, developmental asymmetries in access to nutrition are ubiquitous, and are sufficient to create reproductive division of labour.

Critically, we also assume – in line with the current consensus (Johnson and Linksvayer, 2010; Linksvayer, 2009; West-Eberhard, 1987a; Wilson, 2008) – that the ancestral ant was subsocial (i.e., a progressive provisioner), with a reproductive cycle driven by an ovarian cycle. We propose that this cycle was broken to generate the fixed asymmetry between eusocial queens and workers. Studying the founding of extant ant colonies suggests that the ancestral ant was indeed a progressive provisioner. Colonies are typically (i.e., ancestrally) founded by a single 'semi-claustral' queen who burrows into the ground and lays a batch of eggs before appearing to cease reproduction and focussing on rearing her offspring (see Chapter 1). The 'foundress' forages for them, often hunting small arthropods, until they have grown to adulthood, after which she ceases brood care and instead begins to reproduce again (Hölldobler and Wilson, 1990; Peeters, 2010; Peeters and Ito, 2001; Wheeler, 1910; Wilson, 1971). This reproductive cycle is similar to the cycles of subsocial wasps across the hymenopteran phylogeny. This suggests that this initial queen reproductive cycle may have been the ancestral state, and that the evolutionary innovation was that the foundress's offspring failed to leave her

nest after they attained adulthood. However, not all subsocial wasps have an ovarian cycle that causes the reproductive cycle; some of them instead retain a fully-developed egg during their non-reproductive phase (Kelstrup *et al.*, 2018). We do not yet know whether foundress ant queens typically have an ovarian cycle during their founding phase, and more importantly, whether the ancestral ant queen did. If she did not, it would very likely mean that she also did not have an *ilp2* cycle (as there is currently no evidence in the ants to suggest that *ilp2* levels affect egg retention). This would falsify a substantial part of our model for how the first eusocial colonies evolved. In this case, the transition to eusociality would still require the generation of an *ilp2* asymmetry between queens and workers, but this would be a consequence – rather than a cause – of the evolution of fixed reproductive division of labour and eusociality. However, this asymmetry would have to be generated not by reusing a pre-existing temporal asymmetry in *ilp2* production, but by some other means – perhaps simply as a consequence of variation in developmental nutritional asymmetries. I hope that future work will test these assumptions.

Finally, we did not study the mechanisms that explain how non-reproductive workers decide to remain in their mother's nest, or how they decide to perform various aspects of brood care. Whether insulin signalling is relevant to these processes remains unknown, and explaining them is necessary for a better understanding of the origin of eusociality.

CHAPTER 3: FORAGING AND FEEDING ARE INDEPENDENTLY REGULATED BY SOCIAL AND PERSONAL HUNGER IN THE CLONAL RAIDER ANT

Once ants have evolved to live in colonies, they often evolve adaptations for improved cooperation, and especially to maintain colony homeostasis. For example, colonies must regulate how much they forage (Behmer, 2009), how the acquired food is distributed through the colony, and each colony member must decide how much to eat. Foraging is often a distributed process in social insects; many workers forage, sometimes largely independently of each other (Lanan, 2014). Foraging is also a social behaviour: it is performed for the benefit of the colony. Workers leave the nest to locate food, but instead of consuming it *in situ*, they usually transport it back to the colony. The outcome of a foraging event, then, is that all members of the colony have access to the food. However, each forager must independently assess the colony's hunger (i.e. the internal motivational state of the colony that is necessary and sufficient to cause foraging behaviour, analogous to hunger in solitary animals (Toates, 1986)). After this assessment, she must decide how much, if at all, to forage.

How might an ant forager assess the hunger of her colony? Most obviously, like any solitary organism, she could use her own internal nutritional state – her own hunger – as a proxy for colony hunger. Indeed, the nutritional states of ant workers often correlate with how much they forage (Blanchard *et al.*, 2000; Robinson *et al.*, 2008; Robinson *et al.*, 2012; Silberman *et al.*, 2016). Moreover, starving ant colonies – or just their workers - usually increases their foraging effort (Bazazi *et al.*, 2016; Bernadou *et al.*, 2018; Bernadou *et al.*, 2020; Fowler, 1980; Greenwald *et al.*, 2018; Hölldobler, 1971; Howard and Tschinkel, 1980; Mailleux *et al.*, 2006; Mailleux *et al.*, 2010; McGrannachan and Lester,

2013; Traniello, 1977; von Thienen and Metzler, 2016; Wallis, 1962), although some exceptions have been reported (Dejean, 1986; Fourcassié *et al.*, 2003; Rueppell and Kirkman, 2005). Overall, this shows that in many species, workers use their nutritional states as a measure of colony hunger. However, there are two other potential sources of information she could use (Vowles, 1955): a central internal food store, or signals from the larvae or the other adults in the colony. While a few ant species have central food stores (Hölldobler and Wilson, 1990), many other species do not have organized or long-lasting food stores (Hölldobler and Wilson, 1990; Rueppell and Kirkman, 2005). Previous work in *Ooceraea biroi, Solenopsis invicta,* and *Rhytidoponera metallica* shows that larval signals are also often relevant to colony foraging effort (Cassill and Tschinkel, 1995; Cassill and Tschinkel, 1999; Cornelius and Grace, 1997; Dussutour and Simpson, 2009; Howard and Tschinkel, 1980; Schultner *et al.*, 2017; Ulrich *et al.*, 2016; Ulrich *et al.*, 2018). Clearly, then, in at least some social insect colonies, these other factors may serve as (potentially additional) measures of colony hunger. These measures did not exist and could not have influenced foraging behaviour in the solitary ancestors of ants. It follows that these new signals of hunger likely evolved during or after the transition to eusociality.

The influence of larval signals on colony foraging has been relatively well-studied in *O. biroi*. As I discussed in Chapter 1, *O. biroi* colonies undergo a stereotyped colony reproductive cycle in which the adults lay eggs effectively simultaneously during the reproductive phase. The eggs hatch into larvae within a day of each other, inducing the brood care phase by suppressing adult reproduction and causing them to perform brood care instead (Oxley *et al.*, 2014; Ravary and Jaisson, 2002; Ravary *et al.*, 2006). *O. biroi* colonies do not typically forage in their reproductive phase, when there are no larvae in the colony. Moreover, experimentally increasing the proportion of larvae in a colony

increases colony foraging effort (Ulrich *et al.*, 2016; Ulrich *et al.*, 2018). These data show that, in *O. biroi*, foraging is at least substantially induced by larval communication. However, the extent to which foraging may be induced by other factors – especially by individual worker hunger - remains unknown.

One possibility is that larval signals cause workers to forage by depleting their nutritional state. If this is true, it would suggest a straightforward mechanism for how larval signals evolved to regulate adult foraging behaviour. In this case, larvae would simply have acquired the ability to reduce the adult nutritional state. By making use of ancient, conserved physiological mechanisms that regulate foraging, this would have induced the workers to forage. Here, I ask whether foraging in *O. biroi* is entirely a social behaviour – i.e. to what extent *O. biroi* workers assess colony hunger through their own nutritional states rather than larval signals. I also ask how workers subsequently consume food. Specifically, I ask whether workers use their nutritional states to regulate both foraging and feeding behaviour, and I ask whether larval signals affect their feeding behaviour in addition to their foraging behaviour.

3.1: Food deprivation and lipid measurement

To establish a protocol to deprive ants of food, and to verify that their nutritional states had indeed been depleted, I conducted two preliminary experiments. Specifically, first I asked whether depriving *O. biroi* workers of food for a few days was sufficient to significantly deplete their whole-body lipid levels. I either fed (three times, at two-day intervals) or did not feed newly-eclosed *O. biroi* workers. I then starved all workers for the next three weeks, and quantified their mortality and whole-body lipid (i.e. triacylglycerides (TAG)) levels – a proxy for their overall nutritional state - over the next

month. I found that newly-eclosed workers died within two weeks without feeding, and had reduced lipid within one week of starvation (Fig. 3.1 A and B). Second, I injected 180 one-month old workers each with either 230μM TAG or, as a control, 1x phosphate buffered saline (PBS), and starved them for eight days thereafter, during which I quantified their lipid levels at three timepoints (i.e. on days 2, 4, and 8 after injection). I found that depriving one-month old ants of food for either four or more days significantly depleted their lipid levels compared to two days of food deprivation, and that injecting ants with purified TAG transiently increased their measured lipid levels (Fig. 3.1C). Together, these experiments show that I can reliably detect differences in lipid levels of small groups of ant workers, and that depriving workers of food for a few days is sufficient to deplete their lipid levels.

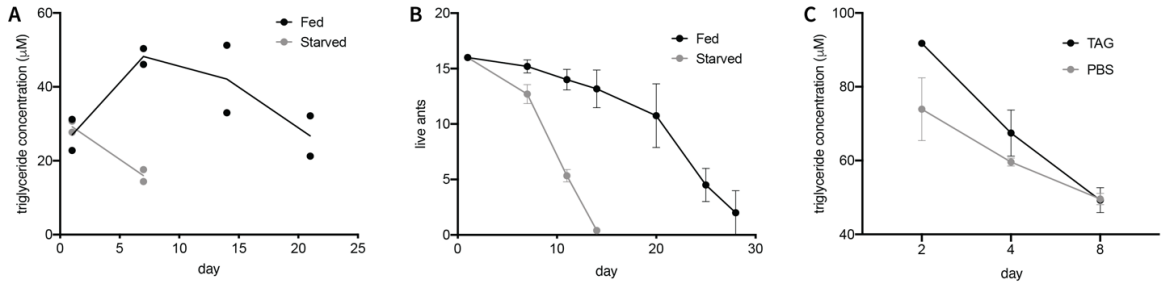


Figure 3.1: Food deprivation depletes worker lipid levels. (**A**) When newly-eclosed ants are starved, their lipid levels (i.e. estimated triacylglyceride concentration) decrease (grey points). Feeding increases their lipid levels (black points at day 7), whereas subsequent starvation decreases their lipid levels (black points after day 7). (**B**) Starving newly-eclosed ants this way kills them within a month. Together, these data show that feeding and starvation have reasonable, detectable effects on ants' nutritional states. (**C**) Starving one-month-old workers for 4 or 8 days significantly diminishes their lipid levels, and injecting purified triglycerides (TAG) into them first transiently increases their lipid levels, showing that a few days of starvation is sufficient to manipulate the ants' nutritional state (n = 4 measurements per treatment per timepoint, two-way ANOVA $p < 0.0001$ for time, $p = 0.049$ for treatment). Error bars in (**B**) and (**C**) represent standard error of the mean.

3.2: The effect of food deprivation on foraging behaviour

Next, I conducted two experiments in which I deprived ants of food and subsequently measured the effect of this deprivation on their foraging behaviour, in (a) homogenous and (b) heterogenous colonies. For these two experiments, I began by establishing a single colony of newly-eclosed ants and larvae from a single source colony in the brood care phase. This ensured the adults were age-matched, and had identical rearing conditions. I permitted this colony to continue through its reproductive cycle naturally, and once it was in its brood care phase, I split it into two colonies of 250 ants each. I added 120 matched larvae to each colony. All ants in each colony were marked on their gasters with one of two colours. One of these colonies was not fed for ten days; the other was fed roughly on alternate days through this ten-day period. I then used these ants to establish experimental colonies for both experiments. For the experiment in which I studied the effects of asymmetric food deprivation on foraging behaviour, I established eight experimental colonies. Each colony consisted of 10 fed ants, 10 food deprived ants, and 20 five-day old larvae from a different source colony (to ensure that larval signals were uniform across all colonies). Ants in each colony were marked with one of two colours corresponding to their deprivation treatment. For the experiment in which I studied the effect of food deprivation on colony-level foraging behaviour, I established four colonies of ants for each treatment. The ants were also marked as before, and in this experiment, all ants in each colony possessed identical marks. Each colony here consisted of 20 uniformly-treated ants, as well as 20 five-day old larvae from a different source colony (to ensure that larval signals were uniform across all colonies). I then studied their behaviour over roughly 6 hours, including periods of tracking first without and then with food in the arena.

Using analyses of automated tracking data (Fig. 3.2A), I found that colonies consisting of fed workers have higher mean distance from the nest than colonies consisting of unfed workers (Fig. 3.2B). Their ants also had travelled further between consecutive frames (Fig. 3.2C), but these colonies did not have a significantly higher mean number of ants outside the nest (Fig. 3.2D). Thus, on two out of three measures, food deprivation appears to decrease – rather than increase - foraging activity. Moreover, although the third measure (i.e. mean number of ants outside the nest) is not significantly different across treatment, this may be due to the low sample size, and the comparison trends in the same direction as the other measures.

I also asked whether differential food deprivation affected the behaviour of ants within a colony. I found that within each colony, fed ants had higher mean distance from the nest than their food-deprived nestmates (Fig. 3.3A). Fed ants also travelled a higher mean distance across consecutive frames (Fig. 3.3B), and I found that, on average, there were more fed ants outside the nest than their food-deprived nestmates (Fig. 3.3C). Thus, according to all three measures, I found that food deprivation decreased the foraging activity of ants both across and within colonies.

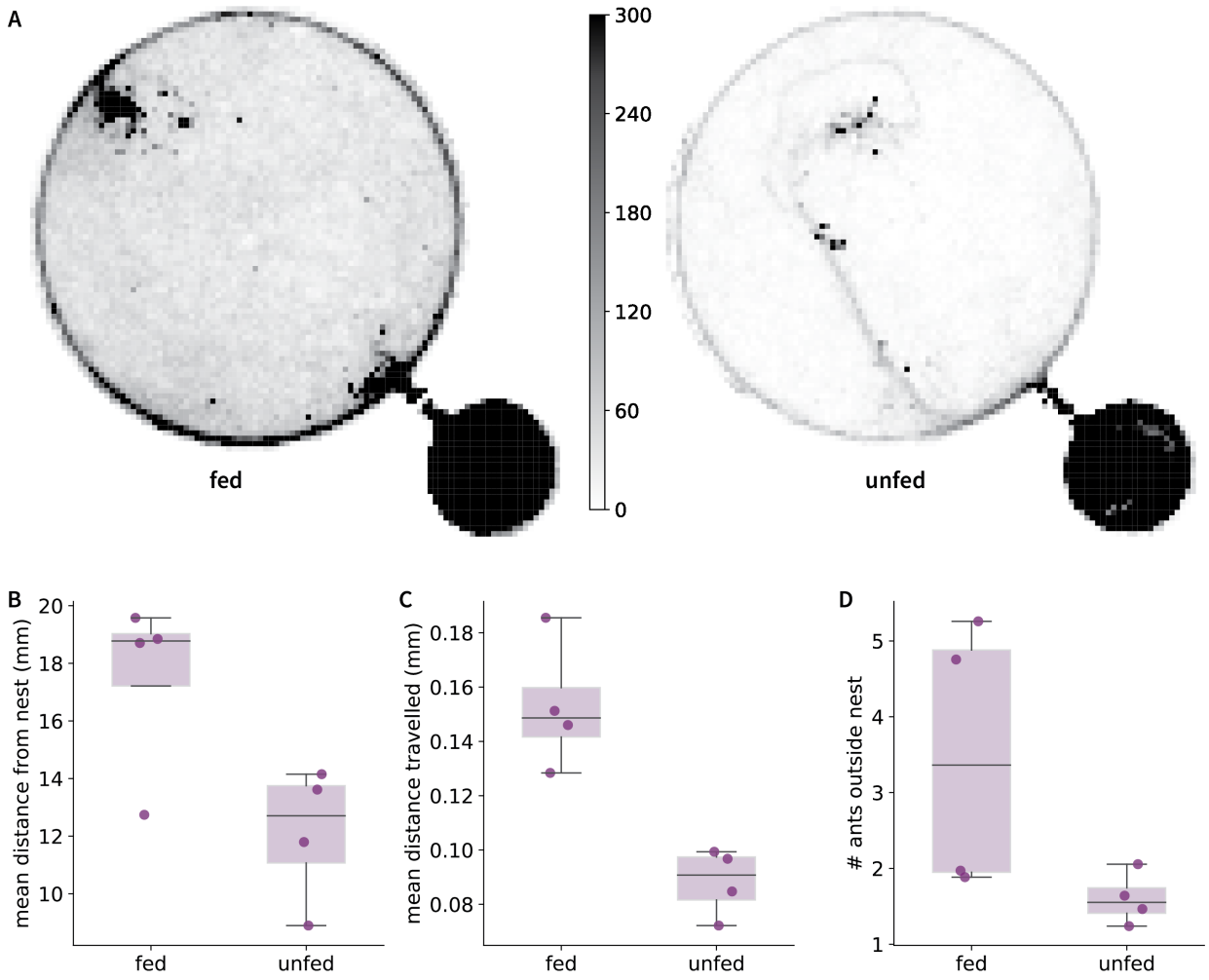


Figure 3.2: Nutritional state affects the foraging behaviour of homogenous colonies. (**A**) A heatmap of the tracked positions of the ants through the timecourse considered for analysis, from one representative colony from each treatment, shows that the colony of fed workers was more active. The scale bar represents the total number of ants detected in each pixel. (**B**) Colonies of fed workers had higher mean distance from the nest than colonies of unfed workers (n = 4, t-test p=0.003), (**C**) travelled a greater mean distance between consecutive frames distance (n = 4, t-test p=0.035), and (**D**) had a higher mean number of ants outside the nest, although this was not significantly different (n = 4, t-test p=0.086).

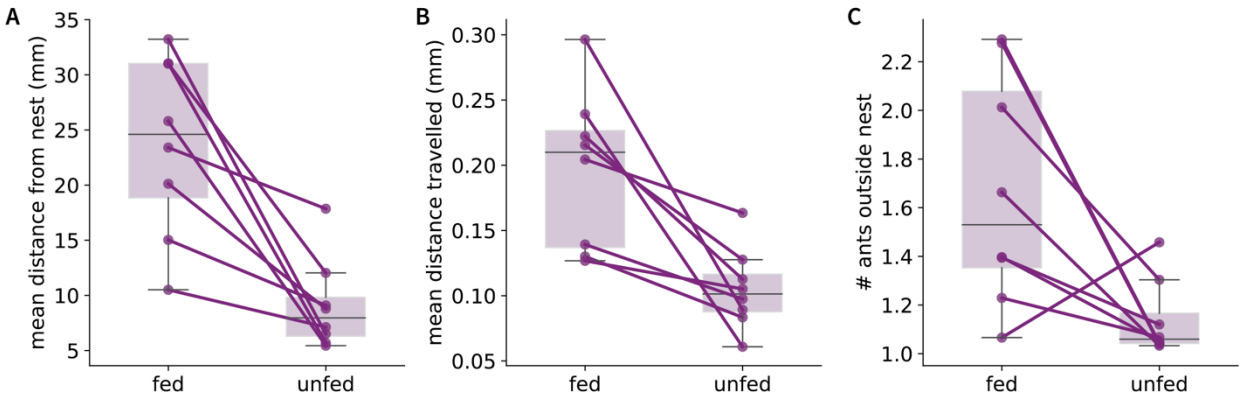


Figure 3.3: Fed ants forage more than their food-deprived nestmates, according to three measures of foraging activity. (**A**) Fed ants have higher mean distance from the nest than their unfed nestmates (n = 8, paired t-test p=0.0029), (**B**) travel further between consecutive frames (n = 8, paired t-test p=0.03), and (**C**) have higher mean number of ants outside the nest (n = 8, paired t-test p=0.007).

3.3: Worker nutritional states through the colony reproductive cycle

To better contextualize our nutritional manipulation experiments, I then asked how the nutritional states (i.e. lipid levels) of the workers in a colony vary over the course of the colony reproductive cycle. For this experiment, I established 16 colonies of 20 ants and 10 larvae each. I set up the colonies when the ants were halfway through their brood care phase (i.e., the ants were 2-3 weeks old). The colonies had access to food virtually throughout the experiment, but they did not forage during their reproductive phase (as expected for *O. biroi*). I cleaned and watered the colonies at regular intervals through the experiment. At four timepoints through the cycle (specifically: on the day that the larvae became pre-pupae, five days later when the ants had all laid eggs, ten days after that when the eggs had all hatched into larvae, and five days after that when the colony was close to the peak of its subsequent brood care phase), I froze all ants from four colonies,

homogenized 16 randomly selected ants within each colony, and extracted and quantified their lipid levels.

I found that workers had the highest lipid levels at the end of the brood care phase (i.e. on the day their larvae became pre-pupae). They then ceased to forage, as expected. Five days later, once the ants had laid eggs, I found that their lipid levels had declined significantly (Fig. 3.4). This decline continued through the entirety of the reproductive phase, during which I did not observe the ants either foraging or feeding. Colonies began to forage roughly four days after the larvae hatched. Quantifying lipid levels six days after the larvae had hatched revealed no further decline in nutritional state (Fig. 3.4), suggesting that the ants were now beginning to eat the food they were retrieving during their foraging. Overall, these data show that the nutritional state of the workers in a colony changes predictably through the colony reproductive cycle, and correlates coarsely with foraging activity. Worker nutritional state appears to be lowest at the start of the brood care phase, and highest at the start of the reproductive phase. However, despite this apparent correlation, the cycles of foraging activity and nutritional state are phase-shifted. Specifically, although the workers' lipid levels decline through the reproductive phase, they do not forage until their eggs hatch into larvae, and although their nutritional state presumably increases through the course of the brood care phase, they continue foraging until their larvae have fully developed.

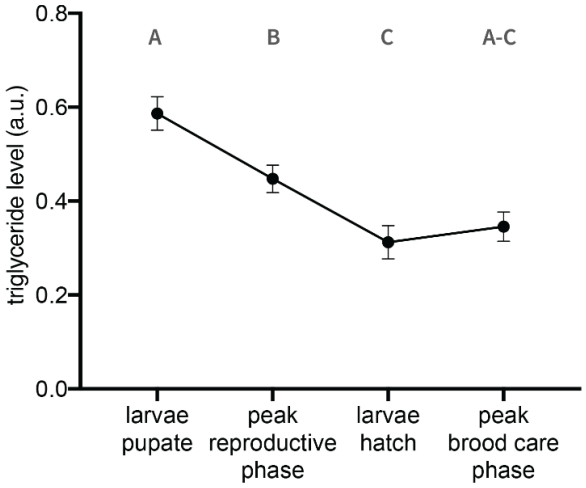


Figure 3.4: Timecourse of lipid levels – a proxy for nutritional state – in adults across the reproductive cycle. During a natural reproductive cycle, workers have maximal nutrition at the end of the brood care phase (when the larvae pupate). They then cease to forage, limiting their access to food. Their nutritional states (represented here by relative TAG levels) continuously decrease through the reproductive phase as they lay eggs but do not eat. This decline only ceases once the eggs hatch into larvae. The larvae then stimulate foraging (between L + 0 and L + 6), upon which the ants once again gain access to food (ANOVA with post-hoc Tukey pairwise comparisons: letters above treatments indicate significant differences at $p<0.05$).

3.4: The effect of worker nutritional state on feeding behaviour

Next, I asked whether the nutritional states of workers affected their feeding – rather than foraging – behaviour. Although foraging and feeding are typically coupled in solitary animals, they may be decoupled in some social insect colonies. To test whether ants fed in proportion to the extent of their food deprivation, I deprived colonies of ants of food for varying amounts of time (i.e., for over a week, for between four and six days, or for less than two days), and gave them access to food for roughly three hours each. I then randomly selected ants from inside the nest, dissected their crops, and quantified

their food consumption. I found that the amount of food ants consumed scaled with the extent of their food deprivation (Fig. 3.5, A and B). In other words, although nutrient deprivation does not cause ants to forage more, it does cause them to feed more.

3.5: The effect of larval signals on worker nutritional state and feeding behaviour

Finally, to ask how larval signals – which induce foraging behaviour - influence worker nutritional state, I performed a 'brood-swap' experiment (Libbrecht *et al.*, 2018). I established 16 experimental colonies of 20 one-month old workers each. All workers were sourced from a single stock-colony that was about to enter the reproductive phase. Half the colonies received 16 five-day old larvae, while the other half did not. Three days later, I froze all colonies, homogenized all adults in each colony, and quantified their lipid levels. I found that ants that had been in contact with larvae for three days did not have significantly different lipid levels to ants that had not been in contact with larvae (Fig. 3.5C).

I then asked whether larval signals affect worker feeding behaviour. To test this, I performed another brood-swap experiment. From a single stock-colony entering the reproductive phase, I established five experimental colonies of 16 one-month old workers each. Half the colonies received 15 six-day old larvae, while the other half did not. Three days later, all colonies were given frozen *S. invicta* pupae infused with 0.5% bromophenol blue for roughly three hours, after which ants were dissected and their feeding was quantified by measuring crop surface-area. I found that ants that had been in contact with larvae for three days did have significantly different crop sizes than ants that had not been in contact with larvae (Fig. 3.5D). Qualitative observations of the hindguts of ants

suggested that there could be a minor increase in total food content in the guts of ants exposed to larvae. However, I could not reliably quantify hindgut food content in my assay, and I did not observe this apparent difference in my previous experiment (i.e., when I measured the effects of food deprivation on feeding behaviour). Together, these data suggest that larvae do not detectably reduce adult nutritional state, and do not substantially (if at all) alter adult feeding behaviour. Thus, larvae induce foraging behaviour but not feeding behaviour, while decreasing nutritional state induces feeding behaviour but not foraging behaviour.

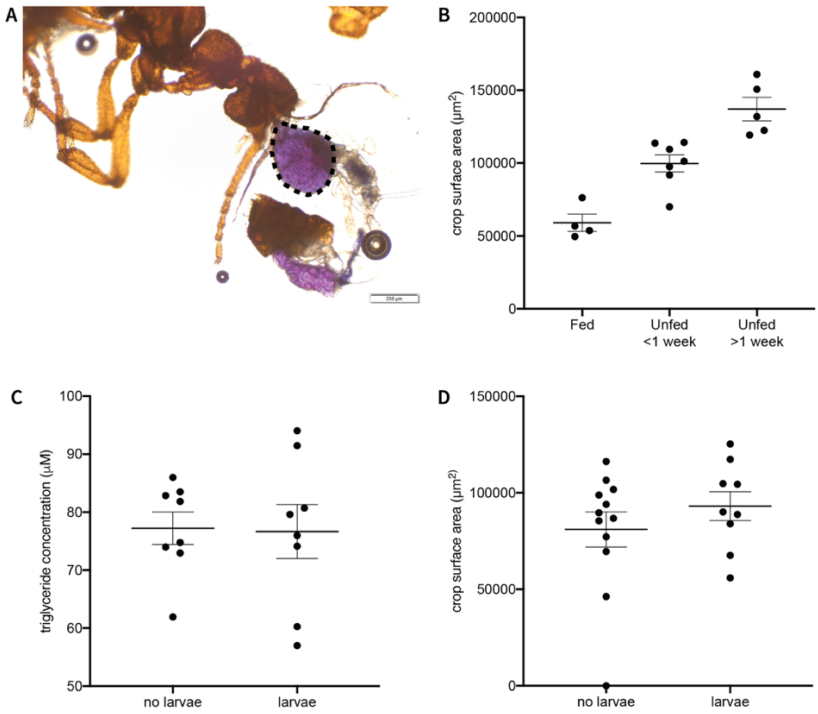


Figure 3.5: Worker nutritional state determines feeding behaviour. (**A**) Lateral view of a dissected *O. biroi* worker that has eaten dyed food. The crop is circled in black. The scale bar represents 2mm. (**B**) The extent to which an ant is starved determines how much food she eats - measured as the surface area of the largest cross-section of the crop ($n \geq 4$, ANOVA with post-hoc Tukey pairwise comparisons all $p < 0.0036$). (**C**) The presence of larvae does not affect the nutritional state of adult workers ($n = 8$, t-test $p = 0.91$), and (**D**) does not affect the amount of food they eat ($n \geq 8$, t-test $p = 0.34$).

3.6: On the control of foraging and feeding behaviour, and its evolution

Overall, these data show that, at least under my experimental conditions, *O. biroi* workers forage less – not more – when they are deprived of food for ten days. It is possible that there are other experimental conditions under which *O. biroi* workers respond differently to food deprivation. For instance, it is conceivable that multiple months of starvation may induce more foraging, or some qualitatively different response. Whilst this is a possibility, my anecdotal observations of the behaviour of colonies starved for multiple months suggest that their activity remains suppressed as their nutritional states decrease.

To the best of my knowledge, there are only three previous reports of food deprivation in ant colonies decreasing foraging activity. (Rueppell and Kirkman, 2005) found that overall activity in *Temnothorax rugulatus* decreased as a function of the extent of starvation. However, (Shaffer, 2014) reports that the workers of this species actually become more active with increased starvation. (Fourcassié *et al.*, 2003) report that *Messor sanctus* workers walk slightly slower when deprived of food. And (Dejean, 1986) found that colonies of *Strumigenys* workers, when starved for over ten days, ceased all activity. Note that these three types of ants are all within the same clade – the lineage that contains the subfamilies Myrmicinae and Formicinae. However, experiments on multiple other species in these subfamilies, as well as from species in the Ectatomminae, Dolichoderinae, and Ponerinae, found that food deprivation increases foraging activity (Bazazi *et al.*, 2016; Bernadou *et al.*, 2018; Bernadou *et al.*, 2020; Fowler, 1980; Greenwald *et al.*, 2018; Hölldobler, 1971; Howard and Tschinkel, 1980; Mailleux *et al.*, 2006; Mailleux *et al.*, 2010; McGrannachan and Lester, 2013; Traniello, 1977; von Thienen and Metzler, 2016; Wallis, 1962). These subfamilies span most of the ant phylogeny (Borowiec *et al.*, 2019). Moreover,

depriving honeybee and paper wasp workers of food also increases their foraging activity (Daugherty *et al.*, 2011; Mayack and Naug, 2013; Toth and Robinson, 2005; Toth *et al.*, 2005). Together, these data suggest that the ancestral ants were also likely to have increased foraging activity in response to food deprivation, and that *O. biroi*'s lack of deprivation-induced-foraging is a novelty within the ants – a trait that evolved after the origin of ant superorganismality.

In any case, my results show that worker nutritional states vary in the course of the colony reproductive cycle, that decreases in nutritional state during the reproductive phase is not sufficient to increase foraging, and that increases in nutritional state during the brood care phase is not sufficient to reduce foraging. Instead, as previously demonstrated (Ulrich *et al.*, 2016; Ulrich *et al.*, 2018), the presence of larvae in the colony largely determines foraging behaviour. My results agree with this finding. When the larvae in the colony pupate (i.e., more specifically, when they eject their meconium and become pre-pupae), the workers largely cease foraging, only resuming when there are once again larvae in the colony. Thus, although experimentally varying worker nutritional state clearly alters their foraging activity (at least in our experimental conditions), this is unlikely to play a substantial role in the regulation of foraging. Instead, larval signals constitute the primary determinant of *O. biroi*'s foraging behaviour.

Why might *O. biroi* foraging be regulated this way? One possibility is its unusual foraging lifestyle. My measurements of foraging and feeding behaviour over the course of the colony reproductive cycle show that *O. biroi* colonies undergo multiple weeks of food deprivation every time they enter the reproductive phase. In the wild, *O. biroi*, like other dorylines, forages in group raids, where the ants collectively raid neighbouring ant colonies and steal their prey (see next chapter). Indeed, many doryline ants exhibit reproductive cycles that are correlated with cycles of foraging effort (Borowiec, 2016;

Gotwald, 1995; Schneirla, 1971). This behaviour requires synchrony among the ants, since raids are likely only successful when performed with coordination (Garnier and Kronauer, 2017; Teseo and Delloro, 2017). Thus, one could reasonably propose that this explains why *O. biroi* only forage in the brood care phase, and why they undergo regular periods of food deprivation. I speculate that this may also explain why larval signals – which constitute a much more effective synchronization mechanism - are the primary determinant of foraging behaviour in many doryline ants.

Finally, although *O. biroi*'s nutritional state does not substantially determine its foraging behaviour, it has retained its ancestral function of regulating feeding behaviour. I found that depriving workers of food causes them to eat more. I also found that larval signals do not reduce the workers' nutritional states, and do not significantly increase their feeding behaviour. Together, my data suggest that foraging and feeding behaviour – which are typically coupled in solitary animals – have been decoupled in *O. biroi*, and are likely regulated largely independently instead. This evolutionary process is analogous to the evolution of centralized regulatory machinery for the control of foraging behaviour in solitary animals. In both cases, the members of the new individual (i.e., cells in the case of some multicellular animals, and workers in the case of some ant colonies) have evolved to use social information to assess the hunger of the individual. In many animals, specific neurons detect nutrient levels in the circulation to assess the animal's hunger, but cells typically 'feed' (i.e., take in circulating nutrition) in proportion to their own nutritional requirements. Similarly, *O. biroi* workers use larval signals as an analogous, centralised measure of colony hunger, but they, like animal cells, feed in proportion to their own hunger. Further work is required to understand how this decoupling occurred over evolutionary time, when larval signals first began to regulate

worker foraging behaviour, and how the mechanisms by which workers assess colony hunger have evolved within the ants.

CHAPTER 4: COLONY EXPANSIONS UNDERLIE THE EVOLUTION OF ARMY ANT MASS RAIDING

Many animal groups, from wildebeest herds to starling murmurations, display complex collective behaviours that emerge from the interactions of individual group members independently following a common set of behavioural rules (Camazine *et al.*, 2001). Ant colonies are no exception, performing some of the most striking feats of coordination known to biologists. For instance, leafcutter ants use an assembly line of foragers, organised in a sequence of discrete morphological castes, to retrieve leaves from trees and to process them before they can be used to fertilise their fungus gardens (Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2011). Weaver ants work in teams to glue leaves together, using silk secreted by their larvae, to build elaborate arboreal nests (Anderson and Franks, 2001; Crozier *et al.*, 2009; Weinstock *et al.*, 2006). And as I mentioned in Chapter 1, army ants raid prey social insect colonies in enormous, tactically sophisticated mass raids. While the members of wildebeest herds or bird flocks or fish shoals participate in these collective movements for selfish reasons (Hamilton, 1971), the collective behaviour of ant workers is altruistic. Many of these behaviours – especially variants of foraging behaviour – are dangerous, and ant workers frequently die in the field (Dejean *et al.*, 2013; Giraldo and Traniello, 2014; Porter and Jorgensen, 1981). This sort of altruistic behaviour is only possible because these colonies behave as individuals; because the fitness interests of the workers are highly aligned with those of the queen.

How such emergent collective behaviours evolve, however, is an open question – in both selfish and altruistic groups of animals. One possibility is that natural selection acts on the neural substrate that encodes the underlying behavioural rules. Across species of social insects, for example, workers may respond differently to local cues during nest

construction, which could translate into different nest architectures (Mizumoto *et al.*, 2019; Theraulaz and Bonabeau, 1995). Such behavioural rules can evolve rapidly, as has been demonstrated via artificial selection experiments on collective movement in guppies (Kotrschal *et al.*, 2020). In principle, an alternative way to modify collective behaviour is to alter group-level parameters, such that the same behavioural rules lead to different collective outcomes. For instance, golden shiners form polarized swarms or milling schools depending on their group size (Tunstrøm *et al.*, 2013). Whether this mechanism is relevant over evolutionary timescales, however, remains unknown. Here I show that army ant mass raiding, one of the most iconic collective phenomena, has evolved from scout-initiated group raiding, and propose that this evolutionary transition in collective behaviour was driven substantially by an increase in colony size, rather than changes in the ants' own behaviour.

Army ants in the subfamily Dorylinae live in huge colonies that contain 10^4 – 10^7 workers, depending on the species. They hunt live arthropods, often other ants, in mass raids (Borowiec, 2016; Gotwald, 1995; Kronauer, 2009; Schneirla, 1971) (see Table 4.1 for definition). Mass raids begin when workers spontaneously and synchronously leave the nest in "pushing parties" (Leroux, 1977; Schneirla, 1933; Schneirla, 1971). At first, small groups of workers hesitantly leave the nest to explore its immediate vicinity. They lay trail pheromone as they walk, returning after only a few steps out. Ants continue to leave the nest, walking further and further out, confidently following their predecessors' trail. When they reach untrodden ground, they also hesitate and turn, spreading outwards along the raid front. Over time, this leads to a dynamic fan of ants traveling outwards, leaving a strong, elongating trail back to the nest in its wake (Leroux, 1977; Schneirla, 1933; Schneirla, 1971). In the species with the largest colonies, the ants at the raid front can be so numerous that the raid advances as a swarm (Schneirla, 1971). At the outset,

the ants have no information about prey location. However, a few scouts search slightly ahead of the raid front, and when they encounter prey, they lay pheromone trail back to the raid front and recruit nestmates for a collective attack (Chadab and Rettenmeyer, 1975). While army ants themselves have been studied extensively (Gotwald, 1995; Kronauer, 2009; Schneirla, 1971), little is known about their cryptic relatives with much smaller colony sizes. Sporadic and usually partial observations suggest that many non-army ant dorylines conduct scout-initiated group raids, in which scouts find prey before recruiting a raiding party (Hölldobler, 1982; Hölldobler and Wilson, 1990). It has therefore been suggested that army ant mass raiding might have evolved from scout-initiated group raiding (Gotwald, 1995; Hölldobler and Wilson, 1990; Wheeler, 1918; Wilson, 1958a; Wilson, 1958b). However, as these species are rarely encountered, no quantitative description of this behaviour is available, a formal evolutionary analysis of foraging behaviour in dorylines is lacking, and the functional relationship between group raiding and mass raiding is unknown.

Table 4.1: Comparison between the two different types of known foraging behaviour in the Dorylinae. Following historical precedent (Berghoff, 2002; Wilson, 1958a; Witte, 2001), I use the terms 'group raid' and 'mass raid' to distinguish two syndromes of raiding behaviour. This table identifies four distinguishing features of each type. Although an ant colony could in principle have a combination of 'group raid' and 'mass raid' features, in practice, such colonies have not been observed in nature (see Appendix B).

(*) Initiation of mass raids is 'spontaneous' only with respect to the discovery of prey. In above-ground species, mass raids are sometimes initiated by sunrise, or follow an apparently circadian rhythm (Rettenmeyer, 1963; Schneirla, 1971; Topoff *et al.*, 1980).

(**) recruitment outside the nest may be followed by further recruitment inside the nest– this classification is hierarchical.

Type	**Raid initiation**	**Initial site of recruitment**	**Number of ants participating in raid**	**Trail bifurcations**
Group raid	Scout-initiated	Inside nest	Dozens to hundreds	No
Mass raid	Spontaneous*	Outside nest (at raid front)**	Thousands to millions	Yes

4.1: The structure of a doryline group raid

I conducted the first study of foraging behaviour in the clonal raider ant, *Ooceraea biroi*. The clonal raider ant is a non-army ant doryline and the only doryline that can be propagated in the laboratory. In the effort to establish this species as an experimental model, high-throughput, automated tracking approaches to monitor individual and collective behaviour have been developed (Gal *et al.*, 2020; Ulrich *et al.*, 2018). This created the unique opportunity for me to study doryline foraging behaviour quantitatively and under controlled laboratory conditions. In a first experiment, I set up nine colonies each of 25 individually tagged ants, and filmed and tracked their foraging behaviour while

offering them a single small fire ant pupa once every twelve hours (for experimental details see Chapter 6). Overall, I analysed tracking data for 31 raids. I found that *O. biroi*, like other non-army ant dorylines, forages in scout-initiated group raids (for ant foraging terminology see Table 4.1). I decompose group raids into six distinct phases (Fig. 4.1 and Fig. 4.2; see Chapter 6 for details). First, in the 'search' phase, one or a few scouts explore the arena.

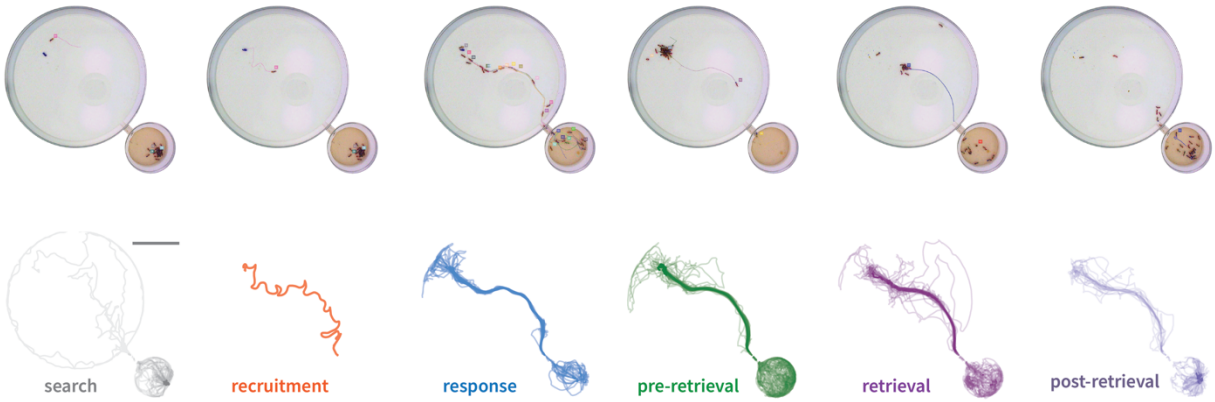


Figure 4.1: The anatomy of a group raid. Snapshots and trajectories of ants at each phase of a representative group raid, separated into six sequential phases. The snapshots show that a short tunnel separates the nest (small circle in lower right of each snapshot) from the foraging arena (large circle in upper left), and the food (blue spot) is at the top right in the first snapshot. The orange track in the 'recruitment' phase depicts the path taken by the recruiting ant, whereas tracks in all other phases depict the paths of all ants in the colony, irrespective of their task. The scale bar represents ~2 cm.

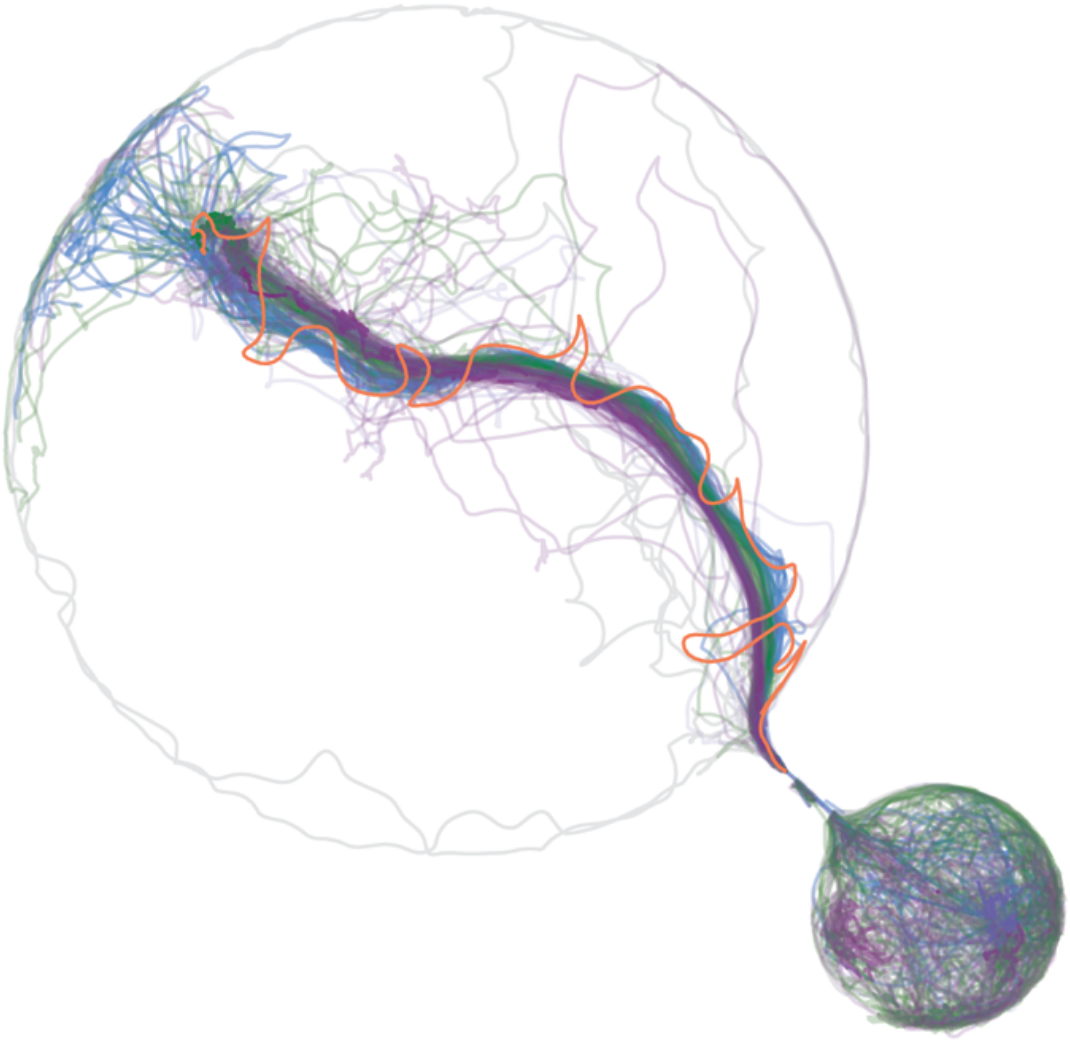


Figure 4.2: Overlay of trajectories from all six phases. The colours here are the same as in the previous figure, and demonstrate that the ants in the response phase (in blue) follow the path of the recruiter (in orange), after which ants continue to reinforce this trail until the end of the raid event.

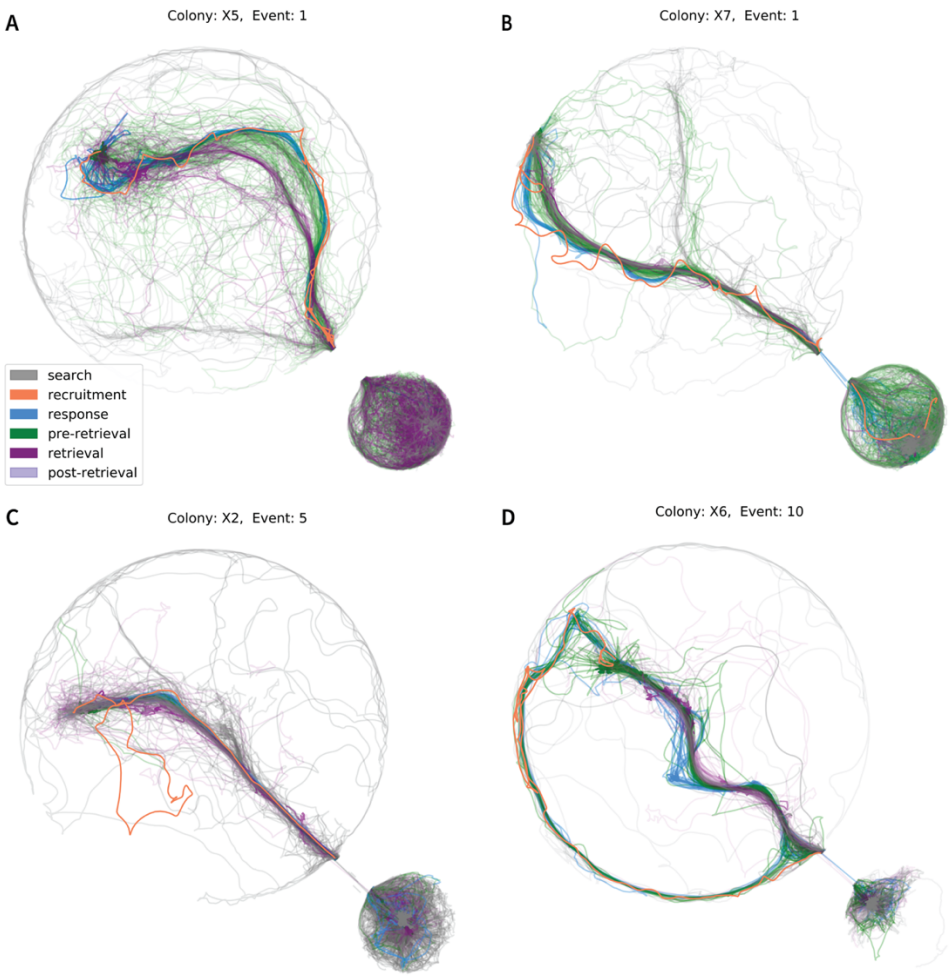


Figure 4.3: Additional examples of raids. These show that the coarse structure of the raid is similar across events, despite differences in details. For instance, in (**A**), the trail shortens discretely during the pre-retrieval phase (with continuous trail shortening in this phase evident in all events). (**B**) is fairly typical. In (**C**), the scout's trajectory homewards is somewhat meandering, looping over itself. However, the responding ants ignore this loop. In (**D**), the response trail bifurcates, with most ants not following their recruiter's path to the food. Careful observation of this event suggests that two ants laid trail pheromone from the food to the nest simultaneously. The scout is defined as the ant that actually initiates the response – i.e., the first ant to enter the nest. When the ants leave the nest in response to her recruitment, they encounter the second recruiting ant near the nest entrance, who appears to have laid the trail that they then largely follow to the food. This event illustrates that colonies of 25 ants may have multiple scouts that do not appear to coordinate their behaviour.

Once a scout has discovered food, she examines it briefly before becoming highly excited. In the 'recruitment' phase, she runs homeward, and as she enters the nest, the ants inside become active. In the 'response' phase, a large proportion of ants inside the nest run towards the scout, exit the nest in single-file, and move towards the food, retracing the scout's homeward trajectory. Most ants then stay on or near the food for a few minutes, while some run back and forth between the food and the nest, which I call the 'pre-retrieval' phase. Next, during the 'retrieval' phase, one to three ants begin to independently drag or carry the food back home, with no apparent help from their nestmates. Finally, in the 'post-retrieval' phase, the remaining ants return to the nest. Raids vary in specific details of their spatial organisation (Fig. 4.3), as well as in their duration (Fig. 4.4A). Variation in the length of the pre-retrieval phase explains most variation in raid length, but its function is currently unknown (Fig. 4.4B).

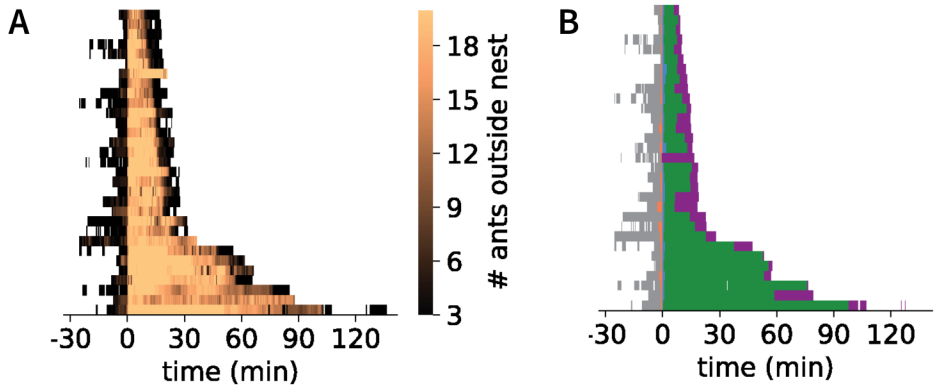


Figure 4.4: The temporal structure of group raids. (**A**) Heatmap showing the number of ants outside the nest over time. 31 raids are sorted vertically by their duration and are aligned to the start of recruitment. (**B**) Representing each phase of each raid by the same colour code as in the previous figures shows that raids vary in length, and that this variation appears to be primarily determined by the length of the pre-retrieval phase. I do not show the 'post-retrieval' phase here, because it has constant length by definition.

To visualise the temporal structure of these raids, I aligned and rescaled each phase of each raid, and quantified three informative features: the number of ants outside the nest, the mean distance from the nest, and the sum of the speeds of all ants (Fig. 4.5). These measures all show that despite variation in spatial and temporal details, the general structure of group raids is highly stereotyped.

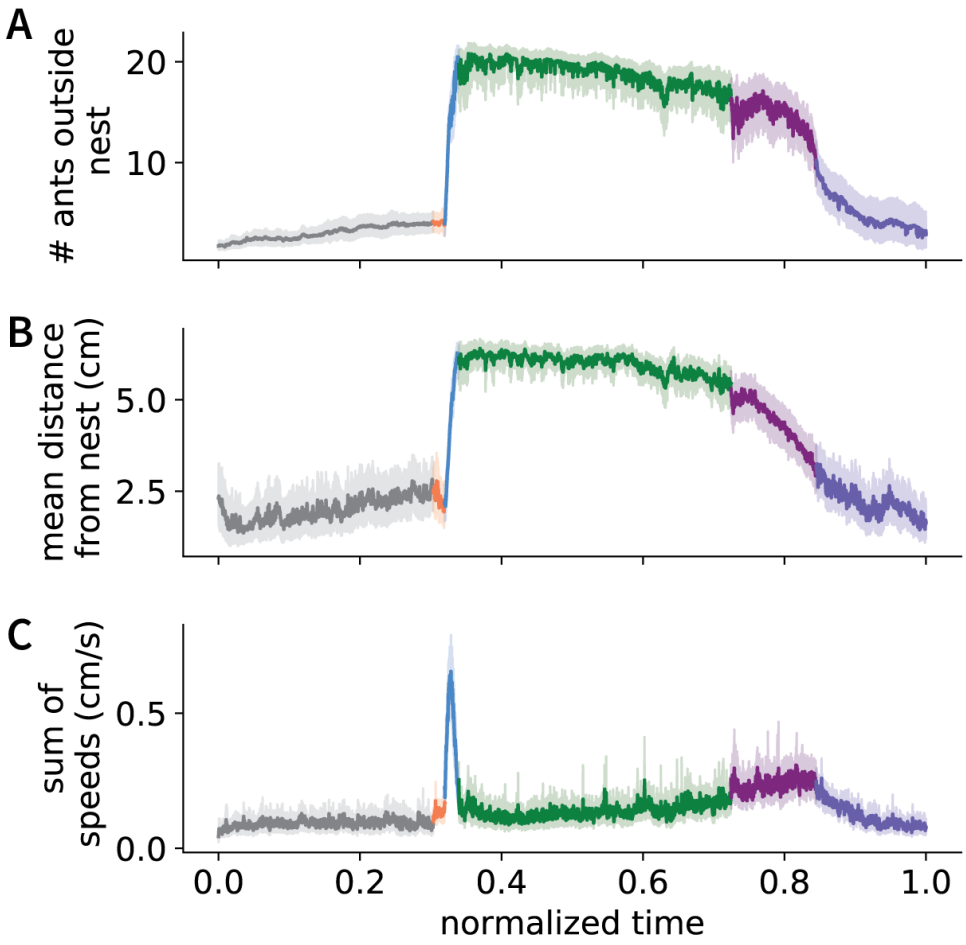


Figure 4.5: Group raids are stereotyped. Aligning and rescaling each phase of each raid (see Chapter 6 for details) and plotting the timecourse of the mean number of ants outside the nest (**A**), their mean distance from the nest (**B**), and the sum of the speeds of all ants (a measure of collective activity) (**C**), shows that the temporal structure of group raids is highly stereotyped. The error bands represent the 95% confidence interval of the mean.

4.2: The determinants of spatial and temporal structure in group raids

What determines this structure in group raids? Based on my own observations, as well as previous work on army ants and two distantly related non-army ant dorylines (Chadab and Rettenmeyer, 1975; Gobin *et al.*, 2001; Hölldobler, 1982; Schneirla, 1971), I hypothesized that at least two distinct, scout-derived signals determine the spatial and temporal structure of group raids. First, I asked how the scout activates nestmates. I conducted an experiment in a modified arena that had a porous wall in the middle of the nest chamber, and separate foraging arenas connected to each nest half (Fig. 4.6A). In each trial, food was placed in one foraging arena, and when a scout with access to that arena located the food, she recruited the ants in her nest half, who formed a column that travelled to the food. Shortly after the scout entered the nest, the ants in the other nest half moved towards the wall separating the two halves (Fig. 4.6, A and B). This suggests that the scout releases a volatile, attractive recruitment pheromone as she enters the nest, rather than activating nestmates by touch, a contact pheromone, or an undirectional volatile pheromone that signals nestmates to exit the nest chamber without conveying spatial information. Second, I asked whether the scout lays a pheromone trail back to the nest during recruitment, and whether that trail is sufficient to guide the responding ants. Scout-initiated raiding has evolved independently on a few occasions in other ant subfamilies, and in several cases the scout is required to lead the raiding party to the target. In other words, here, information about target location resides primarily in the scout, rather than in a pheromone trail (e.g. (Bayliss and Fielding, 2002; Grasso *et al.*, 1997; Longhurst *et al.*, 1979; Mill, 1984; Topoff *et al.*, 1984)). I found that, in *O. biroi*, the scout usually (in 30/31 raids) does not lead the raiding column (Fig. 4.6C). However, the trajectories of the responding ants closely recapitulate the homebound trajectory of the

scout, suggesting that the scout indeed deposits trail pheromone on her way to the nest (Fig. 4.6D). Information about prey location therefore resides exclusively in the scout's trail. This use of pheromones is very similar in two other distantly-related doryline group raiders (Gobin *et al.*, 2001; Hölldobler, 1982), and moreover, is highly reminiscent of recruitment at the raid front in army ant mass raids (Chadab and Rettenmeyer, 1975). Together, this suggests that group- and mass-raiding dorylines all use chemical information in the same way. Given that these group raiders are the closest relatives to army ants, this also suggests that these behaviours – and specifically, the rules for chemical communication - might be homologous.

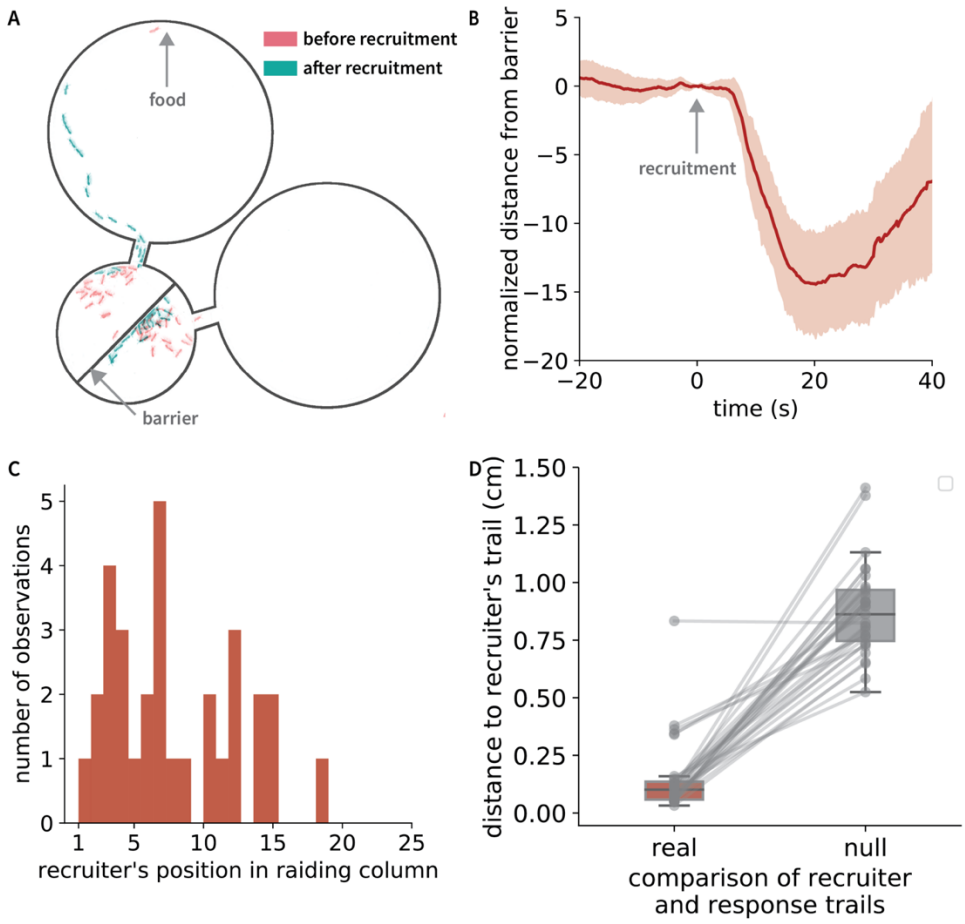


Figure 4.6: A trail and a recruitment pheromone determine the spatial and temporal structure of group raids, respectively. (**A**) The recruitment pheromone is volatile and attractive. The image shows a modified nest with a porous barrier down the middle. On the left side, a scout releases recruitment pheromone, causing the ants to leave the nest. The ants on the right side, meanwhile, run towards the barrier instead of leaving the nest. (**B**) The distance between the barrier and the centre of mass of ants on the side opposite to that of the scout as a function of time since recruitment. The centre of mass travels towards the barrier after recruitment, which shows that the recruitment pheromone is both volatile and attractive (n = 31 raids, error band shows 95% CI of the mean). (**C**) A histogram of the scouts' position in the raiding column shows that scouts do not typically lead raids. (**D**) The outbound trajectories of responding ants are significantly closer to their scout's inbound trajectory than they are to control trajectories of scouts in other group raids, showing that the responding ants indeed follow *their* scout's trail to the food (n = 31 raids, paired t-test $p<10^{-6}$).

4.3: The evolution of doryline raiding behaviour

To systematically understand the evolution of mass raiding, I combined my data on *O. biroi* with published descriptions of doryline biology, and mapped relevant life history traits to a new consensus phylogeny of the Dorylinae (Borowiec, 2019). Maximum likelihood and maximum parsimony ancestral state reconstructions (see Chapter 6 for details) suggest that the ancestral dorylines lived in small colonies, were specialist predators of ants, and indeed conducted scout-initiated group raids (Fig. 4.7; Table 4.2; Appendix B and C). This supports the hypothesis that army ant mass raiding evolved from group raiding as colony size increased, possibly independently in the New World and Old World army ants (Hölldobler and Wilson, 1990; Wilson, 1958a; Wilson, 1958b). It also implies that *O. biroi* might provide mechanistic insight into how these transitions occurred.

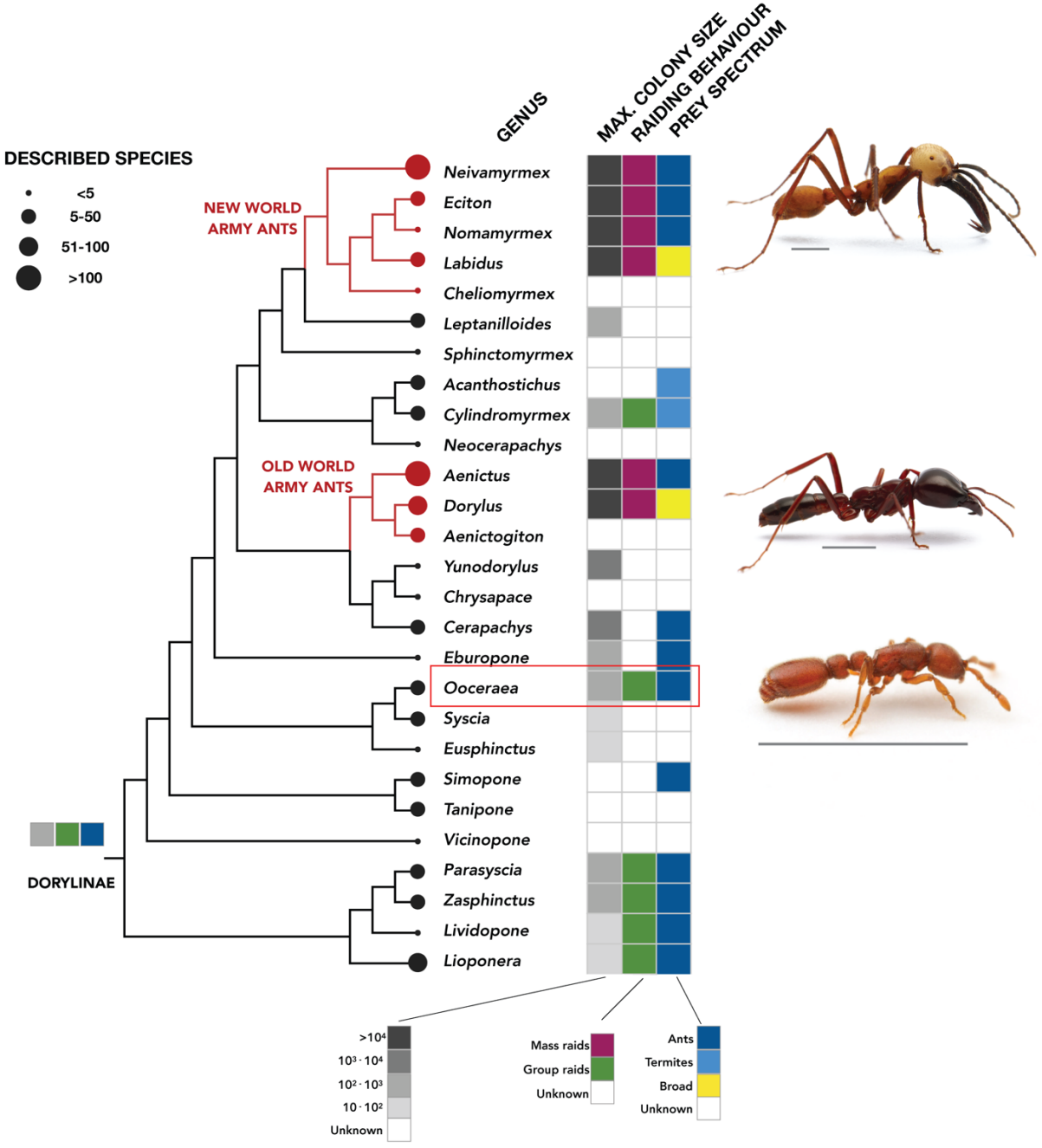


Figure 4.7: Phylogeny of the Dorylinae, showing all extant genera, along with maximum colony size, type of raiding behaviour, and prey spectrum, where known. Ancestral reconstructions on a consensus cladogram (Borowiec, 2019) are shown at the base of the tree (see Appendix C and Chapter 6). Photographs from top to bottom show workers of the army ants *Eciton burchellii* and *Dorylus molestus* (photographs © Daniel Kronauer), as well as the clonal raider ant *Ooceraea biroi* (highlighted by a red box; photograph © Alexander Wild). The scale bars represent ~2mm.

Table 4.2: Proportional likelihoods of each character state for the most recent common ancestor (MRCA) of dorylines from a one-parameter maximum likelihood reconstruction. Most parsimonious states from maximum parsimony reconstructions are shown in blue text.

	Colony size		Raiding behaviour		Prey spectrum	
Doryline MRCA	Small (<5 * 10^4)	0.994	Group raiding	0.93	Ants	0.991
	Big (>5 * 10^4)	0.006	Mass raiding	0.07	Termites	0.004
					Broad	0.004

4.4: How did group raids evolve into mass raids?

To understand the evolutionary transition between group and mass raids, I first considered both to possess the same six-phase structure that I have defined for *O. biroi*'s raids. Since these are homologous behaviours, this is a valid way to view the structure of doryline raids. Moreover, in both group and mass raids, roughly the same sequence of events takes place. Specifically, they both begin with ants searching for food, involve scouts recruiting nestmates to specific targets, involve workers responding to recruitment collectively, and involve workers carrying food back to their own nest before the raiding party returns home (Chadab and Rettenmeyer, 1975; Hölldobler, 1982; Topoff *et al.*, 1980). Thus, each phase of the raid is also homologous between group and mass raids. Intuitively, one might compare the response phase of a group raid with the onset of a mass raid, because these are superficially similar: they both represent columns of ants streaming out of the nest. However, considering the mass raid to have the same sequence of phases as the group raid shows that the onset of a mass raid is actually homologous to the search phase of a group raid (Fig. 4.8). I therefore asked whether *O. biroi* scouts follow

the same basic behavioural rules in their search phase that translate into spontaneous pushing parties in mass raiding army ants.

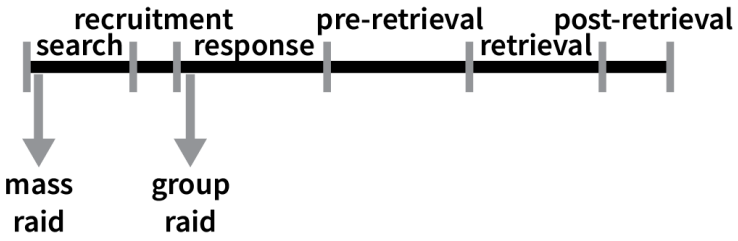


Figure 4.8: Homology in the phases of raids across the Dorylinae. The onset of a mass raid is homologous to the search phase of a group raid, and not to the response phase, despite the superficial resemblance.

4.5: The spatial structure of search in a group raid

First, I analysed our tracking data from colonies of 25 workers to see whether ants incrementally increase their foraging distance by extending previously travelled paths. I found that *O. biroi* often (in 21/31 raids) search an arena that is initially void of trail pheromone in serial excursions (see Chapter 6 for analysis details). Further analysis of these excursions revealed that, on average, early excursions terminate close to the nest, while later excursions terminate farther away (Fig. 4.9A). Additionally, ants walk faster (Fig. 4.9B) and spend longer outside (Fig. 4.9C) in later excursions, and excursions typically begin with trail-following and end with a period of trail-extension prior to reversal (Fig. 4.9D). This behaviour of individual *O. biroi* scouts is highly reminiscent of army ant behaviour at the raid front. In other words, the *O. biroi* scouts' search behaviour is spatially non-random in the specific manner of mass raids.

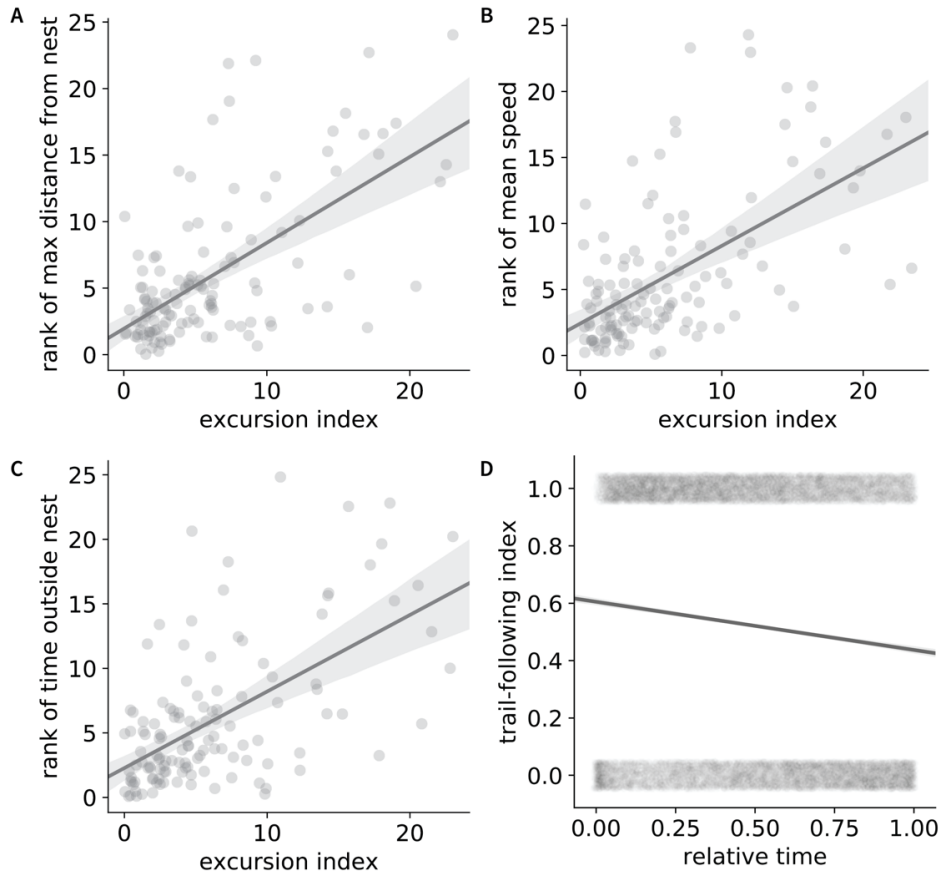


Figure 4.9: *O. biroi* workers in colonies of size 25 search in serial excursions that resemble pushing parties. (**A**) On average, early excursions terminate closer to the nest than later excursions (linear regression $p<0.0001$). (**B**) On average, ants travel faster in later excursions (linear regression $p<0.0001$). (**C**) On average, ants spend longer outside the nest in later excursions (linear regression $p<0.0001$). In panels (A – C), each datapoint represents a single excursion. The y-axis represents the rank of a property of each excursion, and the x-axis represents the index of the excursion - i.e., the number of times an ant has previously conducted excursions during that search phase. (**D**) On average, ants are more likely to follow trail at the beginning of the outbound leg of each excursion than at the end (linear regression $p<0.0001$). Excursions are aligned against each other so that they have the same duration (represented by the x-axis), and the y-axis represents a binary trail-following index (see Chapter 6 for details). Each datapoint represents this trail-following index of a specific ant at a specific timepoint during each outbound excursion leg. In all four panels, the grey line represents the linear regression line of best fit, while the error band around it represents the 95% confidence interval of the mean.

4.6: The temporal structure of search in a group raid

Unlike in army ants, where workers leave the nest *en masse* to go on a raid, *O. biroi* workers typically leave the nest individually during the search phase (Fig. 4.5A). However, this does not exclude the possibility that non-army ant dorylines have mechanisms to synchronize spontaneous nest exits even in the absence of scout-mediated recruitment. To study the temporal structure of search in *O. biroi*, I conducted an experiment with four colonies of size 20, recorded each time an ant exited the nest, and analysed the resulting inter-exit interval distributions (Fig. 4.10A and B; see Chapter 6 for analysis details). To control for the possibility that ants behave differently when food is in the arena, I specifically selected periods when the arena was empty (i.e., the ca. 20 hours after each foraging event each day, resulting in a total of 43 distributions). By comparing the distribution of intervals between subsequent exits to a null distribution (i.e., an exponential distribution expected if the exits occurred in a random, Poisson process), I found that 37/43 distributions deviated significantly from the random expectation. Inspecting the deviations between random and real distributions showed that *O. biroi* workers leave the nest in quick succession more often than expected by chance (Fig. 4.10C). Thus, search in *O. biroi* is both spatially and temporally non-random. Taken together, this suggests that the basic behavioural rules underlying search, recruitment, and response are conserved between army ants and their non-army ant relatives.

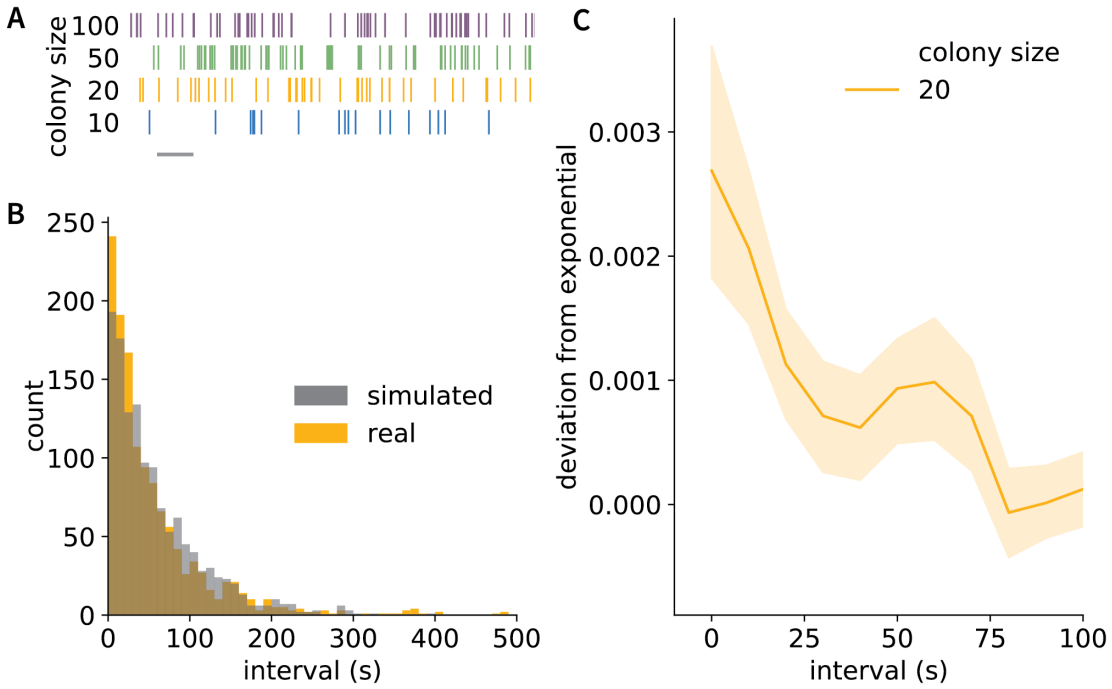


Figure 4.10: Burstiness in spontaneous nest exits. (**A**) Four example sequences of nest exit times, sorted by colony size. The scale bar (in grey) depicts 2 min. (**B**) An example distribution of inter-exit intervals in a colony of size 20. This distribution (in amber) deviates significantly from a simulated exponential distribution (in grey) (Anderson-Darling k-sample test $p<0.001$). (**C**) The deviation in frequency from the theoretical exponential curve for all inter-exit interval sequences from colonies of 20 ants is highest at very short intervals (n = 43 sequences). The amber line represents the mean deviation in frequency, and the error band represents the 95% confidence interval of the mean.

4.7: The effects of colony size on the structure of search behaviour

Army ants live in much larger colonies than non-army ant dorylines, and expansions in colony size within the Dorylinae align perfectly with the evolutionary transition to mass raiding behaviour (Fig. 4.7). Thus, I wondered whether the striking differences between group raiding and mass raiding might simply emerge as a function of colony size. To test this, I established *O. biroi* colonies with 10, 50, or 100 workers,

alongside the colonies of 20 workers described above. Although these colony sizes do not approach those of army ants, this experiment is nonetheless informative regarding the general scaling effects of colony size. As expected, the number of ants participating in the raids increased with colony size (Fig. 4.11). Moreover, across all colony sizes, inter-exit interval distributions typically differed from the random expectation in the same fashion (Fig. 4.12). To test whether ants left the nest in more coordinated bursts as colony size increased, I used detrended inter-exit interval sequences to calculate an autocorrelation-based 'coordination index' of ants leaving the nest, which measures non-randomness of search behaviour and is independent of colony size (see Chapter 6 for analysis details; fig. 4.13A). This index increased markedly as a function of colony size (Fig. 4.13B). Thus, as colony size increases, search behaviour in *O. biroi* begins to resemble the onset of highly bursty, coordinated army ant mass raids. Anecdotally, I also observed events in colonies of ≥50 ants where the spatial and temporal correlations were so strong that they began to qualitatively resemble army ant mass raids. In these events, no apparent recruitment occurred inside the nest. Instead, ants spontaneously formed a column that travelled away from the nest, headed by an obvious pushing party. These observations suggest that, in principle, mass raids could emerge from group raids with increasing colony size.

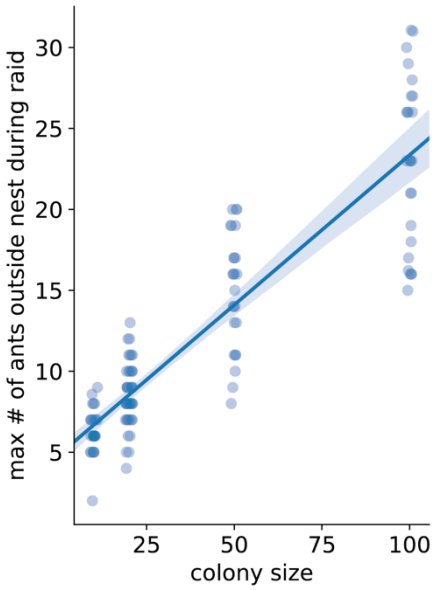


Figure 4.11: The estimated number of ants that participate in raids increases as a function of colony size. The y-axis depicts an estimate of the maximum number of ants outside the nest during raids; the x-axis values are jittered to aid visualization (n = 126 raids, linear regression $p<0.0001$).

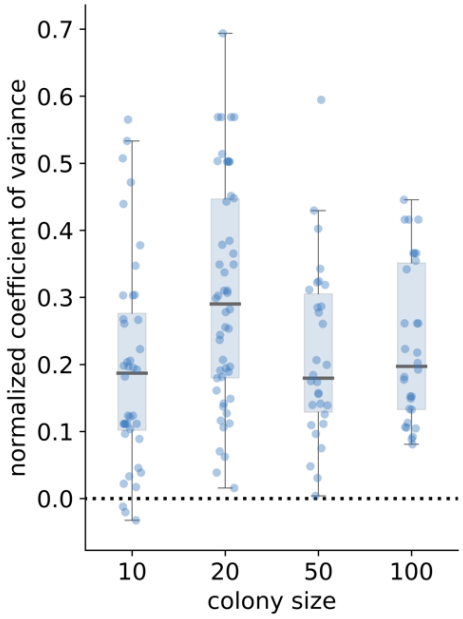


Figure 4.12: Ant inter-exit interval distributions are non-random and bursty. Distributions of normalized coefficients of variance show that across all colony sizes, the normalized coefficient of variance is higher than would be expected for an exponential distribution (this expectation of zero is depicted by the black dotted line). This shows that across all colony sizes, interval distributions are bursty – short exit intervals are overrepresented.

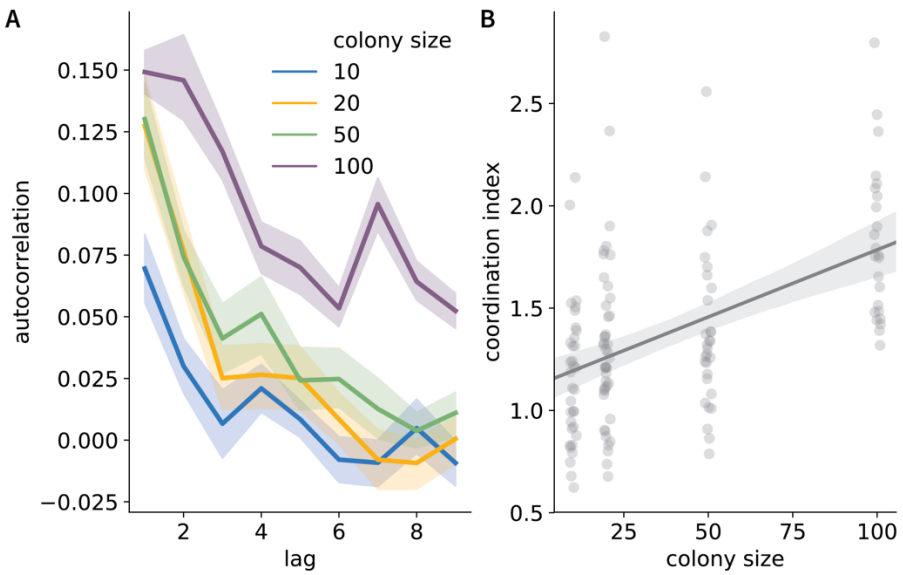


Figure 4.13: As colony size increases, the autocorrelation of spontaneous inter-exit intervals and the number of ants that participate in raids both increase as well. (**A**) The magnitude and lag of the autocorrelation increase with colony size. Dark lines depict mean autocorrelation values for the detrended sequence of inter-exit interval sequences across colony size, and error bands depict standard error of the mean ($n \geq 24$ for each colony size). (**B**) The coordination index of inter-exit intervals increases as a function of colony size ($n = 131$ exit sequences, linear regression $p<0.0001$).

To test whether these scaling effects indeed persist at colony sizes that approach those of army ants, I established two *O. biroi* colonies of roughly 5,000 workers each, an order of magnitude larger than naturally occurring colonies (Tsuji and Yamauchi, 1995), and filmed their raids in large arenas (see Chapter 6 for experimental details). The resulting raids involved thousands of ants and displayed trail bifurcations, simultaneously targeting multiple food sources (Fig. 4.14; Table 4.3). The vast majority of recruitment events now occurred outside the nest and usually at the raid front (43 out of 47). Thus, increasing colony size eventually transforms stereotyped group raids into raids that display all the defining features of army ant mass raids (Table 4.1).

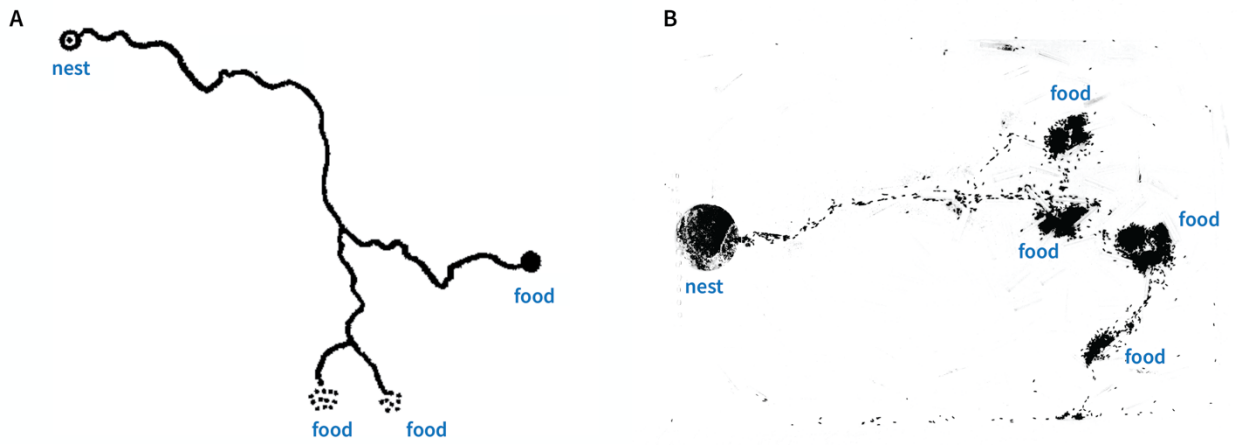


Figure 4.14: Group raids turn into mass raids with increasing colony size. (**A**) Schematic of a mass raid of the army ant *Aenictus laeviceps*, reformatted with modifications from (Schneirla and Reyes, 1966). (**B**) Snapshot (background-subtracted and contrast-enhanced) of an *O. biroi* raid in a colony with ca. 5,000 workers. The raid shows striking similarity to the army ant mass raid depicted in (**A**).

Together, these results suggest that all doryline ants share fundamental rules of search and recruitment behaviour. At small colony sizes, these rules manifest as scout-initiated group raids. However, as colony size increases, either within species or between species across evolutionary time, these rules gradually give rise to spontaneously initiated mass raids in which many ants leave the nest in quick succession, advance in pushing parties, and recruit at the raid front rather than at the nest. The difference between search behaviour in group raiders and mass raiders may thus be largely driven by the scaling effects of increasing colony size. I suggest that this mechanism can explain how army ant mass raiding evolved repeatedly and along strikingly similar trajectories as colony size increased. This constitutes a striking example of an alternative mechanism for the

evolution of collective behaviour that does not necessarily require modification of existing neural circuitry.

Table 4.3: Manually-annotated raids and their features from two *O. biroi* colonies with ~5000 workers each.

		Initial recruitment location								
Event	**Colony**	**First recruitment**	**Second recruitment**	**Third recruitment**	**Fourth recruitment**	**Fifth recruitment**	**Sixth recruitment**	**Seventh recruitment**	**Number of ants in raid**	**Bifurcations?**
1	1	outside	outside	outside	outside	outside	outside	NA	>1000	Yes
2	1	inside	inside	outside	outside	NA	NA	NA	>1000	Yes
3	1	outside	outside	outside	NA	NA	NA	NA	>1000	Yes
4	1	outside	outside	outside	outside	outside	outside	NA	>1000	Yes
5	1	inside	outside	outside	NA	NA	NA	NA	>1000	Yes
6	1	inside	outside	outside	NA	NA	NA	NA	>1000	Yes
7	1	outside	outside	outside	outside	outside	outside	NA	>1000	Yes
8	2	outside	outside	outside	outside	outside	NA	NA	>1000	Yes
9	2	outside	outside	outside	outside	outside	outside	outside	>1000	Yes
10	2	outside	outside	outside	outside	NA	NA	NA	>1000	Yes

CHAPTER 5: DISCUSSION

In Chapter 1, I introduced the worldview that underpins this dissertation – that of hierarchical transitions in individuality. Although much work has been done on the ultimate explanations for these transitions, little is known about how they happen. Here, I chose to study this transition in the ants, because they constitute the paradigmatic example for a second-order transition in individuality – i.e., from multicellular organisms to superorganisms. Through the three studies conducted in Chapters 2-4, using focussed mechanistic investigations of aspects of *O. biroi*'s life history, I have studied major aspects of the evolution of individuality – and superorganismality – in the ants. Together, they have uncovered and explained key aspects of the life history of *O. biroi*, and they help us propose plausible mechanisms for the initial origin, the subsequent maintenance, and the eventual elaboration of eusociality in the ants. Here, I will discuss the implications of some of my findings for the life history of *O. biroi*, as well as for the evolution of eusociality and superorganismality within ants, across independent lineages of eusocial animals, and across fraternal individuals more generally.

5.1: The life history of the clonal raider ant

We knew that *O. biroi*, like many other doryline ants, has a colony reproductive cycle regulated primarily by the larvae. Specifically, the larvae inhibit worker reproduction and induce foraging behaviour (likely via a pheromone), and the extent to which they influence these phenotypes depends on their ratio to the workers in the colony (Ravary and Jaisson, 2002; Ravary *et al.*, 2006; Ulrich *et al.*, 2016). Our work has now shown that larvae regulate this reproductive cycle by producing a signal that

suppresses adult insulin (i.e., *ilp2*) levels and inhibits ovary activation. Moreover, in *O. biroi*, unlike in most other animals, we discovered that insulin signalling no longer honestly represents an ant's true nutritional state. In animals as distant as flies and mammals, insulin levels decrease as the animal's hunger increases, and this allows insulin signalling to regulate metabolism. However, in *O. biroi*, we found that larvae decrease adult insulin expression within two or three days of exposure, but three days of exposure to larvae does not detectably alter adult nutritional state. Larvae also induce foraging behaviour. My data and observations suggest that they do this primarily by dramatically increasing how much the workers search for food – i.e., look for prey nests to raid[15]. In any case, this regulation of the workers' foraging behaviour occurs largely independently of their nutritional state.

If these colonies behave in the wild as they do in the lab, they likely only have access to food during the brood care phase, when they raid prey nests. Once their larvae have pupated, they lay eggs but do not search for food, and then likely undergo weeks – or more – of food deprivation, during which they likely continue to have activated ovaries and elevated insulin levels even as their nutritional levels reduce substantially. Together, this suggests that, in *O. biroi*, the regulation of metabolism by insulin signalling – assuming it really is ancestral – may have been substantially rewired to allow both extended periods of food deprivation as well as non-nutritional control of foraging behaviour. Larval signals probably regulate the reproductive cycle in many other doryline ants as well, and it is possible that this effect is more ancient still. This raises the possibility that this putative rewiring of metabolism may be ancestral to many (and

[15] Whether larvae also increase their probability of recruiting their nestmates, and/or increase the sensitivity of workers to recruitment remains unknown.

perhaps even all) ants. I propose that studying the metabolic causes and consequences of insulin signalling in *O. biroi*, and in ants generally, may be a fruitful avenue of research.

I also found that when *O. biroi* colonies forage, they do so in striking and stereotyped group raids. I believe that this is a significant advance, because it now allows us to conduct controlled experiments of a readily inducible complex collective behaviour in a eusocial species. *O. biroi* is increasingly genetically tractable (Trible *et al.*, 2017), which holds promise for the possibility that we may eventually be able to study the neural circuits – and the computations – that underlie behaviour during group raids. My experiments have shown that aspects of the raids can be experimentally manipulated; for example, nest and arena geometries are amenable to substantial change, and can be used to study the dynamics of pheromone production and perception. For instance, scouts appear to release a single burst of recruitment pheromone inside the nest to initiate a raid. To release this pheromone, they must know that they have entered the nest – a complex decision that we can now begin to study. Precisely how the workers lay pheromone trail also remains unknown. We do not yet know whether the trail laid during recruitment is chemically distinct from trails laid during spontaneous search excursions, whether the trails carry any other information, how workers decide to follow or reinforce trails, or how they choose between bifurcations in trails. Similarly, we do not yet know how workers decide when to begin retrieving the food, and whether this requires communication or consensus. We also do not know how scouts navigate homewards during recruitment. These and other questions can be studied with minimal modification to the assays I developed. And there is potential to further increase the complexity and realism of this behaviour. In the wild, *O. biroi* probably forage underground, travelling through tunnels and crevices in the soil (Daniel Kronauer, pers. comm.). My experiments were largely conducted in relatively open arenas, without tunnels or soil, but this could

easily be altered to study the workers' tunnelling behaviour. Similarly, although I typically used single *S. invicta* pupae as food, this could be replaced with live prey colonies to induce more realistic raids, and to study the *O. biroi* workers' nest-invasion and prey retrieval tactics. In summary, I suspect that we have only just scratched the surface of the true complexity of their behaviour, and that many more surprising discoveries lie in wait.

5.2: The evolution of reproductive division of labour

In Chapter 2, I asked how ants first evolved to live in colonies, and to be eusocial. We found that across the ant phylogeny, reproductive ants in a colony always have higher insulin levels than their non-reproductive nestmates, and that this insulin asymmetry is sufficient to explain the maintenance of the reproductive division of labour. Taking advantage of O. *biroi*'s unusual life history, we proposed a plausible mechanism for the origin of eusociality in ants. These experiments raise additional questions and points about the evolution of social life within the social insects, and its study. In this section, I will highlight five that I think may be of interest to sociobiologists.

First, I believe that our work suggests an approach by which the field may profitably study the mechanisms of social behaviour. Our approach has two distinguishing (advantageous) features: an unbiased, evolutionarily-informed screen, and a focus on the core physiological mechanism that distinguishes adult queens from workers. We began with an unbiased screen to identify candidate regulators of reproduction. Although many other studies have also employed this approach, they have typically faced the problem of too much choice. RNA-Seq experiments generally yield long lists of DEGs. As it is impossible to functionally study more than a few genes in these

lists, one is forced to winnow these lists, choosing candidate genes to continue to study. Such decisions are often made by ranking genes based on the magnitude of the fold change of their expression, or by choosing popular genes or genes for which reagents are readily available. Our comparative approach shows that one can use evolutionary information (here, the extent of conservation of differential expression) to weed out false positives. Although this approach is not fool proof (for instance, it may be sensitive to false negatives, low sample sizes, tissue specificity, and other technical decisions (Morandin *et al.*, 2016)), our work and other similar work (McKenzie *et al.*, 2016) demonstrates its utility. Moreover, I believe that we were fortunate to identify a molecule that, although produced by the brain, appears to act directly – and potently - on the ovaries. The asymmetry in ovarian activity is the key functional difference between queens and workers – it is the most downstream step of the physiological cascade that differentiates the castes. Explaining how this ovarian asymmetry is generated explains the immediate cause of the reproductive division of labour. Some of our colleagues have conducted similar brain transcriptional screens to ours (indeed, we used some of their data). However, interpreting the effects of molecules that act within the brain (rather than outside it, as *ilp2* does) is challenging, given that the proper level of explanation for such molecules lies in their contribution to the computations that specific neural circuits perform (a level of explanation that is currently intractable in social insects). Successive studies that identify different brain molecules cannot usually be interpreted together, and this inhibits synthesis and explanatory progress. I believe that a more promising approach would be to start from the most downstream components of – and therefore interpretable – relevant physiology, and to work upwards, rather than the current approach (of either doing the reverse, or of starting in the middle). More specifically, I suggest that the field may profit more by directly studying the molecular mechanisms of

ovarian control (Khila and Abouheif, 2010), and insulin production (since we have shown that this is a major regulator of ovarian activity).

Second, ants may of course use other modes of reproductive control as well. In a handful of species in the subfamily Myrmicinae, workers develop with no ovaries – they are physiologically incapable of reproduction (Hölldobler and Wilson, 1990; Khila and Abouheif, 2010). In most ant species, queens have larger ovaries than conspecific workers. Their ovaries have more – and longer – ovarioles, and the extent of the asymmetry between queens and workers varies dramatically across the phylogeny. These fixed morphological differences arise in development. Whether they are caused by an insulin asymmetry remains unclear. Moreover, other ant species may use yet other methods to create or amplify reproductive asymmetries, for instance by asymmetrically retaining eggs or regressing their ovaries, by asymmetrically producing trophic (i.e., non-viable) eggs, or by selectively eating worker-laid eggs (Hölldobler and Wilson, 1990; Khila and Abouheif, 2010; Ronai *et al.*, 2016). Such alternative strategies to create distinct reproductive castes may rely on mechanisms of control other than insulin signalling.

Third, we had little reason to expect that independently queenless ants (or more accurately, workers who have independently regained reproductive potential) share any neural mechanism for reproductive regulation. However, we see that *O. biroi*, *D. quadriceps*, and *H. saltator* all share differential expression of *ilp2* between reproductive and non-reproductive workers. This need not have been true, and indeed, may not be true in all queenless ants. For instance, data from *Diacamma sp.* and *Platythyrea sp.* are ambiguous[16] about whether *ilp2* is differentially expressed between reproductive and

[16] Taken at face value, these studies do not find *ilp2* to be differentially expressed. However, the *Diacamma* study has low statistical power, because it uses a very low sample size compounded by multiple comparisons across timepoints. Here, *ilp2* expression trends in the expected direction (i.e. it is

non-reproductive ants (Bernadou *et al.*, 2018; Okada *et al.*, 2017). Whole-body gene expression in *Pristomyrmex sp.* (Araki *et al.*, 2020), meanwhile, finds differential *ilp2* expression between reproductive and non-reproductive ants. More generally, one could conceive of *ilp2*-independent mechanisms by which a worker could activate her ovaries. For instance, increased expression of the insulin receptors, or of something downstream (such as activators of the germline cell cycle) could – in principle – bypass the need for elevated *ilp2*. To ask whether the convergent increase in *ilp2* expression occurs more often than we would expect by chance, we would need increased taxonomic sampling of lineages of reproductive workers, as well as a rigorous and quantitative understanding of the space of alternative mechanisms of ovarian activation and their relative attainability.

Fourth, we do not yet know whether independently eusocial lineages (such as corbiculate bees and vespid wasps) use similar mechanisms to generate their reproductive division of labour. Current data from wasps and bees sometimes – but do not always - find *ilp2* differentially expressed between adult queens and workers (Ferreira *et al.*, 2013; Jedlička *et al.*, 2016; Jones *et al.*, 2017; Patalano *et al.*, 2015; Standage *et al.*, 2016; Toth *et al.*, 2007; Warner *et al.*, 2019), but there is not yet enough data to make generalised claims. This apparent inconsistency – if it turns out to be true - may be explained by the fact that eusociality evolved independently in ants, bees, and wasps. While insulin signalling may have been co-opted repeatedly during social evolution, the details may conceivably differ between independent lineages. Tangentially, in honeybee workers, notch signalling in the ovaries is regulated by queen mandibular pheromone, which suppresses worker reproduction (Duncan *et al.*, 2016). How the sensation of this

higher in reproductives in the appropriate timepoint), but its lack of significance may be a false negative. In the *Platythyrea* study, no information is publicly available regarding expression levels of *ilp2*.

pheromone translates into differential ovarian notch signalling remains unknown, as does the relationship between insulin and notch signalling in ants and bees, and the function of notch signalling in the ovaries of ants. Understanding the mechanistic basis of convergent evolution in reproductive division of labour will require studying the same molecular (and physiological (Ronai *et al.*, 2016)) mechanisms in multiple, independently eusocial lineages.

Finally, we do not know much about how other fraternal individuals – for example, siphonophores, or multicellular organisms such as animals and plants – first evolved. As with social insects, the answer to this question is fundamentally a mechanism that can generate a fixed reproductive division of labour. One notable exception is in multicellular volvocine green algae. In *Volvox carteri*, the differential expression of a transcription factor, *regA*, appears to separate somatic and germ cells. regA is specifically expressed in the soma, where it may suppress genes involved in cell division. The differential expression of *regA* is determined early in development (Michod, 2007; Nedelcu and Michod, 2006). How the unicellular volvocine ancestor of these algae regulated its *regA* expression is unknown. Interestingly, *Chlamydomonas reinhardtii*, a unicellular green alga, has a cyclic life cycle in which cells seem to grow until darkness induces a pause in reproduction. Here, the expression of a gene similar to *V. carteri regA* appears to be induced by darkness. It is plausible that these two cellular states in *C. reinhardtii* are homologous to the germ and somatic lineages of multicellular volvocines. Overall, these data suggest that a gene that, in the ancestor, suppressed reproduction temporally, may have evolved to be differentially expressed in space, creating a fixed reproductive division of labour. This could conceivably be a mechanism for the origin of fraternal individuality in *V. carteri* (Michod, 2007; Nedelcu and Michod, 2006). If this is true, it would bear a striking similarity to the evolution of ant colonies, for which we

propose that a gene (*ilp2*) that may ancestrally have suppressed reproduction temporally evolved to be differentially expressed across the workers in a colony, creating a fixed reproductive division of labour. However, whether such reproductive cycles preceded multicellularity in the volvocines or in other lineages is not yet known.

5.3: The evolution of colony-level nutritional physiology

When a biological entity undergoes a fraternal transition, the level of individuality moves from that entity towards the level of the group, which eventually becomes the new evolutionary individual. Such fraternal individuals often develop higher-order physiology; i.e. systems that regulate coordination and ensure homeostasis at the level of the new individual, analogous to the physiological systems of the entity they descended from (Buss, 1987; Hölldobler and Wilson, 2009; Turner, 2002). In other words, fraternal major evolutionary transitions involve the evolution of mechanisms that regulate this new higher-level physiology. In Chapter 3, I studied one specific instance of this, in the context of the regulation of foraging in *O. biroi*. I found that, unlike the majority of other ant species that have been studied, *O. biroi* workers do not increase their foraging activity when deprived of food. Instead, they rely on larval signals to assess colony hunger.

O. biroi is not the only ant species to use larval signals to assess colony hunger. At least to some extent, other ant species – including *Rhytidoponera metallica* (Dussutour and Simpson, 2008; Dussutour and Simpson, 2009) and *Solenopsis invicta* (Howard and Tschinkel, 1980) - do this too. *S. invicta* workers also use their own nutritional states to regulate their foraging activity; starved workers forage more, and retrieve more food for the colony (Howard and Tschinkel, 1980). In *Pheidole ceres*, workers forage for nutrients that their food stores are lacking (Judd, 2006). Similarly, in honey bees, exposure to larval

pheromone increases the amount of pollen the workers collect (Ma *et al.*, 2018; Pankiw, 2004). Honey bee and bumble bee colonies have pollen stores, and workers assess these stores to regulate their foraging behaviour (Calderone and Johnson, 2002; Camazine, 1993; Kitaoka and Nieh, 2009; Molet *et al.*, 2008). Thus, in honey bees, nutritional states (Mayack and Naug, 2013; Mayack and Naug, 2015; Toth and Robinson, 2005; Toth *et al.*, 2005), larval pheromones, as well as colony food stores collectively determine the workers' foraging effort. This is also very likely true in bumble bee colonies, as well as in ant colonies, although the extent to which each of these three factors determines foraging effort is unknown. It is also interesting that relatively centralised signals of colony hunger (i.e., larval signals and colony food stores) have convergently evolved to help regulate foraging behaviour in bees and ants. I propose that, to assess colony hunger, each worker in these colonies computes some weighted sum of her own nutritional state, the colony's food stores, and larval signals. The set of weights is evidently variable across species, suggesting that - over evolutionary time - colonies can modify their algorithms for assessing hunger by altering this weighting.

The relative importance of larval signals to nutritional state in the first eusocial ants remains unknown. What of the subsocial ancestor? Progressive provisioning (subsocial) wasps likely use larval signals to determine their foraging behaviour (Hunt, 2007; Tinbergen, 1984). However, they hunt insects for their larvae but feed on nectar themselves. Starving parasitoid wasps often makes them forage for nectar (Desouhant *et al.*, 2005; Jacob and Evans, 2001; Lelightle *et al.*, 2010; Lewis and Takasu, 1990; Rasekh *et al.*, 2010). I speculate that in progressive provisioning wasps, and perhaps in the subsocial ancestor of the ants, the wasp foraged for itself when its own nutritional state was reduced, and foraged for the larvae in proportion to larval signals. Indeed, this may represent a social version of the nutrient-specific hungers that many insects (of all degrees

of solitude and sociality) are capable of (Bazazi *et al.*, 2016; Dussutour and Simpson, 2008; Dussutour and Simpson, 2009; Münch *et al.*, 2020; Simpson and Raubenheimer, 2014).

Moreover, as in other ant species (Howard and Tschinkel, 1980; Josens and Roces, 2000), I found that *O. biroi* workers' nutritional states still determine how much they eat. Together, these data suggest that in some ant species, foraging has evolved to be regulated by social, colony-level hunger, while feeding has retained its ancestral mode of regulation. I propose that this is analogous to the evolution of specialised foraging circuits in some animals. Specifically, many solitary animals, such as flies (Dethier, 1976), gastropods (Crossley *et al.*, 2018), nematodes (Avery and You, 2012), and vertebrates (Sternson *et al.*, 2013), possess a centralised nutritional physiology. For instance, they often have circulatory systems such as blood or haemolymph, and within it circulates some proxy of the animal's nutritional state. Within the animal's brain, specialized neural circuits measure this circulating factor – an estimate of how hungry it is - and use this to decide how much it should forage (Fischer and O'Connell, 2017; Pool and Scott, 2014; Sternson *et al.*, 2013). Similar circuits assess hunger to determine how much the animal should eat (Albin *et al.*, 2015; Fischer and O'Connell, 2017; Pool and Scott, 2014; Sohn *et al.*, 2013; Sternson *et al.*, 2013; Yapici *et al.*, 2016). However, given that the earliest animals did not have centralised nervous systems (Budd, 2015; Hejnol and Lowe, 2015; Martín-Durán *et al.*, 2018; Northcutt, 2012), they are unlikely to have had similar centralised circuits that regulated their decision to forage, or to feed, suggesting that the evolution of centralisation here occurred after the fraternal transition to multicellularity.

After consumption, food must be allocated to the various cells and organs of the animal, usually through blood or haemolymph, and each cell must decide – somewhat autonomously – how much circulating nutrition to take in. Cells usually do this by assessing their nutritional needs (their 'cellular hunger'). When they metabolize rapidly,

they also upregulate the expression of glucose transporters to draw more sugar in from outside, and begin to respire anaerobically – a phenomenon known in cancer cells as the Warburg effect (Heiden *et al.*, 2009). Essentially, solitary organisms often possess separate mechanisms to regulate organismal foraging, organismal feeding, and cellular feeding.

In summary, foraging in these solitary organisms is, like in eusocial colonies, a social, cooperative behaviour: specific cells induce foraging by assessing organismal hunger, and the entire organism benefits from the action. I propose that the evolution of relatively centralised machinery for the regulation of foraging occurred either during or after the fraternal transition to individuality, both in multicellular animals and in eusocial colonies. More generally, I suggest that there may be other such similarities in the organisation and evolution of higher-order physiology.

5.4: The evolution of collective foraging behaviour

The evolution of colony-level physiology allows the workers in a colony to better coordinate their behaviour. Although the earliest ant workers were likely solitary foragers, many ant species famously forage collectively. Many of these feats of cooperation – like the mass raids of army ants - are highly risky, and involve the deaths of many thousands of workers. Such behaviour is only possible because these colonies are superorganisms. Although much is known about the structure of some of these collective behaviours, how they evolved has been mysterious. Indeed, we know little about how collective behaviour generally evolves.

In the ants, the study of complex collective behaviour is often challenging in the lab. Typically, the species that perform the most extreme feats of collective behaviour tend to have very large colony sizes, tend not to perform them under controlled

conditions, and tend to be hard to maintain, manipulate, or observe at high resolution. *O. biroi* is an exception, in that it is eminently tractable and amenable to observation and experimental manipulation, colonies are relatively small in the wild, and even colonies of tens of ants are viable and behave normally (Ulrich *et al.*, 2018). Moreover, it is closely related to army ants, whose mass raids constitute one of the most renowned feats of collective behaviour. In Chapter 4, I asked how these army ant raids evolved. To infer their ancestry, I began by studying the foraging behaviour of *O. biroi*. Despite focussed research on colonies of *O. biroi* in the lab for over a decade, we knew very little about the structure of their foraging behaviour. I identified experimental conditions (see Chapter 6) under which *O. biroi* colonies reliably foraged in group raids. I described the structure of group raids quantitatively, and showed that their structure is determined in space and time by trail and recruitment pheromone from a scout ant.

In combination with existing natural history data, I showed that army ant mass raids evolved from ancestral group raids, and this transition – which likely occurred twice – correlates perfectly with two independent expansions in colony size. By experimentally increasing colony size in *O. biroi*, I showed that stereotyped group raids could be transformed into something qualitatively indistinguishable from mass raids. This suggests that the evolution of army ant mass raids can be largely explained as the scaling effects of increasing colony size. In other words, ancestral computations and neural circuits for group raiding behaviour may not need any alteration for mass raids to evolve. This is an unusual mechanism for the evolution of behaviour, which likely typically evolves by altering circuit computations.

Mass raids have evolved independently in the ponerines too – especially within the genus *Leptogenys*, which also features group raiding and solitary foraging species that have smaller colony sizes than their mass raiding relatives (Berghoff *et al.*, 2003; Duncan

and Crewe, 1994; Maschwitz *et al.*, 1989; Witte and Maschwitz, 2000; Witte and Maschwitz, 2002). Group raids are likely to precede mass raids in this genus, but this has not yet been formally tested, and whether increasing colony size explains the evolution of ponerine mass raids remains unknown. The generality of this colony size scaling mechanism is contingent on the specific behavioural rules that the ancestors of mass raiding *Leptogenys* ants used, and it will be interesting to ask how this distant lineage of ants evolved strikingly convergent behaviour.

How did the ancestral doryline ants evolve to forage in group raids? The most recent common ancestor of all ants likely had solitary foragers, as many extant species across the ant phylogeny still do (Lanan, 2014). For group raiding to evolve, the ants must have evolved the ability to produce, secrete, detect, and react to a number of different pheromones, most obviously including trail and recruitment pheromones. Whether the ancestral ants could make these pheromones is not yet known. Similarly, extant ants forage in a number of different ways. Many of these foraging strategies involve recruitment and collective behaviour, and from their phylogenetic distribution (Lanan, 2014), many of them probably evolved independently in multiple different ant lineages. However, not enough is known about the details of foraging behaviour from enough species across the phylogeny to accurately estimate the number of origins of collective foraging, and we do not yet know the sequence of events that led to the evolution of group raiding in the dorylines. Nonetheless, it is clear that this evolutionary process required a number of changes in neural circuit computations, as did the initial evolution of other forms of collective foraging.

More generally, many forms of collective behaviour are sensitive to group-level parameters such as group size. Many ant colonies and fireflies have periodic behaviour, and the extent of their synchronisation depends on the number of participating animals

(Cole, 1991; Dornhaus *et al.*, 2012; Sarfati *et al.*, 2020). Group size also affects other features of collective organisation, including division of labour and decision-making, not just in groups of animals but in groups of cells too (Berdahl *et al.*, 2013; Brahma *et al.*, 2018; Deglincerti *et al.*, 2016; Lee *et al.*, 2009; Morand-Ferron and Quinn, 2011; Simunovic and Brivanlou, 2017; Sumpter *et al.*, 2008; Vicente-Page *et al.*, 2018). As I demonstrated in Chapter 4, and as has been shown in other contexts, both theoretically and empirically, the scaling effects of increasing group size can lead to increased behavioural specialisation, or qualitatively different collective decisions, without any change in the underlying decision rules that each group member follows (Gautrais *et al.*, 2002; Ulrich *et al.*, 2018). In the context of the evolution of individuality, many authors have proposed associations between group size and complexity. Specifically, they have proposed that increasing group size allows the parts of a nascent fraternal individual to specialise further, creating more interdependence between the parts, and eventually, greater individuality (Bonner, 1988; Bonner, 1993; Bourke, 2011; Buss, 1987; McShea, 2001; McShea *et al.*, 2019). This idea is usually phrased in ultimate terms; it is suggested that increasing size selects for increased specialisation, cooperation, and individuality. I suggest that the efficiency of this process may, sometimes, be aided by the proximate mechanism of scaling; i.e., perhaps the number of required mechanistic changes is rather small in these cases, which would provide natural selection access to a readily available substrate on which to act.

5.5: Outlook

The structure of this dissertation is borrowed somewhat from Andrew Bourke's 'Principles of Social Evolution'. Using inclusive fitness theory, he assesses ultimate

explanations for the formation, maintenance, and transformation of social groups into true evolutionary individuals. He argues – as others have done previously (Buss, 1987; Maynard Smith and Szathmáry, 1995; Queller, 1997; Queller, 2000) - that similar evolutionary principles underlie each of these steps in the fraternal transition to individuality.

Irrespective of their recent ancestry or their level of biological organisation, fraternal individuals must all accomplish a largely identical set of tasks. They must evolve mechanisms for reproductive and non-reproductive division of labour. In other words, they must evolve a set of rules that specifies the various component parts of the individual; that determines its 'development'. They must also evolve communication systems to coordinate the behaviour of their various parts, homeostatic mechanisms to ensure that they react appropriately to environmental perturbation, and self- and non-self-discrimination to keep intruders at bay.

The precise molecular genetic mechanisms for the control (and evolution) of these behaviours likely vary across independent origins of individuality, and the extent of this variation between any two lineages likely depends on their divergence time (i.e., more accurately, the extent of their shared developmental constraint). Nonetheless, there may be similarity in the nature of their solutions to each of these problems (and in their proximate evolutionary trajectories), and I discussed these in detail in the preceding sections. To ask - systematically - how common these similarities are, we will require more rigorous manipulative experiments in a few focal species from many independent lineages of fraternal individuals, combined with comparative analyses to place them in their appropriate evolutionary context. My work on the colony reproductive cycle and foraging behaviour in *O. biroi* improves our ability to use this as one such focal species to

understand the mechanisms of cooperative behaviour in the ants, and our understanding of the evolution of superorganismality and individuality more generally.

CHAPTER 6: MATERIALS AND METHODS

Colony maintenance

For all experiments in this dissertation, *Ooceraea biroi* colonies were maintained in the lab at 25°C and >50% humidity in boxes with a plaster of Paris floor. During the brood care phase, experimental colonies were fed with frozen *Solenopsis invicta* brood. All experiments were performed using ants from clonal line B (Kronauer *et al.*, 2012). For most experiments (exceptions are noted where relevant), all ants were one month old, were from the same source colony, and had been reared under the same conditions.

RNA sequencing (RNA-Seq) comparative screen

For this experiment, we compared RNA-Seq data from seven focal ant species: *Dinoponera quadriceps, Ooceraea biroi, Camponotus planatus, Odontomachus ruginodis, Solenopsis invicta, Harpegnathos saltator, and Acromyrmex echinatior*. *C. planatus*, *O. ruginodis*, and *S. invicta* samples were collected specifically for this study, while the data from *D. quadriceps*, *A. echinatior*, *H. saltator* and *O. biroi* were from previously published studies (Gospocic *et al.*, 2017; Li *et al.*, 2014; Libbrecht *et al.*, 2016; Patalano *et al.*, 2015). Details of the life history and experimental sampling and procedures for each species can be found in Appendix A. Code for the comparative screen is available at (Oxley and Chandra, 2018).

Sample collection

We collected multiple queenright colonies of *Odontomachus ruginodis* and *Camponotus planatus* at the Archbold Biological Station near Lake Placid, Florida, in February 2016. Each colony contained a single queen or gyne, and 20-30 workers. We

maintained these colonies in the lab on a diet of sugar water *ad libitum*. After five days all queens and workers were snap-frozen on dry ice and maintained at -80°C until dissected.

Queenright *Solenopsis invicta* colonies were collected in Gainesville, Florida, in November 2014. *S. invicta* colonies were maintained in the lab for several months on the Bhatkar diet (without eggs) (Bhatkar and Whitcomb, 1970) and mealworms. From each of 5 colonies, we collected a reproductively active queen, five reproductively inactive gynes, 20 foraging workers (collected directly from the mealworm and flan feeding trays), and 20 workers from within the nest (collected directly from the brood pile). All queens and workers were snap-frozen on dry ice and maintained at -80°C until dissected. For the RNA-Seq analysis presented here (described in detail below), we treated both gynes and queens as reproductives, and both nurses and foragers as non-reproductives.

Dissection and RNA-Seq

Brains were dissected in 1x PBS at 4°C. Dissected brains were immediately transferred to TRIzol (Invitrogen) and placed on dry ice. The ovaries of each ant were also dissected to assess its reproductive status (see Appendix A). RNA was extracted in TRIzol using RNeasy (Qiagen) purification with DNAse I (Qiagen) on-column digestion, using a previously reported protocol (Libbrecht *et al.*, 2016).

Library preparation and RNA-Seq were performed at the Rockefeller University Genomics Resource Center as follows. For the *O. biroi* dataset (Fig. 2F), 2 ng of total RNA was used to generate full length cDNA (of mRNA) using Clontech's SMART-Seq v4 Ultra Low Input RNA Kit (Cat # 634888). 1 ng of cDNA was then used to prepare libraries using Illumina Nextera XT DNA sample preparation kit (Cat # FC-131-1024). 16 libraries were prepared with unique barcodes and pooled at equal molar ratios. The pool was denatured and sequenced on an Illumina NextSeq 500 sequencer to generate 150 bp

single-end reads following the manufacturer's protocol. For *S. invicta*, 1 ng of total RNA was used to generate full-length cDNA using Clontech's SMART-Seq v4 Ultra Low Input Kit (Cat # 634888), 1 ng of which was then used to prepare libraries using Illumina Nextera XT DNA sample preparation kit (Cat # FC-131-1024). Libraries with unique barcodes were pooled at equal molar ratios. Each pool was sequenced on two lanes on an Illumina HiSeq 2500 sequencer to generate 100 bp single-end reads, following the manufacturer's protocol (Cat #15050107 V03). For *O. ruginodis* and *C. planatus,* 100 ng of total RNA was used to generate libraries using the Illumina TruSeq stranded mRNA LT kit (Cat # RS-122-2101). Libraries prepared with unique barcodes were pooled at equal molar ratios. The pool was denatured and sequenced on an Illumina NextSeq 500 sequencer using high output V2 reagents and NextSeq Control Software v1.4 to generate 150 bp paired-end reads, following the manufacturer's protocol (Cat # 15048776 Rev.E).

Transcriptome Assembly of *Odontomachus ruginodis* and *Camponotus planatus*

Sequences were trimmed using Trimmomatic (Bolger *et al.*, 2014), followed by quality checking with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmed sequences were then assembled using Trinity (Grabherr *et al.*, 2011) with the default settings; i.e. including a minimum contig length of 200 bp and a normalized maximum read coverage of 100.

Prediction and translation of peptide sequences was performed using Transdecoder (2014; http://transdecoder.github.io/). Peptide predictions were trained on the annotated set of *O. biroi* peptides. To assess transcriptome quality, BUSCO assessment was used (Simão *et al.*, 2015), comparing against the BUSCO arthropod database.

***O. ruginodis* TransDecoder Peptide BUSCO assessment:**

2347 (87.7%)	Complete BUSCOs
2111 (78.9%)	Complete and single-copy BUSCOs
236 (8.8%)	Complete and duplicated BUSCOs
180 (6.7%)	Fragmented BUSCOs
148 (5.5%)	Missing BUSCOs
2675	Total BUSCO groups searched

***C. planatus* TransDecoder Peptide BUSCO assessment:**

2413 (90.2%)	Complete BUSCOs
2155 (80.6%)	Complete and single-copy BUSCOs
258 (9.6%)	Complete and duplicated BUSCOs
108 (4.0%)	Fragmented BUSCOs
154 (5.8%)	Missing BUSCOs
2675	Total BUSCO groups searched

Because the *O. ruginodis* and *C. planatus* libraries were sequenced on the same lane, we found low levels of index-switching-like contamination. Specifically, it appears that during demultiplexing some highly expressed genes in *O. ruginodis* were misidentified as belonging to a *C. planatus* sample and *vice versa*. This is a known issue that adds low levels of noise to multiplexed sequencing experiments (Kircher *et al.*, 2012). We estimate that roughly 0.03% - 0.04% of reads in each library in *C. planatus* and *O. ruginodis* are contaminants, in line with previous estimates of contamination caused by index

misassignment. Contaminated reads are normally filtered out when they are aligned to a reference genome. In our case, however, we use the reads to generate *de novo* transcriptomes for *O. ruginodis* and *C. planatus*, and this means that many contaminants are annotated as unique genes. Because we aimed to analyse single-copy orthologs that are conserved across the ants, this constituted a potential issue that could have interfered with our analysis. Specifically, such contamination could have caused some genes to be wrongly classified as having undergone gene duplication in *O. ruginodis* or *C. planatus*. These ortholog groups would thus have been excluded from our analysis. To overcome this potential problem, we identified and removed contaminant transcripts from the annotated transcriptomes before proceeding with our analyses.

To identify specific contaminants in each transcriptome, we selected genes/transcripts that had >90% blastp (Altschul *et al.*, 1990) identity to a gene in the other transcriptome. We then found the best blastp hit to the focal gene in a set of ant and bee species. This set contained the seven ant species in our screen (including the query species: *O. ruginodis* or *C. planatus*, respectively), *C. floridanus*, *Lasius niger*, *Apis mellifera*, *Megachile rotunda*, and *Bombus impatiens*. We then aligned these sequences and constructed maximum likelihood protein phylogenies (using a JTT + gamma substitution model and an automated version of the procedure described in the section on phylogenetic analysis below) for the ortholog groups that these genes were part of. We rooted the resulting trees on the ancestor of all bees, excluding trees that failed to produce a nearly-monophyletic bee grouping, or that did not contain a gene from any of the ponerine or formicine ant species in our alignment. When the alignments contained no bee sequences, we rooted trees on *O. biroi* instead. We used the resulting tree topologies to classify genes as contaminated, contaminating, or clean. Contaminated and contaminating genes were defined as those that produced clades of *O. ruginodis* and *C.*

planatus, in distinct contradiction to the expected species topology (seen in Fig. 1). Contaminated genes fell within the wrong ant subfamily on the phylogeny, while contaminating genes were in the right ant subfamily but had a monophyletic relationship between *O. ruginodis* and *C. planatus*. We also used secondary features of the phylogenies (i.e. the genetic distances between the *O. ruginodis* and *C. planatus* genes in each tree, and the Robinson-Foulds (Robinson and Foulds, 1981) distance between each gene tree and the expected species tree), as well as features of each gene (including the length of its predicted amino acid, its average expression level, etc.) to identify genes that our phylogenetic classification missed. Preliminary analysis (not shown) found that, as expected, contaminated genes (as classified by our phylogenetic analysis) strongly tended to have low expression and short peptide lengths relative to their best hit (i.e. a contaminating gene) in the other species (i.e. in *C. planatus* when the focal gene was from *O. ruginodis*, and *vice versa*). These genes also tended to have extremely low genetic distance (calculated from the ML phylogeny) to their best hit in the other species, and their phylogenies tended to have high Robinson-Foulds distances to the hypothetical species phylogeny. Based on these data, we also classified *O. ruginodis* (or *C. planatus*) genes as contaminated if (a) the gene fell inside a formicine (or ponerine in the case of *C. planatus*) clade and it was >20% shorter and >20% less expressed than its best hit in the other species, or (b) if the gene wasn't in the ponerine (or formicine) clade, had ≥0.6 Robinson-Foulds distance, and had either extremely low genetic distance to their best hit in the other species or was both >20% shorter and >20% less expressed.

Overall, this analysis identified roughly 700 contaminated genes in each transcriptome; i.e. about 3% of annotated transcripts in each transcriptome were contaminated. We removed these genes from the transcriptomes before differential expression analysis. Consequently, they were also excluded from our single-copy

ortholog identification. Orthology identification with contaminations excluded found 40 more single-copy orthologs across the ants. If we had not removed contaminated genes from the transcriptomes, our comparative analysis (described in detail below) would be slightly more conservative. This is because we only analyse single-copy orthologs. The presence of a contaminant causes an ortholog group to appear as though one of its member species possesses two paralogs of the gene, and would thus exclude it from further analysis. Thus, even if our contamination cleanup and transcriptome curation has missed a few contaminants, this would serve to increase the probability that our comparative analysis has false negatives, but not the rate of false positives. Further, we presume that this index misassignment affects all our samples equally. Although we are able to detect and remove contaminated genes when they are found in the transcriptome of a species different from that of their origin, we are unable to identify contaminated reads that hop across two samples of the same species. In principle, this could contribute to the low statistical power of our differential expression analysis, and it could be one reason for the low number of common differentially expressed genes we detect across our seven focal ant species. Despite this, *O. ruginodis* and *C. planatus* have higher numbers of differentially expressed genes than any other species in our screen (Table S1), while *H. saltator* and *D. quadriceps* have very low numbers of differentially expressed genes. Thus, it appears that the latter species explain the low numbers of common differentially expressed genes we detect, and possible contamination in *O. ruginodis* and *C. planatus* does not limit our statistical power.

Differential gene expression analysis

RNA-Seq fastq files for *D. quadriceps*, *A. echinatior*, *H. saltator*, and *O. biroi* were obtained from the NCBI Short Read Archive (PRJNA255520, PRJNA223531,

PRJNA327090, and PRJNA304722, respectively). RNA data from all seven species were hereafter analysed in the same manner. RNA-Seq reads were adapter trimmed with Trimmomatic, quality checked with FastQC, and aligned to their respective genomes using STAR aligner (Dobin *et al.*, 2013). For *O. ruginodis* and *C. planatus*, we used STAR to align reads to their respective curated transcriptomes. Aligned read counts for each gene were calculated using HTSeq (Anders *et al.*, 2015). We used a modified version of HTSeq (https://github.com/oxpeter/htseq-transcriptome) to produce read counts for *O. ruginodis* and *C. planatus*; this was done to ensure that HTSeq did not exclude counts for reads mapping to multiple isoforms of the same predicted gene. Differentially expressed genes were identified using DESeq2 (Love *et al.*, 2014), using a Wald Test to contrast the queen and worker castes, and an FDR q-value cutoff of 0.05.

Single copy ortholog identification

For orthology detection, we downloaded 16 hymenopteran genome annotations from NCBI, including the five focal ant species in our screen for which genome sequences have been published. For each gene, the longest transcript was chosen as the representative transcript for that gene. The inferred peptide sequences corresponding to the longest transcripts for each of the 16 species, as well as the inferred peptide sequences of *O. ruginodis and C. planatus,* were then used as input for OrthoMCL (Li *et al.*, 2003). 17,601 groups of orthologs were identified by OrthoMCL. From these, we identified 5,581 groups in which all seven focal species had one, and only one, gene present. These groups were considered single-copy orthologs across our focal taxa.

ILP2 antibody production

Custom rabbit polyclonal anti-ILP2 designed to recognize an epitope in the B chain was generated and affinity purified by YenZym. YenZym also used ELISA to assess the specificity of the antibody response to the immunizing peptide in vitro. To assess peptide specificity in situ, we pre-incubated the antibody with ILP2 peptide. The subsequent immunostain showed no ILP2 staining, as we would expect if the antibody was bound to synthetic ILP2 and therefore unable to bind to endogenous peptide.

Immunohistochemistry

Whole mount brain stainings as well as ovary and fat body stainings for ILP2 were performed following a similar protocol as in_(McKenzie *et al.*, 2016). Briefly, *O. biroi* tissues were dissected in cold phosphate-buffered saline (PBS), pH 7.4. The tissues were then fixed by incubation in 4% (wt/vol) paraformaldehyde solution in PBS overnight at 4°C. Tissues were washed in PBS once, followed by three 20 min washes in PBS containing 0.5% Triton-X (PBT) at room temperature on a shaker. Samples were blocked in 1% Bovine Serum Albumin (BSA) in PBS for 30 min, washed in PBS 0.01% Tween for 5 min and incubated for either 24 hours at RT or for 48 hours at 4°C (this varied across experiments) with our anti-ILP2 antibody (1:500) containing solution in 1% BSA and 0.5% Triton-X in PBS solution. The next day, samples were washed 3 times for 10 min with PBS Tween (0.01%), incubated with a secondary antibody Alexa Fluor 488 goat anti-rabbit solution (1:250) containing Alexa Fluor 555 or 647 phalloidin (Thermo Fisher Scientific) 1:50 μL of stock solution 6.6 μM) and 4',6-diamidino-2-phenylindole (DAPI) (1:1000) in 1% BSA and 0.5% Triton-X in PBS solution for 2 h and washed five times in PBS. Tissues were mounted with Dako mounting medium between two cover slips separated by a stack of two reinforcement labels (Avery 5720), mounted on a frosted slide, and sealed

using clear nail polish. Tissues were imaged using a Zeiss LSM 880 NLO laser scanning confocal microscope. Images were acquired at a pixel resolution of 1024 x 1024 keeping configuration settings equal within experiments.

For the comparison of workers between the reproductive- and brood care phase, we split an age- and phase- matched colony of ants into two, and desynchronized them by taking the larvae away from one colony and allowing the ants to complete a full reproductive cycle. When the two colonies were in the peak of their reproductive and brood care phases, respectively, we dissected all ant brains, stained them in parallel with anti-ILP2 antibody as described above, and analysed them as described below.

For each comparison of workers and intercastes in both phases of the reproductive cycle, we established a colony of newly-eclosed callow workers and intercastes from a large stock colony. For the experiment in the reproductive phase, we waited approximately four weeks, until the ants were in the middle of their reproductive phase. For the experiment in the brood care phase, we waited roughly five weeks, until the ants had progressed through a reproductive phase and were in the peak of their subsequent brood care phase. We dissected and stained all ant brains in parallel for each experiment.

Image analysis

We imaged immunostained ant brains with a confocal microscope as described above. We then used IMARIS (Bitplane) to semi-automatically segment clusters of insulin-producing cells in each brain image stack. We then used the total intensity of all fluorescent voxels in each image to quantify insulin-producing cell fluorescence intensity. We used the imaging software Zen (Zeiss) to construct 3D projections of the ovaries and FIJI (ImageJ) to construct 3D projections of the brains.

Predicted ILP1 and ILP2 structures in *Ooceraea biroi*

Insulin ortholog and paralog sequences are highly divergent. All members in the insulin superfamily have six characteristic conserved cysteines that form three disulfide bonds. The ILP1 sequence resembles mammalian insulin-like growth factor (IGF) in that it has a short C chain with flanking regions. Furthermore, ILP1 lacks recognizable dibasic amino acid cleavage sites flanking the C chain. Short uncleaved C chains are a common feature of IGF-like peptides (Mizoguchi and Okamoto, 2013; Okamoto *et al.*, 2009). The ILP2 sequence has a longer C chain, as in canonical insulins, that is flanked by dibasic and/or monobasic peptide cleavage sites (Southey *et al.*, 2008; Veenstra, 2000). This suggests that, as in canonical insulins, the C chain is cleaved from the propeptide to form a mature peptide consisting of A and B chains held together by disulfide bonds.

ILP2 synthesis

ILP2 peptide synthesis was performed by Phoenix Pharmaceuticals based on the predicted peptide sequence in *O. biroi*:

A Chain: GIHEECCVNACTISELSSYCGP,

B Chain: SSISAPQRYCGKKLSNALQIVCDGVYNSMF

[Disulfide bonds: A6-A11, A7-B10, A20-B22]

Phoenix Pharmaceuticals performed a mass spectrophotometric analysis that showed that the synthetic peptide had the expected molecular weight. The peptide was >95% pure. The lyophilized peptide was first reconstituted in a minimal volume of water (pH 2.6) and further diluted to a concentration of 100 μM in PBS (pH 7.4). Aliquots were frozen at -80°C until used.

B chain peptide synthesis

Another batch of the B chain of *O. biroi* ILP2 was synthesized separately as an injection control by The Rockefeller University Proteomics Resource Center and verified through liquid chromatography and mass spectrometry. Lyophilized peptides were first reconstituted in a small volume of water (pH 2.6), diluted to a concentration of 100 μM in PBS (pH 7.4), and stored in frozen aliquots at -80°C until used.

ILP2 injections

For each of the experiments involving ILP2 injections, all *O. biroi* ants were collected as callows (i.e., newly eclosed adults) from a single large colony entering the brood care phase. This was to ensure that all ants were of the same age and genotype. We then allowed the ants to progress through a natural colony cycle. We injected ILP2 or the B chain control into one month-old ants in peak brood care phase (i.e. 5 days after the larvae had hatched) and in early reproductive phase (i.e. 2 days after the larvae had pupated). ILP2 and the B chain stock solution were diluted in PBS to reach concentrations of 10 μM and 100 μM. We selected these concentrations based on data from *Aedes aegypti* (Brown *et al.*, 2008), which suggest that the 10-100 μM range is physiologically relevant. We injected approximately 0.1 - 0.2 μL of ILP2 or the ILP2 B chain into each ant. We used a 36 gauge bevelled needle attached to a nanofil syringe (World Precision Instruments, Inc.) for the injections. Each ant was immobilized between silicone pads on the sides of a modified crescent wrench. The needle was inserted dorsally between the first and second tergites of each ant's gaster. In each experiment, we injected 50 ants with ILP2 and 50 ants with the control. Roughly 50% of injected ants died within the first two days after injection. This high initial mortality reflects the fact that injections are technically challenging given the small size of the ants, and that a large proportion of ants are

physically damaged by the injection process. However, we found no difference in mortality rates between treatments, and did not detect differences in long-term survival between uninjected control ants and injected ants that had survived the first two days after injection. For the reported experiments, all ants that were still alive three days after injection were dissected and assayed.

Ovary dissection, staining and mounting

Ants were briefly immersed in 95% ethanol and then transferred to PBS. Ant ovaries were dissected in cold PBS at pH 7.4. They were then fixed by incubation in 4% (wt/vol) paraformaldehyde solution in PBS overnight, washed in PBS and stained with DAPI, and then washed in PBS 5x and mounted in DAKO mounting medium. Ovary images for analysis were acquired with an epifluorescence Olympus BX53 microscope. We measured the largest cross-sectional area of the largest oocyte (or second-largest oocyte) in any ovariole for each ant using the image-processing program Cell Sens Standard. We also counted the total number of follicles in each ant's ovaries. Representative images were acquired using a Zeiss LSM 880 NLO confocal microscope. 3D projections were constructed using Zen imaging software (Zeiss).

Lipid quantification

For all experiments involving quantification of lipid levels, I used a colorimetric serum triglyceride quantification kit (CellBiolabs #STA-396). Ants were homogenized in PBS with 1% Triton X-100, whole-body lipid was extracted and processed using the standard protocol from the kit. All lipid measurements were made using at least two technical replicates.

Tracking chambers

Tracking experiments were conducted in artificial arenas constructed from layers of cast acrylic, with a plaster of Paris floor. Each arena was a square of side 10 cm, in which we laser-cut a nest chamber and a foraging arena, connected to each other by a narrow tunnel (see Fig. 1). The nest chamber had a diameter of 2 cm, the tunnel was ~2 mm wide and ~6 mm long, and the foraging arena had a diameter of 6.5 cm. The floor of the foraging arena was covered with vapor-permeable Tyvek paper to make it less attractive as a nesting site and discourage colonies from emigrating there, while keeping it suitable as a foraging arena. For all experiments in these artificial arenas, ants were introduced to the nest chamber at the start of the reproductive phase. During this period, the tunnel was sealed to prevent ants from entering the foraging arena. 2-4 days after introduction, the ants laid eggs in the nest chamber. Ten days later, the eggs hatched into larvae. 4-6 days after this, when the larvae were in their third or fourth instar, I placed food (i.e., a single frozen *S. invicta* pupa) in the foraging arena, unsealed the tunnel, and filmed the ants foraging.

I filmed colonies at 5-10 Hz and 2592x1944 pixel resolution, using webcams (Logitech C910) in enclosed containers with controlled LED lighting at ~27°C and ~60% humidity.

Behavioural tracking and analysis of food deprivation experiments

For both experiments in which I deprived ants of food and studied their foraging behaviour, I tracked ant positions for roughly six hours spread over the first day after colony establishment (including a period of ~1.5 hours with no food in the arena, and a further period of ~4.5 hours with food in the arena). I chose not to analyse behavioural

data beyond this first foraging event because many ants ate the food they retrieved, and I reasoned that I could no longer confidently infer their nutritional states after this point. All videos for all tracking experiments presented here were tracked using AnTraX (Gal *et al.*, 2020).

Tagged-ant experiment

Nine colonies of ants were established from a single cohort of one-month old ants that were entering the reproductive phase. Each colony consisted of 25 ants, and each ant was tagged with an ordered pair of colour dots that was unique to the colony. Specifically, each ant was painted on her thorax and gaster with one of five colours of oil-paint markers (uni Paint Markers PX-20 and PX-21), a technique previously used by (Ulrich *et al.*, 2018). At the end of the experiment, I counted all larvae, and found that each colony had between 20 and 25 larvae. In other words, the larvae:adults ratio (a known source of variation in colony foraging – see (Ulrich *et al.*, 2016)) was close to 1:1 in all colonies.

For the eight days of the tracking period (i.e., when the larvae were between ~5-13 days old), every 12 hours, I cleaned each foraging arena with water (to remove trail pheromone from the previous foraging event), and placed a single *S. invicta* pupa (infused with 0.05% bromophenol blue to aid visualization) at its far end. I then unsealed the tunnel and allowed the ants to explore the arena. I filmed the arena for roughly four hours thereafter, at 10 frames per second (fps), after which we resealed the arena. For the first five days (i.e., the first ten foraging events), each colony was given a small (worker-destined) *S. invicta* pupa. For the next three days, I presented colonies with large (queen-destined) or small (worker-destined) pupae in alternation. The difference in feeding did not affect the coarse structure of the colonies' foraging behaviour. Here, I do not

differentiate between these foraging events. In some cases, colonies emigrated to the foraging arena. For the next event in such colonies, if the ants had not moved back to the nest chamber, I presented them with a *S. invicta* pupa but did not record foraging. All chambers had their plaster floor watered periodically to saturation.

In sum, I recorded 90 foraging events across nine colonies; each event is defined as a behavioural sequence that begins with the ants searching for food and ends after the food has entered the nest. Of these 90 foraging events, 22 events ended in emigration. In 18 events, the ants appeared to eat the *S. invicta* pupa *in situ* (although we cannot exclude the possibility that they tore it into small pieces before carrying it home, and we cannot be certain that only adults ate the food). The 50 remaining events ended in retrieval – i.e., with the ants transporting the pupa into the nest. In >500 foraging events in subsequent experiments, I never observed emigration again, and only observed a single instance of eating *in situ*, possibly due to subtle differences in experimental design. Thus, I excluded these events from our analysis here. Of the 50 events that ended in retrieval, 19 were excluded from analysis due to failures in data acquisition, poor tracking quality, or cases where the colony was unsettled at the time of food presentation. Our final dataset thus consisted of 31 foraging events from seven colonies.

Annotation of group raid phases

Based on my manual observations of the raids, I identified six discrete, sequential phases of each raid. I defined the 'search' phase as the period beginning at the start of the video, and ending at the time at which the next phase (i.e., 'recruitment') begins. For the group raids that I analyse here, scout ants necessarily located the food during the search phase. The recruitment phase begins when a scout leaves the food and runs homeward, and it ends when the scout recruits her nestmates, which commences the 'response'

phase. The recruitment phase only includes successful recruitment. In some cases, scout ants may run homeward from the food without initiating a response; however, as I cannot judge whether these instances constitute attempted recruitment, I do not use them to define the beginning and end of the recruitment phase. I define the beginning of the response phase as a column of ants leaving the nest, and the end as the moment when the tail of the column reaches the food. This commences the 'pre-retrieval' phase, which ends when ants begin to move the food back home. I define this 'retrieval' phase as beginning when the position of the food has noticeably changed, and ending when the food enters the nest. I define the final phase, 'post-retrieval', as beginning when the food has entered the nest and ending 500 seconds later.

For all raids, I manually annotated the corresponding videos, specifically recording five timepoints that allow us to define these six phases. These timepoints are the time at which a scout leaves the food on her recruitment run, the time at which the leader of the column of ants responding to recruitment enters the foraging arena, the time at which the last ant in the column arrives at the food, the time at which the position of the food begins to change, and the time at which the food enters the nest. In colonies of 25 ants, these timepoints may be recorded with minimal subjectivity, as assessed by repeated annotations of the same raids, and by comparisons of recorded timepoints between observers (data not shown). I also recorded the identities of the scouts that successfully initiated raids, and all ants that contributed to retrieving food.

Visualisation of raid temporal structure

To visualize the temporal dynamics of the average group raid, I rescaled each phase of each raid so that it equals the mean length of the homologous phase over all 31 raiding events. To account for uneven sampling of specific timepoints after rescaling, I

then used interpolation to infer the timecourses of the number of ants outside the nest, their distance from the nest, and the sum of their speeds, over evenly distributed timepoints along each rescaled phase of each raid (Fig. 1, E to G). AnTraX's estimates of the centroids of ants vary slightly from frame to frame, which generates a small amount of tracking noise in the measures of the ants' speed and distance from the nest. I smoothed these measures by applying a 1 second rolling average. This removes small instantaneous variations in the data, eliminating some tracking noise.

Analysis of the scout's position in the raiding column

To ask whether the scout led the raid, I ranked her position in the raiding column in each raid. To do this, I took advantage of the fact that in all analysed raids, the responding ants walked in a single file. I ranked all ants by the time they crossed the halfway mark between the nest and the food. Observations of the videos suggested that changes in the ants' ranks were minimal (i.e., they did not often overtake each other), and selecting alternative points at which to rank the ants did not noticeably alter the distribution of the scout's rank across raiding events (data not shown).

Analysis of trail following during the response phase

To ask whether the ants in the response phase follow the specific trail laid by the scout in the recruitment phase, I asked whether the xy coordinates during their outbound journey were closer to the xy coordinates of the recruiting scout during her inbound journey than expected by chance.

For each raid, let the set of the recruiter's xy coordinates be

$$\{\vec{r}_t^{rec}\}_{t \in \left[t_i^{rec}, t_f^{rec}\right]}$$

where $\vec{r}_t^{rec}$ represents the xy coordinates of the recruiter at time *t*,

t_i^{rec} is the time at the start of the recruitment phase,

and t_f^{rec} is the time at the end of the recruitment phase.

Similarly, the set of all xy coordinates of all responding ants is

$$\{\vec{r}_t^{a}\}_{t \in \left[t_i^{resp}, t_f^{resp}\right], a \in A},$$

where $\vec{r}_t^{a}$ represents the xy coordinates of ant *a* at time *t*,

t_i^{resp} is the time at the start of the response phase,

t_f^{resp} is the time at the end of the response phase,

and A is the set of ants that participate in the response to recruitment.

For each xy coordinate in the response, we then calculated its minimum distance (z_t^a) to the recruiter's track:

$$z_t^a = min_{\hat{t}}\{\left\|\vec{r}_t^{a} - \vec{r}_{\hat{t}}^{rec}\right\|\}_{\hat{t} \in \left[t_i^{resp}, t_f^{resp}\right]}$$

For each raid, we then computed a measure of trail following:

$$Z = \langle z_t^a \rangle_{t \in \left[t_i^{resp}, t_f^{resp}\right], a \in A}$$

If the ants are not following the recruiter's trail, one might still expect Z to have a relatively low value, because the positions of the nest and the food remain constant across each raid (and thus substantially constrain the initial and final xy coordinates of each ant's trajectory). To account for this inherent spatial structure in our null expectation, I compared the set of response xy coordinates to the xy coordinates of scouts from all raids

other than their own. For each set of response coordinates, I thus generated 30 minimum-distance values. I then calculated the mean of this set, to generate a single value, which I then compared to the corresponding true Z value in a paired t-test.

Automatic detection and analysis of excursions in the search phase

I automatically selected complete excursions for all ants in the search phase of each raid. To do this, for each ant, I identified all pairs of transitions across the nest threshold – i.e., sequences of trajectories beginning with a nest exit and terminating in a nest entrance. Each such trajectory was termed an 'excursion'. As a quality filter, and to exclude cases when ants were following the arena wall and/or walking in circles, I excluded excursions in which ants travelled >= 3 times their maximum distance from the nest. For each excursion, I then calculated a number of summary features: its duration, its maximum distance from the nest, and the ant's mean speed. I then ranked these values within each event and plotted the excursion rank versus its index in the event across all events.

To ask how ants follow trails during these excursions, I also selected outbound legs of each excursion. For each xy coordinate in each outbound leg, I classified it as either on- or off-trail, depending on whether it mapped to a previously occupied pixel on a 100x100 pixel binary map (where each pixel represents a square of side 1mm) of all previous ant locations (i.e. excluding the focal excursion) in that search phase. I then rescaled all such binary sequences to be the same length so that I could align the beginning and end of the outbound legs of each excursion. I then tested for a non-zero slope for the regression line between this binary trail-following index and how far in time they were into the outbound leg of each excursion.

Barrier experiment

To study the nature of recruitment, I modified my artificial arenas. I laser-cut cast acrylic porous barriers of 0.8 mm thickness, with multiple holes with a diameter of ~50 um. My preliminary observations showed that ants could not contact each other from across such barriers, but could communicate via volatile pheromones. Each barrier was placed in the middle of a nest that had two foraging arenas, essentially creating two nests separated by this porous barrier. I established colonies of 20 one month old, phase- and genotype-matched ants in each nest half in each of eight replicate nests. The ants laid eggs in each nest half two days later. In the subsequent brood care phase, each day (except for a handful of days interspersed through the experiment when I fed and watered all colonies while preventing them from leaving their nest halves), I placed a single *S. invicta* pupa in the foraging chamber of one nest half of each artificial arena, alternating which half received food each day. In this experiment, a number of colonies often failed to detect the food (because the ants never left their nest). Nonetheless, I recorded 35 instances of foraging in five artificial nests across a two-week period. Of the 35 replicate events, I excluded four events from a single colony from further analysis, because the scout in these events did not enter the nest, or because (in one case) the colony was too active in the search phase for effective recruitment.

As tracking the ants in the dense chamber is impractical, I used an alternative approach to understand the recruitment dynamics in the nest. The background image (an image which includes all image features, but without the ants; see (Gal *et al.*, 2020) for the procedure used to generate these images) was subtracted from each frame in the video, and converted it to grey scale. The value of each pixel, g_i, in this image was taken as the

inverse of the probability that it contains an ant. The centre of mass coordinates for each "half-colony" was then defined as

$$C_x = \frac{1}{n}\sum_{i=1}^{n}(1 - g_i).x_i,\ C_y = \frac{1}{n}\sum_{i=1}^{n}(1 - g_i).y_i$$

where C refers to the centroid's coordinates,

x and y refer to the coordinates of each pixel,

and g refers to the pixel grey-value.

For each frame, the position of the centroid was identified, and its distance to the barrier separating the two nest halves was recorded as the length of the perpendicular from the centroid to the barrier. I then aligned the time series of centroid distance (from the barrier) to the time of recruitment (which I define as the time at which a majority of ants on the scout's nest half are activated and begin to move), and averaged across events.

For the statistical analysis comparing distances before and after the scout releases recruitment pheromone, I manually selected a frame from each video roughly 1-2 seconds before release, and compared the distance of the centroid from the barrier at this timepoint to its distance 20 sec later. To ensure that my manual selection of the initial frame was accurately identifying a time shortly before recruitment, I also measured the dynamics of the number of pixels that were below a threshold grey-value (g_i) intensity (a proxy for the number of ants in the scout's nest half, which we call the 'ant mass') over time, and found that shortly after the initial frame, this 'ant mass' decreased sharply – an indication that the ants in the scout's nest half actually left the nest in response to recruitment pheromone.

Colony size experiment

To ask how increasing colony size altered the structure of search behaviour, I established 3-4 colonies each of 10, 20, 50, or 100 untagged workers. As before, all workers were one month old, and were selected from a single cohort from a large source colony. They were placed in artificial arenas identical to those used in the tagged ant experiment when they were entering the reproductive phase, and laid eggs simultaneously in their new nests shortly thereafter. In the subsequent brood care phase, when their larvae were ~5 days old, I began tracking. Here, every day for 10 days, I gently transferred ants in the foraging arena into the nest, sealed the connecting tunnel, cleaned the foraging arena with water, saturated the plaster base of each colony, and placed food (a single small *S. invicta* pupa) in the foraging arena before reopening the tunnel and starting tracking. Roughly four hours later, I then fed each colony in proportion to their colony size (to control their nutritional states). Specifically, I placed *S. invicta* pupae inside each nest, maintaining a constant 1:10 food items:ants ratio. On rare occasions when a colony did not locate the food in the arena within four hours, I placed it inside the nest. I then continued filming the colony for the next ~20 hours. I repeated this process through the brood care phase, until the larvae had pupated. This experimental design allowed me to study how varying colony size alters the structure of the raid, and more importantly, how it alters the behaviour of ants searching for food when there is no food in the arena – the primary focus of my statistical analyses.

Exit counting analysis and controls

To analyse the temporal structure of search behaviour, the time at which each ant exited the nest (and entered the foraging arena) was recorded. AnTraX was used to track ant movement in the foraging arena. Since the ants were not individually tagged in this

experiment, I did not obtain complete trajectories, but rather a collection of short tracklets, some of which were single-ant and some were multi-ant (Gal *et al.*, 2020). I marked the entrance to the tunnel and filtered all tracklets that originated with an ant emerging from the tunnel (all tracklets that have their first blob overlapping with the entrance mark and have no parent tracklets, or multi-ant tracklets with only one single-ant tracklet parent that start at the tunnel entrance). For each of these tracklets, the first frame was recorded as an "exit time" of one ant. While the false positive rate of this detection process is minimal, the false negative (unrecorded exits) is more substantial, as some cases where ants leave the nest in close proximity, which prevents their segmentation, are recorded as single exits. Nevertheless, for all the analyses described below, these errors work to decrease the reported effect.

Overall, across all colony sizes, I had 150 timeseries of intervals between subsequent nest exits. I excluded 19 samples (i.e., timeseries) that had fewer than 200 total exits from subsequent analysis. As my analysis was focused on short-term activity fluctuation, I detrended each timeseries with third-degree polynomials to account for slow modulations of activity that might correspond to effects such as build-up of colony hunger, circadian cycles, etc. I then assessed the autocorrelation for the first ten lags of each timeseries. The mean autocorrelation was higher for larger colony sizes at most initial lags. To quantify a 'coordination index' C for ants leaving the nest together, I summed the autocorrelation over the first ten lags, and compared this value across samples:

$$C = \sum_{k=1}^{10} \frac{\sum_{i=1}^{N-k}(Y_i - \bar{Y})\,(Y_{i+k} - \bar{Y})}{\sum_{i=1}^{N}(Y_i - \bar{Y})^2}$$

where Y_i refers to the i-th inter exit interval in the detrended sequence,

k refers to the lag,

and N refers to the size of the inter-exit sequence.

Quantifying the number of ants that participate in the raid

As a proxy for the true number of ants involved in raids, I recorded the maximum number of detected blobs outside the nest in any single frame throughout the raid, whether these blobs corresponded to individual or several ants. I removed all counts above the 99.97 percentile to eliminate outliers corresponding to cases of false positive blob detections.

Enlarged *O. biroi* colony experiment

I established two *O. biroi* colonies in the brood care phase with roughly 5000 workers each. Preliminary experiments suggested that colonies of this size settle relatively rapidly, and I found that after 12 hours in a new nest, the colonies behaved qualitatively indistinguishably from colonies that had lived in a nest for arbitrarily long periods. For each foraging event, I anesthetized each colony with CO_2 and transferred it into a new arena (roughly 60cm x 34cm) with a fresh plaster of Paris base and a circular nest chamber (radius 6cm) with a single sealed exit.

O. biroi workers have a strong thigmotactic tendency, and in large, featureless arenas, they spend substantial proportions of time following the outer walls. To ameliorate this effect, I scattered a number of small, transparent acrylic bricks (3cm x 0.3cm x 0.3cm) throughout the arena. Pilot experiments suggested that introducing these bricks inside the arena would enable the workers to follow the short local edges,

diminishing the amount of time they take to locate the food. Additional pilot experiments showed that adding such edges or changing arena size did not qualitatively affect the ants' ability to raid.

Roughly 12-16 hours after introducing each large colony to its new nest, I placed 3-7 piles of fire ant brood far from the nest, and then unsealed the nest exit and allowed each colony to explore the arena. I filmed each colony's foraging behaviour for the next ~24 hours. I repeated this process seven times for one colony and four times for the other, with 1-3 days between subsequent foraging events. Together, I filmed eleven foraging events in the brood care phase in these large arenas, of which I excluded one because the ants were alarmed at the start of filming. I manually annotated the remaining foraging events to assess whether recruitment occurred inside or outside the nest, whether or not recruitment events resulted in bifurcation of the trail, and to estimate approximately how many ants participated in the raid.

Ancestral state reconstructions in the Dorylinae

I used the phylogenetic consensus topology of the Dorylinae from (Borowiec, 2019). I searched the natural history literature on doryline ants to find information on character states for a number of characters: colony size, prey spectrum, and various features of foraging behaviour (raid initiation, recruitment, number of ants in the raid, and trail bifurcation) that are characteristic of either group or mass raiding behaviour (Tables 4.1 and 4.2). Since there is very little evidence from multiple species within each genus (and little quantitative data anywhere in the Dorylinae), I chose to collapse character states for each trait into a genus-level categorical assessment. There were no major ambiguities within any genus.

To infer the ancestral states of foraging behaviour (Appendix C), I classified each genus as either a group raider, a mass raider, or as 'unknown', based on their four foraging characters' states (Table 4.1). There were no inconsistencies across the four characters for any genus - i.e., any species with a character state typical of group raiding had other character states also typical of group raiding, or had no information regarding other character states. Thus, if a genus had at least two known character states, I classified it as either a group or mass raider. I classified genera with information for one or no characters as 'unknown'.

I then reconstructed ancestral states for maximum colony size, prey spectrum, and raiding behaviour using maximum parsimony and maximum likelihood with a one-parameter Markov k-state model, both implemented in Mesquite (Maddison and Maddison, 2019). Given the paucity of character data, I interpret this reconstruction largely qualitatively, ignoring inferred character states for all intermediate nodes except the doryline most recent common ancestor.

Appendix A

Species	*Odontomachus ruginodis*	*Dinoponera quadriceps*	*Ooceraea biroi*
# DEGs with single-copy orthology	2270	171	363
# DEGs	4987	408	784
Number of annotated transcripts	25458	22408	23804
Number of annotated genes in genome	not available	11544	13640
Brood present in colony?	YES	UNKNOWN	YES
Non-reproductive workers	22%	0%	0%
Reproductive workers	N/A	100%	100%
Gynes	50%		
Queens	100%	N/A	N/A
Non-reproductive workers	5	6	4
Reproductive workers	N/A	7	4
Gynes	2		
Queens	3	N/A	N/A
SRA project number	PRJNA472392	PRJNA255520	PRJNA304722
Tissue sample used	Brain	Brain	Brain
Samples & data collected by:	This study	Patalano *et al.* 2015	Libbrecht *et al.*
Fixed reproductive asymmetry?	YES	NO	NO
Queenless?	NO	YES	YES
Multiple reproductives?	Rarely	Never	Always

Species	*Camponotus planatus*	*Solenopsis invicta*	*Acromyrmex echinatior*	*Harpegnathos saltator*
# DEGs with single-copy orthology	2579	1925	1050	16
# DEGs	5304	4116	2262	35
Number of annotated transcripts	17993	21105	20241	22197
Number of annotated genes in genome	not available	15103	12176	12302
Brood present in colony?	YES	YES	UNKNOWN	UNKNOWN
Non-reproductive workers	0%	n/a	unknown	0%
Reproductive workers	N/A	N/A	N/A	100%
Gynes	-	0%	unknown	
Queens	100%	100%	-	N/A
Non-reproductive workers	5	10	6	11
Reproductive workers	N/A	N/A	N/A	12
Gynes	0	7	3	
Queens	5	3	0	N/A
SRA project number	PRJNA472392	PRJNA472392	PRJNA223531	PRJNA327090
Tissue sample used	Brain	Brain	Head	Brain
Samples & data collected by:	This study	This study	Li *et al.* 2014	Gospocic *et al.* 2017
Fixed reproductive asymmetry?	YES	YES	YES	NO
Queenless?	NO	NO	NO	SOMETIMES
Multiple reproductives?	Usually	Often	Often	Sometimes

Summary statistics for RNA-Seq screen, including information about all seven focal ant species.

Appendix B

genus	# species	colony size	ant-eating?	raid initiation	recruitment	# ants in raid	trail bifurcation	raiding behaviour	references
Neivamyrmex	130	100000	Yes	spontaneous	outside nest	many	yes	mass	(Borowiec, 2016; Rettenmeyer, 1963; Topoff *et al.*, 1980)
Eciton	29	100000	Yes	spontaneous	outside nest	many	yes	mass	(Schneirla, 1933; Schneirla, 1934)
Nomamyrmex	2	1000000	Yes	unknown	unknown	many	yes	mass	(Borowiec, 2016; Rettenmeyer, 1963)
Labidus	9	1000000	generalist	unknown	unknown	many	yes	mass	(Borowiec, 2016; Monteiro *et al.*, 2008; Rettenmeyer, 1963)
Cheliomyrmex	4	unknown	unknown	unknown	unknown	unknown	unknown	unknown	(Gotwald, 1971; O'Donnell *et al.*, 2005; Wheeler, 1921)
Leptanilloides	19	100	unknown	unknown	unknown	few	unknown	unknown	(Brandão *et al.*, 1999; Donoso *et al.*, 2006)

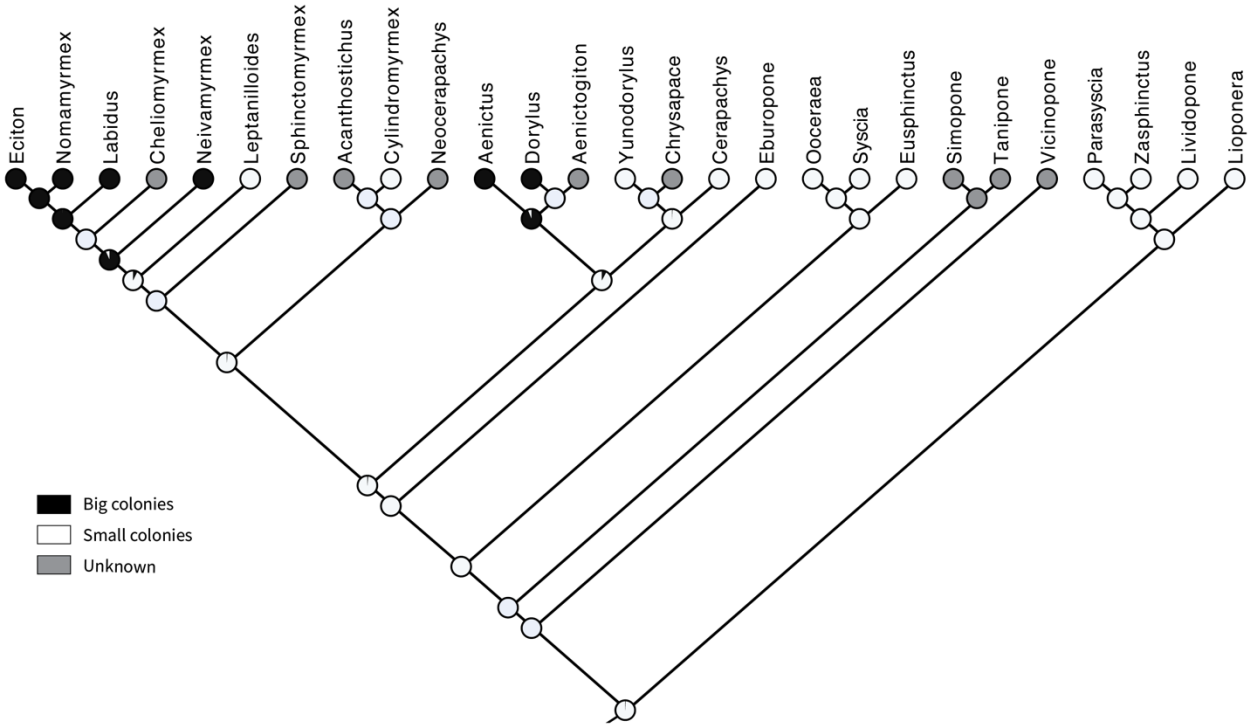
references		(Brown, 1975; MacKay, 1996)	(Gobin *et al.*, 2001; Mariano *et al.*, 2004)		(Borowiec, 2016; Chapman, 1964; Schneirla and Reyes, 1966)	(Berghoff, 2002; Berghoff *et al.*, 2002; Borowiec, 2016)	(Borowiec, 2016)
raiding behaviour	unknown	unknown	group	unknown	mass	mass	unknown
trail bifurcation	unknown	unknown	no	unknown	yes	yes	unknown
# ants in raid	unknown	unknown	few	unknown	many	many	unknown
recruitment	unknown	unknown	inside nest	unknown	unknown	outside nest (inferred)	unknown
raid initiation	unknown	unknown	scout-initiated	unknown	spontaneous	spontaneous	unknown
ant-eating?	unknown	termite predator	termite predator	unknown	Yes	generalist	unknown
colony size	unknown	unknown	100	unknown	10000	1000000	unknown
# species	3	23	10	2	183	60	7
genus	*Sphinctomyrmex*	*Acanthostichus*	*Cylindromyrmex*	*Neocerapachys*	*Aenictus*	*Dorylus*	*Aenictogiton*

references	(Borowiec, 2016)	(Borowiec, 2016)	(Borowiec, 2016)	(Borowiec, 2016)	(Borowiec, 2016)	(Borowiec, 2016)	(Borowiec, 2016)	(Bolton and Fisher, 2012; Borowiec, 2016)	(Borowiec, 2016)
raiding behaviour	unknown	unknown	unknown	unknown	group	unknown	unknown	unknown	unknown
trail bifurcation	unknown	unknown	unknown	unknown	no	unknown	unknown	unknown	unknown
# ants in raid	few	unknown	few	unknown	few	unknown	unknown	unknown	unknown
recruitment	unknown	unknown	unknown	unknown	inside nest	unknown	unknown	unknown	unknown
raid initiation	unknown	unknown	unknown	unknown	scout-initiated	unknown	unknown	unknown	unknown
ant-eating?	unknown	unknown	unknown	Yes	Yes	unknown	unknown	Yes	unknown
colony size	1000	unknown	1000	100	100	10	10	unknown	unknown
# species	4	3	5	1	13	5	2	39	10
genus	*Yunodorylus*	*Chrysapace*	*Cerapachys*	*Eburopone*	*Ooceraea*	*Syscia*	*Eusphinctus*	*Simopone*	*Tanipone*

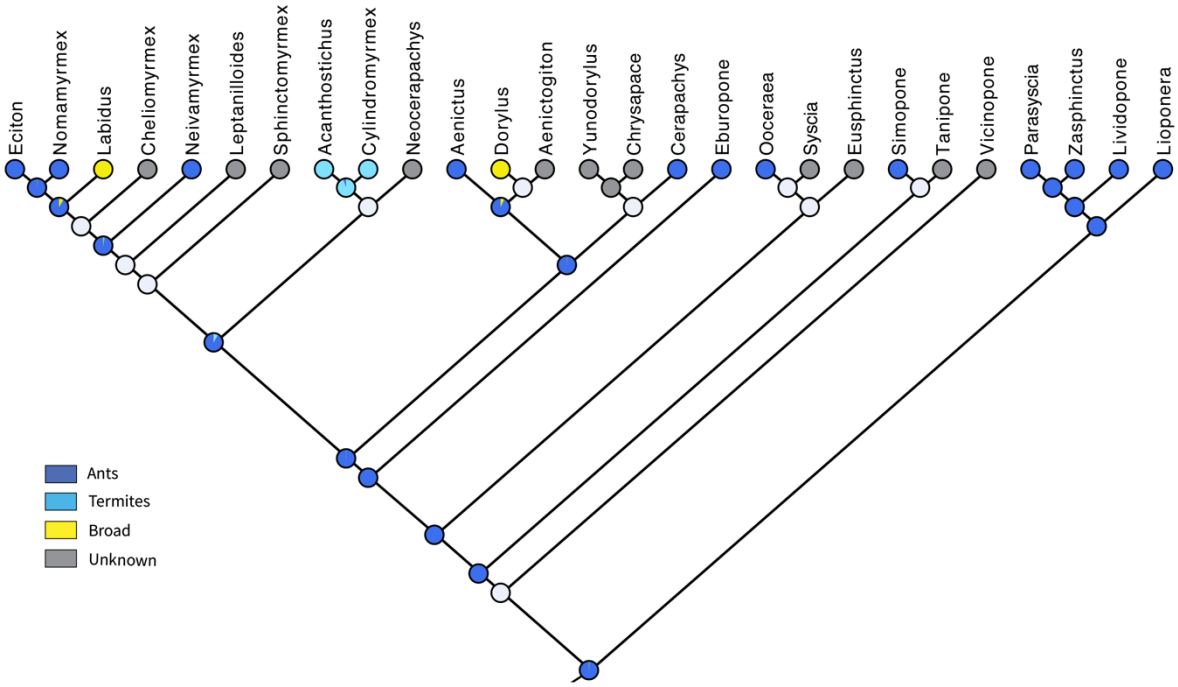
genus	# species	colony size	ant-eating?	raid initiation	recruitment	# ants in raid	trail bifurcation	raiding behaviour	references
Vicinopone	1	unknown	unknown	unknown	unknown	unknown	unknown	unknown	(Borowiec, 2016)
Parasyscia	51	100	Yes	unknown	unknown	few	no	group	(Brown, 1975; Wilson, 1958b)
Zasphinctus	23	100	Yes	unknown	inside nest (inferred)	few	no	group	(Briese, 1984; Buschinger *et al.*, 1989; Clark, 1923; Wilson, 1958b)
Lividopone	1	10	Yes	unknown	unknown	few	no	group	(Borowiec, 2016; Brown, 1975)
Lioponera	74	10	Yes	scout-initiated	inside nest	few	no	group	(Brown, 1975; Clark, 1923; Clark, 1941; Hölldobler, 1982; Wheeler, 1918; Wilson, 1958b)

Classification (from the literature) of colony size, prey spectrum, foraging characteristics, and overall type of foraging behaviour for each extant doryline genus (see Chapter 6 for details of classification). I also list the number of described species for each genus, and whether or not it is classified as an army ant.

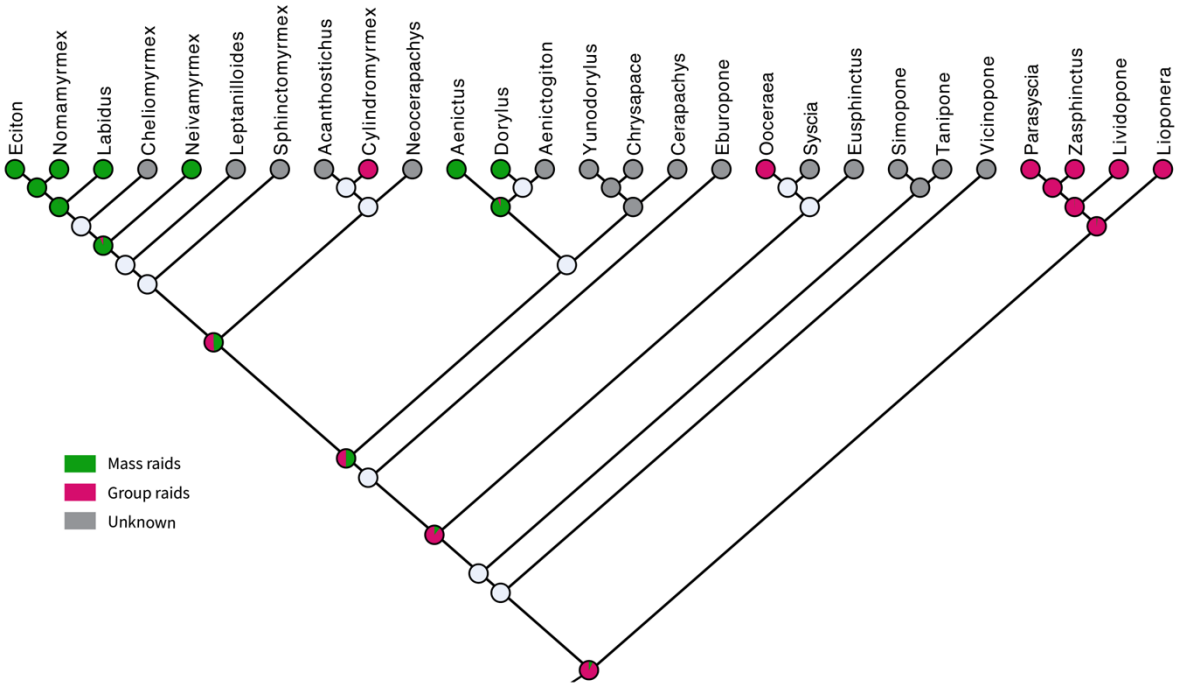
Appendix C



Maximum likelihood ancestral character state reconstruction for colony size. Pie charts at each node of the phylogeny depict the proportional likelihoods of both possible colony size states. The doryline MRCA (at the base of this tree) is highly likely to have had small colonies. As described in Chapter 6, genus-level colony size states were binarised, with colonies above a threshold of $5*10^4$ workers classified as big, while all other colonies were classified as small. See Table 4.2 for proportional likelihoods and maximum parsimony reconstructions.



Maximum likelihood ancestral character state reconstruction for prey spectrum. Pie charts at each node of the phylogeny depict the proportional likelihoods of each possible prey spectrum state. The doryline MRCA (at the base of this tree) is highly likely to have been myrmecophagous (i.e., an ant-predator). See Table 4.2 for proportional likelihoods and maximum parsimony reconstructions.



Maximum likelihood ancestral character state reconstruction for raiding behaviour. Pie charts at each node of the phylogeny depict the proportional likelihoods of both possible raiding behaviour states. The doryline MRCA (at the base of this tree) is highly likely to have been a group raider. See Table 4.2 for proportional likelihoods and maximum parsimony reconstructions.

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